CLINICAL AND BIOLOGICALLY-BASED APPROACHES FOR CLASSIFYING AND PREDICTING EARLY OUTCOMES OF CHRONIC CHILDHOOD ARTHRITIS

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GENERAL ABSTRACT

Background: Juvenile idiopathic arthritis (JIA) comprises a heterogeneous group of conditions that share chronic arthritis as a common characteristic. Current classification criteria for chronic childhood arthritis have limitations. Despite new treatment strategies and medications, some continue to have persistently active and disabling disease as adults. Few predictors of poor outcomes have been identified.

Objectives: This thesis comprises two complementary studies. The objective of the first study was to identify discrete clusters comprising clinical features and inflammatory biomarkers in children with JIA and to compare them with the current JIA categories that have been proposed by the International League of Associations for Rheumatology. The second study aimed to identify predictors of short-term arthritis activity based on clinical and biomarker profiles in JIA patients.

Methods: For both studies we utilized data that were collected in a Canadian nation-wide, prospective, longitudinal cohort study titled Biologically-Based Outcome Predictors in JIA. Clustering and classification algorithms were applied to the data to accomplish both study objectives.

Results: This research identified three clusters of patients in visit 1 (enrolment) and five clusters in visit 2 (6-month). Clusters revealed in this analysis exposed different and more homogenous subgroups compared to the seven conventional JIA categories. In the second study, the presence or absence of active joints, physician global assessments, and Wallace criteria were chosen as outcome variables 18 months post-enrolment. Among 112 variables, 17 were selected as the best predictors of 18-month outcomes. The panel predicted presence or absence of active arthritis, physician global assessment, and Wallace criteria of inactive disease 18 months after diagnosis with 79%, 82%, and 71% accuracy and 0.83, 0.86, 0.82 area under the curve (AUC), respectively. The accuracy and AUC values were higher compared to when only clinical features were used for prediction.

Conclusion: Results of this study suggest that certain groups of patients within different JIA categories are more aligned pathobiologically than their separate clinical categorizations suggest. Further, the research found a small number of clinical and

inflammatory variables at diagnosis can more accurately predict short-term arthritis activity in JIA than clinical characteristics only.

CO-AUTHORSHIP

This dissertation contains two separate manuscripts which were completed and written by Elham Rezaeisarlak in collaboration with her supervisors, Dr. Alan Rosenberg (Department of Pediatrics, College of Medicine, University of Saskatchewan), and Dr. Anthony Kusalik (Computer Science, University of Saskatchewan), and dissertation advisory committee members: Drs. Chandima Karunanayake (Canadian Centre for Health and Safety in Agriculture [CCHSA], College of Medicine, University of Saskatchewan), Nazeem Muhajarine (Community Health and Epidemiology, College of Medicine, University of Saskatchewan), Regina Taylor-Gjevre (Department of Medicine, College of Medicine, University of Saskatchewan), Susan Tupper (Saskatchewan Health Authority), and Donna C. Rennie (CCHSA and College of Nursing, University of Saskatchewan).

Study 1. Clinical and biological features for clustering within a cohort of children with chronic arthritis

Elham Rezaeisarlak conducted data management, interpreted the data, conceptualized and designed the study, and prepared and revised the manuscript; Dr. Rosenberg and Dr. Kusalik contributed to the study concept, design, and interpretation, and reviewed and revised the manuscript. Dissertation advisory committee members contributed to the study concept, results interpretation, and reviewed and revised the manuscript; Dr. Brett Trost and Mr. Daniel Hogan contributed to the study design, data analysis methods, results, interpretation, and reviewed and revised the manuscript.

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Study 2. Biological and clinical predictors of short-term outcomes of JIA

Elham Rezaeisarlak conducted data management, interpreted the data, conceptualized and designed the study, and prepared and revised the manuscript; Dr. Rosenberg and Dr. Kusalik contributed to the study concept, design, and interpretation, reviewed and revised the manuscript. Dissertation advisory committee members contributed to the study concept, results interpretation, reviewed and revised the manuscript; Dr. Brett Trost and Mr. Daniel Hogan contributed to the study design, data analysis methods, results, interpretation, and reviewed and revised the manuscript. The following members from the BBOP group contributed to data collection, and reviewed and revised the manuscript.

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DEDICATED TO

My parents, and my grandfather, Dr. Goudarzi, who offered me unconditional love and instilled in me adventure, curiosity, and freedom.

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LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
ANA	anti-nuclear antibody
AP-1	activation protein-1
AS	ankylosing spondylitis
AUC	area under the curve
BIC	Bayesian Information Criterion
CFS	correlation-based feature selection
CHAQ	childhood health assessment questionnaire
CNTF	ciliary neurotrophic factor
CRP	C-reactive protein
CT-1	cardiotrophin-1
CV	cross-validation
DMARDs	disease-modifying anti-rheumatic drugs
DT	decision tree
EGF	epidermal growth factor
EM	expectation-maximization
ERA	enthesitis-related arthritis
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FGF	fibroblast growth factor
FMF	familial Mediterranean fever
FN	false negative
FP	false positive
FS	feature selection
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GMM	Gaussian mixture model
gp-130	glycoprotein 130
HAQ	health assessment questionnaire
HLA-B27	human leukocyte antigen-B27
HMGB	high mobility group box
ICE	interleukin converting enzyme
IFN	interferon
IL	interleukin
ILAR	International League of Associations for Rheumatology
IP-10	interferon gamma-induced protein 10
JADAS	juvenile arthritis disease activity score
JAQQ	juvenile arthritis quality of my life questionnaire
JCA	juvenile chronic arthritis
JIA	juvenile idiopathic arthritis
JRA	juvenile rheumatoid arthritis
LIDA	laboratory indicators of disease activity
LIF	leukemia inhibitory factor
LOO-CV	leave-one-out cross-validation
MCC	Matthews correlation coefficient
MCP	monocyte chemo-attractant protein

MHC	major histocompatibility complex
MIP	macrophage inflammatory proteins
MMP	matrix metalloproteinases
NK	natural killer
NLLs	NOD-like receptors
NPV	negative predictive value
NSAIDs	nonsteroidal anti-inflammatory drugs
OPG	osteoprotegerin
PAQ	physical activity questionnaire
PC	principal component
PCA	principal component analysis
PGA	physician global assessment
PPCA	probabilistic principal component analysis
PPV	positive predictive value
PRESS	predictive sum of square
PRRs	pattern recognition receptors
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa-B ligand
RANTES	regulated on activation, normal T cell expressed and secreted
RF	rheumatoid factor
ROC	receiver operating characteristic
sIL-6R	soluble receptor of IL-6
sLRP-1	soluble low-density lipoprotein receptor-related protein
SNP	single nucleotide polymorphisms
Th	T-helper
TIMP	tissue inhibitor of metalloproteinase
TLRs	Toll-like receptors
TN	true negative
TNF	tumour necrosis factor
TP	true positive
VEGF	vascular endothelial growth factor
WBC	white blood cell counts

DESCRIPTION OF CHAPTERS

Chapter 1- Overview and rational for the research

The first chapter is a brief overview of backgrounds, rationales, and objectives of studies included in this thesis.

Chapter 2- Background literature review

1- The background literature review provides an overview of previous classifications of childhood arthritis.
2- A review of biomarkers associated with JIA is provided. The most important inflammatory biomarkers involved in the pathophysiology of JIA are explained in this chapter.
3- Gene and human leukocyte antigen (HLA) characteristics in JIA are discussed briefly.
4- A review of suggested JIA outcome measures and predictors of disease activity is provided.
5- Analytic methods, including specific variable selections, clustering and classification algorithms are explained.

Chapter 3- Study 1 manuscript

Chapter 4- Study 2 manuscript

Chapters 3 and 4 are manuscripts of studies 1 and 2, respectively. Each manuscript includes the introduction, methodology, results, discussion and conclusion generated from the study.

Chapter 5- Overall discussion and conclusion

The Discussion and Conclusion chapter provides general, integrated commentary relating to both studies.

CHAPTER 1

OVERVIEW AND RATIONAL FOR THE RESEARCH

1.1 Background

Chronic childhood arthritis is a heterogeneous group of diseases categorized predominantly by clinical manifestations (1). A classification proposed in 2001 by a subcommittee of the International League of Association for Rheumatology (ILAR) denotes the most current system for classifying chronic childhood arthritis (Table 1.1) (2). The impetus for developing the JIA classification system was a desire to establish internationally standardized disease categories to facilitate communication and research collaborations (3).

Classification criteria for chronic childhood arthritis that preceded the ILAR JIA criteria were the American College of Rheumatology (ACR) criteria for Juvenile Rheumatoid Arthritis (JRA) proposed in 1970 (4), and the European League Against Rheumatism (EULAR) criteria for Juvenile Chronic Arthritis (JCA), proposed in 1977 (5).

Both the ACR and EULAR classification systems are based on clinical features at disease onset. As with earlier iterations of childhood arthritis classifications, the utility of the current JIA classification system is limited as it was not devised primarily to predict clinical courses, reliably guide treatment choices, or predict treatment responses. Also, re-classification of a patient might be necessary if clinical manifestations, test results, or pertinent family medical history information change. While JIA categories are intended to be mutually exclusive, some overlaps exist despite the application of exclusion criteria that aim to preserve category purity. In addition, the exclusion criteria can lead to ambiguity and inaccuracies in classifying some patients.

Only two biomarkers, rheumatoid factor (RF) and HLA-B27, are considered in JIA classification (2). The principle premise underlying the research described herein is that a more comprehensive array of biomarkers that could eventually include genomic, proteomic, transcriptomic, immunomic and metabolomic features, when combined with clinical characteristics could yield a more refined, biologically-based classification system. It also can provide insight into chronic childhood arthritis pathogenesis, and aid in predicting disease course and outcomes.

Although not considered in current chronic childhood arthritis classification systems, genomic profiles tend to be distinguishable among JIA categories.

Table 1.1 ILAR JIA classification (2).

JIA classification	Definition and criteria	Exclusion
Systemic Arthritis	Arthritis in one or more joints with or preceded by fever of at least 2 weeks duration that is documented to be daily ("quotidian") for at least 3 days, and accompanied by one or more of the following: -Evanescent (non-fixed) erythematous rash -Generalized lymph node enlargement -Hepatomegaly and/or splenomegaly -Serositis	a, b, c, d.
Oligoarthritis	Arthritis affecting one to 4 joints during the first 6 months of disease. Two subcategories are recognized: 1-Persistent oligoarthritis: Affecting not more than 4 joints throughout the disease course 2-Extended oligoarthritis: Affecting a total of more than 4 joints after the first 6 months of disease	a, b, c, d, e.
Polyarthritis (RF Negative)	Arthritis affecting 5 or more joints during the first 6 months of disease and test for RF is negative	a, b, c, d, e.
Polyarthritis (RF Positive)	Arthritis affecting 5 or more joints during the first 6 months of disease and 2 or more tests for RF at least 3 months apart during the first 6 months of disease are positive	a, b, c, e
Psoriatic Arthritis	Arthritis and psoriasis, or arthritis and at least 2 of the following: -Dactylitis -Nail pitting or onycholysis -Psoriasis in a first-degree relative	b, c, d, e
Enthesitis Related Arthritis	Arthritis and enthesitis, or arthritis or enthesitis with at least 2 of the following: -The presence of or a history of sacroiliac joint tenderness and/or inflammatory lumbosacral pain -The presence of HLA-B27 antigen -Onset of arthritis in a male over 6 years of age -Acute (symptomatic) anterior uveitis -History of ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with inflammatory bowel disease, Reiter's syndrome, or acute anterior uveitis in a first-degree relative	a, d, e
Undifferentiated Arthritis	Arthritis that fulfills criteria in no category or in 2 or more of the above categories	

a. Psoriasis or a history of psoriasis in the patient or first-degree relative.
b. Arthritis in an HLA-B27 positive male beginning after the 6th birthday.
c. Ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with inflammatory bowel disease, Reiter's syndrome, or acute anterior uveitis, or a history of one of these disorders in a first-degree relative.
d. The presence of IgM rheumatoid factor on at least 2 occasions at least 3 months apart.
e. The presence of systemic JIA in the patient.

As examples, certain HLA allotypes and nucleotide polymorphisms confer JIA susceptibility while others appear to be protective (6). Certain single nucleotide polymorphisms (SNP[1]s) are associated with JIA categories (7, 8).

There has been increasing interest in identifying molecules involved in regulating immune responses that relate to susceptibility to, and outcome of JIA. Proteomic data have been studied in different subtypes of JIA with significant associations identified (9-15). The inflammatory process is mediated by an array of innate regulators including interleukins, chemokines, growth factors, and matrix metalloproteinases (MMPs). In the context of JIA, some of these biological markers, such as IL-6 and IL-1 in systemic JIA (16, 17) reflect inflammatory activity while others, like MMPs, receptor activator of nuclear factor kappa-B ligand (RANKL), and Osteoprotegerin (OPG) are predictive of disease outcomes (18, 19). Distinctive cytokine profiles and acute phase protein responses in polyarticular and systemic JIA have been identified (6, 20). This accumulating evidence of biomarker associations with JIA subgroups supports a need to more thoroughly study JIA in the context of clinical and biomarker profiles and to determine if panels of attributes can more precisely distinguish subsets of children with chronic arthritis and predict their disease outcomes.

In addition to considering the importance of clinical and biomarker features for classification of chronic childhood arthritis, we aimed to determine the utility of baseline clinical-biomarker panels for predicting short-term disease course and outcomes. Being able to effectively predict the disease trajectory and eventual outcomes would help inform the timing and aggressiveness of treatment interventions.

Identification of the time frame during which the correct therapeutic choice can change the pathophysiology of disease and improve outcomes is important for optimizing care (21). In JIA early initiation of aggressive treatment, especially in systemic arthritis and polyarticular arthritis categories, results in better outcomes (22). In contrast, patients with mild disease do not require aggressive, potentially harmful, and expensive treatments as more moderate therapies are efficacious. While timing and aggressiveness

[1] SNPs are variations in DNA sequence that occur by changing a single nucleotide in the genome. Each individual has many SNPs, which constitutes a unique DNA pattern for that person.

of treatment interventions in JIA are currently guided by clinical characteristics predominantly, it is conceivable that incorporating biomarker profiling in the therapeutic decision-making process could further enhance treatment effectiveness, minimize adverse effects of therapy and reduce cost.

Different descriptors of JIA states of disease activity have been proposed including, as examples: mild, moderate, or severe disease; inactive disease; minimal disease activity; and remission on/off medication (23-27). ACR criteria defined disease as active/inactive (in remission) in oligoarticular, polyarticular, and systemic JRA by using the number of joints with active arthritis, physician global assessment (PGA), clinical manifestation of systemic arthritis, presence of active uveitis, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, and duration of morning stiffness (26). A composite disease activity scoring system, the juvenile arthritis disease activity score (JADAS), has been developed for assessment of disease status. JADAS includes four variables: number of involved joints, PGA, parent/child ratings of well-being, and ESR or CRP (25, 27). The state of minimal disease activity has been described using number of active joints, PGA, and a parent's global rating of well-being in polyarthritis and oligoarthritis (28). A joint was defined as active in accordance with the definition prescribed in the ILAR JIA classification. Specifically, *"[active] arthritis is swelling within a joint, or limitation in the range of joint movement with joint pain or tenderness, which persists for at least 6 weeks, is observed by a physician, and is not due to primarily mechanical disorders or to other identifiable causes" (2).* In accordance with this definition, an active joint may be effused or not but an effused joint is always active. Wallace *et al*. proposed a set of criteria for inactive JIA including: no joints with active arthritis, no fever, rash, serositis, splenomegaly, or generalized lymphadenopathy attributable to JIA, no active uveitis, normal ESR or CRP, PGA that indicates no disease activity (29).

Clinical and laboratory features have been used to determine disease course and outcome (23, 30). Disease activity states, joint damage, functional ability, and quality of life are characteristics typically applied to determine disease outcome (31-37). Number of active joints at onset, polyarticular onset of JIA, Child Health Assessment Questionnaire (CHAQ) responses, PGA, parent's global assessment, and joint symmetry were

determined to have predictive utility. There is a paucity of information on the use of genetic and immunological characteristics as predictors of JIA outcomes (20, 32, 38).

1.2 Purpose of the study

The purposes of this thesis are: 1) to identify discriminating clusters of clinical and biomarker characteristics and determine how well such clusters align with current JIA categories; and 2) to identify early predictors of JIA outcome based on clinical manifestations and biomarker profile of the patients at first presentation in a JIA inception cohort.

The results could either support the appropriateness of the current JIA categories and predictors or possibly indicate that inflammatory biomarkers might add precision to categorizing and outcome prediction of chronic childhood arthritis.

1.3 Software used for analyses

To realize these aims, clinical and biological data previously collected in a national study, the Canadian Biologically-Based Outcome Predictors (BBOP) in JIA study, were used (39). Data mining algorithms were applied for data analyses. Data were pre-processed to make them suitable for input into data mining algorithms. Raw data are highly susceptible to missing values, outliers, noisy data, and inconsistency. Pre-processing data is an important step to enhance data efficiency. Pre-processing includes several techniques such as cleaning, integration, transformation, and reduction (40). SPSS software (IBM®) was used for pre-processing and data ranking techniques. Several R software packages were used for principal component analysis (PCA) and clustering (study 1), and the software package Waikato Environment for Knowledge Analysis (Weka) was used for predicting JIA outcome (study 2). Weka is open source software with a collection of machine learning algorithms and data pre-processing tools. It is easy to use and a powerful data mining tool (41).

CHAPTER 2

LITERATURE REVIEW

2.1 Juvenile idiopathic arthritis definition and classification

Arthritis, which is inflammation within a joint, is defined as joint swelling or effusion, or the presence of two or more of the following: limitation of range of motion, tenderness or pain on motion, and increased heat in one or more joint (42). Arthritis is the most common chronic childhood conditions. Among the many different forms of childhood arthritis, JIA is the most common class. JIA is a heterogeneous group of chronic childhood arthritis conditions for which the cause is unknown. It is a potentially disabling condition with incidence rate of 7.8/100,000 and a prevalence of 32.6/100,00 in (43).

That chronic arthritis commonly afflicts children is a relatively contemporary realization. However, early archaeological evidence has shown evidence of arthritis in children dating from as early as the 10th century and there are reports of childhood arthritis over the ensuing centuries (44).

Until the late 19th century, arthritis beginning during childhood was considered to be the same disease as adult onset arthritis. However, in 1883 rheumatic diseases in children as a distinctive class of pediatric disease was highlighted at a meeting of the British Medical Association (45, 46). Dr. George Frederic Still, in his 1897 doctoral dissertation, further elucidated the characteristics of chronic arthritis in children and introduced the idea that childhood onset arthritis was distinguishable in many cases from adult onset rheumatoid arthritis (RA) (1). He described the disease based on his observation of 22 children with chronic arthritis and wrote:

> *"The occasional occurrence in children of a disease closely resembling the rheumatoid arthritis of adults has been recognized for several years. The identity of the disease seen in children with that in adults has never, so far as I am aware, been called in question. Although the disease known as rheumatoid arthritis in adults does undoubtedly occur in children, the disease which has most commonly been called rheumatoid arthritis in children differs both in its clinical aspect and in its morbid anatomy from the rheumatoid arthritis of adults; it presents, in fact, such marked differences as to suggest that it has a distinct pathology. The cases hitherto grouped together as rheumatoid arthritis in children include, therefore,*

more than one disease; and it will be shown that there are at least three distinct joint affections which have thus been included under the one head, rheumatoid arthritis" (1).

Still described three types of the disease: 1) chronic childhood polyarthritis indistinguishable from adults arthritis; 2) chronic arthritis associated with systemic manifestations including splenomegaly, lymphadenopathy, and pericarditis; and 3) a disease identical to "*chronic fibrous rheumatism"* (1). As a consequence of Still's reporting, chronic arthritis in children was assigned the eponymous designation, Still's Disease. In 1946, the New York Rheumatism Association replaced the term Still's Disease with the term JRA after reporting 56 cases of chronic childhood arthritis (3).

In 1970, a committee of the ACR proposed the first classification criteria for JRA. The JRA classification system was defined as arthritis in children younger than age 16 years in one joint or more for 6 weeks or longer providing other diagnostic considerations were excluded. The JRA taxonomy included three subtypes, polyarticular, pauciarticular and systemic arthritis. The spondyloarthritis and psoriatic forms of childhood arthritis were considered distinct diseases and were not included in the JRA classification system (4, 47, 48).

The polyarthritis JRA subgroup was defined by the number of involved joints (5 or more); the presence of RF together with polyarthritis was considered to confer a poorer prognosis. RF-positive patients were older, were more likely to be female, had a higher percentage of antinuclear antibody (ANA)-positivity, predominant involvement of the small joints of the hands, a higher frequency of erosions and, in general, a poorer prognosis. Pauciarticular JRA was defined as arthritis in 4 or fewer joints. In general, pauciarticular JRA was considered to have a more favourable prognosis compared to the polyarticular subset. Systemic onset JRA included children with arthritis associated with intermittent fever and systemic manifestations such as rash, lymphadenopathy, hepatomegaly, splenomegaly and serositis. Systemic JRA was recognized to have variable outcomes with some children having a remitting relapsing course, some progressing to chronic polyarthritis as extra-articular manifestations waned, and in some children the disease remitted after a single episode (49).

Concurrent with the ACR classification initiative, the European League Against Rheumatism (EULAR) devised an alternate classification system (5). EULAR proposed

the term juvenile chronic arthritis (JCA) to denote children with chronic arthritis including those having spondyloarthropathies and psoriatic arthritis. EULAR reserved the term JRA for children with polyarthritis associated with RF-positivity (5). Table 2.1 shows the comparisons between ACR and EULAR classification systems.

Table 2.1 Comparison of ACR and EULAR criteria.

Criteria	ACR	EULAR
Name	JRA	JCA
Age at onset	<16 years	<16 years
Duration of arthritis	6 weeks	3 months
Subtypes	Pauciartricular Polyartricular Systemic	Pauciartricular Polyartricular Systemic Juvenile ankylosing pondyloarthritis Juvenile psoriatic arthritis
Rheumatoid Factor positivity	Does not alter	JRA, not JCA
Other causes excluded	Yes	Yes

The latest classification criteria for chronic childhood arthritis, JIA, was proposed by a subcommittee of ILAR in 1994 (50). The impetus for developing the JIA criteria was to establish an internationally standardized disease classification system to facilitate international communication and research collaborations. JIA criteria were revised in 2004 (2) and denote a class of childhood arthritis having an unknown cause developing before the age of 16 years and persisting for at least 6 weeks. The JIA class comprises seven subgroups including 1) systemic arthritis 2) oligoarthritis, 3) RF-negative polyarthritis, 4) RF-positive polyarthritis, 5) psoriatic arthritis, 6) enthesitis-related arthritis (ERA), and 7) undifferentiated (2). The JIA subsets are defined in Table 1.1.

Despite attempts to ensure subgroup homogeneity, some heterogeneity within JIA categories exists. Martini posited that the number of involved joints at onset or during the disease course is not a reliable criterion for defining homogenous subgroups (51). He

proposed that descriptors such as asymmetric arthritis, early onset age, being female, ANA-positivity, and uveitis should be considered as classification criteria rather than number of affected joints (51). Currently, children with those features tend to be clustered into oligoarthritis, polyarthritis or psoriatic arthritis JIA categories. Stoll *et al.* suggested that psoriatic JIA comprises two distinct subsets (52). Specifically, they proposed that younger children with psoriatic JIA are more likely to be female, be ANA-positive, be more likely to progress to polyarthritis, exhibit dactylitis and have small joint involvement. Older patients with psoriatic JIA are more likely to develop enthesitis, axial joint involvement and have persistent oligoarthritis (52, 53).

The spondylarthropathies comprise a group of arthritis characterized by axial and peripheral enthesitis and arthritis, and an association with HLA-B27 (54). Although the spondyloarthropathies typically have their onsets during adolescence and early adulthood, younger children can be affected. Ankylosing spondylitis (AS) is the prototypic spondyloarthropathy. The spondyloarthropathy category also includes undifferentiated spondylitis, reactive arthritis, subsets of psoriatic arthritis, and inflammatory bowel disease related arthritis (54, 55). It is believed that adult onset and childhood onset AS represent a disease continuum with somewhat different clinical expressions; the childhood onset symptoms tend to include peripheral enthesitis and arthritis especially in lower extremities while the adult onset form presents predominantly with axial spine involvement (56-60). Young onset age and female sex are associated with a less favourable AS outcome (56, 59). The ILAR classification system considers ERA and psoriatic arthritis as two distinct subgroups; however, ERA and psoriatic arthritis subgroups can have overlapping features. Thus, some patients would be categorized as undifferentiated arthritis due to overlapping between categories (2).

Childhood arthritis classification systems have been devised based on clinical manifestations predominantly without substantive consideration of the biologic and pathophysiologic basis of the conditions. As new knowledge about the underlying pathophysiology of childhood arthritis emerges there are likely to be new opportunities to refine classification criteria based on both clinical and biologically-based characteristics.

2.2 Predictors of JIA outcomes

JIA is the most common childhood rheumatic disease. It imposes substantial burden on the child's growth and development, quality of life, and future productivity and

is associated with substantial burdens for families and society. A significant proportion of children with JIA develop permanent joint damage resulting in disability. Approximately half of children with JIA will have arthritis as adults (61, 62). Early identification of clinical and biologic characteristics that portend a poor prognosis can help direct the aggressiveness of initial therapy aimed at preventing joint damage and long-term disability. Caution must be taken in identifying patients who benefit from early aggressive therapy to reduce health risks of these treatments.

A number of studies have evaluated short- and long-term JIA outcomes in relation to clinical remission, disability, and radiological damage. Factors such as young onset age, severe arthritis at onset, prolonged active disease, symmetric disease, hip or wrist involvement, and the presence of RF are recognized as predictors of less favourable JIA outcomes (30, 37, 62-69).

The following section summarizes JIA outcome studies in relation to JIA categories.

2.2.1 Systemic JIA

Several short-term outcome studies considered the disease subtypes at onset as a predictive factor. In a retrospective study by Spiegel *et al*. early predictors of systemic JIA poor outcome (destructive arthritis) were identified within 6 months of disease onset. The outcome was measured by severity of joint damage reported by radiologists, and predictors included persistent systemic symptoms such as fever and thrombocytosis (70). The results that predicted the development of a poor functional outcome in the patients was validated by long-term follow-up (71). Male sex, higher number of active joints, and continuing disease activity were identified as risk factors for disability (72). In long-term and short-term studies (there is no definition of long/short-term outcome in the pediatrics rheumatology literature) a significant association was found between elevated levels of fibrin D-dimer and poor functional outcomes of systemic arthritis (73, 74).

Modesto *et al.* described articular outcome in patients with systemic arthritis measured by Helsinki index[2] (HI) and systemic symptoms of the disease. They defined outcomes as HI≥10 a bad articular outcome while HI<10 meant a good prognosis. The

[2] Helsinki Index is an articular index for the classification of patients with systemic onset juvenile chronic arthritis and is a tool for performing a quantification of the number of affected joints (swelling, limitation of motion).

onset predictors of poor outcome were presence of generalized lymphadenopathy, age<8 years and HI>6. The presence of polyarthritis and hip involvement at 6 months were additional indicators of poor prognostic disease (75).

A long-term study of systemic JIA revealed three patterns of disease course: monocyclic, intermittent, and persistent after 5 years follow-up. Fever and active arthritis at 3 months after diagnosis, an ESR>26 mm/hour, and corticosteroid use at 6 months were identified as predictors of a non-monophasic course. Three and 6 months after diagnosis, absence of active arthritis, an ESR of <26 mm/hour, and no requirement for corticosteroid therapy were predictors of an earlier time to remission (76).

In systemic JIA the macrophage migration inhibitory factor (MIF)-173 polymorphism significantly correlates with longer duration of glucocorticoid treatment, higher numbers of joints with active arthritis and limited range of motion, and higher CHAQ scores. MIF-173*C allele is identified as a predictor of poor outcome in systemic JIA (77).

2.2.2 Oligoarthritis

Historically, oligoarthritis was considered a form of JIA with a generally favourable long-term outcome. However, more contemporary studies have indicated that outcomes might not be as generally favourable as once thought (35). In one study, remission was defined as absence of clinical or laboratory evidence of active arthritis for a period of at least 6 months off medication. Thirty six percent of patients with oligoarticular JIA permanently remit off medication, 53% continued to have active disease, and 13% relapse following temporary remission (78).

Age at onset and ANA-positivity are predictors of disability and active disease duration in oligoarticular JIA (68). High ESR and involvement of more than one upper limb joint at onset have been reported as predictive of joint damage and functional disability in oligoarticular JIA (35). Al-Matar *et al.* have shown that early involvement of ankle or wrist disease, symmetrical joint involvement, and an elevated ESR are predictors of extended oligoarticular JIA (30).

2.2.3 Polyarthritis

Patients with polyarthritis JIA with or without RF-positivity have high rates of morbidity and functional disability although RF-positive polyarthritis has a substantially worse prognosis than RF-negative polyarthritis. Morbidity refers to state of being

diseased or may result from adverse effects of therapies, which may impair the quality of life of patients and their families (79). Hyrich *et al*. reported in a cohort of children with polyarthritis JIA 64%, and 40% had CHAQ ≥ 0.75 at diagnosis and 1 year later respectively (80). These patients have low remission rates ranging from 0% to 5% off medications and 65% on medications (33). In another study remission rates of RF-negative and RF-positive polyarthritis JIA 10 years after diagnosis have been reported as 23% and 5%, respectively (62).

Oen *et al.* have shown that male sex is the only predictor for RF-positive polyarthritis JIA that correlates with shorter active disease duration (68). In the same study male sex, older onset age, and rural residence were identified as predictors of good functional outcome for patients with RF-negative polyarthritis JIA (68). No HLA or genetic polymorphisms have been identified as predictors of disease activity in polyarthritis JIA.

2.2.4 Psoriatic JIA

Juvenile psoriatic arthritis is diagnosed when a child has arthritis associated with psoriasis. It can follow an oligoarticular, polyarticular or ERA pattern. The outcome of psoriatic JIA tends to relate to the pattern of joint involvement. Psoriatic JIA long-term outcome studies reveal that 70% of patients still have active disease and 1/3 have functional limitations 5 years after onset (81). Another study showed that after 15 years of disease 33% still require disease-modifying anti-rheumatic drug (DMARD) therapy (82) and a short-term study showed clinical remission in 60% of affected children (52). Flatø *et al.* have identifed early determinants of developing psoriatic JIA including history of psoriasis in the patient or in a first degree relative, dactylitis, ankle or toe arthritis, and HILA-DRB1*11/12 (82). Remission on medication occurs later in the disease course of patients with polyarthritis compared to oligoarthritis and ERA disease patterns (83) .

2.2.5 Enthesitis-Related Arthritis

ERA involves predominantly joints of the lower extremities and the axial skeleton. ERA is more prevalent in adolescent boys. Reported remission rates for ERA have varied from 17% to 60% (32, 61). Sacroiliac (SI) and axial spine involvement occur late in the disease course and can result in limitations in range of motion of the spine (84, 85). Reported frequencies of SI involvement in ERA ranged from 9% to 75%. Severe

disability has been reported to occur in 4% to 52% of ERA patients (84). Evidence of sacroiliitis has been detected by dynamic MRI in 30% of children with ERA, 1 year after disease onset (85).

Early and persistence hip disease, early ankle involvement, and a high number of involved joints during the first 6 months of the disease are predictors of poor functional outcome in ERA (86). Although ERA is strongly associated with HLA-B27, the presence of HLA-DRB1*08, and the absence of HLA-DPB1*02 are predictors of poor outcome (36).

A number of studies have evaluated early predictors of outcomes of a cohort of JIA patients without considering the JIA subtypes. A long-term study aimed to predict three distinct disease outcomes 15 years after onset including: 1) remission according to the ACR definition of remission (26), 2) joint erosion according to radiological findings, and 3) physical disability based on the Stanford Health Assessment Questionnaire (HAQ), and CHAQ (38). Characteristics that were predictive of less favorable remission rates were young age at onset, HLA DRB1*08, positive IgM RF, long duration of elevated ESR, and large number of involved joints within the first 6 months. Predictors of joint erosion were early onset age, a large number of affected joints, positive IgM RF, long duration of elevated ESR, and symmetric arthritis. Predictors of physical disability were female sex, symmetric joint involvement, early hip joint arthritis, long term elevated ESR, and positive IgM RF (38). In a short-term study the outcome was clinically inactive disease according to Wallace *et al.* criteria (29); predictors were identified as active joint count, PGA, patient or parent global assessment of overall well-being, and CHAQ (69).

2.3 Inflammatory biomarkers associated with outcomes considered in the analysis

Genetic differences in the expression levels of a number of important biomarkers can lead to chronic inflammation (29). The measurement of inflammation-related biomarkers in body fluids and synovial tissue has provided insight into the underlying pathophysiology of JIA.

Cytokines are a group of small proteins secreted by a variety of cells including those of the immune system. They modulate acute and chronic inflammation through elaborate cell signalling pathways and interactions. Cytokine is an umbrella term encompassing a large number of chemokines, interferons, interleukins (IL), lymphokines, tumour necrotizing factors (TNFs), and growth factors (87, 88). Although all cell types

are capable of producing cytokines, T-helper (Th) cells and macrophages are the principal sources (89). Three main classification systems for cytokines have been proposed. The first is based on their function and roles in immune response and consist of substances that: 1) induce cells of the adaptive immune response, 2) promote inflammation, and 3) inhibit inflammation (Table 2.2). The second proposed cytokine classification system is based on their action on target cells and include: 1) interleukins, 2) tumour necrosis factors, 3) interferons, 4) colony stimulating factors, and 5) chemokines (90). The third cytokine classification system is based on structure and specifically if an amino acid is or is not located between the first two cysteine residues (CXC and CC respectively) (91). In this review, the first and second cytokine classification systems are discussed.

Functional genetic polymorphisms, including those mediating inflammatory cytokine expression, can alter the gene's structure, function, and resultant phenotype and contribute to risk of polygenic diseases including arthritis (92). In the following discussion, SNPs that influence cytokine expression in the context of JIA are reviewed.

Table 2.2 Classification of cytokines by immune response.

Immune response	Cytokines
Adaptive immunity	IL-2, IL-4, IL-7, IL-9, IL-13 (IL-13R–IL-4R complex), IL-15, IL-21, TSLP (TSLPR–IL-7R complex), GM-CSF, RANTE, RANKL
Pro-inflammatory	IL-1α, IL-1β, IL-1ra, IL-6, IL-6Ra, IL-11, IL-17A-F, IL-25 (IL-17E), IL-18, IL-31, IL-33,IL-36α, IL-36β, IL-36γ, IL-37 and IL-1Hy2, CNTF, CT-1, LIF, OPN, OSM, TNF-α, TNF-β, BAFF, APRI, IFN-α, IFN-β, IFN-ω, IFN-κ, Limitin, IFN-γ, IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), S100A8, S100A9, S100A12, Serum amyloid A
Anti-inflammatory	IL-10, IL-12, IL-19, IL-20, IL-22, IL-23, IL-24, IL-26, IL-27, IL-28, IL-29, IL-35

CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; LIF, leukaemia inhibitory factor; OPN, osteopontin; OSM, oncostatin M; TNF, tumour necrosis factor; TSLP, thymic stromal lymphopoietin; RANKL, Receptor activator of NF-kB ligand; RANTES, regulated on activation, normal T cell expressed and secreted.

2.3.1. Pro-inflammatory cytokines

IL-1, IL-6, TNF-α, S100 and serum amyloid A are the most influential pro-inflammatory cytokines in the context of childhood arthritis (90).

2.3.1.1 IL-1

IL-1 encompasses two distinct proteins, IL-1α and IL-1β, which are respectively encoded by two genes and controlled by specific inhibitors including membrane bound IL-1 receptor antagonist (IL-1Ra), soluble IL-1 receptor type II, and IL-1 receptor accessory protein (93). Monocytes, macrophages, and neutrophils, can produce IL-1 in response to pro-inflammatory stimuli such as cell injury, bacterial products, TNF, and granulocyte-macrophage colony stimulating factor (GM-CSF) (94). Also, induction of pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and NOD-like receptors (NLLs) by viral and microbial agents can lead to excessive IL-1 expression (95, 96). The IL-1 family of proteins are produced as precursors, cleaved by the IL-1-converting enzyme (ICE) or caspase-1 to generate active cytokine (93, 97). IL-1α

activates endothelial cells and macrophages, and induces production of acute phase reactants from the liver. IL-1β stimulates differentiation of CD4+ T cells into Th1 cells and Th17 cell lineages and has pyrogenic effects (98).

The IL-1 family of proteins has nine genes located on chromosome 2 (99). Three IL-1 gene cluster SNPs (rs6712572, rs2071374, and rs1688075), and one IL-1 receptor cluster SNP (rs12712122) have been shown to be associated with risk of developing systemic JIA (100). Ankylosing spondylitis can be associated with IL-1A gene SNPs (rs1800587, rs2856836, rs17561) and these SNPs might account for ethnic variability in the expression of the disease (101). There are reports of IL-1 gene cluster SNP associations with psoriatic arthritis (102).

Inhibiting the action of IL-1 has therapeutic benefits in certain inflammation-mediated diseases. Pascual *et al.* have shown that dysregulated IL-1 production is a major mediator of the inflammatory cascade in systemic JIA, and IL-1 blockade with anakinra (recombinant IL-1Ra) is an effective treatment for the disease (16). However, response to anakinra among patients with systemic JIA is variable; approximately 40% have a favourable response while others have partial or no response (103). Ombrello and colleagues have shown that a variation in the IL-1RN gene influences susceptibility to recombinant IL-1RA therapy in systemic JIA (104).

2.3.1.2 IL-6

Acute and chronic inflammation induces IL-6 production by macrophages, T and B cells, endothelial cells, and tissue fibroblasts. The IL-6 gene is located on human chromosome 2 (99). Its signals are mediated by binding with IL-6 receptor, which is composed of two chains, IL-6R and glycoprotein 130 (gp-130). Gp-130 is a common signal transducing chain for other cytokine receptors. Soluble receptor of IL-6 (sIL-6R) also binds with IL-6, then can be attached to gp-130 (105). The pro-inflammatory functions of IL-6 include inducing fever, activation of endothelial cells, production of acute phase reactants, and B-cell proliferation. It also activates osteoclasts and promotes maturation of megakaryocytes, wound healing, development of Th22 and Th17, endothelial cell activation, fibroblast proliferation, and neuron development (106). Polymorphisms in the 5' flanking region of the IL-6 gene, a change from G to C at position 174, results in suppression of IL-6 transcription (17).

IL-6 and sIL-6R concentrations increase in JIA and are related to the degree of joint destruction (107). Levels of sIL-6R substantially increase in systemic JIA (108). It has been shown that in induced arthritis in mice IL-6 injection causes joint destruction, leucocyte aggregation, apoptosis, and T cell activation (109). Disruption of the IL-6 gene in knockout mice has shown that lack of this protein mitigates arthritis development (110). Many of the clinical features of systemic JIA such as chronic anemia, severe growth retardation, osteoporosis, thrombocytosis, and amyloidosis are related to IL-6 action (111). Tocilizumab, an IL-6R antibody that blocks soluble and membrane-bound IL-6 receptors, is therapeutically beneficial in systemic JIA (112, 113).

2.3.1.3 TNF

The TNF gene-coding region is located within the major histocompatibility complex (MHC) class III, on chromosome 6 and includes three genes. The TNFA gene, encodes TNF-α; TNFB encodes TNF-β, and LTB, encodes lymphotoxin-β (114). TNF-α and TNF-β are similar pro-inflammatory transmembrane glycoproteins belonging to the TNF superfamily. TNF can be cleaved by the metalloprotease TNF-α converting enzyme (TACE) to form soluble TNF that may allow for more widespread cytokine effects (114, 115). The main sources of TNF-α are activated macrophages, monocytes, B and T lymphocytes, and fibroblasts. Activated Th1 cells secrete TNF-β. The TNF superfamily consists of 20 different proteins including CD40L and RANKL. Their biological actions are mediated by binding to p55 and p75 (TNFRI, TNFRII) receptors. All the TNF receptor superfamily of ligands are capable of becoming a secreted form (116). Binding to the TNFRs initiates intracellular signaling leading to transcription of factors such as NF-kB and activation protein-1 (AP-1) that cause the production of inflammatory mediators and anti-apoptotic proteins (117, 118). Although TNFs usually mediate signalling for cell survival, the binding of TNF-α to TNFRI can result in either inflammation or apoptosis. Activation of caspases lead to cell death which is important for self-limitation of cell activation (118). The main effects of TNF are activation of endothelial cells, macrophages, monocytes, neutrophils, induction of the pro-inflammatory activity of fibroblasts, and apoptosis. Similar to an endocrine hormone, TNF-α can circulate in blood and act at distant sites. For instance, TNF-α can stimulate the hypothalamus to induce fever, stimulate hepatocytes to produce acute phase reactants,

and promote metabolic changes leading to cachexia. TNF-α plays a major role in the pathogenesis of sepsis (119, 120).

In RA and JIA patients increased levels of both TNF-α and TNF-β are detected in serum and synovial fluid (121). The presence of a SNP in the TNF-α gene (308 GA/AA and 238 GA) leads to elevation of TNF-α and increased transcriptional activity. This has been associated with a poorer prognosis and a lower response to anti-TNF-α drugs in patients with systemic JIA and RF-positive polyarticular JIA (10, 122). A genetic polymorphism of TNF plays a significant role in oligoarticular JIA; there is a strong association between the intronic 851 TNF SNP and persistence of oligoarticular JIA (123). Synovial tissues of patients with spondyloarthropathies, and persistent oligoarticular JIA express high and low levels of TNF-α, respectively. TNF-α expression in synovial tissues of patients with polyarticular JIA and adult-onset RA have been reported as intermediate. Generally, although expression of TNF-β in synovial tissues are low compared to expression of TNF-α in all groups, it is notably higher in children with polyarticular and ERA JIA (124). TNF-α levels increase in psoriatic plaques, blood, and in the synovial fluid of patients with active psoriatic arthritis and has correlation with disease severity (9, 125-127). There is an association of TNF-α promoter polymorphism at position -238 with psoriasis and psoriatic arthritis (128). In patients with AS, TNF-α, TNFRI, and TNFRII levels are high. After treatment with TNF blockade agents the level of TNFRI falls. In the sacroiliac joints of AS patients abundant TNF-α mRNA near the site of new bone formation can be detected (129).

The significant role of TNF in the pathogenesis of RA has made it an important target for therapy. Anti-TNF therapy is an effective treatment choice in polyarticular JIA patients not responsive to methotrexate (130). An effective strategy to inhibit TNF-α action is to use monoclonal antibodies to block its membrane-bound or soluble receptors (131). Infliximab and adalimumab are chimeric IgG anti-TNF-α monoclonal antibodies that bind to both membrane-bound and soluble TNF-α receptors (132, 133). However, development of neutralizing antibodies against infliximab and adalimumab can reduce the clinical efficacy of the treatments (134-136). Etanercept, a soluble TNF receptor, is the extracellular portion of the human p75 TNF-α receptor fused to the Fc portion of IgG1 (137, 138). Etanercept binds to both TNF-α and lymphotoxin-α, a member of the TNF family that also activates the inflammatory pathway through the TNFRs (139).

Etanercept does not have the potential to induce the formation of neutralizing antibodies (137).

High rates of treatment failure with TNF inhibitors have been reported in systemic JIA (140, 141). ERA subtype patients with peripheral disease who do not respond to DMARDs and those with axial involvement have shown clinical response with anti-TNF inhibitors (142, 143). Psoriatic JIA patients with inadequate response to DMARDs, axial disease, dactylitis and enthesitis are candidates for TNF inhibitor therapy (144). TNF inhibitors are indicated for intractable cases of oligoarticular JIA unresponsive to non-biologic treatment approaches (143).

2.3.1.4 S100

The S100 protein family comprises the largest subgroup within the Ca^{2+}-binding EF-hand (helix E-loop-helix F) protein group (145). Only vertebrates possess S100 genes, which are clustered at chromosome 1q21 and 21q22. Twenty-five proteins have been identified as belonging to the S100 family. They are small molecules, about 10-12kDa, classified into 3 subgroups: 1) proteins which exert intracellular regulatory effects, 2) those with intracellular and extracellular functions, and 3) those with only extracellular regulatory effects (146).

S100 protein can be expressed intracellularly in pathological states while they are absent in normal physiological conditions of the cell. Their expression patterns vary among different S100 proteins (147). These proteins regulate cell proliferation, differentiation, apoptosis, Ca^{2+} homeostasis, energy metabolism, inflammation and migration/invasion through interactions with other proteins. S100 proteins derived from cells of myeloid origin are suggested to be new markers of inflammation (145, 146). A subgroup known as calgranulins including S100A8, S100A9 and S100A12 (also termed phagocyte-specific S100) are highly expressed in monocytes and granulocytes and have been associated with acute and chronic inflammation (148). They are pro-inflammatory mediators when appearing extracellularly. Cryopyrin-associated periodic syndromes, familial Mediterranean fever (FMF), hyper-zincemia and hyper-calprotectinemia, polyarticular JIA, and systemic JIA are autoimmune diseases associated with over expression and dysregulation of calgranulins (149-152).

Increased intracellular calcium concentration stimulates activation of macrophages. Calcium ions initiate changes in calcium-binding proteins (S100A8,

S100A9, and S100A12), which interact with intracellular target structures (153). Expression of S100 proteins in monocytes and macrophages is tissue specific and occurs only during the early stages of cell differentiation (154). In humans, S100A8 and S100A9 assemble as an S100A8/S100A9 heterodimer (155). The distribution of S100A12 in the cytoplasm of granulocytes is similar to S100A8/S100A9, but it is less abundant (156). Intracellular calcium signaling induces S100A12 protein independent of S100A8/S100A9 (157). These proteins are released by stimulated phagocytes partly in response to calcium mediated signaling (158). The first cells targeted by released phagocyte-specific S100 are cells within the endothelial layer. The binding of S100A8/S100A9 and S100A12 to the surface receptors induces various intracellular inflammatory signaling pathways and recruitment of more leukocytes (159, 160). Besides their intracellular effects, S100A proteins activate immune cells. S100A8/S100A9 enhance adhesion of neutrophils to endothelial cells (160). S100A12 exerts chemotactic effects on phagocytes, up-regulates expression of TNFs and IL-1, and increases release of IL-2 (159). Their cytotoxic effects influence the survival or growth of inflammatory cells and homeostasis. S100A8 and S100A9 have direct roles in synovial inflammation and auto-immune disease (151).

Induced arthritis in animal models indicates a direct role of phagocyte-specific S100 proteins in synovitis (161). Accumulation of S100A8 and S100A9 expressing macrophages in the cartilage surface suggests correlation between them and signs of cartilage destruction and direct role of S100A8 and S100A9 in the destructive process of inflammatory arthritis (162). It also has been shown that S100A12 induces synovial inflammation in mice with collagen-induced arthritis (163). Cytokine production and MMP's activation within the synovium depend on interaction of S100A12 with its receptors (163, 164).

S100A8 and S100A9 were first identified in the context of RA. Activated phagocytes expressing S100A8, S100A9 proteins are abundant in inflamed synovium specifically at cartilage destruction and bone erosion sites (165). Synovial fluid concentration of S100A8/S100A9 is 10-fold higher than their serum levels in individual patients with inflammatory arthritis (166). The correlation of serum S100A8/S100A9 concentrations with the arthritis activity and their diagnostic capacity as a marker of synovial inflammation has been confirmed in RA patients (167, 168). Similarly, serum levels of S100A12 correlate well with disease activity (169). S100A12 increases in the

synovial fluid and serum of RA patients while it is undetectable after successful treatment and in patients with osteoarthritis (169, 170).

Phagocyte-specific S100 proteins have also been detected in serum and synovial fluid of JIA patients (171). Similar to RA patients, the serum concentrations of these proteins can be considered as markers of disease activity in childhood arthritis (172). In JIA patients who are judged to have inactive disease by clinical indicators elevated levels of S100A8/S100A9 or S100A12 represent that they are at risk for disease flare (171).

Systemic JIA patients have notably high expression and serum concentrations of S100A8, S100A9 and S100A12 than other JIA subtypes (20-fold higher) because of massive neutrophil activation (171). In addition, these patients show extensive expression of S100A8/S100A9 in the dermal epithelium (172). Therefore, S100A8/S100A9 and S100A12 can be considered as the tool to help differentiate systemic JIA from systemic infections. Potentially, phagocyte-specific S100 proteins might be appropriate targets for new anti-inflammatory therapies.

2.3.1.5 Serum amyloid A

Serum amyloid A (SAA) is a heterogeneous family of proteins which behave as acute phase reactants, and are associated with high density lipoproteins (173). The human SAA consists of 104 amino acid residues with six main isoforms as products of four active genes SAA1, SAA2, SAA3, and SAA4 in the short arm on chromosome 15 (174). SNPs in SAA1 are related to 5 isoforms of SAA1 (SAA1.1 - 1.5), which are associated with FMF, coronary artery diseases, cerebral infarction, and osteoporosis and arthritis related amyloidosis (175, 176). SAA1and SAA2 genes can be activated during acute-phase responses (177). IL-1β, IL-6, TNF-α, and glucocorticoids stimulate expression of the SAA1 gene in hepatocytes. Acute-phase SAA genes expression involves the transcription factors C/EBP, NF-κB, AP2, SAF, Sp1 and STAT3. SAA1 is recognized as a clinical indicator for inflammation (178).

Plasma levels of SAA1 and SAA2 increase dramatically during inflammation and consequently are useful biomarkers of inflammation (179). In JIA patients serum levels of SAA1 increase significantly with a strong positive correlation with the number of active joints (180). Scheinberg, *et al.*, showed that SAA levels are high in children with polyarticular and systemic JIA and increase during disease exacerbation, and decrease during disease remission and after prednisone therapy (181). An adult study has shown

that the serum level of SAA may be a better biomarker for RA disease activity than CRP, especially during treatment with TNF antagonists (182). Elevated levels of A-SAA in synovium of RA patients is associated with increased cartilage degradation (183). These findings support SAA as an indicator of disease activity and outcome predictor in chronic arthritis

2.3.2 Anti-inflammatory cytokines

Anti-inflammatory cytokines control the pro-inflammatory cytokine response including specific cytokine inhibitors and soluble cytokine receptors. They have a crucial role in modulating the inflammatory process (99).

2.3.2.1 IL-10

IL-10 is a homodimer protein (consisting of two identical molecules), mIL-10 and hIL-10, encoded by the IL-10 gene located on chromosome 1 near the IL-19 and IL-20 genes (184). The protein is produced by activated Th2 (helper CD4+), Tc2 (cytotoxic CD8+), Tr1 (regulatory T cell), B cells (185). Other cell types that produce IL-10 are, lymphocytes, monocytes, macrophages, and mast cells (99). The IL-10 receptor has two chains (IL-10R1 and IL-10R2) related to the interferon receptor (IFNR) family (186).

IL-10 plays multiple roles in immune-regulation and inflammation. It suppresses the expression of TNF-α, IL-6 and IL-1, up-regulates endogenous anti-cytokines and down-regulates pro-inflammatory cytokine receptors (187). It stimulates B-cell survival and antibody production, while inhibiting Th1 and Tc1 development. IL-10 also inhibits production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF; chemokines such as monocyte chemoattractant protein (MCP)1, MCP5, RANTE, macrophage inflammatory proteins (MIP)s; and growth factors such as GM-CSF, and granulocyte-colony stimulating factor (G-CSF) (187, 188). IL-10 gene polymorphisms are associated with inflammatory diseases including JIA (189), Behçet's disease, uveitis, systemic lupus erythematosus (SLE) (190), RA, B-cell lymphoma, gastric cancer, and Type 1 diabetes (191-193). Three SNPs in the promoter region of IL-10 at positions -1082(G/A), −819(C/T), and −592(C/A) have been identified (194). A SNP in the IL-10 gene promotor at position -592 increases the risk of developing JIA (187, 195). The G allele at the −1082 position has a negative association with JIA (196). In systemic JIA a low expression of IL10-1082 has been reported (197).

The important anti-inflammatory effect of IL-10 is to inhibit IL-1 and TNF production as they have synergistic and amplifying effects on the inflammatory processes (198, 199). In RA synovial macrophages and T cells produce IL-10 to inhibit production of inflammatory cytokines by synovial cells (200). In animal models of RA, IL-10 reduces clinical manifestations of the disease and suppresses cytokine production (201). Because of the potential effects of IL-10 in suppressing inflammation, targeting IL-10 therapeutically has been considered for treatment of chronic inflammatory diseases. (202).

2.3.3 Adaptive immunity

Genetic background and environmental exposures interact through adaptive immune system responses. Adaptive immunity includes humoral and cell-mediated immunity. T cells produce adaptive immune cytokines after being exposed to a specific antigen.

2.3.3.1 IL-2

IL-2 is important for the proliferation of T and B lymphocytes and natural killer (NK) cells (203). The IL-2 gene is located on chromosome 4 near the gene that encodes IL-21, and has only one allele (204). It is secreted primarily by activated T cells (CD4+ Th0, CD4+ Th1, and CD8Tc1) and dendritic cells (205). The IL-2 receptor has three subunits α (CD25), β (CD122), and γ (CD132). The gamma subunit is shared by IL-4R, IL-7R, IL-9R, IL-15R, and IL-21R. While assembly of the three chains results in a high affinity receptor, integration of two chains (β and γ) produce a receptor with medium affinity (206). Defects in IL-2/IL-2R profoundly affect cell-cell interactions and cell death when the immune system responds to antigens (207). Congenital lack of the gene encoding IL-2R γ chain causes X-linked severe combined immunodeficiency (SCID) (208). The IL-2Rα and IL-2Rβ and a SNP in the IL-2IL12 region (rs1479924) have been identified as a susceptibility loci for oligoarticular or RF-negative polyarticular JIA. (209). These findings suggest a vital role for the IL-2 pathway in JIA pathogenesis.

2.3.4 Matrix metalloproteinases

MMPs are zinc-dependent proteolytic enzymes. They are members of the metzincin group of proteases, which share structurally similar domains, in particular the zinc dependent catalytic domain and the activation peptide (pro-domain). In humans, there are 24 MMP genes that are expressed as inactive pro-proteins. To activate the

protein, the pro-domain is cleaved from the catalytic domain (139). The regulation of MMPs is controlled partly by cytokines, growth factors and tissue inhibitors of metalloproteinases (TIMPs) (210). MMPs participate in tissue remodelling by degrading extra cellular matrix. Important biological processes can be regulated by MMPs including cell migration, cell differentiation, growth, inflammatory processes, neovascularization and apoptosis, processes all operative in the context of arthritis (211).

Most MMPs are expressed in synovial tissue of RA patients predominantly MMP-1, MMP-9, MMP-13, MMP-14, and MMP-15. They are responsible for synovial remodelling and inflammatory tissue destruction (212). MMP-3 is associated with RA disease activity, cartilage breakdown (213), and is a predictor of radiographic disease outcome in RA patients (214). Elevated serum levels of MMP-3 are found in patients with active ankylosing spondylitis (215). Excessive expression of MMPs and low expression of TIMP-1 have been detected in the synovial tissue of JIA patients (210, 212).

Production of MMP-3 and MMP-1 can be induced by IL-1 and TNF in arthritis; thus, these cytokines increase cartilage degradation by inducing collagen-degradation mediators MMP-3 and MMP-1 (216). Gottorno *et al.* reported a significant increase in MMP-3 and MMP-1 in synovial fluid of patients with JIA (217). Peake *et al.* suggested that increased synovial fluid level of MMP-1 is consistent with inflammatory activity in the joint in all JIA subtypes (218). They suggested that degradation of type II collagen occurs early and continues throughout the disease course and that serum MMP-3 is a biomarker of active arthritis in JIA (218). Taken together, identifying the increased expression of some MMPs in RA/JIA synovial tissue and serum may provide a biomarker of diagnostic, prognostic, and therapeutic relevance.

MMP inhibitors have been synthesized (zinc-binding globulins [ZBGs], non-ZBGs inhibitors) for management of osteoarthritis, cancer, and cardiovascular disorders but they exert undesirable musculoskeletal side effects. Gene based therapies of TIMPs are being assessed in animal models (219).

In summary, the available evidence shows that dysregulation and imbalance between pro- and anti-inflammatory biomarkers are important factors in JIA pathophysiology. Growing understanding of the immune and inflammatory pathways in JIA has led to development of new medications that target inflammatory cytokines. As

examples, inhibitors of IL-1, IL-6 and TNF-α have substantially changed the outcome of JIA in systemic and polyarthritis subtypes. Future opportunities will be to recognize specific cytokines as markers of disease activity and attributes that aid in developing biologically-based classification and a more personalized approach to diagnosing and managing individual patients.

2.3.5 Gene and HLA characteristics in JIA

Multiple HLA alleles, different from those in RA patients, are associated with JIA. The reported associations include HLA-B27 with ERA, HLA-DRB1*01, DRB1*08, DRB1*11, DRB1*13, DPB1*02 and DQB1*04 with oligoarticular JIA (220, 221), HLA-DRB1*08 and DPB1*03 with polyarticular RF-negative JIA and DRB1*04, DQA1*03, and DQB1*03 polyarticular RF-positive JIA (221-224). HLA-DRB1*01, and DQA1*0101 are associated with psoriatic arthritis (221), and HLA-DRB1*04 with systemic JIA (224). HLA-DRB1*04 and DRB1*07 have been reported as protective genes for oligoarticular JIA, and DRB1*18:01 and DQB1*06:02-8 have been reported to be protective for all JIA groups (220-222, 225).

Gene expression and genome-wide genotyping have identified loci outside the HLA gene complex associated with different JIA subtypes, particularly PTPN2, PTPN22, STAT4, ANKRD55, IL-2, IL-2RA, IL-21, and SH2B3-ATXN2. The functions of these genes are chiefly regulating production and function of inflammatory biomarkers and their receptors. For instance, the PTPN2 gene modulates the expression of IL-2, IL-4, IL-6, and IFN. SNPs related to PTPN2 (rs7234029 (A>G), and rs2847293 (T>A)) cause impairment in the regulation of inflammatory pathways including joint inflammation (226-228).

Association between early onset oligoarticular and persistent oligoarticular JIA with TNFA, the gene encoding TNF-α, have been reported (123, 229). Systemic JIA is associated with a SNP at position -857 of TNFA (230). TNFA variant is also associated with polyarticular JIA (231). In Norwegian children a SNP in the promoter region of IL-1A, the gene encoding IL-1α, has been shown to be associated with early onset oligoarticular JIA (232). A SNP in the promoter of the IL-6 gene, several variants of the IL-1 gene, and IL-1R gene clusters are associated with systemic JIA (17, 100). An association between JIA and PTPN22 C1858T has been identified in Norwegian, Czech,

and British patients (233-235). Lymphoid tyrosine phosphatase (LYP), which inhibits T-cell activation, is regulated by the PTPN22 gene (236).

2.4 JIA outcome measures

Three major outcomes were investigated in earlier outcome predictor studies including remission, physical function or health status, and joint damage (33, 62, 237, 238). However, there has been some inconsistency in outcome definitions. Remission criteria are influenced by the classification system applied. Time from disease onset to study enrolment and last follow up visit contributes to variability in outcome study protocols making it difficult to precisely compare studies.

Physical function outcome is conventionally measured with specific tools, such as HAQ, CHAQ, JAQQ, or Steinbrocker functional class (239-242). HAQ, a measure of function in adults, consists of 100 questions with five principal dimensions (death, disability, discomfort, drug toxicity, dollar cost), which have been separated into several components. Functional ability is measured by nine components (dressing and grooming, arising, eating, walking, hygiene, reach, grip, outside activity, and sexual activity). Each question is scored from 0 to 3 where 0=without any difficulty, 1=with difficulty, 2=with some help from another person or with a device, and 3=unable to do. The index is calculated by averaging the scores. The other indices also range from 0 to 3. In the dollar cost section, annual medical and surgical costs are calculated. HAQ can be either self or interview administered (240).

CHAQ, an adaptation of the HAQ for use in children, is an effective, valid, and reliable tool to assess childhood arthritis (239). CHAQ evaluates performance of the child's activities in their daily environments. CHAQ is designed for children from 1-19 years old and includes 38 items grouped into 8 domains including physical function, dressing and grooming, arising, eating, walking, hygiene, reach, grip, activities, a pain index, and health status index (overall health status). CHAQ scores items are from 0-3, indicating the magnitude of child difficulty in performance of daily activities during the past week (0=without any difficulty, 1=with some difficulty, 2=with much difficulty, and 3=unable to do) although pain and health status indices are scored on a 10 cm visual analogue scale. CHAQ data is acquired by self-report interview of children ≥ 8 years old, or a parent report for children younger than age 8 (239, 241, 243).

JAQQ is an instrument to assess the health-related quality of life in children with arthritis. It measures physical functioning, emotional well-being, and an array of general symptoms of quality of life in children aged 2-18 years old. JAQQ includes 74 items grouped into four domains including gross motor function, fine motor function, psychosocial function, and systemic or general symptoms. In the revised version of JAQQ a pain scale of 10 cm VAS has been added. JAQQ is self/parent-administered questionnaire with each item rated from 1 to 7 (244, 245).

The Steinbrocker classification is a method for categorizing functional capacity of patients with RA (246). In 1991, ACR revised the Steinbrocker functional classes as follow: Class I=able to perform usual activities of daily living (self-care, vocational, and avocational); Class II=able to perform usual self-care and vocational activities, but limited in avocational activities; Class III=able to perform usual self-care activities but limited in vocational and avocational activities; Class IV=limited in ability to perform usual self-care, vocational, and avocational activities (247).

Anatomical bone and joint outcomes are determined mainly by radiographic assessment of joints (63). Reported structural outcomes include joint erosion, joint space narrowing, and ankyloses. The radiological findings, similar to remission rates, are influenced by the duration of the disease.

Most JIA prediction studies have been retrospective outcome studies. They are difficult to compare as different classification criteria have been used and duration of follow-ups have varied. New medications and treatment strategies have changed the disease outcome substantially, thus the results of earlier long-term studies may no longer be relevant in the context of contemporary therapies (248, 249). There are limited number of studies that have reported the role of an array of inflammatory biomarkers as predictors of disease outcome.

2.5 Overview of statistical analyses methods used in this research

In this section an overview of the application of data mining in medicine and select data mining methods including cross-validation, data reduction and feature selection, a clustering method, and a classification method are discussed.

2.5.1 Data mining and its application in medicine

Coincident with increasing knowledge about JIA pathophysiology and accumulating biologically-based data, methods and tools for analyzing large datasets

have emerged. Data mining is one approach that can help interpret large, complex clinical-biologic datasets in JIA.

Machine learning, a computer science statistical framework that automates the generation of models, can help distill useful information from large amounts of complex data. It has been described by Mitchell as: *"a computer program is said to learn from experience E with respect to some class of tasks T and performance measure P if its performance at tasks in T, as measured by P, improves with experience E"* (250).

Data mining is the process of discovering meaningful hidden patterns within data by applying machine learning techniques. Basic concepts in data mining are description and prediction. By finding useful patterns in a substantial amount of information it is possible to make predictions. There are two data mining methods: unsupervised (description approaches) and supervised (prediction approaches). Unsupervised methods include clustering and association rules; supervised methods include classification and regression. The goal of clustering approaches is to find naturally occurring, interpretable, rational patterns, and associations within the data, while the aim of classification is to construct predictive models (254, 255). Association rules are if/then statements that explore the relationships among variables.

Medicine, like other fields of science, can take advantage of such machine learning approaches. Diseases are mainly categorized (classified) according to their measurable signs and symptoms. For example, an inflamed joint can be described based on the size of an effusion, degree of joint warmth or redness, range of motion, and associated pain without considering the underlying pathophysiologic process that gave rise to the inflammation. Considering inflammatory biomarker profiles in individual patients could inform patient-specific, biologically-based personalized approaches to targeted therapies. In fact, applying supervised learning methods to an integrated biological, genetic, environmental, and clinical dataset could help develop a completely new disease taxonomy that can direct individual patient treatment options.

Machine learning and data mining have provided a unique opportunity for medical scientists to investigate new disease taxonomy leading to accurate diagnosis, targeted therapy, and improved outcome.

Predictive data mining can help solve important problems in research and clinical medicine. By applying predictive data mining, clinicians can use patient information to

predict the course and outcome of disease. Predictive data mining has received great attention in molecular biology and is routinely applied in genomic medicine (256). The majority of predictive genomic studies are related to oncology. In particular, early diagnosis of acute myeloid leukemia and acute lymphoblastic leukemia has dramatically altered patient treatment and outcome. This approach has provided a cancer classification strategy based on only gene expression, which is important for outcome prediction in cancer treatment (257-259).

Combining clinical and gene expression data with unsupervised and supervised learning can further improve classification accuracy and the clinical relevance of the prognostic models (260, 261).

2.5.2 Data pre-processing

Raw data are generally incomplete as they can contain missing values, errors, and outliers, and are susceptible to inconsistency. Prior to applying data mining methods data pre-processing is highly recommended, which encompasses techniques that transform raw data into an understandable format from both numerical and visual standpoints. In data mining a variable is called a feature. For example, for a child with arthritis the features can be listed as number of active joints, sex, age, ESR, and CRP. In this research, variable and features are used interchangeably. Prior to discussing some data pre-processing techniques, it is necessary to understand an important concept known as cross-validation.

2.5.2.1 Cross-validation

Cross-validation (CV) is a commonly used method in data mining and statistics to evaluate models and involves assessing how the results of the analysis will generalize to an independent dataset. Typically, analysts perform *k*-fold CV or leave-one-out CV (LOO-CV). In *k*-fold CV the dataset is randomly split into *k* mutually exclusive subsets of approximately equal size (251, 252). A model is trained using *k*-1 subsets (called traning sets), and is validated on the remaining part of the data (known as test sets). The process repeats *k* times. The overall accuracy of the model is calculated by averaging the performance measures over *k*-folds (252). A common value of *k* is 10 (253). In LOO-CV, at each iteration one observation or one variable is left out from the training set, and the trained model is tested on the one observation or variable that is left out. At the end, overall accuracy of the model is calculated as described above.

2.5.2.2 Dimensionality reduction, principal component analysis

In statistics, data dimension refers to the number of variables. High dimensional data means that the number of variables is high and can exceed the number of observations. There are two fundamental issues associated with multidimensional data: noise and redundancy. Big data can include large amounts of meaningless information known as noise. Noise may be introduced into a variable due to human error such as reporting a subject age as -40. Alternatively, a variable might be a noise. As an example, when evaluating half-life of a drug, weight and height should be removed from the data when body mass index is calculated. Noisy data may provide worthless results with poor accuracy. Redundancy means that the variables are highly correlated. Redundancy issues produce the same information from different points of view and increase the number of degrees of freedom. High dimensionality in a dataset can also make the data difficult to visualize and analyze.

To understand large data, dimensionality reduction methods should be applied (254). In multidimensional datasets there are sets of variables that are uncorrelated with each other, while the variables within each set are correlated with one another. A set of correlated variables can be combined and become a single new variable (255).

PCA, a well-known data reduction method, is a descriptive mathematical procedure introduced in 1901 by Pearson and later by Hotelling, that extracts important variables (in the form of a new set of variables called principal components [PCs]) from a large set of variables (256, 257). PCA extracts low dimensional sets of data with the aim of capturing as much information as possible.

This technique increases interpretability of the dataset while at the same time minimizing information loss. With fewer variables, analysis and visualization become more meaningful. To preserve as much variability as possible, data are transformed linearly. Thus, two assumptions of PCA are: 1) variables should be linearly related to each other, and 2) variables should be correlated to each other to some degree (258). The transformation steps are an iterative procedure to identify combinations of variables with maximum variance and minimum correlation. The eigenvalues and their corresponding eigenvectors are the means for linear transformation of the data. Eigenvectors are the axes (directions) along which a linear transformation performs stretching/compression

changes, and eigenvalues (denoted by λ) are the scalers by which the stretching/compression occurs (259).

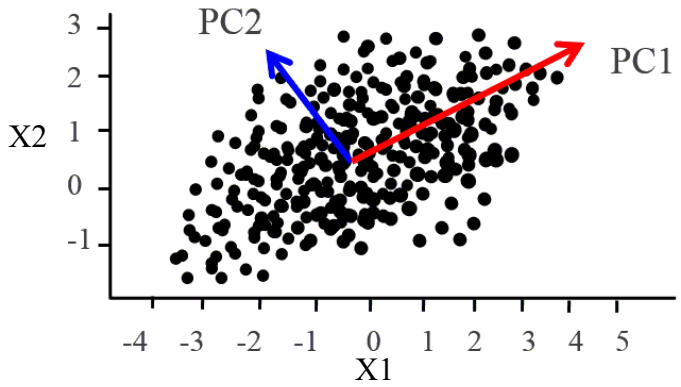

Figure 2.1 A schematic illustration of PCA analysis.
Multidimensional data reconstructed in 2 dimensions. Each vector represents an eigenvector. The PC1 (red arrow) has higher variance than the PC2 (blue arrow). The X1 and X2 axes are geometrical coordinates.

The original data is rotated to find the new axes (eigenvectors) with new coordinates that indicate directions of highest data variance. The axes or new variables are PCs and ordered by amount of their variance (maximum to minimum). The first component, PC1, represents the direction of the highest variance of the data; the second PC represents the highest remaining variance orthogonal to the first component and so forth. In PCA terminology, eigenvectors are termed loadings, and each loading represents one component. The eigenvectors corresponding to the largest eigenvalues are the PCs with the highest variance of the data (260).

For visualization, the first and second component can be plotted against each other to obtain a two-dimensional representation of the data that captures most of the variance (assumed to be most of the relevant information). That approach is useful to analyze and interpret the structure of a dataset (Figure 2.1). Consider a dataset having a number of HLA and gene associations in a group of adult and child patients with chronic inflammatory arthritis. Applying PCA reveals the cluster of HLA and genes of children and adult patients localized to the same PC suggesting an adult counterpart of JIA based on the genetic information related to the disease. As an example, consider that PC1 consists of HLA and genes from children who have developed the following subtypes of JIA: extended oligoarticular, polyarticular RF-positive, and psoriatic arthritis, together

with adults who suffer from polyarthriticular disease. PC2 retains genetic information of persistent oligoarticular, and polyarticular RF-negative JIA along with genetic information of adults with the same condition.

Therefore, PCA analyses create a new dataset of new variables while retaining the important information of the original set. Although PCA has been widely used in genetic studies, it has several limitations. Lack of probabilistic components in PCA confines the potential to extend the scope of application of PCA. As an example, PCA cannot be applied to nominal and ordinal data and does not work well when handling data that is too sparse. It has been shown that the PCs with the larger eigenvalues do not necessarily contain more information (261). The assumption of linearity of relationships among variables is another constraint. Finally, the inability of PCA to deal with missing data restricts its application (256). The shortcomings of PCA can be addressed by integrating a probabilistic approach, resulting in probabilistic PCA (PPCA) (262). In PPCA, the expectation maximization (EM) algorithm estimates the model parameters (for example, mean) through estimation of latent variables within the data, which can deal with missing data (263). The maximum likelihood of the latent variables is equivalent to the principal eigenvectors of conventional PCA (262). EM is described later in this chapter.

An important question is how many PCs should be retained in order to account for most of the variation in the dataset? As eigenvalues are ordered they can be truncated; that is, the first few principal components that account for a desired amount of variance in the original data can be selected. There is no definite method for selecting the number of PCs. One way is to represent the data in scree plot of eigenvalues (264). Figure 2.2 shows a scree plot with the number of eigenvalues ordered from biggest to smallest. The optimum number of components is the number that appears prior to the sharp change in the plot (the elbow) (264).

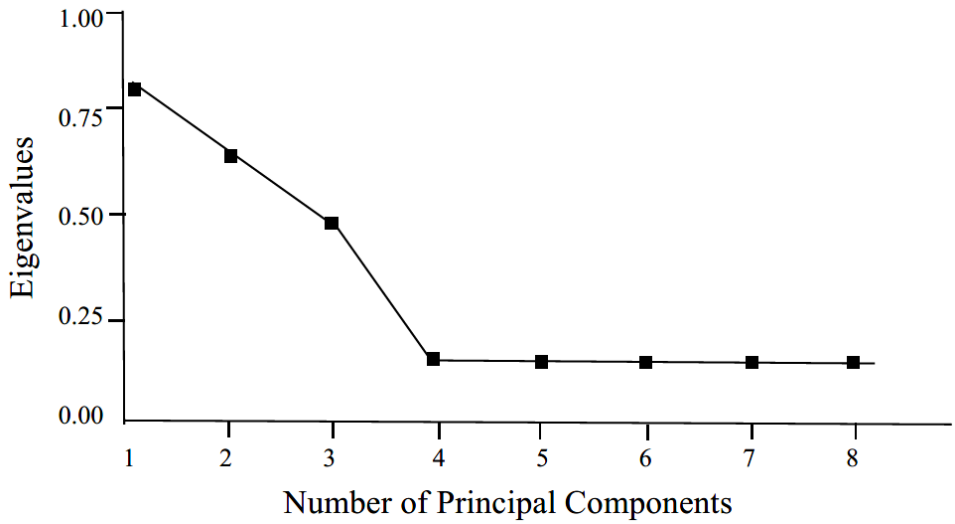

Figure 2.2 Scree plot.
A scree plot displays all eigenvalues in their decreasing order. The appropriate number of components prior to the elbow is 3.

Another method for selecting the optimal number of PCs is calculating a Q^2 index. Q^2 is an index that estimates the external prediction capability of a model and can be used for selecting the number of PCs. The basis of Q^2 is similar to computing the goodness of fit (coefficient of determination) or R^2 in regression models. Because of the mathematical properties of R^2, as the number of factors which can be variables (even noisy ones) increases, the R^2 value increases and therefore it cannot be a criterion for a model's predictive capability (it measures the strength of the least-squares fit to the training set). An R^2 value of 0.7 means that the model accounts for 70% of the variance for the training set. Q^2 is the R^2 value calculated from applying the model to the test set instead of the training set and it may or may not increase when more factors are added. It has a value between 0 and 1; the higher the Q^2, the closer the reconstructed data is to the original data (Figure 2.3) (265-267). It can be interpreted as the ratio of variance that can be predicted independently by the PCA/PPCA. In another words, low Q^2 indicates that the PCA/PPCA model only describes noise and that the model is not a true representative of data structure.

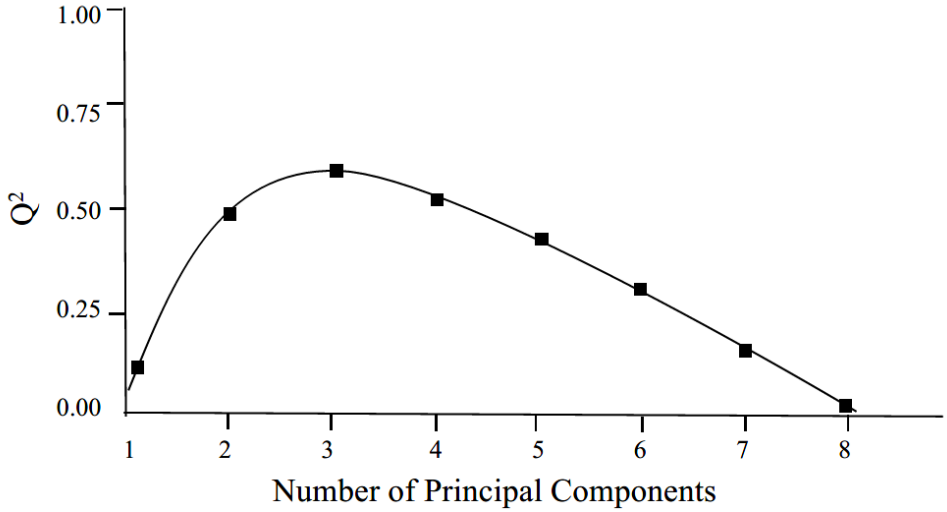

Figure 2.3 A Q^2 plot.
Q^2 plot, the goodness of prediction, (defines the average error of prediction) used for internal cross-validation which allows optimising the choice of number of PCA loadings. In this graph, the appropriate number of PCs is 3.

2.5.2.3 Feature selection

High-dimensional data increases the risk of over-fitting[3] and cannot provide statistically meaningful results due to irrelevant, redundant and noisy data (268). Feature (variable) selection (FS) techniques are essential steps of data pre-processing. FS algorithms attempt to project the original data, which has a large number of features and a small number of subjects, onto a smaller number of variables while preserving as much information as possible (269). FS accelerates learning processes, reduces storage space, facilitates data visualization and understanding, and decreases data dimensionality, which improves prediction accuracy (270).

Many FS techniques have been developed for machine learning and can be categorized into filter and wrapper methods (271). Filter methods are heuristic, fast, and utilize the general characteristics of the data. In comparison, wrappers use learning algorithms to evaluate the utility of feature subsets. Two filter-based approaches that have

[3] Overfitting is a modeling error that occurs when an algorithm fits all or the most of data for the training set, even noise, to generate the model. Thus, the model has high accuracy for a classifier when evaluated on the training set but low accuracy when evaluated on a new test set.

been used in this research are correlation-based feature selection (CFS) and ReliefF. Both are multivariate methods that consider relationships among the features (268).

CFS is based on the rationale "*a good feature subset is one that contains features highly correlated with the class variable (in prediction problems), yet uncorrelated with each other"* (272), and assesses the features' redundancies by use of a correlation that evaluates the predictive ability of subsets of features (273). The algorithm first standardizes variables and then computes correlation coefficients within various composites of variables, and between composites and class variables. The final result is a composite of variables with low inter-correlations, which in turn is highly correlated with the class variable (272).

ReliefF, a multivariate algorithm based on a statistical method instead of heuristic searches, is an instance (observation)-based learning algorithm. In a binomial class variable problem, the algorithm first draws an observation randomly from the training data, and then computes Euclidean distance[4] between the observation and the nearest observation of the same class (nearest hit) and a different class (nearest miss). After *m* iterations, the last step is to give high weight to the feature that discriminates between the nearest miss and the observation while it has the same value for the nearest hit. The only limitation of ReliefF is the inability to recognize redundant features (274).

2.5.3 Clustering

Clustering is an unsupervised pattern recognition method that distributes a set of observations into subsets, denoted as clusters. The goal of clustering algorithms is partitioning the data where a collection of observations within a cluster is similar within the cluster but dissimilar between clusters (275).

There are two distinct clustering methods, hard and soft (276). In hard clustering each observation belongs only to one group, and there is no overlap among clusters. In soft clustering, groups can overlap and a single individual can fall into more than one group with different degrees of belonging. In other words, a single observation could be in several groups at the same time with different probabilities. K-means and Gaussian Mixture Model (GMM) are examples of hard and soft clustering methods, respectively

[4] The Euclidean distance between two points measures the length of a straight segment connecting the two points.

(276). In soft clustering probability measures identify the observations which belong to a cluster. In this thesis, the GMM soft clustering algorithm was used.

2.5.3.1 Gaussian Mixture Model

GMM is a common clustering method; it is alternatively referred to as expectation-maximization (EM) clustering based on optimization strategy. The Gaussian (normal) distribution is bell-shaped. Mean and standard deviation are two characteristics of a probability distribution. The Gaussian distribution formula is termed the probability density function. Using the formula, for a given event X, the associated Y values can be computed, which are the probabilities for X values. GMM refers to multiple Gaussian distributions of multiple hidden bell-shaped curves (277). For a set of events from a distribution with unknown parameters (mean, SD), the probability that an individual event belongs to a specific Gaussian distribution can be computed. The solution to estimate the parameters of the hidden Gaussian distributions is given by the EM algorithm. EM is an iterative mathematical optimization that can compute the maximum likelihood of hidden parameters given observed data points. Each hidden Gaussian distribution represents a cluster in the data (278) (Figure 2.4).

GMM can determine the maximum likelihood estimates of all the distributions' means. If the means are known, then the probability of each data point belonging to one or the other Gaussian distributions can be determined. Thus, the clusters within the data are revealed.

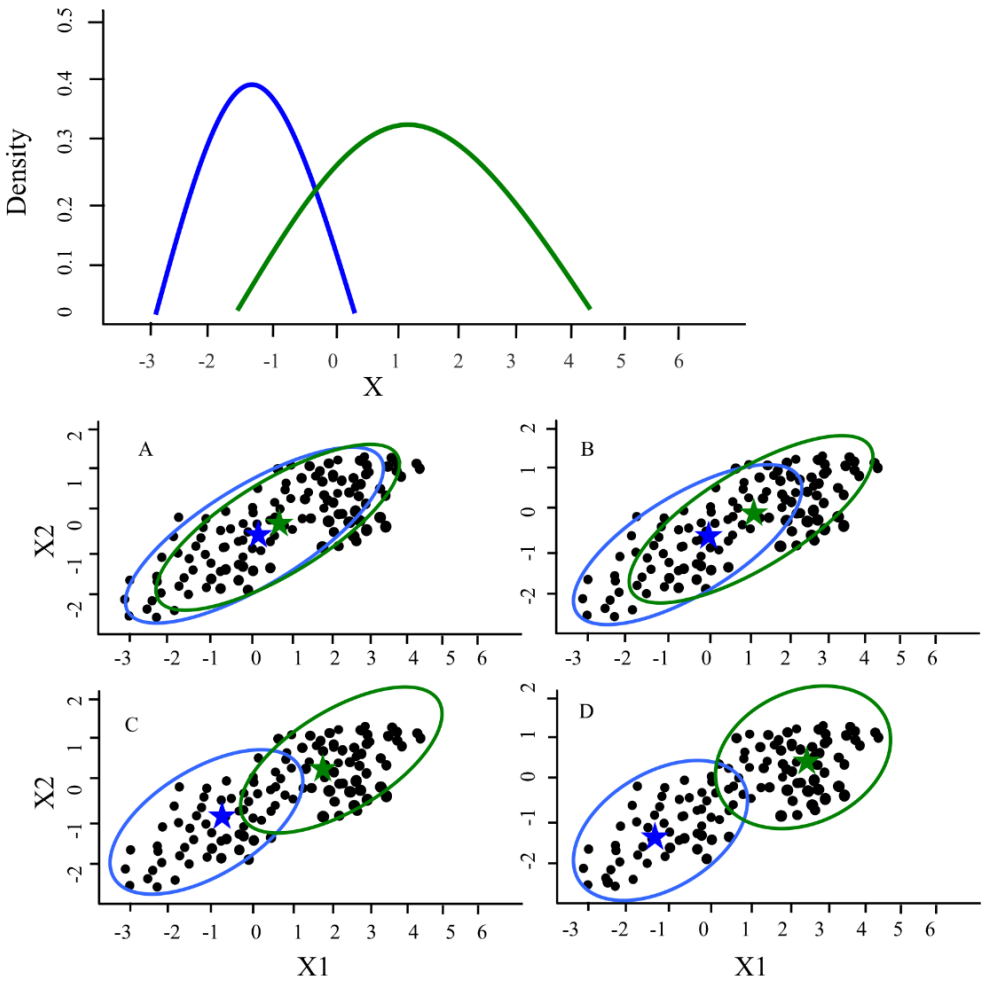

Figure 2.4 Gaussian mixture model.
Two distributions within a dataset are plotted using means and standard deviations (upper graph). GMM starts with initial guesses for means (A), determines the new estimate of means (B), and iterates (C) until convergence (D). Axes are gemetrical co-ordinates. (Figure inspired by Ihler: https://www.youtube.com/watch?v=qMTuMa86NzU)

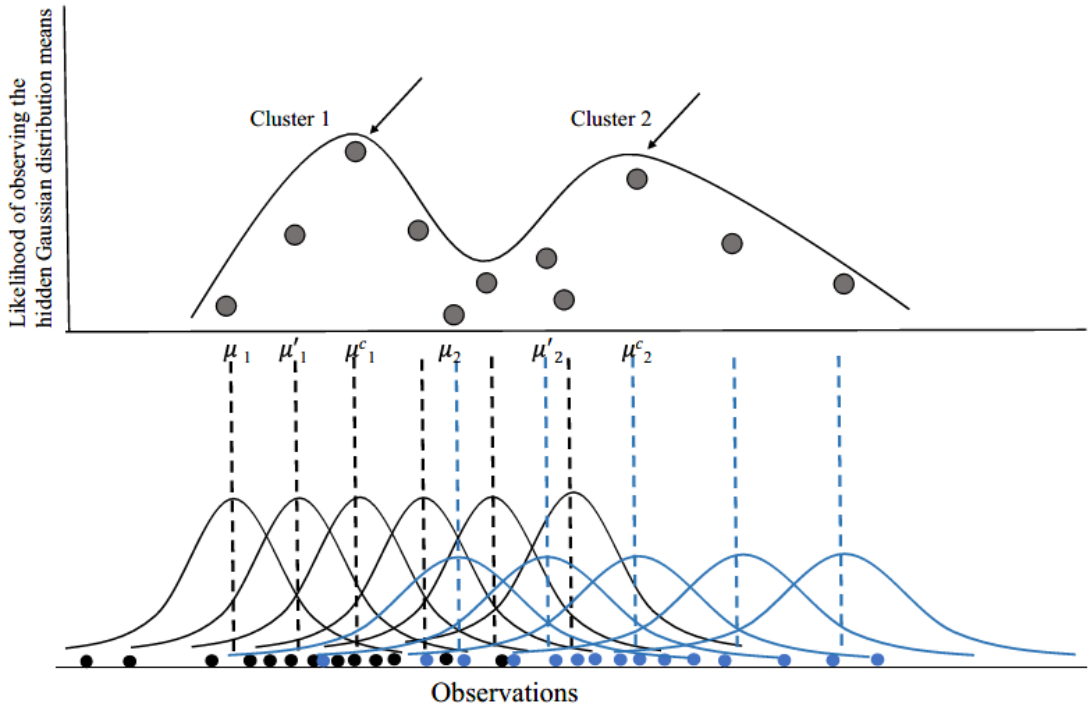

Figure 2.5 Schematic representation of EM algorithm.
Each black dot and blue dot represent an observation in the dataset. The EM algorithm starts with randomly selected parameters (μ_1, μ_2), and iterates up to points of convergence (μ^c_1, μ^c_2). (Figure inspired by Computer Science Department, University of North Carolina: https://www.youtube.com/watch?v=XepXtl9YKwc)

In the EM algorithm a set of random initial means are selected (Figure 2.5). For example in a two-dimensional dataset *h*, assume that the means and covariance of the hidden distributions are initially $\mu_1, \mu_2, {\sigma^2}_1, {\sigma^2}_2$. Then, the algorithm iterates with the E-step and the M-step (Equations 2.1 to 2.3).

E-step:

The expected value $E(z_{ij})$ of each latent variable z is calculated. That is, the probability that data point *i* comes from cluster *j* has either mean μ_1 *or* μ_2 using the following formula:

$$E(z_{ij}) = \frac{P(x = x_i|\mu = \mu_i, {\sigma^2}_i)}{\sum_{i=1}^{n} P(x = x_i|\mu = \mu_j, {\sigma^2}_j)} \quad (2.1)$$

M-step:

A new maximum likelihood hypothesis ($h' = \mu'_1, \mu'_2$) is calculated, using computed latent variable in E-step ($E(z_{ij})$) by the following formula:

$$\mu'_1 = \frac{\sum_{i=1}^{n} E(z_{ij}) x_i}{\sum_{i=1}^{n} E(z_{ij})} \quad (2.2)$$

$$\sigma^{2\prime}{}_1 = \frac{\sum_{i=1}^{n} E(z_{ij}) (x_{i-}\mu_j)^2}{\sum_{i=1}^{n} E(z_{ij})} \quad (2.3)$$

These steps iterate until the difference between two consecutive calculated means becomes small in absolute value, which is the point of convergence. The algorithm stops and the last two means are the estimated maximum likelihood of parameters (means) by which the hidden clusters can be revealed (279, 280). EM is the main element of both PPCA and clustering with GMM, which are used in the first study in this thesis (Chapter 3).

To determine the number of hidden Gaussian distributions required, the Bayesian Information Criterion (BIC) can be applied. BIC is an integrated log likelihood and includes a penalty for including too many parameters in the model (Equation 2.4). The aim of BIC is to quantify the support for one model over others using odds ratios of posteriors of the models that have equal priors[5]. It compares models with differing parameterizations and/or differing numbers of clusters. The larger the value of the BIC is, the stronger the evidence for the model and number of clusters (281, 282).

$$BIC=-2 * log\ likelihood\ (L) + p * log\ (N) \quad (2.4)$$

where log likelihood (L) is the maximized log-likelihood of the data given a particular model, p is the total number of parameters, and N is sample size.

2.5.4 Classification

Classification is a supervised machine learning technique that takes data as input and places it into correct categories based on its features (275). For example,

[5] Prior probability is the probability of an outcome or an event based on the current knowledge before an experiment is performed. The prior probability will be revised as new data or information become available to produce a more accurate measure of the outcome or the event. That revised probability is known as posterior probability.

classification could allow the prediction of whether a JIA patient with either blood marker A or B will develop persistent or extended oligoarthritis subtypes over time. In this example, the blood markers A and B are variables/features and oligoarthritis extended or persistent are categories (class/response variable).

The model first must be trained in order to make precise decisions based on the relationship between features (predictor variables) and known class labels (response variables). The aim of classification is to predict a response variable based on a set of observed predictor variables. It is called supervised machine learning because of the training procedure with the known labeled class. There are unsupervised classification algorithms in which the response variable is not predetermined. Supervised machine learning includes techniques that provide either classification (when the response variable is categorical) or regression (when the response variable is continuous) (283).

2.5.4.1 Classifier

The classification algorithm is known as a classifier. One of the most popular classifiers is decision tree (DT) which can be used for both classification and regression purposes. It recursively divides the observations to generate a model that predicts the value or class of a variable by learning simple decision rules inferred from input variables. A DT consists of internal nodes from which the tree splits into branches, and end branches that do not split further (terminal nodes or leaves). The first node is called the root node (284). The aim of this classifier is to make a tree with low generalization error. The generalization error is the probability of misclassified observations when using a trained model in a new set of data. Although it is not possible to calculate the generalization error for a future dataset, we can estimate it by calculating the testing set error rate of the data to find a desired confidence interval for the generalization error (285).

There are several measures of node impurity, which represents how well the trees split the data. The two most common measures of impurity are the Gini impurity criterion (Gini index) and an entropy measure. Both are measures of uncertainty or misclassification. In other words, they measure how often a randomly chosen observation from the set would be misclassified. Gini impurity and entropy are defined as:

$$Gini\ impurity = 1 - \sum_{k=1}^{m} p_{2k}^2 \tag{2.5}$$

$$entropy = \sum_{k=1}^{m} p_k\, log_2(p_k) \tag{2.6}$$

where there are *m* classes of the response variable indexed by *k=1, 2, 3,…, m,* and p_k is the proportion of observations in the *m*th region that are from the *k*th class. The measures range between 0 and 1. A small value indicates that a node contains observations from a single class. In both the Gini index and entropy measure 0 indicates the preferred lower error rate. The Gini impurity value is small if all of the p_k are close to zero or one, which means a node contains predominantly observations from a single class (pure node). In the two-class case, the two measures are maximized at p_k=0.5 (when observations are equally belong to each class) (286). The value of Gini impurity is always between 0 and 1 regardless of the number of classes; while the value of entropy is larger than 1 if the number of classes is more than 2.

Entropy and Gini impurity can be used to evaluate the quality of a particular split. The measure is calculated before and after the split. The impurity value before the split is subtracted from those after the split, which are weighted by the proportion of observations falling into the classes at each split. The impurity value should be smaller after the split than the value before the split. The best and the next split is chosen by comparing the reduction in the measures across all possible splits (287). The process recursively partitions the remaining training observations until each leaf contains observations from one class (288). These two measures are incorporated into the classification algorithm that is used in this project (the task of growing a DT). Figure 2.6 is an example of a DT.

A DT produces a model that is easy to understand and can achieve high accuracy, but inclines to overfitting (289, 290). Pruning is a method that can handle overfitting. After growing the tree to the full depth, the branches that decrease the generalizability of the model for future data are removed in succession. The dataset is randomly split into a training set and a validation set. The tree is grown on the training portion and then each node of the tree is removed successively. The new classifier (pruned tree) is applied to

the validation set and its accuracy is evaluated. Based on the accuracy measures of the classifier, the algorithm either removes or keeps the node. The process iterates until the point that pruning does not make any further improvement in the classifier accuracy measures (291).

Another drawback of DT is that a small variation in the training set results in a completely different tree. Ensemble methods can handle such tree instability issues. The methods involve combining several models to improve the accuracy and the individual predictions are combined through averaging or voting (289).

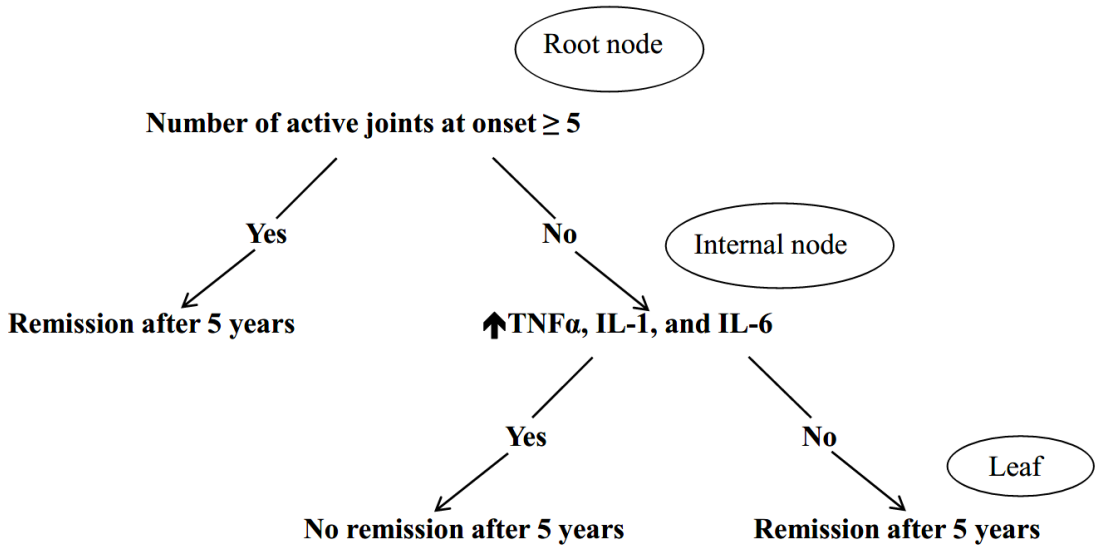

Figure 2.6 A decision tree.

2.5.4.2 Random forest

Ensemble learning6 is an approach that trains and combines multiple learners to solve the same problem. Ordinary machine learning methods learn one hypothesis from training data, while ensemble methods construct a set of hypotheses and combine them. Thus, ensemble methods construct a predictive model by integrating multiple models (292).

Random forest, an ensemble learning technique that overcomes the instability problems of DT, was first proposed by Breiman (293). The algorithm generates many DTs using a

[6] A computer program that uses the data to build a DT is called the learner and the DT is called the classifier.

bootstrap sample of the data (randomly selected subset of observations with replacement). For each bootstrap sample, an unpruned classification or regression tree is grown (273, 293). At each node, instead of using the best split among the features, a subset of variables is selected randomly.

The class is assigned by aggregating the predictions of the trees (majority vote in classification or averaging in regression problems) (293, 294). Random forest can be applied in problems involving more than two classes, can handle noisy variables, and can be used when the number of variables is larger than the number of observations. The unpruned trees result in low bias, and random variable selection results in low correlation among the individual trees (295).

2.5.4.3 Performance evaluation

Evaluation of classifiers involves comparing the classification results against ground truth or another set of results. In this comparison there are four types of metrics, two types of agreement and two types of disagreement. The two forms of agreement are called a true positive (TP) and a true negative (TN). A TP result is a state that appears in actual and predicted sets (observation that is actually positive and predicted positive). A TN result is a state that appears in neither set (observation that is actually negative and predicted negative). The two forms of disagreement are false positive (FP) and false negative (FN). A FP result is a situation that detects the condition when it is absent. FN, opposite of TP,
does not detect the condition when the condition is present. The FP concept is related to type I error and FN is related to type II error used in hypothesis testing. All four types of results can be shown in a confusion matrix (Table 2.3).

Table 2.3 The confusion matrix for comparing two sets of conditions.

		Actual results/classification	
		Yes	No
Predicted results/classification	Yes	TP	FP
	No	FN	TN

Classifier performance evaluations consist of:

$$Sensitivity\ (recall) = \frac{TP}{TP+FN} \tag{2.7}$$

Sensitivity measures the proportion of positive observations that are correctly classified.

$$Specificity = \frac{TN}{TN+FP} \tag{2.8}$$

Specificity measures the proportion of negative observations that are correctly classified.

$$Precision\ (PPV) = \frac{TP}{TP+FP} \tag{2.9}$$

Precision or positive predictive value (PPV) is the proportion of observations identified as positive and are truly positive.

$$Negative\ predictive\ value\ (NPV) = \frac{TN}{TN+FN} \tag{2.10}$$

Negative predictive value (NPV) is the proportion of observations that identify as negative and are truly negative.

$$Accuracy = \frac{TP+TN}{TP+TN+FP+FN} \tag{2.11}$$

Accuracy represents the proportion of observations classified correctly.

$$F\text{-}measure = 2 \times \frac{precision \times recall}{precision + recall} \quad (2.12)$$

F-measure is harmonic mean of the precision (PPV) and recall. It is a measure to see the balance between precision and recall when there is an uneven class distribution (296).

There is a trade-off between FN and FP outcomes. Being more stringent typically results in fewer FPs and more FNs. The opposite is true when one is less stringent. The classifier accuracy measures capture this trade-off, which can be characterized by the receiver operating characteristic (ROC) curve (Figure 2.7). ROC graph is a two-dimensional graph plotting the sensitivity on the y-axis versus (1 – specificity) on the x-axis. The graph shows the cut-off points between benefits (TP) and costs (FP) and the ability of a binary classifier to rank the positive observations against the negative observations. The diagonal line on the graph represents guessing a class randomly. The point (0.5, 0.5) in ROC space shows when a classifier randomly predicts the positive class half of the time. It can be expected to get 50% of the positives and 50% of the negatives correct (i.e., no better than random guessing; see Figure 2.7, line C). Any classifier that appears in the upper left triangle performs better than random guessing and any classifier situated in the lower right triangle performs worse than random guessing. Between two models (for example, A and B in Figure 2.7) that test the same hypothesis, the model with the higher area under its ROC curve is considered the better one (Figure 2.7 comparing the ROC curves of Model A and Model B). A perfect classifier would have an AUC of 1 while random guessing would result in an AUC of 0.5 (297, 298).

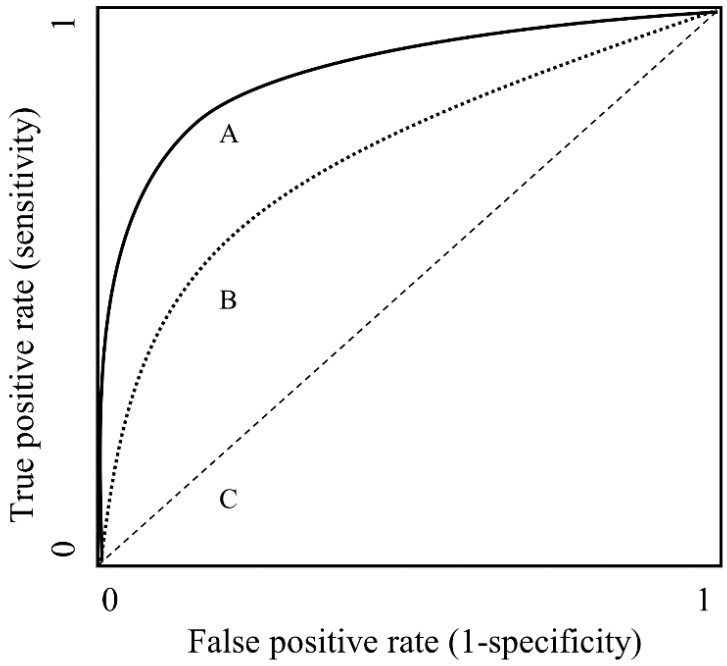

Figure 2.7 ROC curve.
An example of two ROC curves (A and B), and the performance level that could be expected from random guessing (C). In this case, model A is more accurate than B.

In summary, data reduction methods help to transform a large dataset into a small set that is more understandable numerically and visually. Before applying clustering and classification algorithms, the data used in this research was reduced into new sets of variables in the first study (JIA clustering), and for the second study (predicting outcomes), variable selection methods were used. For study 1, clusters should be identified as much as possible by patients' information but in a consice form. For study 2, the idea was to identify a limited number of reliable predictors of disese activity, especialy if they could predict outcomes of all JIA categories. For that reason, feature selection methods were applied in the second study. In study 1, data-driven unsupervised machine learning clustering algorithms were used to reveal hidden patterns that enable categorization of disease based on clinical and biological attributes. Supervised machine learning algorithms help overcome limitations of conventional statistical models and find reliable predictors in a relatively small panel of clinical measures and inflammation-related biomarkers. Because class variables were binomial, classification was used for study 2.

CHAPTER 3

STUDY 1. CLINICAL AND BIOLOGICAL FEATURES FOR CLUSTERING WITHIN A COHORT OF CHILDREN WITH CHRONIC ARTHRITIS

3.1 Abstract

Objective: To identify discrete clusters comprising clinical features and inflammatory biomarkers in children with JIA and to determine cluster alignments with JIA categories.

Methods: A Canadian prospective inception cohort comprising 150 children with JIA was evaluated at baseline (visit 1) and after six months (visit 2). Data included clinical manifestations and inflammation-related biomarkers. PPCA identified sets of composite variables or PCs, from 191 original variables. To discern New Clinical-Biomarker Clusters (Clusters), GMM were fit to the data. Newly-defined Clusters and JIA categories were compared. Agreement between the two was assessed using Kruskal-Wallis tests and heat map plots.

Results: Three PCs recovered 35% (three Clusters) and 40% (five Clusters) of the variance in patient profiles in visits 1 and 2, respectively. None of the Clusters aligned precisely with any of the seven JIA categories, but rather spanned multiple categories. Results demonstrated that the newly defined Clinical-Biomarker Clusters are more homogeneous than JIA categories.

Conclusion: Applying unsupervised data mining to clinical and inflammatory biomarker data discerns discrete Clusters that intersect multiple JIA categories. Results suggest that certain groups of patients within different JIA categories are more aligned pathobiologically than their separate clinical categorizations suggest. Applying machine learning analyses to complex datasets can generate insights into JIA pathogenesis and should contribute to biologically-based refinements in JIA classification.

3.2 Background

JIA is a heterogeneous group of diseases categorized predominantly according to clinical manifestations by ILAR (299, 300).

Only two biomarkers, RF and HLA-B27, are considered when classifying JIA (2). Ravelli *et al.* showed that ANA-positive patients belonging to oligoarticular or polyarticular JIA categories share the same characteristics, suggesting that they represent the same disease (301, 302). However, considering a broader panel of clinical and biologic features was shown to generate childhood arthritis subsets distinguishable from conventional JIA categories (39).

By applying unsupervised data mining algorithms, this present study aimed to identify discrete clusters of patients comprising clinical and inflammatory biomarker attributes and ascertain the extent to which these patient clusters align with currently defined JIA categories. This study extends earlier observations as it assesses a broader array of inflammatory biomarkers and determines changes in cluster composition over time. Applying machine learning analytical frameworks to large clinical and biologic datasets can contribute new insights into JIA pathogenesis and should help inform a future biologically-based JIA classification.

3.3 Methods and data collection

Data were from a Canadian prospective longitudinal inception cohort, The BBOP Study, comprising children with new-onset JIA enrolled consecutively within six weeks of first presentation at a participating pediatric rheumatology centre (N=11 participating sites). Ethics review boards from all sites approved the study (Appendix, supplementary text 1and 2) and informed consent/assent was obtained. The recruitment strategy aimed for a reasonable number of participants in each of the seven JIA categories rather than aspiring to achieve a typical JIA subgroup distribution. To achieve this aim, only participants with polyarthritis or systemic JIA were eligible during the first six months of the enrollment period; after six months and until the end of the two-year enrollment period participants with any JIA category were eligible. Demographic, clinical, functional ability (CHAQ) (239), quality of life (JAQQ) (31) and laboratory data were collected at enrolment and six months later (visits 1 and 2).

Blood was collected in P100 tubes (BD Biosciences) (39, 303) at both visits and plasma stored at -80°C. Biomarkers were assayed by bead-based multiplex or individual

enzyme immunoassays as detailed in Appendix A, supplementary text 2. The biomarker panel was selected to broadly represent Th1 and Th2 pro- and anti-inflammatory cytokines, growth factors, chemokines, and extra-cellular matrix and bone degradation markers. High mobility box 1 protein (HMGB1) and soluble low-density lipoprotein receptor-related protein 1 (sLRP1) were tested as upstream mediators of inflammatory pathways (304-307). Test results for ANA by indirect immunofluorescence assays, RF, and HLA-B27 were from clinical laboratory testing facilities affiliated with each study site and results dichotomized as positive or negative. The biomarkers assayed are listed in Table 3.1.

Table 3.1 Plasma biomarkers measured at enrolment and 6 months after.

Matrix Metalloproteinases	MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, TIMP-1, TIMP-2, TIMP-3, TIMP-4
Interleukins	IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-2p40, IL-2p70, IL-13, IL-15, IL-17, IL-1Rα
Chemokines	OPG, RANKL, RANTES, Exotoxin, IP-10, MCP-1, MIP-1α, MIP-1β
Growth factors	G-CSF, GM-SF, VEGF, EGF, FGF-2
Interferons	IFNα, IFNγ
Tumour necrotizing	TNF-α, TNF-β
Others	Vitamin D, HMBG1, sLRP1

MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinase; IL, interleukins; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; RANTES, regulated on activation normal T cell expressed and secreted; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein; MIP, macrophage inflammatory proteins; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; FGF-2, Fibroblast growth factor; INF, interferons; TNF, tumor necrosis factor; HMGB1, high mobility group box; sLRP-1, soluble low-density lipoprotein receptor-related protein.

3.4 Data analysis

Software and data pre-processing: Statistical analyses were performed using SPSS Statistics Professional, version 23, and R, version 3.2.2. Circos, version 0.63, was

used for generating contingency wheels to depict relationships between New Clinical-Biological Clusters (Clusters) and JIA categories (308). Extreme values were removed using outlier-labeling (309). Data had 20% missing values were imputed using multiple imputation regression (310). Protein concentrations and continuous variables were log- and Z -score- transformed.

Variable selection and dimensionality reduction: PPCA reduced the dimensionality of the dataset from 191 variables, consisting of clinical and biomarker measurements, to a reduced number of PCs (255, 262, 280, 311).

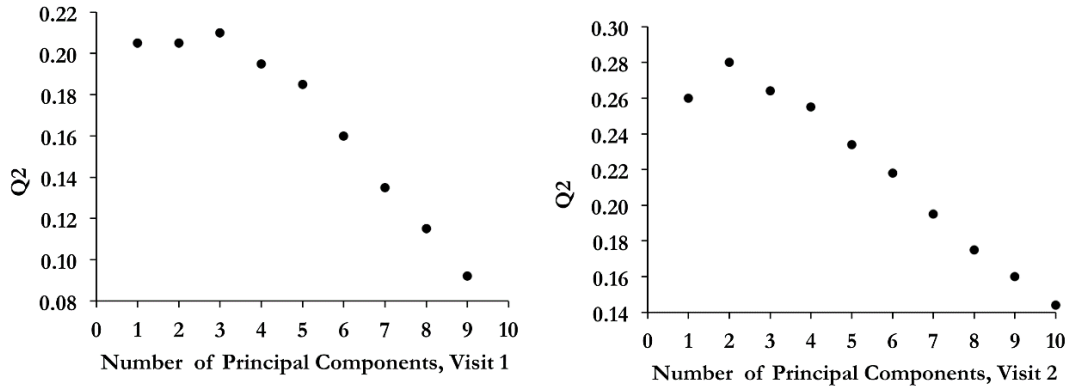

Figure 3.1 Q^2 plots for both visits.
They identify the number of principal components in visit 1 (left) and visit 2 (right). Q^2 is the goodness measure of prediction (defining the average error of prediction) used for internal cross-validation, which allows the identification of the optimal number of PCA loadings.

The number of PCs retained from PPCA was selected by maximizing the Q^2 metric, which reflects how well the original dataset could be reconstructed from the retained PCs. Q^2 was calculated using 10-fold cross-validation and plotted against the number of PCs (Figure 3.1) (312, 313).

To facilitate interpretation and increase the stability of each retained PC, we performed leave-one-variable-out cross-validation with PPCA and if at least 5% of the runs resulted in a contribution (variable contribution -in percentage- involves squaring the variables' loading) less than 2% then the variable was eliminated. In the end, 37 and 38 variables were retained in three PCs in visit 1 and three PCs in visit 2, respectively.

Clustering: To identify Clusters of patients using the PCs, GMMs were applied as provided by the R package mclust (v5.0). Mclust software fits various GMMs (280),

which assume data arise from multiple Gaussian distributions, and uses BIC to select the model with the optimal number of Gaussian kernels and shape constraints (314).

Stability: Homogeneity of Clusters was compared to JIA categories using Kruskal-Wallis test p-values (ranging from 10^{-9} to 10^{-1}) (315). The proportion of variables with p-values less than each threshold was graphed separately for Clusters, JIA categories, clinical variables, and biological variables.

For sensitivity analysis, LOO-CV was performed to assess robustness of Clusters to choices of both variables and patients (252, 316). In the first LOO-CV analysis, each variable was removed sequentially, and the entire analysis was repeated to produce Clusters comprising all but the excluded variable. The second analysis (sensitivity toward patients) was conducted in a similar manner, except that instead of removing variables, 10% randomly selected patients were removed at a time.

3.5 Results

The distributions of JIA categories and demographic characteristics of the 150 patients are shown in Table 3. 2.

Table 3.2 JIA category and demographic characteristics represented in the study cohort.

JIA Category (ILAR Criteria)	N (%)	Female: Male	Age at onset Median (IQR)
Oligoarthritis	42 (28)	27:15	6.0 (2.0-12.0)
Polyarthritis RF+	13 (8.5)	12:1	13.0 (6.0-14.5)
Polyarthritis RF-	50 (33.3)	39:11	11.0 (6.0-14.0)
Psoriatic arthritis	11 (7.3)	7:4	14.0 (9.0-14.0)
ERA	11 (7.3)	7:4	11.0 (5.0-15.0)
Systemic arthritis	16 (10.7)	10:6	6.5 (3.2-13.5)
Undifferentiated arthritis	7 (4.7)	5:2	13.0 (9.0-14.0)
Total	150 (100)	106:44	10.0 (4.7-14.0)

IQR, interquartile range; ERA, enthesitis related arthritis

Three indicators produced by principal components: For visit 1, 3 PCs (PC-1a, PC-1b, and PC-1c) effectively represented the original dataset as reflected by the Q^2 score; for visit 2, the Q^2 score was highest when 2 PCs were chosen, but to be consistent with the first visit, three PCs (PC-2a, PC-2b, and PC-2c) were also retained for visit 2. The three PCs recovered 35% and 40% of the variance from patient profiles in visit 1 and 2, respectively.

As depicted in Figure 3.2, in visit 1, PC-1a comprised only inflammation-related cytokines and growth factors. PC-1b was defined by the number of active and effused joints, PGA, CHAQ scores, levels of CRP, RF positivity, MMPs, TIMPs, and MIP-1α. PC-1c comprised fever, onset age, systemic onset JIA rash, erythrocyte sedimentation rate (ESR), CRP, hemoglobin level, white blood cell (WBC) count, and levels of TIMP-1, epidermal growth factor (EGF), and vitamin D.

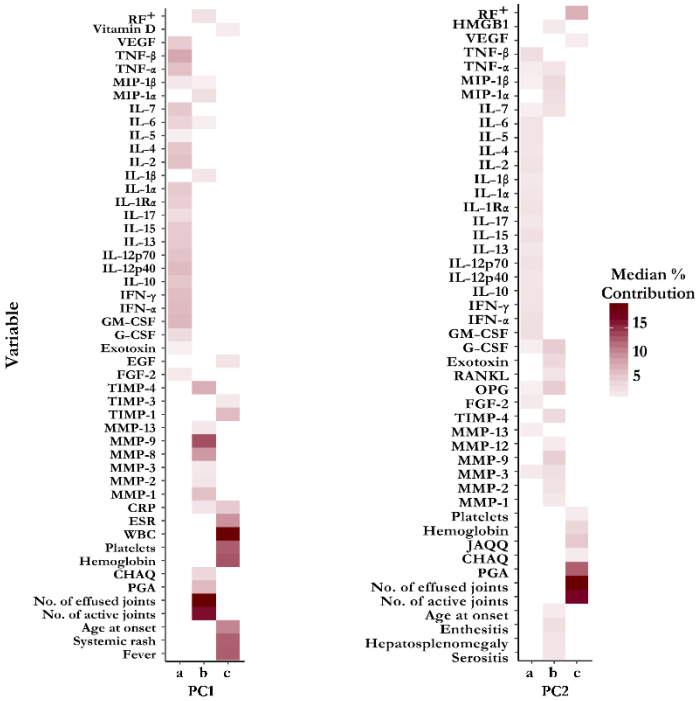

Figure 3.2 Variables contribution.
Normalized principal component (PC) loadings of variables. Variables and the strength of their respective contributions to each of the PCs at visits 1 (left) and 2 (right) are shown. The darker the color, the stronger the contribution that variable makes to the PC.

As shown in Figure 3.2, in visit 2, PC-2a comprised inflammation-related cytokines, growth factors, and MMPs.

PC-2b was defined by clinical manifestations including onset age, serositis, hepatosplenomegaly and enthesitis, levels of MMPs and TIMP-4, inflammatory cytokines, and bone degradation biomarkers. PC-2c comprised the number of active and effused joints, PGA and JAQQ scores, RF positivity, and laboratory measures of disease activity including hemoglobin, platelet count, ESR, CRP, and vascular endothelial growth factor (VEGF).

Three and five Clusters of patients recovered by GMMs: On the basis of the BIC (visit 1 model EVI with BIC=-1710.7 and visit 2 model EEE with BIC=-1698.3), three

and five patient clusters were retained in visit 1 and 2 respectively (Figure 3.3) and designated as V1.1 to V1.3 and V2.1 to V2.5.

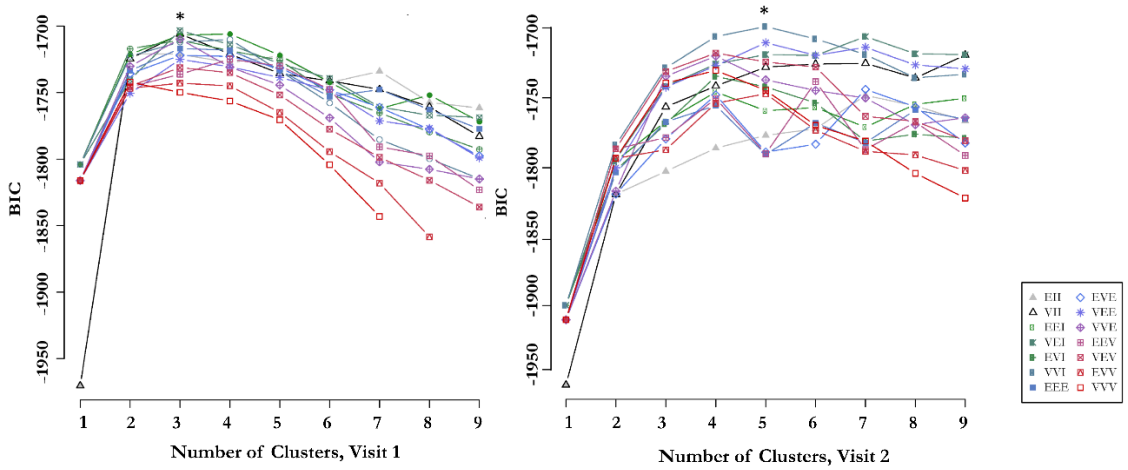

Figure 3.3 The mclust options to select Cluster number and shape.
Bayesian information criterion (BIC) identified three Clusters in visit 1 and five Clusters in visit 2; in visit 1 the model VEI and in visit 2 the model VVI had the highest BICs respectively. Constraints imposed on Clusters by different criteria: EII, spherical, equal volume; VII, spherical, unequal volume; EEI, diagonal, equal volume and shape; VEI, diagonal, varying volume, equal shape; EVI, diagonal, equal volume, varying shape; VVI, diagonal, varying volume and shape; EEE, ellipsoidal, equal volume, shape, and orientation; EVE, ellipsoidal, equal volume and orientation; VEE, ellipsoidal, equal shape and orientation; VVE, ellipsoidal, equal orientation; EEV, ellipsoidal, equal volume and equal shape; VEV, ellipsoidal, equal shape; EVV, ellipsoidal, equal volume; VVV, ellipsoidal, varying volume, shape and orientation; *, indicator of the chosen model.

Visit 1 (Table 3.3): In visit 1, V1.1, the largest Cluster (87 patients) comprised 55 females (63.2%), older children (mean first presentation age, 10.0±4.5 years) and was characterized by having the fewest number of active joints compared to other V1 Clusters (four on average with knees most frequently involved); intermediate levels of laboratory indicators of disease activity (LIDA), including WBC count, platelet count, ESR, and CRP; and low levels of inflammatory cytokines. ANA and HLA-B27 positivity were both most frequent in V1.1.

V1.2 was an intermediate-sized Cluster (45 patients) comprising 33 (73.3%) females and with patients having an average age of 9.6 $\pm$ 5.2 years. This group had the highest number of active joints (14) with wrists predominantly involved, the highest LIDA, and intermediate levels of inflammatory cytokines.

The smallest Cluster in visit 1, V1.3, comprised 18 patients, all female, with a mean age of 7.2 $\pm$ 4.6 years. Patients in this group had an average of six active joints,

predominantly knees, and intermediate levels of LIDAs. This group had the highest levels of inflammatory related cytokines, chemokines, and growth factors and the highest levels of sLRP1, HMGB1 and vitamin D. Appendix A, Table A.1 shows biomarker ranks for each cluster at visit 1.

Visit 2 (Table 3.3): In visit 2, V2.1 was the largest of the five Clusters (45 patients) and included 35 (77.8%) females; patients had a mean age of 11 $\pm$ 4.5 years, and an average of five joints involved, predominantly ankles. This group was characterized by intermediate LIDAs and low levels of plasma cytokines except for OPG.

V2.2 comprised 27 patients of whom 17 (63.0%) were female. Mean age was 10 $\pm$ 4.7 years. An average of two joints were involved, predominantly knees. The group was characterized by intermediate levels of inflammatory cytokines/chemokines with predominance of IL IL-1β, IL-8, IL-6, MCP-1, and MIP-1α.

V2.3 with 13 patients comprised 10 (76.9%) females and had an average age 7.0$\pm$ 5.2 years, an average joint count of six active joints (predominantly ankles), and high LIDAs. Patients had high levels of sLRP1 and HMBG1 and the highest proportion of RF positivity.

V2.4, with 29 patients, included 24 (82.8%) females. The group had an average age of 9$\pm$4.8 years and on average two active joints (predominantly wrist). This group had the highest LIDAs and inflammatory related cytokine levels, and high sLRP1 and HMBG1.

Table 3.3 Cluster characteristics in visits 1 and 2.
ANA, RF, and HLA-B27 are not included in the table due to amount of missing data in visit 2.

	Visit 1			Visit 2				
Cluster	V1.1	V1.2	V1.3	V2.1	V2.2	V2.3	V2.4	V2.5
Number (#)	87	45	18	45	27	13	29	36
Median age yrs. (IQR)	11.0(6.0 -14.0)	12.0(4.0 -15.0)	6.0(2.7 -11.5)	12.4(6.7 -15.6)	12.1(6.0 -14.6)	6.8(2.3 12.0)	9.2(4.0 -13.4)	11.1(6.8 -13.6)
Female:Male	55:32	33:12	18:0	35:10	17:10	10:3	24:5	31:5
Average # of affected joints	4	14	6	5	2	7	2	1
Predominant joint(s) affected	Knees	Wrists	Knees	Ankles	Knees	Ankles	Wrists	Knee
Inflammatory cytokines ranks	Low	Intermediate	High	Low	Intermediate	High	Highest	Low
NSAID treatment (# of patients)	66	39	15	36	23	9	26	28
DMARD treatment (# of patients)	22	18	1	25	10	6	4	20
Biologic treatment (# of patients)	0	0	0	2	2	0	0	5
Intra-articular steroid treatment (# of patients)	9	2	4	2	4	3	3	2

NSAID, non-steroidal anti-inflammatory; DMARD, disease modifying anti-rheumatic drug; Biologic, biologically-based therapies; IQR, interquartile range.

V2.5, with 36 patients, was the second-largest Cluster in visit 2. The group included 31 (86.1%) females, had an average age of 10 $\pm$ 4.1 years, the lowest number of active joints (one on average) with a high rate of knee involvement. The group had low inflammation-related cytokine levels and had the highest frequency of ANA-positivity. Appendix A, Table A.2 lists biomarker ranks for each Cluster at visit 2.

Clinically meaningful patterns: Clusters were compared to JIA categories using, chi-square test, Circos (Figure 3.4), and the Kruskal-Wallis test (Figure 3.6). The analyses demonstrated that the Clusters did not align consistently with JIA categories (chi-square $p<0.001$) and that PC scores of patients in each Cluster were more homogenous than in each JIA category, especially for the first PC, which represents inflammatory biomarkers.

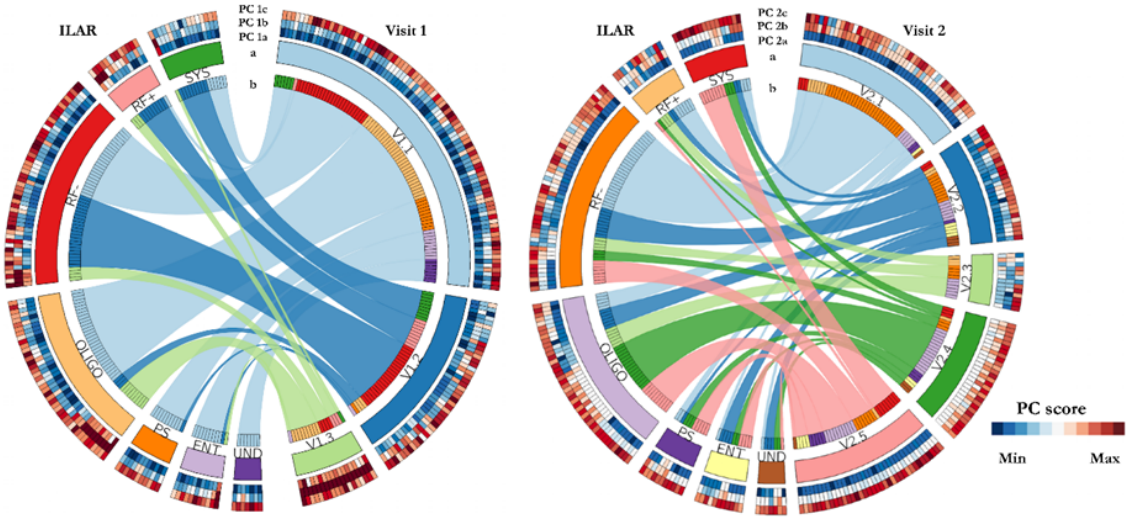

Figure 3.4 Contingency wheel plots comparing JIA and Clusters.
The contingency wheel plots depict the relationships between Clusters (right semicircle) and JIA categories (left semicircle) at visit 1 (left circle) and visit 2 (right circle). Individual patient scores for each of the three principal components (PCs) are depicted as stacks of three rectangles in the three outermost layers of the wheel (labeled as PC 1/2 a, b, c). The color scale in each of the three rectangles comprising the stacks from each individual patient indicates the magnitude of patient scores for each PC in accord with the gradient color scale legend. Each Cluster and each JIA category, shown in layer a, is distinguished by a different color. The right-hand side of the innermost layer (b) of each Circos figure illustrates the alignment of each individual patient (represented by the patient's JIA category color) within the respective Clusters. Similarly, the left side of each Circos figure illustrates the alignment of each individual patient (represented by the Cluster color) within the respective ILAR-defined JIA categories. Colored ribbons link clusters and JIA subtypes. Numbers of patients are proportional to the width of the ribbons; thus, thicker ribbons depict that more patients are shared between newly defined Clusters and JIA category.

In visit 1 (Table 3.3), patients in four of the seven JIA were predominantly assigned to V1.1; specifically, all seven of those in the undifferentiated category (100%), 10 of 11 (90.9%) in the psoriatic arthritis group, 9 of 11 (81.8%) in the ERA group and 30 of 42 (71.4%) in the oligoarticular group aligned with V1.1. The majority of patients with oligoarthritis (30 of 42 patients; 71.4%) and nearly half of those with RF-negative polyarthritis (24 of 50 patients; 48%) were assigned to the V1.1. Five of the seven JIA categories had some patients with strong associations with variables in PC-1a and PC-1b, PCs comprising predominantly inflammatory cytokines and MMPs/TIMPS; in contrast, all patients with strong PC-1a and PC-1b associations clustered in one group (V1.3) in the new clustering scheme.

Table 3.4 Percentages of patients in each JIA category and their distribution into Clusters in visit 1 and 2.

Disease Subtype (ILAR Criteria)	Visit 1 Clusters				Visit 2 Clusters					
	V1.1	V1.2	V1.3	Total	V2.1	V2.2	V2.3	V2.4	V2.5	Total
Systemic arthritis	4%	6%	1%	11%	2%	1%	0%	2%	5%	11%
Polyarthritis RF+	1%	6%	2%	9%	4%	1%	2%	1%	1%	9%
Polyarthritis RF-	16%	15%	3%	33%	18%	6%	3%	2%	5%	33%
Oligoarthritis	20%	2%	6%	28%	3%	4%	4%	10%	7%	28%
Psoriatic arthritis	7%	1%	0%	7%	1%	1%	0%	2%	3%	7%
Enthesitis related arthritis	6%	1%	1%	7%	1%	3%	0%	1%	3%	7%
Undifferentiated arthritis	5%	0%	0%	5%	1%	2%	0%	1%	1%	5%
Total	58%	30%	12%	100%	30%	18%	9%	19%	24%	100%

Large subsets of patients with RF-negative polyarthritis grouped to V2.1 (Table 3.3). Few patients with psoriatic arthritis, ERA, and undifferentiated arthritis had high levels of inflammatory cytokines after six months; they grouped into either V2.3 or V2.4. Figure 3.5 shows how visit 1 clusters split to constitute visit 2 clusters. Next, we assessed homogeneity of Clusters relative to JIA category using chi-square ($p<0.001$) and Kruskal-Wallis tests (Figure 3.6). Relative to JIA category, both visit 1 Clusters and Visit 2 Clusters had a higher proportion of variables that were statistically significant at any p-value threshold (Figure 3.6, upper graph). When considering only clinical variables, visit 2 clusters had the highest proportion followed by JIA category and visit 1 Clusters (Figure 3.6, middle graph). Lastly, when considering biologic variables only, both clusters had a markedly larger proportion of statistically significant variables at any p-value threshold relative to JIA category (Figure 3.6, lower graph).

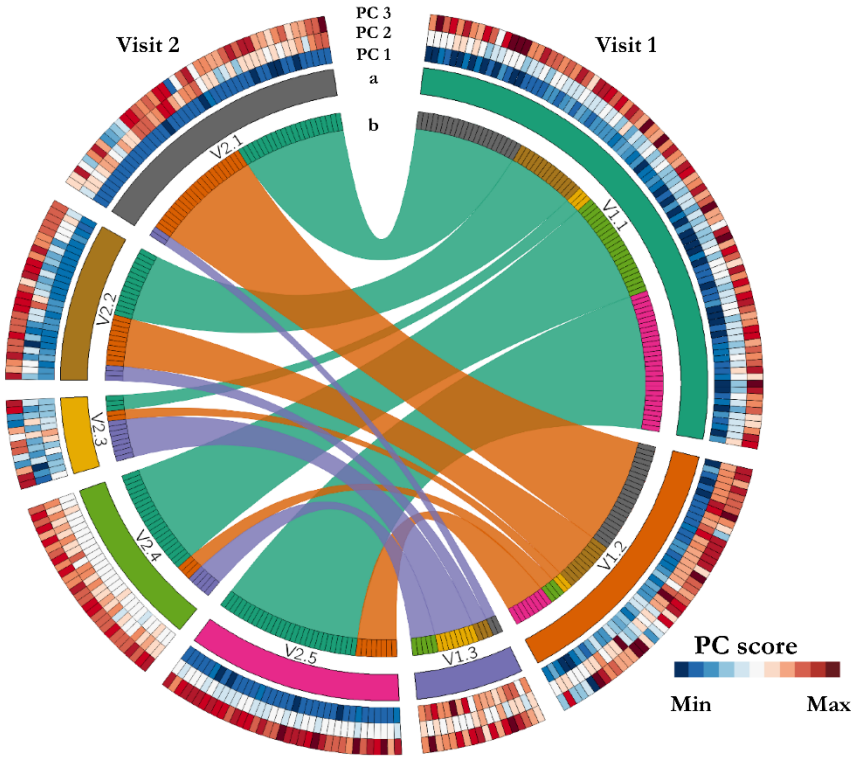

Figure 3.5 Contingency wheel comparing visit 1 and visit 2.
The contingency plot depicts the relationship between Clusters at enrollment (visit 1) and Clusters at six months after enrollment (visit 2). See Figure 3.4 for description of wheel elements.

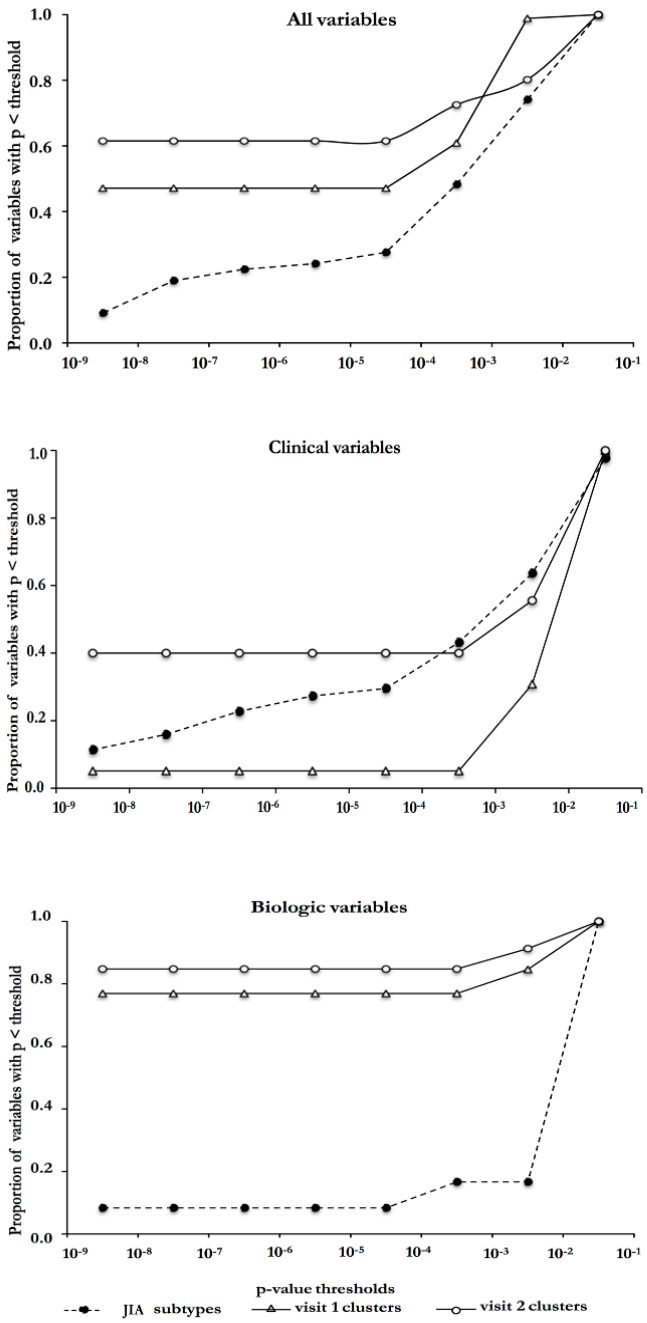

Figure 3.6 Kruskal-Wallis p-values.
Proportion of Kruskal-Wallis p-values ≤ 0.05 for Clusters in visit 1 and 2 tended to be higher than for the JIA categories when considering all variables (upper graph) and biological data (lower graph). JIA categories have a higher proportion of Kruskal-Wallis p-values ≤ 0.05 compared to the visit 1 Clusters and lower values than the visit 2 while considering only clinical variables (middle graph).

These results demonstrate that Clusters are more homogeneous than JIA categories and that homogeneity improves from visit 1 to visit 2 (which might be in part

due to treatment response). For example, profiles characterized by intermediate levels of cytokines (Figure 3.5) were almost exclusively aligned with V2.4, a Cluster containing patients from all seven JIA categories. This suggests that, with respect to inflammatory cytokines, subsets of patients from different JIA categories are more concordant than their distinctive JIA categorizations might imply.

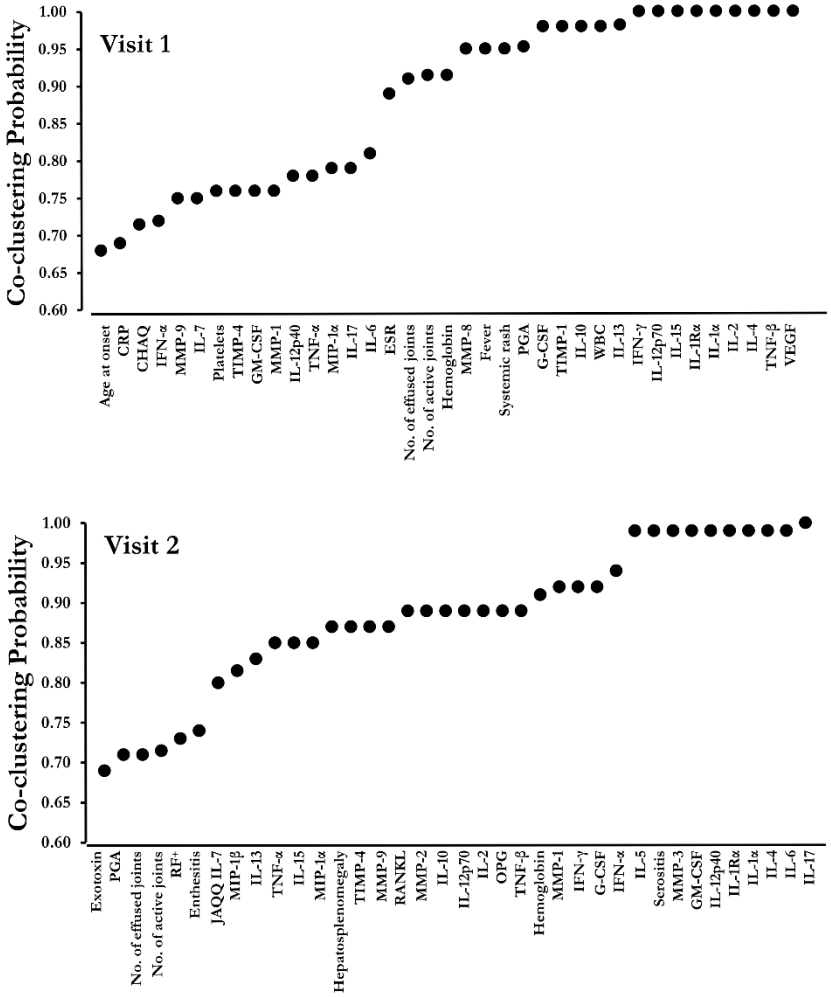

Figure 3.7 Variable sensitivity analysis.
Leave-One-Variable-Out Cross-Validation (LOVO-CV), visit 1 (upper) and visit 2 (lower). By removing one variable at a time and measuring the co-clustering probability, the Clusters remain the same with median of 93% in visit 1 and 89% in visit 2.

Sensitivity analysis determined how robust clustering was to removal of different variables and patients. In variable sensitivity analysis, the entire analysis was repeated 37 times for visit 1 and 38 times for visit 2, each time holding back one variable. Then co-clustering probabilities were computed. Results indicate that in visit 1, 70%-100%

(median=93%) and in visit 2, 69%-100% (median=89%) of the time patients remained in the same Clusters (Figure 3.7).

Sensitivity analysis for patients showed that visit 1 and visit 2 were approximately equally sensitive to removal of 10% of patients; in visit 1, 73%-94% (median=84%) and in visit 2, 74%-92% (median=86%) of the time patients remained in the same Clusters (Figure 3.8).

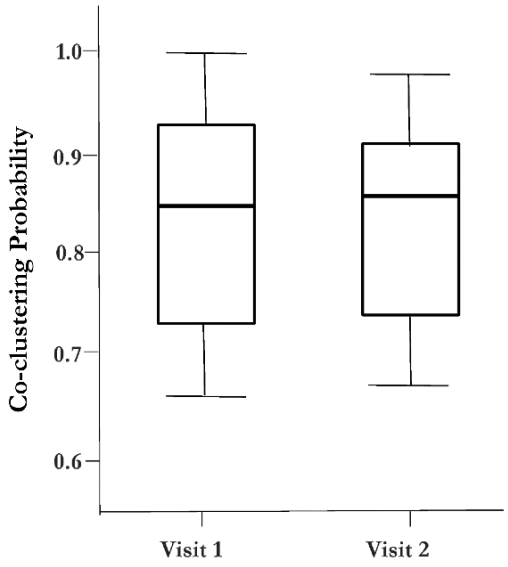

Figure 3.8 Subject sensitivity analysis.
Boxes show the co-clustering probability (calculated using 100 iterations) after removal of 10% of subjects at visit 1 (left) and visit 2 (right). In visit 1, patients remain co-clustered with median of 84%, and in visit 2, with median of 86%.

3.6 Discussion

Using data-driven, machine learning analytical approaches in a JIA cohort, discrete Clusters arise comprising clinical and inflammatory biomarker attributes that tend to intersect multiple JIA categories and that change from the time of diagnosis to 6 months later. Our results suggest that certain pathobiologic processes are shared among JIA categories and fluctuate during the course of the disease.

PC-1a of visit 1, comprised mostly pro-inflammatory cytokines (ILs, IFNs, GM-CSF, and TNF-β), demonstrating the role of inflammatory cytokines in the pathophysiology of chronic childhood arthritis early in the disease. Anti-inflammatory cytokines including IL-10, IL-2, and IL-4 were also expressed in PC-1a. PC-1b constituted clinical features including number of joints with active arthritis and

parent/patient/physician assessment of overall well-being and functional ability. In addition, PC-1b identified significant correlations among MMP-1, -8, and -9, TIMP-4, and MIP-1α. Our findings and previous reports of expression of MMP-1 and -3, and TIMP-1 in JIA synovial fluid and correlation with disease activity (218, 317) suggests that type II collagen degradation, mediated partly by MMP-1 and -3, can begin early in some patients and might portend a poorer prognosis (218). PC-1c was characterized by fever, systemic rash, low hemoglobin and elevated acute phase reactants, all features of systemic JIA (318).

In visit 2, plasma inflammatory cytokine levels were retained in PC-2a, although their contribution was weaker than in visit 1 (Figure 3.2). PC-2b showed associations of both systemic JIA and ERA features with IL-7 and TNF-α. IL-7 promotes Th1 and Th17 activation and production of pro-inflammatory mediators MIP-1, MIP-3, MCP-5, and TNF-α (319-321) in addition to promoting osteoclastogenesis by up-regulating RANKL (322, 323). Although systemic JIA and ERA are clinically distinguishable, bone degradation seen in some patients with systemic JIA and in some with ERA could be mediated by common osteoclastogenic pathways in both conditions. PC-2c grouped clinical measures of disease activity including numbers of active and effused joints and parent/patient/physician assessments of overall well-being and functional ability.

When considering inflammatory biomarkers along with clinical features, Cluster assignments are dynamic; patients aligned with one of three Clusters at enrollment but to one of five Clusters six months later, reflecting alterations of clinical and inflammatory processes over time. These temporal changes could be a consequence of treatment interventions and/or inherent modulations of inflammatory processes.

In visit 1, 100%, 78%, and 42% of ANA-positive patients with psoriatic arthritis, oligoarthritis, and RF-negative polyarthritis, respectively, were retained in Cluster-1(V1.1). Ravelli *et al.* suggested that some patients with similar characteristics can be assigned to different JIA categories (324). For example, ANA-positive female patients classified as oligoarthritis, RF-negative polyarthritis, and psoriatic arthritis are more similar than their different designations might suggest (51, 324). Our findings tend to support this idea although ANA was not a determinant variable in our models (324).

In this study, variable and subject sensitivity analysis indicated that Clusters described are robust to small variations in data. In visit 1, removal of any of 15 of 37

variables resulted in insignificant disruption of clustering. Our results are in accord with those of Eng *et al.* in a study that considered a smaller subset (n=102 compared to our with n=150) of the enrolment BBOP cohort and a smaller number of biomarkers (18 compared to our 48) (39).

Sensitivity analysis shows that the Clusters are robust and unaffected by data perturbation. Nine biological variables (INF-γ, IL-12p70, IL-15, IL-1Rα, IL-1α, IL-2, IL-4, TNF-β, and VEGF) in visit 1, and one (IL-17) in visit 2 had co-clustering probability equal to 1, which indicates that removal of each of them individually cannot affect the clustering scheme. The IL-2/IL-21 gene locus 4q27 is associated with susceptibility to JIA (325). Variant loci of IL-2, IL-2RA, and IL-2RB are associated with oligoarticular and RF-negative polyarticular JIA (325, 326). The role of IL-2 in defining disease groups by sensitivity analysis at visit 2 supports a role for IL-2 in the immunopathogenesis of JIA.

This study did not include other biomarkers relevant to JIA, such as S100 and serum amyloid A (180), genomic markers (broad HLA typing, genetic polymorphisms), gene expression, or metabolomics profiling. Applying the same machine learning analytical frameworks to a broader array of clinical and biologic features should help further elucidate underlying pathogenic processes and might aid in refining disease classification.

We did not investigate reasons for changing profiles over time. Future studies are required to ascertain how treatments influence clinical-biomarker profiles. Panels comprising a small number of clinical-biomarker attributes could then be applied to predict and detect treatment responsiveness and provide more conceived rational, biologically-directed personalized treatment at a lower cost.

Biomarker levels can be influenced by diurnal variations and physical activity (327), variables not controlled for in this research. Further, in addition to our sensitivity analyses and cross-validation, generalizability of the reported PCs requires validation in an independent cohort.

3.7 Conclusion

In JIA, data-driven machine learning algorithms uncover distinctive Clusters comprising clinical and biomarker attributes. Considering biomarker profiles with clinical characteristics can contribute to understanding JIA pathogenesis and may lead to refining

subgroup classifications. We anticipate that this type of data-driven classification of patients will ultimately allow for a more precise personalized approach to diagnosis, prognostication,and treatment of children with JIA.

CHAPTER 4

STUDY 2. BIOLOGICAL AND CLINICAL PREDICTORS OF SHORT-TERM OUTCOMES OF JIA

4.1 Abstract

Objective: The objective of this study was to identify early predictors of short-term arthritis activity in JIA using clinical and biomarker profiling.

Methods: Clinical and inflammatory biomarker data were collected in a prospective longitudinal cohort of 96 newly-diagnosed children with JIA. Presence or absence of active joints, PGA, and Wallace criteria were chosen as outcome variables 18 months post-enrolment. Correlation-based feature (variable) selection and ReliefF were used for feature selection. A random forest was trained to predict outcomes based on the selected features.

Results: From the original 112 features, 17 effectively predicted outcome after 18 months. The variables included onset age, wrist/foot involvement, number of active and effused joints, systemic JIA rash, white blood cell count, erythrocyte sedimentation rate, platelet counts, and plasma levels of eight inflammatory biomarkers (IL-1α, IL-10, IL-15, IL-17, IL-12p70, TIMP-4, GM-CSF, and VEGF). The panel predicted presence or absence of active arthritis, physician global assessment, and Wallace criteria of inactive disease after 18 months with 79%, 82%, and 71% accuracy and 0.83, 0.86, 0.82 AUC, respectively. The accuracy and AUC values were higher compared to when only clinical features were used for prediction.

Conclusion: This study showed that a small number of clinical and inflammatory features at diagnosis can more accurately predict short-term arthritis activity in JIA than clinical characteristics only. Considering clinical features together with a broader array of biomarkers should yield more refined prediction of future arthritis activity and guide more rationally-conceived, biologically-based early JIA treatment strategies.

4.2 Background

JIA encompasses a heterogeneous group of diseases categorized predominantly by clinical manifestations including the number of affected joints and the presence of certain extra-articular features (328). Only two biological variables, RF and HLA-B27 are considered in the JIA classification system. The intent of the JIA taxonomy is to assign patients, for research purposes, with similar characteristics at onset to categories presumed to share similar pathophysiology, treatment responses, and outcomes. However, even within the same JIA category patients exhibit different disease courses and outcomes. Thus, JIA category assignment alone does not always reliably predict which children are destined for a favourable or unfavourable outcome (61, 62).

In general, studies in the era of biologically-based pharmacotherapies indicate improving outcomes (329). Nearly half of children with JIA are estimated to have inactive disease within a year after diagnosis when biologics are used sparingly (330). More generous use of biologics results in up to 80% of JIA patients having inactive disease (331, 332). However, recommending a biologic in a child with JIA requires judicious assessment of baseline disease characteristics and severity and an informed expectation that outcomes will improve with the chosen therapy. Previous studies have identified predominantly clinical predictors of poor prognosis (30, 37, 68, 71). Improving the effectiveness with which JIA outcomes can be predicted early in the disease course by encompassing clinical characteristics with biomarker profiling could further refine patient selection for early aggressive treatment. There are limited studies that have evaluated the utility of a broad array of inflammatory biomarkers together with clinical characteristics for predicting JIA outcomes (333, 334). Our objective was to identify, in a JIA inception cohort, panels of clinical and biomarker attributes that could predict short-term disease activity as reflected by presence of active arthritis, PGA, and Wallace criteria (29).

4.3 Methods and materials

Data collection: Data were from The BBOP Study. Ethics review boards at the 11 participating sites approved the study. BBOP data included 282 clinical characteristics and 48 plasma inflammatory biomarkers. Study participants were diagnosed according to ILAR classification (328). Prior to enrollment, subjects had not received systemic therapies beyond non-steroidal anti-inflammatory medications and/or methotrexate. From the entire BBOP cohort of 186 participants 96 were selected for the current study based

on availability of complete outcome data at the 18-month follow-up visit. Demographic (Table 4.1), clinical, and laboratory data were collected prospectively at enrolment (visit 1) and 18 months later (visit 2). Pediatric rheumatologists conducted a joint examination at each assessment and documented the number of active joints and number of effused joints. The pediatric rheumatologist also completed a physician global assessment of disease activity using a horizontal 10 cm visual analogue scale from 0=no disease activity to 10=maximum disease activity.

In accord with previously described standardized protocols peripheral blood was collected in P100 tubes (BD Biosciences) and plasma stored at -80°C until assayed (303). The list of biomarkers included in the panel is shown in Table 3.1.

Table 4.1 Patient's characteristics at the first visit.

	Outcomes (number)	M/F	Median age yer. (IQR)	Mean/SD active joint	Mean/SD PGA	Mean/SD ESR
Systemic arthritis	1 (n=11)	5/6	8.0(5.0-14.0)	5±5	4.6±2.0	21±12
	2 (n=11)	5/6	8.0(5.0-14.0)	4±6	5.0±2.0	51±50
	3 (n=6)	3/3	6.0(4.5-12.0)	6±6	4.0±2.0	29±24
Oligoarthritis	1 (n=17)	5/12	10.5(4.5-14.0)	2±2	3.1±2.0	24±20
	2 (n=21)	8/13	8.0(4.0-13.5)	2±2	3.0±2.0	25±23
	3 (n=9)	3/6	14.0(5.5-15.0)	3±4	3.0±2.0	32±21
Polyarthritis (RF-negative)	1 (n=32)	5/27	11.0(4.2-14.0)	9±8	4.4±2.0	28±24
	2 (n=33)	5/28	11.0(5.5-14.0)	10±8	4.5±2.0	31±26
	3 (n=20)	3/17	10.0 (6.0-14.0)	8±8	4.0±2.0	28±26
Polyarthritis (RF-positive)	1 (n=10)	2/8	13.0(7.5-15.0)	18±11	4.8±2.0	38±30
	2 (n=10)	1/9	12.0(7.2-15.0)	17±11	4.3±2.0	41±30
	3 (n=6)	1/5	12.5(4.2-14.7)	10±10	5.0±3.0	18±12
Psoriasis	1 (n=6)	2/4	9.0(5.20-14.5)	11±7	5.3±2.0	27±22
	2 (n=6)	2/4	9.0(5.25-14.5)	8±7	4.5±2.0	27±22
	3 (n=4)	1/3	9.0(3.75-13.5)	8±9	6.0±1.0	14±7
Enthesitis related arthritis	1 (n=7)	4/3	13.0(9.0-15.0)	6±5	3.2±3.0	33±28
	2 (n=7)	4/3	13.0(9.0-15.0)	6±5	3.2±3.0	43±37
	3 (n=6)	3/3	12.5(9.0-14.2)	7±3	4.0±2.0	36±18
Undifferentiated	1 (n=5)	1/4	14.0(10.0-15.0)	3±2	3.7±2.0	20±16
	2 (n=5)	1/4	14.0(10.0-15.0)	3±2	3.7±2.0	20±16
	3 (n=3)	0/3	15.0(14.0-15.0)	12±14	5.0±1.0	20±23

M, male; F, female; IQR, interquartile range; SD, standard deviation; outcome 1, active joint; outcome 2, PGA; outcome 3, Wallace criteria.

Biomarkers assayed are described in supplementary text 2. ANA test results were from indirect immunofluorescence assays performed at clinical laboratory facilities at each study site and results dichotomized as positive or negative; ANA patterns and titers were not recorded.

4.4 Outcome indicators

Three independent outcome measures were considered to define inactive and active disease 18 months after diagnosis. The first outcome indicator was based on the presence or absence of active arthritis (n=88), with active arthritis defined as the presence of intra-articular swelling and/or limitation of joint motion with one or more of the following: swelling, warmth, pain on motion, tenderness. The second indicator was based on PGA score (n=93), with active disease defined as PGA>1cm and inactive disease defined as PGA≤1 cm. The third was based on Wallace criteria of active/inactive disease (n=54) (29).

4.5 Feature selection

FS can be applied to a large dataset to select the optimal features for class prediction and it is valuable for analyzing high-dimensional data (that is, datasets in which the number of features exceeds the number of subjects) (268). By eliminating redundant and irrelevant features, FS techniques improve prediction accuracy. FS aims to project the original data, which has a large number of features, onto a smaller number of features while preserving the most important information. In this study, we used two filter-based FS approaches: CFS and ReliefF (335). In filter methods, features are selected on the basis of their scores in various statistical tests by looking at the properties of the data (336). CFS and ReliefF are multivariate methods that consider relationships among the features. CFS is based on the rationale "*a good feature subset is one that contains features highly correlated with the class, yet uncorrelated with each other"* (272). It assesses both redundancy of features by applying correlation algorithms and the predictive ability of a subset of features (273). ReliefF chooses the features that are distinct among different classes (337). The basic idea of ReliefF is to select subjects randomly, compute their nearest neighbors (nearest subjects), and to identify features that discriminate the subject from neighbors of different classes. Specifically, ReliefF randomly draws a subject (A) and then identifies its two nearest neighbors: one from the same class (nearest hit, H) and the other from the different class (nearest miss, M). It then calculates differences between features from subjects A and H and between A and M. A desirable scenario is when the subjects A and M have different values of a particular feature and that feature discriminates two subjects with different class values. If subjects

A and H have similar values of the individual feature, then that feature does not separate two subjects with the same class values.

To estimate a weight for each feature (f), the algorithm uses the following probability equation:

$$Weight\ (f) = P(different\ value\ of\ (f) | different\ class) - P(different\ value\ of\ (f) | same\ class) \quad (4.1)$$

The operation is iterative and gives more weight to the features that discriminate the subject from the neighbors of a different class (338). High-ranked features identified by both CFS and ReliefF were selected for further analysis.

4.6 Predicting outcome in JIA based on clinical and biological features

To determine how well a constellation of selected features predicts JIA outcome, the random forest classification algorithm was applied (339). In the dataset used to derive the random forest algorithm, each patient represented a subject. A prediction model was trained using 90% of the data (training set) randomly, and then the model was tested on the remaining 10% of the data (test set). This procedure is iterative and is called10-fold cross-validation. The ultimate goal of the random forest classification algorithm was to maximize the predictive accuracy of the trained model on the new data (290).

The random forest algorithm generates many decision trees (each of which predicts the class value by learning simple decision rules inferred from the selected features) from randomly selected subsets of the subjects and features (339). There are two assumptions: first, most of the trees correctly predict the class for most of the subjects, and second, the trees make mistakes at different places. According to these assumptions, the algorithm conducts voting for each of the classes and collectively ranks the importance of features in predicting the correct class (294, 339).

By default, Weka (data mining software) injected randomness into the training procedure by randomly selecting log2 (number-of-features+1) subjects from the dataset prior to training each decision tree.

The mean decrease in accuracy of a feature was determined during the cross-validation. A single feature was excluded from the test set then accuracy rate of the

model was calculated. The feature was considered more important if its removal caused a large decrease in the model accuracy measurements.

4.7 Data analysis

Statistical analyses were performed using SPSS Statistics Professional v23, and R v3.2.2. Weka was used for feature selection and prediction (340). The outlier-labeling rule was applied to identify and remove extreme values (309). Protein concentrations and continuous features were log and Z-score transformed.

For each outcome, ROC curves were plotted, where the *y-axis* represents true positive rate (TPR, sensitivity/recall) and the *x-axis* represents false positive rate or 1− specificity (FPR). Each outcome was evaluated based on the area under its ROC curve (AUC) where a value of 1 represents perfect discrimination and 0.5 represents performance at chance level. AUC is a threshold-independent measure of overall classification accuracy. Accuracy, precision, recall, and F-Measure were calculated for each model as additional indicators of classifier performance.

The performances of our classifiers were compared to the results of the same analysis when using clinical-only features and the results reported by Oen et al (69). The latter included predominantly clinical predictors at enrollment including: age, sex, JIA subtype, pain score, active joint count, number of active joints with limited range of movement, PGA, patient or parent global assessment of overall well-being measured on a 10-cm visual analog scale, CHAQ, JAQQ, ESR, and c-reactive protein CRP (69).

4.8 Results

From an initial set of 112 features, uninformative and redundant features were removed using CFS and ReliefF. As a result, 31 features were selected. The final sets of features for predicting each outcome are shown in Table 4. 2 In addition to clinical disease manifestation, eight inflammation-related biomarkers including pro-inflammatory IL-1α, IL-10, IL-15, IL-12p70, IL-17, TIMP-4, GM-CSF, and VEGF were identified as predictors. Wrist joint involvment and IL-12p70 were common predictors among all outcomes. Foot joint involvment, number of active joints and number of effused joints, white blood cell count (WBC), ESR, and IL-1α were shared as predictors by both outcomes 1 and 2 (Table 4.2). The selected features were used as input for the random forest algorithm.

Eighteen months after diagnosis, 33%, 37%, and 26% of patients had active disease in defined outcomes 1, 2, and 3, respectively. For each outcome, the random forest classifier was trained. Characteristics of the classifiers' performances are listed in Table 4.3 and illustrated in ROC curves (Figure 4.1). Classifiers predicted arthritis activity outcome, PGA, and Wallace outcomes with 79%, 82%, and 71% accuracy, respectively. When considering presence/absence of active joint as the outcome, the classifier achieves a higher specificity. In contrast, other performance measures (AUC, sensitivity, precision, and F-measure) were slightly higher when PGA was used as the disease outcome. Wallace outcome had the lowest predictive performance.

Table 4.2 Predictive features.
Features at first presentation predictive of active arthritis, physician global assessment (PGA), and Wallace criteria outcome at 18 months.

Outcome 1(Active arthritis)	Outcome 2 (PGA)	Outcome 3 (Wallace criteria)
# of active joints	# of active joints	Wrist
# of effused joints	# of effused joints	Platelet
Systemic rash	Foot	IL-10
Foot	Wrist	IL-12p70
Wrist	WBC	GM-CSF
Age	ESR	
WBC	IL-1a	
ESR	IL-12p70	
IL-1a	IL-17	
IL-12p70	TIMP-4	
IL-15		
VEGF		

IL, interleukin; VEGF, vascular endothelial growth factor; TIMP-4, tissue inhibitor of metalloproteinase; GM-CSF, granulocyte macrophage colony-stimulating factor; WBC, white blood cell counts; ESR, erythrocyte sedimentation rate.

Table 4.3 Performance measures of classifiers.
Combined clinical and biological predictors and clinical predictors only for the outcome 1 (active arthritis) and the outcome 2 (Physician Global Assessment [PGA]), and the outcome 3 (Wallace criteria).

Performance measures	**Classifiers**	**Outcomes**		
		Active arthritis (n=88)	PGA (n=93)	Wallace criteria (n=54)
AUC	**Clinical-Biological**	**0.83**	**0.86**	**0.82**
	Clinical	0.76	0.79	0.67
F-measure	**Clinical-Biological**	**0.79**	**0.82**	**0.7**
	Clinical	0.71	0.75	0.65
Generalization error	**Clinical-Biological**	**0.23**	**0.20**	**0.29**
	Clinical	0.27	0.26	0.35
Accuracy (CI)	**Clinical-Biological**	**79% (0.317-0.142)**	**82% (0.281-0.118)**	**71% (0.477-0.222)**
	Clinical	72% (0.362-0.177)	74% (0.349-0.170)	65% (0.399-0.160)
Precision (CI)	**Clinical-Biological**	**79% (0.654-0.871)**	**82% (0.732-0.914)**	**72% (0.384-0.758)**
	Clinical	73% (0.615-0.839)	75% (0.596-0.814)	65% (0.384-0.758)
Specificity (CI)	**Clinical-Biological**	**76% (0.670-0.878)**	**82% (0.708-0.904)**	**57% (0.509-0.823)**
	Clinical	72% (0.609-0.839)	70% (0.603-0.817)	57% (0.458-0.798)
Sensitivity (CI)	**Clinical-Biological**	**79% (0.716-0.920)**	**82% (0.732-0.914)**	**71% (0.615-0.974)**
	Clinical	73% (0.615-0.842)	75% (0.684-0.889)	65% (0.483-0.867)

AUC, area under the curve; CI, confidence interval.

The same analysis was applied to the clinical characteristics of the patients. Table 4.4 shows the clinical predictors of the three outcomes. The performances of clinical predictors are shown in the Table 4.3, which alone were not as satisfying as performances of clinical and biological predictors combined.

The performances of the identified classifiers in Table 4.2 were compared with those of the previous study (clinical predictors) reported by Oen *et al.* (Figure 4.1) (69). Results indicate that the former predicts outcomes more accurately than the latter.

Table 4.4 predictors of the three outcomes.
Features at first presentation predictive of active arthritis, physician global assessment (PGA), and Wallace criteria at 18 months when applying the models on clinical-only features.

Outcome 1(Active arthritis)	Outcome 2 (PGA)	Outcome 3 (Wallace criteria)
Family history of arthritis	Family history of arthritis	# of active joints
# of joints with enthesitis	# of joints with enthesitis	# of effused joints
# of active joints	# of active joints	Ankle
# of effused joints	# of effused joints	Wrist
Morning stiffness	Systemic rash	PGA
Sex	Sex	
Fever	Fever	
Ankle	Ankle	
Wrist	Wrist	
Foot	Foot	
Knee	Age	
Age	PGA	
PGA	Platelet	
Platelet	WBC	
WBC	ESR	
ESR		

WBC, white blood cell; ESR, erythrocyte sedimentation rate.

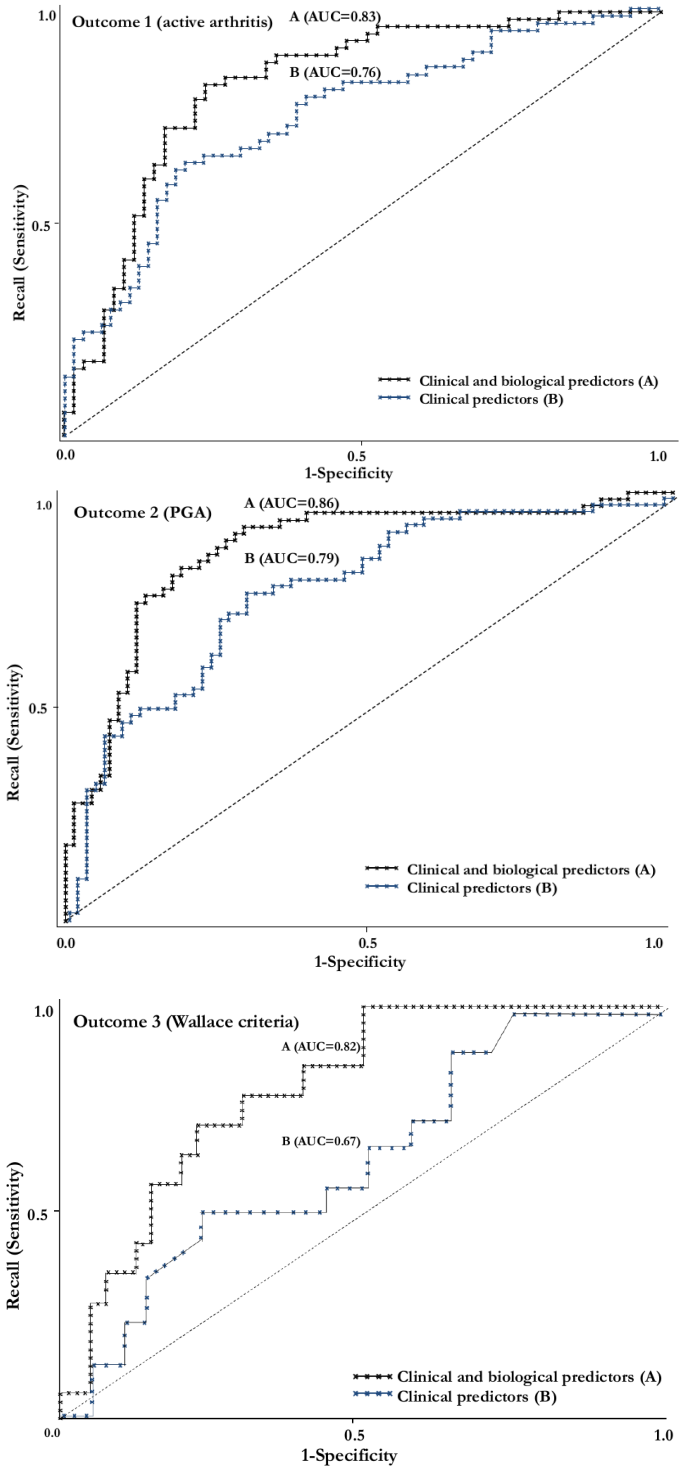

Figure 4.1 Receiver Operating Characteristic (ROC).
ROC curve for clinical and biological predictors (A) and clinical predictors (B) for outcome 1 (active arthritis-upper), outcome 2 (PGA-middle), and outcome 3 (Wallace criteria-lower). The diagonal line denotes the expected performance of a tool that uses random guessing.

4.9 Discussion

Using a composite panel of clinical and biomarker features in JIA patients at first presentation, we found improved prediction of short-term disease outcomes compared to historic clinical features alone.

The panel was developed from a set of clinical and biomarker attributes by applying feature selection and random forest techniques. Random forest is a robust machine learning classification algorithm that can investigate prediction power of features in a compound (quantitative or categorical) and high-dimensional dataset. It is among the most accurate methods of classification and permits both a measure of the relative importance of features and prediction. Previous studies have focused on the utility of clinical or laboratory characteristics of patients separately (341). Among clinical and laboratory predictors of JIA outcomes previously reported (36, 38, 62, 68, 69) our analysis confirmed only active joint count, effused joint count, wrist involvement, age at disease onset, systemic JIA rash, and ESR, and added foot joint involvement, WBC, and platelet counts as the clinical predictors in the composite panel.

The cytokine profile we identified is pertinent to JIA. IL-1, a pathogenic cytokine in systemic JIA (100) is among the predictive biomarkers, an observation that aligns with systemic JIA rash, a sign of active disease, being among the clinical predictors. It has been suggested that IL-15 may trigger the overproduction of IL-17 in joints of rheumatoid arthritis patients (342). IL-17 expressing T cells are abundant in JIA joints and correlate with number of involved joints (343). Increased IL-17 levels in synovial fluid of patients with ERA correlate with disease activity (12). IL-12p70 promotes the induction and activation of both Th1-cells and Th17-cells, key mediators in the pathophysiology of JIA (344). Yamasaki *et al.* showed that VEGF is an indicator of disease activity in oligoarticular and polyarticular JIA in remission and can be employed as a marker for guiding, tapering, or discontinuing treatment (345). Ramamurthy *et al.* reported an association between experimental systemic arthritis in rats and elevated gingival tissue MMPs that was reversed with TIMP-4 gene therapy (346). GM-CSF stimulates the production of macrophages and is an inflammatory mediator in JIA. Therapeutic antibodies targeting the GM-CSF receptor chain may be a viable therapeutic option in treatment-resistant JIA (347, 348).

Van Dijkhuizen *et al.* showed that in a cohort of JIA patients outcomes could not reliably predict inactive disease in the entire cohort using clinical, cytokine, and microbiome inputs (334). However, when certain JIA categories were considered separately (oligarticular, RF-negative polyarthritis and ANA-positive), prediction of inactive disease was moderately robust. In this current study, we did not investigate predictors for individual JIA categories as the number in each group was small.

If we use only clinical characteristics, a higher number of predictors was needed to reach acceptable test performances but considering a combination of clinical and biomarker features resulted in a higher classifier performance than clinical data only.

Somewhat unexpectedly, systemic JIA rash but not systemic JIA fever was a predictive clinical feature, and IL-1α but not TNF-α a predictive biomarker. In the analytical frameworks applied, elimination of features having equal importance (fever and systemic rash and TNF-α and IL-1α) might account for the unanticipated exclusion of certain features. Finally, it should be noted that JIA ILAR category was not retained as a predictor in any of the models. This observation could suggest that JIA categories might not align precisely with category-specific pathobiological process that mediates outcomes.

Medications started at enrollment and continued to 18 months included nonsteroidal anti-inflammatory drugs (NSAIDs) for 89 (95%), DMARDs for 57 (50%), prednisone for 25 (29%), and intra-articular corticosteroid injections in 7 (7%) patients. Biologically-based medications were prescribed at approximately 6 months post-enrollment in 18 patients (19%). As there were insufficient numbers of patients to stratify into on/off a particular treatment group, effects of medication on outcomes were not assessed in this study.

The results of this study, if confirmed in an independent JIA validation cohort, could help inform the development of a clinically useful tool for early prediction of JIA outcomes and thereby aid in treatment selection. We found that readily accessible clinical measures alone had reasonable performance statistics. However, while adding biomarkers improved accuracy and should add a more personalized approach to assessing individual patients, reliable biomarker analyses are not easily accessed in current routine clinical settings. Until such time as evidenced-based, comprehensive, and personalized biomarker assessments become integrated into usual clinical care a two-step approach to

prognostication and treatment selection could be applied. Under such a model clinical feature could be considered first in all patients and then, if indicated, targeted biomarker assessments undertaken with reference to the respective clinical contexts.

4.10 Study limitation

Fluctuations in biomarkers can be influenced by diurnal variations, physical activity, sleep, and food intake (327), features that were not controlled for in this study. Recent studies suggest joint ultrasound features are predictive of inactive disease (349); however, we did not include imaging as a potential outcome predictor. Further, this study did not include a validation cohort; the generalizability of the results requires validation in an independent cohort of children with JIA.

Our study did not include genetic markers (HLA and single nucleotide polymorphisms, as examples) or gene expression and metabolomics profiling. Considering these additional biologic markers could further enhance and refine panels of outcome predictors. In this study, we used three clinical indicators of disease activity; a broader array of outcome measures and longer duration of follow-up should further strengthen the reliability of clinical-biomarker predictive panels.

4.11 Conclusion

Supervised machine learning algorithms are enabling us to overcome limitations of conventional statistical models especially when large datasets are available in relatively small study populations. We proposed a model that can evaluate the predictive ability of a relatively small panel of clinical measures and inflammation-related biomarkers simultaneously. We have shown that combined clinical and biological measures of JIA shortly after diagnosis can be used to predict clinically important 18-month outcomes.

CHAPTER 5

OVERALL DISCUSSION AND CONCLUSION

The studies presented in this thesis were designed to develop and assess new approaches for categorizing and predicting outcomes of chronic childhood arthritis. Biologically-based characteristics of JIA patients in concert with clinical disease manifestations were used to identify and characterize distinctive subgroup clusters and ascertain their alignments with conventional JIA taxonomy (Chapter 3). These same patient characteristics were investigated as short-term JIA outcome predictors (Chapter 4). Since Chapters 3 and 4 each includes a discussion section, this present chapter provides a general overview and discussion of sample characteristics, methods, and findings of both studies in the context of the literature reviewed in Chapter 1. Finally, this chapter includes concluding remarks relating to the entire study's strengths, limitations and implications for clinical practice and future research.

5.1 Study participant characteristics

Data were derived from a Canadian prospective longitudinal inception cohort (The BBOP Study) comprising children with new-onset JIA who were enrolled within six weeks of first presentation to the pediatric rheumatology care service. Initially 186 JIA participants were enrolled in the BBOP study. However, some participants did not complete all BBOP study elements. As a consequence, 150 participants were included in the categorization study (Study 1; Chapter 3) and 96 were included in the prediction study (Study 2; Chapter 4) (Table 5.1). Due to BBOP selection criteria explained in the following section (5.2) the prevalence of BBOP patients by design differ from the typical distribution of JIA categories in North American JIA clinic populations.

Table 5.1 The number of participants in each JIA category in Studies 1 and 2 compared to the JIA category distribution in typical clinical populations (350).

	Clustering (n=150) Male/Female=46/104	Prediction (n=96) Male/Female=29/67	JIA prevalence in North America
Oligoarthritis	42 (28%)	22 (23.0%)	50.0%-80.0%
Polyarthritis RF+	13 (8.5%)	11 (11.5%)	15.0%
Polyarthritis RF-	50 (33.3%)	34 (.36%)	20.0%
Psoriatic arthritis	11 (7.3%)	7 (7.5%)	7.0%
ERA	11 (7.3%)	6 (6.5%)	1.0%-7.0%
Systemic arthritis	16 (10.7%)	11 (12.0%)	5.0%-15.0%
Undifferentiated arthritis	7 (4.7%)	5 (5.4%)	No data

ERA, enthesitis related arthritis.

5.2 Sample characteristics

The demographic characteristic of participants in both studies the categorization study (Study 1) and the prediction study (Study 2) were similar (Table 3. 2). Females predominate in JIA cohorts (female/male ratios ranging from 2 to 6:1 depending on the JIA category) as was the case in our study population (350, 351).

Oligoarticular and RF-positive polyarthritis JIA categories tend to predominantly affect girls while ERA has a higher frequency in boys. Sex distribution within JIA subgroups in study 1 cohort follows the expected trend (Table 5. 2).

In North America 50-80% of JIA patients are affected by oligoarthritis while 15%-20% of patients have polyarticular subtypes. However, because of BBOP's recruitment strategy, the number of patients with oligoarthritis in our cohorts is proportionately lower than those having polyarthritis. BBOP aimed for a reasonable number of participants in each of seven JIA subgroups rather than aspiring to achieve a typical JIA subgroup distribution. To achieve this, only participants with polyarthritis or systemic JIA, the least common categories, were eligible during the first six months of the enrollment period; after six months and until the end of the two-year enrollment period participants with any JIA subtype were eligible.

Table 5.2 Sex distribution of JIA categories in study 1.

	Oligoarthritis	Polyarthritis RF+	Polyarthritis RF-	Psoriatic arthritis	ERA	Systemic arthritis	Undifferentiated
Female	27(64.3%)	12(92.3%)	30(78.0%)	7(63.6%)	4(36.4%)	10(62.5%)	5(71.4%)
Male	15(35.7%)	6(7.7%)	1(22.0%)	4(36.4%)	15(63.6%)	6(37.5%)	2(28.6%)

ERA, enthesitis related arthritis.

In the current studies the average age distribution of the patients in JIA categories were oligoarthritis (mean 4.8), RF-positive polyarthritis (mean 11.4), RF-negative polyarthritis (mean 6.2), psoriatic arthritis (mean 9), ERA (mean 11.4), systemic arthritis (mean 7.4), undifferentiated arthritis (mean 7.4). Mean and Median age in the BBOP study are: oligoarthritis (mean 7, median 6), RF-positive polyarthritis (mean 10, median 13), RF-negative polyarthritis (mean 10, median 11), psoriatic arthritis (mean 12, median 13), ERA (mean 10, median 12), systemic arthritis (mean 9, median 9), undifferentiated arthritis (mean 12, median 13).

In summary, the sex ratio and ages of participants in both studies are representative of the typical JIA populations. The distribution of JIA categories, by design, differed from the typical JIA population.

5.3 Data pre-processing

The analyses began by pre-processing data including dealing with missing values, removing outliers, and log- and Z-transformation. There were cases where missing values were concentrated in certain variables. MMP7, a biomarker for which there was substantial missing data (<60% available data), was removed from the dataset. Missing values of the other variables were distributed randomly. Biomarker variables (continuous variables) had missing values completely at random. To retain as much data as possible in the analysis, multiple imputation was done using SPSS. Multiple imputation was done for variables with ≤40% missing values. To impute missing data in several variables a multivariate model should be fitted to all of the variables with missing values. SPSS default method was used, which scans the data to determine the best imputation method. For continues variables linear regression is the default method.

Resolving missing values is important for generating correct hypothesis testing and making valid inferences, while in predictive models' accuracy is the main concern.

In normal conditions, blood concentrations of cytokines are low or undetectable. However, there is no reliable information about upper limits of normal for respective cytokines under various physiological states influenced, for example, by diurnal variations, physical activity, nutrition, or sample collection, processing and storage. In the presence of inflammation, extracellular and intracellular cytokine concentrations increase.

Data pre-processing is an important step before analysing biological data. There were some data points in cytokine levels that diverge from the overall pattern of the data. They had influential effect on regression analysis and cause the coefficient of determination to be bigger (R^2=0.963 with outliers and R^2=0.388 without outliers). Thus, outliers were removed. Although removal of outliers improves data stability there was no evidence in our study that the biomarker outlier values were a consequence of technical issues or other artifactual influences rather than accurate biomarker measures.

Data reduction and variable selection techniques are another class of data transformation. For the clustering study, PPCA was applied and for the prediction study CFS and ReliefF methods were used. We used a probabilistic PCA algorithm for data reduction. The conventional PCA method for data reduction defines a linear projection of the data and cannot handle categorical or binomial data; PCA cannot be applied to mixed datasets comprising various data types. Due to lack of association with a probabilistic model, the scope of applications for using PCA is limited and can fail to reveal latent data structure as large data may comprise a mixture of two or more Gaussian distributions with common covariance. By using PPCA, variables were reduced into three uncorrelated components, which were used for clustering. In the prediction model, we needed to find variables that possess reliable predictive power that can be simply implemented in the clinical setting. As the number of variables retained in the PCs was large, feature selection methods were applied.

In summary, data pre-processing methods used in this study enabled us to create a smaller dataset that was easier to work with while still yielding robust and informative results.

5.4 Principal components

Three PCs have been retained, each containing variables with maximum correlation. The first PCs from visit 1 and 2 (PC1a and PC2a) were explained by levels of

pro/anti-inflammatory cytokines, interferons, and growth factors. The biomarkers retained in the first PC in both visits comprised: G-CSF, GM-CSF, VEGF, IFN-γ, IFN-α, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1α, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, TNF-α, and TNF-β.

Cytokines mediating Th1 and Th2 immune responses are retained in the first PC. Although these two cytokine pathways tend to be antagonist to each other our data suggest a positive correlation between these two pathways at enrolment and also 6 months later.

As an example of inflammatory joint disease, adult patients with early arthritis who develop RA have a distinct but transient synovial fluid cytokine profile, which does not persist. In early RA synovial fluid elevated levels of IL-1, IL-2, IL-4, IL-13, IL-15, and IL-17 are found within 3 months after symptom onset, compared with early arthritis patients who do not develop RA (352). IL-6 was present in all type of all inflammatory arthritis (352). Our results show high levels of both Th1 and Th2 cytokines, which change over time and are more biased toward Th1 when the disease is well-established. Longer-term follow-up studies should help reveal changes in cytokine profiles in different JIA categories over time.

An essential mechanism in the pathogenesis and persistence of RA, and probably JIA, is angiogenesis (353, 354). Oxygen and nutrients necessary for metabolism of high metabolic cells involved in the arthritic process are delivered *via* new vascularization. Growth factors, mainly VEGF, induce angiogenesis which starts very early in the arthritis process (353). Biomarkers that modulate angiogenesis (353). Biomarkers that modulate angiogenesis have been associated with pathogenesis, severity, and progression of JIA (354). VEGF correlates with the degree of inflammation in JIA patients (354). Serum levels of G-CSF and GM-CSF, which regulate hematopoiesis, are elevated in RA and correlate with measures of disease. Comparable to studies in RA, early imbalance of grow factors are evident from our results (355). Future studies are required to determine if levels of growth factors in JIA reflect disease activity.

Clinical characteristics of JIA together with PGA were grouped in the second PC at enrolment and the third PC at the 6-month visit. At enrolment, clinical data and levels of MMPs, MIP, and TIMP were retained together in one PC, while at the 6-month visit, the same cytokines along with specific clinical findings of systemic arthritis retained in

one PC. Clinical findings in patients with active systemic JIA (such as serositis and hepatosplenomegaly) grouped with MMPs suggesting that these cytokines might be markers reflective of disease activity in systemic JIA. The regulation of MMP-1, 3 and TIMP1 in synovial fluid of JIA patients has previously been studied. The finding revealed that regardless of JIA category and age groups, degradation of type II collagen is present early in the disease (218). MMPs pathways mediate cartilage and bone remodelling (19, 356).

At enrolment, the third PC retained some of the features of systemic disease (skin rash and fever) together with conventional measures of disease activity such as WBC, ESR, CRP, and platelet count while the same measures grouped with the number of active and effused joints and PGA 6 months later. Again, these findings show that conventional measures of disease activity are reliable; however, their importance might fluctuate in various stages of the chronic arthritis.

In summary, the PCs recovered three important aspects of JIA, clinical manifestations, underlying biology, and laboratory markers. Three PCs were used as new variables for clustering patients in the first study.

5.5 Clustering

The aim of this study was to identify panels of clinical and biomarker attributes that could define homogenous chronic childhood arthritis disease categories. According to ACR and ILAR criteria, disease duration of 6 months is required to determine the JRA/JIA disease categories based predominantly on clinical manifestations. When considering biological factors in conjunction with clinical features for classifying disease subtypes a similar temporal trajectory to assigning subtypes is required; we found patients aligned with one of three Clusters at enrollment but were assigned to one of five Clusters six months later.

The purpose of a disease classification system is to identify differences among patients using measurable, discretely defined metrics. Although clinical manifestations are measurable, they can be prone to inter-observer variations. Considering both clinical and biological characteristics when categorizing patients should lead to a more refined disease taxonomy.

The finding of three Clusters at enrolment and five Clusters six months later is consistent with the current notion that time is required for children with chronic arthritis

to settle into more representative subcategories of the disease. Augmenting clinical characteristics with biomarker profiling at the time of diagnosis should contribute to informing more rationally conceived, biologically-based treatment interventions resulting in mitigation of disease progression.

Children with RF-negative polyarthritis seem to have two clinical trajectories. Disease course and manifestations of ANA-positive, RF-negative polyarthritis patients are comparable to those with the oligoarthritis subset (357). New Clusters in visit one also divided RF-negative polyarthritis patients mainly into two subgroups (Figure 4.4); almost half were aligned with oligoarticular patients in Cluster-1a while the other half grouped with RF-positive and systemic arthritis patients in Cluster-1b. The statistical differences that distinguished the two subsets of RF-negative patients included number of active and effused joints, CRP, MMP-1, 8, 9, TIMP-4, EGF, GM-CSF, IP10, TNF-α, IL-6, IL-1α, and IL-1Ra. The subset of RF-negative patients in Cluster-1a has fewer involved joints and lower levels of inflammatory biomarkers compared to those that grouped in Cluster-1b. Only 4 of 50 patients with RF-negative arthritis who had the highest levels of inflammatory biomarkers grouped into Cluster-1c. These three subsets of the patients did not have significantly different frequencies of ANA-positivity. The findings reveal that at least two distinct subsets, defined by clinical and biomarker features, can be discerned from within the conventional RF-negative polyarthritis JIA category.

In Visit 1, when considering ANA-positive patients with psoriatic arthritis, oligoarthritis, RF-negative polyarthritis, and undifferentiated JIA, 100%, 78%, 42%, and 57% respectively were retained in Cluster-1a. The dataset included 66 ANA-positive patients at visit 1; of these 57% were grouped in Cluster-1a, 28% in Cluster-1b, and 13.6% in Cluster-1c. Earlier report have posited that ANA-positive patients assigned to different JIA categories actually constitute a homogeneous patient population with similar characteristics (51, 301, 302); our results support this suggestion as ANA-positive patients in our cohort tended to align together (51).

Patients received a single medication or a combination of medications including NSAIDs (mainly Naproxen, n=120), DMARDs (only Methotrexate, n=41), and corticosteroids (oral, n= 41or intra-articular, n=29). Only two patients in our cohort were treated with biologic agents. There were different responses to the same treatment

regimen, even within each apparently homogenous JIA category. For example, of two patients with RF-positive polyarthritis who had received Naproxen and Methotrexate during the first 6 months, one Clustered into the group with high levels of biomarkers, higher measures of disease activity, and higher number of active joints, while the other experienced mild disease activity after 6 months and Clustered into a group with similar characteristics. Thus, disease course and response to therapy might not be consistently predictable in the context of the current JIA taxonomy. Having a classification system based on the underling pathophysiology of the disease might be expected to lead to development of more biologically-based, personalised treatment interventions.

5.6 Predicting outcome

The importance of biomarkers in the pathophysiology of JIA lead us to investigate whether a composited panel of clinical and biomarker variables in patients at disease onset could predict short-term disease outcomes. A number of studies have elucidated predictors of JIA prognosis. Adib *et al.* (2005), and Dijkhuizen *et al.* (2018) noted substantial variances in the prognosis even within JIA categories (23, 334). However, to reduce that variance Wallace *et al.* developed and validated a set of criteria for disease remission (26, 29, 358, 359). However, these criteria were derived mainly in relation to polyarticular, oligoartricular, and systemic JIA.

Clinical measures of active arthritis such as joint swelling, warmth, tenderness and pain on motion, together with PGA are applied as indicators of disease activity and outcome variables in almost all JIA predictor studies. Consequently, in our study, we defined outcomes as 1) presence/absence of clinical manifestations of active arthritis, 2) PGA, and 3) Wallace criteria. The first two outcome measures are easily determined in the clinic setting and are more responsive than functional ability and laboratory measures (360). Responsiveness is an element of validity and defined as how a clinical measure is sensitive to change over time or between groups (361).

Wallace outcome criteria is an accepted outcome measure among clinicians and include both absence of active joints and PGA score together with no fever, rash, serositis, splenomegaly, or generalized lymphadenopathy attributable to JIA, no active uveitis, and normal ESR or CRP (29).

A number of potential outcome predictors have been suggested by various studies. The most commonly suggested predictors are disease activity parameters, sex, age, active

disease duration and severity, JIA categories, HLA type, ANA, RF, WBC, ESR, CRP, and patients' socio-economic status (37, 67-69, 71, 76, 78, 85, 362-364). However, some of these variables, such as demographic and laboratory measures, are not valuable predictors because they show too much variability. Disease activity parameters, such as the number of active joints at onset, PGA, the parent or patient global assessment, CHAQ score, and symmetric joint involvement were identified as valuable outcome predictors. Other potential predictors such as cytokine levels in blood or synovial fluid, and genetic markers, like HLA and SNPs in genes associated with the immune system are seldom applied as outcome predictors in the clinical setting. Many of the predictor studies were retrospective and used univariate analysis, consequently. Thus, they were prone to selection bias and failed to effectively exclude confounding factors.

In a prospective cohort study, Guzman et al predicted JIA severe disease course by assessment of quality of life, pain, medication requirements, patient-reported side effects, and active joint counts (365). They identified four disease courses in JIA based on variables derived from clinical experience. In 2011, ACR published recommendations for JIA treatment informed by features putatively predictive of a poor prognosis. Their predictors were evidence-based and were shown to correlate with outcome but were not applied to predict outcome (231).

Earlier studies analyzed clinical or laboratory characteristics separately and used univariate analysis which describes the linear relationship between two variables. A number of investigations were correlation studies that did not identify predictors; correlation analysis simply detects an association between two variables, which may reflect that they are related to an unknown factor, or another variable.

In study 2, the predictive powers of clinical, laboratory, and inflammatory biomarkers have been evaluated using random forest, a robust classification algorithm with good accuracy (293, 295). Random forest is the best choice when the number of predictor variables is greater than the number of subjects. Logistic regression has less power to deal with this situation as the degrees of freedom increase dramatically including higher-order interactions in the model (366). Random forest provides a measure of the relative importance of variables that is helpful for selecting a small number of key predictors.

The classification tree algorithm rapidly selects significant features resulting in a classification tree with binary split criteria and enables automatic classification, for instance lung cancer patients and control subjects based on their individual genetic profile. Logic regression is a generalized regression methodology for predicting the outcome in classification and regression problems based on Boolean combinations of logic variables. Even though a logic regression is able to include continuous covariates, the predictors must be binary in order to be considered as a Boolean combination. This can be somewhat limiting when compared to other tree-based classifiers.

Results of the current study identify factors that are predictive of active arthritis and PGA 18 months after the first presentation of JIA including number of active and effused joints, wrist and foot joint involvement, age, ESR, WBC, systemic rash, IL-1α, IL-10, IL-17, IL-15, IL-12p70, VEGF, GM-CSF, and TIMP-4.

Number of active joints is the most prominent clinical manifestation of the disease and considered as a criterion for JIA classification, prediction and disease activity outcome measure (2, 38, 61, 69). Al-Matar *et al.* and Magin-Manzoni *et al.* have shown that wrist involvement is among the best predictors of long-term IJA outcome predictors (30, 63). Age at onset, wrist involvement, and number of active joints during the first 6 months of disease, ESR, and systemic arthritis manifestations were previously reported as outcome predictors in JIA (36, 38, 62, 68, 69). Our results are in concordance with these studies.

JIA categories and age at onset usually served as proxies for one another. For example, oligoarthritis has a peak age range of 2-4 years, RF-positive polyarthritis disease and ERA occur mostly during late childhood or adolescence, RF-negative polyarthritis has biphasic distribution with a peak in early childhood (2-4 years of age) and later peak (6-12 years of age), and psoriatic also with a biphasic age distribution (early peak at 2-4 years of age and later peak at 9-11 years of age). Systemic JIA has no particular age predominance. Young age at onset has been reported as a predictor of persistent disease and joint erosions (38). In addition, associations between certain HLA allotypes and onset age have been reported. HLA-DR11 and HLA-DR13, are more often observed in patients with younger onset age (less than 6 years old), while HLA-B27 and HLA-DR4, and are associated with protection early in life but with increased risk of disease later in childhood (224). The disease-predisposing HLA-DRB1/DPB1 alleles

(including: DRB1*0801; DQA1*0400; DQB1*0402, DRB1*1103/4; DQA1*0500; DQB1*0301, and DRB1*1301; DQA1*0103; DQB1*0603) were observed in 78% of patients with JIA onset age <6 years. Frequency of these alleles occurred in 68% of patients with oligoarticular JIA with disease onset age≥6 years. DRB1*0801 was reported with increased frequency in JIA children with polyarthritis whose onset was 6 years or older (367).

ESR is a marker of both systemic and organ-specific inflammation. It is one of the ACR core set criteria for definition of improvement. In a Nordic population-based JIA study, ESR showed strong correlation with disease activity in JIA (27). It also has been identified as a predictor of persistent disease and joint erosions in long term studies (38). Long duration of elevated ESR within the first 6 months is a risk factor for the absence of remission at follow-up (69). Long duration of elevated ESR within the first 6 months is a risk factor for the absence of remission at follow-up (69). Elevated ESR>35 mm at disease onset is reported to be a predictor for the occurrence of uveitis 2-3 years later (368, 369).

The study 2 indicates that arthritis involving foot and wrist, together with eight biomarkers (IL-1α, IL-10, IL-15, IL-17, IL-12p70, TIMP-4, GM-CSF, and VEGF) collectively predict short-term arthritis activity in JIA.

Imbalance in the pro/anti-inflammatory cytokines is the main underlying pathogenic process in arthritis. IL-1 is an important biomarker in the pathogenesis of arthritis (370). In systemic JIA dysregulation of IL-1 production plays a critical pathogenic role. Pascual *et al.* have shown that the serum of patients with systemic JIA up-regulates the expression of IL-1α and IL-1β genes by healthy peripheral blood mononuclear cells and treatment with IL-1Ra efficiently treats the disease (16).

Biologically active pro IL-1α, which is released from cells in systemic JIA, is a main activator of acute inflammatory responses. IL-1α is important in arthritis development and progression; levels of membrane-bound IL-1α correlate with the severity of arthritis in a mouse model. RA patients who have higher levels of anti-IL-1α antibodies develop less destructive joint disease. SNPs in IL-1R2, IL-1α, IL-1F10 and IL-1RN genes are linked to systemic JIA (100). Ravindran *et al.* found a higher frequency of the IL-1α polymorphism in adult Caucasian patients with psoriatic arthritis (371). Rahman *et al.* noted that the IL-1 gene, with at least 2 independent regions, appears to be

a high-priority susceptibility locus in psoriatic arthritis (100, 102). IL- 1 and TNF-α enhance cartilage degradation by inducing MMP-3 and MMP-1 expression, but not TIMP expression (372). Agarwal *et al.* noted that ERA and polyarticular JIA patients have elevated IL-17 levels in their synovial fluid, which correlated with measures of disease activity. They suggested that IL-17 might play an important role in pathogenesis of synovitis in these patients. IL-17 also specifically induces MMP expression in ERA synovial fibroblasts, without inducing TIMP, suggesting a role of this cytokine in cartilage destruction (12). The T cell subset that produces IL-17, IL-21, and IL-22 (the Th17 subset) is more abundant in the joints of JIA patients compared with their blood. There are significantly higher numbers of Th17 cells type in the synovial infiltrate in the joints of children with extended oligoarticular JIA than in those with persistent oligoarticular JIA (343). There is a notion that IL-15 may trigger the overproduction of IL-17 in joints of rheumatoid arthritis patients. IL-12p70 promotes the induction and activation of both Th1-cells and Th17-cells and IL-12B (a subunit of IL-12p70) gene was associated with the development and disease severity of ankylosing spondylitis in adults (344, 373).

Vignola *et al.* noted that VEGF levels in synovial fluid of JIA patients are higher than serum. They suggested that this factor may have a major role in the outgrowth of hyperplastic pannus and tissue damage in JIA (374). A strong correlation between serum VEGF levels and disease activity in polyarticular patients has been found suggesting the importance of VEGF in joint inflammation (375). Yamasaki *et al.* showed that VEGF is an indicator of disease activity in oligoarticular and polyarticular JIA in remission. They suggested that this biomarker can be employed as a marker for guiding tapering or discontinuing treatment (345).

There are a limited number of studies that considered biomarkers and genes together as predictors of outcome in JIA. Oen *et al.* found significant correlations between pain and IL-6 genotypes; between PGA and IL-10 genotypes; and between joint space narrowing on early radiographs and TGF-1and IL-10 genotypes using univariate analysis. In the same study, multivariate analyses revealed that only IL-6 genotype was significantly correlated with pain scores (20). Another genetic study revealed that polymorphism of RANTES gene is associated with an early relapse of childhood arthritis after clinical remission (374, 376).

Overall, the results of this study show the contributions that clinical and biologic profiles can have in predicting short-term JIA outcomes as indicated by arthritis activity, PGA, and Wallace criteria.

5.7 Limitations

Small sample size, particularly at the 6-month visit (study 1) and 18-month visit (study 2), limited the robustness of the analysis. For example, there was not enough data for indicators of functional capacity or quality of life 18 months after enrolment to consider as a potential JIA outcome. Due to sampling method during the first few months of the data collection, our cohort, by design, was not precisely representative of a typical JIA population. The variance of our study population from a typical JIA population might influence our results. For example, ANA was not identified as an important predictor variable. In contrast to the typical JIA population our cohort had more polyarthritis than oligoarthritis patients.

We did not include HLA, genetic information, and radiologic measures of joint damage as potential outcome predictors. Lack of a validation cohort reduced the generalizability of the results. A longer duration of follow up and larger sample size could potentially increase the predictive power of the analysis.

larger sample size could potentially increase the predictive power of the analysis.

In addition to sensitivity analyses and cross-validation, the generalizability of the PCs and Clusters need to be evaluated by applying them in an independent cohort of children with chronic arthritis. To improve generalizability of the results, the validation cohort should be ethnically diverse, a goal that can best be achieved by multi-centered, international collaborations. In the present cohort, 75% of the study participants were of European lineage.

Biomarker profiling is increasingly recognized as important for understanding and managing inflammatory diseases such as JIA. However, accurately detecting and quantifying biomarkers can be compromised by fluctuations in biomarker measures influenced by sample collection, processing and storage (303) and by influences of physiologic diurnal variations, physical activity, sleep, and food intake (377). Circadian variations in inflammatory biomarkers have not been evaluated in the context of childhood arthritis. A number of parameters can affect reliable measurements of circulatory levels of cytokines such as, timing of sampling, handling, storage, and

processing. Cytokines have a diurnal rhythm (378). IL-1, IL-6, TNF-α and IFN-γ peak early in the morning (379). Exercise also has an impact on the blood level of cytokines, for example physical activity increases release of IL-6 from muscle cells (380). The diurnal rhythm and exercise effect have not been considered in the current study. Determining whether variations in biomarker levels result from physiologic fluctuations or reflect disease activity requires further study.

Plasma and serum should be separated soon after blood draw and be frozen at -80°C within 1 hour after blood draw. Interruption in sample processing may cause degradation, absorption, or cellular production of cytokines (381). Another consideration is the type of the blood collection tubes. Sodium heparin tubes show more consistent cytokine recovery than EDTA tubes. In this study, P100 tubes were used, which contain spray-dried K2EDTA anticoagulant (377). Earlier studies have shown that the integrity of cytokine measures are retained over longer periods of time using P100 tubes (303, 305-307).

5.8 Conclusion

Emerging insights into underlying pathobiologic processes in JIA provide opportunity to predict and measuer disease outcoms. Supervised machine learning algorithms provide opportunities to overcome limitations of conventional statistical models especially in rare diseases with small numbers of patients and large amounts of data. Machine learning analytical frameworks can evaluate the predictive ability of a relatively small panel of clinical measures and inflammation-related biomarkers simultaneously.

5.9 Future research

Characterizing a broad array of biomarkers could inform refinements in JIA classification, treatment, and outcome prediction. We need to investigate methods that are reliable, simple to perform, economically reasonable, and robust for integrating biomarkers measurements into clinical practice. We have shown that clinical and biological measures of JIA shortly after diagnosis can be used to categorize and predict clinically important outcomes. Nevertheless, the present results suggest that further study of inflammatory biomarkers along with clinical manifestations of JIA in relation to patient outcome is warranted as they may prove to be useful prognostic markers.

There remains a need for the further evaluation of biomarkers and methods of selecting candidate biomarkers for JIA classification and outcome prediction. They need to be tested and validated in large patient cohorts. Reliable biomarker tests may assist with aiding treatment choices at disease onset, predicting response to medication and thus contribute to improving outcomes. They also can help to accurately identify patients who can safely stop medication once biological remission is reached. Another goal of biomarker profiling in childhood arthritis is to help to minimize adverse effects of treatments. To fulfill these goals, multi-centre and international collaborations are needed.

We hope to continue this work with a larger cohort of JIA patients and with longer follow up to validate and extend these results. Then it will be possible to explore more thoroughly the utility of clinical and biomarker characteristics together to help refine approaches for diagnosing, managing, and predicting courses of JIA.

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APPENDIX A

Supplementary text 1

Biomedical Research Ethics Board, University of Saskatchewan: #07-86; *Clinical Research Ethics Board*, University of British Columbia: #H07-01204; Health Research Ethics Board, University of Alberta: #6984; Biomedical Research Ethics Board, University of Manitoba: #H2007:111; Research Ethics Board, Hospital for Sick Children: #1000011118; Research Ethics Board, Children's Hospital of Eastern Ontario: #09-16E; Biomedical Research Ethics Board, McGill University: #PED-07-020; Research Ethics Committee, Université Laval: #123.05.09; Institutional Ethics Committee of Research Involving Humans, University of Sherbrooke: #07-119; IWK Health Centre Research Ethics Board: #1001241; Human Investigation Committee, Memorial University: #06.047.

Supplementary text 2

Cytokine, chemokine, growth factor, and metalloproteinase plasma levels were assayed by bead-based immunoassays. Product codes for analytes (Milliplex, Milllipore Sigma) were as follows: RANKL (HBN51K1RANKL), RANTES (HCYTOMAG-60K-01), OPG (HBN1B-51K-01), TIMP-1/2 (HTIMP1-54K-02), TIMP-3/4 (HTIMP2-54K-01), MMP-3/12/13 (HMMP1-55K-03), MMP-1/2/7/9/10 (HMMP2-55K-05), MMP-8 (HSP2MAG-63K-01), 29-plex cytokine/chemokine panel (HCYTMAG-60K-PX29), and FGF-2 (HCYTOMAG-60K-01). All bead-based analytes were analyzed on a Luminex100 LabMAP system (Luminex, Austin, TX; Analytical Facility for Bioactive Molecules, Hospital for Sick Children, Toronto) according to manufacturer's instructions. sLRP1 was assayed in duplicate by ELISA as follows: 96-well micro-titer plates (Microlon, Greiner Bio-One Inc., Monroe, NC USA) were coated with 100 µl per well of monoclonal antibody specific for sLRP1 (clone α2-MRα2; Genway Biotech, San Diego, CA, USA), 1 µg/ml diluted in carbonate-bicarbonate (15mM Na_2CO_3, 35mM $NaHCO_3$) with overnight incubation at 4°C. Plates were washed 3 times with 0.1% phosphate buffered saline (PBS; 0.14M NaCl, 1.5mM KH_2PO_4, 2.7mM KCl, 18.9mM Na_2HPO_4) containing 0.1% Tween 20 (PBST) and then 100 µl of plasma diluted 1/500 (or 1/1000, as needed to bring the sample ELISA values within the standard curve) was added to duplicate wells. After incubation at 37°C for 1 hour the plates were washed 3 times with PBST and 100 µl biotin-labeled anti-human LRP1 (Pierce, Thermo Scientific, and

Waltham, MA, USA) diluted 1:2500 in PBST was added. The plates were incubated for 1 hour at 37°C, then washed 3 times with PBST. 100 µl of horseradish peroxidase (HRP)-conjugated Avidin (Vector Laboratories, Burlingame, CA, USA) were then added at a 1:5000 dilution for 30 minutes at room temperature. Plates were washed 3 times in PBST and 100 µl of the substrate (2 mM ortho-phenylenediaminine 0.02M citric acid, 0.05 M Na_2HPO_4, 0.012% H_2O_2) added. After a 30-minute incubation at 20°C, the reactions were terminated by addition of 100 µl of 4M H_2SO_4. Optical densities were measured at 492 nm (Universal Microplate Reader EL800, Bio-Tek Instruments Inc. Winooski, VT, USA). Concentrations of sLRP1 were calculated based on a standard curve, which had a sensitivity of 1 ng/ml to 100 ng/ml. The standard curve was generated using sLRP1 purified by affinity chromatography, using anti-sLRP (Genway Biotech, San Diego, USA) linked to Pierce NHS-Activated Agarose Slurry (Thermo Scientific, Waltham, USA). HMGB1 and vitamin D assays were performed as previously described (382, 383).

A.1 Biomarkers that have a significantly different ranking in visit 1 Clusters.

Biomarkers	Clusters	Mean Rank	Chi-Square	P value
MMP-8	V1.1 V1.2 V1.3	60 88 98	19.2	0.000
MMP-10	V1.1 V1.2 V1.3	68 76 103	9.5	0.009
MMP-13	V1.1 V1.2 V1.3	73 69 108	11.5	0.003
TIMP-4	V1.1 V1.2 V1.3	68 78 98	7.4	0.025
FGF-2	V1.1 V1.2 V1.3	66 72 128	30.4	0.000
RANKL	V1.1 V1.2 V1.3	71 71 107	10.7	0.005
EGF	V1.1 V1.2 V1.3	64 83 96	10.8	0.005
Exotoxin	V1.3 V1.1 V1.2	60 81 120	29.6	0.000

GCS-F	V1.1 V1.2 V1.3	55 85 129	46.7	0.000
GM-CSF	V1.1 V1.2 V1.3	51 88 136	63.6	0.000
IFN-α	V1.1 V1.2 V1.3	52 85 139	63.3	0.000
IFN-γ	V1.1 V1.2 V1.3	53 84 139	60.9	0.000
IL-10	V1.1 V1.2 V1.3	52 86 137	60.4	0.000
IL-12p40	V1.1 V1.2 V1.3	51 88 136	64.0	0.000
IL-12p70	V1.1 V1.2 V1.3	59 77 138	48.3	0.000
IL-13	V1.1 V1.2 V1.3	53 84 138	58.8	0.000
IL-15	V1.1 V1.2 V1.3	53 86 134	56.0	0.000
IL-17	V1.1 V1.2 V1.3	59 78 135	44.5	0.000
IL-1Ra	V1.1 V1.2 V1.3	47 93 135	76.4	0.000
IL-1α	V1.1 V1.2 V1.3	52 85 139	61.1	0.000
IL-1β	V1.1 V1.2 V1.3	57 86 115	31.6	0.000
IL-2	V1.1 V1.2 V1.3	53 84 137	58.8	0.000
IL-4	V1.1 V1.2 V1.3	58 79 138	51.4	0.000
IL-5	V1.1 V1.2 V1.3	59 81 126	35.9	0.000

IL-6	V1.1 V1.2 V1.3	48 92 134	69.9	0.000
IL-7	V1.1 V1.2 V1.3	58 79 137	49.1	0.000
IL-8	V1.1 V1.2 V1.3	61 82 112	22.2	0.000
IP-10	V1.1 V1.2 V1.3	63 82 108	17.4	0.000
MCP-1	V1.1 V1.2 V1.3	64 81 103	13.0	0.002
MIP-1α	V1.1 V1.2 V1.3	54 88 123	44.4	0.000
MIP-1β	V1.1 V1.2 V1.3	56 84 129	44.8	0.000
TNF-α	V1.1 V1.2 V1.3	55 83 135	51.7	0.000
TNF-β	V1.1 V1.2 V1.3	52 85 141	65.5	0.000
VEGF	V1.1 V1.2 V1.3	55 82 139	56.6	0.000
HMGB-1	V1.1 V1.2 V1.3	68 76 103	9.4	0.009
sLRP-1	V1.1 V1.2 V1.3	64 81 103	12.9	0.002

A.2 Biomarkers that have a significantly different ranking in visit 2 Clusters.

Biomarkers	Clusters	Mean Rank	Chi-Square	P value
MMP-1	V2.1 V2.2 V2.3 V2.4 V2.5	81 56 70 108 59	27.5	0.000
MMP-2	V2.1 V2.2 V2.3 V2.4 V2.5	52 79 90 78 95	22.4	0.000
MMP-3	V2.1 V2.2 V2.3 V2.4 V2.5	76 58 74 130 45	68.2	0.000
MMP-8	V2.1 V2.2 V2.3 V2.4 V2.5	68 83 80 109 51	30.5	0.000
MMP-9	V2.1 V2.2 V2.3 V2.4 V2.5	77 62 53 122 55	47.6	0.000
MMP-12	V2.1 V2.2 V2.3 V2.4 V2.5	65 59 94 119 59	43.0	0.000
MMP-13	V2.1 V2.2 V2.3 V2.4 V2.5	50 68 101 127 62	65.2	0.000
TIMP-1	V2.1 V2.2 V2.3 V2.4 V2.5	86 51 76 77 79	11.6	0.021
TIMP-3	V2.1 V2.2 V2.3 V2.4 V2.5	75 70 79 104 57	19.5	0.001

TIMP-4	V2.1 V2.2 V2.3 V2.4 V2.5	87 64 58 106 53	30.8	0.000
FGF-2	V2.1 V2.2 V2.3 V2.4 V2.5	52 72 126 123 52	76.1	0.000
OPG	V2.1 V2.2 V2.3 V2.4 V2.5	71 60 65 131 53	61.6	0.000
RANKL	V2.1 V2.2 V2.3 V2.4 V2.5	78 64 85 120 42	54.9	0.000
EGF	V2.1 V2.2 V2.3 V2.4 V2.5	72 76 87 94 59	11.7	0.020
Exotoxin	V2.1 V2.2 V2.3 V2.4 V2.5	54 83 96 106 64	31.1	0.000
GCS-F	V2.1 V2.2 V2.3 V2.4 V2.5	42 96 119 119 52	85.5	0.000
GM-CSF	V2.1 V2.2 V2.3 V2.4 V2.5	49 82 118 133 42	102.0	0.000
IFN-α	V2.1 V2.2 V2.3 V2.4 V2.5	42 90 134 126 45	109.2	0.000
IFN-γ	V2.1 V2.2 V2.3	43 90 131	108.8	0.000

	V2.4 V2.5	127 44		
IL-10	V2.1 V2.2 V2.3 V2.4 V2.5	38 94 127 127 49	112.4	0.000
IL-12p40	V2.1 V2.2 V2.3 V2.4 V2.5	39 93 129 123 49	104.1	0.000
IL-12p70	V2.1 V2.2 V2.3 V2.4 V2.5	42 85 113 135 50	105.4	0.000
IL-13	V2.1 V2.2 V2.3 V2.4 V2.5	40 96 132 124 44	112.9	0.000
IL-15	V2.1 V2.2 V2.3 V2.4 V2.5	41 88 114 134 48	110.3	0.000
IL-17	V2.1 V2.2 V2.3 V2.4 V2.5	40 87 117 129 53	98.3	0.000
IL-1Ra	V2.1 V2.2 V2.3 V2.4 V2.5	48 84 112 129 47	88.5	0.000
IL-1α	V2.1 V2.2 V2.3 V2.4 V2.5	43 86 133 125 47	102.8	0.000
IL-1β	V2.1 V2.2 V2.3 V2.4 V2.5	52 84 104 126 48	72.8	0.000
IL-2	V2.1	40	107.7	0.000

	V2.2 V2.3 V2.4 V2.5	88 122 130 49		
IL-3	V2.1 V2.2 V2.3 V2.4 V2.5	59 65 70 123 68	45.3	0.000
IL-4	V2.1 V2.2 V2.3 V2.4 V2.5	39 90 119 131 50	110.2	0.000
IL-5	V2.1 V2.2 V2.3 V2.4 V2.5	41 92 96 135 51	100.9	0.000
IL-6	V2.1 V2.2 V2.3 V2.4 V2.5	48 92 118 129 39	103.1	0.000
IL-7	V2.1 V2.2 V2.3 V2.4 V2.5	42 96 132 114 51	90.3	0.000
IL-8	V2.1 V2.2 V2.3 V2.4 V2.5	55 85 89 121 52	54.5	0.000
IP-10	V2.1 V2.2 V2.3 V2.4 V2.5	63 68 102 119 53	47.8	0.000
MCP-1	V2.1 V2.2 V2.3 V2.4 V2.5	60 80 73 104 69	18.9	0.001
MIP-1α	V2.1 V2.2 V2.3 V2.4	53 94 107 119	72.8	0.000

	V2.5	43		
MIP-1β	V2.1 V2.2 V2.3 V2.4 V2.5	48 93 119 118 47	78.8	0.000
TNFα	V2.1 V2.2 V2.3 V2.4 V2.5	49 90 112 120 49	73.4	0.000
TNF-β	V2.1 V2.2 V2.3 V2.4 V2.5	40 89 118 135 47	114.8	0.000
VEGF	V2.1 V2.2 V2.3 V2.4 V2.5	55 69 139 111 54	66.8	0.000
HMGB-1	V2.1 V2.2 V2.3 V2.4 V2.5	63 83 86 94 68	11.9	0.018
Vitamin-D	V2.1 V2.2 V2.3 V2.4 V2.5	64 71 76 98 75	11.0	0.027
sLRP-1	V2.1 V2.2 V2.3 V2.4 V2.5	56 75 121 97 67	32.1	0.000