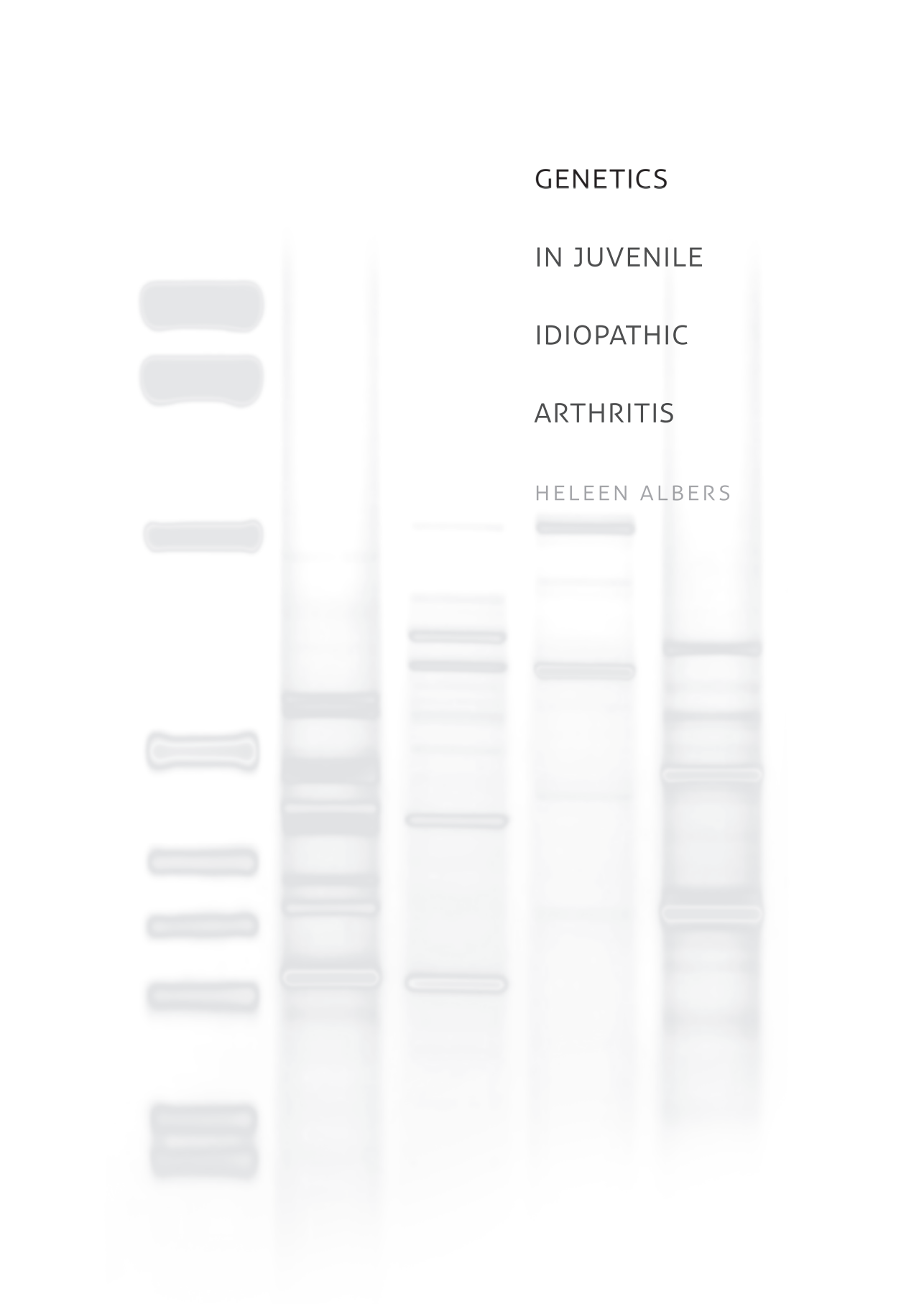
GENETICS IN JUVENILE IDIOPATHIC ARTHRITIS

HELEEN ALBERS



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GENETICS IN JUVENILE IDIOPATHIC ARTHRITIS

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"It is more important to know what sort of person has a disease than to know what sort of disease a person has"

Hippocrates

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

In this thesis the uncommon childhood disease Juvenile Idiopathic Arthritis (JIA) was studied. In a large cohort of JIA patients the association of genetic markers with the susceptibility to JIA was investigated. Associated markers would represent the genetic predisposition for developing disease, but could also be helpful in elucidating the pathophysiology of JIA, which is still largely unknown. Additionally, genetic markers have been studied in relation to the course of disease in order to identify prognostic markers for disease severity. Finally the role of genetic parameters in the ability to predict the response to treatment was examined with the aim of serving the pediatric rheumatologist in selecting the best individual treatment.

Definition and classification of JIA

About 100 years ago the first attempt was made to classify chronic arthritis in children, although the first report of juvenile arthritis by Boticelli dates back to 1483.[1] From the 1940's in Europe a classification of Juvenile Chronic Arthritis (JCA) was used, as defined by the European League against Rheumatism (EULAR).[2] In North America the nomenclature of the American Rheumatism Association (ACR), defining Juvenile Rheumatoid Arthritis (JRA), has been adhered to.[3] In the following years more distinct phenotypes were recognized, for example juvenile onset of spondyloarthritis, arthritis associated with psoriasis and progression of oligoarthritis into a polyarticular course. In 1994 the International League of Associations for Rheumatology (ILAR) was founded and created a worldwide consensus on re-classifying juvenile arthritis as Juvenile Idiopathic Arthritis (JIA). The first version of this JIA classification was proposed in 1994[4] which was subsequently revised, incorporating the results of several studies validating these classification criteria.[5;6]

JIA is defined as arthritis of unknown etiology that begins before the sixteenth birthday and persists for at least 6 weeks with other known conditions having been excluded. JIA encompasses a group of heterogeneous diseases that are characterized by chronic arthritis. The ILAR classification is based predominantly on clinical features and some laboratory parameters and has the intention to define homogeneous subtypes (Table 1). The major criterion is the number of affected joints present at disease onset.

Systemic JIA accounts for 10-15% of patients with JIA. Nowadays systemic JIA is considered to be an auto-inflammatory disease or syndrome due to the activation of the innate immune system and the prominent role of IL-1 and IL-6.[7] The course of disease can be monocyclic, can follow a relapsing course or be continuously active. The subtypes oligoarthritis (persistent and extended) and rheumatoid factor (RF) negative polyarthritis share many clinical features. Antinuclear antibodies (ANA) are predominantly present in these subtypes. Oligoarthritis has a higher prevalence in the Western world and comprises up to 50% of the JIA patients. JIA-associated uveitis is an extra-articular manifestation, which mainly develops in patients with oligoarthritis (10-30%) and will be discussed in more detail later on. Almost 20% of the total group of JIA patients has RF-negative polyarthritis. RF-positive polyarthritis is the juvenile equivalent of adult rheumatoid arthritis (RA) and is present in only a small percentage (about 5%) of JIA patients. It is the only subtype in which antibodies to cyclic citrullinated peptides (anti-CCP) are found.[8] Psoriatic arthritis (5-10% of all JIA patients) represents a heterogeneous group of patients. Two categories seem to be present: patients with psoriasis and arthritis resembling enthesitis related arthritis and patients that have early-onset, ANA positive oligoarthritis and also psoriasis.[9] Enthesitis related arthritis accounts for 5-10% of JIA patients and is an undifferentiated spondyloarthritis. Most patients are HLA-B27 positive, which is also related to more active joints involved.[10]

Many features have not been included in the ILAR classification and are subject of discussion.[11] Some of these parameters are: age at onset, detailed description of arthritis (smaller or larger joints/ symmetric or asymmetric joint involvement), total number of joints affected, presence of ANA,[12-14] presence of (chronic) anterior uveitis and (family history of) psoriasis.[15;16] In the near future genetic, immunologic, genome wide mRNA-expression and proteomic studies might reveal parameters that could also be incorporated into the JIA classification or even lead to a novel classification. As described, the classification of chronic arthritis in children is still evolving. The ultimate goal is to determine biologically distinct subtypes with a predictable response to treatment and outcome. However this situation is still far from being realized.

Table 1. The ILAR classification of the different categories (or subtypes) of JIA[6]

Subtype		Definition	Exclusion criteria*	Distribution#
Systemic JIA		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: 1. Evanescent (nonfixed) erythematous rash 2. Generalized lymph node enlargement 3. Hepatomegaly and/or splenomegaly 4. Serositis	a,b,c,d	10-15%
Oligoarthritis	Persistent oligoarthritis	Arthritis affecting one to 4 joints during the first 6 months of disease and affecting not more than 4 joints throughout the disease course	a,b,c,d,e	50%
	Extended oligoarthritis	Arthritis affecting one to 4 joints during the first 6 months of disease and affecting a total of more than 4 joints after the first 6 months of disease	a,b,c,d,e	
RF-negative polyarthritis		Arthritis affecting 5 or more joints during the first 6 months of disease; test for RF is negative	a,b,c,d,e	20%
RF-positive polyarthritis		Arthritis affecting 5 or more joints during the first 6 months of disease; 2 or more tests for RF at least 3 months apart during the first 6 months of disease are positive	a,b,c,e	5%
Psoriatic arthritis		Arthritis and psoriasis, or arthritis and at least 2 of the following: 1. Dactylitis 2. Nail pitting or onycholysis 3. Psoriasis in a first-degree relative	b,c,d,e	5-10%
Enthesitis related arthritis		Arthritis and enthesitis, or arthritis or enthesitis with at least 2 of the following: 1. The presence of or a history of sacroiliac joint tenderness and/or inflammatory lumbosacral pain 2. The presence of HLA-B27 antigen 3. Onset of arthritis in a male over 6 years of age 4. Acute (symptomatic) anterior uveitis 5. 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	5-10%
Undifferentiated arthritis		Arthritis that fulfills criteria in no category or in 2 or more of the above categories.		

RF: rheumatoid factor

*) Exclusion criteria:

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.

#) Distribution in patients with a Caucasian European ethnicity

Prevalence of JIA

Worldwide the prevalence of JIA has been variably described, with a prevalence varying between 15/100.000 and 150/100.000.[17] Prevalence seems to be higher in the more northern countries of the northern hemisphere, as compared to countries lying closer to the equator. Remarkable is a difference in the distribution of subtypes; in the "Western world" the oligoarticular subtypes are the most frequent, whereas polyarthritis predominates in countries such as India, New Zealand and South Africa.[18] It should be noted this variation could be explained by methodological differences (like diagnostic difficulty of JIA, change in diagnostic criteria over time and variation in study design) or accessibility to health care. However this difference in prevalence of JIA subtypes might well be caused by differences in genetic background. Even when corrected for geographical differences, the genetic background (or ethnicity) is still related to the distribution of JIA subtype.[19] At this moment worldwide research is in progress to collect more data concerning the epidemiology of JIA (EPOCA study).[20]

Course of disease in JIA

Remission

Until 2004 each reported study of remission rates in JIA used a different definition of remission, making comparison of clinical outcome and efficacy of treatment difficult. An international consensus project has attempted to develop a definition of clinical remission and inactive disease. This project is still on-going.[21-23] The criteria for clinical inactive disease in oligoarthritis (persistent and extended), polyarthritis (RF negative and positive) and systemic JIA are: no joints with active disease, no fever, rash, serositis, splenomegaly or generalized lymphadenopathy attributable to JIA, no active uveitis (defined by the SUN Working Group), ESR or CRP level within normal limits or if elevated not attributable to JIA, physician's global assessment of disease activity score of best possible on the scale used and duration of morning stiffness <= 15 minutes.[22] The preliminary definition of clinical remission on medication is met when clinical inactive disease is present for a minimum of six continuous months with the patient on medication. Clinical remission is defined as clinical inactive disease that is present for a minimum of 12 continuous months with no use of medication.[21] Several studies using these definitions demonstrated only a small percentage of patients (approximately 30%) reaching clinical remission and a large percentage of patients with persistent active arthritis.[24;25] Consistently observed in different studies is that patients with persistent oligoarthritis have a more favorable outcome with a higher percentage of clinical remission and inactive disease (but varying from 43-84%).[24-27] The course of disease in the persistent oligoarthritis subtype is less progressive compared to both ex-

tended oligoarthritis and RF-negative polyarthritis. Patients with polyarticular JIA, and especially RF-positive polyarthritis, have a more progressive course with less remission and inactive disease.[24-26;28] Likewise, a recent prospective study with a follow-up of 17 years showed a large variability in disease course between the different subtypes, with remission overall present in only 40% of the JIA patients.[29]

Pattern of disease activity

The course of disease in JIA follows an unpredictable pattern of episodes with different levels of active disease and episodes of disease quiescence. This pattern of disease activity was described in more detail for the first time by Wallace et al,[30] who also introduced the term "cumulative time spent in a state of active or inactive disease". This outcome measure is the only one that takes the fluctuating character of disease activity in JIA into account. Patients with persistent oligoarthritis spend almost 60% of the time in a state of inactive disease, whereas patients with extended oligoarthritis or RF-negative polyarthritis have inactive disease only 30-36% of their time.[28;30] These outcomes are consistent with the higher percentage of continuous active disease described in extended oligoarthritis and RF-negative polyarthritis compared to persistent oligoarthritis.[27]

The aim of treatment is achieving a total absence of disease activity. However in patients with clinical remission, a biological disturbance is still present.[31;32] Different biomarkers (such as phagocyte activation marker S100A12 and myeloid-related protein MRP8/14) are under investigation for their role in defining immunological quiescence of JIA.[33]

Radiological damage

Many different scoring systems for radiological assessment of damage of the joints have been used in JIA; the Steinbrocker score (the most original radiologic score in RA), the Sharp/van der Heijde score and the Poznanski score.[34-36] Because of the lack of scoring systems for JIA patients, the Dijkstra composite score has been developed to describe radiological features of patients with JIA in a standardized manner.[37;38] Also specific for JIA is the Juvenile Arthritis Damage Index (JADI) that has been developed for articular (JADI-A) and extra-articular damage (JADI-E). This measure uses information obtained by physical examination and by a brief review of the patient's clinical history.[39] This scoring system correlates with radiological damage.[39] Most recently, a MRI scoring system (JAMRIS) for evaluating disease activity in the knee has been developed.[40]

Due to these different scoring systems and various study designs, the percentage of patients with radiological damage varies widely between 12% and 60%.[41-44] The

RF-positive polyarticular JIA patients have the most articular damage. A prolonged disease activity is associated with more radiologic damage, underlining the need of aggressive treatment inducing clinical remission at an early stage.[41;44;45]

JIA- associated uveitis

The most common extra-articular manifestation in JIA, is JIA-associated uveitis or chronic anterior uveitis.[46] It is also called silent uveitis because of the lack of symptoms, until complications cause symptoms of visual loss. This type of uveitis is associated with oligoarthritis, RF-negative polyarthritis and psoriatic arthritis, but is especially related to ANA positive, early-onset oligoarthritis (10-30%).[46-49] JIA-associated uveitis mostly develops during the first years after diagnosis of JIA, but can also precede the symptoms of arthritis or develop only many years after the JIA onset. Guidelines for ophthalmologic screening in patients with JIA have been formulated, taking the different risk factors into account.[50-52]

The course of JIA-associated uveitis is fluctuating and not always related to the activity level of arthritis. Uveitis that is already present at first screening and most likely preceded symptoms of arthritis, together with sustained inflammation of the eye are factors associated with complications and visual loss. Several complications of uveitis can be seen; cataract, band keratopathy, posterior synechiae, glaucoma, ocular hypotonia and macular edema. Recent studies reported complications, mainly cataract, in 15-20% of the uveitis patients and a few patients developed blindness. Both frequent screening and an altered therapeutic regime, with more aggressive medication, has led to a decrease in complication rate and a better outcome.[53-55]

Treatment of JIA-associated uveitis is based on a step-up approach and consists of topical or/and systemic glucocorticoids, followed by systemic methotrexate or azathioprine. When treatment fails adalimumab, infliximab, cyclosporin A or abatacept are administered as last step.[56;57]

Treatment of JIA

In the last decades, the treatment of JIA has changed dramatically. In 1981treatment of JIA was dominated by salicylate and non-steroidal anti-inflammatory drugs (NSAIDs). Treatment modalities such as gold, antimalarials or penicillamine were used in progressive polyarthritis and glucocorticoids only in selected patients with ongoing disease.[58]

The latest JIA treatment recommendations of the American College of Rheumatology, dating from 2011 (with an update concerning systemic JIA in 2013), are focused on disease modifying anti-rheumatic drugs (DMARDs) like methotrexate (MTX) and TNF-alpha-inhibitors for treating polyarticular JIA in an early stage as well as treating oligoarthritis following the use of intra-articular steroids (IAS) or NSAIDs.[59;60] The use of

IAS still has an important role in the treatment of oligoarthritis, however the long term benefits seem limited.[61]

DMARDs

In JIA the most frequently used non-biological DMARD is MTX, followed by Sulfasalazine. The biological DMARD that is mainly used in JIA is the TNF-alpha-inhibitor etanercept.

Methotrexate

Methotrexate (MTX) has an anti-proliferative effect, by acting as a folate antagonist interfering in (amongst others) purine and pyrimidine biosynthesis. It is also involved in an increased adenosine release, that has an immune-suppressive effect,[62] and acts as an inhibitor of cytokine production induced by T-cell activation.[63] Randomized controlled trials have proven the efficacy of a weekly low dose of MTX (10 mg/m^2) in the different subtypes of JIA (polyarticular JIA,[64] systemic JIA and extended oligoarthritis[65]). It is described that in recent years about 75% of all patients with JIA have used MTX during the first year of treatment.[66,67] When remission is reached, the time to withdrawal of MTX doesn't seem to have an influence on the relapse-rate or time to relapse.[68] No evidence-based guidelines for withdrawing MTX are available. MTX will be discussed in more detail in the General Discussion (Pharmacogenetics) section.

Etanercept

TNF-alpha is a pro-inflammatory cytokine that plays a (major) role in the pathogenesis of JIA. Soluble TNF-alpha-receptor acts as an inhibitor by binding TNF-alpha. Etanercept (Enbrel) is a fully human dimeric fusion protein consisting of the extracellular portion of the human TNF-alpha-receptor linked to the Fc portion of human lgG1. Clinical studies have first been performed in RA and thereafter the efficacy and safety has been investigated in polyarticular JIA in a double-blind study.[69] The dosage of etanercept is 0.4 mg per kilogram of body weight and it is given subcutaneously by injection twice weekly or at 0.8 mg per kilogram once weekly. Combining etanercept with prevailing DMARDs leads to a good response,[70;71] whereas etanercept as mono-treatment seems to be less effective.[72] In recent years, applying etanercept early in the disease course is not common, as only 5% of the patients have started in the first year after diagnosis.[66] Besides the effective use in polyarthritis, with a response to treatment in 74% of the patients,[69] etanercept results in a good response in extended oligoarthritis as well with a response defined by ACR 30 in 88.6% of the patients.[73] Although not registered for persistent oligoarthritis, it seems a justifiable option when treatment with IAS and MTX has failed.[74] Long-term efficacy (up to 8 years) and safety has been described in JIA.[75-80] Different registries have been established to monitor the efficacy and side

effects of treatment with etanercept in patients with JIA (German[76]; Dutch[79]; British[81] and Polish registry[82]). Several factors have been proposed as prognostic factors for a good response; a lower baseline disability score, the use of fewer DMARDs prior to initiating etanercept and a younger age at onset.[83;84] Successful withdrawal from etanercept has been described,[85] but also early flares in a substantial proportion of patients after discontinuation of treatment.[86] Several side effects have been suggested, such as infectious disease (for example tuberculosis), malignancies and the development of inflammatory bowel disease. However the relation with etanercept has not been clarified.

Other TNF-alpha-inhibitors such as adalimumab (Humira), a (humanized) monoclonal antibody against TNF-alpha and Infliximab (Remicade), a (human-mouse) chimeric monoclonal antibody against TNF-alpha, are less frequently used in JIA. Adalimumab especially seems to be the TNF-alpha-inhibitor of choice when JIA-associated uveitis is present.[87] Infliximab is used in high dosage in case of refractory or highly active arthritis and seems to be effective without serious side effects (most importantly infections) or infusion reactions.[88] Long-term data concerning the use of infliximab or adalimumab are lacking.[89]

Other biologic DMARDs

In JIA subtypes, besides systemic JIA, biologic DMARDs (or biologicals) other than TNF-alpha inhibitors, are not frequently used. One of the biologicals sometimes used in polyarticular JIA is abatacept. Abatacept is a recombinant fusion protein comprising the extracellular part of human CTLA-4 connected to a modified Fc part of IgG-1. It selectively modulates the T-cell co-stimulation, inhibiting the T-cell activation. It is proven effective in JIA patients not responding to previous treatment.[90;91] Tocilizumab, an interleukin-6 receptor inhibitor, has recently been proven effective and safe for treating patients with polyarticular JIA who failed to respond to MTX.[92]

In clinical practice, a large variability is observed in the treatment of patients with different subtypes of JIA. Various approaches for treating polyarticular JIA can be differentiated, such as a step up approach (non-biologic DMARD followed by a biologic DMARD), non-biologic and biologic DMARD combined, and direct use of a biologic DMARD.[93] The use of an early aggressive treatment (for example combining MTX and anti-TNF-alpha plus glucocorticoids versus MTX alone) seems to be more beneficial.[94;95] Also a large variation in treating mono-arthritis has been described, varying from IAS alone, a combination of IAS and NSAIDs or even NSAIDs with non-biological DMARD (mainly MTX).[96] This illustrates that the choice of best treatment has still not been resolved. An ability to better predict the course of disease and the response to treatment

would be helpful in guiding the pediatric rheumatologist in his/her decision about the best individual treatment.

Prognostic factors

In the last decades numerous studies have been performed to identify prognostic factors for the outcome of JIA. Outcome has been defined in different ways; as joint erosions, persistent disease and physical disability. Many factors have been suggested as prognostic factor: time to diagnosis, young age at onset, female sex, large number of affected joints, greater severity of arthritis or higher disability at onset (C-HAQ), symmetric disease, hip/wrist involvement, long duration of elevated erythrocyte sedimentation rate or inflammatory markers and a positive RF.[29;97-101] Of all these factors, only the different JIA subtypes have consistently been associated with a different rate of remission and can be regarded as having some prognostic value. Also a prolonged active disease in the first 6- 12 months seems to be associated with a worse long-term outcome.[29;98] However this is not a predictive factor that is present at disease onset. The aim of treatment is to reduce the time with active disease, as early in the disease course as possible. Prognostic factors or a predictive model that can be determined at disease onset are still needed.

Etiology

JIA is thought to be an autoimmune disease, although the pathogenesis remains largely unknown.[102-104] The initial trigger is supposed to be a self-antigen, that has not been identified, but cartilage-derived autoantigens (like aggrecan, fibrillin and MMP3) might be involved.[105] Following an autoreactive trigger, different parts of the immune response are activated such as (antigen-specific) T-cell response (T-helper-1/ T-helper-17 cells), activation of neutrophils, expansion of regulatory T-cells and the release of cytokines and chemokines. In JIA, as well as in other autoimmune diseases, the possible role of regulatory T-cells has been described.[106;107] Regulatory T-cells are supposed to maintain self-tolerance and suppress inflammation. Interleukin (IL)-2 plays an important role in the development and function of regulatory T-cells through its interaction with CD25 (IL2-receptor-alpha). At the site of inflammation increased levels of regulatory T-cells are consistently reported, although their exact contribution in the local inflammatory environment remains unclear.[108] Besides regulatory T-cells, also T-helper-17 cells have been reported to play a central role in the initiation and maintenance of the autoimmune reaction in JIA.[109;110] T-helper-17 cells produce the pro-inflammatory cytokines IL17 and IL22. An imbalance between pro-inflammatory T-helper-1/T-helper-17 cells and anti-inflammatory regulatory T-cells seems to be essential for developing JIA. The

persistent inflammation of the synovium eventually leads to cartilage destruction and bone erosions.
Systemic JIA has similarities with autoinflammatory syndromes. An overproduction of IL6 is present and patients have a unique IL1 signature. Treatment with tocilizumab (a humanized recombinant anti-IL6 receptor antibody) has been successful[111;112] and clinical trials show effect of IL1 blockade.[113]

Complex disease

JIA is thought to be a complex disease, in which interaction of environmental factors and multiple genetic factors exists.[114]

Environmental factors

A causal association between environmental factors and the development of JIA remains difficult. The trigger of the (auto)immune reaction might be a (viral) infection. Seasonal variation, suggesting an infectious etiology, is described only in systemic JIA, with a higher incidence in spring and summer. Stressful life events, physiological factors, perinatal factors, several viral and bacterial (such as streptococcal) infections have all been related to some degree to JIA and described in detail.[17]

Genetic factors

Familiar risk

A higher occurrence and similar clinical features of disease in affected siblings and families are suggestive for a genetic trait. As early as 1939 juvenile arthritis has been described in monozygotic twins.[115] Most of the (monozygotic) twins and affected sib pairs that have been studied have a concordance for disease subtype and course of disease.[116-120] Clinical features of juvenile arthritis are the same in familial cases and sporadic cases.[120;121] The relative risk (lambda) for first-degree family members has been estimated in small study cohorts to be 15-30.[114;117;118] Recently the relative risk was calculated using a large cohort of JIA patients combined with data from a population database and showed a relative risk in siblings of 11.6, declining to 5.8 in first cousins.[122] The risk for JIA attributable to familial factors was about 13%. These data strengthen the hypothesis that genetic factors play a role in the pathogenesis of JIA.

Ethnicity and gender

Differences in phenotypes of JIA are observed in different ethnic groups. African and Asian patients are more likely to have polyarticular JIA with a higher incidence of a positive RF.[123;124] In Caucasian patients (European and North American) the oligoarticu-

lar subtype is more common. Oligoarticular JIA with positive ANA and JIA-associated uveitis is even more rare in the non-Caucasian population.[125] As in other autoimmune diseases, a female preponderance is seen in JIA, with a female:male ratio of 7:1.

Clustering of autoimmune diseases in JIA families

Clustering of multiple autoimmune diseases in one family, and even in one patient, has been known for many years. The pattern of clustering of the 5 major autoimmune diseases, amongst which is RA, has been described and reviewed in detail.[126;127] Several studies have described the familial autoimmunity in JIA families. All studies indicate a higher prevalence of autoimmunity in JIA families compared to controls.[128-131]

Genetic association studies

The aim of genetic studies is to identify associations between genetic variations (like a single nucleotide polymorphism (SNP), microsatellite, variable number tandem repeat, insertions/deletions) and phenotypic traits. In order to establish a genetic association, different types of studies can be performed.[132]

In a family based study (the transmission disequilibrium test (TDT)), the transmission of a genetic marker from parents to their offspring is studied. It is expected that when a genetic variation is associated with disease, the transmission of that variation to affected offspring is more frequent than expected by Mendelian laws. In this type of study, the potential confounding effect of population stratification is avoided because parents act as controls for the cases (affected offspring). To generate enough power to detect associations with only small effects, the same number of cases is needed as in a case-control study, together with DNA of both parents.

In a case-control association study the difference in frequency of a genetic marker is studied between cases with a specific trait and controls lacking this trait. If a genetic marker is significantly more/less frequent in cases, than there is an association with the specific trait. When the genetic marker is the causal variant, a direct association is revealed. However most of the time a genetic marker is not the causal variant, but is in linkage disequilibrium (LD) with nearby (and possible causal) variants situated in one LD block (that is located between two recombination hot spots) and is called an indirect association. In case SNPs have been studied, the genetic marker of such an indirect association is called a tag SNP, representing an LD block. In case-control studies admixture of the population (population stratification) should be avoided, because it can cause false positive or false negative associations.

Different types of case-control association studies can be performed. In a candidate gene study the selected genetic markers are located in genes of specific interest and are thought to play a role in the mechanism that is investigated. In studies focusing

on the susceptibility of disease, genes are selected that might be involved in the pathogenesis and in studies that focus on the severity of disease, genes can be selected from pathways involved in ongoing inflammation. In genome wide association studies (GWAS) multiple genetic markers (covering a small fraction of the total sequence variation) are studied without underlying hypothesis, which could result in discovery of totally new and unexpected associations. Unfortunately a large cohort of cases and controls is needed to be able to correct for multiple testing. Nowadays the Human Genome Project has identified about 10 million common SNPs. Because of the LD, it is estimated that about 300,000 to 600,000 (tag)SNPs will cover the genetic variation between individuals.[133]

The aim of genetic association studies is to identify a genetic association, replicate/validate this association in an independent cohort and examine the functional consequence of this genetic marker (or associated causal variant). Thereafter the role in pathogenesis of disease (leading to better diagnosis and classification) or possible therapeutic consequences may be investigated.

Genetics association studies in JIA

HLA

The first genetics factor that has been studied in JIA was the human leukocyte antigen (HLA), because of its major role in T-cell immunity and because it is polymorphic and easily typed.[134] There are common HLA associations in the overall JIA patient group, but also different patterns of HLA association in different JIA subgroups are described. These subgroups do not always follow the ILAR classification.[135;136] Nowadays many genetic factors have been studied, however HLA remains to have the strongest association in JIA.[137]

Non-HLA associations

From 1998 onwards multiple case-control candidate gene association studies have been performed in JIA in selected genes (like IL-6, TNF-alpha, IL-1, IL-10, MIF). In most of these studies only a small cohort was available for study (median of 130 cases vs 276 controls)[138] and, therefore, these studies were underpowered to detect associations with a small effect size. The positive associations that were detected were often difficult to replicate. Systematic review of these studies identified that about 100 loci had been investigated until 2008.[138] However at that point in time (which was also the starting point of our study) only 5 associations were independently confirmed; PTPN22, MIF, SLC11A1 (NRAMP1), TNFA, and WISP3.

In recent years more loci associated with JIA have been replicated ; STAT4, TNFAIP3, IL2RA, TRAF1/C5, and VTCN1.[139] In addition to candidate gene studies, genome wide association studies (GWAS) have also been performed in JIA. The first GWAS revealed (besides HLA) a new association; VTCN1.[140] VTCN1 was discovered and replicated in an independent cohort. The GWAS published in 2012 revealed 3q13 as a novel susceptibility locus for JIA.[141] The most recent study using large scale genotyping focused on different regions of interest using dense genotyping on an Immunochip. This study revealed 14 new loci of interest.[137]
PTPN22 is a good example of an association in JIA that has been replicated in multiple independent cohorts (in both candidate gene studies and GWAS) and this will therefore be discussed in more detail.

PTPN22

PTPN22 encodes lymphoid-specific phosphatase, which is an inhibitor of the T-cell activation, preventing spontaneous activation and restricting the response to antigen. A functional SNP was discovered in codon 620, changing arginine into tryptophan, leading to a more efficient inhibition of T-cell activation that could have a part in failure to delete autoreactive T-cells during thymic selection or insufficient activity of regulatory T-cells.[142] In a candidate gene approach the association of this SNP was tested in diabetes mellitus type 1 (DM1) and a positive association of the tryptophan variant (allele 1858T) was found and replicated in an independent cohort.[143] Functional studies show that only lymphoid-specific phosphatase with Arg620 (allele 1858C) forms a complex with the C-terminal Src kinase (CSK), whereas lymphoid-specific phosphatase with Trp620 (allele 1858T) does not bind to CSK, resulting in a reduced T-cell receptor-mediated signaling.[144]
When the candidate gene study in DM1 was performed, a large genome wide association study of putative functional SNPs in RA was simultaneously conducted,[145] identifying PTPN22 as a risk factor for RA. PTPN22 (allele 1858) has since then been found to be associated with multiple autoimmune diseases such as SLE, celiac disease, Crohn's disease and thyroid autoimmunity. A positive association with JIA was first described in 2005[146] followed by mostly positive associations in several cohort studies (some of them with different ethnicities).[147-150] In the first genome wide association study in JIA, the association with PTPN22 was only modest, most likely because the SNPs that had been genotyped, had only low correlation with the causal variant.[140]
The precise implication of this altered function of lymphoid-specific phosphatase is still under investigation.[151;152] Lymphoid-specific phosphatase is currently being investigated as a possible drug target for treatment of autoimmunity.[144;153]

Common autoimmune susceptibility loci

Because of the familial clustering of autoimmune diseases it has always been thought that shared immunological pathways exist. When data from GWAS of multiple autoimmune diseases were compared (not including JIA), a large overlap in genetic susceptibility became clear.[154] Distinct shared immunological pathways were revealed; T-cell differentiation (e.g. IL10, IL18RAP, STAT3, STAT4, IL2RA), immune-cell signaling (e.g. CTLA4, PTPN22) and innate immunological response and TNF-alpha-signaling (e.g. TRAF/C5, TNFAIP3). Shared susceptibility genes encoding cytokines and chemokines were consistently found, together with shared loci with an (as yet) unknown function, that might include transcription factor binding sites.[155]

THIS THESIS

The aim of this thesis is to identify on the one hand non-HLA genetic factors that are associated with the susceptibility to develop JIA (part A) and additionally identify clinical and genetic factors that are associated with the differences in the course of disease and the response to treatment (part B).

In order to address these questions, a new independent cohort of Caucasian JIA patient has been created through collaboration with multiple rheumatology referral centers in North-Western Europe. Both DNA and detailed data on the course of disease and use of medication have been collected.

Several case-control candidate gene association studies have been performed in this new cohort of JIA patients comparing them to a population of healthy controls. Some of these studies only tested one hypothesis, such as TRAF1/C5 (Chapter 2) and the 4q27 locus (Chapter 3), based on the latest (at that time) discovered associations in RA (which of all the autoimmune diseases mostly resembles JIA). In Chapter 4 large scale genotyping is described, involving genes/ loci that have already been associated with JIA (in order to replicate associations) or other autoimmune diseases (in order to identify common autoimmune susceptibility loci that also concern JIA) or are involved in immune-regulation (in order to discover new associations).

To capture the fluctuating pattern of different levels of disease activity during the course of JIA, the parameter "percentage of active disease (in the first two years after disease onset)" has been used to define the course of disease. Both clinical (Chapter 5) and genetic factors (Chapter 6) have been studied in relation to the course of disease. Due to the fact that MTX is the most used DMARD in JIA, clinical and genetic factors associated with the response to MTX are of major interest and are explored in Chapter 7.

Associations with the susceptibility and the severity of JIA might reveal pathways involved in the pathogenesis of JIA and could bring to light important lead points for treatment of JIA. Clinical and genetic factors that are associated with the course of disease or the response to treatment could act as predictive parameters, alone or combined in a predictive model.

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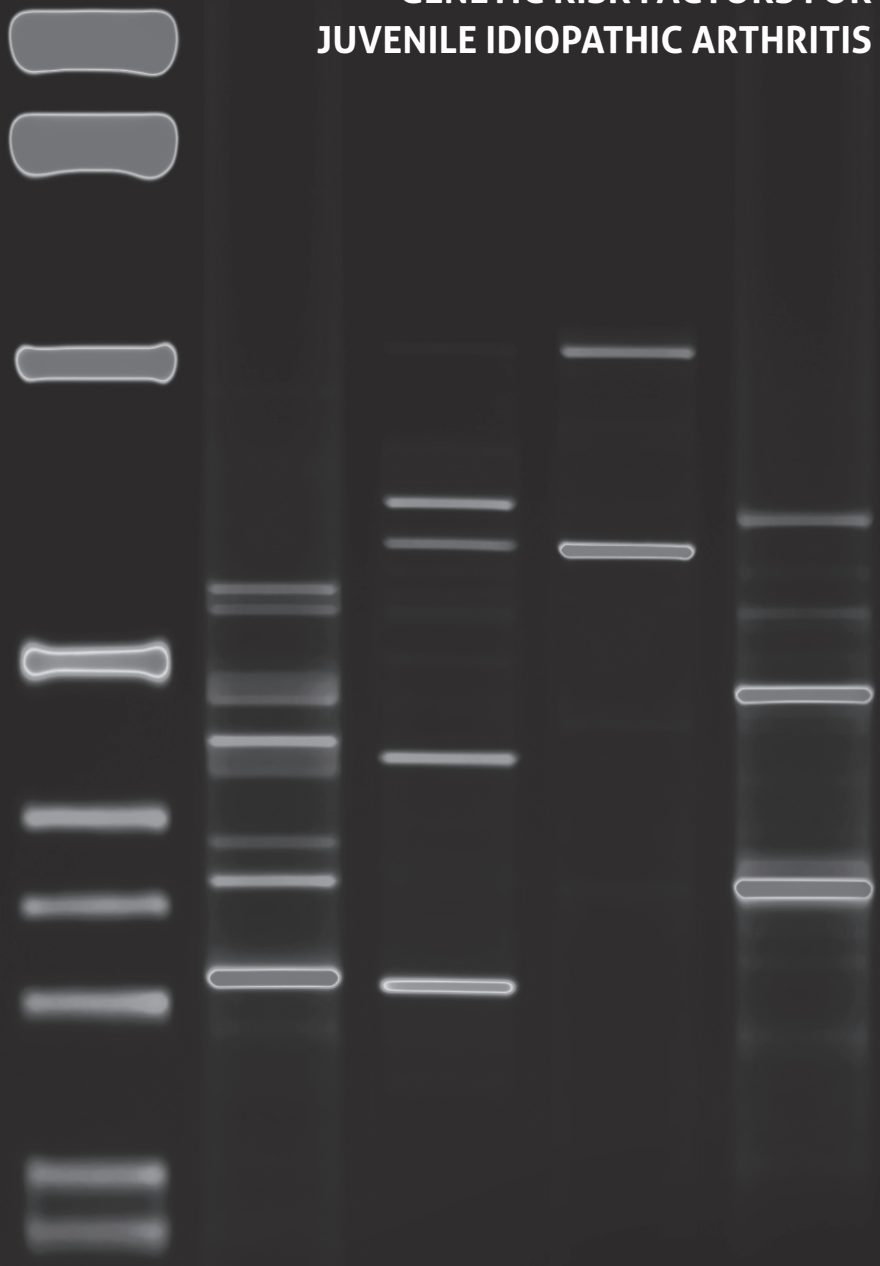
PART A

GENETIC RISK FACTORS FOR JUVENILE IDIOPATHIC ARTHRITIS



CHAPTER 2

THE *TRAF1/C5* REGION IS A RISK FACTOR FOR POLYARTHRITIS IN JUVENILE IDIOPATHIC ARTHRITIS

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*H.M. Albers *[1], F.A.S. Kurreeman*[1], J.J. Houwing- Duistermaat[1], D.M.C. Brinkman[1], S.S.M. Kamphuis[2], H.J. Girschick[3], C. Wouters[4], M.A.J. van Rossum[5], W. Verduyn[1] , R.E.M. Toes[1], T.W.J. Huizinga[1], M.W. Schilham[1], R. ten Cate[1].*

**) contributed equally*

1) Leiden University Medical Center, Leiden, the Netherlands
2) Erasmus Medical Center- Sophia Children's Hospital, Rotterdam, the Netherlands
3) University of Wuerzburg, Wuerzburg, Germany
4) University Hospital Gasthuisberg, Leuven, Belgium
5) Academic Medical Center- Emma Children's Hospital and Reade, location Jan van Breemen, Amsterdam, the Netherlands

ABSTRACT

Objective

Juvenile Idiopathic Arthritis (JIA) is a chronic disorder in which both genetic and environmental factors are involved. Recently we identified the *TRAF1/C5* region (located on chromosome 9q33-34) as a risk factor for Rheumatoid Arthritis (RA) ($p_{combined}$= 1.4 x 10^{-8}). In the present study the association of the *TRAF1/C5* region with the susceptibility to JIA was investigated.

Methods

A case-control association study was performed in 338 Caucasian JIA patients and 511 healthy individuals. We genotyped SNP rs10818488 as a marker for the *TRAF1/C5* region.

Results

The A-allele was associated with the susceptibility to Rheumatoid Factor (RF) negative polyarthritis with an 11% increase in allele frequency (OR 1.54, 95% CI 1.09- 2.18; p= 0.012). This association was stronger when combining subtypes with a polyarticular phenotype (OR 1.46, 95% CI 1.12- 1.90; p= 0.004). In addition, we observed a trend towards an increase in A-allele frequency in patients with extended oligoarthritis versus persistent oligoarthritis (49%, 38% respectively); p=0.055.

Conclusion

Apart from being a well replicated risk factor for RA, *TRAF1/C5* also appears to be a risk factor for the RF negative polyarthritis subtype of JIA and, more generally, seems to be associated with subtypes of JIA characterized by a polyarticular course.

INTRODUCTION

Juvenile Idiopathic Arthritis (JIA) is defined as arthritis of unknown etiology that persists for at least 6 weeks and begins before the age of 16 years. It is the most common chronic inflammatory rheumatic disease in childhood.[1] In 1997 the International League of Associations for Rheumatology (ILAR) formulated criteria for the classification of 7 different subtypes of JIA based on clinical and laboratory features.[2,3]
Although the pathogenesis and etiology are still poorly understood, JIA is thought to be an autoimmune disease in which genetic and environmental factors play a role. Evidence for the importance of a genetic component includes the ethnic variability in the incidence of different subtypes of JIA, a female preponderance, an increased sibling recurrence rate (λ_s) of 15 and the association with HLA- and non-HLA genes (like PTPN22).[3-5] Still little is known about which genetic factors are involved in the susceptibility to JIA and the severity of JIA. Identification of these genetic factors could help to understand the pathogenesis of JIA and could be of use to identify patients with an unfavorable prognosis in an early stage.
Recently we identified the *TRAF1/C5* region (located on chromosome 9q33-34) as a genetic risk factor for RA, using a candidate gene approach.[6] A similar finding was made in a genome-wide study in RA.[7] *TRAF1* is encoding the TNF-receptor-associated factor 1 and *C5* is encoding the complement component 5. Inspired by these results we have studied whether variability in the *TRAF1/C5* region also affects the susceptibility to JIA.

MATERIALS AND METHODS

A case-control association study was performed in 338 Caucasian JIA patients from pediatric rheumatology centers in the Netherlands (54%), Belgium (24%) and Germany (22%). Genotype frequencies of 511 healthy unrelated Dutch adult controls were available out of the 524 controls previously described by Kurreeman et al. due to random genotyping failure.[6] All JIA patients (72% female, 28% male) were diagnosed according to the revised ILAR criteria.[2] The inclusion of patients focused on persistent (39%) and extended (14%) oligoarthritis and RF-negative (22%) and RF-positive (5%) polyarthritis because of their relative homogeneous phenotypes. Patients included had a follow-up of at least two years. Informed consent from all patients and/or parents and approval from each institutional review board were obtained. DNA was isolated from blood samples (20%) or mouthswabs (80%).

One tagging SNP (rs10818488) was genotyped as it revealed the strongest association in RA and none of the other tagging SNPs in the 65kb block provided additional information.[6] Rs10818488 is highly linked with rs3761847 (r^2= 1, data from HAPMAP) and rs2900180 (r^2= 0.66), which were associated with RA as well.[7] Rs10818488 was genotyped using a PCR-RFLP method as described.[6] Each 96-wells plate contained 2 positive and 2 negative controls. 8% of the samples were run in duplicate and we observed a concordance rate > 98%.
Differences in genotype frequencies between cases and controls were assessed using the Cochran-Armitage Trend test. Allelic odds ratios (OR) with 95% confidence interval (CI) as well as the genotype-specific odds ratios were computed. Case and control genotype frequencies were in Hardy –Weinberg-equilibrium. Statistical analysis was performed with SPSS 14.0. A p-value of <0.05 was considered statistically significant.

RESULTS

JIA is a heterogeneous disease consisting of several subtypes. As it is best to investigate genetic risk factors in well-defined phenotypic groups, we have analyzed the genotypes of the rs10818488 SNP in the different subtypes of JIA as well as in the overall group of JIA-patients as shown in Table 1.
Frequencies in patients with persistent oligoarthritis, systemic JIA and in the overall JIA patient-group did not differ from those in controls. In extended oligoarthritis and RF positive polyarthritis (the equivalent of RA) a trend towards an increased A-allele frequency was observed (49%, 50% resp. vs 41% in controls). However, although we do observe an 8-9% increase in the A allele, we possess limited power to detect significant differences. In RF negative polyarthritis patients we found a significant increase in the A-allele by 11% (Allelic OR 1.54, 95% CI 1.09- 2.18) when compared to controls. Homozygotes for the susceptibility allele (AA) had an OR of 2.51 (95% CI 1.23- 5.14) compared to the homozygotes of the protective allele (GG), whereas heterozygotes had an OR of 1.50 (95% CI 0.81- 2.77). Gender was not a significant covariate when performing a regression analysis (p=0.124).
Since extended oligoarthritis, RF negative polyarthritis and RF positive polyarthritis have a considerable phenotypic overlap and share a polyarticular course of disease, we also analyzed these subtypes grouped together to determine whether the *TRAF1/C5* region predisposes to a polyarticular disease course. The A-allele was significantly increased in this combined group (51% in cases, 41% in controls), with an allelic OR 1.46 (95% CI 1.12 -1.90), a genotypic OR (AA vs GG) of 2.25 (95% CI

Table 1. Genotype and allele frequencies in different subtypes of JIA versus controls

Diagnosis [1]	n	Allele frequency	Genotype frequency				Allelic OR (95% CI)	Genotypic OR* (95% CI)	
		A	AA	AG	GG	p°		AA	AG
Controls	511	0.41	79 (0.16)	265 (0.52)	167 (0.33)				
Persistent Oligoarthritis	133	0.38	18 (0.14)	65 (0.49)	50 (0.38)	0.297	0.87 (0.66- 1.14)	0.76 (0.42- 1.39)	0.82 (0.54- 1.24)
Extended Oligoarthritis	48	0.49	10 (0.21)	27 (0.56)	11 (0.23)	0.136	1.36 (0.89- 2.06)	1.92 (0.78- 4.71)	1.55 (0.75- 3.20)
RF negative Polyarthritis	73	0.52	19 (0.27)	38 (0.51)	16 (0.22)	**0.012**§	**1.54** (1.09- 2.18)	**2.51** (1.23- 5.14)	1.50 (0.81- 2.77)
RF positive Polyarthritis	18	0.50	5 (0.28)	8 (0.44)	5 (0.28)	0.288	1.42 (0.73- 2.75)	2.11 (0.60- 7.51)	1.01 (0.32- 3.13)
Systemic JIA	41	0.37	3 (0.07)	24 (0.59)	14 (0.34)	0.375	0.82 (0.51- 1.30)	0.45 (0.13- 1.62)	1.1 (0.54- 2.1)
All JIA patients	338	0.44	59 (0.17)	179 (0.53)	100 (0.30)	0.281	1.11 (0.91- 1.35)	1.25 (0.82- 1.90)	1.13 (0.83- 1.54)

1) Diagnosis according to the revision ILAR classification[2]
°) p-value of the Cochran-Armitage Trend test
§) p-value of <0.05 is considered statistically significant
*) GG as reference genotype

1.29- 3.90) and a p-value (Cochran-Armitage Trend test) of 0.004 which remains significant after bonferonni correction (p<0.013).
Since persistent and extended oligoarthritis are clinically similar at disease onset, but differ in their course and outcome, we tested the hypothesis that these two subtypes would also differ in their genetic predisposition. Intriguingly, we detected a borderline significant difference between these two subtypes when compared directly to each other (Allelic OR 1.57, 95% CI 0.98- 2.51; p=0.055).
Together, these data indicate that the A-allele predisposes predominantly to subtypes of JIA characterized by a polyarticular course, indicating that this allele does not associate with JIA as such, but rather with a particular phenotype of JIA.

DISCUSSION

In this study we report for the first time an association between the *TRAF1/C5* region and JIA. The A-allele of rs10818488 was significantly associated with the susceptibility to RF negative polyarthritis. However, patients with RF negative polyarthritis have a considerable phenotypic overlap with patients with extended oligoarthritis and RF positive polyarthritis, in having a polyarticular course of disease. Intriguingly, rs10818488 seems to be associated with this polyarticular phenotype and this difference remained significant after bonferroni correction for multiple tests (p<0.013).
Although we cannot formally exclude the possibility that population- stratification may play a role in this study, we did not observe any statistically significant differences in the minor allele frequencies of the patients from the Netherlands, Belgium and Germany. Additionally, analyzing the Dutch population independently did not alter the results (e.g. RF negative polyarthritis vs controls: Allelic OR 1.66 (95% CI 1.10- 2.51), genotypic OR (AA vs GG) 3.05 (95% CI 1.25- 7.44).
At the clinical level, it is also important to make a distinction between persistent oligoarthritis and extended oligoarthritis in order to predict the probability of the development of a polyarticular disease course and adjust the medical treatment accordingly. Comparison of patients with persistent and extended oligoarthritis revealed a borderline significant difference in A-allele frequency. This may indicate that the *TRAF1/C5* region may eventually be helpful in predicting the development of an extended course in patients with oligoarthritis. Extended studies of this polymorphism may confirm its relevance as a predictive marker.
It is presently unclear how the association between the *TRAF1/C5* region and disease susceptibility can be explained biologically. The associated polymorphism is highly linked to the *TRAF1* gene as well as the 3' untranslated region of the C5 gene. Acti-

vated complement-component-5 acts as a strong chemoattractant for neutrophils, and a deregulated activity may contribute to the perpetuation of inflammation. In JIA, complement activation is occasionally observed, especially in active polyarthritis.[8] On the other hand, TNF-receptor-associated factor-1 (TRAF-1) plays an essential role in the intracellular TNF-signaling pathway and is possibly a negative regulator of TNF-signalling.[9] Evidence for the importance of TNF in JIA is suggested by the effectiveness of treatment directed against TNF-alpha, especially in subtypes with a polyarticular course.[10] However, future research will be necessary to confirm the genetic association and investigate functional consequences of the associated allele and could reveal further insight in the pathogenesis of polyarticular disease in JIA.
Apart from being a well replicated risk factor for RA, *TRAF1/C5* also appears to be a risk factor for the RF negative polyarthritis subtype of JIA and, more generally, seems to be associated with subtypes of JIA characterized by a polyarticular course.

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CHAPTER 3

THE AUTOIMMUNE 4Q27 LOCUS IS ASSOCIATED WITH JUVENILE IDIOPATHIC ARTHRITIS

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H. M. Albers,[1] F. A. S. Kurreeman,[1] G. Stoeken-Rijsbergen,[1] D. M. C. Brinkman,[1] S. S. M. Kamphuis,[2] M. A. J. van Rossum,[3] H. J. Girschick,[4] C. Wouters,[5] R. K. Saurenmann,[6] E. Hoppenreijs,[7] P. Slagboom,[1] J. J. Houwing-Duistermaat,[1] W. Verduijn,[1] T. W. J. Huizinga,[1] R. ten Cate,[1] R. E. M. Toes,[1] and M. W. Schilham[1]

1) Leiden University Medical Center, Leiden, the Netherlands
2) Erasmus University Medical Center- Sophia Children's Hospital, Rotterdam, the Netherlands
3) Academic Medical Center- Emma Children's Hospital and Reade, location Jan van Breemen, Amsterdam, the Netherlands
4) University of Wurzburg, Wurzburg, Germany
5) University Hospital Gasthuisberg, Leuven, Belgium
6) Zurich University Children's Hospital, Zurich, Switzerland
7) Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

ABSTRACT

Objective

Juvenile Idiopathic Arthritis (JIA) is characterized by chronic arthritis and an autoimmune etiology. In several autoimmune diseases, amongst which rheumatoid arthritis, an association has been found with the 4q27 locus. In this study the possible role of the 4q27 locus in JIA has been investigated.

Materials & Methods

A case-control association study was performed in a total of 655 Caucasian JIA patients and 791 healthy controls in two independent sample sets. The rs6822844 marker in the 4q27 locus was genotyped.

Results

In the first and largest sample set a 5% decrease in T-allele frequency was observed in patients compared to controls (allelic OR 0.72, 95% CI 0.55- 0.95; p= 0.019), together with a decrease of 2.5% in T-allele frequency in the second sample set (allelic OR 0.81, 95% CI 0.61- 1.09; p=0.169). The combined dataset generated an OR of 0.76 (95%CI 0.62- 0.93; p= $7.08*10^{-3}$). When analyzing the different JIA subtypes individually, a significant decrease was seen in the subtypes with a polyarticular course of disease (extended oligoarthritis; p= 0,019 and RF negative polyarthritis; p=0,038).

Conclusion

The 4q27 locus, previously reported to be associated with rheumatoid arthritis, type 1 diabetes, celiac disease and psoriatic arthritis, is also associated with susceptibility to juvenile idiopathic arthritis.

INTRODUCTION

Juvenile Idiopathic Arthritis (JIA) is a group of heterogeneous disorders characterized by chronic arthritis diagnosed in children less than 16 years of age.[1] Seven different subtypes can be distinguished according to the ILAR classification.[2] The subtypes oligoarthritis (persistent and extended) and rheumatoid factor (RF) negative polyarthritis are considered the most homogeneous subtypes with shared phenotypic features. Systemic JIA has a more distinct phenotype resembling an autoinflammatory syndrome. Although the precise etiology is still unknown, JIA is considered to be an autoimmune disease.

Genetic studies in autoimmune diseases have revealed the presence of shared common autoimmune susceptibility loci.[3] In JIA, associations have been described with the *MHC* locus, *PTPN22* and *TRAF1/C5*.[4-6] The 4q27 locus, a region of strong linkage disequilibrium (LD) including the genes encoding interleukin 2 (*IL2*) and interleukin 21 (*IL21*), has been associated with celiac disease, rheumatoid arthritis, type 1 diabetes and psoriatic arthritis.[7-9] To answer the question whether the 4q27 locus is also associated with JIA we genotyped rs6822844, which can be used as a proxy for the 4q27 haplotype that is associated with autoimmune disease,[8] in JIA patients and controls.

MATERIAL & METHODS

Patient population

A case-control association study was performed in two independent sample sets consecutively. These sample sets consisted of Caucasian JIA patients, recruited from pediatric rheumatology centers in the Netherlands (n=327), Belgium (n= 96), Germany (n=95) and Switzerland (n=137) and healthy Dutch adult controls, who were randomly selected by the section Immunogenetics and transplantation Immunology of the Leiden University Medical Center, the Netherlands. The first sample set contained 328 JIA patients and 465 healthy controls, and the second sample set consisted of 327 JIA patients and 326 controls. All JIA patients (69% female, 31% male) were diagnosed according to the revised ILAR classification.[2] The inclusion of patients focused on the oligoarthritis (persistent and extended) and rheumatoid factor (RF) negative polyarthritis subtypes, because of their homogeneous phenotypes. The overall JIA patient group included 44% persistent oligoarthritis, 13% extended oligoarthritis, 24% RF negative polyarthritis, 3% RF positive polyarthritis, 11% systemic JIA patients and 5% patients with other JIA subtypes. Because of the small sample size in the RF positive

polyarthritis patients and JIA patients with "other" subtypes, these groups were excluded from further subanalysis. All patients had a self-reported European Caucasian ethnicity. Written informed consent was obtained from all patients and/or parents and the institutional review boards from all participating centers approved the study.

DNA and genotyping

DNA was collected by means of a blood sample (12% of cases and all controls) or a mouthswab (88% of cases). Genotyping was performed using MassArray matrix-assisted laser desorption ionization time-of-flight mass spectrometry, according to the protocols recommended by the manufacturer (Sequenom, San Diego, California, USA). Each 384-well plates contained 8 positive controls (CEPH DNA), 8 negative controls and 10% duplicates. The error rate was less than 1%. Random genotyping failure occurred in 3% cases and in 1% controls.

Statistical analysis

Because of the adherence to an additive model and the lack of evidence for a recessive model, we compared cases and controls using allelic odds ratios (OR) with 95% confidence interval (CI). The common OR of the two independent sample sets combined was calculated using the Mantel-Haenszel test. There was no heterogeneity between the two sample sets (Breslow-Day test $p<0.05$). Case and control genotype frequencies did not deviate from Hardy Weinberg equilibrium. All statistical analysis was performed with SPSS 14.0. A p-value of <0.05 was considered statistically significant.

RESULTS

To test for association of the 4q27 region with JIA, rs6822844 was typed in two independent sample sets of patients and controls, consecutively. In the first and largest sample set the T-allele frequency was significantly decreased from 20% in controls to 15% in patients (allelic OR 0.72, 95%CI 0.55- 0.95; p=0.019). The same trend in decrease in T-allele frequency from 18% to 15% was observed in the second sample set, although did not reach statistical significance (allelic OR 0.81, 95% CI 0.61- 1.09; p=0.169). The common OR of sample set 1 and 2 combined showed a positive association with JIA ($p=7.08x10^{-3}$) (Table 1).

As it is important to investigate genetic risk factors in homogeneous, well-defined phenotypic groups, we also analyzed association in the different JIA subtypes (Table 2). Although a trend towards a decreased T-allele frequency was observed in persistent oligoarthritis and systemic JIA, only the subtypes with a polyarticular course of

disease (extended oligoarthritis; p= 0,019 and RF negative polyarthritis; p=0,038) showed a significant decrease in T allele frequency.

Table 1. Genotype and allele frequencies (rs6822844) in JIA patients and controls in two independent sample sets

		n	Genotype frequency n (%)			Allele frequency n (%)	Allelic OR (95% CI)°	p
			GG	GT	TT	T		
Set 1	Controls	460	293 (63.7)	152 (33.0)	15 (3.3)	182 (19.8)		
	JIA patients	311	224 (72.0)	80 (25.7)	7 (2.3)	94 (15.1)	0.72 (0.55- 0.95)	0.019*
Set 2	Controls	323	218 (67.5)	95 (29.4)	10 (3.1)	115 (17.8)		
	JIA patients	324	233 (71.9)	85 (26.2)	6 (1.9)	97 (15.0)	0.81 (0.61- 1.09)	0.169
Combined data sets							0.76 (0.62- 0.93)	7.08 $x10^{-3}$ *

* p-value <0.05 considered significant
° in the combined dataset the Mantel-Haenszel OR is used.

Table 2. Analysis of allele-frequencies in the different subtypes of JIA in the overall cohort of cases and controls

Diagnosis•	n	T-allele	Allelic OR (95% CI)	p
Control	783	0.190		
Persistent oligoarthritis	275	0.165	0.85 (0.65- 1.10)	0.207
Extended oligoarthritis	83	0.114	0.55 (0.34- 0.91)	0.019*
RF negative polyarthritis	151	0.139	0.69 (0.49- 0.98)	0.038*
Systemic JIA	69	0.152	0.77 (0.47- 1.24)	0.280

* p-value <0.05 considered significant
• Diagnosis according to the revised ILAR classification (2)

DISCUSSION

This study shows for the first time a positive association of the 4q27 locus (rs6822844) in JIA, where a protective effect of the T-allele was observed. When testing the different JIA subtypes individually, a decrease in T-allele frequency was observed in all subtypes. Interestingly, only in the JIA subtypes that share a polyarticular phenotype this decrease was significant. A similar observation was made for the recently identified association with the *TRAF1/C5* region indicating a common genetic constitution underlying the polyarticular phenotype.[4]

With patients originating from different European countries, population stratification cannot completely be ruled out. However no significant variance in allele- frequency in control populations from Western Europe has been reported[7;8] and the allele- frequency observed in our control population was very similar to previously reported frequencies. In addition, when comparing the allele-frequencies in the cases originating from the different European countries, no significant difference was found (p=0,77). Moreover, the underrepresentation of the minor T-allele and the 4q27 effect size is similar as described in other autoimmune diseases such as RA and T1D.[8]

The 4q27 locus consists of a large region of strong linkage disequilibrium (LD) encoding the genes *KIAA1109*, *TENR*, *IL2* and *IL21*. Both *IL2* and *IL21* are likely candidates for association with susceptibility to JIA, as both cytokines are involved in immune activation and regulation pathways.

The IL2 pathway, in which the interaction between IL2 and the IL2-receptor-alpha (IL2RA) is central, is involved in T-cell proliferation and regulation.[10] Mice deficient in *Il2*, have T-cells with impaired proliferation and effector function in vitro and develop lethal autoimmunity.[11] Not only the 4q27 locus, but also the *IL2RA* locus has been associated with several autoimmune diseases,[12;13] indicating an important role of the IL2-pathway in immune regulation and maintenance of self-tolerance.

IL21 is involved in a wide range of immunological processes. IL21 appears to play a role in autoimmunity by both influencing the cellular immune response through inhibition of suppression by CD4+ regulatory T- cells and generating Th17 cells, as well as by influencing the humoral response.[14]

Further analysis of the immunologic pathways involved in JIA may be helpful in identifying the causal gene in this locus. Moreover, sequencing, fine-mapping and extensive testing of variants of this region will be required to narrow down the region of association and identify the associated gene as well as functional testing of all the linked variants associated with disease.

In conclusion, like rheumatoid arthritis, type 1 diabetes, celiac disease and psoriatic arthritis, also JIA is associated with the 4q27 locus. The identification of the 4q27 locus as a risk factor for JIA contributes to the collected evidence that one of the genes in this region plays a role in autoimmune diseases in general. In addition, our data indicate that the 4q27 locus contributes to the genetic susceptibility to JIA and warrants further research into the biological pathways explaining this association.

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CHAPTER 4

CD226 (*DNAM-1*) IS ASSOCIATED WITH SUSCEPTIBILITY TO JUVENILE IDIOPATHIC ARTHRITIS

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H.M. Albers[1], T.H.C.M. Reinards[1*], D.M.C. Brinkman[2], S.S.M. Kamphuis[3], M.A.J. van Rossum[4], H.J. Girschick[5], C. Wouters[6], E.P.A.H. Hoppenreijs[7], R.K. Saurenmann[8], A.Hinks[9], J.A. Ellis[10], E. Bakker[1], W. Verduijn[1], P. Slagboom[1], T.W.J. Huizinga[1], R.E.M. Toes[1], J.J. Houwing-Duistermaat[1], R. ten Cate[1#], M.W. Schilham[1#]*

**) shared first authorship*
#) shared last authorship
1) Leiden University Medical Center, Leiden, The Netherlands
2) Rijnland Hospital, Leiderdorp, the Netherlands
3) Erasmus Medical Center- Sophia Children's Hospital, Rotterdam, the Netherlands
4) Academic Medical Center- Emma Children's Hospital and Reade, location Jan van Breemen, Amsterdam, the Netherlands
5) Vivantes Children's Hospital, Berlin-Friedrichshain, Germany
6) University Hospital Gasthuisberg, Leuven, Belgium
7) St Maartenskliniek and Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
8) Zürich University Children's Hospital, Zürich, Switzerland,
9) Manchester Academic Health Science Centre- The University of Manchester, Manchester, United Kingdom
10) The University of Melbourne, Melbourne, and Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Australia

ABSTRACT

Objectives

Juvenile idiopathic arthritis (JIA) is considered a complex genetic autoimmune disease. We investigated the association of genetic variants previously implicated in JIA, autoimmunity and/or immunoregulation, with susceptibility to JIA.

Methods

A genetic association study was performed in 639 JIA patients and 1613 healthy controls of North-West European descent. Ninety-three single nucleotide polymorphisms (SNPs) were genotyped in a candidate gene approach. Results of the entire JIA patient group (all subtypes) were compared to results obtained, alternatively, with a clinically homogeneous patient group including only oligoarticular and rheumatoid factor (RF) negative polyarticular JIA patients (n=493). Meta-analyses were performed for all SNPs that have been typed in other Caucasian JIA cohorts before.

Results

SNPs in or near *PTPN22*, *VTCN1*, the *IL2-IL21* region, *ANKRD55*, and *TNFA* were confirmed to be associated with JIA ($p<0.05$), strengthening the evidence for involvement of these genes in JIA. In the majority of these replicated SNPs, effect sizes were larger when analysing a homogeneous patient cohort than when analysing all subtypes. We identified two novel associations with oligoarticular and RF negative polyarticular JIA: *CD226* rs763361 (OR 1.30, 95%-CI 1.12-1.51, p=0.0006) and *CD28* rs1980422 (OR 1.29, 95%-CI 1.07-1.55, p=0.008). Meta-analyses including reported studies confirmed the association of both SNPs with susceptibility to JIA (OR 1.16, p=0.001 and OR 1.18, p=0.001, for rs763361 and rs1980422 respectively).

Conclusions

The *CD226* gene has been identified as novel association with JIA, and a SNP near *CD28* as a suggestive association. Both genes are probable candidate risk factors since they are involved in costimulation of T cells.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease in childhood. Prevalence numbers vary from 4 to 400 per 100,000 children.[1] JIA comprises a heterogeneous group of conditions that share chronic arthritis with onset before the age of sixteen. Seven distinct subtypes have been defined by the International League of Associations of Rheumatologists (ILAR) based on clinical characteristics and laboratory parameters.[2] However, phenotypic overlap between subtypes does exist, particularly between oligoarthritis (persistent and extended) and rheumatoid factor (RF) negative polyarthritis. These subtypes are only distinguished on the basis of the number of affected joints at onset and during the course of the disease.[3] A proportion of these patients have circulating antinuclear antibodies (ANA) and are specifically at risk for developing JIA-associated uveitis.[3]

The pathogenesis of JIA is not well understood. It is considered an autoimmune disease in which a deregulated T cell response towards an, as yet unidentified, self-antigen causes joint inflammation.[4] In most subtypes, synovial inflammation, which eventually leads to bone erosion, is associated with an overproduction of pro-inflammatory cytokines, such as TNF-α and IL-17.[5-9] JIA is a complex trait in which both genetic and environmental factors seem to be involved. Ethnic differences in epidemiologic studies,[1,10] as well as an increased risk of JIA for relatives of patients (sibling recurrence risk ratio λ_S of 12),[11-13] form evidence for genetic contribution to the risk of JIA.

It has become increasingly clear that autoimmune diseases cluster in individuals and families.[14,15] In line with this, genetic variations have been identified that are associated with more than one autoimmune disease, like rheumatoid arthritis (RA), type 1 diabetes mellitus, autoimmune thyroid disease, inflammatory bowel disease, systemic lupus erythematosus, multiple sclerosis, and also JIA.[16-20] These results imply the existence of general genetic susceptibility to autoimmune diseases.

Identification of genetic risk variants could contribute to understanding of disease pathways, improve diagnosis of (subtypes of) JIA, and ultimately improve prognosis by providing new targets for therapy. Both candidate gene and genome-wide association studies (GWAS) have been performed to elucidate the genetic basis of JIA. Compared to more common autoimmune diseases, until recently JIA cohorts were small and heterogeneous. Only few genetic associations had been replicated, such as *PTPN22* and the major histocompatibility complex (MHC) region.[21,22] Other (suggestive) JIA susceptibility loci have been reported, but not confirmed, such as *ANKRD55* on 5q11.[22] Nevertheless, replication of these loci is essential to exclude false positive associations. Therefore we investigated in a Caucasian JIA cohort

genetic loci previously implicated in JIA. Additionally, we investigated the association of genetic loci implicated in autoimmunity and/or immunoregulation with JIA. Analyses were performed in both a large but relatively heterogeneous JIA patient cohort (including all subtypes), and a smaller but phenotypically more homogeneous patient group (including only the persistent and extended oligoarticular and RF negative polyarticular subtypes).

METHODS

Subjects

DNA was available from 639 JIA patients with all subtypes, recruited through seven collaborating paediatric rheumatology referral centres in The Netherlands, Belgium, Germany, and Switzerland. All patients were of self-reported or parent-reported North-West European Caucasian descent. JIA cases were classified according to the revised ILAR criteria.[2] The patient group contained 263 patients with persistent oligoarthritis, 88 with extended oligoarthritis, and 142 with RF negative polyarthritis, resulting in a homogeneous group of 493 patients (Table 1).

DNA samples from healthy Caucasian controls were collected from three sources. See online supplementary methods for a detailed description of the control panels. Of the 93 markers that were successfully typed in the JIA patients, 40 were typed in 869 controls and 53 in 1319 controls (supplementary Tables S1, S2).

All patients and controls provided informed consent. The institutional review boards of all participating centres approved this study.

Genotyping

We genotyped single nucleotide polymorphisms (SNPs) adopting a candidate gene approach. The choice for specific genes and/or SNPs was based on previous reports suggesting involvement in JIA, other autoimmune diseases and/or immunoregulation. 93 SNPs located in 57 genes/loci passed quality control, listed in supplementary Table S2. See online supplementary methods for a detailed description of genotyping methods.

Statistical analysis

Allele frequencies were compared between JIA cases and controls. Allelic odds ratios (OR) and 95% confidence intervals (CI) were calculated using the allelic case-control association test in PLINK.[23] These ORs correspond to the genotypic ORs of an additive model. We differentiated between loci that have been associated with JIA before ('JIA replication loci'; 36 of 93 successfully typed SNPs) and loci that have not been impli-

Table 1
Patient characteristics of the JIA cohort

	n	(%)
Total cohort	639	
Gender		
Female	439	(69)
Male	200	(31)
Origin		
The Netherlands	324	(51)
Belgium	94	(15)
Germany	93	(15)
Switzerland	128	(20)
Subtype		
Persistent oligoarthritis	263	(41)
Extended oligoarthritis	88	(14)
RF negative polyarthritis	142	(22)
RF positive polyarthritis	22	(3)
Systemic JIA	73	(11)
Psoriatric arthritis	4	(<1)
Enthesitis related arthritis	3	(<1)
Undifferentiated JIA	44	(7)
ANA status		
Positive	280	(44)
Negative	229	(36)
Inconclusive/unknown	130	(20)
Family history [a]		
AID in 1st degree relative	69	(16)
AID in 1st or 2nd degree relative	169	(38)

RF: rheumatoid factor; ANA: antinuclear antibodies; AID: autoimmune disease
a) Family history known of 442 patients

cated in JIA before (57 SNPs) (supplementary Table S2). For analysis of 'replication loci' we included patients with all JIA subtypes, to conform to reported studies that revealed these JIA loci. Because there was a prior probability that these loci would be associated with JIA in our study too, a p value < 0.05 was considered significant for these SNPs. We also performed the association analyses including only the most homogeneous JIA subtypes (persistent and extended oligoarthritis and RF negative polyarthritis, n=493). For the other 57 SNPs we analysed these 493 homogeneous JIA patients by comparing them as a group to controls. To adjust for multiple testing, a Bonferroni correction should be applied to the results for these 57 SNPs, leading to a significance threshold

of p < 0.001. Additionally, we performed ILAR subtype-specific case-control analyses (within the homogeneous patient group) for all 93 SNPs, and also compared all ANA positive patients within this group to controls (supplementary Table S3).
Meta-analyses were performed for all SNPs that have been investigated in JIA before (supplementary Tables S4, S5). We included reported genetic association studies in Caucasian case-control cohorts (including a mixed set of JIA subtypes), in which the same SNPs have been typed. We excluded studies for which data necessary to calculate allelic ORs were not available. Not all JIA replication loci were included for meta-analyses because of these inclusion and exclusion criteria. In case of overlapping individuals in reported studies, we only included the study with the largest JIA cohort. For meta-analyses of rs1980422 near *CD28* and rs763361 in *CD226*, we performed analyses in homogeneous (oligoarticular and RF negative polyarticular) JIA patients.[18,24,25] Because of the small number of studies included in the meta-analyses, a fixed-effects model was used in a Mantel-Haenszel test.
All statistical analyses were performed with use of the software PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).[23]

RESULTS

Replication of JIA loci

Thirty-six SNPs that have been previously reported to be associated with JIA were investigated in our entire JIA cohort consisting of 639 patients with all seven different subtypes. To conform to reported studies, all ILAR subtypes were included. Nine of 36 reported SNPs were confirmed to be associated (p < 0.05): *VTCN1* rs10923217, *KIAA1109* rs4505848, *IL21* rs1398553, *ANKRD55* rs6859219, *TNFA* rs1799724, rs1800750, rs361525, and rs1800610, and *MIF* rs755622 (Table 2). Because we propose to perform association studies only in a patient cohort as homogeneous as possible, additional analyses were limited to only oligoarthritis and RF negative polyarthritis (n=493). Although limiting the patient group led to fewer cases, all associations were still significant (Table 2). By performing analyses in the smaller, more homogeneous JIA cohort, the reported JIA SNPs *PTPN22* rs2476601 and *TNFA* rs1800629 were additionally confirmed. In the majority of these replicated associations, analysis in the more homogeneous patient cohort led to larger effect sizes. For all SNPs that had been investigated in Caucasian JIA cohorts before, a meta-analysis was performed. For 23 of 36 replication loci, appropriate data for meta-analyses were available, of which *PTPN22* rs2476601, *VTCN1* rs10923217, rs6669320, rs10923223 and rs12046117, *PTPRC* rs10919563, *AFF3* rs1160542, *CCR5* rs333, *TNFA* rs1799724,

Table 2. Association analyses of reported JIA loci

							All JIA subtypes		*Only homogeneous JIA subtypesb*			
Chr	**Position[a]**	**Gene/ region**	**SNP**	**Minor allele**	**MAF controls**	**MAF cases**	**OR (95% CI)**	**P (allelic)**	**MAF cases**	**OR (95% CI)**	**P (allelic)**	**Reference**
1	114377568	*PTPN22*	**rs2476601**	A	0.10	0.12	1.26 (1.00-1.59)	0.051	0.13	**1.32 (1.03-1.69)**	**0.02702**	[54]
1	117685992	*VTCN1*	rs6673837	A	0.20	0.21	1.04 (0.86-1.24)	0.7101	0.20	0.99 (0.82-1.21)	0.9474	[55]
1	117690758	*VTCN1*	rs2358817	T	0.08	0.07	0.83 (0.63-1.10)	0.1933	0.07	0.88 (0.65-1.19)	0.4164	[55]
1	117711911	*VTCN1*	rs2358820	A	0.07	0.06	0.89 (0.66-1.20)	0.4467	0.07	0.92 (0.67-1.26)	0.602	[55]
1	117730048	***VTCN1***	**rs10923217**	C	0.48	0.52	**1.20 (1.04-1.40)**	**0.01358**	0.52	**1.21 (1.03-1.42)**	**0.02079**	[55]
1	117730623	*VTCN1*	rs6669320	A	0.15	0.13	0.89 (0.72-1.11)	0.3032	0.13	0.89 (0.70-1.12)	0.3117	[55]
1	117746573	*VTCN1*	rs10923223	C	0.15	0.16	1.16 (0.95-1.42)	0.1571	0.16	1.09 (0.88-1.36)	0.4383	[55]
1	117751365	*VTCN1*	rs12046117	T	0.13	0.14	1.11 (0.89-1.37)	0.3522	0.13	1.04 (0.82-1.32)	0.7252	[55]
1	198700442	*PTPRC*	rs10919563	A	0.13	0.12	0.91 (0.74-1.13)	0.3853	0.11	0.86 (0.68-1.08)	0.195	[20]
2	100832155	*AFF3*	rs1160542	G	0.45	0.46	1.05 (0.92-1.21)	0.4699	0.46	1.05 (0.90-1.22)	0.5177	[18]
2	100835734	*AFF3*	rs10865035	A	0.46	0.47	1.05 (0.91-1.23)	0.4924	0.47	1.05 (0.89-1.24)	0.5762	LD[18]
2	113590390	*IL1B*	rs1143634	A	0.25	0.24	0.93 (0.79-1.11)	0.4424	0.24	0.95 (0.79-1.14)	0.5542	[56]
2	204732714	*CTLA4*	rs231775	G	0.37	0.35	0.91 (0.78-1.06)	0.2112	0.35	0.92 (0.78-1.09)	0.3251	LD[29]
3	46414947	*CCR5*	rs333	del	0.10	0.09	0.83 (0.65-1.05)	0.1155	0.08	0.81 (0.62-1.06)	0.1168	[57]
4	123132492	***KIAA1109***	**rs4505848**	G	0.36	0.40	**1.23 (1.07-1.42)**	**0.004019**	0.40	**1.20 (1.03-1.41)**	**0.01901**	[58]
4	123348345	*ADAD1*	rs11732095	G	0.08	0.07	0.90 (0.68-1.17)	0.4229	0.07	0.91 (0.68-1.23)	0.5508	LD[58]
4	123514528	*IL2-IL21*	rs4492018	A	0.24	0.21	0.85 (0.72-1.00)	0.05315	0.21	0.88 (0.73-1.05)	0.1495	LD[58]
4	123548068	***IL21***	**rs1398553**	T	0.33	0.38	**1.24 (1.07-1.43)**	**0.003359**	0.38	**1.22 (1.05-1.43)**	**0.0109**	LD[58]
5	55438580	***ANKRD55***	**rs6859219**	A	0.22	0.18	**0.77 (0.64-0.92)**	**0.003175**	0.17	**0.74 (0.61-0.90)**	**0.002953**	LD[22]
6	31542482	***TNFA***	**rs1799724**	T	0.10	0.13	**1.37 (1.09-1.73)**	**0.007318**	0.13	**1.40 (1.09-1.79)**	**0.007489**	LD[59]
6	31542963	***TNFA***	**rs1800750**	A	0.02	0.01	**0.37 (0.17-0.80)**	**0.008935**	0.01	**0.36 (0.15-0.86)**	**0.01635**	LD[59]
6	31543031	***TNFA***	**rs1800629**	A	0.17	0.15	0.87 (0.71-1.06)	0.172	0.14	**0.79 (0.63-0.99)**	**0.04234**	[60]

Table 2. Association analyses of reported JIA loci (continued)

							All JIA subtypes		*Only homogeneous JIA subtypesb*			
Chr	**Position[a]**	**Gene/ region**	**SNP**	**Minor allele**	**MAF controls**	**MAF cases**	**OR (95% CI)**	**P (allelic)**	**MAF cases**	**OR (95% CI)**	**P (allelic)**	**Reference**
6	31543101	***TNFA***	**rs361525**	A	0.05	0.03	**0.56 (0.37-0.83)**	**0.003987**	0.03	**0.55 (0.35-0.86)**	**0.007526**	[59]
6	31543827	***TNFA***	**rs1800610**	A	0.10	0.13	**1.34 (1.06-1.69)**	**0.01332**	0.13	**1.38 (1.08-1.77)**	**0.009952**	LD[59]
6	31544189	*TNFA*	rs3093662	G	0.06	0.04	0.75 (0.53-1.05)	0.09238	0.04	0.75 (0.52-1.09)	0.1328	LD[59]
6	138006504	*TNFAIP3*	rs6920220	A	0.21	0.20	0.98 (0.82-1.18)	0.8275	0.20	0.98 (0.80-1.19)	0.821	[16]
7	128594183	*TNPO3*	rs10488631	C	0.10	0.10	1.00 (0.79-1.26)	0.9903	0.10	1.03 (0.80-1.33)	0.8078	[20]
10	6053163	*IL2RA*	rs12722605	T	0.14	0.15	1.03 (0.85-1.25)	0.7694	0.15	1.05 (0.85-1.30)	0.6622	LD[22]
10	6099045	*IL2RA*	rs2104286	G	0.25	0.25	0.97 (0.82-1.15)	0.7435	0.23	0.91 (0.76-1.10)	0.3382	[61]
10	6114660	*IL2RA*	rs41295061	A	0.09	0.09	1.02 (0.81-1.30)	0.8551	0.09	1.02 (0.79-1.32)	0.8834	[61]
16	11179873	*CLEC16A*	rs12708716	G	0.35	0.34	0.94 (0.82-1.09)	0.4245	0.33	0.92 (0.78-1.07)	0.2729	LD[62]
16	11249329	*CLEC16A*	rs6498169	G	0.34	0.36	1.08 (0.93-1.24)	0.3049	0.37	1.09 (0.93-1.28)	0.2653	[62]
22	24236392	***MIF***	**rs755622**	C	0.21	0.16	**0.69 (0.57-0.84)**	**0.0002126**	0.15	**0.67 (0.54-0.83)**	**0.0002084**	[28]
22	37544245	*IL2RB*	rs3218258	T	0.28	0.28	0.99 (0.85-1.15)	0.8765	0.27	0.97 (0.82-1.15)	0.7135	LD[22]
22	37544810	*IL2RB*	rs3218253	T	0.28	0.28	1.01 (0.87-1.18)	0.8677	0.28	0.99 (0.84-1.17)	0.9164	LD[22]
22	37551607	*IL2RB*	rs743777	G	0.34	0.33	0.97 (0.84-1.12)	0.6348	0.33	0.95 (0.81-1.12)	0.5386	LD[22]

Chr: chromosome; MAF: minor allele frequency; OR: odds ratio; CI: confidence interval; LD: in linkage disequilibrium with reported JIA locus

a) Base-pair position is based on NCBI dbSNP build 136

b) Patient group limited to oligoarticular (persistent and extended) and RF negative polyarticular JIA patients

TNFAIP3 rs6920220, *TNPO3* rs10488631, *IL2RA* rs2104286, and *CLEC16A* rs6498169 were significant (supplementary Table S4).

Novel JIA loci

An additional 57 SNPs, not reported to be associated with JIA before, located in or near autoimmune loci, were investigated in the homogeneous patient cohort. One SNP was strongly associated with JIA, *CD226* rs763361 (OR 1.30, 95% CI 1.12-1.51, p=0.0006). This SNP has not been reported to be associated with JIA before and represents a novel association (Table 3). The effect is particularly prominent in the persistent oligoarthritis patients (OR 1.39, p=0.0008) (supplementary Table S3). *CD226* rs763361 has been investigated before in two other Caucasian JIA cohorts and a non-significant trend of this SNP towards association with JIA (all subtypes) was reported (p=0.13[18]; p=0.059[25]). We performed a meta-analysis combining the results from our study and the published data. To limit clinical heterogeneity between study cohorts, only patients with oligo-articular and RF negative polyarticular JIA were included in this meta-analysis. The meta-analysis revealed a combined association of this *CD226* variant with JIA, OR 1.16, p=0.001 (supplementary Table S5, Figure S1).

Rs1980422 near *CD28* was revealed as a suggestive association (OR 1.29, 95% CI 1.07-1.55, p=0.008) which was not significant after correction for multiple testing (Table 3). The effect of this SNP is particularly prominent in ANA-positive patients (OR 1.52, p=0.0004) (supplementary Table S3). This SNP was investigated before in a GWAS in Caucasian (oligoarticular and RF-negative polyarticular) JIA patients and a trend towards association was reported (p=0.04).[24] Also for this SNP we performed a meta-analysis combining the results of the present and the reported study, which resulted in a combined association of rs1980422 with JIA, OR 1.18, p=0.001 (supplementary Table S5, Figure S1).

Table 3
Polymorphisms in immune related genes associated with homogeneous subtypes of JIA[a]

Chr	Position[b]	Gene/ region	SNP	Minor allele	MAF controls	MAF cases	OR (95% CI)	P (allelic)
2	204610396	*CD28*	rs1980422	C	0.22	0.27	1.29 (1.07-1.55)	0.008079
18	67531642	*CD226*	rs763361	T	0.47	0.54	1.30 (1.12-1.51)	0.0006295

Chr: chromosome; MAF: minor allele frequency; OR: odds ratio; CI: confidence interval

[a] Homogeneous subtypes include oligoarticular (persistent and extended) and RF negative polyarticular JIA patients. Only SNPs that are (suggestively) associated with p<0.05 are listed.

[b] Base-pair position is based on NCBI dbSNP build 136

Pooling our results with published non-significant associations (24 of 57 SNPs) revealed a potential novel association of *PRKCQ* rs4750316 with JIA (OR 0.90, p=0.01) (supplementary Table S5).

DISCUSSION

We investigated the role of 93 genetic markers previously associated with JIA or involved in autoimmunity or immunoregulation in a large Caucasian JIA cohort. We identified the *CD226* gene as a novel association with JIA, and a SNP near *CD28* as a suggestive association.

While this study was powered (>=80%) to detect associations with an effect size (OR) >= 1.4, for SNPs with a minor allele frequency >= 0.10 at an alpha of 0.05, replication is the golden standard in genetic association studies and essential to exclude false-positive results. Collecting a large, homogeneous patient cohort which generates sufficient power is challenging in JIA, given the relatively low prevalence. A recent large study in JIA with dense genotyping of immune-related disease loci, which was published whilst this study was in progress, has also shown that increasing sample size improves power to detect true JIA loci.[22] Although meta-analyses have limitations in case of publication bias and clinical heterogeneity between cohorts, they are also valuable for evaluating potentially false-positive or false-negative associations. Pooling of our and published results provides additional evidence for associations of 13 SNPs in 9 loci previously implicated in JIA. Furthermore, pooling of non-significant results from several studies suggests association of rs4750316 near *PRKCQ* with JIA, which is also an RA-locus.[26,27] However, heterogeneity within a cohort (e.g. by including clinically different JIA subtypes) can be a pitfall leading to false-negative results.

We compared results obtained by analysing the entire JIA patient group in a case-control study to, alternatively, only JIA patients with the two clinically most similar ILAR subtypes (persistent and extended) oligoarthritis and RF negative polyarthritis). Although limiting the inclusion to two JIA subtypes resulted in smaller patient numbers, the study had enough power to confirm well-established JIA loci, e.g. *PTPN22* and *TNFA*. In the majority of replicated SNPs, effect sizes were larger when analysing only the homogeneous cohort. This argues in favour of restricting analyses to a homogeneous patient cohort, as has also been performed in two recent large studies in JIA.[22,24]

The associated loci *PTPN22* (rs2476601), *VTCN1* (rs10923217), *KIAA1109* (rs4505848), *IL21* (rs1398553), *ANKRD55* (rs6859219), and *TNFA* (rs1799724,

rs1800750, rs1800629, rs361525, rs1800610) were replicated in this study strengthening their involvement in JIA. Four of these were confirmed in a meta-analysis: *PTPN22* rs2476601, *VTCN1* rs10923217, and *TNFA* rs1799724 and rs361525. An additional nine JIA replication SNPs, not confirmed by this study, were significantly associated in the meta-analysis. All of these genes are also associated with RA and/or other autoimmune diseases and are probably involved in the regulation of the immune response. All genes except *ANKRD55* have obvious roles in immune processes. It should be noted that the C allele of rs755622 in the promoter region of *MIF* (encoding the proinflammatory cytokine macrophage migration inhibitory factor) is associated with protection to JIA in our study, but this conflicts with results from other JIA cohorts that suggest association of the C allele with susceptibility.[28-31] The reason for these opposing results is not clear, but the minor allele frequency (MAF) of this SNP in our control cohort is comparable to the MAF in other North-West European control cohorts.[29,32-34] Genetic studies of *MIF* in RA and inflammatory bowel disease have yielded similarly opposing results,[29,35-40] indicating that the role of this SNP in JIA and other autoimmune diseases is still unclear. The other investigated JIA loci, of which *IL2RA* and *IL2RB* reached genome-wide significance in a previous report, were not confirmed in our cohort, which might be due to insufficient power to detect modest risk loci.[22] A SNP in *IL2RA* was significant in the meta-analysis, underlining the importance of combining data from different JIA cohorts.

One of the strongest replicated associations with JIA is rs6859219, located at locus 5q11, in *ANKRD55*, an ankyrin repeat domain-containing gene with unknown function. This region has been recently identified by dense genotyping of immune-related disease loci in JIA patients.[22] Although nearby genes *IL6ST* and *IL31RA* encode proteins involved in immunity, SNPs in these genes are not in linkage disequilibrium with the associated SNP in *ANKRD55*. Ankyrin repeat domains are common protein structures and mediate protein-protein interactions. Although the precise function of *ANKRD55* is not known, it is specifically expressed in resting CD4+ T cells (http://www.amazonia.transcriptome.eu), which is interesting when a role in autoimmunity is presumed. Rs6859219 was previously found to be associated with RA in a GWAS and with multiple sclerosis.[41-43]

In addition to the confirmation of previously identified associations, two novel susceptibility loci for the most common JIA subtypes were discovered. *CD28* rs1980422 is only weakly associated, but is interesting because of its location between two immune related genes: *CD28* (approximately 10 kb away) and *CTLA4* or cytotoxic T-lymphocyte antigen-4 (approximately 129 kb away), a gene that is associated with multiple autoimmune diseases including RA, with conflicting results in JIA.[18,21,29,44,45] There is minimal linkage disequilibrium between rs1980422 and

several SNPs in *CTLA4*. We also investigated the well-established general autoimmunity SNP rs231775 in *CTLA4*, but this SNP was not associated with JIA in this study. Both gene products have opposing roles in T cell activation. CD28 is expressed on the T cell surface and involved in costimulation of T cells. CTLA4 is expressed by T cells upon activation by antigen presenting cells. It functions as an attenuator of T cell activation by competing with CD28 for shared ligands (CD80 and CD86). Rs1980422 near *CD28* was associated with RA in a GWAS.[46] In a recent GWAS in JIA, this SNP was tested, but not significantly associated with JIA after correction for multiple testing.[24] Combining these results with ours in a meta-analysis resulted in a significant association. The *CD28* region has not been identified as a significant JIA susceptibility locus in a recent, large association study in which the ImmunoChip was used, but interestingly, this ImmunoChip study as well as the JIA GWAS revealed a strong association of SNPs in the region of *CD80*, coding for a ligand of CD28, with JIA.[22,24] The implication of three components of this costimulatory pathway, CD80, CTLA4 and CD28, in JIA and other autoimmune diseases is supportive for a role in autoimmune pathogenesis.

The most strongly associated novel SNP rs763361 is located in *CD226*, which encodes CD226 or DNAX accessory molecule 1 (DNAM-1). DNAM-1 is a type 1 membrane protein belonging to the Ig-supergene family. It is mainly expressed on T and NK cells and is involved in the adhesion and costimulation of these cells via its ligands CD112 and CD155.[47,48] The *CD226* region has not been identified as a (genome-wide significant) JIA susceptibility locus in the large association study with use of the ImmunoChip, which also captures *CD226*.[22] Nevertheless, a meta-analysis of our study with two previous candidate gene studies confirmed the association with JIA.[18,25] Furthermore, recent genetic studies have reported an association of this non-synonymous SNP (Gly307Ser) with susceptibility to multiple autoimmune diseases, as type 1 diabetes mellitus, autoimmune thyroid disease, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus, denoting it as a general autoimmunity locus.[49,50] A fine-mapping study of the 18q22 region, in which the SNP lies, was performed in type 1 diabetes mellitus and multiple sclerosis patients by exonic resequencing and tag SNP mapping.[49] This study pointed out the SNP as a probable causal variant. However, this study cannot exclude other (rare) variants in linkage disequilibrium with rs763361 being the true causal variant. If rs763361 would be correlated with altered expression and/or signaling, this could explain the contribution of this variant to autoimmunity. DNAM-1 deficient mice show impaired control of viral infections and less cytotoxic activity against tumors compared to wild type mice, suggesting a form of immunodeficiency when CD226 function is impaired.[51,52] In another report in which the role of CD226 in experimental autoimmune

encephalomyelitis (EAE, a model for multiple sclerosis) was studied, application of a monoclonal antibody against CD226 led to delayed onset and reduced severity of EAE.[53] This is suggestive for a role of CD226 in the development of autoimmune disease. By contrast, it is apparently contradictory that individuals that were homozygous for a *CD226* haplotype associated with susceptibility to systemic lupus erythematosus expressed lower *CD226* transcript levels and lower surface proteins on T cells and NK cells.[50] Replication in independent, homogeneous cohorts and additional fine-mapping and functional studies are needed to clarify the pathogenic implications of variation in these loci.

In summary, our data generate renewed interest for a role in JIA of two biological pathways that aid priming of T cells: the costimulatory mechanism involving CD28, CTLA4 and CD80/CD86, and the *CD226* gene, encoding the accessory molecule DNAM-1, which is a novel JIA susceptibility locus. This does not only contribute to knowledge of JIA pathogenesis, but targeting these T cell stimulating processes might also be of therapeutic interest.

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SUPPLEMENTARY DATA

Supplementary methods

Control subjects

1130 controls were healthy blood bank donors (758 randomly selected by the Immunogenetics and Transplantation Immunology (ITI) section of the Department of Immunohematology and Bloodtransfusion, and 372 by the Laboratory for Diagnostic Genome Analyses (LDGA) at Leiden University Medical Center, all from the region of Leiden, The Netherlands), 372 controls were anonymized individuals that requested genetic counselling at the LDGA regarding a monogenic disease in their families, but tested negative for this single genetic defect, and 111 controls were recruited via participating patients and their families (but unrelated) (supplementary Table S1). Due to limited availability of DNA, genetic markers were genotyped in different control cohorts. Of the 93 markers that were successfully typed in the JIA patients, 40 were typed in 869 controls and 53 in 1319 controls (supplementary Tables S1, S2).

DNA and genotyping

DNA was isolated from buccal swabs or a blood sample. We performed genotyping of 112 SNPs in 65 genes/loci by iPLEX MassARRAY according to the manufacturer's recommendations (Sequenom, San Diego, California, USA). Only SNPs exceeding a 90% call rate and no evidence for deviation from Hardy-Weinberg equilibrium in the control population ($p > 0.005$) were used for further analysis. 93 of 112 SNPs (83%) located in 57 genes/loci passed quality control, listed in supplementary Table S2. SNP call rates per individual exceeded 90%.

Supplementary Table S1
Control subjects

Set	Source	n	Type of control subjects
1	ITI	464	Healthy blood donors
2	ITI	294	Healthy blood donors
3	LDGA	372	Healthy blood donors
4	LDGA	372	Healthy relatives of monogenic disease patients
5	JIA families	111	Healthy unrelated acquaintances of JIA patients

ITI: Immunogenetics and Transplantation Immunology section; LDGA: Laboratory for Diagnostic Genome Analyses

Supplementary Table S2 Allele and genotype frequencies in oligoarticular (persistent and extended) and RF negative polyarticular JIA patients versus controls

Chr	Position[a]	Gene/ region	SNP	Minor allele	MAF controls	MAF cases	OR (95% CI)	P (allelic)	Genotype counts controls	Genotype counts cases	Typed in control sets[c]	Reported JIA locus[d]
									11 / 12 / 22[b]	11 / 12 / 22[b]		
1	2553624	*TNFRSF14-MMEL1*	rs3890745	G	0.32	0.30	0.90 (0.77-1.06)	0.2137	126 / 568 / 570	47 / 187 / 231	1, 3, 4, 5 (n = 1319)	no
1	12091210	*TNFRSF8-MIIP*	rs946461	T	0.26	0.29	1.15 (0.98-1.36)	0.09358	84 / 480 / 668	41 / 196 / 240	1, 3, 4, 5 (n = 1319)	no
1	12252955	*TNFRSF1B*	rs1061622	G	0.23	0.24	1.03 (0.85-1.24)	0.7678	44 / 309 / 492	34 / 160 / 281	1, 2, 5 (n = 869)	no
1	32729702	*LCK*	rs1004420	T	0.17	0.17	0.98 (0.80-1.19)	0.8087	46 / 332 / 839	11 / 135 / 314	1, 3, 4, 5 (n = 1319)	no
1	32743866	*LCK*	rs695161	C	0.48	0.47	0.97 (0.83-1.13)	0.6721	295 / 592 / 345	108 / 233 / 135	1, 3, 4, 5 (n = 1319)	no
1	114377568	*PTPN22*	rs2476601	A	0.10	0.13	1.32 (1.03-1.69)	0.02702	10 / 150 / 689	9 / 104 / 363	1, 2, 5 (n = 869)	yes
1	117685992	*VTCN1*	rs6673837	A	0.20	0.20	0.99 (0.82-1.21)	0.9474	40 / 265 / 544	19 / 154 / 302	1, 2, 5 (n = 869)	yes
1	117690758	*VTCN1*	rs2358817	T	0.08	0.07	0.88 (0.65-1.19)	0.4164	6 / 125 / 699	0 / 69 / 400	1, 2, 5 (n = 869)	yes
1	117711911	*VTCN1*	rs2358820	A	0.07	0.07	0.92 (0.67-1.26)	0.602	4 / 112 / 731	1 / 59 / 406	1, 2, 5 (n = 869)	yes
1	117730048	*VTCN1*	rs10923217	C	0.48	0.52	1.21 (1.03-1.42)	0.02079	201 / 397 / 239	132 / 232 / 109	1, 2, 5 (n = 869)	yes
1	117730623	*VTCN1*	rs6669320	A	0.15	0.13	0.89 (0.70-1.12)	0.3117	27 / 194 / 625	12 / 94 / 341	1, 2, 5 (n = 869)	yes
1	117746573	*VTCN1*	rs10923223	C	0.15	0.16	1.09 (0.88-1.36)	0.4383	13 / 221 / 614	15 / 119 / 341	1, 2, 5 (n = 869)	yes
1	117751365	*VTCN1*	rs12046117	T	0.13	0.13	1.04 (0.82-1.32)	0.7252	11 / 197 / 638	10 / 107 / 356	1, 2, 5 (n = 869)	yes
1	198700442	*PTPRC*	rs10919563	A	0.13	0.11	0.86 (0.68-1.08)	0.195	23 / 268 / 935	5 / 95 / 370	1, 3, 4, 5 (n = 1319)	yes
1	206946897	*IL10*	rs1800896	C	0.50	0.47	0.90 (0.77-1.06)	0.2119	219 / 408 / 219	92 / 238 / 115	1, 2, 5 (n = 869)	no
1	207015957	*IL19*	rs2243191	T	0.20	0.23	1.17 (0.96-1.43)	0.1209	27 / 283 / 536	21 / 155 / 261	1, 2, 5 (n = 869)	no
1	207038686	*IL20*	rs1400986	T	0.16	0.14	0.86 (0.69-1.08)	0.1975	23 / 230 / 595	7 / 122 / 344	1, 2, 5 (n = 869)	no
2	100832155	*AFF3*	rs1160542	G	0.45	0.46	1.05 (0.90-1.22)	0.5177	255 / 629 / 384	95 / 241 / 131	1, 3, 4, 5 (n = 1319)	yes
2	100835734	*AFF3*	rs10865035	A	0.46	0.47	1.05 (0.89-1.24)	0.5762	269 / 631 / 367	88 / 174 / 108	1, 3, 4, 5 (n = 1319)	yes
2	103070568	*IL18RAP*	rs917997	T	0.22	0.24	1.14 (0.94-1.37)	0.1782	45 / 283 / 519	29 / 172 / 272	1, 2, 5 (n = 869)	no
2	113537223	*IL1A*	rs17561	A	0.30	0.29	0.92 (0.77-1.09)	0.3289	74 / 366 / 406	32 / 194 / 226	1, 2, 5 (n = 869)	no
2	113542960	*IL1A*	rs1800587	A	0.31	0.28	0.89 (0.74-1.07)	0.2285	74 / 369 / 404	29 / 169 / 205	1, 2, 5 (n = 869)	no
2	113590390	*IL1B*	rs1143634	A	0.25	0.24	0.95 (0.79-1.14)	0.5542	57 / 306 / 485	20 / 185 / 269	1, 2, 5 (n = 869)	yes
2	113594867	*IL1B*	rs16944	A	0.33	0.35	1.11 (0.94-1.31)	0.2315	79 / 395 / 370	61 / 211 / 203	1, 2, 5 (n = 869)	no

Supplementary Table S2 Allele and genotype frequencies in oligoarticular (persistent and extended) and RF negative polyarticular JIA patients versus controls

Chr	Position[a]	Gene/ region	SNP	Minor allele	MAF controls	MAF cases	OR (95% CI)	P (allelic)	Genotype counts controls	Genotype counts cases	Typed in control sets[c]	Reported JIA locus[d]
2	162856148	*DPP4*	rs2268894	C	0.45	0.47	1.07 (0.91-1.25)	0.4192	164 / 437 / 247	99 / 246 / 130	1, 2, 5 (n = 869)	no
2	191835596	*STAT1*	rs3771300	C	0.48	0.50	1.10 (0.94-1.27)	0.2321	281 / 608 / 337	118 / 238 / 118	1, 3, 4, 5 (n = 1319)	no
2	191843445	*STAT1*	rs13010343	A	0.14	0.13	0.96 (0.77-1.20)	0.7449	29 / 287 / 924	10 / 107 / 354	1, 3, 4, 5 (n = 1319)	no
2	191845725	*STAT1*	rs1547550	C	0.35	0.34	0.95 (0.82-1.12)	0.5685	158 / 536 / 519	62 / 197 / 212	1, 3, 4, 5 (n = 1319)	no
2	191855521	*STAT1*	rs7562024	T	0.40	0.39	0.95 (0.82-1.11)	0.5502	203 / 579 / 447	71 / 221 / 174	1, 3, 4, 5 (n = 1319)	no
2	204610396	*CD28*	rs1980422	C	0.22	0.27	1.29 (1.07-1.55)	0.008079	65 / 433 / 759	30 / 144 / 203	1, 3, 4, 5 (n = 1319)	no
2	204732714	*CTLA4*	rs231775	G	0.37	0.35	0.92 (0.78-1.09)	0.3251	126 / 375 / 347	44 / 240 / 184	1, 2, 5 (n = 869)	yes
3	46414947	*CCR5*	rs333	del	0.10	0.08	0.81 (0.62-1.06)	0.1168	17 / 224 / 1028	5 / 68 / 392	1, 3, 4, 5 (n = 1319)	yes
3	58556841	*FAM107A*	rs13315591	C	0.07	0.08	1.13 (0.86-1.50)	0.3795	5 / 170 / 1092	2 / 71 / 397	1, 3, 4, 5 (n = 1319)	no
4	26108197	4p15	rs874040	G	0.31	0.29	0.93 (0.79-1.09)	0.3556	112 / 534 / 594	43 / 189 / 243	1, 3, 4, 5 (n = 1319)	no
4	123132492	*KIAA1109*	rs4505848	G	0.36	0.40	1.20 (1.03-1.41)	0.01901	170 / 567 / 538	73 / 222 / 166	1, 3, 4, 5 (n = 1319)	yes
4	123348345	*ADAD1*	rs11732095	G	0.08	0.07	0.91 (0.68-1.23)	0.5508	4 / 182 / 1057	3 / 57 / 388	1, 3, 4, 5 (n = 1319)	yes
4	123514528	*IL2-IL21*	rs4492018	A	0.24	0.21	0.88 (0.73-1.05)	0.1495	72 / 464 / 740	19 / 161 / 283	1, 3, 4, 5 (n = 1319)	yes
4	123548068	*IL21*	rs1398553	T	0.33	0.38	1.22 (1.05-1.43)	0.0109	148 / 527 / 556	69 / 225 / 183	1, 3, 4, 5 (n = 1319)	yes
5	55438580	*ANKRD55*	rs6859219	A	0.22	0.17	0.74 (0.61-0.90)	0.002953	55 / 441 / 774	14 / 131 / 320	1, 3, 4, 5 (n = 1319)	yes
5	96124330	*ERAP1*	rs30187	T	0.32	0.35	1.14 (0.97-1.34)	0.1018	139 / 510 / 574	55 / 224 / 196	1, 3, 4, 5 (n = 1319)	no
5	135287029	*LECT2*	rs31517	A	0.36	0.37	1.03 (0.88-1.20)	0.7415	166 / 561 / 514	63 / 223 / 191	1, 3, 4, 5 (n = 1319)	no
6	31540141	*LTA*	rs2239704	A	0.39	0.36	0.89 (0.75-1.05)	0.1671	131 / 386 / 324	58 / 222 / 192	1, 2, 5 (n = 869)	no
6	31540313	*LTA*	rs909253	G	0.34	0.35	1.07 (0.90-1.26)	0.4406	103 / 365 / 378	62 / 210 / 202	1, 2, 5 (n = 869)	no
6	31540784	*LTA*	rs1041981	A	0.33	0.35	1.09 (0.93-1.29)	0.2906	101 / 361 / 384	61 / 212 / 200	1, 2, 5 (n = 869)	no
6	31542482	*TNFA*	rs1799724	T	0.10	0.13	1.40 (1.09-1.79)	0.007489	11 / 145 / 691	10 / 105 / 356	1, 2, 5 (n = 869)	yes
6	31542963	*TNFA*	rs1800750	A	0.02	0.01	0.36 (0.15-0.86)	0.01635	0 / 30 / 818	0 / 6 / 465	1, 2, 5 (n = 869)	yes
6	31543031	*TNFA*	rs1800629	A	0.17	0.14	0.79 (0.63-0.99)	0.04234	29 / 228 / 591	9 / 114 / 353	1, 2, 5 (n = 869)	yes
6	31543101	*TNFA*	rs361525	A	0.05	0.03	0.55 (0.35-0.86)	0.007526	3 / 77 / 769	0 / 26 / 449	1, 2, 5 (n = 869)	yes
6	31543827	*TNFA*	rs1800610	A	0.10	0.13	1.38 (1.08-1.77)	0.009952	11 / 145 / 693	8 / 108 / 357	1, 2, 5 (n = 869)	yes

Supplementary Table S2 Allele and genotype frequencies in oligoarticular (persistent and extended) and RF negative polyarticular JIA patients versus controls

Chr	Position[a]	Gene/ region	SNP	Minor allele	MAF controls	MAF cases	OR (95% CI)	P (allelic)	Genotype counts controls	Genotype counts cases	Typed in control sets[c]	Reported JIA locus[d]
6	31544189	*TNFA*	rs3093662	G	0.06	0.04	0.75 (0.52-1.09)	0.1328	4 / 91 / 752	2 / 38 / 430	1, 2, 5 (n = 869)	yes
6	57012930	*ZNF451*	rs3734738	A	0.21	0.21	0.99 (0.82-1.19)	0.9212	53 / 428 / 772	20 / 158 / 290	1, 3, 4, 5 (n = 1319)	no
6	106568034	*PRDM1*	rs548234	C	0.32	0.35	1.13 (0.96-1.33)	0.1366	112 / 574 / 548	54 / 213 / 191	1, 3, 4, 5 (n = 1319)	no
6	138006504	*TNFAIP3*	rs6920220	A	0.21	0.20	0.98 (0.80-1.19)	0.821	41 / 269 / 538	25 / 139 / 301	1, 2, 5 (n = 869)	yes
6	159482521	*TAGAP*	rs394581	C	0.28	0.27	0.97 (0.82-1.15)	0.7544	103 / 500 / 670	33 / 188 / 246	1, 3, 4, 5 (n = 1319)	no
6	167534290	*CCR6*	rs3093023	A	0.44	0.46	1.07 (0.91-1.26)	0.3993	244 / 624 / 400	82 / 186 / 116	1, 3, 4, 5 (n = 1319)	no
7	75442759	*CCL24*	rs2302005	T	0.22	0.21	0.93 (0.78-1.12)	0.4639	55 / 400 / 703	14 / 171 / 292	1, 3, 4, 5 (n = 1319)	no
7	75442855	*CCL24*	rs2302004	C	0.44	0.42	0.94 (0.81-1.09)	0.4046	245 / 592 / 405	82 / 234 / 158	1, 3, 4, 5 (n = 1319)	no
7	92246744	*CDK6*	rs42041	G	0.26	0.25	0.95 (0.80-1.13)	0.5815	96 / 464 / 708	28 / 177 / 262	1, 3, 4, 5 (n = 1319)	no
7	128594183	*TNPO3*	rs10488631	C	0.10	0.10	1.03 (0.80-1.33)	0.8078	7 / 228 / 1023	5 / 83 / 382	1, 3, 4, 5 (n = 1319)	yes
9	34743681	*CCL21*	rs951005	C	0.15	0.16	1.08 (0.88-1.34)	0.4482	24 / 295 / 849	7 / 136 / 334	1, 3, 4, 5 (n = 1319)	no
9	139775146	*TRAF2*	rs7048473	C	0.26	0.25	0.95 (0.80-1.13)	0.5385	78 / 487 / 691	30 / 171 / 269	1, 3, 4, 5 (n = 1319)	no
9	139787453	*TRAF2*	rs2811761	G	0.21	0.21	0.99 (0.83-1.20)	0.9556	59 / 405 / 774	22 / 155 / 296	1, 3, 4, 5 (n = 1319)	no
9	139815053	*TRAF2*	rs10781522	G	0.38	0.38	1.00 (0.86-1.17)	0.9959	176 / 602 / 488	77 / 198 / 192	1, 3, 4, 5 (n = 1319)	no
9	139821068	*TRAF2*	rs3750512	C	0.38	0.37	0.96 (0.82-1.12)	0.6014	180 / 570 / 479	74 / 203 / 199	1, 3, 4, 5 (n = 1319)	no
10	6053163	*IL2RA*	rs12722605	T	0.14	0.15	1.05 (0.85-1.30)	0.6622	32 / 295 / 916	12 / 117 / 340	1, 3, 4, 5 (n = 1319)	yes
10	6099045	*IL2RA*	rs2104286	G	0.25	0.23	0.91 (0.76-1.10)	0.3382	76 / 472 / 695	29 / 139 / 252	1, 3, 4, 5 (n = 1319)	yes
10	6114660	*IL2RA*	rs41295061	A	0.09	0.09	1.02 (0.79-1.32)	0.8834	6 / 219 / 1049	4 / 78 / 384	1, 3, 4, 5 (n = 1319)	yes
10	6393260	*PRKCQ*	rs4750316	C	0.19	0.19	0.99 (0.82-1.20)	0.9552	39 / 394 / 794	16 / 148 / 306	1, 3, 4, 5 (n = 1319)	no
11	36525293	*TRAF6*	rs540386	T	0.13	0.11	0.83 (0.66-1.05)	0.1194	26 / 285 / 941	4 / 99 / 364	1, 3, 4, 5 (n = 1319)	no
11	71709272	*IL18BP*	rs3814721	C	0.06	0.07	1.18 (0.85-1.64)	0.3216	1 / 95 / 751	0 / 63 / 408	1, 2, 5 (n = 869)	no
11	71710478	*IL18BP*	rs2298455	C	0.12	0.12	0.96 (0.75-1.24)	0.7774	11 / 179 / 656	7 / 90 / 355	1, 2, 5 (n = 869)	no
11	71714078	*IL18BP*	rs1541304	T	0.03	0.02	0.87 (0.52-1.46)	0.5935	1 / 43 / 805	0 / 22 / 454	1, 2, 5 (n = 869)	no
11	112035458	*IL18*	rs1946518	T	0.40	0.40	1.00 (0.85-1.18)	0.9851	137 / 395 / 312	64 / 233 / 158	1, 2, 5 (n = 869)	no
11	117869670	*IL10RA*	rs2229113	A	0.31	0.30	0.97 (0.81-1.16)	0.7345	85 / 327 / 394	40 / 205 / 227	1, 2, 5 (n = 869)	no

Supplementary Table S2 Allele and genotype frequencies in oligoarticular (persistent and extended) and RF negative polyarticular JIA patients versus controls

Chr	Position[a]	Gene/ region	SNP	Minor allele	MAF controls	MAF cases	OR (95% CI)	P (allelic)	Genotype counts controls	Genotype counts cases	Typed in control sets[c]	Reported JIA locus[d]
12	6450945	*TNFRSF1A*	rs767455	C	0.42	0.40	0.92 (0.79-1.09)	0.3416	161 / 389 / 295	80 / 220 / 173	1, 2, 5 (n = 869)	no
12	6451590	*TNFRSF1A*	rs4149570	A	0.40	0.42	1.06 (0.90-1.25)	0.4766	151 / 377 / 315	87 / 213 / 164	1, 2, 5 (n = 869)	no
12	57968715	*KIF5A*	rs1678542	C	0.37	0.37	1.02 (0.87-1.19)	0.8226	135 / 599 / 442	66 / 217 / 184	1, 3, 4, 5 (n = 1319)	no
16	11179873	*CLEC16A*	rs12708716	G	0.35	0.33	0.92 (0.78-1.07)	0.2729	148 / 594 / 515	61 / 192 / 217	1, 3, 4, 5 (n = 1319)	yes
16	11249329	*CLEC16A*	rs6498169	G	0.34	0.37	1.09 (0.93-1.28)	0.2653	145 / 585 / 539	68 / 205 / 194	1, 3, 4, 5 (n = 1319)	yes
16	27448401	*IL21R*	rs3093341	G	0.10	0.08	0.80 (0.62-1.05)	0.1069	12 / 227 / 1005	1 / 77 / 399	1, 3, 4, 5 (n = 1319)	no
16	67189486	*TRADD*	rs11574518	T	0	0	*not polymorphic*		0 / 0 / 1143	0 / 0 / 450	1, 3, 4, 5 (n = 1319)	no
17	32594568	*CCL2-CCL7*	rs8079244	C	0	0	*not polymorphic*		0 / 0 / 1236	0 / 0 / 473	1, 3, 4, 5 (n = 1319)	no
17	40447401	*STAT5A*	rs7217728	C	0.31	0.29	0.91 (0.77-1.07)	0.2662	102 / 581 / 570	51 / 174 / 245	1, 3, 4, 5 (n = 1319)	no
17	40461003	*STAT5A*	rs2293154	A	0.18	0.18	1.05 (0.86-1.27)	0.6606	32 / 378 / 849	23 / 120 / 313	1, 3, 4, 5 (n = 1319)	no
18	67531642	*CD226*	rs763361	T	0.47	0.54	1.30 (1.12-1.51)	0.0006295	290 / 623 / 361	132 / 237 / 97	1, 3, 4, 5 (n = 1319)	no
19	44515514	*ZNF230*	rs12753	A	0.14	0.15	1.05 (0.85-1.30)	0.6291	22 / 304 / 905	10 / 121 / 346	1, 3, 4, 5 (n = 1319)	no
20	43280231	*ADA*	rs6031698	A	0	0	*not polymorphic*		0 / 0 / 844	0 / 0 / 472	1, 2, 5 (n = 869)	no
20	44746982	*CD40*	rs1883832	T	0.25	0.25	0.99 (0.82-1.19)	0.9027	57 / 306 / 484	31 / 172 / 273	1, 2, 5 (n = 869)	no
21	34640788	*IL10RB*	rs2834167	G	0.24	0.27	1.14 (0.95-1.37)	0.1566	61 / 293 / 494	39 / 174 / 254	1, 2, 5 (n = 869)	no
22	24236392	*MIF*	rs755622	C	0.21	0.15	0.67 (0.54-0.83)	0.0002084	37 / 283 / 526	10 / 121 / 334	1, 2, 5 (n = 869)	yes
22	37544245	*IL2RB*	rs3218258	T	0.28	0.27	0.97 (0.82-1.15)	0.7135	99 / 514 / 655	31 / 196 / 243	1, 3, 4, 5 (n = 1319)	yes
22	37544810	*IL2RB*	rs3218253	T	0.28	0.28	0.99 (0.84-1.17)	0.9164	98 / 506 / 652	33 / 195 / 242	1, 3, 4, 5 (n = 1319)	yes
22	37551607	*IL2RB*	rs743777	G	0.34	0.33	0.95 (0.81-1.12)	0.5386	143 / 552 / 543	43 / 225 / 207	1, 3, 4, 5 (n = 1319)	yes

Chr: chromosome; MAF: minor allele frequency; OR: odds ratio; CI: confidence interval

a) Base-pair position is based on NCBI dbSNP build 136

b) 11: homozygous for the minor allele; 12: heterozygous; 22: homozygous for the major allele

c) See supplementary Table S1 for details of control sets

d) See Table 2 for references

All presented results are derived from analyses with the homogeneous patient group (persistent or extended oligoarticular and RF negative polyarticular JIA) compared to controls.

Supplementary Table S3 Allele frequencies and associations per JIA subgroup versus controls

Gene/region	SNP	Minor allele	Controls n = 869 / 1319[a]	JIA homogeneous[b] (n = 493) vs controls			Persistent oligoarthritis (n = 263) vs controls			Extented oligoarthritis (n = 88) vs controls			RF negative polyarthritis (n = 142) vs controls			ANA positive JIA[c] (n = 254) vs controls		
			MAF	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p
TNFRSF14-MMEL1	rs3890745	G	0.32	0.30	0.90	0.2137	0.31	0.92	0.4347	0.29	0.85	0.347	0.30	0.90	0.4611	0.28	0.83	0.08291
TNFRSF8-MIIP	rs946461	T	0.26	0.29	1.15	0.09358	0.26	0.99	0.9563	0.33	1.40	0.0428	0.32	1.32	0.04465	0.30	1.20	0.09404
TNFRSF1B	rs1061622	G	0.23	0.24	1.03	0.7678	0.23	1.00	0.9709	0.22	0.91	0.6237	0.26	1.17	0.2849	0.26	1.16	0.2131
LCK	rs1004420	T	0.17	0.17	0.98	0.8087	0.18	1.03	0.8286	0.17	0.98	0.9097	0.16	0.88	0.4721	0.17	0.96	0.7445
LCK	rs695161	C	0.48	0.47	0.97	0.6721	0.46	0.94	0.5339	0.50	1.09	0.6046	0.47	0.95	0.6838	0.45	0.90	0.2833
PTPN22	rs2476601	A	0.10	0.13	1.32	0.02702	0.11	1.08	0.6468	0.14	1.51	0.07322	0.16	1.67	0.00493	0.13	1.39	0.03457
VTCN1	rs6673837	A	0.20	0.20	0.99	0.9474	0.21	1.07	0.5881	0.19	0.90	0.5935	0.19	0.92	0.6078	0.21	1.03	0.8411
VTCN1	rs2358817	T	0.08	0.07	0.88	0.4164	0.08	1.00	0.9886	0.08	1.00	0.9936	0.05	0.61	0.0817	0.08	0.94	0.7407
VTCN1	rs2358820	A	0.07	0.07	0.92	0.602	0.08	1.07	0.7114	0.07	0.96	0.8891	0.04	0.61	0.1135	0.07	1.06	0.7725
VTCN1	rs10923217	C	0.48	0.52	1.21	0.02079	0.51	1.12	0.2585	0.49	1.07	0.6787	0.58	1.50	0.002179	0.52	1.20	0.07851
VTCN1	rs6669320	A	0.15	0.13	0.89	0.3117	0.13	0.88	0.4157	0.13	0.83	0.4585	0.14	0.92	0.6767	0.14	0.94	0.6633
VTCN1	rs10923223	C	0.15	0.16	1.09	0.4383	0.17	1.21	0.1692	0.16	1.08	0.7348	0.13	0.89	0.5622	0.18	1.28	0.07157
VTCN1	rs12046117	T	0.13	0.13	1.04	0.7252	0.14	1.11	0.473	0.14	1.07	0.7836	0.12	0.90	0.6181	0.16	1.26	0.1072
PTPRC	rs10919563	A	0.13	0.11	0.86	0.195	0.12	0.96	0.7804	0.10	0.76	0.2868	0.10	0.73	0.1446	0.12	0.94	0.6871
IL10	rs1800896	C	0.50	0.47	0.90	0.2119	0.47	0.88	0.2208	0.45	0.82	0.2266	0.50	1.00	1	0.49	0.94	0.5658
IL19	rs2243191	T	0.20	0.23	1.17	0.1209	0.21	1.10	0.4737	0.25	1.36	0.1071	0.23	1.19	0.2811	0.22	1.15	0.2677
IL20	rs1400986	T	0.16	0.14	0.86	0.1975	0.16	0.98	0.857	0.17	1.04	0.8427	0.10	0.57	0.007092	0.15	0.90	0.4437
AFF3	rs1160542	G	0.45	0.46	1.05	0.5177	0.44	0.95	0.6405	0.52	1.35	0.06018	0.47	1.07	0.582	0.46	1.06	0.5746
AFF3	rs10865035	A	0.46	0.47	1.05	0.5762	0.45	0.97	0.7889	0.54	1.37	0.08522	0.47	1.03	0.8378	0.47	1.04	0.7041
IL18RAP	rs917997	T	0.22	0.24	1.14	0.1782	0.23	1.07	0.5657	0.27	1.34	0.1124	0.24	1.15	0.3743	0.25	1.17	0.1966
IL1A	rs17561	A	0.30	0.29	0.92	0.3289	0.29	0.91	0.4294	0.29	0.94	0.7177	0.28	0.90	0.4967	0.28	0.90	0.3559

Supplementary Table S3 Allele frequencies and associations per JIA subgroup versus controls (continued)

Gene/region	SNP	Minor allele	Controls n = 869 / 1319[a]	JIA homogeneous[b] (n = 493) vs controls			Persistent oligoarthritis (n = 263) vs controls			Extented oligoarthritis (n = 88) vs controls			RF negative polyarthritis (n = 142) vs controls			ANA positive JIA[c] (n = 254) vs controls		
			MAF	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p
IL1A	rs1800587	A	0.31	0.28	0.89	0.2285	0.29	0.95	0.6497	0.25	0.74	0.1427	0.28	0.89	0.4431	0.27	0.84	0.1471
IL1B	rs1143634	A	0.25	0.24	0.95	0.5542	0.23	0.92	0.5044	0.23	0.89	0.5441	0.25	1.02	0.8818	0.23	0.91	0.4422
IL1B	rs16944	A	0.33	0.35	1.11	0.2315	0.33	1.01	0.9639	0.39	1.32	0.09227	0.37	1.18	0.2235	0.35	1.12	0.3072
DPP4	rs2268894	C	0.45	0.47	1.07	0.4192	0.47	1.06	0.5513	0.41	0.86	0.3464	0.50	1.24	0.105	0.46	1.04	0.6856
STAT1	rs3771300	C	0.48	0.50	1.10	0.2321	0.47	0.96	0.7153	0.51	1.15	0.3818	0.55	1.35	0.01999	0.48	1.02	0.8538
STAT1	rs13010343	A	0.14	0.13	0.96	0.7449	0.14	0.99	0.9477	0.12	0.86	0.5314	0.14	0.98	0.9254	0.15	1.09	0.5533
STAT1	rs1547550	C	0.35	0.34	0.95	0.5685	0.35	0.98	0.8245	0.34	0.93	0.6744	0.33	0.93	0.5853	0.34	0.97	0.744
STAT1	rs7562024	T	0.40	0.39	0.95	0.5502	0.39	0.95	0.6424	0.43	1.14	0.4157	0.36	0.85	0.2286	0.40	1.01	0.9559
CD28	rs1980422	C	0.22	0.27	1.29	0.008079	0.26	1.23	0.09935	0.27	1.30	0.1918	0.29	1.39	0.03501	0.30	1.52	0.0004481
CTLA4	rs231775	G	0.37	0.35	0.92	0.3251	0.35	0.90	0.3164	0.36	0.94	0.716	0.36	0.95	0.6931	0.35	0.92	0.4572
CCR5	rs333	del	0.10	0.08	0.81	0.1168	0.09	0.83	0.2842	0.06	0.57	0.08363	0.09	0.92	0.721	0.09	0.86	0.3884
FAM107A	rs13315591	C	0.07	0.08	1.13	0.3795	0.08	1.15	0.4292	0.06	0.90	0.7552	0.09	1.25	0.3376	0.09	1.30	0.1396
4p15	rs874040	G	0.31	0.29	0.93	0.3556	0.27	0.84	0.09887	0.36	1.28	0.1325	0.28	0.90	0.4424	0.30	0.95	0.6689
KIAA1109	rs4505848	G	0.36	0.40	1.20	0.01901	0.38	1.13	0.2286	0.41	1.26	0.1603	0.42	1.31	0.03709	0.40	1.20	0.07744
ADAD1	rs11732095	G	0.08	0.07	0.91	0.5508	0.07	0.90	0.6068	0.04	0.48	0.07923	0.09	1.20	0.4245	0.07	0.96	0.8327
IL2-IL21	rs4492018	A	0.24	0.21	0.88	0.1495	0.20	0.81	0.08452	0.25	1.05	0.8018	0.22	0.89	0.4681	0.22	0.92	0.4964
IL21	rs1398553	T	0.33	0.38	1.22	0.0109	0.38	1.21	0.05882	0.40	1.31	0.0934	0.38	1.20	0.178	0.38	1.25	0.03146
ANKRD55	rs6859219	A	0.22	0.17	0.74	0.002953	0.18	0.81	0.09507	0.19	0.84	0.3994	0.14	0.57	0.002153	0.16	0.69	0.005596
ERAP1	rs30187	T	0.32	0.35	1.14	0.1018	0.34	1.09	0.4044	0.36	1.19	0.2774	0.36	1.20	0.163	0.35	1.12	0.2729
LECT2	rs31517	A	0.36	0.37	1.03	0.7415	0.37	1.05	0.6601	0.34	0.93	0.6575	0.37	1.06	0.6833	0.36	1.02	0.8649
LTA	rs2239704	A	0.39	0.36	0.89	0.1671	0.38	0.98	0.8285	0.30	0.68	0.02545	0.35	0.88	0.3353	0.37	0.94	0.5656

Supplementary Table S3 Allele frequencies and associations per JIA subgroup versus controls (continued)

Gene/region	SNP	Minor allele	Controls n = 869 / 1319[a]	JIA homogeneous[b] (n = 493) vs controls			Persistent oligoarthritis (n = 263) vs controls			Extented oligoarthritis (n = 88) vs controls			RF negative polyarthritis (n = 142) vs controls			ANA positive JIA[c] (n = 254) vs controls		
			MAF	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p
LTA	rs909253	G	0.34	0.35	1.07	0.4406	0.34	1.01	0.9397	0.39	1.25	0.1703	0.35	1.07	0.617	0.37	1.13	0.2434
LTA	rs1041981	A	0.33	0.35	1.09	0.2906	0.34	1.04	0.7436	0.40	1.33	0.08439	0.35	1.07	0.6184	0.37	1.16	0.1618
TNFA	rs1799724	T	0.10	0.13	1.40	0.007489	0.13	1.39	0.03609	0.13	1.39	0.1626	0.14	1.43	0.06627	0.15	1.58	0.002279
TNFA	rs1800750	A	0.02	0.01	0.36	0.01635	0.01	0.34	0.06219	0.01	0.65	0.5485	0.00	0.20	0.08317	0.00	0.23	0.02768
TNFA	rs1800629	A	0.17	0.14	0.79	0.04234	0.12	0.68	0.01005	0.15	0.87	0.5175	0.16	0.97	0.8566	0.14	0.80	0.1244
TNFA	rs361525	A	0.05	0.03	0.55	0.007526	0.03	0.52	0.02582	0.04	0.83	0.6328	0.02	0.44	0.04588	0.02	0.36	0.002901
TNFA	rs1800610	A	0.10	0.13	1.38	0.009952	0.13	1.42	0.02286	0.13	1.31	0.2737	0.13	1.37	0.1157	0.15	1.61	0.001418
TNFA	rs3093662	G	0.06	0.04	0.75	0.1328	0.04	0.75	0.2412	0.05	0.88	0.7177	0.04	0.67	0.2213	0.03	0.55	0.02543
ZNF451	rs3734738	A	0.21	0.21	0.99	0.9212	0.21	0.97	0.8117	0.23	1.12	0.5601	0.20	0.95	0.7468	0.22	1.06	0.622
PRDM1	rs548234	C	0.32	0.35	1.13	0.1366	0.33	1.05	0.644	0.38	1.28	0.1353	0.36	1.19	0.1948	0.36	1.20	0.0787
TNFAIP3	rs6920220	A	0.21	0.20	0.98	0.821	0.19	0.92	0.5275	0.22	1.08	0.7053	0.21	1.02	0.8938	0.20	0.98	0.8617
TAGAP	rs394581	C	0.28	0.27	0.97	0.7544	0.28	1.00	0.9931	0.28	1.00	0.9958	0.26	0.91	0.5282	0.27	0.97	0.7971
CCR6	rs3093023	A	0.44	0.46	1.07	0.3993	0.47	1.12	0.3127	0.44	1.01	0.9509	0.45	1.04	0.7957	0.43	0.98	0.8225
CCL24	rs2302005	T	0.22	0.21	0.93	0.4639	0.19	0.86	0.2088	0.23	1.06	0.7666	0.22	1.00	0.9886	0.21	0.93	0.5674
CCL24	rs2302004	C	0.44	0.42	0.94	0.4046	0.38	0.81	0.03051	0.49	1.27	0.1343	0.44	1.02	0.8712	0.42	0.95	0.642
CDK6	rs42041	G	0.26	0.25	0.95	0.5815	0.26	1.02	0.8385	0.26	1.00	0.9918	0.22	0.80	0.15	0.25	0.95	0.6539
TNPO3	rs10488631	C	0.10	0.10	1.03	0.8078	0.11	1.15	0.3883	0.09	0.98	0.9295	0.08	0.85	0.498	0.10	1.10	0.5706
CCL21	rs951005	C	0.15	0.16	1.08	0.4482	0.17	1.15	0.2895	0.15	1.02	0.9258	0.15	1.00	0.992	0.17	1.17	0.2316
TRAF2	rs7048473	C	0.26	0.25	0.95	0.5385	0.25	0.97	0.8145	0.24	0.89	0.5493	0.24	0.93	0.6308	0.25	0.95	0.6402
TRAF2	rs2811761	G	0.21	0.21	0.99	0.9556	0.21	0.98	0.8389	0.20	0.93	0.7283	0.22	1.07	0.6615	0.23	1.11	0.3989
TRAF2	rs10781522	G	0.38	0.38	1.00	0.9959	0.38	1.03	0.7762	0.37	0.99	0.9326	0.37	0.96	0.7444	0.39	1.06	0.5554

Supplementary Table S3 Allele frequencies and associations per JIA subgroup versus controls (continued)

Gene/region	SNP	Minor allele	Controls n = 869 / 1319[a]	JIA homogeneous[b] (n = 493) vs controls			Persistent oligoarthritis (n = 263) vs controls			Extented oligoarthritis (n = 88) vs controls			RF negative polyarthritis (n = 142) vs controls			ANA positive JIA[c] (n = 254) vs controls		
			MAF	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p
TRAF2	rs3750512	C	0.38	0.37	0.96	0.6014	0.37	0.96	0.6644	0.37	0.96	0.7817	0.37	0.97	0.7972	0.38	1.03	0.7936
IL2RA	rs12722605	T	0.14	0.15	1.05	0.6622	0.16	1.11	0.4539	0.15	1.07	0.7598	0.14	0.93	0.6884	0.15	1.06	0.6882
IL2RA	rs2104286	G	0.25	0.23	0.91	0.3382	0.24	0.94	0.582	0.19	0.71	0.09939	0.25	1.02	0.9141	0.23	0.92	0.4751
IL2RA	rs41295061	A	0.09	0.09	1.02	0.8834	0.09	0.98	0.8898	0.10	1.14	0.6107	0.09	1.02	0.9163	0.09	1.00	0.993
PRKCQ	rs4750316	C	0.19	0.19	0.99	0.9552	0.18	0.93	0.5832	0.21	1.13	0.5353	0.20	1.03	0.8562	0.22	1.15	0.2461
TRAF6	rs540386	T	0.13	0.11	0.83	0.1194	0.12	0.84	0.2368	0.11	0.83	0.4601	0.11	0.82	0.3398	0.11	0.76	0.08008
IL18BP	rs3814721	C	0.06	0.07	1.18	0.3216	0.07	1.18	0.4299	0.07	1.33	0.3517	0.06	1.09	0.7532	0.07	1.31	0.1792
IL18BP	rs2298455	C	0.12	0.12	0.96	0.7774	0.12	1.03	0.856	0.13	1.06	0.817	0.10	0.80	0.3006	0.13	1.07	0.6699
IL18BP	rs1541304	T	0.03	0.02	0.87	0.5935	0.02	0.74	0.4001	0.02	0.86	0.7822	0.03	1.11	0.798	0.02	0.76	0.4337
IL18	rs1946518	T	0.40	0.40	1.00	0.9851	0.42	1.11	0.3339	0.38	0.94	0.7224	0.36	0.86	0.2847	0.39	0.95	0.6597
IL10RA	rs2229113	A	0.31	0.30	0.97	0.7345	0.28	0.89	0.2876	0.34	1.17	0.353	0.31	1.01	0.9266	0.32	1.03	0.7612
TNFRSF1A	rs767455	C	0.42	0.40	0.92	0.3416	0.42	0.99	0.9141	0.37	0.82	0.2178	0.39	0.88	0.3469	0.39	0.89	0.2598
TNFRSF1A	rs4149570	A	0.40	0.42	1.06	0.4766	0.43	1.12	0.2806	0.43	1.12	0.484	0.39	0.93	0.5849	0.41	1.05	0.6677
KIF5A	rs1678542	C	0.37	0.37	1.02	0.8226	0.35	0.92	0.4395	0.42	1.25	0.1688	0.39	1.07	0.6106	0.37	0.99	0.8935
CLEC16A	rs12708716	G	0.35	0.33	0.92	0.2729	0.36	1.01	0.9414	0.36	1.02	0.8991	0.28	0.70	0.01183	0.31	0.82	0.05822
CLEC16A	rs6498169	G	0.34	0.37	1.09	0.2653	0.35	1.01	0.9101	0.37	1.10	0.5513	0.40	1.25	0.09136	0.38	1.18	0.1082
IL21R	rs3093341	G	0.10	0.08	0.80	0.1069	0.07	0.72	0.0696	0.10	1.04	0.8741	0.08	0.82	0.3732	0.08	0.77	0.1394
TRADD	rs11574518	T	0	0	Not polymorphic													
CCL2-CCL7	rs8079244	C		0	Not polymorphic													
STAT5A	rs7217728	C	0.31	0.29	0.91	0.2662	0.27	0.83	0.08635	0.28	0.86	0.3998	0.34	1.12	0.4273	0.30	0.94	0.5391
STAT5A	rs2293154	A	0.18	0.18	1.05	0.6606	0.18	1.00	0.9989	0.21	1.23	0.302	0.18	1.02	0.9117	0.19	1.11	0.4299

Supplementary Table S3 Allele frequencies and associations per JIA subgroup versus controls (continued)

Gene/region	SNP	Minor allele	Controls n = 869 / 1319[a]	JIA homogeneous[b] (n = 493) vs controls			Persistent oligoarthritis (n = 263) vs controls			Extented oligoarthritis (n = 88) vs controls			RF negative polyarthritis (n = 142) vs controls			ANA positive JIA[c] (n = 254) vs controls		
			MAF	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p	MAF	OR	p
CD226	rs763361	T	0.47	0.54	1.30	0.0006295	0.55	1.39	0.0008022	0.52	1.23	0.194	0.51	1.19	0.1818	0.53	1.27	0.01752
ZNF230	rs12753	A	0.14	0.15	1.05	0.6291	0.15	1.09	0.549	0.20	1.53	0.03052	0.11	0.73	0.1149	0.17	1.26	0.07806
ADA	rs6031698	A	0	0	Not polymorphic													
CD40	rs1883832	T	0.25	0.25	0.99	0.9027	0.24	0.95	0.6524	0.22	0.88	0.4875	0.27	1.14	0.3614	0.24	0.97	0.8199
IL10RB	rs2834167	G	0.24	0.27	1.14	0.1566	0.28	1.21	0.09756	0.21	0.83	0.339	0.28	1.23	0.1563	0.27	1.12	0.3515
MIF	rs755622	C	0.21	0.15	0.67	0.0002084	0.18	0.82	0.1387	0.11	0.47	0.002125	0.13	0.53	0.0009787	0.15	0.66	0.002712
IL2RB	rs3218258	T	0.28	0.27	0.97	0.7135	0.26	0.92	0.4651	0.23	0.76	0.1478	0.32	1.22	0.1579	0.25	0.84	0.1144
IL2RB	rs3218253	T	0.28	0.28	0.99	0.9164	0.27	0.96	0.69	0.23	0.77	0.1579	0.32	1.22	0.1449	0.25	0.85	0.1534
IL2RB	rs743777	G	0.34	0.33	0.95	0.5386	0.32	0.91	0.3628	0.30	0.82	0.2601	0.37	1.12	0.3797	0.30	0.85	0.1258

MAF: minor allele frequency; RF: rheumatoid factor; ANA: antinuclear antibodies

a) Typed control sets per SNP are listed in Supplementary Table S2

b) Including oligoarthritis (persistent and extended) and RF negative polyarthritis patients

c) Including persistent oligoarthritis (n=129), extended oligoarthritis (n=60), and RF negative polyarthritis (n=65) patients

Supplementary Table S4
Meta-analyses of reported JIA loci*

SNP (minor allele)							
***PTPN22* rs2476601 (1858) (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** meta-analysis
Viken 2005	Norway	320	555	*A-allele associated with disease, but data to calculate allelic OR not available*			
Hinks 2005	UK	661	595	17%	1.53	(1.2 - 2)	
Seldin 2005	Finland	230	1400	5%	1.17	(0.9 - 1.5)	
Cinek 2006	Czech Republic	130	400	7%	2.35	(1.61 - 3.42)	
Pazár 2008	Hungary	150	200	3%	1.13	(0.66 - 1.95)	
Thompson 2010	USA, Germany, Czech Republic	949	1214	27%	1.67	(1.38 - 2.01)	
Ellis 2013	Australia	200	341	5%	1.45	(0.93 - 2.27)	
Dimopoulou 2013	Greece	128	221	2%	2.23	(1.03 - 4.84)	
Kaalla 2013	USA (overlap not reported)	636	733	18%	1.29	(1.02 - 1.62)	
Reinards et al.	*present study*			16%	1.32	(1.03 - 1.69)	
pooled					**1.50**		**8.95E-16**
***VTCN1* rs6673837 (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** meta-analysis
Hinks 2009 II	UK	654	1847	35%	1.16	(1 - 1.37)	
Thompson 2012	USA	810	3040	43%	0.95	(0.82 - 1.09)	
Reinards et al.	*present study*			22%	0.99	(0.82 - 1.21)	
pooled					1.03		0.5485
***VTCN1* rs2358817 (T)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** meta-analysis
Hinks 2009 II	UK	654	1847	27%	0.68	(0.52 - 0.89)	
Thompson 2012	USA	812	3056	51%	1.12	(0.92 - 1.36)	
Reinards et al.	*present study*			22%	0.88	(0.65 - 1.19)	
pooled					0.93		0.3035

Supplementary Table S4 (continued)
Meta-analyses of reported JIA loci*

SNP (minor allele)							
***VTCN1* rs2358820 (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2009 II	UK discovery	249	184	10%	0.40	(0.23 - 0.68)	
	UK validation	321	2024	9%	0.45	(0.26 - 0.78)	
Thompson 2012	USA	814	3058	58%	1.10	(0.9 - 1.36)	
Reinards et al.	*present study*			24%	0.92	(0.67 - 1.26)	
pooled					0.88		0.1227
***VTCN1* rs10923217 (C)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2009 II	UK	654	1847	59%	1.17b	(1.02 - 1.33)	
Reinards et al.	*present study*			41%	1.21	(1.03 - 1.42)	
pooled					**1.19**		**0.001113**
***VTCN1* rs6669320 (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2009 II	UK	654	1847	62%	0.80	(0.67 - 0.97)	
Reinards et al.	*present study*			38%	0.89	(0.7 - 1.12)	
pooled					**0.83**		**0.01299**
***VTCN1* rs10923223 (C)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2009 II	UK	654	1847	58%	1.45	(1.2 - 1.75)	
Reinards et al.	*present study*			42%	1.09	(0.88 - 1.36)	
pooled					**1.29**		**0.0005872**
***VTCN1* rs12046117 (T)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2009 II	UK	654	1847	48%	1.58	(1.29 - 1.94)	
Ellis 2013	Australia	200	341	16%	1.15	(0.81 - 1.64)	
Reinards et al.	*present study*			36%	1.04	(0.82 - 1.32)	
pooled					**1.29**		**0.0003681**

Supplementary Table S4 (continued)
Meta-analyses of reported JIA loci*

SNP (minor allele)							
***PTPRC* rs10919563 (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2012	UK, USA	1611[a]	12719[a]	80%	0.88	(0.78 - 0.99)	
Thompson 2012	USA	*overlap with Hinks 2012*					
Reinards et al.	*present study*			20%	0.86	(0.68 - 1.08)	
pooled					**0.88**		**0.01388**
***AFF3* rs1160542 (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2010 II	UK	915	2967	40%	1.25	(1.13 - 1.39)	
Thompson 2012	USA	810	3054	34%	1.11	(0.99 - 1.24)	
Ellis 2013	Australia	200	341	7%	1.16	(0.91 - 1.49)	
Reinards et al.	*present study*			19%	1.05	(0.9 - 1.22)	
pooled					**1.16**		**1.31E-05**
***CTLA4* rs231775 (+49) (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Miterski 2004	Germany	197	362	13%	0.92	(0.71 - 1.19)	
Suppiah 2005	UK (Northern Ireland)	72	475	7%	0.76	(0.53 - 1.09)	
Prahalad 2008	USA (>90% of Northern European ancestry)	650	345	25%	0.95	(0.79 - 1.15)	
Thompson 2010	USA, UK	809[a]	3521[a]	22%	0.98	(0.84 - 1.15)	
Reinards et al.	*present study*			32%	0.92	(0.78 - 1.09)	
pooled					0.93		0.1224
***CCR5* rs333 (delta32) (del)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Lindner 2007	Norway	515	645	22%	0.82	(0.63 - 1.07)	
Hinks 2010 III	UK	983	3121	56%	0.79	(0.66 - 0.94)	
Reinards et al.	*present study*			22%	0.81	(0.62 - 1.06)	
pooled					**0.80**		**0.0005273**

Supplementary Table S4 (continued)
Meta-analyses of reported JIA loci*

SNP (minor allele)							
***TNFA* rs1799724 (-857) (T)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p meta-analysis**
Zeggini 2002 II	UK	*no evidence of association with disease, but no exact data available*					
Miterski 2004	Germany	170	415	28%	1.10	(0.74 - 1.63)	
Reinards et al.	*present study*			72%	1.40	(1.09 - 1.79)	
pooled					**1.31**		**0.01207**
***TNFA* rs1800629 (-308) (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p meta-analysis**
Ozen 2002	Czech Republic	159	100	8%	1.48	(0.94 - 2.33)	
Zeggini 2002 II	UK	138	75	5%	2.12	(1.2 - 3.7)	
Miterski 2004	Germany	122	312	10%	1.13	(0.76 - 1.68)	
Schmeling 2006	Germany (overlap not reported)	228	196	11%	0.78	(0.53 - 1.13)	
Cimaz 2007	France (cases and controls), Italy (cases)	107	630	*no evidence of association with disease, but no exact data available*			
Mourão 2009	Portugal	114	117	*no significant differences between cases and controls, but data to calculate allelic OR not available*			
Kaalla 2013	USA	628	729	35%	0.82	(0.66 - 1.01)	
Reinards et al.	*present study*			32%	0.79	(0.63 - 0.99)	
pooled					0.91		0.1609
***TNFA* rs361525 (-238) (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p meta-analysis**
Ozen 2002	Czech Republic	159	100	22%	2.02	(1.28 - 3.19)	
Zeggini 2002 II	UK	137	76	6%	0.41	(0.17 - 0.95)	
Miterski 2004	Germany	130	375	6%	0.71	(0.29 - 1.77)	
Schmeling 2006	Germany (overlap not reported)	228	196	7%	1.30	(0.58 - 2.93)	
Cimaz 2007	France (cases and controls), Italy (cases)	107	630	*no evidence of association with disease, but no exact data available*			
Kaalla 2013	USA	638	749	35%	0.66	(0.46 - 0.95)	
Reinards et al.	*present study*			23%	0.55	(0.35 - 0.86)	
pooled					0.83		0.08965

Supplementary Table S4 (continued)
Meta-analyses of reported JIA loci*

SNP (minor allele)							
***TNFAIP3* rs6920220 (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p meta-analysis**
Prahalad 2009	USA (>90% of Northern European ancestry)	441	619	15%	1.30	(1.05 - 1.61)	
Hinks 2010	UK	873	3644	42%	1.16	(1.02 - 1.31)	
Thompson 2010	USA (overlap not reported)	809[a]	531[a]	19%	0.94	(0.78 - 1.13)	
Thompson 2012	USA	*overlap with Thompson 2010*					
Ellis 2013	Australia	200	341	7%	1.04	(0.77 - 1.41)	
Reinards et al.	*present study*			17%	0.98	(0.8 - 1.19)	
pooled					**1.09**		**0.03309**
***TNPO3* rs10488631 (C)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p meta-analysis**
Hinks 2012	UK	1179	5176	78%	1.20	(1.05 - 1.37)	
Reinards et al.	*present study*			22%	1.03	(0.8 - 1.33)	
pooled					**1.16**		**0.01277**
***IL2RA* rs2104286 (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p meta-analysis**
Hinks 2009	UK	593	>3000a	20%	0.76	(0.66 - 0.88)	
Prahalad 2009	USA (>90% of Northern European ancestry)	438	634	11%	1.04	(0.85 - 1.27)	
Thompson 2010 initial cohort	USA, UK (overlap not reported)	809a	3521a	25%	0.76	(0.66 - 0.86)	
Thompson 2010 replication cohort	USA, Germany, Czech Republic (overlap not reported)	1015a	1569a	26%	0.96	(0.84 - 1.1)	
Thompson 2012	USA	*overlap with Thompson 2010*					
Ellis 2013	Australia	200	341	5%	0.87	(0.64 - 1.18)	
Reinards et al.	*present study*			13%	0.91	(0.76 - 1.1)	
pooled					**0.86**		**8.21E-06**

Supplementary Table S4 (continued)
Meta-analyses of reported JIA loci*

SNP (minor allele)							
***IL2RA* rs41295061 (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** $_{\text{meta-analysis}}$
Hinks 2009	UK	619	3614	58%	0.80	(0.63 - 1)	
Reinards et al.	*present study*			42%	1.02	(0.79 - 1.32)	
pooled					0.89		0.1646
***CLEC16A* rs12708716 (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** $_{\text{meta-analysis}}$
Skinningsrud 2010	Norway	507	2109	36%	0.89	(0.77 - 1.03)	
Thompson 2010	USA	809[a]	531[a]	31%	0.98	(0.84 - 1.16)	
Thompson 2012	USA	*overlap with Thompson 2010*					
Reinards et al.	*present study*			33%	0.92	(0.78 - 1.07)	
pooled					0.93		0.09463
***CLEC16A* rs6498169 (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** $_{\text{meta-analysis}}$
Skinningsrud 2010	Norway	498	2110	28%	1.19	(1.03 - 1.37)	
Thompson 2012	USA	814	3056	41%	1.11	(0.99 - 1.25)	
Ellis 2013	Australia	200	341	8%	1.03	(0.79 - 1.33)	
Reinards et al.	*present study*			23%	1.09	(0.93 - 1.28)	
pooled					**1.12**		**0.002768**
***MIF* rs755622 (-173) (C)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** $_{\text{meta-analysis}}$
Donn 2002	UK	526	259	15%	1.86	(1.36 - 2.55)	
Miterski 2004	Germany	150	390	14%	1.21	(0.88 - 1.66)	
Kaalla 2013	USA	638	742	39%	1.06	(0.88 - 1.29)	
Reinards et al.	*present study*			32%	0.67	(0.54 - 0.83)	
pooled					1.01		0.8222

Supplementary Table S4 (continued)
Meta-analyses of reported JIA loci*

SNP (minor allele)							
IL2RB **rs743777 (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** meta-analysis
Thompson 2012	USA	812	3051	67%	1.02	(0.91 - 1.14)	
Reinards et al.	*present study*			33%	0.95	(0.81 - 1.12)	
pooled					1.00		0.9405
IL1B **rs1143634 (3954) (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** meta-analysis
Cimaz 2007	France (cases+controls), Italy (cases)	107	630	*A-allele protective for disease, but exact data not available*			
Reinards et al.	*present study*				0.95	(0.79 - 1.14)	
no meta-analysis performed							
TNFA **rs1800750 (-376) (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**p** meta-analysis
Zeggini 2002 II	UK	*no evidence of association with disease, but no exact data available*					
Reinards et al.	*present study*				0.36	(0.15 - 0.86)	
no meta-analysis performed							

OR: odds ratio; CI: confidence interval
Patient group of the present study limited to oligoarticular (persistent and extended) and RF negative JIA patients References are listed in Supplementary Table S6.s
a) Exact number of succesfully typed individuals not known
b) Minor allele unclear

Supplementary Table S5
Meta-analyses of loci previously tested in JIA (but not significantly associated*)

SNP (minor allele)							
CD28 rs1980422 (C)[b]	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Thompson 2012	USA	814	3058	69%	1.14	(1 - 1.29)	
Reinards et al.	*present study*			31%	1.29	(1.07 - 1.55)	
pooled					**1.18**		**0.001411**
CD226 rs763361 (T)[b]	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2010 II	UK	600	3494	53%	1.07	(0.94 - 1.21)	
Ellis 2013	Australia	158	341	11%	1.22	(0.93 - 1.59)	
Reinards et al.	*present study*			36%	1.30	(1.12-1.51)	
pooled					**1.16**		**0.001115**
***TNFRSF14-MMEL1* rs3890745 (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Thompson 2010	USA, UK	809[a]	3521[a]	63%	0.92	(0.81 - 1.04)	
Thompson 2012	USA	*overlap with Thompson 2010*					
Reinards et al.	*present study*			37%	0.90	(0.77 - 1.06)	
pooled					**0.91**		**0.07282**
***TNFRSF1B* rs1061622 (G)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Zeggini 2002	UK	435	261	35%	1.25	(0.97 - 1.61)	
Reinards et al.	*present study*			65%	1.03	(0.85 - 1.24)	
pooled					**1.10**		**0.2057**
***LCK* rs695161 (C)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Thompson 2012	USA	813	3057	64%	0.98	(0.88 - 1.1)	
Reinards et al.	*present study*			36%	0.97	(0.83 - 1.13)	
pooled					**0.98**		**0.5911**

Supplementary Table S5 (continued)
Meta-analyses of loci previously tested in JIA (but not significantly associated*)

SNP (minor allele)							
IL10 **rs1800896 (-1082) (C)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Crawley 1999	UK	435	274	28%	1.07	(0.86 - 1.32)	
Donn 2001	UK (overlap not reported)	348	239	24%	0.83	(0.66 - 1.05)	
Reinards et al.	*present study*			48%	0.90	(0.77 - 1.06)	
pooled					**0.93**		**0.1908**
IL18RAP **rs917997 (T)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Hinks 2010 IV	UK	987	2931	71%	1.00	(0.89 - 1.13)	
Reinards et al.	*present study*			29%	1.14	(0.94 - 1.37)	
pooled					**1.04**		**0.4693**
IL1A **rs1800587 (-889) (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
McDowell 1995	Norway	269	99	6%	1.34	(0.94 - 1.9)	
Donn 2001	UK	330	236	11%	0.75	(0.57 - 0.97)	
Cinek 2004	Czech Republic	130	102	4%	1.19	(0.78 - 1.81)	
Thompson 2012	USA	814	3058	55%	1.04	(0.93 - 1.18)	
Reinards et al.	*present study*			23%	0.89	(0.74 - 1.07)	
pooled					**0.99**		**0.825**
IL1B **rs16944 (-511) (A)**	**origin**	**nr of cases**	**nr of controls**	**weight**	**OR**	**(95% CI)**	**$p_{meta\text{-}analysis}$**
Cinek 2004	Czech Republic	130	103	14%	1.29	(0.86 - 1.95)	
Reinards et al.	*present study*			86%	1.11	(0.94 - 1.31)	
pooled					**1.13**		**0.116**

Supplementary Table S5 (continued)
Meta-analyses of loci previously tested in JIA (but not significantly associated*)

SNP (minor allele)							
STAT1 rs7562024 (T)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	814	3058	67%	1.00	(0.9 - 1.12)	
Reinards et al.	*present study*			33%	0.95	(0.82 - 1.11)	
pooled					**0.98**		**0.7298**
FAM107A rs13315591 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	814	3056	64%	0.92	(0.75 - 1.14)	
Reinards et al.	*present study*			36%	1.13	(0.86 - 1.5)	
pooled					**0.99**		**0.9204**
4p15 rs874040 (G)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	811	3057	67%	1.03	(0.92 - 1.16)	
Reinards et al.	*present study*			33%	0.93	(0.79 - 1.09)	
pooled					**0.99**		**0.9008**
ERAP1 rs30187 (T)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Hinks 2011	UK	1054[a]	5200[a]	71%	1.02	(0.92 - 1.13)	
Ellis 2013	Australia	200	341	8%	1.06	(0.82 - 1.37)	
Reinards et al.	*present study*			21%	1.14	(0.97 - 1.34)	
pooled					**1.05**		**0.2089**
LECT2 rs31517 (T)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	814	3057	65%	0.92	(0.82 - 1.03)	
Reinards et al.	*present study*			35%	1.03	(0.88 - 1.2)	
pooled					**0.96**		**0.3354**

Supplementary Table S5 (continued)
Meta-analyses of loci previously tested in JIA (but not significantly associated*)

SNP (minor allele)							
PRDM1 rs548234 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{\text{meta-analysis}}$
Hinks 2012	UK	1170	8001	43%	0.96	(0.88 - 1.06)	
Thompson 2012	USA	813	3056	37%	1.03	(0.92 - 1.16)	
Reinards et al.	*present study*			20%	1.13	(0.96 - 1.33)	
pooled					**1.02**		**0.6309**
TAGAP rs394581 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{\text{meta-analysis}}$
Hinks 2012	UK	1175	2617	70%	1.00	(0.9 - 1.12)	
Reinards et al.	*present study*			30%	0.97	(0.82 - 1.15)	
pooled					**0.99**		**0.8646**
CCR6 rs3093023 (A)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{\text{meta-analysis}}$
Hinks 2012	UK	1002	8128	49%	0.96	(0.87 - 1.05)	
Thompson 2012	USA	806	3047	35%	1.08	(0.97 - 1.21)	
Reinards et al.	*present study*			16%	1.07	(0.91 - 1.26)	
pooled					**1.02**		**0.5788**
CDK6 rs42041 (G)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{\text{meta-analysis}}$
Hinks 2010	UK	926	2962	53%	1.03	(0.92 - 1.16)	
Thompson 2010	USA, UK (overlap not reported)	809[a]	3521[a]	27%	0.99	(0.87 - 1.13)	
Thompson 2012	USA	*overlap with Thompson 2010*					
Reinards et al.	*present study*			20%	0.95	(0.8 - 1.13)	
pooled					**1.00**		**0.9404**

Supplementary Table S5 (continued)
Meta-analyses of loci previously tested in JIA (but not significantly associated*)

SNP (minor allele)							
CCL21 rs951005 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Hinks 2012	UK	999	7947	72%	0.91	(0.8 - 1.04)	
Reinards et al.	*present study*			28%	1.08	(0.88 - 1.34)	
pooled					**0.96**		**0.43**
PRKCQ rs4750316 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Hinks 2010	UK	943	3500	42%	0.88	(0.77 - 1)	
Thompson 2012	USA	814	3058	33%	0.87	(0.75 - 1)	
Ellis 2013	Australia	200	341	6%	0.88	(0.63 - 1.23)	
Reinards et al.	*present study*			19%	0.99	(0.82 - 1.2)	
pooled					**0.90**		**0.01012**
TRAF6 rs540386 (T)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	814	3051	69%	1.10	(0.94 - 1.28)	
Reinards et al.	*present study*			31%	0.83	(0.66 - 1.05)	
pooled					**1.01**		**0.8841**
TNFRSF1A rs767455 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Hinks 2013	UK	987	5194	74%	0.98	(0.89 - 1.08)	
Reinards et al.	*present study*			26%	0.92	(0.79 - 1.09)	
pooled					**0.97**		**0.4013**
TNFRSF1A rs4149570 (A)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	812	3055	37%	1.02	(0.91 - 1.14)	
Hinks 2013	UK	929	5191	46%	0.94	(0.85 - 1.04)	
Reinards et al.	*present study*			18%	1.06	(0.9 - 1.25)	
pooled					**0.99**		**0.7597**

Supplementary Table S5 (continued)
Meta-analyses of loci previously tested in JIA (but not significantly associated*)

SNP (minor allele)							
KIF5A rs1678542 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Hinks 2010	UK	941	3530	42%	0.91	(0.82 - 1.01)	
Thompson 2010	USA, UK	809[a]	3521[a]	33%	1.00	(0.89 - 1.13)	
Thompson 2012	USA	*overlap with Thompson 2010*					
Ellis 2013	Australia	200	341	7%	0.96	(0.74 - 1.24)	
Reinards et al.	*present study*			19%	1.02	(0.87 - 1.19)	
pooled					**0.96**		**0.2652**
IL21R rs3093341 (G)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	814	3057	66%	0.99	(0.82 - 1.2)	
Reinards et al.	*present study*			34%	0.80	(0.62 - 1.05)	
pooled					**0.92**		**0.3058**
STAT5A rs7217728 (C)	origin	nr of cases	nr of controls	weight	OR	(95% CI)	$p_{meta\text{-}analysis}$
Thompson 2012	USA	812	3058	65%	0.96	(0.85 - 1.08)	
Reinards et al.	*present study*			35%	0.91	(0.77 - 1.07)	
pooled					**0.94**		**0.2319**

OR: odds ratio; CI: confidence interval
* Patient group of the present study limited to oligoarticular (persistent and extended) and RF-negative JIA patients References are listed in Supplementary Table S6
a) Exact number of succesfully typed individuals not known
b) Patient group of all included studies limited to oligoarticular (persistent and extended) and RF-negative JIA patients

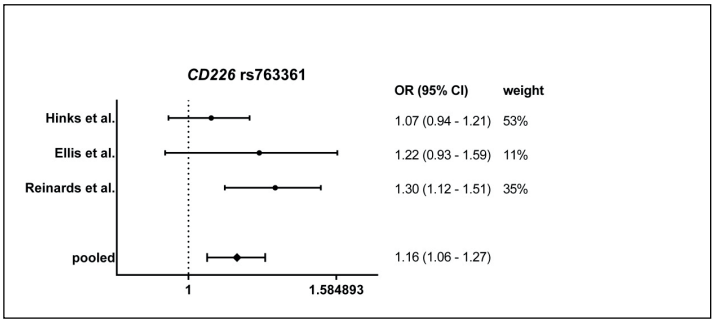
SupplementaryTable S6. References of studies analysed for meta-analysis (supplementary Tables S4 and S5)

Cimaz 2007[1]	Cimaz R, Cazalis MA, Reynaud C et al. IL1 and TNF gene polymorphisms in patients with juvenile idiopathic arthritis treated with TNF inhibitors. *Ann Rheum Dis 2007; 66(7):900-904.*
Cinek 2004[2]	Cinek O, Vavrincova P, Striz I et al. Association of single nucleotide polymorphisms within cytokine genes with juvenile idiopathic arthritis in the Czech population. *J Rheumatol 2004; 31(6):1206-1210.*
Cinek 2006[3]	Cinek O, Hradsky O, Ahmedov G et al. No independent role of the -1123 G>C and+2740 A>G variants in the association of PTPN22 with type 1 diabetes and juvenile idiopathic arthritis in two Caucasian populations. *Diabetes Res Clin Pract 2007; 76(2):297-303.*
Crawley 1999[4]	Crawley E, Kay R, Sillibourne J et al. Polymorphic haplotypes of the interleukin-10 5′ flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. *Arthritis Rheum 1999; 42(6):1101-1108.*
Dimopoulou 2013[5]	Dimopoulou DG, Zervou MI, Trachana M et al. Investigation of juvenile idiopathic arthritis susceptibility loci: results from a Greek population. *Hum Immunol* 2013; 74(9):1194-1198.
Donn 2001[6]	Donn RP, Barrett JH, Farhan A et al. Cytokine gene polymorphisms and susceptibility to juvenile idiopathic arthritis. British Paediatric Rheumatology Study Group. *Arthritis Rheum* 2001; 44(4):802-810.
Donn 2002[7]	Donn R, Alourfi Z, De BF et al. Mutation screening of the macrophage migration inhibitory factor gene: positive association of a functional polymorphism of macrophage migration inhibitory factor with juvenile idiopathic arthritis. *Arthritis Rheum* 2002; 46(9):2402-2409.
Ellis 2013[8]	Ellis JA, Chavez RA, Pezic A et al. Independent replication analysis of genetic loci with previous evidence of association with juvenile idiopathic arthritis. *Pediatr Rheumatol Online J* 2013; 11(1):12.
Hinks 2005[9]	Hinks A, Barton A, John S et al. Association between the PTPN22 gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: further support that PTPN22 is an autoimmunity gene. *Arthritis Rheum* 2005; 52(6):1694-1699.
Hinks 2009[10]	Hinks A, Ke X, Barton A et al. Association of the IL2RA/CD25 gene with juvenile idiopathic arthritis. *Arthritis Rheum* 2009; 60(1):251-257.
Hinks 2009 II[11]	Hinks A, Barton A, Shephard N et al. Identification of a novel susceptibility locus for juvenile idiopathic arthritis by genome-wide association analysis. *Arthritis Rheum* 2009; 60(1):258-263.
Hinks 2010[12]	Hinks A, Eyre S, Ke X et al. Overlap of disease susceptibility loci for rheumatoid arthritis and juvenile idiopathic arthritis. *Ann Rheum Dis* 2010; 69(6):1049-1053.
Hinks 2010 II[13]	Hinks A, Eyre S, Ke X et al. Association of the AFF3 gene and IL2/IL21 gene region with juvenile idiopathic arthritis. *Genes Immun* 2010; 11(2):194-198.
Hinks 2010 III[14]	Hinks A, Martin P, Flynn E et al. Association of the CCR5 gene with juvenile idiopathic arthritis. *Genes Immun* 2010; 11(7):584-589.
Hinks 2010 IV[15]	Hinks A, Martin P, Flynn E et al. Investigation of type 1 diabetes and coeliac disease susceptibility loci for association with juvenile idiopathic arthritis. *Ann Rheum Dis* 2010; 69(12):2169-2172.
Hinks 2011[16]	Hinks A, Martin P, Flynn E et al. Subtype specific genetic associations for juvenile idiopathic arthritis: ERAP1 with the enthesitis related arthritis subtype and IL23R with juvenile psoriatic arthritis. *Arthritis Res Ther* 2011; 13(1):R12.
Hinks 2012[17]	Hinks A, Cobb J, Sudman M et al. Investigation of rheumatoid arthritis susceptibility loci in juvenile idiopathic arthritis confirms high degree of overlap. *Ann Rheum Dis* 2012; 71(7):1117-1121.
Hinks 2013[18]	Hinks A, Martin P, Thompson SD et al. Autoinflammatory gene polymorphisms and susceptibility to UK juvenile idiopathic arthritis. *Pediatr Rheumatol Online J* 2013; 11(1):14.

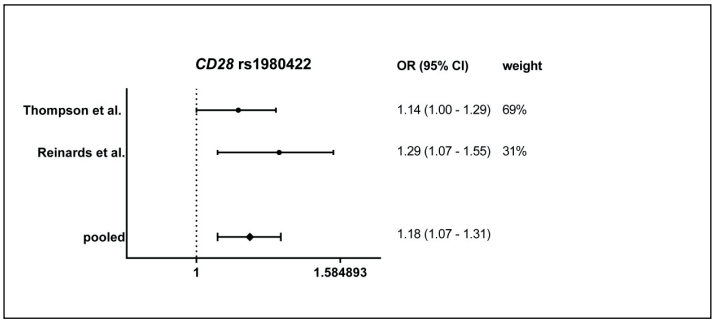
Kaalla 2013[19]	Kaalla MJ, Broadaway KA, Rohani-Pichavant M et al. Meta-analysis confirms association between TNFA-G238A variant and JIA, and between PTPN22-C1858T variant and oligoarticular, RF-polyarticular and RF-positive polyarticular JIA. *Pediatr Rheumatol Online J* 2013; 11(1):40.
Lindner 2007[20]	Lindner E, Nordang GB, Melum E et al. Lack of association between the chemokine receptor 5 polymorphism CCR5delta32 in rheumatoid arthritis and juvenile idiopathic arthritis. *BMC Med Genet* 2007; 8:33.
McDowell 1995[21]	McDowell TL, Symons JA, Ploski R et al. A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism. *Arthritis Rheum* 1995; 38(2):221-228.
Miterski 2004[22]	Miterski B, Drynda S, Boschow G et al. Complex genetic predisposition in adult and juvenile rheumatoid arthritis. *BMC Genet* 2004; 5:2.
Mourão 2009[23]	Mourao AF, Caetano-Lopes J, Costa P et al. Tumor necrosis factor-alpha -308 genotypes influence inflammatory activity and TNF-alpha serum concentrations in children with juvenile idiopathic arthritis. *J Rheumatol* 2009; 36(4):837-842.
Ozen 2002[24]	Ozen S, Alikasifoglu M, Bakkaloglu A et al. Tumour necrosis factor alpha G-->A -238 and G-->A -308 polymorphisms in juvenile idiopathic arthritis. *Rheumatology (Oxford)* 2002; 41(2):223-227.
Pazár 2008[25]	Pazar B, Gergely P, Jr., Nagy ZB et al. Role of HLA-DRB1 and PTPN22 genes in susceptibility to juvenile idiopathic arthritis in Hungarian patients. *Clin Exp Rheumatol* 2008; 26(6):1146-1152.
Prahalad 2008[26]	Prahalad S, Bohnsack JF, Whiting A et al. Lack of association of functional CTLA4 polymorphisms with juvenile idiopathic arthritis. *Arthritis Rheum* 2008; 58(7):2147-2152.
Prahalad 2009[27]	Prahalad S, Hansen S, Whiting A et al. Variants in TNFAIP3, STAT4, and C12orf30 loci associated with multiple autoimmune diseases are also associated with juvenile idiopathic arthritis. *Arthritis Rheum* 2009; 60(7):2124-2130.
Schmeling 2006[28]	Schmeling H, Wagner U, Peterson A et al. Tumor necrosis factor alpha promoter polymorphisms in patients with juvenile idiopathic arthritis. *Clin Exp Rheumatol* 2006; 24(1):103-108.
Seldin 2005[29]	Seldin MF, Shigeta R, Laiho K et al. Finnish case-control and family studies support PTPN22 R620W polymorphism as a risk factor in rheumatoid arthritis, but suggest only minimal or no effect in juvenile idiopathic arthritis. *Genes Immun* 2005; 6(8):720-722.
Skinningsrud 2010[30]	Skinningsrud B, Lie BA, Husebye ES et al. A CLEC16A variant confers risk for juvenile idiopathic arthritis and anti-cyclic citrullinated peptide antibody negative rheumatoid arthritis. *Ann Rheum Dis* 2010; 69(8):1471-1474.
Suppiah 2005[31]	Suppiah V, O'doherty C, Heggarty S et al. The CTLA4+49A/G and CT60 polymorphisms and chronic inflammatory arthropathies in Northern Ireland. *Exp Mol Pathol* 2006; 80(2):141-146.
Thompson 2010[32]	Thompson SD, Sudman M, Ramos PS et al. The susceptibility loci juvenile idiopathic arthritis shares with other autoimmune diseases extend to PTPN2, COG6, and ANGPT1. *Arthritis Rheum* 2010; 62(11):3265-3276.
Thompson 2012[33]	Thompson SD, Marion MC, Sudman M et al. Genome-wide association analysis of juvenile idiopathic arthritis identifies a new susceptibility locus at chromosomal region 3q13. *Arthritis Rheum* 2012; 64(8):2781-2791.
Viken 2005[34]	Viken MK, Amundsen SS, Kvien TK et al. Association analysis of the 1858C>T polymorphism in the PTPN22 gene in juvenile idiopathic arthritis and other autoimmune diseases. *Genes Immun* 2005; 6(3):271-273.
Zeggini 2002[35]	Zeggini E, Thomson W, Alansari A et al. Tumour necrosis factor receptor II polymorphism and juvenile idiopathic arthritis. *Rheumatology (Oxford)* 2002; 41(4):462-465.
Zeggini 2002 II[36]	Zeggini E, Thomson W, Kwiatkowski D et al. Linkage and association studies of single-nucleotide polymorphism-tagged tumor necrosis factor haplotypes in juvenile oligoarthritis. *Arthritis Rheum* 2002; 46(12):3304-3311.

Supplementary Figure S1 Meta-analysis of novel JIA susceptibility loci

a. Meta-analysis of *CD226* rs763361, restricted to oligoarticular and RF negative JIA patients of Caucasian origin

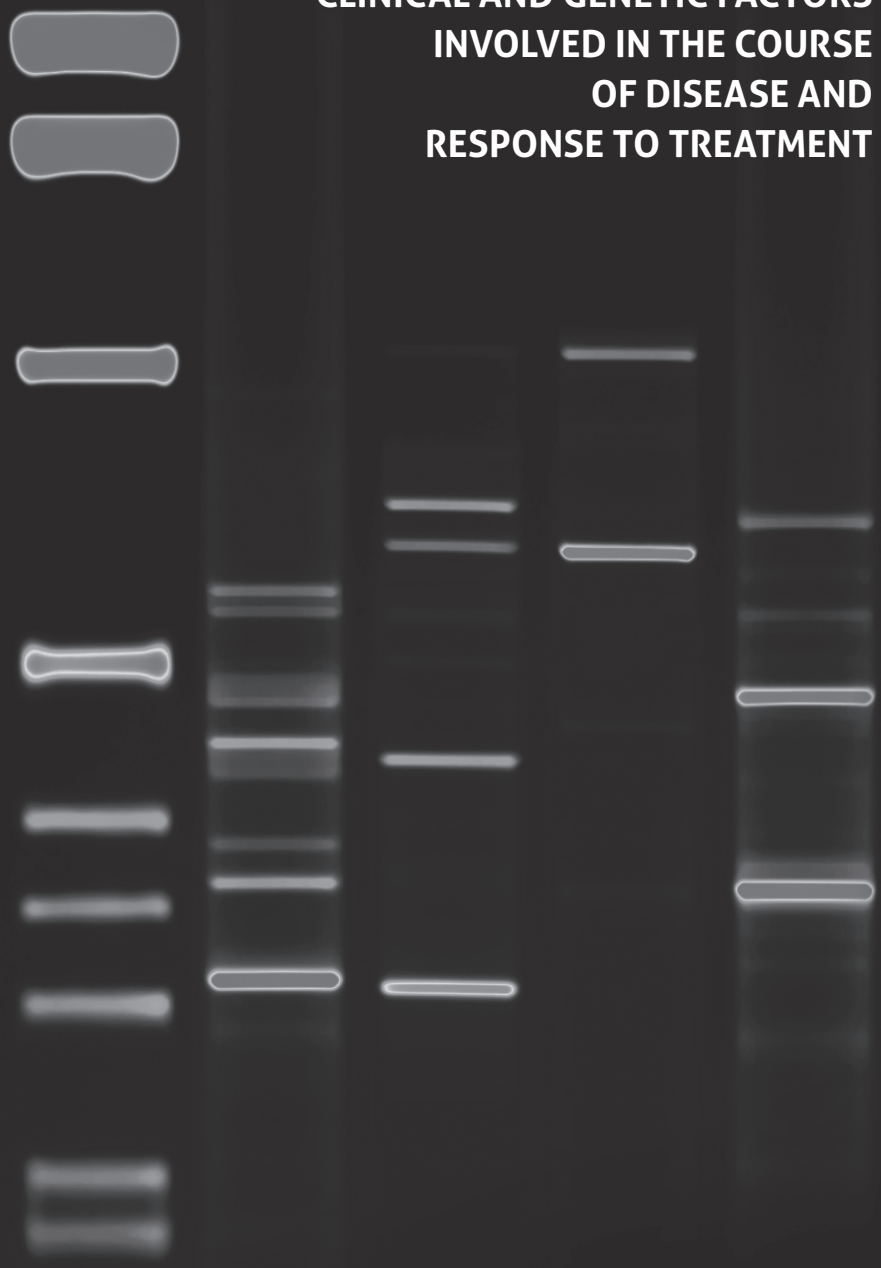


b. Meta-analysis of *CD28* rs1980422, restricted to oligoarticular and RF negative JIA patients of Caucasian origin



PART B

CLINICAL AND GENETIC FACTORS INVOLVED IN THE COURSE OF DISEASE AND RESPONSE TO TREATMENT



CHAPTER 5

THE CLINICAL COURSE AND PROGNOSTIC VALUE OF DISEASE ACTIVITY IN THE FIRST TWO YEARS IN DIFFERENT SUBTYPES OF JUVENILE IDIOPATHIC ARTHRITIS

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H.M. Albers [1], D.M.C. Brinkman[1], S.S.M. Kamphuis[2], L.W.A. Suijlekom-Smit[2],M.A.J. van Rossum[3], E.P.A.H. Hoppenreijs[4], H.J. Girschick[5], C. Wouters[6], R.K. Saurenmann[7], J.J. Houwing-Duistermaat[1] T.W.J. Huizinga[1], M.W. Schilham[1], R. ten Cate[1]

1) Leiden University Medical Center, Leiden, the Netherlands.
2) Erasmus Medical Center- Sophia Children's Hospital, Rotterdam, the Netherlands.
3) Academic Medical Center- Emma Children's Hospital and Reade, location Jan van Breemen, Amsterdam, the Netherlands
4) Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands
5) University of Wuerzburg, Wuerzburg, Germany.
6) University Hospital Gasthuisberg, Leuven, Belgium.
7) Zurich University Children's Hospital, Zurich, Switzerland

ABSTRACT

Objective

Juvenile Idiopathic Arthritis is a heterogeneous disease involving chronic arthritis. The clinical course is characterized by a fluctuating pattern of active and inactive disease. We have described in detail the clinical course in different JIA subtypes in the first two years after diagnosis and studied its relation to disease activity in the following years.

Methods

Detailed clinical data on different parameters describing the disease activity in sequential time periods covering the first two years after diagnosis were retrieved from the charts of 311 JIA patients and compared between subtypes. In a cohort of 146 patients the relation of these different clinical variables to the course of disease in the following three years was evaluated.

Results

The percentage of time with active disease in the first two years differed significantly between subtypes. In all subtypes a broad spectrum of activity was observed. The time with active disease in the first two years was the most significant factor associated with the duration of active disease in the following years.

Conclusion

In this study, different percentages of time with active disease have been observed between JIA subtypes in the first two years. The cumulative duration of activity varied widely within each subtype. Regarding the prognosis of the individual patient, the clinical course in the first two years appears to be predictive of the clinical course in the following years. Patients that have less time with active disease in the first two years are not likely to develop unremitting clinical course later on.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a heterogeneous group of disorders characterized by chronic inflammation of the joint. Seven different subtypes have been defined by the International League of Associations for Rheumatology (ILAR) based on clinical and laboratory parameters.[1] Within JIA, persistent oligoarthritis, extended oligoarthritis and rheumatoid factor (RF) negative polyarthritis are the most homogeneous subtypes with a large phenotypic overlap.[2] Although the remission rate is the highest in persistent oligoarthritis,[3] the precise clinical course of the different subtypes is still unclear.

Prediction of the clinical course of the disease could be helpful for choosing the optimal treatment strategy for an individual patient. However, at this moment no clinically useful prognostic factors are known.[4;5] The outcome of JIA regarding disease activity and remission has frequently been studied in the different subtypes, but unfortunately different studies have used a large variety of definitions of remission and thereby generated data that are difficult to compare.[3-6] Several years ago a preliminary definition of remission has been formulated, defining different states of inactivity; inactive disease, clinical remission on medication and clinical remission off medication.[7] Furthermore, almost all studies in JIA concerning outcome have used a cross-sectional study design, while Wallace et al clearly showed that JIA is a disease with fluctuating disease activity that can best be described by analysis of sequential time periods.[8] By using sequential time periods, the cumulative time with active or inactive disease can be determined and used as outcome measure.[8;9] Already different studies have shown that a prolonged disease activity is related to a poor outcome regarding radiographic damage.[10;11] Moreover it seems to be important to distinguish between different states of activity, because reaching a state of minimal disease activity (MDA) is related to less damage to the joint (amongst others) compared to not reaching MDA.[12]

In this study we will describe the clinical course of the first two years after diagnosis in different subtypes of JIA by analyzing sequential episodes of disease activity. Moreover, we have studied the different parameters of disease activity assessed in the first two years after diagnosis in relation to the clinical course in the following years to identify clinical prognostic factors for the individual patient.

MATERIALS AND METHODS

Patient population

A cohort of 640 JIA patients, with oligoarthritis, polyarthritis and systemic JIA, originating from 7 different pediatric rheumatology referral centers in the Netherlands, Belgium, Germany and Switzerland and who were diagnosed after 1 January 1991, was observed retrospectively. For this study patients were excluded when no clinical data on disease activity were available (n= 144), when the follow-up was less than 2 years (n=158), if re-classified with psoriatic arthritis, JIA with enthesitis and undifferentiated JIA (n=6) and if an ongoing uveitis was present with only a mild course of arthritis (n=3). This resulted in a JIA cohort of 311 patients that were included in the analysis of the clinical course of the first two years after diagnosis. In order to identify prognostic factors for the clinical course in the following years, only patients with a follow-up of >= 5 years were included (n=146), excluding 165 patients with a follow-up of >2 years and < 5 years. All patients were diagnosed or (re)classified according to the ILAR classification and had a self- or parental-reported Caucasian ethnicity.[1] The characteristics of the JIA patient cohort are listed in Table 1.

The ethical review boards of all participating centers gave their approval to this study and informed consent was obtained from all patients and/or parents.

Table 1. Patients' characteristics[#]

		n	(%)
Total JIA population		311	
Subtype:			
Persistent oligoarthritis		124	(39.9)
Extended oligoarthritis		56	(18.0)
RF- negative polyarthritis		91	(29.3)
RF- positive polyarthritis		9	(2.9)
Systemic JIA		31	(10.0)
Female		214	(68.8)
Follow-up (year)	(median; min-max)	4.8	2.0- 15.9
Age at onset (year)	(median; min-max)	4.1	0.6- 15.5
ANA	positive	163	(52.4)
	negative	109	(35.0)
	other*/ unknown	38	(12.2)
Uveitis	positive	47	(15.1)
	negative	225	(72.3)
	unknown	32	(10.3)

#) Parameters listed in numbers and percentages unless otherwise indicated

*) If antinuclear antibodies are not consistently present or detected

Clinical data

Each patient's chart was reviewed retrospectively and the disease activity (according to different parameters) at all visits during the complete disease course was evaluated. We assumed that the patient stayed in the same state of disease activity until the next visit. Patients visited the pediatric rheumatologists with an interval of 6 months or less in case of inactive disease and with an interval of 3 months or less when disease was active. Due to the retrospective nature of this study, the follow-up of the patients has not been uniform; patients with a remitting course of disease had fewer visits to the pediatric rheumatologist than patients with an unremitting course of disease. However, when patients in an inactive state developed complaints the subsequent visit was usually placed forward. Therefore, in individual patients, mainly the state of active disease might be over-estimated.

Different parameters were used indicating disease activity; state of disease activity (active or inactive disease), the subjective physicians' global assessment of disease activity (categorized as: 0 = inactive disease, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe disease) and the number of joints with arthritis (categorized as: 0 = no joints, 1 = monoarthritis (1 joint) , 2 = oligoarthritis (>=1 – 4 joints), 3 = polyarthritis (>=4- <=10 joints), 4 = severe polyarthritis (> 10 joints). No joint score was used for systemic JIA, because of the systemic features that are involved in JIA. Inactive disease was defined as absence of active arthritis, no systemic features, normal ESR (when available) and a physicians' global assessment indicating inactivity (category 0). The activity of JIA-associated uveitis was not incorporated in the definition of inactivity, because of missing data about the detailed ophthalmologic follow-up. However, to minimize the possible bias this could cause, we excluded patients (n=3) that had a known ongoing uveitis with only short periods of arthritis.

Furthermore, data on the use of different medication (yes/no) in the first two years were reviewed and grouped as: the use of non-steroidal anti-inflammatory drugs (NSAIDs) alone; intra-articular steroids (IAS) alone or combined with NSAIDs ; sulfasalazine (SSZ) alone or in combination with NSAIDs; the use of Methotrexate (MTX) (started <= 6 months (early) or >6 months (late) after diagnosis) combined with any other (or no other) drugs besides Etanercept; Etanercept in combination with MTX and any other (or no other) drugs; other types or combinations of treatment.

Beside data on disease activity and medication, also data on the presence or absence of antinuclear antibodies (ANA), age at onset and gender were collected.

Parameters of activity

In order to evaluate the clinical course of the different subtypes, for each patient the first two years after diagnosis were analyzed. For this period, the percentage of time

with active disease was calculated together with the percentage of patients that had reached clinical remission within these first two years. Clinical remission (yes/no) was defined as the presence of an episode of inactivity lasting >= 6 months. Additionally, the mean physicians' global assessment and the mean joint score were calculated for each individual patient for the time the disease was active in the first two years (later referred to as the physicians' global assessment and joint score). The percentage of time with active disease that was evaluated as mild by the physicians (category 1) was used as measure for mild active disease. These different parameters of disease activity were compared between the different subtypes.

To study the prognostic value of different clinical parameters assessed in the first two years, the percentage of time with active disease in the following three years (third, fourth and fifth year (3-5) after diagnosis) was calculated to determine the clinical course later on in the disease. The percentage of time with active disease during the years 3-5 was used to define remitting and unremitting clinical course. Remitting clinical course was defined as: percentage of time with active disease between 0 and 35%, whereas unremitting clinical course was defined as: percentage of time with active disease >=65%. Patient with active disease during more than 35% and less than 65% of the time were considered as having an intermediate clinical course. Following the same definition, patients were also categorized into remitting, intermediate and unremitting course according to their percentage of active disease in the first two years after onset.

Statistical analysis

When analyzing the parameters of activity in the first two years in different subtypes, non-parametric tests (Kruskal Wallis, Mann-Whitney U test) were used because of the non-normal distribution of different variables in the overall population and some subtypes. Comparing the different parameters in patients with a remitting and unremitting clinical course, a Pearsons' Chi-square or Mann-Whitney U test were used depending on the tested variable. A p-value of <0.05 was regarded as statistically significant.

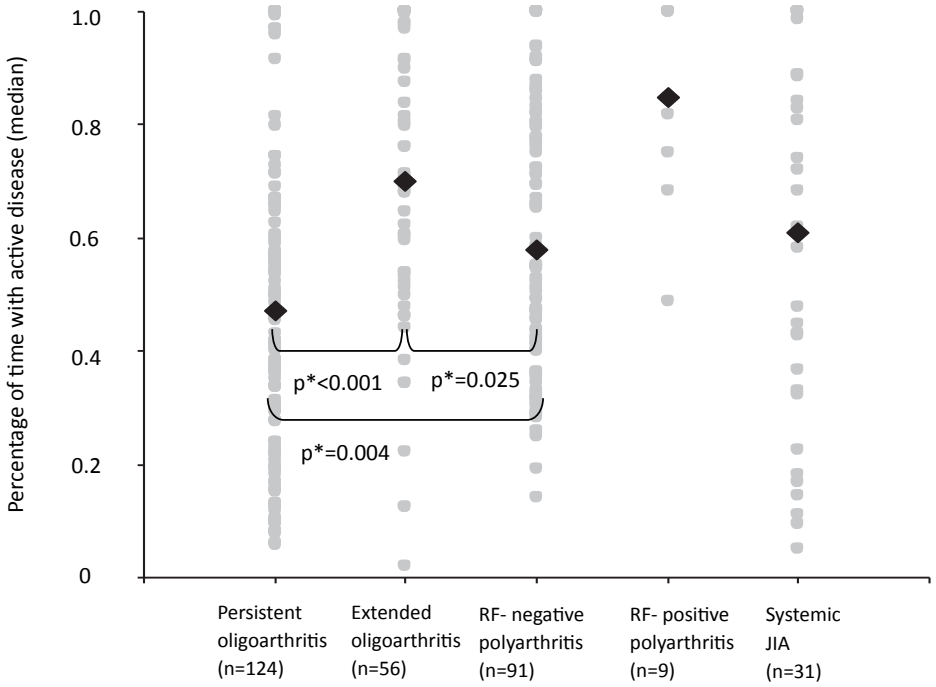
Positive and negative predictive values were calculated for the remitting and unremitting category comparing the clinical course in the first two years after onset with the clinical course in the years 3-5 in order to estimate the predictive value of the clinical course in the first two years. Data were analyzed using SPSS 16.0.

RESULTS

Percentage of time with active disease in the first two years

The percentages of time with active disease in the first two years after diagnosis in the overall JIA cohort as well as in the individual subtypes are listed in Table 2 and shown in Figure 1. The median percentage of time with active disease in the overall JIA cohort is 57%, meaning that half of the patients have more than 14 months of active disease (cumulative) during the first two years. The percentage of time with active disease is significantly different between the subtypes (p=0.031). Analyzing the three subtypes with the most phenotypic overlap (persistent oligoarthritis, extended oligoarthritis and rheumatoid factor (RF) negative polyarthritis), the lowest median percentage of time with active disease is observed in patients with persistent oligoarthritis (47%) compared to extended oligoarthritis (70%; p <0.001) and RF- negative polyarthritis (58%;

Figure 1. The percentage of time with active disease in the first two years after diagnosis in the different subtypes.



♦ median percentage of time with active disease per subtype of JIA

*) p-value of Mann- Whitney U test comparing two subtypes (persistent oligoarthritis vs extended oligoarthritis, extended oligoarthritis vs RF-negative polyarthritis, persistent oligoarthritis vs RF-negative polyarthritis)

p=0.004), whereas the median percentage of time with active disease in extended oligoarthritis is the highest and significantly different from RF-negative polyarthritis (p=0.025). In patients with systemic JIA the median percentage of active disease is comparable to patients with RF-negative polyarthritis (61%; p=0.67). Patients with RF-positive polyarthritis have the highest median percentage of time with active disease (85%), but because of the small sample size (n=9) no reliable conclusion can be drawn from these data.

Clinical remission in the first two year

When an episode of inactive disease lasted >= 6 months, this was regarded as an episode of clinical remission. The percentage of patients within the different subtypes who reached clinical remission in the first two years is listed in Table 2. Patients with extended oligoarthritis have a significantly lower percentage of clinical remission (36%) compared to both persistent oligoarthritis (69%; p< 0.001) and RF-negative polyarthritis (60%; p= 0.004). No difference between persistent oligoarthritis and RF-negative polyarthritis patients was observed (p=0.22). In systemic JIA, 58% of the patients have reached clinical remission in the first two years. Inherent to the high percentage of time with active disease, the percentage of clinical remission is low (11%) in RF-positive polyarthritis. Only 84 patients had a continuous period of inactive disease for >= 12 months in these first two years and 34 patients (of which 68% persistent oligoarthritis patients) were off medication for >= 12 months in that episode.

Physicians' global assessment of disease activity, percentage of mild active disease and the joint score in the first two years

The physicians' global assessment (reviewed at times when the disease was active in the first two years) in the overall JIA cohort and in the different subtypes is listed in Table 3. No significant differences in the median physicians' global assessment of disease activity were observed when comparing only persistent oligoarthritis, extended oligoarthritis and RF-negative polyarthritis (p=0.58). This means that although the cumulative time of active disease is different in these three subtypes, the physician evaluates the severity of disease activity similar at times when the disease is active. No association between the percentage of time with active disease and the physician's global assessment is observed in the analysis of the total JIA cohort (data not shown).
The percentage of time in which the physicians' global assessment is evaluated as mild (category 1) is also listed in Table 3. No significant differences in percentages of mild active disease are observed comparing JIA subtypes.
Due to the ILAR classification that is based on differences in the number of affected joints, the median joint score during active disease in the first two years is signifi-

Table 2 The percentage of time with active disease and the percentage of patients that have reached clinical remission in the first two years after diagnosis in the overall JIA cohort and the different subtypes.

	Percentage of time with active disease		Clinical remission °	
	median (range)	p[1]	%	p[2]
Overall JIA cohort (n=311)	0.57 (0.02-1.0)	0.031 (1-5)	0.58	< 0.001 (1-5)
1.Persistent oligoarthritis (n=124)	0.47 (0.06- 1.0)	<0.001 (1vs 2)	0.69	<0.001 (1 vs 2)
2.Extended oligoarthritis (n=56)	0.70 (0.02- 1.0)	0.025 (2 vs 3)	0.36	0.004 (2 vs 3)
3.RF- negative polyarthritis (n=91)	0.58 (0.14- 1.0)	0.004 (1vs 3)	0.60	0.218 (1 vs 3)
4.RF- positive polyarthritis (n=9)	0.85 (0.49- 1.0)		0.11	
5.Systemic JIA (n=31)	0.61 (0.05- 1.0)	0.67 (3 vs 5)	0.58	0.269 (1 vs 5)

°) percentage of patients that have reached clinical remission in the first two years after diagnosis

1) p-value of Kruskal-Wallis test in all subtypes (1-5) or Mann- Whitney U test comparing two subtypes (persistent oligoarthritis vs extended oligoarthritis, extended oligoarthritis vs RF-negative polyarthritis, persistent oligoarthritis vs RF-negative polyarthritis) indicated by numbers (in brackets).

2) p-value of Pearson-Chi-square comparing the percentage of clinical remission in all subtypes (1-5) and comparing the subtypes indicated by numbers (in brackets).

cantly different between the subtypes (Table 3). Patients with an extended oligoarthritis have a significant higher median joint score than persistent oligoarthritis patients, although both subtypes have <= 4 joints involved in the first 6 months. This indicates that extended oligoarthritis not only affects more than 5 different joints during the disease course, but that on the average more joints are active at the same time.

These data show that the clinical course, indicated by the percentage of time with active disease in the first two years after diagnosis, is different in the JIA subtypes. Especially the clinical course of persistent oligoarthritis is significantly different from extended oligoarthritis, which seem to be two separate entities. However within each subtype an individual patient can have a wide range of activity varying from only a short time of active disease to ongoing disease activity. This already shows that the subtype JIA on itself is not valid as a prognostic factor for the individual patient.

Prognostic factors for the clinical course in years 3-5 after diagnosis

In patients with a follow-up of >= 5 years (n=146) the percentage of time with active disease was evaluated during the years 3-5 (third, fourth and fifth year) after diagnosis and patients were categorized into a remitting (0-35% time with active disease), intermediate (35-65%) or an unremitting clinical course (65-100%). The different parameters of disease activity measured in the first two years, the use of medication in the first two years together with subtype, age at onset, gender and ANA status were

Table 3 The physicians' global assessment, the percentage of time with mild active disease and joint score in the first two years after diagnosis, all reviewed only over the time when disease was active.#.

	Physicians' global assessment*		Percentage of time with mild active disease		Joint score°	
	median (range)	p[1]	median (range)	p[1]	median (range)	p[2]
Overall JIA cohort (n=266/261)	1.74 (1.0- 3.38)	0.031 (1-5)	0.33 (0- 1.0)	0.203 (1-5)		
1.Persistent oligoarthritis (n= 97/95/ 124)	1.70 (1.0- 2.62)	0.583 (1-3)	0.41 (0- 1.0)	0.885 (1-3)	1.30 (1.0- 2.31)	<0.001 (1 vs 2)
2.Extended oligoarthritis (n=49/48/56)	1.74 (1.0- 3.0)		0.34 (0- 1.0)		1.82 (0.61- 3.0)	0.023 (2 vs 3)
3.RF negative polyarthritis (n=80/78/91)	1.74 (1.0- 2.72)		0.33 (0- 1.)		2.45 (1.69- 4.0)	0.012 (3 vs 4)
4.RF positive polyarthritis (n= 9/9/9)	2.00 (1.08- 2.73)		0 (0- 0.92)		3.00 (1.37- 3.0)	
5.Systemic JIA (n= 31/31/31)	1.87 (1.07- 3.38)		0.18 (0- 0.93)			

*) The physicians' global assessment of disease activity is categorized as: 0 = inactive disease, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe disease.

°) The joints score is categorized as: 0 = no joints, 1 = monoarthritis (1 joint) , 2 = oligoarthritis (>=1 – 4 joints), 3 = polyarthritis (>=4- <=10 joints), 4 = severe polyarthritis (> 10 joints) and in case of systemic JIA an additional category 5 = systemic features.

1) p-value of Kruskal-Wallis test in all subtypes (1-5) or in persistent oligoarthritis, extended oligoarthritis and RF-negative polyarthritis (1-3).

2) p-value of Mann-Whitney U test. Subtypes that are being compared are indicated by numbers (in brackets).

Table 4. Patients' characteristics and comparison of the different clinical and demographic parameters between patients with a remitting course and an unremitting course #

		n	Remitting course*	Unremitting course*	p
All JIA subtypes•		146	62 (42.5)	44 (30.1)	
Subtype°					0.418[1]
Persistent oligoarthritis		43	25 (58.1)	10 (23.3)	
Extended oligoarthritis		44	15 (34.1)	14 (31.8)	
RF negative polyarthritis		39	15 (38.5)	12 (30.8)	
RF positive polyarthritis		4	0 (0)	4 (100)	
Systemic JIA		16	7 (43.8)	4 (25.0)	
Age at onset (years), median (range)		146	2.94 (0.62-12.64)	4.17 (1.23- 14.2)	0.203[2]
Gender	female	108	46 (42.6)	33 (30.6)	
	male	38	16 (42.1)	11 (28.9)	0.96[1]
ANA†	positive	83	37 (44.6)	22 (26.5)	0.437[1]
	negative	34	12 (35.3)	13 (38.2)	
Percentage active disease in first two years, median (range)		106	45.8 (6.22- 100)	100 (47.4- 100)	**<0.001[2§]**
Clinical remission (>= 6 months) in first 2 years (yes/no)		69	45 (65.2)	8 (11.6)	**<0.001[1§]**
Clinical remission off mediation (>= 12 months) in the first 2 years (yes/no)		10	9 (90)	0	**<0.001[1§]**
Physicians'global assessment in first two years, median (range)[&]		122	1.70 (1.0- 3.0)	1.74 (1.0- 2.72)	0.373[2]
Percentage mild active disease		119	0.34 (0- 1.0)	0.37 (0- 1.0)	0.941[2]
Medication in first two years:					
NSAIDs only		35	21 (60.0)	8 (22.9)	0.052[1]
IAS [3]		29	19 (65.5)	4 (13.8)	**0.017[1§]**
SSZ[3]		9	3 (33.3)	1 (11.1)	0.171[1]
MTX[4]		47	14 (29.8)	19 (40.4)	0.074[1§]
MTX early		13	5 (38.5)	6 (46,2)	0,396&
MTX late		34	9 (26.5)	13 (38,2)	
Etanercept[5]		6	1 (16.7)	4 (66.7)	0.120[1]
Systemic glucocorticoids[6]		39	11 (28.2)	17 (43.6)	**0.042[1§]**

NSAIDs= non-steroidal anti-inflammatory drugs, IAS= intraarticular steroids, SSZ= sulfasalazine, MTX= methotrexate

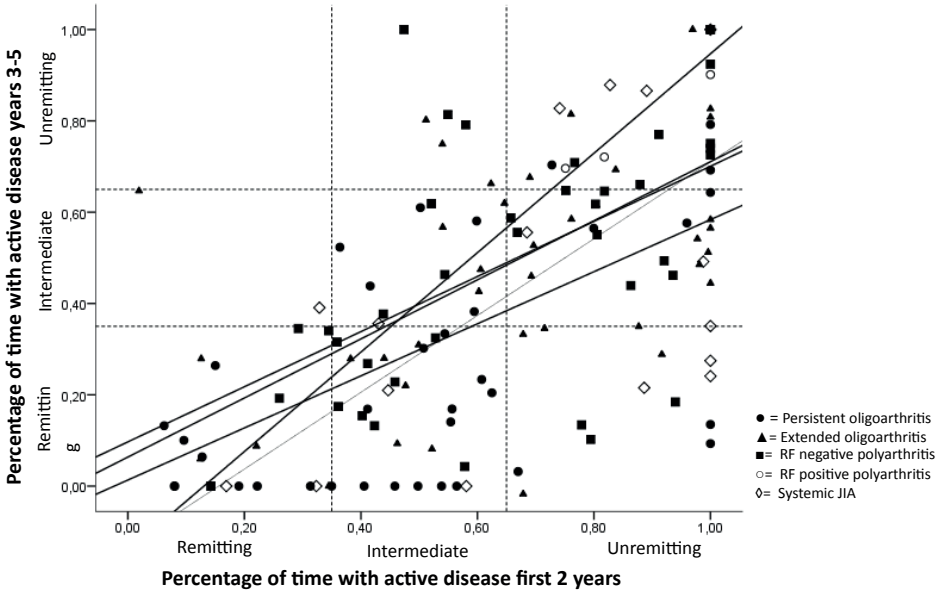
#) Parameters listed in number (percentage) unless otherwise indicated.
*) Remitting and unremitting course based on the disease activity in the years 3-5 after diagnosis.
•) According to the revised ILAR classification (1)
°) Patients with RF-positive polyarthritis not included in the analysis of subtypes because of the low number of patients.
1) p-value of Pearson's Chi-square
2) p-value of Mann Whitney U test
†) Analysis of ANA only in persistent oligoarthritis, extended oligoarthritis and RF- negative polyarthritis.
§) p-value of <0.05 statistical significant
&) calculated over the time when disease was active
3) monotherapy or combined with NSAIDs
4) monotherapy or combined with other drugs besides Etanercept
&) Difference between MTX early and late
5) always combined with MTX
6) monotherapy or combined with other drugs

compared between the group of patients with a remitting and unremitting clinical course in years 3-5 in order to evaluate their relation to the clinical course (Table 4).
A remitting clinical course during the years 3-5 was observed in 42.5% of the patients compared to 30.1% of patients with an unremitting disease course. No differences in age at onset, gender and ANA status were observed between patients with a remitting course and those with an unremitting course. When analyzing the different subtypes, the patients with RF-positive polyarthritis were excluded because of their small sample size. No difference in the percentage of remitting and unremitting clinical course was observed between the remaining subtypes. When the use of medication in the first two years was analyzed, it appeared that patients who had received only intra-articular steroids (combined with NSAIDs) as treatment showed more often a remitting clinical course ($p=0.017$), while patients that were treated with systemic glucocorticoids had a higher percentage of unremitting clinical course ($p=0.042$).
Most important, already in the first two years after diagnosis the percentage of time with active disease was significantly lower and the percentage of patients reaching clinical remission significantly higher in patients who had a remitting clinical course in the following years (years 3-5) ($p<0.001$). During the first two years, there were no differences in physicians' global assessments or percentage of time periods with mild active disease observed between the patient groups with a remitting or unremitting course in year 3-5. This might indicate that reaching a state of inactive disease instead of a diminished disease activity should be the goal of treatment in JIA.

Predictive value of the percentage of active disease during the first 2 years

Figure 2 shows the percentage of time with active disease in the first 2 years plotted against the activity in the years 3-5. A significant correlation between the two variables is observed both in the overall JIA population as well as in each subtype ($p <0,05$; data not shown). The percentage of active disease in the first two years was categorized into remitting, intermediate and unremitting course (following the same definition as used for years 3-5), and compared to the clinical course in years 3-5 (Figure 2 and Table 5). Furthermore the positive and negative predictive values of having a remitting or unremitting course in the first two years after diagnosis in relation to the course in years 3-5 are listed in Table 5. The positive predictive value of having a remitting disease in the first two years of disease was 90.9%, whereas the negative predictive value of having an unremitting clinical course in the first two years is 91.3%.
Analyzing the clinical variables assessed in the first two years and their association with activity of the longer term (years 3-5) resulted in a significant association

Figure 2. The percentage of time with active disease in the first 2 years after diagnosis plotted against the percentage of time with active disease in the three following years (n=146).



------- lines indicating the criteria for a remitting, intermediate and unremitting clinical course

_____ regression lines of the different subtypes (all p<0.05)

Table 5. Categorized clinical course of first two years against the clinical course of the following three years

Activity first 2 years	Activity year 2-5			Positive predictive value	Negative predictive value
	Remitting	Intermediate	Unremitting		
Remitting	20 (90.9)	2 (9.1)	0 (0)	**90.9**	66.1
Intermediate	28 (59.6)	13 (27.7)	6 (12.8)		
Unremitting	14 (18.2)	25 (32.5)	38 (49.4)	49.4	**91.3**

between the time with active disease in the first 2 years and the duration of active disease in the following three years. Patients with a remitting course in the first two years are not likely to develop a more severe course, whereas patients categorized as having no unremitting course are not likely to develop an unremitting course. It seems that the disease activity (category) in the first two years can be used as predictive factor for the disease in the following years.

DISCUSSION

This study shows that the clinical course, indicated by the percentage of time with active disease in the first two years after diagnosis, is different in the JIA subtypes. Patients with extended oligoarthritis seem to have the most severe clinical course with respect to time with active disease and not achieving remission, compared to patients with persistent oligoarthritis and RF-negative polyarthritis and should be regarded as a separate entity. This higher percentage of active disease in extended oligoarthritis might be due to less aggressive treatment; significant less patients with extended oligoarthritis received methotrexate in the first two years (38%), compared to RF- negative polyarthritis patients (65%; p=0.002). This suggests that extended oligoarthritis patients should be treated more aggressively and/or earlier in their clinical course.

The observed disease activity in the RF-positive polyarthritis patients is the highest of all, but because of the small number of patients included in this study, no conclusions can be drawn concerning this subtype. The percentage of time with active disease in the different subtypes in this cohort of JIA patients is similar to the percentages of active disease that are described by Wallace et al and Oen et al.[8;9] These similarities suggest that the percentage of active disease is representative for the different subtypes and a reproducible measure for sequential disease activity during the course of the disease.

In the systemic JIA subtype it is already known that a broad spectrum of disease activity with a monocyclic or intermittent course as well as an unremitting disease courses exists. Moreover, in all subtypes a broad spectrum of disease activity is observed in individual patients, varying from a small percentage of time with active disease to no inactive episodes at all. For the individual patient it would be more relevant to identify clinical prognostic factors that are associated with a remitting or unremitting clinical course.

In this study different clinical parameters have been tested for their association with the clinical course in the years 3-5 after diagnosis. We have chosen assess these different parameters in the first two years after diagnosis. We assumed that a minimum of two years are required after diagnosis to treat the patient with the optimal medication and evaluate its effectiveness and therefore this period might give a good representation of the disease activity after onset in the individual patient. In all subtypes the percentage of active disease in the first 2 years after diagnosis is strongly associated with the clinical course in the following three years and can reliably be used to identify patients with a remitting or a non-unremitting clinical course. We have defined the clinical course based on the percentage of time with active disease; patients with an unremitting course having active disease 65-100%

of the time, thus only a short time with disease quiescence. It should be noted that no detailed information about the activity of a JIA-associated uveitis was available, so patients with inactive disease could be having an active uveitis. However, patients with only mild arthritis and an ongoing severe uveitis were excluded from the study. In contrast to the study of Oen et al, in this study no associations between subtype, ANA status and age at onset and the clinical course were observed.[13] Association with the types and symmetry of joints involved at disease onset was not studied because of the lack of detailed data on joint involvement.
Our data show that in the overall JIA population up to 30% of the patients have an unremitting clinical course in the years 3-5 after diagnosis, indicating that the treatment regime used in this patient cohort is not sufficient to induce long lasting disease quiescence. However patients included in this study having a follow-up of >= 5 years were diagnosed before May 2003 and only 6 patients used Etanercept and no patients used other biologicals. In the last years more patients have efficiently been treated with Etanercept and other biologicals like IL1-receptor antagonist or anti IL-6.[14] Future studies should be performed to evaluate the clinical disease course of patients treated with these drugs.

In conclusion, differences in percentages of active disease between JIA subtypes have been observed, but more important; within each subtype individual patients can have both a remitting or unremitting clinical course. For the individual patients the clinical course in the first two years after diagnosis is clearly related to the clinical course in the following three years. Studies in rheumatoid arthritis show that reaching sustained remission early in the course of disease by using a more aggressive initial treatment is related to an improved radiographic outcome and less joint damage.[15-17] This study shows that a lower percentage of active disease in the first two years is related to a mild course of disease afterwards. Also in JIA, reaching a state of clinical remission early in the course of disease should be the aim for treating patients and therefore a more aggressive initial treatment might be necessary. Future studies are needed to identify prognostic factors that could already be determined at disease onset and could reliably point out the patients that are at risk of an unremitting disease course and should be treated with early aggressive treatment.

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CHAPTER 6

GENETIC VARIATION IN *VTCN1* (B7-H4) IS ASSOCIATED WITH COURSE OF DISEASE IN JUVENILE IDIOPATHIC ARTHRITIS

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H.M. Albers[1], T.H.C.M. Reinards[1*], D.M.C. Brinkman[2], S.S.M. Kamphuis[3], M.A.J. van Rossum[4], E.P.A.H. Hoppenreijs[5], H.J. Girschick[6], C. Wouters[7], R.K. Saurenmann[8], E. Bakker[1], W. Verduyn[1], P. Slagboom[1], T.W.J. Huizinga[1], R.E.M. Toes[1], J.J. Houwing-Duistermaat[1], R. ten Cate[1#], M.W. Schilham[1#]*

**) shared first authorship*
#) shared last authorship
1)Leiden University Medical Center, Leiden, the Netherlands
2) Rijnland Hospital, Leiderdorp, the Netherlands
3) Erasmus Medical Center- Sophia Children's Hospital, Rotterdam, the Netherlands
4) Academic Medical Center- Emma Children's Hospital and Reade, location Jan van Breemen, Amsterdam, the Netherlands
5) St Maartenskliniek and Radboud University Nijmegen Medical Centre, Nijmegen
6) Vivantes Children's Hospital, Berlin-Friedrichshain, Germany
7) University Hospital Gasthuisberg, Leuven, Belgium
8) Zürich University Children's Hospital, Zürich, Switzerland

ABSTRACT

Objective

The course of disease in Juvenile Idiopathic Arthritis (JIA) is unpredictable with episodes of activity and remission. In order to identify predictive factors, 93 SNPs, JIA subtype, age at onset and ANA status were studied in relation to disease course.

Methods

Genetic and clinical parameters were analysed in a cohort of 272 Caucasian patients with persistent oligoarthritis (n=129), extended oligoarthritis (n=57) and rheumatoid factor negative polyarthritis (n=86). Categories of disease course (remitting (n=65), intermediate (n=96) and unremitting (n=111)) were designed based on the cumulative time spent in active disease in the first two years.

Results

Univariate analysis revealed association of the course of disease with JIA subtype ($p=5.7*10^{-5}$) and three SNPs; *VTCN1* rs10923223 ($p=4.4*10^{-5}$), *VTCN1* rs12046117 (p=0.017) and *CDK6* rs42041 (p=0.038). In a subsequent multivariate ordinal logistic regression analysis, *VTCN1* rs10923223 (OR 0.41, 95%-CI 0.26-0.63) and JIA subtype (OR 3.8, 95%-CI 2.0-7.2; OR 2.5, 95%-CI 1.4-4.2, for extended oligoarthritis and RF-negative polyarthritis versus persistent oligoarthritis, respectively) were the strongest independent factors for course of disease.

Conclusion

This study provides evidence that *VTCN1*, encoding B7-H4, is associated with course of disease in selected subtypes of JIA. *VTCN1* might be useful in predicting the course of disease.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a heterogeneous group of disorders characterized by chronic inflammation of the joint(s). JIA is thought to be an autoimmune disease in which the immune response is deregulated.[1] Clinical course and outcome are of great importance to patients, parents and physician. In outcome studies using a variety of criteria for remission, an overall remission rate of 40% has been reported. The highest remission rate was consistently observed in persistent oligoarthritis compared to extended oligoarthritis and rheumatoid factor (RF)-negative polyarthritis.[2-4] Because the course of disease is unpredictable and fluctuating in all JIA subtypes with episodes of active and inactive disease, the cumulative time spent in a state of active disease is an accurate measure for the disease activity over time.[4-6] Since it is unethical to study patients who do not receive treatment, course of disease is inevitably defined for patients receiving treatment if and when necessary. The percentage of active disease in the first years is not only predictive for the course of disease in the following years,[2, 6] but a prolonged disease activity is also related to damage to the joints or functional impairment.[7] The aim of treatment is to minimize the time spent in active disease.

No clinical parameters or biomarkers are available at disease onset to predict the course of disease.[8] Genetic markers would be ideal as predictive factors, already present at disease onset and not influenced by disease activity or medication. Recently, a number of genetic susceptibility factors for JIA (e.g. *HLA*, *PTPN22*, *PTPN2* and *IL2RA*) have been described and replicated by means of genome-wide and candidate gene association studies.[9-11] Some of these genetic risk factors are also involved in other autoimmune diseases.

The aim of this study was to identify genetic factors associated with severity of JIA. The relationship between single nucleotide polymorphisms (SNPs) in candidate genes involved in immunoregulation and autoimmunity and the percentage of active disease in the first two years after diagnosis was investigated.

MATERIALS AND METHODS

Patient population

Two hundred and seventy-two Caucasian JIA patients with persistent oligoarticular, extended oligoarticular and RF-negative polyarticular JIA of whom both DNA and clinical data were available, were studied. Patients, recruited from eight centers throughout North-Western Europe, were diagnosed between 1991 and 2006 and had a follow-up

of at least two years. Informed consent was obtained from all patients and/or parents and the review boards of the participating centers approved this study (Table 1).

Clinical data

All hospital visits during the first two years after diagnosis have been retrospectively evaluated to determine the state of disease activity at each visit. Inactive disease was defined as the absence of clinically active arthritis, the lowest possible physician's global assessment and an ESR <20 mm/hr (following a modified definition of clinically inactive disease).[12] The cumulative time spent in active disease in the first two years was determined and formed the basis for the categories of disease course:[6] remitting (<= 35% active disease), intermediate (35-65% active disease) and unremitting (>= 65% active disease) (Table 1).

Genetic factors

112 SNPs in 65 genetic regions were genotyped, because of their association with JIA, other autoimmune diseases and/or their role in immunoregulation (Supplementary Table S1).

Statistical analysis

The three categories representing course of disease were used as outcome parameter. Univariate analysis was performed with the clinical parameters JIA subtype, age at onset and ANA status using Chi-square and Kruskal-Wallis tests, and with the genetic parameters comparing the genotype distribution of each SNP between the categories of disease course with a linear by linear trend test. The linkage disequilibrium of associated SNPs ($p<0.05$) was studied using parental data (Haploview).[13]

Parameters with a p-value <0.05 in the univariate analysis were analyzed by multivariate ordinal logistic regression. Odds ratios and 95% confidence intervals (CI) are presented. A p-value <0.001 was considered significant after Bonferroni correction for multiple testing (57 successfully typed genes/loci). ROC curves were plotted for two models with different parameters predicting course at disease onset (Supplementary Table S2 and Figure S1). A case-control analysis was performed with the SNPs that were associated with course of disease ($p<0.05$), to evaluate their role in susceptibility to JIA (Supplementary Table S3). Data were analyzed using IBM SPSS Statistics 20.

Table 1. Characteristics of the JIA cohort

	n	(%)
Total cohort[a]	272	
Country[b]		
The Netherlands	138	(50.7)
Belgium	45	(16.5)
Germany	49	(18.0)
Switzerland	40	(14.7)
Subtype		
Persistent oligoarthritis	129	(47.4)
Extended oligoarthritis	57	(21.0)
RF negative polyarthritis	86	(31.6)
Gender		
Female	190	(69.9)
Male	82	(30.1)
ANA		
Positive	153	(56.3)
Negative	86	(31.6)
Inconclusive/unknown	33	(12.1)
Course of disease[c]		
Remitting course	65	(23.9)
Intermediate course	96	(35.3)
Unremitting course	111	(40.8)
Medication[d]		
Intra-articular steroids (n=166)	63	(38.0)
Sulfasalazine (n=218)	73	(33.5)
Methotrexate (n=238)	97	(40.8)
Etanercept (n=233)	4	(1.7)
	median	**range**
Percentage of active disease[e]		
Total cohort	54.9	(2-100)
Remitting course	22.1	(2-35)
Intermediate course	49.8	(36-65)
Unremitting course	91.1	(65-100)
Age at onset	3.98	(0.6-16.2)

a) Written informed consent from patients and parents was obtained after a personalised informative letter from the local investigator and the coordinating centre, in their native language. The majority of patients participated by taking a buccal swab at home and sending this to the coordinating centre. They authorised storage and analysis of their DNA for this and further JIA related research.

b) Paediatric rheumatologists in the participating centres (The Netherlands n=5, Belgium n=1, Germany n=1, and Switzerland n=1) are all members of the Paediatric Rheumatology European Society (PRES).

c) Remitting clinical course: percentage of active disease in the first two years <= 35%; intermediate clinical course: percentage of active disease in the first two years >35 and <65%; unremitting clinical course: percentage of active disease in first two years >=65%.

d) Treatment was started in the first two years after diagnosis. Notable is that not of all patients the detailed data on use of medication are known (n).

e) Percentage of active disease in the first two years after diagnosis.

RESULTS

93 of 112 SNPs (83%) located in 57 genes/loci were successfully genotyped in 272 patients (Supplementary Table S1). This cohort consisted of 65 patients with a remitting course of disease, 96 patients with an intermediate course, and 111 patients with an unremitting course (Table 1).

In univariate analysis JIA subtype, but not ANA status or age at onset, was significantly associated with the course of disease; the persistent oligoarthritis patients had more intermediate and remitting disease course, whereas the majority of extended oligoarthritis and RF-negative polyarthritis patients followed an unremitting

Table 2. Univariate analysis of clinical and genetic parameters with the categories representing disease course as outcome parameter[a].

Subtype			Remitting	Intermediate	Unremitting	p
Persistent oligoarthritis			45 (0.35)	48 (0.37)	36 (0.28)	
Extended oligoarthritis			6 (0.11)	17 (0.30)	34 (0.60)	
RF-negative polyarthritis			14 (0.16)	31 (0.36)	41 (0.48)	$5.7*10^{-5}$ [b]
ANA						
Positive			21 (0.24)	28 (0.33)	37 (0.43)	
Negative			33 (0.22)	59 (0.39)	61 (0.40)	0.65[b]
Age at onset (median, range)			4.0 (1.0-15.0)	3.2 (0.6- 14.9)	4.3 (1.0- 16.2)	0.49[c]
Gene	**SNP**	**Genotype**	**Remitting**	**Intermediate**	**Unremitting**	**p[d]**
CDK6	rs42041	00	31 (49.2)	55 (59.8)	69 (65.1)	
		01	27 (42.9)	32 (34.8)	33 (31.1)	
		11	5 (7.9)	5 (5.4)	4 (3.8)	0.038
		MAF (G)	0.29	0.23	0.19	
VTCN1	rs10923223	00	33 (51.6)	63 (67.0)	88 (79.3)	
		01	26 (40.6)	29 (30.9)	22 (19.8)	
		11	5 (7.8)	2 (2.1)	1 (0.9)	$4.4*10^{-5}$
		MAF (C)	0.28	0.18	0.11	
VTCN1	rs12046117	00	40 (62.5)	64 (69.6)	88 (79.3)	
		01	23 (35.9)	27 (29.3)	22 (19.8)	
		11	1 (1.6)	1 (1.1)	1 (0.9)	0.017
		MAF (T)	0.20	0.16	0.11	
LCK	rs695161	00	22 (33.8)	27 (28.4)	28 (25.7)	
		01	33 (50.8)	53 (55.8)	51 (46.8)	
		11	10 (15.4)	15 (15.8)	30 (27.5)	0.051
		MAF (C)	0.41	0.45	0.51	

a) Only the SNPs of interest with a p-value <0.1 are shown.
b) p-value of Pearson Chi-square test
c) p-value of Kruskal-Wallis test
d) p-value of linear by linear association (trend test) with one degree of freedom

disease course ($p=5.7*10^{-5}$) (Table 2). Comparing the genotype distribution in the three categories reflecting course of disease revealed three SNPs that were associated ($p<0.05$). Two of these SNPs were located in the *VTCN1* gene, rs10923223 ($p=4.4*10^{-5}$) and rs12046117 ($p=0.017$) and a third association was found with *CDK6* rs42041 ($p=0.038$) (Table 2). The minor alleles of the three SNPs were associated with a remitting course. None of the other investigated SNPs were associated, except a trend towards association with *LCK* rs695161 ($p=0.051$). Because two of the associated SNPs (rs10923223 and rs12046117) were located in the *VTCN1* gene, the linkage disequilibrium was studied. The two SNPs were highly correlated ($D'=0.97$; $r^2=0.77$). Therefore we have included only *VTCN1* rs10923223 in further analyses.
In multivariate ordinal regression analysis, JIA subtype (extended oligoarthritis and RF-negative polyarthritis, with persistent oligoarthritis as reference; extended oligoarthritis OR 3.8, 95%-CI 2.0-7.2, $p=6.3*10^{-5}$; RF-negative polyarthritis OR 2.5, 95%-CI 1.4-4.2, $p=9.0*10^{-4}$) and the genetic factors *VTCN1* rs109232203 (OR 0.41, 95%-CI 0.26-0.63, $p=5.8*10^{-5}$) and *CDK6* rs42041 (OR 0.66, 95%-CI 0.44-0.97, $p=0.036$) were independently associated with course of disease (Table 3). The addition of *VTCN1* rs10923223 and *CDK6* rs42041 to a model predicting course of disease at diagnosis in which only subtype at onset is included, increases the AUC of an ROC curve from 0.57 to 0.67 (Supplementary Table S2 and Figure S1).

Table 3. Multivariate ordinal logistic regression of associated clinical (subtype) and genetic parameters (*VTCN1*, *CDK6*).

Subtype	B	OR	95%CI	p
Persistent oligoarthritis vs. extended oligoarthritis	1.33	3.8	2.0-7.2	$6.3*10^{-5}$
Persistent oligoarthritis vs. RF-negative polyarthritis	0.90	2.5	1.4-4.2	$9.0*10^{-4}$
VTCN1 rs10923223	-0.90	0.41	0.26-0.63	$5.8*10^{-5}$
CDK6 rs42041	-0.42	0.66	0.44-0.97	0.036

DISCUSSION

In this study we describe that genetic variations in *VTCN1* (rs10923223 and rs12046117) and in *CDK6* (rs42041) are associated with the course of disease in JIA. Our data show a protective effect of the minor allele of all three SNPs on the course of disease. This effect is independent of the association that exists between the JIA subtype and the course of disease. Because CDK6 is involved in cell-cycling, the association of disease

course with *CDK6* rs42041 is not unlikely. However, this association is not significant after correction for multiple testing.

Interestingly, the association of *VTCN1* with the course of disease is in line with the described function of the encoded protein. *VTCN1,* V-set domain containing T cell activation inhibitor 1, encodes B7-H4, a member of the B7 co-signaling molecule family expressed on antigen presenting cells.[14,15] Ligation of B7-H4 has an inhibitory effect on T-cell proliferation and production of cytokines.[14,15] Because of its inhibiting effects on immune responses B7-H4 has been associated with prognosis in cancer and autoimmunity.[16,17]

In B7-H4 deficient mice an exacerbation of collagen induced arthritis was observed.[18] An agonistic soluble B7-H4Ig suppressed the progression of collagen induced arthritis and improved the progression of experimental autoimmune encephalomyelitis.[18,19] Targeting of this inhibitory B7-H4 pathway might be of therapeutic interest, similar to the CTLA4 fusion protein (Abatacept) in rheumatoid arthritis.

In our cohort no significant association of *VTCN1* with the susceptibility to JIA was observed (Supplementary Table S3), but instead an association of the minor allele of rs10923223 with a less severe course of disease (Table 2). It is conceivable that the minor allele is associated with a gain of function of *VTCN1*, leading to a stronger inhibitory signal to activated T cells. This hypothesis implies that *VTCN1* does not contribute to initiation but rather to the course of the disease. At present it is very difficult to predict the course of disease at the moment of diagnosis. The addition of *VTCN1* and *CDK6* to the predictive model for disease course increased the AUC (Supplementary Table S2 and Figure S1). Although the total predictive power is still limited it suggests that genetic information may contribute to prognosis in the case of JIA.

It is remarkable that the well-established JIA loci *PTPN22*, 4q27 *(IL2-IL21)*, 5q11 *(ANKRD55)* and *TNFA* were not associated with course of disease, although they were associated with susceptibility in our cohort as well *(Reinards et al. manuscript in preparation).*[9-11, 20] This suggests that genes involved in the development of disease are not necessarily involved in the progression of disease. Replication of our data remains necessary since patient numbers are relatively small when comparing categories of disease course. However, if association of *VTCN1* with course of disease will be confirmed, these data support the development of therapeutic tools based on interfering with this receptor ligand interaction in JIA and maybe other autoimmune diseases.

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SUPPLEMENTARY DATA

ROC curve

To give more insight into the predictive value for disease course of the parameters that can be used at disease onset (when persistent oligoarthritis cannot be distinguished from extended oligoarthritis yet), a ROC curve was plotted. The predictive value of an ordinal logistic regression model with subtype at onset (oligoarthritis versus polyarthritis at presentation) as only parameter was plotted (Figure S1a), followed by a model of the subtype at onset together with the genetic parameters with an independent effect in the multivariate analysis (Figure S1b).

Control population

A case-control analysis was performed with the SNPs that were associated with course of disease ($p<0.05$), to evaluate their role in developing JIA.

The control population, used to determine allele frequencies of *VTCN1* rs10923223 and rs12046117 in healthy individuals, consisted of blood bank donors that were randomly selected by the Immunogenetics and Transplantation Immunology Section at Leiden University Medical Center, The Netherlands (n=758), or that were recruited via participating patients and their families (but unrelated) (n=111). Because there was not sufficient DNA for all of these controls to type all SNP's, 744 additional individuals were included in case of *CDK6* rs42041. These individuals were blood bank donors (n=372) or requested genetic counselling at the Laboratory for Diagnostic Genome Analyses, Leiden University Medical Center, regarding a monogenic disease in their families, but tested negative for this single genetic defect (n=372).

All controls were unrelated, of Caucasian descent, above the age of 18 years, and gave informed consent for anonymized (further) use of their (left over) DNA for scientific purposes, and for anonymized use of their DNA in a biobank.

Supplementary Table S1
Genetic variants selected for association analysis with course of disease in JIA

Chromosome	Position*	Gene/region	SNP	Minor allele
1	2553624	*TNFRSF14-MMEL1*	rs3890745	G
1	12091210	*TNFRSF8-MIIP*	rs946461	T
1	12252955	*TNFRSF1B*	rs1061622	G
1	32729702	*LCK*	rs1004420	T
1	32743866	*LCK*	rs695161	C
1	114377568	*PTPN22*	rs2476601	A
1	117685992	*VTCN1*	rs6673837	A
1	117690758	*VTCN1*	rs2358817	T
1	117711911	*VTCN1*	rs2358820	A
1	117730048	*VTCN1*	rs10923217	C
1	117730623	*VTCN1*	rs6669320	A
1	117746573	*VTCN1*	rs10923223	C
1	117751365	*VTCN1*	rs12046117	T
1	198700442	*PTPRC*	rs10919563	A
1	206946897	*IL10*	rs1800896	C
1	207015957	*IL19*	rs2243191	T
1	207038686	*IL20*	rs1400986	T
2	100832155	*AFF3*	rs1160542	G
2	100835734	*AFF3*	rs10865035	A
2	103070568	*IL18RAP*	rs917997	T
2	113537223	*IL1A*	rs17561	A
2	113542960	*IL1A*	rs1800587	A
2	113590390	*IL1B*	rs1143634	A
2	113594867	*IL1B*	rs16944	A
2	162856148	*DPP4*	rs2268894	C
2	191835596	*STAT1*	rs3771300	C
2	191843445	*STAT1*	rs13010343	A
2	191845725	*STAT1*	rs1547550	C
2	191855521	*STAT1*	rs7562024	T
2	204610396	*CD28*	rs1980422	C
2	204732714	*CTLA4*	rs231775	G
3	46414947	*CCR5*	rs333	del
3	58556841	*FAM107A*	rs13315591	C
4	26108197	4p15	rs874040	G
4	123132492	*KIAA1109*	rs4505848	G
4	123348345	*ADAD1*	rs11732095	G
4	123514528	*IL2-IL21*	rs4492018	A
4	123548068	*IL21*	rs1398553	T

Supplementary Table S1 (continued)
Genetic variants selected for association analysis with course of disease in JIA

Chromosome	Position*	Gene/region	SNP	Minor allele
5	55438580	*ANKRD55*	rs6859219	A
5	96124330	*ERAP1*	rs30187	T
5	135287029	*LECT2*	rs31517	A
6	31540141	*LTA*	rs2239704	A
6	31540313	*LTA*	rs909253	G
6	31540784	*LTA*	rs1041981	A
6	31542482	*TNFA*	rs1799724	T
6	31542963	*TNFA*	rs1800750	A
6	31543031	*TNFA*	rs1800629	A
6	31543101	*TNFA*	rs361525	A
6	31543827	*TNFA*	rs1800610	A
6	31544189	*TNFA*	rs3093662	G
6	57012930	*ZNF451*	rs3734738	A
6	106568034	*PRDM1*	rs548234	C
6	138006504	6q23	rs6920220	A
6	159482521	*TAGAP*	rs394581	C
6	167534290	*CCR6*	rs3093023	A
7	75442759	*CCL24*	rs2302005	T
7	75442855	*CCL24*	rs2302004	C
7	92246744	*CDK6*	rs42041	G
7	128594183	*TNPO3*	rs10488631	C
9	34743681	*CCL21*	rs951005	C
9	139775146	*TRAF2*	rs7048473	C
9	139787453	*TRAF2*	rs2811761	G
9	139815053	*TRAF2*	rs10781522	G
9	139821068	*TRAF2*	rs3750512	C
10	6053163	*IL2RA*	rs12722605	T
10	6099045	*IL2RA*	rs2104286	G
10	6114660	*IL2RA*	rs41295061	A
10	6393260	*PRKCQ*	rs4750316	C
11	36525293	*TRAF6*	rs540386	T
11	71709272	*IL18BP*	rs3814721	C
11	71710478	*IL18BP*	rs2298455	C
11	71714078	*IL18BP*	rs1541304	T
11	112035458	*IL18*	rs1946518	T
11	117869670	*IL10RA*	rs2229113	A
12	6450945	*TNFRSF1A*	rs767455	C
12	6451590	*TNFRSF1A*	rs4149570	A

Supplementary Table S1 (continued)
Genetic variants selected for association analysis with course of disease in JIA

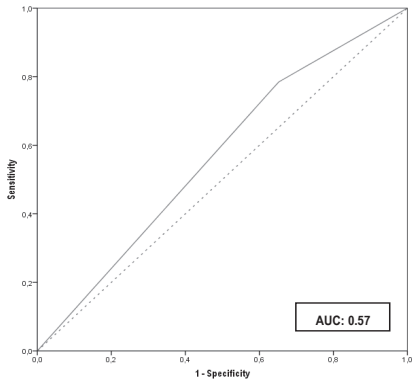
Chromosome	Position*	Gene/region	SNP	Minor allele
12	57968715	*KIF5A*	rs1678542	C
16	11179873	*CLEC16A*	rs12708716	G
16	11249329	*CLEC16A*	rs6498169	G
16	27448401	*IL21R*	rs3093341	G
16	67189486	*TRADD*	rs11574518	T
17	32594568	*CCL2-CCL7*	rs8079244	C
17	40447401	*STAT5A*	rs7217728	C
17	40461003	*STAT5A*	rs2293154	A
18	67531642	*CD226*	rs763361	T
19	44515514	*ZNF230*	rs12753	A
20	43280231	*ADA*	rs6031698	A
20	44746982	*CD40*	rs1883832	T
21	34640788	*IL10RB*	rs2834167	G
22	24236392	*MIF*	rs755622	C
22	37544245	*IL2RB*	rs3218258	T
22	37544810	*IL2RB*	rs3218253	T
22	37551607	*IL2RB*	rs743777	G

*) Base-pair position is based on NCBI dbSNP build 136

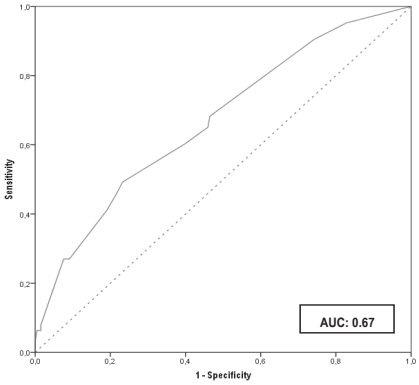
112 SNPs in 65 loci have been genotyped by the iPLEX MassARRAY platform according to the manufacturer's recommendations (Sequenom, San Diego, California, USA), of which these 93 SNPs in 57 loci passed quality control criteria. Only SNPs exceeding a 90% call rate were used for further analysis. SNP call rates per individual exceeded 90%.

Supplementary Figure S1
ROC curves of predictive models for course of disease

a. Only subtype at onset included as predictor



b. Subtype at onset and genetic parameters included as predictors



AUC: area under the (solid) curve
The dashed line represents the reference.

Supplementary Table S2
Clinical and genetic parameters as predictors for course of disease plotted in a ROC curve

Figure	Parameters included	AUC*
S1a	Subtype at onset#	0.57
S1b	Subtype at onset#, *VTCN1* rs10923223, and *CDK6* rs42041	0.67

*) Area under the curve (AUC) reflecting the ability to distinguish between remitting, intermediate and unremitting disease
#) Subtype at onset comparing oligoarthritis (persistent and extended oligoarthritis) to (RF-negative) polyarthritis

Supplementary Table S3
Genotype frequencies of the SNPs of interest in JIA cases compared to healthy controls

Gene	**SNP**		**n**	**MAF***	**Genotype**			**p#**
					00 (%)	01 (%)	11 (%)	
VTCN1	rs10923223	Cases	269	0.17	184 (68)	77 (29)	8 (3)	
		Controls	856	0.14	622 (73)	221 (26)	13 (2)	0.10
VTCN1	rs12046117	Cases	266	0.15	191 (72)	72 (27)	3 (1)	
		Controls	848	0.13	640 (76)	197 (23)	11 (1)	0.30
CDK6	rs42041	Cases	261	0.23	155 (59)	92 (35)	14 (5)	
		Controls	1274	0.26	713 (56)	465 (37)	96 (8)	0.19

*) MAF: minor allele frequency
#) p-value of linear-by-linear association (trend test with one degree of freedom)

CHAPTER 7

TIME TO TREATMENT IS AN IMPORTANT FACTOR FOR THE RESPONSE TO METHOTREXATE IN JUVENILE IDIOPATHIC ARTHRITIS

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H.M. Albers[1], J.A.M. Wessels[1], R.J.H.M. van der Straaten[1], D.M.C. Brinkman[1], L.W.A. Suijlekom-Smit[2], S.S.M. Kamphuis[2], H.J.Girschick[3], C. Wouters[4], M. Schilham[1], S. le Cessie[1], T.W.J. Huizinga[1], R. ten Cate[1], H.J. Guchelaar[1].

1)Leiden University Medical Center, Leiden, the Netherlands
2) Erasmus Medical Center- Sophia Children's Hospital, Rotterdam, the Netherlands
3)University of Wurzburg, Wurzburg, Germany
4) University Hospital Gasthuisberg, Leuven, Belgium

ABSTRACT

Objective

Methotrexate (MTX) is the most commonly used disease modifying antirheumatic drug in Juvenile Idiopathic Arthritis (JIA). At present no reliable prediction of individual response to MTX can be made. Identification of clinical and genetic factors that influence the response to MTX could be helpful in realizing the optimal treatment for individual patients.

Materials & methods

A cohort of 128 JIA patients treated with MTX was studied retrospectively. Eleven clinical parameters and genotypes of 6 single nucleotide polymorphisms in 5 genes related to the mechanism of action of MTX were compared between MTX responders and non-responders using a multivariate regression analysis.

Results

The time from diagnosis to start MTX treatment, the physician's global assessment at baseline and the starting dosage were significantly associated with the response to MTX at 6 months after initiation. Patients with a shorter time from diagnosis to start MTX and a higher disease activity according to the physician, but with a lower MTX dosage showed increased response. The effect of the starting dosage on MTX response seemed to be mainly due to the influence of the systemic JIA subtype. The time from diagnosis to start MTX treatment and physicians' global assessment at baseline were highly correlated. Therefore, the precise effect size of each independent variable could not be determined.

Conclusion

In children with JIA, the time from diagnosis to start MTX appears to be an important factor for the MTX response. Our results suggest that earlier start of MTX treatment will lead to an increased response.

INTRODUCTION

Juvenile Idiopathic Arthritis (JIA) is the most common chronic rheumatic inflammatory disorder in childhood, with an incidence of around 10 per 100.000 children.[1] JIA is defined as arthritis of unknown etiology that persists for more than 6 weeks and with an onset before the age of sixteen years. Seven different subtypes have been defined according to the criteria of the International League of Associations for Rheumatology (ILAR).[2]

Methotrexate (MTX) is the most commonly used disease modifying antirheumatic drug (DMARD) in JIA, especially in the treatment of polyarticular JIA.[3] The efficacy of MTX has been shown in randomized placebo controlled trials and in subsequent clinical use.[4;5] The response rate of MTX, prescribed in a weekly standard dose of 8.5-12.5 mg per body surface area (m^2), is about 65% at 4-6 months after initiation of therapy.[4;6-8]

The precise mechanism of action of MTX remains unclear, although it is thought that MTX inhibits the de novo synthesis of purine and pyrimidine, essential components of DNA and RNA.[9;10] Thereby it inhibits the proliferation of cells, amongst which T-lymphocytes. Additionally it has been shown that the anti-inflammatory effects of MTX are mediated by an increased adenosine-release. More recently research groups have reported that genetically based differences contribute to MTX efficacy since polymorphisms in genes involved in the purine and pyrimidine synthesis have been associated with response to MTX in JIA and RA.[11-13]

However in JIA, reliable predictors for the response to MTX are yet unknown. Factors identifying JIA patients with a high likelihood to respond to MTX therapy would be very helpful for achieving the optimal treatment for individual patients in an early stage of the disease and thereby preventing damage to the joints on the long term. Therefore, the aim of this study is to identify clinical and genetic factors that are associated with the response to MTX in patients with JIA.

METHODS & MATERIALS

Patient population

The patients in this analysis are a retrospectively observed cohort of children diagnosed with JIA that were recruited from 4 pediatric rheumatology referral centers in The Netherlands, Belgium and Germany. Clinical data were collected from 347 patients of which also DNA was available. Forty-four percent of these patients were treated with MTX (n=152) and 128 patients fulfilled the inclusion criteria for this study. Twenty-four

patients were excluded because: at start of MTX> 18 years (n=5), start MTX < 6 months ago (n=5), use of MTX because of JIA associated uveitis (n=3) and missing data about follow-up (n=11). No statistical significant differences were found with regard to subtype, age at onset and gender between the 128 genotyped patients and the total group of patients receiving MTX (p>0.05). Patients with undifferentiated JIA (n=5), JIA with enthesitis (n=4) and psoriatic arthritis (n=1) were grouped together in the subgroup "other JIA". 97 Per cent of the patients were of European white ethnicity based on self-report. Written informed consent was obtained from all patients and/or parents together with approval of each institution's medical ethics board.

Clinical data

Demographic and clinical data, together with detailed information about the use of MTX and co-medication were collected from the patients' chart. At the time point of start and at 6 months after initiation of MTX, the following parameters were scored: the physician's global assessment of disease activity, the amount of joints with arthritis (defined by swelling, not due to bony enlargement, or if no swelling is present, limitation of motion accompanied either by pain on motion and/or tenderness) (32 joint count) and the erythrocyte sedimentation rate (ESR). The physician' global assessment was scored at a five point scale (1 no, 2 mild, 3 moderate, 4 severe and 5 very severe activity). In addition, the joint score was divided into categories with 0 no arthritis, 1 monoarthritis (1 joint), 2 oligoarthritis (2-4 joints), 3 polyarthritis (5-10 joints), 4 severe polyarthritis (>11 joints) and in systemic JIA patients an additional category (5) was used when systemic features were present.

Definition of response

The response to MTX was defined as follows: improvement in physician's global assessment of 1 or more categories together with an equal or improved joint score measured from baseline to 6 months after the start of MTX. The ESR was not incorporated in the definition of response because of the large number of missing data. Patients were considered non-responders to MTX if they did not fulfill the criteria of response.

Pharmacogenetics

Six single nucleotide polymorphisms (SNPs) in 5 candidate genes, related to the mechanism of action of MTX, were selected taking the following criteria into consideration: validated SNP, SNP -preferably- causing non-synonymous amino acid change, indications for clinical relevance from previous publications[11-13] and a preferred minimal genotype frequency of approximately 10%. These SNPs were located in the genes adenosine monophosphate deaminase (AMPD1) (34C>T; rs17602729), amino-

imidazole carboxamide ribonucleotide transformylase (ATIC) (347C>G; rs2372536), inosine triphosphate pyrophosphatase (ITPA) (94C>A; rs41320251) methylenetetrahydrofolate reductase (MTHFR) (677C>T; rs1801133 and 1298A>C; rs1801131) and in methylenetetrahydrofolate dehydrogenase (MTHFD1) (1958G>A; rs2236225). Genotyping was performed using real-time polymerase chain reaction (PCR) with Taqman technique according to protocols provided by the manufacturer (Taqman, Applied Biosystems, Foster City, CA, USA). 5-10% of samples were genotyped in duplicate. The mean for overall success rate was 96%. All 6 SNP genotype frequencies showed Hardy-Weinberg equilibrium.

Statistical analysis

Clinical variables considered relevant for the response to MTX at 6 months after initiation were: subtype of JIA, age at start MTX, time from diagnosis to start MTX (time-to-start MTX in months), disease activity at start MTX (physician's global score, joint score), ESR, starting dosage MTX (milligrams per body surface area per week), use of intra-articular steroids and/or sulfasalazine (SSZ) and/or other DMARDs before MTX (yes/no), use of systemic steroids before MTX (yes/no), use of systemic steroids during MTX treatment (yes/no) and use of SSZ during MTX treatment (yes/no). These variables were compared between responders and non-responders by the Student's t-test, Mann-Whitney U test or Chi-square test depending on the tested variable. Differences in genotype distribution between responders and non-responders were tested in a two-by-two cross tabulation by carrier analysis with a two-sided Chi-square test. MTHFR 677C>T and MTHFR 1298A>C were only tested as number of copies of the MTHFR1298A-677C haplotype. With the sample size of 55 non-responders and 73 responders, an increase in frequency of 2 haplotype copies (MTHFR1298A-677C) from 12% to 34% could be detected with 80% power and 95% confidence. Variables with a p-value of < 0.1 between responders and non-responders were considered relevant for influencing the response to MTX either by a true effect of by confounding. Therefore, variables with p-value <0.1 were included in the multiple binary logistic regression analysis with response as dependent. Additionally the univariate odds ratios (with 95% confidence interval) of these variables were calculated to illustrate the confounding effect of the different variables. ESR was not included in the multivariate analysis because of the large number of missing data. All statistical analyses were performed using SPSS 14.0. Variables with p-value <0.05 in the multivariate regression analysis were considered statistical significant.

RESULTS

Description of the patient population

The clinical and demographic characteristics for responders and non-responders are presented in Table 1. In our cohort (n=128) the response rate at 6 months after initiation of MTX was 44% in persistent oligoarthritis, 69% in extended oligoarthritis, 61% in RF negative polyarthritis, 82% in RF positive polyarthritis and 32% in systemic JIA, whereas the response rate in the overall JIA population with these subtypes combined was 57% (Table 1). In addition the comparison of the clinical and demographic characteristics of the MTX responders and MTX non-responders is listed in Table 1.

Table 1.
Clinical and demographic characteristics of 128 JIA patients, according to their response to MTX[#]

	MTX non-responder % (n)	MTX responder % (n)	p-value[*]
Total group of JIA patients	43 (55)	57 (73)	
Diagnosis:[1]			0.050 [P]
Persistent oligoarthritis	56 (10)	44 (8)	
Extended oligoarthritis	31 (8)	69 (18)	
RF negative polyarthritis	39 (17)	61 (27)	
RF positive polyarthritis	18 (2)	82 (9)	
Systemic JIA	68 (13)	32 (6)	
Other JIA•	50 (5)	50 (5)	
Female:male	74:26 (41:14)	79:21 (58:15)	0.51 [P]
Age at start MTX (years), mean (range; sd[2])	7.9 (1.9- 15.9; 3.7)	7.7 (1.3- 17.4; 4.3)	0.82 [t]
Time-to-start-MTX in months, median (range)	16.3 (0.0- 150)	9.5 (0.0- 94.8)	0.074 [MW]
Medication:			
Starting dosage MTX (mg/m^2) mean (range;sd)	10.4 (4.5- 23.7; 4.7)	8.6 (1.8- 16.5; 3.3)	0.018 [t]
SSZ,IAS or other DMARDs used before start MTX (yes/no)	58 (32)	56 (41)	0.82 [P]
Systemic steroids used before start MTX (yes/no)	31 (17)	16 (12)	0.053 [P]
SSZ used during MTX (yes/no)	33 (18)	32 (23)	0.88 [P]
Steroids used during MTX (yes/no)	35 (19)	52 (38)	0.048 [P]
Disease activity at start MTX:			
ESR[2] (mm/h), median (range) (n=67)	30 (7-137)	25 (2-107)	0.040 [MW]
Physician's global assessment			0.000 [lin]
1 Inactive	7 (4)	0 (0)	
2 Mild	16 (9)	4 (3)	
3 Moderate	55 (30)	56 (41)	
4 Severe	22 (12)	48 (28)	

5 Very severe	0 (0)	1 (1)	
Joint score			0.40 [lin]
0 None	7 (4)	0 (0)	
1 Monoarthritis	2 (1)	4 (3)	
2 Oligoarthritis	35 (19)	31 (23)	
3 Polyarthritis	45 (25)	51 (37)	
4 Severe polyarthritis	0 (0)	11 (8)	
5 Systemic features	11 (6)	3 (2)	

sd: standard deviation, SSZ: sulfasalazine, IAS: intra-articular steroid, DMARD: disease modifying anti rheumatic drug, ESR: erythrocyte sedimentation rate
#) all variables are presented as percentage (number of patients) unless indicated otherwise
•) other JIA consists of undifferentiated JIA (n=5), JIA with enthesitis (n=4) and psoriatic arthritis (n=1)
*) p-value of different statistical test comparing these clinical variables between the non-responders and responders.
[1] Diagnosis JIA according to the revised ILAR criteria(2)
[2] ESR not included in further analysis because of the large number of missing data
[P] p-value of Pearson Chi-square
[MW] p-value of Mann-Whitney U Test
[t] p-value of Student's t-test
[lin] p-value of linear-by- linear association

Genetic analysis

The genotype frequencies and the MTHFR haplotype frequencies in this population are presented in Table 2. Comparing MTX responders with non-responders in a haplotype-carrier analysis, a statistically significant difference in number of MTHFR1298A-677C haplotype copies was found. Non-responders showed more frequently none or 1 copy of the MTHFR1298A-677C haplotype when compared to responders (p-value= 0.039). All other pharmacogenetic association analyses showed no significant differences.

Table 2.
Genotype and (MTHFR 1298A-677C) haplotype frequencies in MTX non-responders and MTX responder %(n).

	AMPD1 34 C-T			**ATIC 347 C-G**			**ITPA 94 C-A**			**MTHFD1 1958 G-A**			**MTHFR 1298A-677C***	
	C/C	**C/T**	**T/T**	**C/C**	**C/G**	**G/G**	**C/C**	**C/A**	**A/A**	**G/G**	**G/A**	**A/A**	**0+1**	**2**
MTX non-responder	77 (40)	23 (12)	0	42 (23)	49 (27)	9 (5)	91 (50)	9 (5)	0	24 (13)	53 (29)	24 (13)	89 (47)	11 (6)
MTX responder	66 (44)	32 (21)	2 (1)	49 (35)	38 (27)	13 (9)	85 (58)	13 (9)	2 (1)	30 (21)	49 (35)	21 (15)	74 (50)	26 (18)
p°	0.183			0.748			0.279			0.503			**0.039**	

*) The number of MTHFR1298A-677C haplotype copies
°) p-value of linear-by-linear association

Univariate and multivariate analysis of variables in relation to MTX response

Variables with a p-value of <0.1 (Table 1 and 2) were considered of influence of the response to MTX and were analyzed univariately and thereafter included in a multivariate regression analysis to correct for confounding effects (Table 3).

In the multivariate regression analysis, the time-to-start MTX, the baseline physician's global assessment and the starting dosage of MTX were significantly associated with the response to MTX at 6 months after initiation. No confounding effect of the included variables on the effect of time-to-start MTX on response was observed. Briefly, responders started earlier with MTX and had a higher disease activity at baseline based on the physician's global assessment. What is more remarkable is that responders received a lower starting dosage. However, the starting dose MTX was highly influenced by the subtype of JIA (ANOVA p<0.001), especially by the systemic JIA patients who receive a higher starting dosage and have a decreased response. Repeating the multivariate analysis without the systemic JIA subtype resulted in a significant association of the time-to-start MTX and baseline physician's global assessment with MTX response (data not shown) and no effect of the starting dose on the MTX response was observed (OR 0.89, 95%CI 0.76- 1,05; p= 0.166).

Table 3 Univariate and multivariate regression analysis of clinical and genetic factors with MTX response in JIA patients (n=118) as dependent variable.

	Univariate analysis		**Multivariate analysis[1]**	
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**
JIA subtype*		0.069		0.441
Persistent oligoarthritis	1.00 (reference)			
Extended oligoarthritis	2.81 (0.81- 9.80)	0.104	3.41 (0.62- 18.7)	0.159
RF negative polyarthritis	1.99 (0.65- 6.03)	0.226	0.76 (0.18- 3.15)	0.700
RF positive polyarthritis	5.63 (0.94- 33.8)	0.059	1.41 (0.16- 12.4)	0.757
Systemic JIA	0.58 (0.15- 2.21)	0.422	0.68 (0.09- 5.12)	0.712
Other JIA	1.25 (0.27- 5.89)	0.778	0.92 (0.14- 6.2)	0.933
Time-to-start-MTX (months)	0.98 (0.97- 0.996)	0.013	0.97 (0.95- 0.996)	**0.021**
Starting dosage MTX (mg/m^2)	0.89 (0.81- 0.98)	0.023	0.84 (0.73- 0.97)	**0.021**
Steroid use during MTX (yes/no)	2.06 (1.00- 4.23)	0.050	2.34 (0.86- 6.34)	0.095
Steroid use before MTX (yes/no)	0.44 (0.19- 1.02)	0.056	0.43 (0.12-1.6)	0.205
Physician's global assessment at start	2.6 (1.5- 4.7)	0.001	2.4 (1.1- 5.1)	**0.026**
MTHFR-haplotype^o	2.8 (1.03- 7.7)	0.043	2.3 (0.68- 7.7)	0.178

* JIA subtype as category with persistent oligoarthritis as reference subtype.

^{o} 0 or 1 haplotype (MTHFR1298A-677C) copies versus 2 haplotype copies.

1 total R^2 (Cox & Snell): 0.27

Regarding the time-to-start MTX (Table 3), it is evident that the use of intra-articular steroids, SSZ and/or other DMARDs prior to MTX influences time-to-start MTX. Although the use of prior treatment was not related to MTX response (Table 1), we analyzed whether the association of time-to-start MTX was independent of the use of prior treatment and a true effect for MTX response. Therefore the effect of time-to-start MTX on response was assessed including only patients with prior treatments. This repeated multivariate analysis showed that the time-to-start MTX was still significantly associated to the response to MTX (OR 0.96, 95%CI 0.94-0.99; p= 0.017).

Finally, the time-to-start-MTX is strongly correlated to the physicians' global assessment at start MTX, meaning that patients with an increased disease activity are treated earlier. Interestingly, our data showed that the physicians' global assessment at baseline also reflects the disease activity as measured from diagnosis to start MTX (data not shown). Although the precise effects size of time-to-start MTX and the physicians' global assessment at baseline cannot be individually determined, from a clinical point of view it is important to observe that earlier treatment is related to increased response rates.

DISCUSSION

In this retrospectively observed cohort of JIA patients, the time-to-start-MTX, the physician's global assessment at baseline and the starting dosage are significantly associated with the response to MTX at 6 months. Patients with an earlier start of MTX and an increased disease activity show an increased response. Our finding that a lower starting dosage MTX is associated with increased response is mainly due to the systemic JIA patients, who receive a higher starting dose and show decreased response.

Although treatment with intra-articular steroids, sulfasalazine and/or other DMARDs prior to MTX is an important determinant for the delay in starting MTX, only including patients with prior treatments to MTX therapy still showed that an early start of treatment with MTX was significantly associated with an increased response. This indicates that our data were not biased by population selection.

Our data show that patients with an increased disease activity receive MTX earlier after diagnosis, which may partially reflect confounding by indication. Therefore, the independent effects of a higher physicians' global assessment at baseline and the decreased time-to-start MTX on response cannot be determined in this analysis. The best evidence for these associations remains the replication in controlled trials with JIA patients.

Because of the different treatment strategies and small numbers of patients in the different JIA subtypes, only associations with MTX response in the general JIA population were observed and no conclusion about the influences of response in individual subtypes can be drawn.

With regard to the genetic parameters, only the MTHFR 1298A-677C haplotype showed a significant decrease in lower copy number in non-responders. This finding is consistent with recent-onset rheumatoid arthritis where a higher number of haplotype copies was related to increased response to MTX.[13] Remarkably, no other significant genetic associations with the response to MTX were detected. This may be due the small sample size resulting in an increased probability to obtain false negative findings (type 2 error).

Our data analysis used a composite measure for response including the physician's global assessment and the joint score. No frequent parent/ patient global assessment of overall well-being and information about limitation of joints could be retrospectively obtained from the patients' chart. Although the definition of improvement as developed by Giannini et al.[14] includes 6 core set variables, the two variables that were included in our definition of improvement are sensitive instruments for measuring change, with the subjective assessment of disease activity by the physician as the most responsive instrument.[15] The independent factors are able to measure an improvement of 53-60%.[16] Combined in our definition of improvement it generated a 57% response. Since improvement of the physician's global assessment is part of our response definition and a higher physician's global assessment at baseline is associated with response, the response rates in our cohort might be partly due to regression to the mean and an effect of unequal distance between the categories and will not fully reflect the true effect size of MTX.

According to the distribution of subtypes, this retrospectively observed cohort is comparable to the clinical practice described by Brunner et al, indicating that no non-random exclusion of subtypes has occurred.[3] In the different subtypes the response rates in this study are comparable to previous reported efficacy of MTX, except for persistent oligoarthritis.[4-8] In the persistent oligoarthritis cohort of Brik et al an response rate of 90% has been reported in patients receiving early treatment (maximum of 4.3 months), whereas our patients with persistent oligoarthritis had a response rate of 44% and were treated after a mean of 22.2 months (range 0-104 months; sd 27.4).[17] This difference in response rate underlines that also in persistent oligoarthritis early treatment with MTX may be an important factor for efficacy.

In summary, in this JIA population, the time-to-start MTX is an important and independent factor for the response to treatment. Already in RA it has been shown that early treatment in the "widow of opportunity" is associated with an improved

clinical outcome and less radiographic damage.[18] Although it is thought that JIA patients may show similar favorable effects of early treatment, our study is the first to illustrate that JIA patients have increased responses to MTX when treated earlier. Patients with a good clinical response to other DMARDs than MTX were not included in this study. Therefore, no conclusion about the effectiveness of MTX compared to other DMARDs or the association between time-to-start-MTX and other DMARDs in JIA can be drawn.

In RA it has been shown that the onset of disease and immunologic events predate the symptoms by many years. The activation of RA is believed to be a multifactorial process that is followed by an ongoing progression of inflammation leading to bone damage already in the first year after diagnosis.[19] Chronis arthritis in JIA patients probably has as similar early onset preceding the symptoms and a subsequent progression of inflammation. The increased response to early treatment with MTX, that is described in RA and shown in JIA in this study, might reflect the fact that MTX can suppress early stages of inflammation, but that the mechanism of action is less sufficient to control well-established chronic inflammation.

It is clinically highly relevant that reducing the time-to-start MTX may lead to an increased response. Future prospective studies are needed to replicate these findings and reveal the exact window of opportunity for JIA. More importantly future studies are needed to determine if an increased early response leads to less joint damage on the long-term.

In conclusion, our study shows the time-to-start-MTX appears to be an important factor for the MTX response. Our results suggest that earlier initiation of MTX treatment will lead to an increased response.

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CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of this thesis was to identify genetic factors associated with the susceptibility to JIA (part A) and to reveal clinical and genetic parameters involved in determining the course of disease and the response to treatment (part B).

PART A. GENETIC RISK FACTORS FOR JUVENILE IDIOPATHIC ARTHRITIS

Genetics involved in JIA

Part of the evidence that genetic factors are involved in the pathogenesis of JIA, is the increased relative risk for family members of patients to develop JIA. It is reported that 1-5% of the JIA population is part of an affected sib pair.[1,2] In our cohort of JIA patients, 7 sib pairs were included (2% of the total cohort) of which 2 were twins (Table 1). Five out of 7 sib pairs had a similar JIA subtype with a comparable age at onset. It has been described that most of the (monozygotic) twins and sibpairs not only have a concordance in JIA subtype, but also in their course of disease,[3] reflecting genetic factors that are involved in coping with ongoing inflammation. The concordance of the course of disease in our sibpairs, however, is not very obvious.

Table 1. Affected sib pairs included in the JIA cohort.

Sib pair	Gender	Diagnosis	Age at onset (yr)	Course of disease
1	F	Persistent oligoarthritis	Not known	Not known
	M	RF-negative polyarthritis	2.5	Not known
2	M	RF-negative polyarthritis	4.25	Unremitting
	M	RF-negative polyarthritis	2	Remitting
3	F	RF-negative polyarthritis	13.75	Not known
(twin)	F	RF-negative polyarthritis	14.75	Not known
4	F	Persistent oligoarthritis	2.25	Intermediate
	F	Persistent oligoarthritis	3.5	Remitting
5	F	RF-negative polyarthritis	3.75	Remitting
	M	RF-negative polyarthritis	3.25	Intermediate
6	F	Persistent oligoarthritis	3.75	Unremitting
(twin)	F	Persistent oligoarthritis	4	Unremitting
7	F	Persistent oligoarthritis	1.75	Intermediate
	M	RF-negative polyarthritis	4.5	Unremitting*

* limited follow-up of 16 months.

Genetic association studies in JIA

Genetic association studies have been performed in many diseases in order to identify risk factors for developing disease and pathways involved in the pathogenesis. This research project was designed to perform genetic association studies in JIA. The focus was on revealing associations with SNPs in non-HLA loci. Multiple SNPs have been studied in cases and controls of North-West European descent. The genes and genetic regions that are described in this thesis have been selected based on previously described associations in JIA and other autoimmune diseases, and their involvement in immune regulation. Several associations have been identified in this JIA cohort; these include newly discovered associations in JIA, in case of TRAF1/C5 (Chapter 2), the 4q27 locus (Chapter 3) and the common autoimmune loci CD226 and CD28 (Chapter 4), as well as replications of previously described associations in JIA (Chapter 4).

Novel genetic associations in JIA

TRAF1/C5

The association of the TRAF1/C5 locus and autoimmunity was first described in RA as a result of a candidate gene study[4] and an independent GWAS[5] in the Caucasian population. Thereafter, more studies have been performed on the association between TRAF1/C5 and RA and other autoimmune diseases like systemic lupus erythematosus, alopecia areata and celiac disease in both Caucasians and other populations.[6-9] We were one of the first research groups to reveal the association between polyarticular JIA and TRAF1/C5 (Chapter 2). At almost the same time the association with TRAF1/C5 was described in a smaller cohort of 67 JIA patients.[10] In other JIA cohorts the association has been confirmed[11-13] although an association has not been found in all studies.[14] A meta-analysis should be performed including all published studies of TRAF/C5 in JIA to confirm this association.

The locus on chromosome 9, that is associated with several autoimmune diseases, contains two genes; TRAF1, encoding tumor necrosis factor receptor-associated factor 1 and C5, encoding complement factor 5. Both genes have a role in immunity and could be involved in the pathogenesis of JIA. Activated complement-component-5 acts as a strong chemoattractant for neutrophils, and a deregulated activity may contribute to the perpetuation of inflammation. In JIA, complement activation is occasionally observed, especially in active polyarthritis.[15] On the other hand, TNF-receptor-associated factor-1 (TRAF-1) plays an essential role in the intracellular TNF-signaling pathway and is possibly a negative regulator of TNF-signaling.[16] Evidence for the importance of TNF-alpha in JIA is suggested by the effectiveness of treatment directed against TNF-alpha, especially in subtypes with a polyarticular course.[17]

However, additional research investigating the functional consequences of the associated allele is required and could provide further insights in the pathogenesis of polyarticular disease in JIA.

To explore the TRAF-1 pathways in more detail, additional genes involved in the TNF-signaling pathway have been incorporated in later genotyping (Chapter 4); these included TNFA, TNFRSF1A , TNFRSF1B , TNFAIP3, TNFRSF14-MMEL1, TNFRSF8-MIIP, TRAF-2, TRAF-6, CD40 (=TNFRSF5). An association has been replicated only for TNFA, and no other new associations were discovered or established associations replicated. Additionally we have investigated polymorphisms in complement-related genes (C1QA, C1QB, C1QC, C1Qlocus, C3, CFB, C4BPA) in a smaller cohort but similarly did not find any association (unpublished data).

4q27

The genetic association of the 4q27 region (also called the KIAA1109-TENR-IL2-IL21 locus) with autoimmune immune disease was first described in celiac disease (GWAS)[18] which was shortly afterwards followed by an association with diabetes mellitus type I (GWAS)[19] and RA.[20] The association with RA was confirmed in both the Caucasian population as well as in other ethnicities. [21, 22] As described in this thesis (Chapter 3) we were the first to describe the association of 4q27 (rs6822844) with JIA. The association of this locus with JIA has been replicated in different cohorts. [23-25] In addition to celiac disease, DM1, RA and JIA, this locus has been associated with further autoimmune diseases like psoriasis, psoriatic arthritis and ulcerative colitis. Therefore this locus can be considered a general autoimmune locus.

The 4q27 locus contains the genes *KIAA1109*, *TENR*, *IL2* and *IL21* that are in strong linkage disequilibrium (LD). Two of the genes in this region are not likely to have a role in autoimmunity; the function of the protein coded by *KIAA1109* is still unknown and the protein TENR (testis nuclear RNA-binding protein, also called adenosine deaminase domain-containing protein 1 (ADAD1)) has been described in mice as being transcribed exclusively in the testis and is suggested to have a role in testis-specific nuclear posttranscriptional processes. However, most interesting are the genes *IL2* and *IL21*, which are both very likely candidates to play a part in the pathogenesis of autoimmunity.

Interleukin 2

IL2

The response to IL2 is mediated through the interaction with the IL2-receptor complex: IL2R-alpha (CD25), IL2R-beta (CD122) and IL2R-gamma (CD132). This IL2-receptor

complex can be trimeric (CD25/CD122/CD132), having a high affinity for IL2 and which is expressed by regulatory T-cells or can be dimeric (CD122/CD132) having an intermediate affinity and which is more broadly expressed on T-cells, natural killer cells and monocytes. Stimulation of the receptor complex by IL-2 induces cytokine release (IL10 and others), through the activation of STAT5.[26] IL2 plays an important role in the proliferation and differentiation of T-cells[27] and is crucial for the development and function of regulatory T-cells in the periphery. Regulatory T-cells suppress the excessive immune response to self-antigens (in autoimmunity) and foreign antigens (in infection).

Immune complexes of IL2 and anti-IL2 monoclonal antibodies (IL2/JES6-1) can induce expansion of regulatory T-cells, binding the high-affinity receptor complex. Mice pre-treated with IL2/JES6-1 develop less collagen induced arthritis. In these mice expansion of regulatory T-cells was seen, causing suppressed levels of antibodies to collagen type II and inhibition of T-helper-17 cells and IL17 production.[28] Although still under investigation as such, the IL2 immune complex might have a role in treating autoimmune diseases like RA and JIA.

Determining the serum levels of IL2 is not feasible because of its limited half-life, so study of the relation between polymorphisms in IL2 (4q27) and serum levels of IL2 will not yield meaningful results.

IL2-receptor

Genetic polymorphisms in IL2RA/CD25 (located on 10p15), that is part of the high affinity IL2-receptor, have at first been associated with DM1[29] and thereafter with multiple other autoimmune diseases such as Graves' disease,[30] multiple sclerosis[31] and RA.[32] An association with JIA has also been described.[33, 25] IL2RA is therefore considered a general autoimmune locus. Although less investigated, association with IL2RB (CD122, located on 22q13) has been described in JIA[25] and RA[34] and has been associated with a more erosive form of arthritis.[35]

More importantly, the genotype-phenotype correlation of polymorphisms in IL2RA has been studied, revealing variable surface levels of CD25 (on CD4+ memory cells), most likely reflecting a transcriptional difference depending on the underlying haplotype.[36] In a recent study, the association between IL2RA polymorphisms and the function of regulatory T-cells was investigated. It was shown that the presence of a disease-associated IL2RA haplotypes lead to a diminished IL2 responsiveness, resulting in lower levels of FOXP3 expression by regulatory T-cells and a reduction in their ability to suppress proliferation of autologous memory CD4+ cells.[37]

Concluding from these data, the IL2-pathway seems to play an important role in the pathogenesis of autoimmune disease (and thus JIA), partly through its effect on

regulatory T-cells. Genetic variation is not only associated with disease susceptibility, but also functional consequences of this genetic variation have been brought to light.

Interleukin 21

IL21 binds to the IL21-receptor (IL21R) that is present on a variety of immune and non-immune cells. Activation of the IL21R results in a pro-inflammatory and immune stimulatory response. Its plays a significant role in inducing the differentiation and expansion of T-helper-17 cells, which play a critical role in the pathogenesis of RA.[38] An increased level of IL21 is associated with increased disease activity in RA.[39] In mice, blocking IL21 activity by IL21R Fc fusion protein (a potent neutralizing reagent for IL21) reduces collagen-induced arthritis and reverses the adjuvant-induced arthritis in Lewis rats.[40] The autoimmune K/BxN mouse deficient of IL21R ((IL21R^{-}/$^{-}$) K/BxN) does not develop arthritis.[41]

Underlining the role of IL21 in autoimmunity are the positive associations with genetic polymorphisms in IL21R. An association between SLE (rs3093301),[42] Hashimoto's thyroiditis (rs3093301 and rs2285452)[43] and IL21R was described. Even more important are genetic associations that are related with an alteration of function. Genetic polymorphisms in IL21 have been studied in SLE in relation to serum levels of IL21. The A allele of rs2055979 was more frequently present in patients compared to controls and this allele was associated with higher levels of IL21 within the patient group.[44] However it should be mentioned that these polymorphisms are situated in the large LD block on 4q27 and might not only reflect change of function of the IL21 gene.

These data strongly suggest that IL21 and IL21R are involved in the pathogenesis of autoimmune disease and targeting IL21 may have a role in treating RA, JIA and other autoimmune diseases.

To reveal more associations of the 4q27 locus and the IL2 and IL21 pathway genes, additional polymorphisms have been typed in a second round of genotyping. These polymorphisms, mainly included in Chapter 4, are summarized in Table2.

Common autoimmune susceptibility loci

Clustering of autoimmune diseases in JIA families

Several studies have indicated a higher prevalence of autoimmunity in JIA families compared to controls.[45-47] It is described that in 21.4% of the JIA families an autoimmune disease is present in first degree family members that is increased compared to the incidence in the normal population.[45] Another study described that in 110 JIA

Table 2. Additional polymorphisms in the 4q27 loci, IL2RA and IL21R

Gene*	rs	OR	p
IL21/ADAD1	rs11732095	0.91 (0.68-1.23)	0.5508
IL21	rs1398553	1.22 (1.05-1.43)	0.0109
IL21	rs4492018	0.88 (0.73-1.05)	0.1495
IL21/KIAA1109	rs4505848	1.20 (1.03-1.41)	0.01901
IL2RA	rs12722489	failed	
IL2RA	rs12722605	1.05 (0.85-1.30)	0.6622
IL2RA	rs2104286	0.91 (0.76-1.10)	0.3382
IL2RA	rs41295061	1.02 (0.79-1.32)	0.8834
IL2RA	rs791589	failed	
IL2RA#	rs11594656	0,94 (0,79- 1,12)	0,486*
IL2RA#	rs10795791	1.23 (1.062- 1.43)	0,0062*
IL2RB	rs3218253	0.99 (0.84-1.17)	0.9164
IL2RB	rs3218258	0.97 (0.82-1.15)	0.7135
IL2RB	rs743777	0.95 (0.81-1.12)	0.5386
IL21R	rs11074861	failed	
IL21R	rs3093341	0.80 (0.62-1.05)	0.1069
IL21R	rs8057432	failed	

*) Polymorphisms have been discussed in Chapter 4, except for IL2RA rs11594656, rs10795791 and the SNP that have failed genotyping.

#) IL2RA rs11594656 and rs10795791 have been investigated in a smaller cohort and data have not been published.

families, 81 families had at least one relative with an autoimmune disease (73.7%), compared to 33.3% in control families. Of all the JIA relatives, 12.6% had an autoimmune disease (predominantly Hashimoto thyroiditis), compared to 4.0% of relatives of the control population. [46] Huang et al showed comparable results, namely that 11.8% of the relatives of 110 JIA families (first, second and third degree family members) had an autoimmune disease.[47]

We have performed a family survey by a questionnaire, asking about the prevalence of 11 autoimmune diseases in the family and indicating which family member was affected (first degree, second degree of more remote). The following autoimmune diseases were included: psoriasis, Bechterew's disease, uveitis, RA, JIA, inflammatory bowel disease (Crohn's disease and ulcerative colitis), DM1, thyroid disease, multiple sclerosis, systemic lupus erythematosus, celiac disease. It should be mentioned that the diagnoses had not been medically verified and may contain misinterpretations, for example osteoarthritis recorded as RA. Of 412 JIA families information about the family history was available. A total of 301 cases of autoimmune disease were present in 223 families (54.1% of all JIA families). Autoimmune diseases were pres-

Table 3. Number of different autoimmune diseases present in 412 JIA families.

Number different AID	1st degree	1st and 2nd degree	In any relative
0	329	217	189
1	74	149	162
2	7*	37	46
3	2	8	13
4	0	1	2
AID positive	83 (12.9%)	195 (47.3%)	223 (54.1%)

*) the combination of psoriasis and RA in 3 families, other combinations were JIA and DM, Bechterew's disease and DM, RA and IBD, Bechterew's disease and IBD.

ent in the first or second degree family member in 195 (47.3%) families and in only the first degree in 83 (12.9%) families (Table3). Psoriasis and RA were the main conditions present in family members. The distribution of the different autoimmune diseases is illustrated in Table 4. Unfortunately the total number of (unaffected) relatives in these families is not known.

Table 4. Distribution of autoimmune disease in 412 JIA families.

	1st degree	2nd degree	> 2nd degree	degree unknown	Total (%)
Psoriasis	35	35	4	3	77 (25.6)
Bechterew's disease	5	10	2	1	18 (6.0)
Uveitis	0	0	0	0	0
Rheumatoid arthritis	14	62	18	3	97 (32.2)
Juvenile idiopathic arthritis	13	9	5	2	29 (9.6)
Inflammatory bowel disease	8	11	2	1	22 (7.3)
Insuline dependent diabetes	7	12	4	2	25 (8.3)
Thyroid disease	9	12	1	2	24 (8.0)
Multiple sclerosis	1	3	0	0	4 (1.3)
Systemic lupus erythematosus	1	3	1	0	5 (1.7)
Celiac disease	0	0	0	0	0
Total (% of total)	93 (30.9)	157 (52.2)	37 (12.3)	4 (4.7)	301

Replication of (common) autoimmune loci and JIA loci

In JIA several studies have been performed analyzing common autoimmune susceptibility loci found in RA and other autoimmune diseases. [14,48,11,23,49,24,50,12,25] In Chapter 4

the replication of multiple common autoimmune loci in JIA is reported. Genes/loci that have been replicated are VTCN1, 4q27, TNFA, PTPN22 (the latter only in a more homogeneous cohort) and ANKRD55. Although no significant association was identified in our case-control association study, a meta-analysis incorporating already published data of other cohorts revealed additional associations of SNPs in PTPRC, AFF3, CCR5, TNFAIP3, TNPO3, IL2RA and CLEC16A and JIA. The overlapping polymorphisms that have been investigated in our cohort and in the papers investigating common autoimmune susceptibility loci in JIA, are listed in Table 5. Unfortunately genotyping of some significant polymorphisms in the highly interesting genes STAT 4 (rs7574865, rs8179673), TNFAIP3 (rs10499194) and IL2RA (rs12722489 and rs791589) have failed. We did not include PTPN2, COG6, C12orf30, C3orf1, ANGPT or CD247 in our study.

ANKRD55

As more often is the case in discovery of new susceptibility loci (such as in PTPN22 and

Table 5. Sample sizes requirements for genetic association studies

MAF	10%		20%		30%		40%	
	cases	controls	cases	controls	cases	controls	cases	Controls
OR								
2.0	223	446	137	274	113	226	107	214
1.5	703	1406	415	830	332	664	304	608
1.3	1751	3502	1016	2032	798	1596	719	1438

Table shows number of cases and controls that need to be typed to detect associations at a significance value of 0.05, power of 80%, for various minor allele frequencies, and odds ratios. A case:control ratio of 1:2 is assumed. OR: odds ratio; MAF: mino allele frequency

TRAF-1/C5), the association of ANKRD55 (rs6859219) with JIA was investigated at the same time in two different cohorts in various ways. While in the UK cohort, combined with the US and German cohort, dense genotyping was performed in regions of interest by Immunochip,[25] at the same time a candidate gene approach was pursued in our cohort. In both studies an association with JIA was revealed.

ANKRD55 was first discovered and replicated in a GWAS in RA[51] and is also associated with multiple sclerosis.[52] ANKRD55 is located at 5q11 and is an ankyrin repeat domain-containing gene with unknown function. Nearby genes are IL6ST and IL31RA (encoding proteins involved in immunity) but 13 haplotype-tagging SNPs in the IL6ST–IL31RA cluster were not in linkage disequilibrium with rs6859219.[52]

(Common) autoimmune loci as novel associations in JIA

Besides the replication of common autoimmune/JIA loci, we have also revealed novel associations with common autoimmune loci, which had not yet been discovered earlier in JIA. We reported the association of CD226 with JIA (in the selected subtypes oligoarthritis and RF-polyarthritis) and a trend towards association of a locus near CD28. Both genes have been analyzed before in JIA, but with no significant association as outcome. We have performed a meta-analysis including these data, to generate an overall odds ratio, strengthening proof of association. Meta-analysis of CD226 showed an overall positive association for CD226 in JIA, underlining the role of this gene in JIA. In addition, CD28 also seems to be a novel association in JIA.

Meta-analysis of our (non-significant) data with results from previous case-control association studies in JIA, that generated similar results lacking association, revealed a possible additional association with PRKCQ (rs4750316)

CD226

The association with autoimmunity of CD226 was first described in DM1,[19] followed by the association with multiple autoimmune diseases such as multiple sclerosis, RA and autoimmune thyroiditis[53] in both European and non-European patients.[54] The strongest association is found with the non-synonymous SNP Gly307Ser (rs763361), although the function consequence of this polymorphism is not yet clear. CD226 (also known as DNAX accessory molecule 1, DNAM-1) is a 67-kDa type I membrane protein involved in the adhesion and co-stimulation of T-cells and NK cells. CD226 binds to two different cell surface ligands; CD155 (poliovirus receptor) and CD112. CD226 might have a role in differentially regulating the proinflammatory (T-helper -1/T-helper-17)/anti-inflammatory (T-helper-2) balance.

We were the first to demonstrate a significant association with JIA (in the selected subtypes oligoarthritis (persistent and extended) and RF-negative polyarthritis), although this polymorphism has been studied before with various results. Ellis et al found a trend towards significance in their Australian cohort (including all JIA subtypes).[12] CD226 was included in a Immunochip analysis,[25] and already studied in a smaller (overlapping) UK cohort , using imputed data,[23] but in these studies no association with JIA was found. Analyzing the pooled data by means of a meta-analysis provided an overall odds ratio of 1.16 (p= 0.001115) that was significant.

CD28

The second novel susceptibility locus identified in our JIA cohort is CD28 (rs1980422), although after correction for multiple testing the significance was low. CD28 has been studied before by various methods; in a candidate gene approach,[50] in a GWAS[55] and

Table 5. Genes/ loci that have been investigated in our cohort in relation to other JIA cohorts

Immunological pathway		Our results		Hinks, 2013☆[25]	Ellis, 2013[12]	Hinks, 2012[50]	Thompson, 2010[24]	Hinks, 2010[11]	(AFF3)[23]	Hinks CCR5[49]	CLEC16A[48]	Hinks, 2009[33]	Prahalad 2009[14]	Hinks, 2009[80]
Gene/ locus	SNP	OR (95% CI)	P (allelic)											
T-cell differentiation														
IL18RAP	rs917997	1.14 (0.94-1.37)	0.1782				ns	ns						
PRDM1	rs548234	1.13 (0.96-1.33)	0.1366			ns								
Immune cell signalling														
PTPN22	rs2476601	1.32 (1.03-1.69)	0.02702	p= 3.19 × 10−25	OR 1.62; p= 0.006		OR 1.54 ; p= 3.28E-04							
VTCN1	rs6673837	0.99 (0.82-1.21)	0.9474											OR; 1.16 p= 0.05
VTCN1	rs2358817	0.88 (0.65-1.19)	0.4164											OR 0.68;; p= 0,005
VTCN1	rs2358820	0.92 (0.67-1.26)	0.602				ns							OR 0.45; p= 0.003
VTCN1	rs10923217	1.21 (1.03-1.42)	0.02079											OR 1.17; P=0.02
VTCN1	rs6669320	0.89 (0.70-1.12)	0.3117											OR 0.8; p=0.02
VTCN1	rs10923223	1.09 (0.88-1.36)	0.4383											OR 1.45; P=0.0001
VTCN1	rs12046117	1.04 (0.82-1.32)	0.7252		ns		ns							OR 1.58; P=1 x 10E-6
PTPRC	rs10919563	0.86 (0.68-1.08)	0.195			OR 0.67; p= 2.6 X10E-7								
AFF3	rs1160542	1.05 (0.90-1.22)	0.5177						OR 1.25; p= 2.05 X 10E-5					

Table 5. Genes/ loci that have been investigated in our cohort in relation to other JIA cohorts (continued)

Immunological pathway		Our results		Hinks, 2013☆[25]	Ellis, 2013[12]	Hinks, 2012[50]	Thompson, 2010[24]	Hinks, 2010[11]	(AFF3)[23]	Hinks CCR5[49]	CLEC16A[48]	Hinks, 2009[33]	Prahalad 2009[14]	Hinks, 2009[80]
CD28	rs1980422	1.29 (1.07-1.55)	0.008079			ns								
ERAP1	rs30187	1.14 (0.97-1.34)	0.1018		ns		ns							
TAGAP	rs394581	0.97 (0.82-1.15)	0.7544			ns								
CDK6	rs42041	0.95 (0.80-1.13)	0.5815				ns					ns		
PRKCQ	rs4750316	0.99 (0.82-1.20)	0.9552		ns		ns					OR 0.88; p= 0.05		
CD226	rs763361	1.30 (1.12-1.51)	0.0006295		OR 1.21; p= 0.059		ns		ns					
Innate immune system and TNF-signalling														
TNFRSF14-MMEL1	rs3890745	0.90 (0.77-1.06)	0.2137				ns							
TRAF6	rs540386	0.83 (0.66-1.05)	0.1194			OR1.13; p=0.06								
CCR5	rs333	0.81 (0.62-1.06)	0.1168							OR 0.79; P=0.006				
CCR6	rs3093023	1.07 (0.91-1.26)	0.3993			ns								
CCL21	rs951005	1.08 (0.88-1.34)	0.4482			ns								
Unknown function														
FAM107A/ 3p14	rs13315591	1.13 (0.86-1.50)	0.3795			ns								

Table 5. Genes/ loci that have been investigated in our cohort in relation to other JIA cohorts (continued)

Immunological pathway		Our results		Hinks, 2013☆[25]	Ellis, 2013[12]	Hinks, 2012[50]	Thompson, 2010[24]	Hinks, 2010[11]	(AFF3)[23]	Hinks CCR5[49]	CLEC16A[48]	Hinks, 2009[33]	Prahalad 2009[14]	Hinks, 2009[80]
6q23	rs6920220	0.98 (0.80-1.19)	0.821				ns					OR 1.16; p= 0.02	OR 1.30; p= 0.015	
ANKRD55	rs6859219	0.74 (0.61-0.90)	0.002953	p= 2.73 × 10−11☆										
7q23	rs10488631	1.03 (0.80-1.33)	0.8078			OR 1.20; p=0.009	ns							
KIF5A	rs1678542	1.02 (0.87-1.19)	0.8226		ns		OR 1.46; p= 2.55E-02					ns		
CLEC16A	rs12708716	0.92 (0.78-1.07)	0.2729				ns				ns			
CLEC16A	rs6498169	1.09 (0.93-1.28)	0.2653		ns		OR 1.12; p= 6.53E-02				OR (1.03 - 1.37); P= 0.016			

was included in an Immunochip analysis.[25] None of the other analyses provided any evidence of association. However when combining data from the GWAS with our data in a meta-analysis, the common odds ratio showed a positive association for CD28 (rs1980422) with JIA.
Association with CD28 has been described in RA[56,51] and in DM1.[57] The polymorphism studied (rs1980422) is located between CD28 and CTLA4. CD28, like CTLA4, binds to their shared ligands CD80 and CD86 and has a costimulatory function on T-cell activation. Interestingly, an association with CD80 has also been revealed in JIA recently.[25]

Other loci studied

Besides common autoimmune loci we included a study of some genes involved in immune cell signaling, but they did not show any association. These genes are: LCK, LECT2, DPP4, ADA, ZNF230, ZNF451, CCR6 and CCL24.

Limitations of genetic association studies

Power and sample size

As is seen in the last decades of genetic association studies in JIA, the relative risk of the associated polymorphisms is only moderate to small. For example the odds ratio (OR) of our findings range from about 1.29 to 1.51 and the minor allele frequency (MAF) in controls of the investigated polymorphism is 0.1- 0.47 (TRAF1/C5: MAF 0.41, OR 1.46 (1.12- 1.90), 4q27: MAF 0.18-0.20, OR 0.76 (0.62- 0.93)(1/0.76 = 1.31), CD226: MAF 0.47, OR 1.30 (1.12-1.51), CD28: MAF 0.22, OR 1.29 (1.07-1.55), ANKRD55: MAF 0.22, OR 0.74 (0.61-0.90) (1/0.74= 1.35), PTPN22: MAF 0.10, OR 1.32 (1.03-1.69), TNFA (rs1799724): MAF 0.10, OR 1.40 (1.09-1.79)).
Different cohort sizes that are needed to detect an association at a significance value of 0.05 and with a power of 80% detection are listed below (by Prahalad 2008)[58] (Table ..). As indicated, in our cohort of 639 cases and 1319 controls we hardly have power to detect a small OR in polymorphisms that have a low MAF.
The size of our cohort is roughly comparable to other JIA cohorts that have been used for genetic studies in the last few years (Table 6). In order to increase power to detect associations with a small effect size, cohorts are being combined nowadays. Ideally, several large independent cohorts should be available to replicate genetic findings.
Besides the relative small number of JIA patients that are included in each JIA cohort, it is striking to notice that only a limited number of independent JIA cohorts

Table 6. Sample size, ethnicity and included JIA subtypes in the different JIA cohorts engaged in genetic association studies in JIA.

	Cases	Subtypes included	Ethnicity cases	Controls	Ethnicity controls
Our cohort (Chapter 4)	639	All JIA subtypes (ao 493 oligoarthritis and RF-polyarthritis)	North-West European	869/ 1319	North-West European
Ellis, 2013[12]	324	All subtypes (not specified)	Australian (200 European descent)	568	Australian (341 European descent)
Hinks, 2013[25]	2816*	Oligoarthritis and RF-polyarthritis	UK (772), US (1596) and Germany (448); all European descent)	13056	US (4048), UK (8,530), German (478)
Thompson 2012 (GWAS)[55]	Discovery 814** Replication: 1728	Oligoarthritis and RF-polyarthritis	Discovery: European/American Replication: European (US, UK, Germany)	Discovery: 658 +2400 Replication: 6997	Discovery: UK Caucasian Replication: Welcome Trust Case Control Consortium2 (WTCCC 2)
Hinks 2012[50]	Discovery: 1242 *** Validation: 813	UK: all JIA subtypes US: Oligoarthritis and RF-polyarthritis	Discovery: UK Caucasian Validation: US Caucasian	Discovery: 4281 + 5380 Validation US: 3058	Discovery: UK Caucasian (ao WTCCC 2) Validation: US European Caucasian
Hinks 2010 [59,49,23,11]	1054***	All JIA subtypes (ao 627 oligoarthritis and RF-polyarthritis)	UK Caucasian	3129	UK Caucasian
Skinningsrud, 2010[48]	509	All JIA subtypes (ao 303 oligoarthritis and RF-polyarthritis)	Norwegian caucasian	2149	Norwegian caucasian
Thompson, 2010[24]	Discovery: 809** Replication: 1015	Oligoarthritis and RF-polyarthritis	Discovery: US European American Replication: US Caucasian and German	Discovery: 3521 Replication: 1569	Discovery: WTCCC Replication: US and German
Prahalad 2009[14]	445	All JIA subtypes (ao 301 Oligoarthritis and RF-polyarthritis)	US with North European ancestry	643	US with North European ancestry
Hinks 2005 (PTPN22)[60]	661***	All subtypes	UK	595	UK

UK: United Kingdom, US: United States of America

*) German and US cohort overlap with cohort Tompson[24,55], UK cohort overlap with Hinks [50]

**) overlap with Thompson 2010[24]

***) overlap with Hinks 2010 [59,49,23,11] and 2005 [60]

exist. This underlines once more the difficulty in creating a large patient cohort of a relatively rare disease.

Phenotypic heterogeneity

JIA is a heterogeneous group of diseases characterized by chronic arthritis. Based on clinical features and laboratory parameters a classification into different subtypes has been made. Some of these subtypes have very distinct features, such as systemic JIA. More evidence arises that systemic JIA can be regarded as an autoinflammatory disease. JIA with enthesitis and psoriatic JIA each have specific characteristics. Only one category of psoriatic arthritis has some resemblance with early-onset ANA positive oligoarthritis. The subtypes oligoarthritis (persistent and extended) and RF-negative polyarthritis share the most clinical features and have a consistent overlap in genetic associations. Because of the phenotypic homogeneity of these subtypes, we have chosen to perform our genetic association study of new JIA loci (Chapter 4) only in a group of patients with oligoarticular and RF-negative polyarticular JIA. The same approach has been followed in the US cohort[55] that has also limited inclusion to these selected subtypes and in the most recent dense genotyping study with the Immunochip.[25]
Between these subtypes (oligoarthritis and RF-negative polyarthritis) no consistent pattern of genetic association can be seen. In some cases the subtypes with a polyarticular course (extended oligoarthritis and RF-negative polyarthritis) seem to have more similarities in their genetic associations, but this was not a consistent result for all associations (Chapter 4).

Ethnic/ geographical background

In our cohort we have included patients form the Netherlands, Belgium, Germany and Switzerland, all with a self/parent reported Caucasian ethnicity. We have included only the patients with a North-West European background, taking the genetic variation due to immigration in Europe into account. A low average level of genetic differentiation among Europeans has been described in 3000 Europeans with a close correspondence between genetic and geographic distances.[61] Therefore we regard our cohort as having a similar genetic background.
When admixture within a study population exist population stratification can take place. For a proportion of the SNPs (52/93), we had the possibility to compare our control MAFs to results of another Dutch control cohort, which are comparable. This other Dutch cohort has been checked and corrected for population outliers.[62] MAFs that were not publicly available have been obtained by personal communication. Because of the comparable MAFs of the control populations, we think it is unlikely

Table 7. Both case-control analysis en TDT listed for selected genes*

Chr	Position[a]	Gene/region	SNP	Minor allele	MAF controls	MAF cases	Case-controle analysis		Transmission disequilibrium test					
							OR (95% CI)	P (allelic)	T[1]	U[2]	OR	L95	U95	P
1	2553624	*TNFRSF14-MMEL1*	rs3890745	G	0.32	0.30	0.90 (0.77-1.06)	0.2137	**126**	123	1.024	0.799	1.313	0.8492
1	12091210	*TNFRSF8-MIIP*	rs946461	T	0.26	0.29	1.15 (0.98-1.36)	0.09358	136	120	1.133	0.8866	1.449	0.3173
1	12252955	*TNFRSF1B*	rs1061622	G	0.23	0.24	1.03 (0.85-1.24)	0.7678	103	108	0.9537	0.7281	1.249	0.7307
1	32729702	*LCK*	rs1004420	T	0.17	0.17	0.98 (0.80-1.19)	0.8087	73	87	0.8391	0.6148	1.145	0.2684
1	32743866	*LCK*	rs695161	C	0.48	0.47	0.97 (0.83-1.13)	0.6721	168	163	1.031	0.8309	1.279	0.7835
1	114377568	***PTPN22***	**rs2476601**	A	0.10	0.13	**1.32 (1.03-1.69)**	**0.02702**	72	51	1.412	0.9862	2.021	0.05829
1	117685992	*VTCN1*	rs6673837	A	0.20	0.20	0.99 (0.82-1.21)	0.9474	89	114	0.7807	0.5917	1.03	0.07932
1	117690758	*VTCN1*	rs2358817	T	0.08	0.07	0.88 (0.65-1.19)	0.4164	41	42	0.9762	0.6348	1.501	0.9126
1	117711911	*VTCN1*	rs2358820	A	0.07	0.07	0.92 (0.67-1.26)	0.602	31	35	0.8857	0.5462	1.436	0.6225
1	117730048	***VTCN1***	**rs10923217**	C	0.48	0.52	**1.21 (1.03-1.42)**	**0.02079**	150	151	0.9934	0.7925	1.245	0.954
1	117730623	*VTCN1*	rs6669320	A	0.15	0.13	0.89 (0.70-1.12)	0.3117	56	66	0.8485	0.5943	1.211	0.3653
1	117746573	*VTCN1*	rs10923223	C	0.15	0.16	1.09 (0.88-1.36)	0.4383	84	71	1.183	0.8626	1.623	0.2964
1	117751365	*VTCN1*	rs12046117	T	0.13	0.13	1.04 (0.82-1.32)	0.7252	71	59	1.203	0.852	1.7	0.2926
1	198700442	*PTPRC*	rs10919563	A	0.13	0.11	0.86 (0.68-1.08)	0.195	62	73	0.8493	0.6054	1.191	0.3438
1	206946897	*IL10*	rs1800896	C	0.50	0.47	0.90 (0.77-1.06)	0.2119	136	157	0.8662	0.6885	1.09	0.2199
1	207015957	*IL19*	rs2243191	T	0.20	0.23	1.17 (0.96-1.43)	0.1209	100	98	1.02	0.7723	1.348	0.887
1	207038686	*IL20*	rs1400986	T	0.16	0.14	0.86 (0.69-1.08)	0.1975	81	79	1.025	0.7521	1.398	0.8744
2	100832155	*AFF3*	rs1160542	G	0.45	0.46	1.05 (0.90-1.22)	0.5177	142	163	0.8712	0.6956	1.091	0.2292
2	100835734	*AFF3*	rs10865035	A	0.46	0.47	1.05 (0.89-1.24)	0.5762	126	140	0.9	0.7075	1.145	0.3907
2	103070568	*IL18RAP*	rs917997	T	0.22	0.24	1.14 (0.94-1.37)	0.1782	119	114	1.044	0.8074	1.35	0.7432
2	113537223	*IL1A*	rs17561	A	0.30	0.29	0.92 (0.77-1.09)	0.3289	117	119	0.9832	0.7618	1.269	0.8964
2	113542960	*IL1A*	rs1800587	A	0.31	0.28	0.89 (0.74-1.07)	0.2285	99	114	0.8684	0.6634	1.137	0.3041
2	113590390	*IL1B*	rs1143634	A	0.25	0.24	0.95 (0.79-1.14)	0.5542	111	107	1.037	0.7955	1.353	0.7865
2	113594867	*IL1B*	rs16944	A	0.33	0.35	1.11 (0.94-1.31)	0.2315	134	141	0.9504	0.7502	1.204	0.6729
2	162856148	*DPP4*	rs2268894	C	0.45	0.47	1.07 (0.91-1.25)	0.4192	159	165	0.9636	0.775	1.198	0.7389

Table 7. Both case-control analysis en TDT listed for selected genes* (continued)

Chr	Position[a]	Gene/region	SNP	Minor allele	MAF controls	MAF cases	Case-controle analysis				Transmission disequilibrium test			
							OR (95% CI)	P (allelic)	T[1]	U[2]	OR	L95	U95	P
2	191835596	*STAT1*	rs3771300	C	0.48	0.50	1.10 (0.94-1.27)	0.2321	167	160	1.044	0.8403	1.296	0.6987
2	191843445	*STAT1*	rs13010343	A	0.14	0.13	0.96 (0.77-1.20)	0.7449	68	88	0.7727	0.5631	1.06	0.1093
2	191845725	*STAT1*	rs1547550	C	0.35	0.34	0.95 (0.82-1.12)	0.5685	145	146	0.9932	0.7893	1.25	0.9533
2	191855521	*STAT1*	rs7562024	T	0.40	0.39	0.95 (0.82-1.11)	0.5502	159	139	1.144	0.911	1.436	0.2466
2	204610396	*CD28*	rs1980422	C	0.22	0.27	1.29 (1.07-1.55)	0.008079	111	88	1.261	0.9536	1.669	0.103
2	204732714	*CTLA4*	rs231775	G	0.37	0.35	0.92 (0.78-1.09)	0.3251	134	167	0.8024	0.6392	1.007	0.05716
3	46414947	*CCR5*	rs333	del	0.10	0.08	0.81 (0.62-1.06)	0.1168	44	53	0.8302	0.5566	1.238	0.3608
3	58556841	*FAM107A*	rs13315591	C	0.07	0.08	1.13 (0.86-1.50)	0.3795	41	30	1.367	0.8534	2.189	0.1917
4	26108197	4p15	rs874040	G	0.31	0.29	0.93 (0.79-1.09)	0.3556	128	141	0.9078	0.7146	1.153	0.428
4	123132492	***KIAA1109***	**rs4505848**	G	0.36	0.40	**1.20 (1.03-1.41)**	**0.01901**	161	135	1.193	0.9488	1.499	0.1307
4	123348345	*ADAD1*	rs11732095	G	0.08	0.07	0.91 (0.68-1.23)	0.5508	40	41	0.9756	0.6311	1.508	0.9115
4	123514528	*IL2-IL21*	rs4492018	A	0.24	0.21	0.88 (0.73-1.05)	0.1495	105	125	0.84	0.648	1.089	0.1872
4	123548068	***IL21***	**rs1398553**	T	0.33	0.38	**1.22 (1.05-1.43)**	**0.0109**	153	138	1.109	0.8808	1.396	0.3792
5	55438580	***ANKRD55***	**rs6859219**	A	0.22	0.17	**0.74 (0.61-0.90)**	**0.002953**	89	109	0.8165	0.6171	1.08	0.1552
5	96124330	*ERAP1*	rs30187	T	0.32	0.35	1.14 (0.97-1.34)	0.1018	144	151	0.9536	0.759	1.198	0.6836
5	135287029	*LECT2*	rs31517	A	0.36	0.37	1.03 (0.88-1.20)	0.7415	148	152	0.9737	0.7765	1.221	0.8174
6	31540141	*LTA*	rs2239704	A	0.39	0.36	0.89 (0.75-1.05)	0.1671	133	174	0.7644	0.6099	0.9579	0.01928
6	31540313	*LTA*	rs909253	G	0.34	0.35	1.07 (0.90-1.26)	0.4406	148	135	1.096	0.8682	1.384	0.4397
6	31540784	*LTA*	rs1041981	A	0.33	0.35	1.09 (0.93-1.29)	0.2906	154	133	1.158	0.9181	1.46	0.2151
6	31542482	***TNFA***	**rs1799724**	T	0.10	0.13	**1.40 (1.09-1.79)**	**0.007489**	93	71	1.31	0.9618	1.784	0.08581
6	31542963	***TNFA***	**rs1800750**	A	0.02	0.01	**0.36 (0.15-0.86)**	**0.01635**	3	6	0.5	0.125	1.999	0.3173
6	31543031	***TNFA***	**rs1800629**	A	0.17	0.14	**0.79 (0.63-0.99)**	**0.04234**	77	92	0.837	0.6183	1.133	0.2486
6	31543101	***TNFA***	**rs361525**	A	0.05	0.03	**0.55 (0.35-0.86)**	**0.007526**	17	24	0.7083	0.3805	1.318	0.2743
6	31543827	***TNFA***	**rs1800610**	A	0.10	0.13	**1.38 (1.08-1.77)**	**0.009952**	90	69	1.304	0.9532	1.785	0.09583
6	31544189	*TNFA*	rs3093662	G	0.06	0.04	0.75 (0.52-1.09)	0.1328	28	30	0.9333	0.5577	1.562	0.7928
6	57012930	*ZNF451*	rs3734738	A	0.21	0.21	0.99 (0.82-1.19)	0.9212	90	109	0.8257	0.6246	1.092	0.178

Table 7. Both case-control analysis en TDT listed for selected genes* (continued)

Chr	Position[a]	Gene/region	SNP	Minor allele	MAF controls	MAF cases	Case-controle analysis				Transmission disequilibrium test			
							OR (95% CI)	P (allelic)	T[1]	U[2]	OR	L95	U95	P
6	106568034	*PRDM1*	rs548234	C	0.32	0.35	1.13 (0.96-1.33)	0.1366	138	119	1.16	0.9075	1.482	0.2359
6	138006504	*TNFAIP3*	rs6920220	A	0.21	0.20	0.98 (0.80-1.19)	0.821	92	88	1.045	0.7805	1.4	0.7656
6	159482521	*TAGAP*	rs394581	C	0.28	0.27	0.97 (0.82-1.15)	0.7544	125	122	1.025	0.7984	1.315	0.8486
6	167534290	*CCR6*	rs3093023	A	0.44	0.46	1.07 (0.91-1.26)	0.3993	134	143	0.9371	0.7403	1.186	0.5887
7	75442759	*CCL24*	rs2302005	T	0.22	0.21	0.93 (0.78-1.12)	0.4639	97	98	0.9898	0.7475	1.311	0.9429
7	75442855	*CCL24*	rs2302004	C	0.44	0.42	0.94 (0.81-1.09)	0.4046	165	156	1.058	0.8498	1.316	0.6154
7	92246744	*CDK6*	rs42041	G	0.26	0.25	0.95 (0.80-1.13)	0.5815	109	114	0.9561	0.7353	1.243	0.7378
7	128594183	*TNPO3*	rs10488631	C	0.10	0.10	1.03 (0.80-1.33)	0.8078	44	58	0.7586	0.5127	1.123	0.1657
9	34743681	*CCL21*	rs951005	C	0.15	0.16	1.08 (0.88-1.34)	0.4482	77	77	1	0.7291	1.371	1
9	139775146	*TRAF2*	rs7048473	C	0.26	0.25	0.95 (0.80-1.13)	0.5385	103	108	0.9537	0.7281	1.249	0.7307
9	139787453	*TRAF2*	rs2811761	G	0.21	0.21	0.99 (0.83-1.20)	0.9556	103	101	1.02	0.775	1.342	0.8886
9	139815053	*TRAF2*	rs10781522	G	0.38	0.38	1.00 (0.86-1.17)	0.9959	149	142	1.049	0.8338	1.32	0.6816
9	139821068	*TRAF2*	rs3750512	C	0.38	0.37	0.96 (0.82-1.12)	0.6014	151	136	1.11	0.8807	1.4	0.3759
10	6053163	*IL2RA*	rs12722605	T	0.14	0.15	1.05 (0.85-1.30)	0.6622	79	83	0.9518	0.6994	1.295	0.7533
10	6099045	*IL2RA*	rs2104286	G	0.25	0.23	0.91 (0.76-1.10)	0.3382	108	117	0.9231	0.7106	1.199	0.5485
10	6114660	*IL2RA*	rs41295061	A	0.09	0.09	1.02 (0.79-1.32)	0.8834	52	39	1.333	0.8803	2.02	0.173
10	6393260	*PRKCQ*	rs4750316	C	0.19	0.19	0.99 (0.82-1.20)	0.9552	92	86	1.07	0.7973	1.435	0.6529
11	36525293	*TRAF6*	rs540386	T	0.13	0.11	0.83 (0.66-1.05)	0.1194	57	83	0.6867	0.4902	0.9621	0.02799
11	71709272	*IL18BP*	rs3814721	C	0.06	0.07	1.18 (0.85-1.64)	0.3216	39	32	1.219	0.7636	1.945	0.4061
11	71710478	*IL18BP*	rs2298455	C	0.12	0.12	0.96 (0.75-1.24)	0.7774	60	72	0.8333	0.5916	1.174	0.2963
11	71714078	*IL18BP*	rs1541304	T	0.03	0.02	0.87 (0.52-1.46)	0.5935	14	12	1.167	0.5396	2.522	0.6949
11	112035458	*IL18*	rs1946518	T	0.40	0.40	1.00 (0.85-1.18)	0.9851	140	146	0.9589	0.7605	1.209	0.7227
11	117869670	*IL10RA*	rs2229113	A	0.31	0.30	0.97 (0.81-1.16)	0.7345	118	142	0.831	0.651	1.061	0.1366
12	6450945	*TNFRSF1A*	rs767455	C	0.42	0.40	0.92 (0.79-1.09)	0.3416	134	162	0.8272	0.658	1.04	0.1036
12	6451590	*TNFRSF1A*	rs4149570	A	0.40	0.42	1.06 (0.90-1.25)	0.4766	154	146	1.055	0.8411	1.323	0.6442
12	57968715	*KIF5A*	rs1678542	C	0.37	0.37	1.02 (0.87-1.19)	0.8226	133	152	0.875	0.6933	1.104	0.2604

Table 7. Both case-control analysis en TDT listed for selected genes* (continued)

Chr	Position[a]	Gene/region	SNP	Minor allele	MAF controls	MAF cases	Case-controle analysis				Transmission disequilibrium test			
							OR (95% CI)	P (allelic)	T[1]	U[2]	OR	L95	U95	P
16	11179873	*CLEC16A*	rs12708716	G	0.35	0.33	0.92 (0.78-1.07)	0.2729	109	138	0.7899	0.6144	1.015	0.065
16	11249329	*CLEC16A*	rs6498169	G	0.34	0.37	1.09 (0.93-1.28)	0.2653	151	132	1.144	0.9057	1.445	0.2587
16	27448401	*IL21R*	rs3093341	G	0.10	0.08	0.80 (0.62-1.05)	0.1069	45	49	0.9184	0.6127	1.376	0.6799
16	67189486	*TRADD*	rs11574518	T	0	0	*not polymorphic*		0	1	0	0	nan	0.3173
17	32594568	*CCL2-CCL7*	rs8079244	C	0	0	*not polymorphic*		0	0	NA	NA	NA	NA
17	40447401	*STAT5A*	rs7217728	C	0.31	0.29	0.91 (0.77-1.07)	0.2662	124	127	0.9764	0.7624	1.25	0.8498
17	40461003	*STAT5A*	rs2293154	A	0.18	0.18	1.05 (0.86-1.27)	0.6606	102	88	1.159	0.8715	1.542	0.3098
18	67531642	*CD226*	rs763361	T	0.47	0.54	1.30 (1.12-1.51)	0.0006295	148	168	0.881	0.7063	1.099	0.2606
19	44515514	*ZNF230*	rs12753	A	0.14	0.15	1.05 (0.85-1.30)	0.6291	81	88	0.9205	0.6807	1.245	0.5903
20	43280231	*ADA*	rs6031698	A	0	0	*not polymorphic*		0	0	NA	NA	NA	NA
20	44746982	*CD40*	rs1883832	T	0.25	0.25	0.99 (0.82-1.19)	0.9027	122	107	1.14	0.8795	1.478	0.3216
21	34640788	*IL10RB*	rs2834167	G	0.24	0.27	1.14 (0.95-1.37)	0.1566	120	106	1.132	0.8718	1.47	0.3517
22	24236392	***MIF***	**rs755622**	C	0.21	0.15	**0.67 (0.54-0.83)**	**0.0002084**	81	94	0.8617	0.6402	1.16	0.3258
22	37544245	*IL2RB*	rs3218258	T	0.28	0.27	0.97 (0.82-1.15)	0.7135	120	101	1.188	0.9118	1.548	0.2012
22	37544810	*IL2RB*	rs3218253	T	0.28	0.28	0.99 (0.84-1.17)	0.9164	122	99	1.232	0.9453	1.606	0.1218
22	37551607	*IL2RB*	rs743777	G	0.34	0.33	0.95 (0.81-1.12)	0.5386	146	139	1.05	0.8327	1.325	0.6784

*) genes described in Chapter 4
1) T: Tranmission of minor allel to offspring
2) U: Minor allele untransmitted to offspring

that potential population outliers in our control cohort have distorted our association results.

When the genetic variation within a subpopulation differs from the rest of the (case or control) population, regardless of the presence of disease (or trait to be investigated), it can cause false positive or false negative associations. In order to avoid population stratification, a TDT analysis (a family based study) can be conducted. In case of a TDT analysis, patient-parents trios are investigated and parents act as controls for their affected offspring.

In our cohort, a TDT test was performed with the SNPs that were investigated in Chapter 4 (data not published) and the results are listed in Table 7. Three hundred twenty eight parent-patient trios were available for analysis in the cohort consisting of oligoarthritis and RF-negative polyarthritis. In LTA (rs2239704) and TRAF6 (rs540386) a significant overtransmission of the minor allele is demonstrated compared to the transmission expected based on Mendelian laws, indicating an association with disease. However no association was found in the case-control association study with these SNPs. Remarkable is the trend toward association in the TDT analysis seen in PTPN22 (rs6673837), VTCN1 (rs6673837), CTLA4 rs231775), TNFA (rs1799724), TNFA (rs1800610) and CLEC16 (rs12708716); all SNPs that have been associated with JIA in previous studies. This shows that a TDT test generates similar results and can be additive to (or replace) a case-control association study. However it is challenging to create a cohort with the same number of patients that are needed for a case-control study, but with also (DNA of) both parents available.

What have genetic association studies brought us?

Unfortunately, little is known about the genetic contribution to the susceptibility of JIA. It is estimated that 30% of the JIA risk can be explained by (common) genetic variations.[25;55;63] In JIA the HLA-locus is estimated to explain 8-13% of the overall heritability.[25;55;63] All other identified risk factors have only a small effect size.

More is known about the genetic contribution to RA, a comparable autoimmune disease, also characterized by chronic arthritis. The genetic contribution to RA is estimated to be 65%.[64;65] The contribution of the established risk loci to the total heritability to RA is thought to be 33-47%.[51] The HLA-locus and PTPN22 are the most important genetic risk factors in RA.[66;67] In RA, an additional 20% of disease risk could be explained by the thousands of additional SNPs embedded in GWAS results.[68]

Although the contribution of each of the associated SNPs to the susceptibility to JIA is only modest and cannot be used to predict the occurrence of JIA in a healthy population, these genetic associations can generate more knowledge about the

immunological pathways involved in the pathogenesis of JIA and its overlap with other autoimmune diseases. Also these (modest) genetic associations can contain major lead points for developing new treatments and thereby possibly improving the outcome of disease in patients with JIA.

PART B. CLINICAL AND GENETIC FACTORS INVOLVED IN THE COURSE OF DISEASE AND RESPONSE TO TREATMENT

Prognostic markers for clinical course

Genetic association studies have revealed some of the risk factors that are involved in developing JIA and have offered potential targets for new therapeutic options. However, the physician is dealing with an individual patient and has to inform the patient/parents and decide about the optimal treatment, preferably taking into account the course that the disease will follow. It would be ideal if at onset of the disease, markers were available that could help in predicting the course of disease and response to treatment, possibly leading to tailor made therapy.

To address the question of predictive factors, clinical parameters have first been investigated with regards to an association with the course of disease (Chapter 5). Subsequently genetic markers were studied for their ability to function as a prognostic marker for predicting the course of disease in JIA (Chapter 6).

Clinical prognostic markers for the course of disease

The clinical course of JIA has a fluctuating character with unpredictable episodes of active disease and disease quiescence. In order to capture these fluctuations, the sequential episodes of active and inactive disease have been studied and the percentage of time with active disease has been calculated for the first 2 (or 5 years) after disease onset (Chapter 5).

In the first two years after diagnosis this percentage of time with active disease differs significantly between JIA subtypes ($p=0.031$); patients with persistent oligoarthritis have the lowest percentage of time with active disease (47%) and patients with extended oligoarthritis have the highest percentage of time with active disease (70%) in the first two years after onset (generating a p-value of <0.001 when comparing these two subtypes). Within this, only the cumulative time with active disease seems to be related to the JIA subtype, but not the physicians' global assessment of the level of disease activity. The percentage of time with active disease in the different subtypes in this cohort of JIA patients is similar to the percentages of active disease that have been described previously in an independent JIA cohort,[69;70] suggesting

that the percentage of time with active disease is representative for the different JIA subtypes and seems a reproducible measure for disease activity during the course of the disease. In our cohort, the percentage of time with active disease (divided arbitrarily into categories of course of disease; remitting, intermediate, unremitting) in the first two years is predictive for the course of disease in the following years. This can therefore be used as overall marker for predicting the subsequent course of disease.

This parameter "percentage of time spent in active disease" differs from the parameter "reaching clinical remission (at least 6 months of inactive disease) on medication", that has been proposed and validated in recent years.[71] In our cohort of 272 patients, 164 (60,3%) patients had inactive disease for >= 6 months during the first two years (on medication). Sixty five (39.6%) of these patients were classified as having a remitting disease course, 80 (48.8%) had an intermediate course of disease, but 19 (11.6%) patients were classified as having an unremitting disease course (Table 8).

Table 8. The cohort classified according to the different parameters indicating course of disease.

	Remitting course of disease	Intermediate course of disease	Unremitting course of disease	
Clinical remission*	65	80	**19**	164
No clinical remission	0	16	92	108
	65	96	111	272

*) Clinical remission (on medication) defined as a continuous period of inactive disease of at least 6 months with the use of medication (ref) that has taken place in the first two years after disease onset.

These 19 patients had an episode of inactive disease for 6 months, but were active for about 1 ½ year in their first two years after diagnosis and considered unremitting in our analysis. If reaching clinical remission (on medication) was used as parameter defining course of disease, these patients would have been classified as having a favorable course. Considering prognosis in terms of damage to the joint, every moment that a patient has (clinically) inactive disease is advantageous and thus is important. The aim of treatment is to minimize the time spent in a state of active disease, in order to prevent damage to the affected joints. According to Wallace et al [70] who studied the fluctuating pattern of the course of JIA, patients experience many episodes of inactive disease, that not always (only in 48%) lead to clinical remission.

The JIA subtype is related to the clinical course in the first two years, however the JIA subtype cannot be determined at onset. Oligoarthritis can evolve into extended

oligoarthritis or be defined as persistent oligoarthritis only after 6 months (by definition). However once evolved into extended oligoarthritis, the course of disease (defined by the percentage of time with active disease in the first two years) is the least favorable compared to persistent oligoarthritis and RF-negative polyarthritis. This high percentage of time with active disease in extended oligoarthritis might be due to a less aggressive treatment or a delayed use of DMARDs. Patients with extended oligoarthritis receive less methotrexate in the first two years (38%) compared to those with RF-negative polyarthritis (65%). Extended oligoarthritis should be regarded as a separate entity and a more aggressive therapeutic approach might be beneficial. There seems to be a particular need for prognostic factors predicting the evolvement into extended oligoarthritis, that has great impact on the course that the disease will follow.

Genetic prognostic markers for the course of disease

The same genetic markers as described in Chapter 4 were investigated in the cohort of 272 JIA patients of which the course of disease was determined. These genes/loci were selected based on their role in (general) autoimmunity and immunoregulation. Different categories of course of disease (remitting, intermediate, unremitting course), based on the percentage of active disease in the first two years after disease onset, were used as outcome parameter. Both univariate and multivariate analyses revealed VTCN1 (rs10923223) as a genetic marker associated with the course of disease, although replication of these data is still essential (Chapter 6). A modest association has been found with CDK6 (rs42041), although not statistically significant.

VTCN1

The gene VTCN1 (V-set domain containing T cell activation inhibitor 1) is located on chromosome 1 (1p13) and encodes B7-H4, a member of the B7 co-signaling molecule family.[72] Binding to an as yet unknown receptor, B7-H4 has an inhibitory effect on T-cells by decreasing proliferation and cytokine production.[72-74] The involvement of B7-H4 in tumor immunity has been described in multiple types of cancer.[75] A higher expression of B7-H4 has been related to a more progressive disease and decreased survival, because of its potential inhibitory effect on T-cells involved in the anti-tumor response in (amongst others) renal cell carcinoma, ovarian cancer, melanoma, gastric and esophagus tumors.[76] Important is the possible involvement in autoimmunity, where a loss of negative regulation might be crucial. In mice deficient of B7-H4, an increased severity of collagen induced arthritis is observed. Use of B7-H4Ig suppresses the progression of collagen induced arthritis, even in established arthritis.[77] B7-H4 is expressed in the synovium, indicating a local influence of its inhibitory function.[78] This

inhibitory B7-H4 pathway might be of therapeutic interest, (similar to the CTLA4 Ig results in RA).[79] The genetic association of VTCN1 and several autoimmune diseases (JIA, RA and SLE) underline the importance of B7-H4 in autoimmunity.[80-82] However associations with susceptibility in JIA have been inconsistent. A positive association has been discovered in a GWAS and replicated in an independent cohort study.[80] Equally, in our candidate gene study (Chapter 4) a modest association was found. However in two other cohort studies no association with VTCN1 was detected.[12;24] Of more clinical importance is that we describe, for the first time, the association of VTCN1 with the progression of JIA. The minor allele of especially rs10923223 is related to a more remitting disease. Possibly, this variation causes a gain of inhibitory function of B7-H4, leading to a better autoimmune regulation. Additional studies are needed to relate the genetic variation of VTCN1 to the co-inhibitory function of B7-H4. However our results contribute to the hypothesis that the B7-H4 pathway can be a new therapeutic target (for example agonistic B7-H4Immunoglobulinfusion proteins) in JIA. Finally, this polymorphism might be useful as a predictive factor for the course of disease in JIA.

CDK6

A modest association of CDK6 gene (rs42041) with a more remitting disease has been discovered, although not statistically significant after correcting for multiple testing. CDK6 (cyclin-dependent kinase 6) is a regulator of the cell cycle progression and as such might be involved in regulation of disease progression. It is therefore still a gene of interest and further investigation is needed to identify this gene as truly associated with the course of disease.

Combining clinical and genetic markers

Both clinical and genetic parameters seem to be associated with the clinical course of JIA. When plotting the predictive value of the parameters that are available at disease onset in a ROC curve, we see almost no effect of JIA subtype alone, probably due to the fact that no differentiation can be made (at onset) between persistent oligoarthritis and extended oligoarthritis. However when adding genetic markers (VTCN1 rs10923223 and CDK6 rs42041) the area under the curve increases. This suggests that a predictive model involving clinical and genetic parameters can be constructed and might be useful for the physician to guide the individual (therapeutic) approach. Additional studies in independent (prospective) cohort studies are needed to validate a predictive model.

Pharmacogenetics

Besides predicting the course of disease, it is also beneficial for optimizing the individual therapeutic regime to be able to predict the response to treatment. Methotrexate

(MTX) has been the most important (non biological) disease modifying anti-rheumatic drug used in the last years. Variations in genes involved in the folate and adenosine pathways could be predictive for the response to MTX. Both clinical and genetic parameters have been studied in relation to the response to MTX.

Methotrexate

Clinical predictors for the response to MTX have been described in several reports, revealing an early start of treatment as the most consequently performing predictive parameter.[83-85] Consistent with previous (and following) reports, our study also revealed that the time to start of treatment is an important factor for the response to MTX (Chapter 7); early treatment seems to be more effective.

In addition to clinical parameters, the genetic variation in genes involved in the cell entry, folate and adenosine pathway could also have a role in predicting the response to MTX treatment in JIA. In RA, the association of genetic polymorphisms in genes coding for folate pathway enzymes and genes involved in the adenosine pathway and the response to treatment has been described.[86;87] In JIA, a polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene (A1298C) has been associated with efficacy of MTX.[88] Following this reported association, several studies have been performed yielding different results. SNPs in the SLC16A7 gene,[89] inosine triphosphate pyrophosphatase gene (ITPA), 5-aminoimidazole-4-carboxamide ribonucleotide transformylase gene (ATIC),[90] ABCB1 rs1045642, ABCC3 rs4793665, and SLC19A1 rs1051266[91] were associated with response to MTX and also a possible effect of SNPs in methionine synthase reductase, multidrug resistance 1 (MDR-1/ABCB1), multidrug resistance protein 1 (MRP-1/ABCC1) and proton-coupled folate transporter (PCFT)[92] were reported. However other studies, including our report, have failed to detect any effect.[93;94]

Overall these reports show that the pharmacogenetics concerning the genes involved in the cell entry, folate and adenosine pathways are far from elucidated. One of the major concerns with regards to these studies is the small patient cohorts, ranging from 58 to 287 patients (in total), that only have sufficient power to detect associations with a large effect. It is possible that associations with a relatively small effect have gone undetected, because of the lack of power. Furthermore no official criteria defining response to treatment are available or were consistently used. A preliminary definition for improvement has been proposed,[95] but not validated or widely implemented in studies involving response to MTX. Comparing different criteria evaluating response to MTX (by ACR and EULAR) demonstrated a good agreement.[96]

On the one hand, the aim is to create a prediction model for response to MTX, involving genetic polymorphism, clinical parameters and biomarkers (such as MRP8/14)[97] that are associated with response to treatment. A prediction model could be helpful in choosing the optimal treatment strategy for the individual patient. At the other hand, by investigating the (change of) function of genetic polymorphisms that are associated with response to MTX, the working mechanism of MTX can be further elucidated, possibly leading to more specific lead points of treatment.

Limitations

Even more than in studying genetic polymorphisms regarding susceptibility to JIA, the issue of small sample sizes (lacking power to detect associations with low risk) in association studies with course of disease or response to treatment, is vast. To create a cohort of which detailed information about the course of disease is known, including information about all different episode of active and inactive disease in the first years after diagnosis, together with detailed use of medication, is challenging. Access to a detailed patient file is needed. Perhaps when electronic patient dossiers become fully available in the future, this would facilitate this process. The same number of patients (per category that is investigated) is needed in these type of studies compared to investigating genetic risk factors for developing disease (see before). But in this type of studies the total patient cohort is divided into subgroups that are compared to each other, so at least twice the number of patients is needed. Replicating result of such studies is also more challenging, because of the (internationally and between centers) differences in approach towards defining course of disease and response to treatment. Compared to genetic association studies only concerning susceptibility to JIA, investigating genetic associations with the course of disease or response to treatment will yield results that are of interest to the clinician and could be beneficial with regards to informing the patient/ parents about the course of disease and treating the individual patient in the best way possible.

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CHAPTER 9

FUTURE PLANS

FUTURE PLANS

Increasing the size of the JIA cohort

Although it is not the most scientific challenging goal, it is of utmost importance for the power of the following studies (for example candidate gene studies or genotyping by Immunochip) to increase the size of the JIA cohort by collaborating with more European rheumatology referral centers. It has been discussed that only the three subtypes that have a phenotypic overlap, should be included with a similar genetic/ geographic background. Not only information about the subtype, but also detailed information about the course of disease and use of medication should be gathered. Collaborating with other JIA cohorts will increase the number of patients, however independent cohorts should still remain in order to allow for replication of the associations found.

Functional studies

The associations with JIA that have been revealed in this thesis are located either in loci with an unknown function (ANKRD55), or in loci with more than one gene which could be involved (in locus TRAF1/C5 the genes *TRAF1* or *C5* and in locus 4q27 the genes *IL2* or *IL21*) and in loci/genes that are more likely to be directly involved in the pathogenesis or progression of JIA (VTCN1, CD226, CD28, PTPTN22, TNFA). Of all of these associations, the functional (non-synonymous) variant in PTPN22 seems the most obvious one of which the function should have shortly become clarified. However, after some years the functional consequences of PTPN22 (1858T) are still under investigation.[1;2] This illustrates the difficulty in translating a genetic association to a functional difference that is related to disease or disease severity.

Because of also its association with the course of disease and therefor its clinical relevance, the functional consequences of the different genetic variations in VTCN1 should also be studied. In different studies, several polymorphisms in this gene are associated with the susceptibility of JIA. These seem to be indirect associations, with the causal variant not yet known. Because as yet no change in coding for amino-acids is known, a 3D image of an altered protein, to provide more insight in the consequences of such a different amino acid, cannot be prepared. However, the polymorphic gene can be expressed differently, thus the gene expression of the different genetic variations should also be studied, especially because a higher expression has been related to a more progressive disease in certain types of cancer. Because B7-H4 (the gene product of *VTNC1*) has an inhibitory effect on T-cells, the effect of the different VTCN1-gene products on T-cell stimulation should be studied by means of T-cell proliferation assays and levels of cytokine production. This should be done in healthy persons to rule out the influence of disease activity. Bypassing

the more subtle changes in function and ignoring the association of certain genetic variations, one might consider applying agonists or antagonists and evaluating the outcome of this extreme intervention, for example in collagen induced arthritis. More insight in the altered function of VTCN1 in patients with a more remitting course could be a lead point for developing drugs interfering in the VTCN1-pathway. Not only in the case of VTCN1, but also of the other genes/ loci associated with JIA, functional consequences should be studied by means of expression studies, measuring levels of gene product and levels of proteins involved in the pathway of concern and in more functional studies such as proliferation assays.

Increasing the investigated pathways

In the work presented in this thesis the focus has been on replication of the genetic associations known in JIA and other autoimmune disease and investigating some genes that are part of a number of pathways that could be involved in JIA. Because ours is a new cohort of Caucasian JIA patients, reproduction in such an independent cohort is important for reinforcing the already known associations. At the same time replication of these associations shows that our cohort is comparable to others in the generation of similar results. The following step will then be to expand the number of pathways to be investigated. For example, the IL17/ IL23/ IL12/ IFN-gamma pathway also plays a major role in JIA. Associations of genes involved in this pathway and the susceptibility to and the severity of JIA should be studied. This could be done by means of a candidate gene study because of the limited number of genes involved.
However more pathways involved in autoimmunity can be studied simultaneous by an Immunochip assay and this should be considered. The SNPs incorporated on the immunochip are dense selected tagging SNPs in specific genetic loci.

Genome wide association study

In addition to studying selected genes or pathway, a study without prior hypothesis can be undertaken. In a genome wide association study (GWAS), polymorphisms throughout the entire genome may thus be identified. The advantage is that unexpected loci can be revealed. The disadvantage is the large number of patients that are needed (partly because of the low effect sizes that are expected). In JIA such a study has been undertake twice. Surprising associations were revealed. In the first GWAS the associations with VTCN1 were brought to light and the second revealed 3q13 as a novel susceptibility locus that needs to be investigated in more detail to identify an associated gene.

Pharmacogenetics

To develop more insight in determinants of the response to treatment, genetic associations in genes involved in the specific pathways should be investigated. The pharmacogenetics of the MTX pathway has already been discussed. Nowadays treatment with anti-TNF has a big part in treating ongoing disease. Genes involved in the TNF-pathway should also be investigated in relation to response (comparing allele frequencies between responders and non-responders). Ideally the response to drugs that are being developed based on genetic findings (such as might be the case in PTPN22 or VTCN1), should be studied in relation to the varying genetic background of a patient.

Predictive model for the course of disease and response to treatment

Nevertheless, the most important still is the individual patient, which should be treated in the best possible way. In this thesis many parameters have been raised and discussed that could be part of a predictive model for the course of disease in the individual patient. These parameters are either clinical parameters (such as the subtype of JIA), or genetic factors (such as VTCN1, or additional genes that will be tested in future studies), but may also be biomarkers resulting from future functional studies. Similar models should be developed concerning the response to treatment (MTX or anti-TNF), similarly involving clinical and genetic parameters and possible biomarkers.

Association with specific characteristics of JIA

Many patients with predominantly oligoarticular JIA develop JIA-associated uveitis. Because this type of uveitis has few clinical symptoms, a stringent follow-up of eye-involvement is required. Genetic associations with JIA-associated uveitis could be helpful in predicting uveitis. Genetic variations that are associated with the progression of uveitis are of clinical relevance for patients with uveitis. However creating a study patient group with JIA-associated uveitis of sufficient size is already challenging, therefore to include sufficient patients to compare mild uveitis patients with progressive uveitis patients seems hardly feasible.

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CHAPTER 10

SUMMARY

SUMMARY

Juvenile idiopathic arthritis (JIA) is a non-common disease in children that can persist into adulthood. JIA consist of a heterogeneous group of disorders characterized by chronic arthritis. Following the ILAR criteria several subtypes can be distinguished based on the number of joints that are affected at presentation and during the course of disease, and on some blood values. The JIA subtypes oligoarthritis (persistent and extended) and rheumatoid factor (RF) negative polyarthritis are the most common within the group of JIA. These subtypes share phenotypic features and have a similar course of disease. In all subtypes the course of disease follows an unpredictable pattern of episodes with active disease alternating with episode of inactive disease. Prolonged episodes of active disease will lead to (irreversible) damage of the joints. At this point, no reliable prediction can be made about the course that the disease will follow in the individual patient.

The goal of treatment is to bring the ongoing arthritis to a halt shortly after disease onset to avoid damage to the joints. In recent years the treatment of JIA has shifted towards a more aggressive treatment with the use of potent anti-rheumatic medication in earlier stages of the disease. The level of response to medication differs between patients. No reliable prediction regarding the response to medication can be made, although this would be essential for choosing the optimal treatment for the individual patient.

JIA is a complex disease

JIA is considered to be an auto-immune disease, although the precise pathophysiology is not clear. The immune reaction against an unknown agent causes an ongoing inflammation of the joint, leading towards destruction of cartilage and bone. Several factors are believed to be involved in the pathogenesis of JIA. Besides environmental factors, also genetic factors seem to be important. Twin and family studies show that siblings of patients with JIA have a higher risk of developing (the same type of) JIA. In recent years several genetic studies have been performed trying to reveal an association between genetic variants (for example single nucleotide polymorphisms (SNPs)) and JIA by comparing the frequency of the variant in patient to the frequency in healthy persons. If the frequency of the genetic variant varies significantly between patients and controls, the genetic variant is considered associated with the disease. Replication of an association in an independent JIA cohort makes an association stronger and is essential. Multiple associations have been replicated in independent JIA cohorts, like HLA, PTPN22, STAT4, TNFAIP3, IL2RA. It is estimated that 30% of the JIA risk can be explained by (common) genetic variations.

Aim of this thesis

The goal of this research project is to discover genetic associations with JIA. Genetic factors that are associated with the development of JIA can give more insight in the pathogenesis and might indicate lead points for new treatment strategies. Of more clinical relevance are the genetic factors that are associated with the course of disease or with the response to certain medication. These genetic associations might serve as a predictive tool, leading to a better understanding of the individual disease and more optimal individualized treatment.

In order to perform genetic association studies, a new and independent JIA cohort has been created including 639 patients from North-West Europe (the Netherlands, Belgium, Germany and Switzerland) of Caucasian origin. The focus has been on patients with oligoarthritis (persistent and extended) and RF-negative polyarthritis, because of their similar clinical phenotype. Several genetic polymorphisms (SNPs) have been studied in this JIA cohort. The frequencies of SNPs have been compared between JIA patients and healthy Caucasian (Dutch) individuals in order to discover association with the susceptibility to JIA.

Additionally, detailed clinical information regarding the progression of disease and the use of medication has been collected for studying the association of genetic polymorphisms with the course of disease or the response to medication. The percentage of active disease in the first two years has been selected as outcome measure regarding course of disease. Patient with a mild (remitting) disease (< 35% of the time with active disease in the first two years) were compared to patients with an intermediate (35-65% active disease) and with a severe (non-remitting) disease (>65% active disease) to study association with the course of disease. Genetic association with response to methotrexate (MTX) was studied by comparing the frequency of selected genetic polymorphisms between MTX responders and MTX non-responders. The response to MTX was based on the improvement of the (subjective) physician score combined with the decrease in number of affected joints after 6 months of treatment.

For these genetic studies multiple genes and loci have been selected. Genes/loci have been selected based on a known association with JIA in order to replicate this association in a new and independent cohort. Also genes/loci have been selected that have been associated with other autoimmune diseases, especially with rheumatoid arthritis that shares many features with JIA, in order to strengthen the hypothesis of a shared common auto-immune susceptibility. Furthermore, genes have been selected that are involved in immune-regulation following an educated guess.

Genetic associations with the susceptibility to JIA

In this cohort we have discovered new associations in JIA in the genes/loci TRAF1/C5 (chapter 2), 4q27 (chapter 3), CD226 and CD28 (chapter 4). These genes have already been associated with other auto-immune diseases and might be part of a shared common auto-immune susceptibility. Also known JIA associations were replicated in our JIA cohort; VTCN1, 4q27, TNFA, PTPN22 and ANKRD55. By performing a meta-analysis involving our results and already published results, additional positive (overall) associations were revealed with PTPRC, AFF3, CCR5, TNFAIP3, TNPO3, IL2RA and CLEC16A.

TRAF1/C5 (chapter 2)

The genetic locus TRAF1/C5 includes both genes encoding TRAF-1 (TNF-receptor-associated factor-1) and complement 5 (C5). Genetic information of this locus is transmitted in total to the next generation without recombination with its sister chromosome. This phenomenon is called linkage disequilibrium (LD). The positive association of JIA with this locus could reflect an association with TRAF1 as well as with C5 (or non-coding DNA). Both genes are likely to play a role in the pathogenesis of JIA. TRAF-1 is part of the TNF-pathway that seems to be of high importance in JIA, considering the effect of anti-TNF treatment in JIA. Complement 5 has a role in attracting neutrophils and dysregulation could be involved in the development of JIA. Following our result, the association of TRAF-1/C5 and JIA has been studied in other cohorts with conflicting results. Additional (more detailed) research of this locus is needed.

4q27 (chapter 3)

The 4q27 locus has already been associated with multiple auto-immune diseases, but for the first time an association with JIA has been discovered in our cohort. The 4q27 locus contains several genes of which two genes are highly likely to be involved in the pathogenesis of JIA. These genes encode interleukin 2 (IL2) and interleukin 21 (IL21) and are inherited together (LD). Interleukin 2 is involved in the proliferation and differentiation of T-cells and in particular the regulatory T-cells. These regulatory T-cells have a role in regulating or inhibiting the inflammatory response and dysfunction can lead to an excessive (auto)immune-response. Genetic variation in the gene coding the IL2-receptor (*IL2RA*) is associated with multiple autoimmune diseases, including JIA, underlining the importance of the IL2 pathway in autoimmunity. IL21 is involved in the differentiation and expansion of T-helper-17 cells. These T-helper-17 cells seem to play a central role in JIA and have a pro-inflammatory effect. In several autoimmune diseases the association with IL21-receptor has been described. In recent years the association of 4q27 and JIA has been replicated in several cohorts.

*CD226 (DNAM-1)(*chapter 4*)*

For the first time an association of CD226 and JIA is described in chapter 6. However CD226 has already been associated with other auto-immune diseases. CD226 (known as DNAX accessory molecule 1 or DNAM-1) is involved in the co-stimulation of T-cells and NK cells. Therefore, CD226 could have a role in the regulation of the balance between the pro- and anti-inflammatory immune response. In other JIA cohorts this association has been studied, but no association was found. However, performing a meta-analysis with our results and all published data, an overall positive association is revealed.

Genetic association with the course of JIA (chapter 5 and 6)

For the first time genetic association with the course of disease, defined by the percentage of active disease in the first two years, has been studied in this cohort. The same polymorphisms that were selected for studying the association with the susceptibility to JIA were used for discovering association with the course of disease. Detailed clinical data necessary for determining the clinical course of disease were available for 272 patient with oligoarthritis (persistent and extended) and RF-negative polyarthritis (out of the 639 patient). First the predictive value of several clinical parameters was determined (chapter 5). This showed that the JIA subtypes have a different course of disease; patients with persistent oligoarthritis have a favorable course with a low percentage of active disease in the first two years, whereas patients with an extended oligoarthritis have the highest percentage of active disease. Because the percentage of active disease in the first 2 years corresponded with the percentage of active disease in the three following years, this parameter was regarded a suitable outcome measure reflecting course of disease.

When analyzing the association with course of disease, both clinical parameters (subtype) and genetic factors were included. Multivariate analysis showed that VTCN1 (rs10923223) is associated with the course of disease, regardless of JIA subtype (chapter 6).

VTCN1 has already been associated with susceptibility to JIA and other auto-immune diseases (with varying results), but seems to have a prominent role in the progression of the inflammatory response in JIA. *VTCN1* encodes B7-H4 that is involved in the co-stimulation of T-cells and inhibits the immune-response. Our data show that the minor allele is associated with a lower percentage of active disease in the first two years (remitting disease). This allele might enhance the function of VTCN1 leading towards a stronger inhibition of the immune response. Additional studies concerning the functional consequence of this VTCN1 variation will be essential. However until the precise role of VTCN1 will be clarified, the genetic association might be of

use in predicting the course of disease and helping the physician in choosing the optimal treatment strategy. Further prospective study should be conducted to verify the predictive value of VTCN1.

Genetic association and the response to methotrexate (chapter 7)

Both clinical and genetic factors have been studied in relation to the response to MTX in a cohort of 55 MTX non-responders and 73 MTX-responders. Several polymorphisms in genes involved in the folate and adenosine pathway have been selected. A multivariate analysis showed that the time to start MTX (the time from diagnosis to start MTX) is associated with response to MTX; treatment in more early stages of the disease has a better response. No association with genetic factors was found. Because of the small group size the lack of association with genetic factors might be due to low power to detect smaller effects.

These first genetic studies in this new (Caucasian) JIA cohort, including patients with similar phenotypic features, have led to discovery of new association (TRAF1/C5, 4q27, CD226 and CD28) and replication of known associations. Of clinical importance is the association of VTCN1 with the course of disease that might already be useful as factor predicting the course of disease and guiding the physician in the choice of treatment. Future plans following the results of this thesis will be to perform functional studies to elucidate the role of the VTCN1 polymorphism and its inhibitory effect on the immune-response. Possibly, this might have therapeutic consequences. Furthermore the size of the cohort should be enlarged to enhance the power of our research. Especially increasing the cohort of patients with a known detailed course of disease would enable discovery of clinical useful associations.

CHAPTER 11

NEDERLANDSE SAMENVATTING

NEDERLANDSE SAMENVATTING

Juveniele idiopathische artritis (JIA), ofwel jeugdreuma, is een niet veel voorkomende ziekte bij kinderen, die kan blijven bestaan tot in volwassenheid. Het is een verzamelnaam voor aandoeningen die gekenmerkt worden door een chronische gewrichtsontsteking. Deze groep is volgens de ILAR classificatie onderverdeeld in meerdere subtypen op basis van het aantal gewrichten dat is aangedaan bij het begin van de ziekte, maar ook gedurende het ziektebeloop, en enkele laboratoriumwaarden. De subtypen oligoartritis (persisterend of extended) en reumafactor negatieve polyartritis zijn het meest voorkomend en zijn binnen de totale groep van jeugdreuma vergelijkbaar in hoe de ziekte zich gedraagt.
Het beloop van de ziekte in alle subtypen bestaat uit onvoorspelbare episoden van ziekteactiviteit in verschillende gradaties, afgewisseld met inactieve ziekte. Hoe langer de ziekte actief is, hoe meer kans op beschadiging van de aangedane gewrichten. Op dit moment is niet te voorspellen wat de prognose van jeugdreuma is in de individuele patiënt.
Therapie is erop gericht de ziekteactiviteit in een zo vroeg mogelijk stadium rustig te krijgen om schade aan de gewrichten te voorkomen. In de laatste jaren is behandeling van jeugdreuma steeds agressiever geworden, met gebruik van meer potente antireumatische medicatie toegepast in een vroeger stadium van de ziekte. De respons op de verschillende soorten medicatie kan per patiënt verschillend zijn. Er zijn op dit moment ook nog geen goede voorspellers voor de respons op therapie, terwijl dat wel aanvullend zou zijn voor de keuze voor meest optimale individuele therapie.

JIA als multifactoriële aandoening

JIA is een auto-immuunziekte, hoewel het precieze ontstaansmechanisme van de ziekte nog onduidelijk is. Ook waartegen de immuunreactie zich precies richt is niet bekend, maar wel is duidelijk dat de aanhoudende ontstekingsreactie in het gewricht zorgt voor schade aan het kraakbeen en het bot. Er wordt gedacht dat verschillende factoren een rol spelen in het ontstaan van jeugdreuma. Naast mogelijke omgevingsfactoren, lijkt een genetische aanleg belangrijk te zijn. In tweeling- en familie-studies wordt gezien dat jeugdreuma meer voorkomt bij de andere helft van een aangedane tweeling en ook bij broers en zussen van patiënten met jeugdreuma.
Er zijn de afgelopen jaren veel studies verricht naar de associatie van genetische factoren en het ontstaan van jeugdreuma. Dit zijn vooral studies waarin de frequentie van bepaalde genetische variaties (bv single nucleotide polymorphisms; SNPs) vergeleken wordt tussen patiënten met jeugdreuma en gezonde controle personen.

Als in patiënten met JIA de genetische variatie significant meer of minder voorkomt dan in controles, is de genetische variatie geassocieerd met JIA. Naast HLA, zijn meerdere genen (bv PTPN22, STAT4, TNFAIP3, IL2RA) bij herhaling en in onafhankelijke JIA patiëntengroepen geassocieerd met jeugdreuma. Het totale aandeel dat genetische factoren hebben in de aanleg voor jeugdreuma is geschat op 30%.

Doel van dit proefschrift

Het doel van het onderzoek beschreven in dit proefschrift is om genetische associaties in JIA te onderzoeken. Genetische factoren die van invloed zijn op het ontstaan van JIA in een patiënt geven informatie over de pathogenese, maar kunnen ook belangrijke aanknopingspunten zijn voor nieuwe therapieën. Genetische factoren die geassocieerd zijn met het beloop van JIA of met de respons op bepaalde medicatie kunnen mogelijk voorspellende waarde hebben. Het is voor de behandelend (kinder)reumatoloog van klinisch belang om te kunnen voorspellen hoe de ziekte zich zal gedragen en wat de respons op therapie zal zijn. Informatie aan de patiënt en ouders kan hierdoor geoptimaliseerd worden en de keuze voor therapie (en tijdstip van starten van therapie) kan geïndividualiseerd worden, met mogelijk een snellere remissie tot gevolg.

Om deze studies uit te voeren is een nieuw en onafhankelijk JIA cohort gecreëerd, waarin 639 patiënten uit Noord-West Europa (Nederland, België, Duitsland en Zwitserland) met een caucasische etniciteit zijn geïncludeerd. Voornamelijk patiënten met oligoartritis (persisterend en extended) en reumafactor negatieve polyartritis zijn geselecteerd om de patiëntengroep een zo veel mogelijk overeenkomstig fenotype te geven. De frequentie van verschillende genetische polymorfismen (SNPs) is vergeleken tussen deze JIA patiënten en gezonde (caucasische) controles (afkomstig uit Nederland) om te kijken naar genetische associatie met het ontstaan van de ziekte. Van deze JIA patiënten is gedetailleerde informatie over het beloop van hun ziekte verzameld. Als maat voor de ernst van het beloop van de ziekte is er gekeken naar hoeveel procent van de tijd de patiënten een actieve ziekte hebben gehad in de eerste twee jaar na de diagnose. Patiënten met een mild beloop van de JIA (< 35% van de tijd actieve ziekte) zijn vergeleken met patiënten met een intermediair beloop van de ziekte (35-65% van de tijd actieve ziekte) en een meer ernstig beloop (>65% van de tijd actieve ziekte) om te kijken naar genetische associatie met het beloop van de ziekte. Binnen deze groep van JIA patiënten is er ook gekeken naar patiënten die methotrexaat (MTX) hebben gebruikt. Om te kijken naar betrokkenheid van genetische factoren op de respons op MTX werd de frequentie van geselecteerde SNPs in patiënten met een goede respons op MTX (gedefinieerd als een verbetering in subjectieve score door de behandelend kinderreumatoloog samen met een gelijk blijven of verbeteren van het aantal gewrich-

ten wat aangedaan is in de eerste 6 maanden na het starten van MTX) vergeleken met patiënten die geen respons vertoonden op MTX.
Voor de genetische studies naar associaties met het ontstaan van de ziekte en het beloop van de ziekte zijn meerdere genen/loci geselecteerd. Dit zijn aan de ene kant genen waarvan al een associatie bekend is met het ontstaan van JIA met als doel de associatie te repliceren in ons onafhankelijke cohort en zo de associatie mogelijk te versterken. Aan de andere kant zijn genen geïncludeerd die al geassocieerd zijn met (meerdere) auto-immuunziekten, waaronder reumatoïde artritis, wat veel overeenkomstige kenmerken heeft met JIA, en die deel uitmaken van een gemeenschappelijke genetische vatbaarheid voor autoimmuniteit. Daarnaast zijn ook genen geselecteerd die betrokken zijn bij de immuunregulatie.

Genetische associaties met het ontstaan van JIA

In ons cohort hebben we een associatie gevonden met genen die nog niet eerder geassocieerd waren met JIA. Dit zijn de genen/loci TRAF1/C5 (hoofdstuk 2), 4q27 (hoofdstuk 3), CD226 en CD28 hoewel de laatste met een lage significantie (beide beschreven in hoofdstuk 4). Dit zijn allemaal genen die al wel geassocieerd waren met andere autoimmuunziekten. Daarnaast zijn verschillende genen die al geassocieerd waren met JIA in ons cohort gerepliceerd; VTCN1, 4q27, TNFA, PTPN22 en ANKRD55. Meta-analyse van onze uitkomsten samen met studies die eerder gedaan waren in JIA (maar vergelijkbaar met onze studie geen associatie lieten zien) toonde alsnog een (gezamenlijke) associatie van JIA met PTPRC, AFF3, CCR5, TNFAIP3, TNPO3, IL2RA en CLEC16A.

*TRAF1/C5 (*hoofdstuk 2*)*

TRAF1/C5 is een genetisch locus wat zowel de genen TRAF-1 (TNF-receptor-associated factor-1) als C5 (complement 5) omvat. Genetische associaties in dit locus worden gezamenlijk overgeërfd naar een volgende generatie, zonder dat er uitwisseling plaatsvindt met genetisch materiaal op het zuster chromosoom. Dit wordt linkage disequilibrium (LD) genoemd. Een associatie van JIA met dit TRAF1/C5 locus kan dus zowel een associatie zijn met het TRAF-1 gen als met complement 5. Beide genen zouden een potentiele rol kunnen hebben in het ontstaan van JIA. TRAF-1 is onderdeel van de TNF-pathway, dat mede door het effect van anti-TNF behandeling in JIA, een belangrijke rol lijkt te hebben. Daarnaast is complement 5 betrokken bij het aantrekken van neutrofielen en dysregulatie hiervan zou een voortgaande ontsteking kunnen veroorzaken. In latere studies in andere cohorten is deze associatie met TRAF1/C5, die wij als eerste hebben beschreven, bevestigd, maar niet consequent. Meer onderzoek is nodig naar de precieze rol die de TRAF1/C5 locus heeft in JIA.

4q27 (hoofdstuk 3)

In ons JIA cohort is als eerste de associatie tussen de 4q27 locus en JIA beschreven. De 4q27 locus bevat meerdere genen die mogelijk een belangrijke rol hebben in de ontwikkeling van JIA. Dit zijn de genen die coderen voor interleukine 2 (IL2) en interleukine 21 (IL21). Deze zijn in LD met elkaar en worden gezamenlijk overgeërfd. IL2 heeft een rol in de proliferatie en differentiatie van T-cellen, waaronder regulatoire T-cellen. Deze regulatoire T-cellen hebben een rol in het reguleren of dempen van een ontstekingsreactie en ontregeling van hun functie kan een excessieve ontstekingsreactie tot gevolg hebben. Genetische variaties in de IL2-receptor (IL2RA) zijn geassocieerd met meerdere autoimmuunziekten, waaronder JIA. Dit benadrukt de rol van de IL2-pathway in JIA. IL21 speelt een rol in de differentiatie en expansie van T-helper-17 cellen. Deze T-helper-17 cellen spelen een belangrijke rol in het ontstaan van JIA en hebben een pro-inflammatoir effect. In meerdere autoimmuunziekten zijn associaties met SNPs in het IL21-receptor gen beschreven. Zowel IL2 als IL21 zijn betrokken bij de pathogenese van JIA en zijn beide mogelijke kandidaten die de genetische associatie met dit locus weerspiegelen. In verschillende andere cohorten is de associatie met de 4q27 locus gerepliceerd.

CD226 (DNAM-1) (hoofdstuk 4)

De associatie van CD226 met verschillende autoimmuunziekten was al bekend, maar de associatie met JIA is als eerste gevonden in ons JIA cohort. CD226 (ook bekend als DNAX accessory molecule 1 of DNAM-1) is betrokken bij de co-stimulatie van T-cellen en NK-cellen en zou een rol kunnen hebben in de regulatie van de balans tussen de pro-inflammatoire en anti-inflammatoire immuunrespons. In andere JIA patiëntengroepen is de associatie met CD226 ook onderzocht, waarbij er geen associatie gevonden werd. Echter als alle resultaten samen worden geanalyseerd in een meta-analyse, komt er een significante associatie met JIA naar voren.

Genetische associatie met het beloop van JIA (hoofdstuk 5 en 6)

In ons cohort is voor het eerst gekeken naar de associatie van genetische factoren met het beloop van JIA, gedefinieerd aan de hand van het percentage actieve ziekte in de eerste twee jaar. Dezelfde SNPs werden geselecteerd als in de studie naar de associatie met het ontstaan van JIA. Van de groep van 639 patiënten, was er van 272 patiënten gedetailleerde informatie aanwezig om het beloop van JIA te kunnen bepalen. Allereerst zijn verschillende klinische parameters onderzocht in relatie tot het voorspellen van het beloop van de ziekte (hoofdstuk 5). Hieruit kwam naar voren dat de verschillende subtypen JIA een ander beloop hebben; de persisterende oligoartritis heeft het laagste percentage actieve ziekte in de eerste twee jaar, terwijl extended oligoartritis het

hoogste percentage actieve ziekte heeft. Tevens werd beschreven dat het percentage ziekteactiviteit in de eerste twee jaar representatief is voor de ziekteactiviteit in de jaren die daar op volgen en dus een geschikte parameter lijkt om de ernst van de ziekte mee weer te geven. Bij de analyse van genetische factoren en het beloop van JIA is er gecorrigeerd voor het subtype. De multivariate analyse toont dat VTCN1 (rs10923223) geassocieerd is met het beloop van de ziekte (hoofdstuk 6), ongeacht het subtype. Dit gen was eerder al geassocieerd met het ontstaan van JIA (met wisselende resultaten) en andere autoimmuunziekten, maar lijkt vooral een aandeel te hebben in de verdere progressie van de ontstekingsreactie in JIA. VTCN1 codeert voor B7-H4, wat betrokken is bij de costimulatie van T-cellen en een inhiberend effect heeft op de immuunrespons. Het allel wat het minste voorkomt (minor allel) is geassocieerd met een lager percentage ziekte in de eerste twee jaar. Mogelijk zorgt dit allel voor een toegenomen functie en dus een sterkere inhibitie van de immuunrespons. Meer onderzoek is nodig naar de functionele consequenties van de genetische variatie in VTCN1. Echter zonder dat er functionele consequenties bekend zijn, kan dit polymorfisme mogelijk wel van waarde zijn in het voorspellen van het beloop van de ziekte en daarmee de (kinder) reumatoloog ondersteunen in het bepalen van de beste individuele therapie en informeren van de ouders en patiënt. Prospectieve studies zijn nodig om de mogelijk voorspellende waarden van VTCN1 te kunnen toetsen.

Genetische associatie en de respons op MTX (hoofdstuk 7)

Zowel klinische parameters als genetische parameters zijn onderzocht in relatie tot de respons op MTX in een groep van 55 non-responders en 73 responders. Verschillende genen die betrokken zijn bij de folaat en adenosine pathways zijn geselecteerd. In een multivariate analyse kwam de tijd tot het starten van behandeling naar voren als de belangrijkste parameter geassocieerd met respons op MTX; hoe sneller je de behandeling start, hoe beter de respons. Er was geen associatie met genetische parameters, wat mogelijk zou kunnen komen door de kleine groep patiënten, waarbij er weinig power is om associaties met een klein effect aan te kunnen tonen.

Deze eerste genetische studies in dit nieuwe cohort met (caucasische) JIA patiënten (met een zo veel mogelijk overeenkomstig fenotype) hebben allereest een aantal nieuwe associaties in JIA aangetoond (TRAF1/C5, 4q27, CD226 en CD28) en daarnaast replicatie van reeds beschreven associaties. Van klinische waarde is de associatie van VTCN1 met het beloop van de ziekte, omdat dit mogelijk als voorspeller kan dienen voor de ziekteactiviteit in de individuele patiënt, waarmee rekening gehouden kan worden met de keuze van therapie. Tevens zou dit een belangrijk aanknopingspunt kunnen zijn voor de ontwikkeling van nieuwe therapie.

Een belangrijk doel voor de toekomst is om dit cohort verder uit te breiden, zodat er meer power is om genetische associaties aan te kunnen tonen. Vooral het uitbreiden van de groepen waarbij het beloop van de ziekte bekend is, vormt daarbij een uitdaging. Daarnaast is het verder uitwerken van de functie van VTCN1 in het kader van regulatie van de ziekteactiviteit van klinisch belang omdat dit mogelijk therapeutische consequenties heeft.

LIST OF ABBREVIATIONS

AID	autoimmune disease
ANA	anti-nuclear antibodies
DM1	diabetes mellitus type 1
DMARD	disease modifying anti-rheumatic drugs
GWAS	genome wide association study
HLA	human leukocyte antigen
IAS	intra-articular steroids
IL	interleukin
ILAR	International League of Associations for Rheumatology
JIA	International League of Associations for Rheumatology
LD	linkage disequilibrium
MAF	minor allele frequency
MS	multiple sclerosis
MTX	methotrexate
NSAID	non-steroidal anti-inflammatory drugs
OR	Odds ratio
RA	rheumatoid arthritis
RF	rheumatoid factor
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
TDT	transmission disequilibrium test

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CURRICULUM VITAE

Heleen Marion Albers was born on the 1st of November 1975 in Leidschendam, the Netherlands. She attended secondary school at College 't Loo in Voorburg and passed her gymnasium exam in 1994. From 1994-1995 she studied Medical Biology at the University of Amsterdam. She studied Medicine at the Leiden University from 1995-2000 and obtained her medical degree in 2002. Thereafter she worked as a resident (ANIOS) at the Reinier de Graaf Gasthuis in Delft. In September 2003 she started a PhD project at the Department of Pediatrics (Prof Dr. R.M. Egeler) and the Department of Rheumatology (Prof. Dr. .W. Huizinga) at the Leiden University Medical Center on identifying genetic associations in juvenile idiopathic arthritis. The research project was combined with a clinical training in pediatric rheumatology supervised by Dr. R. ten Cate. In July 2005 she started her clinical training in pediatrics at the Leiden University Medical Center and the Reinier de Graaf Gasthuis. From 2005-2012 she followed an alternating course of clinical training and PhD research, leading to this thesis. In November 2012 she was registered as a pediatrician. As from April 2013 she is working as a general pediatrician at the Van Weel- Bethesda Ziekenhuis in Dirksland. She is married and has two sons.