Temporomandibular joint (TMJ) arthritis in Juvenile Idiopathic Arthritis (JIA):
Clinical management and analyses of TMJ synovial fluid.
© Heming Olsen-Bergem, 2015

Series of dissertations submitted to the Faculty of Dentistry, University of Oslo

ISBN 978-82-8327-012-9

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Cover: Hanne Baadsgaard Utigard.
Print production: John Grieg AS, Bergen.

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To Maria and Anders Kristian
Joy of my life!
## Contents

ACKNOWLEDGEMENTS ............................................................................................................. 5
ABBREVIATIONS .................................................................................................................. 7
LIST OF PAPERS .................................................................................................................. 9
INTRODUCTION .................................................................................................................... 10
THE TEMPOROMANDIBULAR JOINT (TMJ) ........................................................................ 11
  TEMPOROMANDIBULAR JOINT SYSTEM DISORDERS .................................................... 12
JUVENILE IDIOPATHIC ARTHRITIS .................................................................................... 14
  DEFINITION AND CLASSIFICATION CRITERIA ............................................................ 14
  EPIDEMIOLOGY ................................................................................................................. 15
  ETIOLOGY .......................................................................................................................... 16
  PATHOGENESIS ................................................................................................................ 17
    The innate and the adaptive immune system ................................................................. 17
    Cellular immune reaction ............................................................................................. 18
    Autoantibodies and clinical laboratory features .......................................................... 18
    Arachidonic acid .......................................................................................................... 19
    Reactive oxygen species (ROS) .................................................................................... 19
    Neuropeptides .............................................................................................................. 19
    Genetics and environmental factors ............................................................................ 19
    Cytokines ...................................................................................................................... 20
    Bone biomarkers in inflammatory disease .................................................................. 21
    Microbial flora and arthritis ......................................................................................... 24
  TREATMENT ................................................................................................................... 25
  JIA AND THE TEMPOROMANDIBULAR JOINT (TMJ) .................................................... 26
    JIA - TMJ growth and growth disturbances ................................................................ 27
    JIA – TMJ treatment ...................................................................................................... 28
AIMS OF THE STUDY ....................................................................................................... 29
MATERIAL, METHODS AND METHODOLOGICAL CONSIDERATIONS .................. 30
  MATERIAL ...................................................................................................................... 30
  METHODS – BACTERIA DETECTION AND PROTEIN ANALYSIS ................................ 32
  METHODOLOGICAL CONSIDERATIONS .................................................................... 33
SUMMARY OF RESULTS ................................................................................................. 34
  PAPER I .......................................................................................................................... 34
  PAPER II ........................................................................................................................ 34
  PAPER III ....................................................................................................................... 35
DISCUSSION .................................................................................................................... 36
  BACTERIAL INFLUENCE ............................................................................................... 36
  REGARDING CYTOKINES AND BONE MARKERS ....................................................... 37
  CLINICAL IMPLICATIONS ............................................................................................ 39
CONCLUSIONS ............................................................................................................... 42
Acknowledgements

Over the many years working with this thesis, there are many people, whom deserve my gratitude. I have been so fortunate to be working with people with knowledge, experience, enthusiasm and know-how, which they have been willingly sharing. I am grateful to all colleagues and friends at The Dental Faculty and at Oslo University Hospital.

I am grateful for the funding and working facilities for this project provided by The Faculty of Dentistry at the University of Oslo, and for the outstanding cooperation and support from the Department of Rheumatology at The National Hospital, Oslo University Hospital (OUS). I would very much like to thank Senior Consultant Dr. Med. Odd Vinje, Professor Berit Flåtø and the staff at Section for Children Rheumatology at OUS for giving me the opportunity to examine children and adolescents with JIA, and for support and interest with this project.

I would like to thank all my supervisors and co-authors for their valuable contributions. Without their constructive help and criticism and endurance this project would not have been possible.

I would first and foremost like to express my sincere gratitude to my main supervisor and mentor Professor Tore Bjørnland for his outstanding enthusiasm and support, for sharing his extensive knowledge, for his educational support and know-how. I am grateful to him for introducing me to this project, for his belief in me, for being a source of inspiration, and most of all for his friendship.

I am most grateful to my supervisor and co-author Professor Janne Elin Reseland for sharing her impressive knowledge within the complex and vast field of immunology and bone regeneration, and for challenging me to perform my very best at all time.

I would like to express my sincere thanks to my supervisor and co-author Associate Professor Jørn A. Aas for sharing his intellect and enthusiasm for life in general, and for microbiology in specific. I am grateful for his inspiration and for his friendship.

I would like to thank my co-author Senior Engineer Anne Karin Kristoffersen for her outstanding knowledge and skilled inspiration within the field of microbiology at Department of Oral Biology.
I would like to thank all my colleagues at Department of Oral Surgery and Oral Medicine, Professor Pål Barkvoll, Senior Professor Hans Reidar Haanæs (whom started the research and treatment of JIA at the department), Professor Janicke C. Liaaen Jensen, Associate Professor Bente Brokstad Herlofson, Assistant Professor Hanne Kleven Ingstad and Assistant Professor Marianne Tingberg. It is a privilege to be working with you all! I would also like to thank the staff and the trainees for their interest and support, as well as their professional attitude combined with a great sense of humor, making every day at Department of Oral Surgery and Oral Medicine great!

I am most grateful to Sigvard Kopp (Karolinska Institute) and Per Alstergren (Malmö University, Faculty of Odontology) for inspiration and enthusiastically sharing their great knowledge at the very beginning of my PhD-candidacy.

Finally, my sincere thanks goes to my family, Anders Kristian and Maria, my children, for always believing in me and for supporting me when I had to work late in the evenings. My outmost respect and gratitude goes to Anne Kristine Bergem, my wife, mentor and best friend, for her inspiration, loving care and constant support. You are a continuous inspiration!
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAOP</td>
<td>American Academy of Orofacial Pain classification</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ANA</td>
<td>Antinuclear antibodies</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CARRA</td>
<td>Childhood Arthritis and Rheumatology Research Alliance</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase (COX1, COX2)</td>
</tr>
<tr>
<td>CPLs</td>
<td>Circulating pathogenic-like lymphocytes</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DC/TMD</td>
<td>Diagnostic Criteria for temporomandibular disorders</td>
</tr>
<tr>
<td>DMARDs</td>
<td>Disease-Modifying Anti-Rheumatic Drugs</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen (gene locus)</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal (-axis)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>ILAR</td>
<td>International League of Associations for Rheumatology</td>
</tr>
<tr>
<td>JIA</td>
<td>Juvenile Idiopathic Arthritis</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide (bacterial)</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated (NF-κB) protein kinase</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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</table>
MIO  Maximum incisal opening
MMPs  Matrix metalloproteinases
MRI  Magnetic resonance imaging
NK  Natural killer (cells)
NSAIDs  Non-Steroidal Anti-Inflammatory Drugs
OPG  Osteoprotegerin
OPN  Osteopontin
PDGF  Platelet-derived growth factor
PGE2  Prostaglandin E2
PIO  Pain incisal opening
PTH  Parathyroid hormone
RA  Rheumatoid Arthritis
RANK  Receptor activator for nuclear factor kappa B
RANKL  Receptor activator for nuclear factor kappa B ligand
RDC/TMD  Research diagnostic criteria for temporomandibular disorders
rDNA  ribosomal DNA
ReA  Reactive arthritis
RF  Rheumatoid factor
Tcyt  Cytotoxic T-cells
TH1  T-helper-1-cells
TH2  T-helper-2-cells
TH17  T-helper-cells producing IL-17
Treg  Regulatory T-cells
TMD  Temporomandibular disorder/ temporomandibular dysfunction syndrome
TMJ  Temporomandibular joint
TNF-α  Tumor necrosis factor-alpha
VAS  Visual analog scale
List of papers

The following articles (Papers I-III) are submitted in partial fulfillment of the requirements for the degree Philosophiae Doctor (Ph.D.) at the Faculty of Dentistry, University of Oslo, Oslo, Norway. This thesis is based on clinical work at the Institute of Clinical Dentistry, Faculty of Dentistry and at Oslo University Hospital, The National Hospital, and on experimental work at Clinical Oral Research Laboratory and Department of Oral Biology, Oslo, Norway. In the text the papers will be referred to by their Roman numerals.


Introduction

This thesis is the result of a curiosity, as it should be, towards the complexity and the challenge in treating temporomandibular joint (TMJ) disorders, especially in children with arthritis.

Originally this was meant to be a clinical study, although in an early epiphany, we understood this was an opportunity to look into the fundamentals of the disease, or maybe diseases, as we experienced improvement of TMJ symptoms in a child receiving antibiotics for osteomyelitis of the jaw. Could bacteria possibly be present within the TMJ, and, if so, influence upon the symptoms and the development of the joint disease?

In this thesis I am starting with the description of the TMJ and its many functions and possible dysfunctions, followed by up-to-date information of juvenile idiopathic arthritis (JIA). JIA is most likely a heterogenic group of arthritis in children and adolescents, and hence, there are many different opinions with regard to classification, etiology, epidemiology, and pathology. The same goes for TMJ disorders, implicating a diverse and somewhat scattered field of research.

There is still much unknown with regard to rheumatic disease, immune responses, bone affecting factors, control mechanisms, pain responses, immune responses to bacteria, and more. In this thesis, some of the resent findings and knowledge are described, and compared with our new findings.
The Temporomandibular Joint (TMJ)

The temporomandibular joint system is responsible for movement of the mandible, and is involved in speech, chewing, swallowing, kissing, mood expressions and more. This system consists of two joints (TMJs), the mandible, chewing muscles, supra- and infra-hyoid muscles, tendons and ligaments, surrounding soft tissues, the tongue and the teeth in both the mandible and the maxilla. The temporomandibular joint is a unique joint in many aspects. Anatomically the TMJ is a di-arthrodial joint (two joints connected by the mandible) with both rotation and translation movements dictated by associated muscles and limited by ligaments. This unique movement is not only controlled by the joint morphology, but also by the dentition/jaws at the other end of the lever system. The articular disc is composed of dense fibrous connective tissue, and is following the movement of the joint, although, because it is a separate structure, may move independently, and may lead to disc displacements with/without clicking, obstruction of TMJ movements and (traumatic) joint inflammation. Lining the inner aspect of all synovial joints, including the TMJ, are articular cartilage and synovium. TMJ cartilage is composed of a fibrocartilage (chondrocytes, collagen fibers, water and ground substance) adapted to take shearing forces as compared to the compressive forces that act on the hyaline cartilage in the knee joint. In contrast to the knee joint, the temporomandibular joint is lined by dense, avascular, fibrous connective tissue (not hyaline cartilage), a movement consisting of both rotation and translation of the joint, and the connection of bilateral joints by the mandible. The temporomandibular joint is the most frequently used joint of the human body, involved in speaking, yawning, eating, swallowing and other mouth moving activities (Nitzan 2003, Tanaka and van Eijden 2003, Öberg et al. 1971, Colombo et al. 2008). Many refer to the temporomandibular joint as “the forgotten joint” in pediatric rheumatology (Arabshahi and Cron 2006), although the general inflammatory disease frequently affects it, and may induce pain, decreased movement and function, and growth disturbances, affecting the face contour, and hence the appearance of the growing child. Pain and reduced movement of the jaws may lead to problems with eating. This may lead to changes in food intake, favoring soft carbohydrate-rich food, with influence upon diseases, such as tooth caries and diabetes, among others (Welbury et al. 2003). Children with arthritis (JIA) are at an increased risk of developing emotional problems, typically depression (Stevanovic and Susic 2013). In this, changes in facial appearance and problems with normal jaw function (social eating, kissing, pain, and more) may be a part of the problem.
Temporomandibular joint system disorders

TMD (Temporomandibular dysfunction syndrome, temporomandibular joint disorder) is a widely used term for a number of different disorders affecting the temporomandibular joint and/or its supportive tissues (muscles, tendons), and in many cases, also the teeth (teeth grinding and pressing) and the tongue (tongue hyperactivity). TMD, is, in the author’s opinion, with the support of Dr. D. Laskin and others, an outdated term, not suitable for correct diagnosis and treatment of each patient’s specific disorder. Instead, one should focus the diagnosis and the treatment on each pathological finding, bearing in mind these are most often multifactorial disorders involving most often both physiological and psychological contributing factors, and hence, in need of a multi-disciplinary examination, diagnosis and treatment.

Disorders in the muscles due to muscle hyperactivity and/or dysfunctional activity are often very painful (myalgia), and may mask an underlying joint disorder. Typical hyperactivities are tongue trusting, teeth grinding and/or teeth pressing, but also habits (such as biting on fingernails and similar activities) and unnatural movements (in order not to provoke clicking of the joints), and many more. Trauma, diseases, and genetic syndromes may also involve TMJ muscles, resulting in myalgia, myositis, and voluminous changes.

Disorders in the TMJ may be degenerative (osteoarthritis), inflammatory (arthritis), disc pathology (clicking, jaw locking, and disc induced inflammation/arthritis), capsular pathology (enthesitis, fibromyalgia), hamartomas, and benign and malign disorders. Arthritis may be induced by trauma (accidents, violence, and microtrauma induced by disc problems or...
osteoarthritis), inflammatory disorders (rheumatism, connective tissue disorders, bleeding disorders and more), and other diseases. TMJ disorders may (but certainly not always) be painful, and may influence upon normal jaw function (Greene and Laskin (ed), Treatment of TMDs, Quintessence 2013, Manfredini, Current concepts on temporomandibular disorders, Quintessence 2010).

In the case of TMD, the search for a causal factor is often frustrating and unproductive, and the current classification schemes are not based on the etiology of symptoms. At present, the classification systems most widely adopted in the literature are the American Academy of Orofacial Pain (AAOP) classification (De Leeuw 2008) and the Research Diagnostic Criteria for Temporomandibular disorders (RDC/TMD, Dworkin and LeResche 1992), and the recently introduced Diagnostic Criteria for temporomandibular disorders (DC/TMD, Schiffman et al. 2014).

Tongue impressions and mild attrition of teeth in a 22-year-old female. Patient complained of extensive, continuous pain. (Private photo, by permission of patient)
Juvenile Idiopathic Arthritis

Definition and classification criteria

Juvenile idiopathic arthritis (JIA) is an umbrella-term describing a heterogeneous group of conditions characterized by chronic arthritis beginning before the age of 16 years, persisting for at least 6 weeks, and having no other identifiable cause (ILAR/Petty 2001). Considered as a whole group, JIA is the most common rheumatologic condition of childhood and consists of subtypes including oligoarticular, polyarticular, and systemic onset. The highest frequency of onset occurs between one and three years of age (Cassidy and Petty 2005) and oligoarticular JIA is the most common subtype (Woo and Colbert 2009, Oen and Cheang 1996). Polyarticular JIA affects a variety of patients with a wide spectrum of etiologic risk factors, unique disease course, and therapeutic challenges. Children with polyarticular JIA tend to have a more refractory course in comparison to those with fewer affected joints. Due to a prolonged course of active disease, they are at increased risk for joint damage, resulting in poorer functional outcomes and decreased quality of life (Ringold et al. 2009).

The nomenclature to define childhood arthritis has changed several times over the decades. Due to the different classification systems for JIA (Table 1), there are difficulties in comparing results from different research groups. The latest definition, as put forth by ILAR in 1997 and later revised in 2001, divides JIA into seven subgroups. The intent of this current classification system was to create consistency among international providers in order to identify children with similar characteristics for the purposes of research towards epidemiology, pathogenesis, and treatment strategies. Debate, however, is still ongoing as to whether this current scheme is too inclusive and should be further redeveloped with emphasis more on antibody presence, age of symptom onset, and symmetry of arthritis (Martini 2012).

There have been publications fueling this discussion. Antinuclear antibodies (ANA) positive patients with oligoarticular JIA share similar features with ANA positive, RF-negative polyarticular JIA patients with early onset, asymmetry and frequent uveitis (Ravelli et al. 2005 and 2011), while ANA-negative patients with polyarticular disease tend to present older with a cumulative, symmetrical arthritis, and with a different pattern of joint involvement that more commonly involves the hips and shoulders. Some authors suggest RF-positive status should be a subset of its own regardless of the numbers of joints involved (Sailer et al. 1997).
Age appear to be an independent risk factor as younger RF-negative children tend to develop a more aggressive disease course compared to RF-negative children who present later in childhood or early adolescence (Greenwald et al. 2013).

<table>
<thead>
<tr>
<th>Table 1 Classification of chronic arthritis of childhood</th>
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<tr>
<td>Organizational criteria</td>
</tr>
<tr>
<td>Criteria name</td>
</tr>
<tr>
<td>Age of onset</td>
</tr>
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<td>Duration</td>
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</tbody>
</table>
| Subsets | 1. Pauciarticular: <5 joints  
2. Polyarticular: >4 joints, RF-  
3. Systemic arthritis with characteristic fever  
4. Juvenile rheumatoid arthritis: >4 joints, RF+  
5. Juvenileankylosing spondylitis  
2. Oligoarthritis (pauciarticular disease): <5 inflamed joints  
2. Oligoarthritis: a)Persistent, b)Extended  
3. Polyarthritis:RF-  
4. Polyarthritis:RF+  
5. Psoriatic arthritis  
6. Enthesitis-related arthritis  
7. Undifferentiated: a)fits no other category, b)fits more than one category |

RF-, rheumatoid factor negative; RF+, rheumatoid factor positive.

Epidemiology

JIA has a yearly incidence in Norway of 14-23/100 000 children (Riise et al. 2008). The incidence of juvenile chronic arthritis (EULAR criteria) in Norway, Finland and Sweden has been reported in 11-23/100 000 children (Berntson et al. 2003, Moe and Rygg 1998,
Kunnamo et al. 1986, Gare and Fasth 1992), which is higher than rates reported from other European countries (Ravelli and Martini 2007, Prieur et al. 1987, Kiessling et al. 1998). The disease is more frequent in girls than in boys, the ratio varying between 3:2 and 2:1. Studies have found remission in about 40-60% of patients at follow-up, with the disease either at a dormant stage or being “burned out” (Flatø et al. 2003, Berntson et al. 2003). Still, in a recent study of JIA disease progression, 34% of the patients with JIA had active disease after 30 years and 28% had a high symptom state of pain and/or movement limitations (Selvaag et al. 2014).

**Etiology**

The etiology of JIA is largely unknown. JIA is considered to be a multifactorial autoimmune disease. Many associations between subsets of JIA and HLA or non-HLA molecules have been described (Glass et al. 1999, Prahalad 2004, Rosen et al. 2003, Prahalad and Glass 2008). The HLA region has been estimated to account for up to 13% of genetic risk in JIA (Hinks et al. 2013) as compared to up to approximately 30% in RA (Gorman and Criswell 2002). HLA-B27-association in enthesitis-related arthritis has been reported (Thomson et al. 2002), while HLA-A2 is seen in younger female patients with oligoarticular JIA (Murray et al. 1999).

Speculations have been made of a possible association with infectious agents at a low level, but data are scarce. Post- and parainfectious arthritis, however is better described in the literature. This comprises a heterogenic group including viral arthritis, acute rheumatic fever, post-streptococcal arthritis and reactive arthritis (arthritis following genitourinary tract or gastrointestinal tract infections) of a specific organism (-s). Some authors have used reactive arthritis (ReA) describing a non-septic arthritis developing after an extra-articular infection with typically *Chlamydia, Yersinia, Salmonella, Shigella* or *Campylobacter* (Aho et al. 1985, Keat 1983). Reiter’s syndrome is a triad of arthritis, conjunctivitis, and urethritis (or cervicitis). In the more recent criteria from 1995 (Kingsley and Sieper 1996) ReA consist of a typical peripheral arthritis in addition to evidence of a preceding infection. *Chlamydia* and enterobacteria arthritis seem to be frequent in Norwegian adults (Kvien et al. 1994).

Lyme arthritis (Lyme disease, Lyme borreliosis) is so far the only arthritis with a proven epidemiologic link and a single microbial agent, *B. burgdorferi* (Steere et al. 1977, Pancewicz et al. 2009).
Pathogenesis

The innate and the adaptive immune system

The immune system may be divided into two cooperative systems, the innate immune system and the adaptive immune system. The innate immune system is evolutionary the oldest one, and is the only immune system in non-vertebrates, and has a more primitive, non-specific response to pathogens. This immune system is partly based upon anatomical barriers, such as mucosa and skin, upon the secretion of chemical barriers (saliva, tears, sweat, gastric acid, and many more), upon the equilibrium of the normal bacterial flora, and upon immune cells, mainly phagocytic cells. The innate system is the major response system to intracellular pathogens, such as virus, leading to secretion of IFN-γ and activation of NK-cells. Phagocytosis (via Toll-like receptors) and antigen presentation, opsonization of infectious agents, binding to apoptotic cellular debris, activation of sentinel cells, chemokines, cytokines and complement, and release of histamine, resulting in inflammation, vasodilation, and pain are also a part of the innate immune system (Iwasaki and Medsithov 2015, Artis and Spits 2015). The adaptive immune system consists of the humoral immune system (B-lymphocytes/B-cells, antibodies) and the cell-mediated/cellular immune system (T-lymphocytes/T-cells), and is responsible for creating immunological memory (Hirano et al. 2011, Danilova 2012). All cells with a nucleus are capable of presenting antigen through the major histocompatibility complex (MHC), but professional antigen-presenting cells, such as dendritic cells, B-cells and macrophages, are equipped to prime naïve T-cells (Raphael et al. 2014). Naïve T-cells differentiate into cytotoxic T-cells (Tcyt, CD8+), T-helper cells 1 (TH1, CD4+, IFN-γ-production, leading to cellular immunity by stimulation of macrophages, B-cell opsonization and complement-fixing antibodies), T-helper cells 2 (TH2, CD4+, IL-4, IL-5, IL-13, leading to B-cell production of non-cytolytic antibodies and humoral immunity), T-regulatory cells (Treg, CD4+, regulatory effects, such as maintaining self-tolerance, suppressing asthma and allergies, inducing oral tolerance and maternal tolerance to fetus, and regulating immune response upon plasma cells, B-cells and T-cells, among others), and T-helper cells 17 (TH17, IL-17, tissue inflammation, and has been linked to autoimmunity, transplant rejection and cancer, among others) (Paul WE (ed.), Fundamental immunology 2012, Bogen (ed), Immunologi, 2007). There seems to be a different pathogenesis between
systemic onset JIA involving the innate immune system, while the other subclasses seems to involve the adaptive immune system.

The pathogenesis of both JIA and RA is complex involving different parts of the immune system: the cellular immune reaction, autoantibodies, cytokines, arachidonic acid metabolites, reactive oxygen species and neuro-peptides.

**Cellular immune reaction**

T-cells seem to be important in the immune reaction in RA and JIA. TH2-cells are frequent in the synovial membrane in RA and in JIA (Spreafico et al. 2014). In this study circulating pathogenic-like lymphocytes (CPLs) were demonstrated to produce an increased amount of IFN-γ, TNF and IL-17, and hereby fuel the inflammation. CPLs are found in excessive amounts in active JIA and RA, and are believed to escape from the site of autoimmune reaction, possibly from tertiary lymphoid tissues developing within the autoimmune area in chronic autoimmune disease (Pitzalis et al. 2014). CPLs may be a biological marker for the autoimmune disease activity in JIA and RA, and may also in part explain the unresponsiveness to therapy as seen in some patients with JIA and RA.

**Autoantibodies and clinical laboratory features**

IgM-, IgA- and IgG-auto-antibodies with specificity for Fc-part of IgG (Rheumatoid factor/RF) is found in most RA-patients, in approximately 80% during the disease development. RF is not specific for RA, and is also increasingly present with age, but is almost always present in extra-articular manifestations of RA. Immune complexes with RF are mostly found in synovial fluid and synovial tissue, with local activation of complement and inflammation. Clinically, Waaler’s reaction and RF-latex-test are frequently used tests of IgM RF. IgG and IgA RF are most likely more related to disease activity than IgM RF. These tests are highly sensitive, but less specific, with as many as 10% of healthy individuals being positive. Detection tests for antibodies binding cyclic citrullinated peptides (a-CCP) are more specific (98%), maintaining a high sensitivity (70%). (Pødenphant (ed), Reumatologi, 2006, Bogen (ed), Immunologi, 2007)

Very few studies have focused on identifying clinical and laboratory features that may predict the evolution into chronic arthritis among patients with early onset arthritis. Patients in a
Finnish study with a disease duration of chronic arthritis exceeding two weeks had low level of C-reactive protein (CRP) and an elevated IgG level and no fever (Kunnamo et al. 1987), while in a Norwegian study, a high platelet count, a normal neutrophil white blood cell count, a frequent HLA-B27 association, and a positive ANA were detected (Riise et al. 2008). In a recent study of long-term follow-up, physician’s global assessment, number of active joints, ESR and CRP all improved significantly from 15 to 30 years follow-up, although patient’s total assessment did not (Selvaag et al. 2014).

**Arachidonic acid**

Prostaglandins are a group of active lipid compounds, derived from fatty acids, having a hormone-like effect in humans. They are involved in development and regulations of local inflammation and bone destruction. Prostaglandins are partly produced by cyclooxygenases (COX), and COX2 stimulation by pro-inflammatory mediators, such as IL-1, TNF, IL-17 and arachidonic acid metabolites, will result in production of many prostaglandins. Prostaglandin E2 (PGE2) believed to be the most bioactive (Akaogi et al. 2006, Pødenphant (ed) 2006).

**Reactive oxygen species (ROS)**

ROS are normal byproducts of the normal metabolism of oxygen, and are involved in cell signaling and homeostasis. In environmental stress and inflammation, ROS levels increase dramatically. ROS is highly reactive, and may induce cell-membrane damage, tissue necrosis, inflammation and oxidative post-translational changes. These changes may induce dysfunction in both T- and B-cell regulation, and may induce changes in immune tolerance, leading to autoimmunity (Ryan et al. 2014).

**Neuropeptides**

Neuropeptides with pro-inflammatory abilities, such as Substance P and Calcitonin gene-related peptide, are released from nerve-endings in response to inflammation. Substance P may induce inflammation by effects upon granulocytes and mast-cells, histamine release, cytokine synthesis and PGE2 production and activation (Sun and Bhatia, 2014).

**Genetics and environmental factors**

Different mutations within and beyond the HLA region may induce effects such as failure to downregulate T-cell activation (Hinks et al. 2005), failure to remove potentially autoreactive
T-cells during thymic selection (Vang et al. 2005), and failure in the regulation of interleukin-12 (IL-12) responsiveness (Zhao et al. 2013). IL-12 is also involved in the growth and function of T-cells and natural killer (NK) cells, and stimulates the production of TNF-α and IFN-γ.

In the setting of a genetically susceptible individual, environmental influences are hypothesized to contribute to JIA pathogenesis. Breastfeeding has been suggested to have a protective effect on development of rheumatoid factor (RF), and is associated with a decreased risk of adult-onset RA (Young et al. 2007, Jacobson et al. 2003). Tobacco smoke as been shown to be a strong risk factor in the development of RA in adults (Wilson et al. 1999). In one retrospective study assessing risk factors for RF-positivity in children, HLA-DR4-negative children who were RF-positive were five times more likely to have been exposed to environmental tobacco smoke compared to RF-negative children (Young et al. 2007). Tobacco has a direct effect on the immune system leading to a reduction in the number of NK-cells and abnormalities in T-lymphocyte function (George et al. 1997).

Psychosocial factors, such as stress, has been demonstrated in JIA patients to both increase IL-6 production, IL-6 being a potent cytokine in the pathogenesis of JIA, and to increase the expression of adrenergic receptors in mononuclear cells in blood (Roupe van der Voort et al. 2000).

**Cytokines**

Cytokines serve as the mediators of cellular differentiation (growth), inflammation, immune pathology, and regulation of immune response. A well-regulated immune response demands a suitable balance between pro-inflammatory and anti-inflammatory cytokines. The classic, well-known pro-inflammatory cytokines include TNF-α, IL-1β, IL-6, IFN-γ and IL-17 among others. Pro-inflammatory cytokines are involved in the differentiation and activation of pathogenic cells (like TH17), migration of pathogenic cells into the joint, angiogenesis, development and activation of osteoclasts, and in bone damage during the course of autoimmune arthritis (Astry et al. 2011, Gaffen et al. 2014). Anti-inflammatory cytokines include IL-4 and IL-10, and IL-27 in rheumatoid disease.

**TNF-α** is mainly produced by macrophages, T-cells, NK-cells, mast cells, endothelial cells and in adipose tissue (Clark 2007), and are released in large quantities when these cells are exposed to lipopolysaccharide or IL-1β, which upon binding to TNF receptor can drive the cell to apoptosis or activate the MAPK-pathway, leading to cell survival, chemotaxis of
immune cells and other inflammatory processes (Kant et al. 2011). TNF-α is believed to be a key regulator of IL-6 production, and of local synovitis (Matsuno et al. 2002).

**IL-6** is mainly produced by dendritic cells and macrophages, and contributes to arthritis pathogenesis by differentiation of TH17 cells, osteoclast differentiation through expression of RANKL, and production of other pro-inflammatory mediators and tissue-degrading enzymes (Yoshida and Tanaka 2014, Calabrese and Rose-John 2014). Interestingly, IL-6, when activated by other pathways than MAPK, may act as an anti-inflammatory cytokine, with inhibitory effects on TNF-α and IL-1 and activation of IL-10. IL-6 may also act in a hormone-like manner in muscles (a myokine) mobilizing substrate for the muscle (Petersen and Pedersen 2005). Both TNF-α and IL-6 is believed to modulate insulin resistance. IL-6 is persistently increased in systemic inflammatory states like in obesity or type 2 diabetes, and may induce insulin resistance, whereas transient increases of IL-6 contributes to normal glucose homeostasis (Rabe et al. 2008).

**IL-1β** is produced by tissue macrophages, blood monocytes and dendritic cells and exhibits a pro-inflammatory function involving cell differentiation and proliferation, and apoptosis. IL-1β, IL-17 and TNF-α works synergistically as pro-inflammatory mediators. IL-1β also induces COX2, thus contributing to inflammatory pain hypersensitivity. IL-1β is together with IL-6 considered the key cytokines in systemic JIA (Sikora and Grom 2011).

**IL-17** is mainly produced by TH17 cells, and the expression is increased (especially IL-17A) in inflammatory arthritis. IL-17A is involved in the differentiation of naïve T cells into pathogenic T cells, the migration of pathogenic cells into the synovium, the increased survival of synoviocytes, angiogenesis, osteoclast differentiation and MMP secretion leading to bone and cartilage damage (Hot and Miossec 2011, Shabgah et al. 2014).

**IL-32** is produced by lymphocytes in the synovium and synovial fibroblasts of arthritic joints. It has been shown to correlate with the severity of rheumatoid arthritis, as well as with the expression of other pro-inflammatory cytokines, including TNF-α and IL-1β (Alsaleh et al. 2010).

**IL-34** is expressed in the synovium and fibroblast-like synoviocytes in rheumatoid arthritis and has been implicated in macrophage differentiation, osteoclastogenesis and enhancement of bone resorption (Chen et al. 2011).

**Bone biomarkers in inflammatory disease**

The data regarding bone biomarkers in inflammation is scarce. In a recent review article (Jadon et al. 2015) regarding bone biomarkers in psoriatic arthritis an association was found
with regard to MMP and M-CSF among others, and an uncertain association with OPG and ALP. Only ten studies were included in this review.

**Osteopontin (OPN)** is expressed in many cell types and tissues including pre-osteoblasts, osteoblasts, osteocytes (Chen et al. 2014), fibroblasts, macrophages and T-cells (Lund et al. 2009), among others, and is involved in physiological and pathological events, such as biomineralization, tissue remodeling, obesity, diabetes, diabetic nephropathy, liver disease, cardiovascular disease and inflammation. OPN is upregulated by cytokines like IL-1 and TNF-α (Gao et al. 2005), and enhances TH1 and inhibits TH2 cytokine expression, inducing a possible cytotoxic response. Macrophage expression of IL-12 and inhibition of IL-10 is induced, thereby stimulating inflammatory responses and decreasing anti-inflammatory signaling pathways (Ashkar et al. 2000).

**Leptin and other adipokines.** Leptin is a hormone secreted mainly by adipocytes, which is a modulator in food intake, body fat composition, insulin activity, thermogenesis, angiogenesis and the immune system (Stenvinkel et al. 1999). Leptin can stimulate chondrocytes to secrete higher levels of key mediators in cartilage degradation such as TNF-α, IL-1, IL-6 and other (Toussirot et al. 2007), and in osteoarthritis, synovial leptin was much higher than in a matched plasma sample, indicating a more important local role for leptin in the bone metabolism regulation (Presle et al. 2006). Leptin has been a highly investigated adipokine with studies indicating a role among many in multiple myelomas (Reseland et al. 2009), neuroinflammation (Aguilar-Valles et al. 2015), and in the oral region studies have indicated a role in periodontitis (Shi et al. 2015), peri-implantitis (Wohlfahrt et al. 2014) and in periapical granulomas (Martín-González et al. 2015). Leptin is also known to suppress the HPA-axis.

Other adipokines, adiponectin, visfatin and resistin are studied with regard to their role in osteoarthritis, suggesting both protective and destructive roles for different adipokines. Adiponectin is believed to be anti-inflammatory by stimulating release of IL-10 and IL-1RA (Chen el al. 2006), it stimulates osteoblasts and thereby the inflammatory mediators IL-6 and IL-8 (Luo et al. 2005). By contrast, adiponectin also stimulates RANKL production and inhibits OPG production, which in turn indirectly activates osteoclasts (Luo et al. 2006). In insulin resistance and obesity most adipokines, not adiponectin, normally are elevated.

**Parathyroid hormone (PTH)** is secreted from the parathyroid glands mainly in order to raise blood calcium concentration, as a response to low serum calcium, increased serum phosphate or decreased serum magnesium. PTH regulates serum calcium concentrations by effects on bone, kidneys (increased reabsorption of calcium and magnesium) and intestines (enhances
absorption of calcium by increasing activation of vitamin D in the kidneys). PTH indirectly stimulates osteoclasts in order to release calcium from the bone, by binding to osteoblasts and osteocytes, and thereby enhancing the expression of RANKL and inhibit the expression of osteoprotegerin (OPG). RANKL/RANK is an osteoclast stimulator, while OPG inhibit osteoclast activation by binding RANKL.

The regulation of blood calcium concentration is complex, involving among others PTH and osteocalcin. Recently, the observations of oxidized PTH counteracting non-oxidized PTH, has suggested PTH analysis to be a confounding factor with the commercial kits available (Mazzaferro et al. 2014), illustrating obstacles and difficulties within this field of research.

**Osteoprotegerin (OPG) and RANKL/RANK/OPG-axis.** OPG is a decoy receptor protein that prevents the interaction of RANKL/RANK by binding RANK, and is expressed by a variety of cells including osteoblasts, osteocytes and cardiovascular cells, and is involved in the regulation of bone metabolism and vascular function (Schoppet et al. 2002). BMP-2, vitamin D3, calcium, angiotensin II, estrogens and PDGF stimulate OPG, while inhibitors of OPG includes PTH, glucocorticoids, prostaglandin E2, basic fibroblast growth factor (bFGF) and immunosuppressant drugs. OPG is capable of inducing both osteoporosis and osteopetrosis in transgenically over-/underexpression in mice (Simonet et al. 1997). In rheumatoid arthritis (RA), RANKL is highly expressed in the synovium, and is believed to be largely responsible for RA-related bone destruction (Takayanagi 2012). In RA, the source of RANKL appears to be synovial fibroblasts, and cytokines IL-17, IL-1, TNF-α, and IL-6 is involved (Takayanagi 2012). The bone destruction in osteoporosis following long-term RA appears to be different from the periarticular process. Xu et al. (2012) found age and CRP to be independent risk factors for osteoporosis in RA. RA patients had lower plasma levels of OPG and higher levels of RANKL, and thus a significantly lower OPG/RANKL ratio than the control group, indicating a shift in bone homeostasis towards osteoclastic activity.

**Adrenocorticotropic hormone (ACTH)** is produced in the pituitary gland and stimulates secretion of glucocorticoid hormones from the adrenal cortex cells. The ACTH receptor, although mainly expressed on adrenal cell, may also be expressed on osteoblasts (Zhong et al. 2005), and is believed to have a protective role of the osteoblast and in protection of glucocorticoid-induced osteonecrosis (Zaidi et al. 2010).

**Osteocalcin (OC)** is bone-derived hormone regulating energy metabolism. It is a cell specific small protein secreted by osteoblasts, but is also released and activated by osteoclasts, due to a low pH in the resorption lacuna (Ferron et al. 2010). Osteocalcin knockout mice had decreased levels of insulin, decreased insulin sensitivity, and were abnormally fat (Lee et al. 2010).
Studies suggest that osteocalcin and insulin increase each other’s activity or secretion in a feed forward loop, and hence there must be negative regulators of osteocalcin to maintain glucose homeostasis, and leptin is one such negative regulator. Chronic use of glucocorticoids has been associated with the development of glucose intolerance, insulin resistance, diabetes and dyslipidemia (Gounarides et al. 2008). In addition, glucocorticoids have adverse effects on bone by suppressing osteoblast activity, and thereby osteocalcin secretion, and this effect could partially explain the detrimental effect of glucocorticoids on glucose metabolism (Brennan-Speranza et al. 2012).

**Microbial flora and arthritis**

Humans are dependent upon bacteria in order to survive, and coexist in a delicate steady state situation with these “old friends”. In contrast, many bacteria exhibit antigen capable of inducing a strong immune response involving every section of the immune system. A typical antigen is lipopolysaccharide (LPS), which is an important part of the bacterial wall in gram-negative bacteria, such as *Enterobacter, Neisseria* and more, LPS being recognized through binding to LPS-binding protein and Toll-like receptor 4. In gram-positive bacteria, typical antigens are lipoteichoic acid (LTA), lipoproteins and peptidoglycans, while there are antigens linked to specific abilities of bacteria, such as flagelline in bacteria with flagella, and lipoarabinomannan in Mycobacteriaceae. LPS-complex binding to Toll-like receptor 4 induces a powerful activation of macrophages and dendritic cells, with release of IL-1, IL-6 and TNF, among others. LTA bound to targets can interact with circulating antibodies and activate the complement cascade to induce a passive immune kill phenomenon. It also triggers the release from neutrophils and macrophages of reactive oxygen and nitrogen species, acid hydrolases, highly cationic proteinases, bactericidal cationic peptides, growth factors, and cytotoxic cytokines, which may act in synergy to amplify cell damage. (Bogen (ed), Immunologi, 2006 and Jawetz (ed), Medical Microbiology 2013).

Normal intestinal microbiota can induce chronic arthritis in RA. This is what is proposed in a review paper from 2003 (Toivanen 2003). Previously, microbes have been suggested as an environmental factor in the etiology of rheumatoid arthritis (Bennett 1978, Midtvedt 1987). Anaerobic bacterial DNA and high levels of antibodies against these bacteria have been detected in the serum and large joint (not TMJ) synovial fluid from patients with RA (Moen et al. 2003 and 2006, Ogrendik et al. 2005). Bacterial DNA in large joint SF has previously been shown to be present in both arthritis patients and in healthy controls (Kempsell et al. 2000), indicating that not all bacterial species induce local joint inflammation. In the study by Moen
et al., pathogenic bacteria known to induce an inflammatory response in oral tissue were found in synovial fluid, both in RA and in the control group. In light of these data, and others, there are indications of bacteria playing a possible role in the initiation and progression of arthritis. The data regarding findings of bacterial DNA and possible consequences in arthritis, not being reactive arthritis, are scarce and further studies are needed.

JIA is by definition idiopathic, that is, with no identifiable cause. Nevertheless, several microbial agents, particularly viruses, have been associated with the onset of JIA. There have been studies indicating a correlation with JIA and influenza A (Pritchard et al. 1988), rubella (Chantler et al. 1985), parvovirus B19 (Gonzalez et al. 2007), and Epstein-Barr virus (Aghighi et al. 2007), but the results are controversial, especially due to the fact that these are common infections to which many people have been exposed.

**Treatment**

Currently, there is no cure for JIA. The goal of therapy includes disease remission, pain control, improved functioning, preservation of normal growth, and preservation of long-term joint damage while balancing the side effects of medications (Hayward and Wallace 2009, Oberle et al. 2014).

Treatment recommendations have been recently published by ACR (Beukelman et al. 2011) and by CARRA (Ringold et al. 2014), however, variability in treatment of JIA remains.

The most commonly used class of medicine is **NSAIDs**. NSAIDs block prostaglandin formation via inhibition of COX-1 and COX-2, leading to decreased analgesic and anti-inflammatory response. NSAID monotherapy is, according to ACR recommendations, indicated for up to 2 months depending on disease activity. NSAIDs are often used together with DMARDs and/or biologic therapy.

**Glucocorticoids** are mostly used as intra-articular injections in patients with either few involved joints or as adjunct therapy in patients on other immunosuppressive agents. Systemic glucocorticoids are not routinely recommended for treatment of JIA. The recommended steroid is triamcinolone hexacetonide, and expected improvement should last for at least 4 months (Zulian et al. 2004).

**DMARDs** have historically been second-line therapy for JIA following a course of NSAIDs. Methotrexate has anti-inflammatory and immunomodulatory properties by means of inhibition of dihydrofolate reductase, necessary for DNA-synthesis. Other DMARDs, such as
leflunimide, sulfasalazine, cyclosporine, tacrolimus, thalidomide, and more, have been studied in JIA patients, but these medications is not routine therapy, although they may be used in refractory disease.

**Biologics**, or biologic DMARDs are a group of medicines aiming to target cells or selectively block specific cytokines. This group consists of TNF inhibitors (etanercept, infliximab, adalimumab), Abatacept (blocks T-cell activation), IL-6 inhibitor (tocilizumab), Rituximab (binds B-cell receptor, depleting B-cells), and IL-1 inhibitors (anakinra, canakinumab, rilonacept). TNF inhibitors are in the ACR guidelines recommended after incomplete response to methotrexate.

Patients with JIA today are treated more aggressively with earlier use of methotrexate in higher concentrations and more frequent use of biologics, adjusted to JIA subtype, and with individual differences. Though highly effective, the biologics are expensive, and there are reports on hypersensitivity reactions, as well as concerns regarding risks of infections and malignancies (Klein and Horneff 2009).

Commonly, patients with JIA also get adjunctive treatment with physiotherapy, acupuncture, exercise, psycho-motoric physiotherapy, psychological treatment and others, but data regarding the effect of this is scarce. Surgical corrective treatment may also be necessary (Cassidy and Petty 2005).

**JIA and the temporomandibular joint (TMJ)**

Studies have reported TMJ arthritis often to be without swelling and without symptoms from the TMJ (Arabshahi and Cron 2006, Ringold and Cron 2009), although other studies have reported significantly higher pain score in JIA of both the TMJ and the masticatory muscles. In a study by Koos et al. in 2014, MRI diagnosed TMJ arthritis in 80% of patients with JIA (not classified in subtypes), with 25% exhibiting unilateral symptoms, and 55% bilateral symptoms. Clinical examination alone does not seem sufficiently sensitive in detecting TMJ arthritis, and MRI is recommended, thereby helping to prevent undertreatment and overtreatment (Koos et al. 2014, Müller et al. 2009). MRI is found to be capable of diagnosing TMJ arthritis in 75% of all cases, while ultrasonography was neither sufficiently sensitive (true positive rate) nor specific (true negative rate) (Müller et al. 2009, Weiss et al. 2008). Imaging studies have reported very high frequencies of TMJ involvement. The TMJ is considered one of the most frequently involved joints in patients with JIA (Küseler et al.
1998, Pedersen et al. 2008, Ringold and Cron 2009), and TMJ abnormalities were detected in long-term studies (Arvidsson et al. 2009 and 2010). Pathologic changes in the TMJ is the result of a biochemical cascade involving hypoxia and stimulation of synovium, condyle, disc, and localized vasculature, resulting among other in production and release of inflammatory cytokines and proteases.

**JIA - TMJ growth and growth disturbances**

The growth cartilage of the TMJ is positioned just beneath the fibrous articular layer, and is adaptive to the functional load of the joints, undergoing atrophic change in the absence of function, and regaining its endochondral capability upon reestablished functional demands (Glineburg et al. 1982). Bone erosions, osteopenia, soft-tissue swelling, lymphocyte infiltration into the joint area, and uniform joint space loss generally characterize inflammatory arthritis. The extent of synovial macrophage infiltration correlates strongly with the degree of joint erosion in arthritis (Yanni et al. 1994), macrophages being a source of TNF and IL-1. IL-1 and TNF promote recruitment of neutrophils. Inflammatory cells, such as macrophages, release pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-8 (Wess et al. 2005, Choy et al. 2001).

Synovial macrophages may differentiate into osteoclasts after RANKL stimulation (Adamopoulos et al. 2006). The RANKL/RANK/OPG axis is believed to tightly regulate osteoclast formation and bone resorption. RANKL-induced osteoclastogenesis is inhibited by osteoprotegrin (OPG) (Simonet et al. 1997, Gillespie 2007).

The regulation of bone formation and bone resorption is complex and not fully understood. IL-27 is an interleukin that negatively regulates osteoclast formation and bone resorption, although its role in inflammatory arthritis remains puzzling. As an example, IL-27 has been suggested to be both pro-inflammatory (Cox et al. 2011) and anti-inflammatory (Tanida et al. 2011).

Craniofacial growth disturbances have been reported in approximately 50% of patients with JIA (Arvidsson et al. 2010, Kjellberg 1998), and TMJ involvement has been considered the most important cause of abnormal craniofacial growth (Stabrun et al. 1988, Kjellberg 1998). Micrognathia has been reported in 18-27% (Rönning et al. 1994, Arvidsson et al. 2010) and
has previously been associated with large structural changes of the TMJs (Larheim and Haanæs 1981).

**JIA – TMJ treatment**

Reducing pain, regain normal function and prevent growth disturbances are main goals in treatment of TMJ involvement in JIA. Early diagnosis and treatment are essential to prevent substantial orthodontic and possibly orthognathic surgery (Pedersen 1998). Besides systemic treatment as mentioned previously, local treatment may also be indicated. Treatment of the TMJ and its supporting tissues is complex and multifactorial, and there is yet much to learn. Reduction of orofacial symptoms has been shown using stabilization splints (Stoustrup et al. 2014), intra-articular steroid injections (Stoll et al. 2012). There is an ongoing debate with regard to positive and negative local effects of glucocorticoids (Stoll et al. 2012, Stoustrup et al. 2013).

Treatment of orofacial pain in JIA with physiotherapy, acupuncture, chiropractic treatment and other treatments are performed, but with little, if any, scientific basis. There is no consensus on the treatment of TMJ pathology and dentofacial deformities in patients with JIA (te Veldhuis et al. 2014).
Aims of the study

The overall hypothesis for this thesis was: Bacterial DNA in TMJ synovial fluid stimulates the local immune response, and hence both the local treatment response and the bone homeostasis in juvenile idiopathic arthritis. The overall aim of the studies of this thesis was to extend our knowledge on treatment of temporomandibular joint arthritis in juvenile idiopathic arthritis, and the correlation to synovial fluid content of immunoglobulins, bone markers and bacterial DNA.

A)
What are the effects of intra-articular TMJ arthrocentesis with and without use of steroids upon patients with JIA?
Will aspiration and thereby changing the composition of the TMJ SF improve TMJ pain and function?
Is the effect of glucocorticoids in combination with arthrocentesis better than arthrocentesis alone?
For how long could a possible improvement last?

B)
What are immunological and microbial contents of the synovial fluid of the TMJ?
Is the TMJ aseptic in JIA?
In what degree are cytokines and bone parameters expressed and present in the TMJ SF in JIA?
Are there any correlations between clinical parameters and SF contents, with regard to bacterial DNA, immunological parameters, or both?
Are there any differences in levels of cytokines and bone markers with regard to age and/or JIA versus RA?
Material, methods and methodological considerations

Material

A total of 94 JIA patients were referred for possible TMJ problems at Oslo University Hospital, Rikshospitalet, Oslo, Norway. Of these, 41 patients had a combined clinical examination and MRI strongly indicating TMJ arthritis. 20 of these patients did not agree to participate, mainly due to long travel distances, patients being referred from a large geographic area, leaving 21 patients (38 joints) included in the study. There was no control group due to ethical considerations.
Joints were randomly selected for either arthrocentesis with glucocorticoid or arthrocentesis alone. Recordings of jaw function and pain, spontaneous and upon palpation, as well as previous treatment, on-going treatment and general medical history were performed at baseline (before intervention) and at two follow-ups (mean, 3 and 8 months). Pain assessments were performed using both VAS (scale, 0-10 cm and number, 0-100) and a visual faces pain scale.

![Visual faces pain scale, Bieri D et al. Pain 1990, 41: 139-50.](image)

Ultrasound-guided arthrocentesis (push and pull technique, Alstergren 2003) were performed in general anesthesia in a sterile operation field. Bacteria skin samples from the injection site were taken before and after skin disinfection. Samples were immediately frozen at -80°C for later analyses.

![Picture showing arthrocentesis with the push-and-pull technique using Behepan®/salic water.](image)
In paper II and III we analyzed synovial fluid from 30 patients (54 TMJs), 20 children with JIA, and 10 adults with either RA (n=5) or JIA (n=5). Adults were included to serve as control for age as a confounding factor. All adults were referred from Diakonhjemmet Hospital, Oslo, Norway.

Synovial fluid was analyzed for bacterial DNA using 16S rDNA PCR, and protein analysis were performed using BCA protein assay and Luminex/XMAP technology.

Methods – bacteria detection and protein analysis

The advent of DNA sequencing has made it possible to sequence genes from a high number of different organisms. The gene 16S rDNA is the most widely used component from the translation apparatus for phylogenetic analysis. 16S occur in all bacteria and the genes are long enough to be informative and short enough to allow easy sequencing (Wade 2002). 16S rDNA was amplified from clinical samples by the polymerase chain reaction (PCR). PCR is a method based upon thermal cycling generating DNA single strands, and enzymatic replication using heat-stable DNA polymerases, primers and DNA oligonucleotides (deoxynucleoside triphosphate, dNTP). To check if the PCR generated the anticipated DNA fragment (Amplicon), gel purification for size separation is performed, followed by sequencing leading to bacteria species identification. PCR is a safe method with a high accuracy level, although problems do occur. I high concentration of the DNA template may lead to packed DNA with possible false priming and obstruction of the polymerases. A low concentration of the DNA template may increase the risk for contamination and loss of a substantial amount of the sample due to clotting, adsorption, and chemical/enzymatic degradation. Troubles may also occur due to inadequate amount of oligonucleotides, primers, polymerases, Mg$^{2+}$, and due to failure to maintain the correct thermal cycle.

Analysis of interleukins and bone markers was performed using Luminex/XMAP multiplex. This assay enables the possibility of conjugate biomolecules to the surface of specific beads to capture analytes of interest. XMAP beads are coated with a reagent specific to a particular bioassay, and are color-coded to be specifically identified by the Luminex analyzer.

Luminex multiplex system was our procedure of choice due to the possibility to get multiple analyses from very small samples. In addition to the different parameters in our test, there are other factors, which would have been interesting to analyze as well, for instance IL-17 and RANKL. In all assays with multiple antibodies there is a possibility for cross-reactions, possibly leading to an incorrect result of either a false high/positive result or a false
low/negative result. In our study, comparisons were made of individual changes or in comparison of groups, and thereby reducing the effect of this possible problem. One experienced person performed all analyses at the same time, in order to eliminate possible problems with the Luminex method, and great care was taken not to contaminate samples during the laboratory procedures, working under sterile conditions, and using standards as control for contamination.

Methodological considerations

The selection of patients included in the study considered both symptoms from the TMJ, problems with jaw function, and a positive MRI. Patients with silent arthritis were not included.

Evaluation of treatment strategies in TMJ arthritis is difficult. Local anatomy, such as innervation, bilateral joint movement, supportive tissue, and joint anatomical considerations of disc displacements and more must be considered in the evaluation of pain and function. Intervention in a TMJ may influence the contralateral joint, either as a direct mechanical response, or by systemic (hematologic) influence. In this study, intervention was performed in both TMJs in most patients. One might consider intervention in only one joint, leaving the other as a control, but besides the ethical consideration of not treating an inflamed joint, there are still the systemic and local effects to be considered. This must be considered when evaluating the results of two different treatments in the TMJ in a patient. In this study, evaluation of the treatment is based on subjective findings of pain and function, and objective measurements of mandibular movement. In case some children were unable to understand VAS, a pain facial detection scale was included in the study. Although some patients were as young as 6-8 years old, there was no discrepancy between VAS and the facial detection scale. Other methods for evaluation, like algometers, ultrasound, MRI and others, were considered, but were not suitable for this study. Patients in this study are (post-PhD) intended to be included in a long-term follow up study, with clinical and MRI evaluations.

SF was collected using a push/pull-technique involving vitamin B (Behepan®, red colour) in order to verify presence of synovial fluid by spectrophotometry. Vitamin B is known to improve certain inflammatory conditions, but this is mostly in cases with an on-going lack of
this vitamin. Little is known of the local effect of vitamin B in the TMJ. Concerns with the push-and-pull technique are discussed in Paper II.

All SF samples were frozen at -80°C, and care was taken not to defrost the samples unnecessarily. Still, there is always the possibility of degradation and changes in both bacterial DNA and in proteins (cytokines and bone markers).

Summary of results

Paper I

Synovial bacterial DNAs were detected in more than 60% of patients.
In 30 patients (54 joints) we detected possible contamination of TMJ SF in 6 patients, whom were excluded from this study.
14 different bacterial species were detected, *Pseudomonas fluroscens* being the most frequent bacteria.
There was a tendency towards a greater bacterial diversity in children.

TNF-α, ACTH and adiponectin content (Paper II) were elevated in TMJs with bacterial DNA.
Patients with detected bacterial DNA reported less pain and better jaw function.

Paper II

ACTH levels in TMJ SF were significantly lower in JIA compared to adults with RA, and correlated with IL-1 and several bone markers.
In TMJ SF there was a tendency towards a higher IL-1, TNF-α, adiponectin and osteocalcin in JIA than in adults with either JIA or RA, while IL-6, OPG, leptin, OPN, PTH, and insulin tended to be lower in children than adults.

Total protein concentration tended to be higher in children, and tended to be lower in those with the longest duration of TMJ symptoms (negative correlations).
TNF-α and OPG were lower in patients with a tendency to higher pain levels. This is highly interesting, TNF-α being a target in general medication in both JIA and RA, and OPG, believed to be a biomarker in pain syndromes (Krämer et al. 2014). Leptin was higher in RA than in JIA, and even higher in JIA in adults.

**Paper III**

Patients included in this study were generally severely affected with regard to joint pain and function at baseline. There was a significant improvement from baseline to both first and second follow-up with regard to MIO, PIO, lateral excursion (measurements of function), and with regard to VAS function (subjective). There was a significant reduction in pain upon palpation and of pain in general (VAS). There was no difference with regard to treatment method upon the improvements as mentioned above.
Discussion

Bacterial influence

As previously mentioned in this thesis, the etiopathogenesis in JIA is not completely understood, and infections may be part of this development. Previous studies have indicated bacterial DNA as a possible initiating or contributing factor to joint disease (Deng and Tarkowski 2000, Zhang et al. 2000), and an increase in TNF-α and IL-6 has previously been associated with the presence of Chlamydia trachomatis in tissue samples from the TMJ (Henry et al. 2007).

The most frequently detected bacterial DNA in our study was Pseudomonas spp., and P. fluorescens in particular, although there was a large bacterial diversity, especially in JIA. In P. fluorescens strains, genome sequences have found a diversity of integrated elements, such as genomic islands and phage-like regions (Mavrodi et al. 2009), exemplifying the evolutionary possibility in bacteria strains to induce new pathogen types. These adapting and developing ability of bacteria also make it difficult to correctly classify bacteria, while different subtypes of a bacteria strain may have different pathogenicity and immune-stimulating properties (Silby et al. 2011).

JIA patients have been found to be three times more susceptible to bloodstream infections and severe infections as compared to healthy controls (Salonen et al. 2014, Beukelman et al. 2012). This 3-fold risk for infections was irrespective of immunosuppressive therapy, and use of methotrexate and/or TNF inhibitors did not increase the risk for serious infections, although use of glucocorticoids gave an increased risk for infections. In spite of this there was still a low incidence of infections in JIA, with only 5 patients with serious infection of a total 1604 JIA patients (Salonen et al. 2014). In these studies the bacteria causing the infections were not of strains with increased microbial resistance, typical strains were Streptococcus pneumonia, Staphylococcus aureus, Escherichia coli and Fusobacterium necrophorum. S. aureus is also more frequently found in tonsils in JIA, as compared to patients with non-JIA tonsillitis. These bacteria are mainly found in the core of the tonsils, rendering a microbial swab tests inefficient (Gul et al. 2007, Austrauskiene et al. 2009). S. aureus has previously been shown to induce expression of prostaglandin E2, RANKL and IL-6 on osteoblast leading to bone erosions (Somayaji et al. 2008, Spelling et al. 2008). Other bacteria, such as Streptococcus pyogenes, may also induce this osteoclastogenesis (Okahashi et al. 2003).
Upregulation of RANKL in infected osteoblasts appears to involve a COX2-mediated PGE2-dependent pathway (Somayaji et al. 2008). Osteoblast COX2 expression is induced by several bone resorbing, inflammatory factors, such as IL-1 and IL-6 (Miyaura et al. 2003). COX2-activation of prostaglandin induces pain and inflammation.

Bacteria may stimulate pain by other pathways as well. There is a very rapid increase in ACTH following injections with bacterial lipopolysaccharide (LPS), and LPS may also induce production of pro-inflammatory cytokines, such as IL-1, IL-2, IL-6 and TNF in the brain and the pituitary gland, but also the anti-inflammatory cytokines IL-10, IL-13 and IL-RA. In the periphery these inhibit the inflammatory response induced by the pro-inflammatory cytokines. Limited studies indicate that these anti-inflammatory cytokines antagonize the action of the pro-inflammatory cytokines in the brain as well as the hypothalamic-pituitary-adrenal (HPA) response to infection (Wong et al. 1997).

In a study by Chiu et al. (2013), bacteria were shown to modify the immune response by directly stimulating nociceptors. Why this is different in the present study is unknown. One could speculate whether this might be due to the autoimmune origin of the inflammation, or bacterial stimulation of inflammatory mediators not implicated in mediating pain, or maybe a steady state situation?

**Regarding cytokines and bone markers**

ACTH was higher in SF in patients with detected bacterial DNA in our study, and lower in children than adults. Cytokines induced by bacterial DNA may act locally on nociceptors to induce pain, but the most common pain-inducing pathway is indirect, stimulating the release of other agents such as prostaglandins. In acute phase, cytokines appear to induce sensitization via receptor-associated kinases and phosphorylation of ion channels, whereas in chronic inflammation transcriptional up-regulation of receptors and secondary signaling become more important (Opree and Kress 2000). These different pathways in acute and chronic phases needs to be further studied with regard to rheumatoid arthritis and JIA; all the while these diseases are chronic inflammatory diseases with acute and sub-acute inflammatory stages. Both disease duration and activity state may be important factors influencing on the different pain modulating pathways. The elevated ACTH in SF in our study may indicate a bacteria-induced shift in the pain-inducing pathway possibly involving the HPA-axis. Leptin is known to regulate stress-induced HPA-axis responses leading to release of ACTH and corticosterone, among others (Roubos et al. 2012). In our study leptin is
higher in adults than in children with JIA, although no correlation to pain intensity was found. This is in contrast with previous findings of an association between levels of leptin and pain (Lübbeke et al. 2013). Possibly, there are different mechanisms by which leptin in a joint may induce pain, and possibly are being influenced by other factors counteracting leptin locally. Inflammation, and the release of cytokines (and serotonin, histamine, bradykinin and others) may result in peripheral- and central sensitization. In peripheral sensitization cytokines may stimulate pain by ways of neurogenic inflammation, direct sensitization of nerve endings, indirectly by stimulation of nerve growth factor, and other pathways (Kopp S 2001).

Antibodies against TNF reduce hyperalgesia in inflammatory models, and anti-TNF treatment in RA is followed by substantial reductions in pain score (Mani et al. 1998). More modest pain reduction is seen with regard to anti-IL-1 and anti-IL-6 (Bresnihan et al. 1998, Xu et al. 1997), possibly indicating TNF having a more important role in pain modulation. In our study, TNF seemed to be more important in boys with regard to pain, possibly indicating a gender difference with regard to pain mechanisms. In boys, TNF tended to be lower when the pain expression was highest, which are interesting, although difficult to explain with the current knowledge in the literature.

Besides NSAIDs and methotrexate, TNF-inhibitors are the most common treatment in JIA and in adult rheumatoid disease. TNF-inhibitors are reported in studies as being effective in improving the patients’ symptoms and complaints. In contrast, TNF-α is most often reported to be low in local synovial fluid as compared to serum. In our study, TNF-α was lower in TMJ SF in patients reporting higher pain levels, and in patients with bacterial DNA in SF. In a study by Matsuno et al. (2002) TNF-α is “proved” to be a key molecule in the control of the inflammatory changes in RA synovium. They found TNF-α capable of controlling IL-6 production, but did not find any effects on bone or cartilage produced by TNF-α. JIA joint inflammation is not uncommonly silent, and there may be other signaling pathways responsible for pain stimulation. Presence of bacterial DNA in SF may be an etiologic factor in initiation of arthritis, it may be secondary to an already existing pathological condition, or it may be, in fact, naturally present in the joint. If bacterial DNA in SF is capable of changing the immune response from TNF, this may possibly lead to a reduction in IL-6 production, and possibly to a shift in a more destructive pathway, maybe with less pain induction. This is however, highly speculative, and is beyond the current knowledge of the different pathways of the immune system, and of the different role of each cytokine in this different pathways. At present, there is a growing understanding of the importance of the normal bacterial flora, and its regulation of the immune system. Bacteria are highly adaptive and will respond to local
and general changes. The gut-brain effects from microbiome-changes related to stress, health and disease are highly investigated at present (Moloney et al. 2014). There are some concerns with regard to changes of microbial flora due to modern living, and this influence on disorders such as allergies, inflammatory diseases, autoimmunity, depression and anxiety (Rook et al. 2014).

The anti-inflammatory cytokine IL-10 generally promotes a Th2 type immune response (humoral immunity, B-cells) by inhibiting Th1 cytokine production and cell-mediated immunity (phagocytes), and hence, reduces the tissue destruction (Ouyang et al. 2011). IL-10 also decreases brain inflammation and protects the nerve tissue from LPS-induced inflammation and damage (Qian et al. 2006). IL-10 may play an important role in pain induction and/or pain regulation in rheumatoid diseases, as it does in peripheral neuropathies, although the mechanism are not yet fully understood, and may be specific to different types of nerve-affecting pathologies (Khan et al. 2015).

Osteoprotegerin (OPG) was low in patients reporting most pain in this study, a finding in contrast with previous studies focusing on OPG as a biomarker in pain syndromes (Krämer et al. 2014). Low OPG may be induced by a local factor, such as PTH, IL-6-feedback, or other regulatory mechanisms, but may be induced by methotrexate as well, although the data are few and inconclusive. We tested RANKL in SF in JIA, but possibly due to very small samples left, no RANKL were detectable in our studies, which is unfortunate, OPG/RANKL ratio being highly important in order to suggest a response favoring bone degradation or not. Anti-TNF agents have been shown to increase OPG (Catrina et al. 2006), and glucocorticoids have been shown to decrease synovial RANKL in human RA (Magrykiannakis et al. 2006). Methotrexate, as used by most patients in this study, has been shown to decrease lymphocyte and macrophage infiltration in the synovium, reduce expression of RANKL and RANK, decrease the synovial RANKL/OPG ratio, and to decrease osteoclast formation and bone resorption. Local inflammation may regulate synovial RANKL and RANK expression, but these are probably not completely dependent on local inflammation (Revu et al. 2013).

**Clinical implications**

In paper III we found little difference in treatment outcome of adding steroids to arthrocentesis. Besides the importance of treating TMJ arthritis in children, in order to reduce pain and establish normal growth and function, one must also consider the side-effects of this
treatment. Arthrocentesis is a minor trauma to the TMJ and its surrounding tissues, and may generate adverse changes in the joint. Glucocorticoids not only have anti-inflammatory effects, one must also consider the local effect upon the bone homeostasis (Brennan-Speranza et al. 2012). In addition, the general load of medicines, such as glucocorticoids, methotrexate and biologics, to the developing child must be considered. This concerns both physiological stress (disease and treatment) and psychological load, and comprises a huge number of influencing factors, such as pain, altered function, sleep, comorbid pain conditions, growth, genetics, drug side-effects, and many more, and is a wide topic outside the scope of this thesis.

In several studies radiographic TMJ abnormalities have been described, and studies with TMJ injections in patients with JIA have been reported (Stoll et al. 2012, Stoustrup et al. 2013). However, prospective treatment studies of TMJ arthrocentesis in JIA are necessary, and this was performed in Paper III. The children with TMJ arthritis and JIA in this study were severely affected with the condition; nevertheless, a single intervention seemed to give good relief from TMJ symptoms and it improved TMJ function for at least eight months. Principally, a comparative group of non-treated patients should have been included in order to more favorably evaluate our results. This was not performed for ethical reasons, which is a weakness in the present study. Growth disturbances as described by Arvidsson et al. (2010) were seen in only two of our patients. The reason for this may be the early introduction of methotrexate in these patients.

Serological samples performed at the same moment as the SF sampling would have been a strengthening of this study. Comparison of blood and SF with regard to the laboratory analyses of bacterial DNA, cytokines and bone markers could possibly have given some answers with regard to the presence of SF bacterial DNA and its origin, and to local versus systemic effects of the cytokines and bone markers. 8 months follow-up sampling and control MRI with comparison of the development of all markers would have been highly interesting, but were not possible due to ethical reasons and geographical considerations.

In our study, JIA patients were both oligoarticular (n=10) and polyarticular (n=10), not counting the TMJ. Including the TMJ would have rendered all patients as having polyarticular JIA. We found no difference between these two groups, neither with regard to the TMJ symptoms and function, nor with regard to SF analyses. One child had systemic JIA, but had no divergent results as compared to the other children. Still, with so many unknown
influencing factors, such as presence and influence of bacterial DNA in SF, and all the different pathways in inflammation, bone homeostasis, and pain modulation, one must strive to perform this kind of clinical research as homogenic as possible. This is very difficult in such a heterogenic group of children with arthritis, problems starting with the classification of the diseases, and with the systemic and local effects upon the growing joints. We believe, however, the inclusion of JIA patients with the most significant pain and problems with jaw function in combination with a positive MRI, to be a positive strength in this study. To the best of our knowledge no previous study has neither measured cytokines and bone markers, nor has bacterial DNA and its diversity been detected in SF in JIA.

In spite of many years and many papers of research with regard to etiology, pathogenesis and treatment of JIA, RA and TMJ problems, little is still known in order to solve the center of all this research, that is people with this diseases and the everyday problems it may cause.

Research on rheumatic disorders raises questions, such as

- Are the patients correctly classified into a group of people with the same etiology and pathogenic response? That is, is the research performed on children and adults with the same etiology, genetic basis and inflammatory response resulting in TMJ arthritis?
- In what degree do the environmental factors and local non-immunologic factors affect the development of, and progression of TMJ arthritis?
- With the lack of a possibility to classify the degree of local arthritis, will this influence upon the degree of improvement?
- In what degree are bacteria able to induce arthritis? Are there any differences between the different bacterial species, or with different basic structures of bacteria, or their expressed antigens? Are the bacteria present in synovial fluid in arthritis causative, homed to an already damaged tissue, or previously present without pathogenic influence?
- Treatment with anti-TNF is common, and generally very successful in reducing symptoms in JIA and RA. At the same time, there are increasingly reports showing low TNF values in both serum and synovial fluid. What then, are the mechanisms of this positive anti-TNF response?
In what degree is the contralateral TMJ influenced by both unilateral TMJ arthritis and its treatment? Are these responses the same when this TMJ is pathologically influenced as well?

These questions, and many more, exemplify the complexity in performing research on diseases involving the immune system. In addition, there are other factors to be considered, developmental, psychological, physiological and social factors.

Conclusions

- Bacterial DNA from 14 different species was found in 60% of patients with JIA and RA, and these patients reported better jaw function and less pain compared to patients with arthritis and no bacterial DNA present (Paper I).
- The TMJ is not aseptic. It is possible to contaminate the joint in arthrocentesis, but we have also shown presence of bacterial DNA in the joint without contamination (Paper I).
- TNF-α and ACTH were elevated in TMJs with bacterial, and lower in JIA compared to adults (Paper I and II).
- TNF-α and OPG were lower in patients with a tendency to higher recorded pain levels (Paper II).
- With regard to age, there was a significant correlation with regard to total protein concentration (higher in children), leptin (higher in adults), and ACTH (lower in children with JIA than adults with JIA, but not lower than in RA) (Paper II).
- With regard to gender, there was a higher total protein concentration in boys. TNF, and in part IL-1, was negatively correlated to pain in boys, but not in girls (Paper II).
- A single intervention in the TMJ with either arthrocentesis or arthrocentesis in combination with local glucocorticoids is capable of significantly reducing pain and improving function in children with JIA and TMJ arthritis (Paper III).
- There was no difference upon improvement when comparing arthrocentesis with and without glucocorticoids (Paper III).
- We registered improvement of a single TMJ intervention even after 8 months (Paper III).
Bacterial DNA in TMJ SF may influence upon the local inflammatory response, and may affect both bone homeostasis as well as local treatment. Further studies are needed in order to better understand both presence of bacteria in human tissue, the immune response upon both inflammation and bone homeostasis, and thereby a better and more precise treatment of TMJ arthritis in JIA and RA.

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