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The role of miR-150 in the physiopathology of oligoarticular juvenile idiopathic arthritis

Diploma thesis

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Statutory declaration

I declare that the enclosed diploma thesis is my original author work. All literature and other sources of information, that I used while processing, are listed in the „references“ section and they are cited properly.

Hradec Králové, 5.5.2019

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Abstract

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Title of diploma thesis:

The role of miR-150 in the physiopathology of oligoarticular juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatoid disease affecting children, and its pathological mechanisms are still poorly understood. Innate and adaptive immunity including myeloid cells play a major role in these processes. Epigenetic deregulations along with non-coding microRNAs have been reported in many inflammatory diseases. Moreover, preliminary results obtained by the research group of Prof. Florence Apparailly showed accumulation of intermediate monocytes along with the high expression of miR-150 in the synovial fluid of children affected by oligoarticular JIA. Based on these findings a hypothesis has been postulated suggesting that miR-150 could have a role in the pathogenesis of this disease and in the regulation of monocyte differentiation and function. To study the impact of miR-150 on monocytes from the peripheral blood of healthy donors, transfection experiments were performed to neutralize miR-150. The phenotype of the cells was analysed by flow cytometry. In parallel, *in silico* analysis was carried out to find putative target genes using miRNA databases. RT-qPCR experiments were performed to analyse the expression of these genes in miR-150-modified monocytes. A decrease of intermediate monocytes was seen in 4 out of 6 transfection experiments with miR-150 inhibitor compared with the control. We also found 10 genes down-regulated and 27 up-regulated, 7 of them with statistical significance ($p < 0.05$). Comparing both *in silico* and *in vitro* experimental data, the results suggest that CCR2 might be a direct target of miR-150-5p in human monocytes. Taken together, we suggest that miR-150 might play a significant role in the pathogenesis of oligoarticular JIA by influencing the egress of monocytes from the bone marrow and their homing to inflamed tissues.

Abstrakt

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Název diplomové práce:

Role miR-150 v patofyziologii oligoartikulární juvenilní idiopatické artritidy

Juvenilní idiopatická artritida (JIA) je nejčastější chronické onemocnění revmatoidního charakteru postihující děti, přesto jsou mechanismy jeho patofyziologie z velké části neprozkoumány. Zásadní roli hrají v těchto procesech složky vrozené a získané imunity, včetně buněk myeloidní řády. U zánětlivých onemocnění byly popsány epigenetické deregulace zahrnující i nekódující mikroRNA. Mimoto, předběžné výsledky získané týmem Prof. Florence Apparailly ukázaly akumulaci přechodných monocytů a zároveň vyšší expresi miR-150 v synoviální tekutině dětí postižených oligoartikulární JIA. Na základě těchto objevů vznikla hypotéza naznačující určitou roli miR-150 v patofyziologii tohoto onemocnění, přesněji v regulaci diferenciaci monocytů a jejich funkcí. Za účelem prostudování účinku, který má miR-150 na monocyty, bylo provedeno několik transfekcí klasických monocytů (separovaných z periferní krve zdravých dárců) inhibitorem miR-150. Buňky byly následně označeny příslušnými protilátkami a podrobeny průtokové cytometrii se záměrem kvantifikace jednotlivých podtypů monocytů ve vzorku. Současně byl proveden in silico experiment, ve kterém byly využity miRNA databáze k nalezení potenciálních cílů miR-150. Pro analýzu genové exprese v monocytech transfekovaných miR-150 inhibitorem byla zvolena metoda RT-qPCR. Pokles přechodných monocytů byl zřetelný u 4 ze 6 transfekcí miR-150 inhibitorem ve srovnání s kontrolními vzorky. 10 genů ze 48 zkoumaných mělo potlačenu expresi a 27 mělo expresi zvýšenu, z toho 7 se statistickou významností ($p < 0.05$). Při srovnání in silico a in vitro experimentů data ukazují, že CCR2 by mohl být přímým cílem miR-150-5p v lidských monocytech. Zmíněné výsledky naznačují, že by miR-150 mohla opravdu hrát značnou roli v patofyziologii oligoartikulární JIA mobilizací monocytů z kostní dřeně a chemotaxí do tkání postižených zánětem.

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1 List of abbreviations

Ab – antibody

ANA – antinuclear antibodies

CD – cluster of differentiation (e.g. CD80)

cDNA – complementary deoxyribonucleic acid

CT – cycle threshold

DGCR8 – DiGeorge syndrome chromosomal/critical region 8

DMARDs – disease-modifying antirheumatic drugs

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

ESR - erythrocyte sedimentation rate

FACS – fluorescence-activated cell sorter

FBS – fetal bovine serum

hsa – homo sapiens/human

IgM – immunoglobulin M

IL – interleukin

ILAR – International League of Associations for Rheumatology

JIA – juvenile idiopathic arthritis

miR/miRNA – micro RNA (ribonucleic acid)

MRI – magnetic resonance imaging

mRNA – messenger RNA

NSAIDs – nonsteroidal anti-inflammatory drugs

Opti-MEM – improved Minimal Essential Medium

PBMC – peripheral blood mononuclear cell

PBS – phosphate-buffered saline

pre-miRNA – miRNA precursor

pri-miRNA – primary miRNA

RA – rheumatoid arthritis

RF – rheumatoid factor

RISC – RNA-induced silencing complex

RNase – ribonuclease

RPMI – Roswell Park Memorial Institute

RT – reverse transcription

RT-qPCR – real-time polymerase chain reaction, also known as quantitative polymerase chain reaction

SD – standard deviation

SF – synovial fluid

TLDA – TaqMan Low-Density Array

TNF – tumour necrosis factor

UTR – untranslated region

2 Introduction

Juvenile idiopathic arthritis (JIA) is a wide term that describes a clinically heterogeneous group of rheumatoid disorders of unknown etiology affecting children under 16 years of age. It is one of the most common and aggressive forms of inflammatory disease affecting joints of children.

Arthritic impairments occurring in children are difficult to characterize. Mechanisms involved in these diseases are poorly understood, which results in a constant revision of the classification. The few studies that are published highlight deregulation of innate and adaptive immunity including myeloid cells that are known to play a major role in inflammatory reactions. Impairments of monocyte accumulation have been reported in children with JIA, suggesting an involvement of these cells in the exacerbated inflammation responsible for joint destruction (Gaur et al. 2017). Epigenetic deregulations have also been identified in inflammatory diseases, including microRNAs (miRNAs) which are key regulators of gene expression (Stylianou 2019, Álvarez-Errico & Ballestar 2016). However, their implication in JIA remains largely unexplored.

The goal of this project is to better characterize the pathophysiological mechanisms occurring in children affected by idiopathic arthritis. To do so, specific interest is drawn toward miR-150, a miRNA which is highly expressed in the synovial fluid (SF) of children affected by oligoarticular JIA, according to the preliminary results. The future objective is then to understand the effect of this non-coding RNA on the function of monocytes from the SF of JIA patients.

3 Theoretical part

3.1 Juvenile idiopathic arthritis

3.1.1 Definition and classification

Juvenile idiopathic arthritis is a group of heterogeneous inflammatory diseases defined as arthritis of unknown etiology that starts before 16 years of age and persists for at least 6 weeks. According to the International League of Associations for Rheumatology (ILAR), there are 7 different subtypes of JIA (such as systemic arthritis, oligoarthritis, polyarthritis rheumatoid factor (RF)-positive, polyarthritis RF-negative, enthesitis-related arthritis, psoriatic arthritis and the last category that consists of patients with JIA who do not match any of the previous types or fit more than one). However, the classification and definitions of JIA subtypes are still being debated in order to make it more accurate and up-to-date with recent findings (Petty et al. 2004, Martini et al. 2018).

3.1.2 Epidemiology

JIA is one of the most common causes of chronic rheumatic disease in childhood that leads to short- and long-term disability. The incidence in European and North American countries varies from 2 to 20 per 100 000 people. The epidemiology of JIA differs throughout the world. In fact, a wide variation occurs in the prevalence of the diverse disease subtypes across different regions. Oligoarthritis is the most common type in Western countries while in India, New Zealand and South Africa it is the polyarthritic subtype that occurs the most and the systemic JIA predominates in Asia. The incidence also differs in the matter of age and sex according to the specific subtype. More detailed information on this subject is provided by the international study EPOCA (Epidemiology, treatment, and outcome of Childhood Arthritis) that has enrolled around 9000 JIA patients from more than 40 countries (Giancane et al. 2016, Consolaro et al. 2012).

3.1.3 Physiopathology

The cause of JIA is assumed to be multifactorial and the causative factors are still poorly understood but seem to include both genetic and environmental elements. The heterogeneity of JIA subtypes complicates the unanimous pathogenesis description. It is assumed that a genetically susceptible person could develop an uncontrolled immune response towards a self-antigen to an unknown environmental trigger of a self-perpetuating loop, which results in activation of both innate and adaptive immunity causing tissue damage and inflammation (Ravelli & Martini 2007, Prakken et al. 2011).

3.1.4 Clinical features

Patients affected by JIA regardless of the type often suffers from morning stiffness and stiffness after a long period of inactivity such as sitting or lying down for a larger amount of time. They also complain about morning arthralgia which usually gets better throughout the day (Jacobson et al. 2018).

Systemic arthritis has proportional representation among other subtypes within North America and Europe of about one-tenth. It affects equally both boys and girls and does not have a specific age of onset. In general, the patients at the time of the diagnosis show signs of serious illness, they are often fatigued and experience feverish conditions. According to ILAR criteria, systemic JIA is characterized as arthritis accompanied by fever lasting at least 2 weeks with a characteristic course with one or two fluctuations per day exceeding 39°C followed by a rapid return to baseline or below it. Additionally, at least one of the following symptoms is present: typical rash described as pink salmon (Figure 1), generalized lymphadenopathy, hepatomegaly, splenomegaly, or serositis. Myalgia and abdominal or chest pain may also occur and during the fever peak, it may be intense. During the early stage of the disease, arthritis might not be present, but it develops within a few weeks or months. As for laboratory deviations, leucocytosis, thrombocytosis, significant erythrocyte sedimentation rate (ESR) and C-reactive protein elevated concentration are present. Anaemia is also common and in some cases even a severe one (Ravelli 2015, Cassidy 2011).



Figure 1 Typical rash of systemic-onset disease in an 8-year-old boy (Ravelli & Martini 2007)

Oligoarthritis is described as arthritis that affects four or fewer joints during the first 6 months. It accounts for 50-80% of the total white children's population in North America and Europe affected by JIA. The patients are not systemically ill, and except for chronic uveitis, which affects almost 20% of those children, extraarticular symptoms are rather rare. The knee affection can sometimes cause growth abnormalities, resulting in one leg being longer than the other. There are two types of oligoarthritis, a persistent type if the number of affected joints remains below the limit of four, or extended type, if arthritis expands to more than four joints after first 6 months. Oligoarthritis has an early onset characterized by asymmetric joint affection (onset peaks between 2 and 4 years of age), female predilection and high frequency of positive antinuclear antibodies (ANA). The affliction has mainly an impact on smaller joints (fingers, toes and wrists, less often on elbows and temporomandibular joints), while hips and shoulders are rarely affected in this subtype. Affected joints are swollen, with the absence of redness, they are often warm and usually not very painful (Figure 2). In most than half of the patients, only one joint is affected. Acute phase reactants are often within the range of physiological values or just slightly elevated, except for the

ESR, which can be increased significantly. In approximately 80% of patients, ANAs are detected, which represents a risk of developing iridocyclitis (Ravelli 2015, Cassidy 2011, Macaubas et al. 2009).



Figure 2 Swelling of the right knee in a girl with oligoarthritis (Ravelli & Martini 2007)

RF-positive polyarthritis is an arthritis with later age of onset affecting 5 or more joints during the first 6 months and has at least 2 positive immunoglobulin M (IgM) RF tests measured at least 3 months apart. It is characterized by a symmetric polyarthritis of small joints of the limbs, wrists, affecting mainly adolescent girls. Cervical spine and the temporomandibular joints are also often affected. The most common extra-articular features detected in 30% of patients in the first year of the disease are rheumatoid nodules. The most common locations of these knots are flexor tendon sheaths, elbows, Achilles tendons and the soles of the feet (Ravelli 2015).

RF-negative polyarthritis is characterized as arthritis affecting 5 or more joints in the first period of 6 months, but unlike RF-positive, IgM RF is not present. RF-negative polyarthritis is the most diverse group of all the subtypes of JIA. It could be more subdivided into 3 groups, the first one is very similar to oligoarthritis, but unlike oligoarthritis, RF-negative polyarthritis affects more than 4 joints. The second group resembles the RF-negative polyarthritis seen in adults and has an onset at school age. The last subgroup is arthritis known as 'dry synovitis' characterized by not very significant joint swelling but prominent

stiffness. The third group often has a bad response to treatment and can have a destructive character (Ravelli 2015).

Enthesitis-related arthritis is associated with human leukocyte antigen (HLA)-B27, but without signs of RF or ANA. It is arthritis manifested mainly in boys after the 6th year of life, it affects, in particular, the lower extremities and hip joints are no exception. A typical sign, as the name of the subtype suggests, is enthesitis associated with arthritis. Enthesitis is most commonly found in the heel bone insertions of the Achilles tendon, the plantar fascia, and the tarsal area (Ravelli 2015).

Psoriatic arthritis differs from the other subtypes in the presence of arthritis and characteristic psoriatic rash or two of the following features: family history of psoriasis in a first-degree relative, dactylitis and nail pitting or onycholysis (Ravelli 2015).

Undifferentiated arthritis includes patients who do not meet the criteria of other types of JIA or who fall under more than one category. Thus, it is practically impossible to describe the clinical signs of this subgroup in sum (Ravelli 2015).

3.1.5 Diagnosis

The diagnosis of JIA is mainly based on the method of exclusion and is closely linked to clinical features of the subtypes of JIA mentioned above. A complete medical examination is needed alongside with an overview of personal and family history with an emphasis on pain and joint stiffness (Giancane et al. 2016).

The imaging of structural joint damage and growth disturbances is carried out either by conventional radiography, ultrasonography or by magnetic resonance imaging (MRI), which is the only procedure with the ability to assess all features of the synovial disease. MRI has the advantage of direct visualization of synovitis, cartilage and early erosive lesions (Giancane et al. 2016).

At this time there is no specific biomarker that could confirm the diagnosis of JIA. Anyway, several laboratory examinations can be used to support the

diagnosis, to confirm the presence of inflammation and to monitor the toxicity of therapy. High level of leukocytes, platelets and elevated ESR and C-reactive protein indicate the inflammation and the “acute phase response”. More disease-related markers include antibodies such as rheumatoid factors, anti-cyclic citrullinated peptide antibodies and antinuclear antibodies. A synovial fluid examination has also a significant role in JIA diagnosis (Petty et al. 2016).

3.1.6 Disease-related complications

If not treated or the treatment is insufficient, JIA can result in many health complications such as uveitis, anaemia, chronic pain, growth disturbances, pericarditis and arthritis-based joint destruction (Jacobson et al. 2018).

Uveitis is typically asymptomatic ocular inflammation, except for enthesitis-related arthritis, where it is presented as painful pink eye. It is manifested as inflammation of the iris and ciliary body and if left untreated it may result in eye damage such as cataracts, glaucoma and even in total blindness (Jacobson et al. 2018, Cassidy 2011, Crayne et al. 2018).

Growth abnormalities are presented as generalized, resulting in growth retardation (reported in cca. 50% of adult patients, affected by JIA in childhood), or localized growth disturbances. Both pro-inflammatory cytokines (tumour necrosis factor (TNF) alpha, interleukin (IL)-1, IL-6) and systemic glucocorticoids are responsible for the reduced height by targeting the insulin-like growth factor. In the case of localized abnormalities, the growth is in the vast majority accelerated, most likely because of the hyperaemia caused by the inflammation. On the other hand, the chronic inflammation can also cause the premature closure of the epiphyseal plate, which therefore results in growth retardation and shorter limbs (Cassidy 2011, Crayne et al. 2018).

Joint destruction occurs in patients with uncontrolled arthritis. It may lead to contractures, limit the range of motion, cause bone deformities, loss of cartilage with the result of significant disability. It is more common in the polyarticular type of arthritis (Crayne et al. 2018).

3.1.7 Treatment

The management of the treatment of JIA patients consists of pharmacological and non-pharmacological measures. Nowadays, JIA cannot be cured, but disease control is commonly achieved. The goal is to attain disease remission, to have control over the pain, minimize movement limitations and prevent systemic complications. Normal nutrition, physical and occupational therapy and psychological development are no less important. In some cases, orthopaedic surgery takes place in the disease management (Petty et al. 2016).

There are four major pharmacologic groups that are being used in the therapy of JIA. The first class consists of nonsteroidal anti-inflammatory drugs (NSAIDs) that are the most commonly used medications in the treatment of JIA including naproxen, ibuprofen and indomethacin. NSAIDs have both anti-inflammatory and analgesic effect by the blockade of production of prostaglandins (Jacobson et al. 2018).

The second group is formed by glucocorticoids, either as local or systemic therapy. Intra-articular glucocorticoid injections mainly containing triamcinolone hexacetonide/acetone are earmarked for patients with localized disease. The less used option (due to their adverse effects) are systemic glucocorticoids prednisone and methylprednisone, which now have in the management of JIA more of a historical meaning (Jacobson et al. 2018).

Nonbiologic disease-modifying antirheumatic drugs (DMARDs) are a class of JIA pharmacotherapy that slows the progression and prevents long-term morbidity. The most commonly used nonbiologic DMARDs are methotrexate, leflunomide and sulfasalazine (Jacobson et al. 2018).

Biologic DMARDs are the most studied treatments and represent a very promising option in the management of JIA. They are administered either via the intravenous or subcutaneous routes. They act specifically either by inhibiting TNF- α (etanercept, adalimumab and infliximab), inhibiting T-cell activation by binding to cluster of differentiation (CD80 or CD86) of antigen-presenting cells (abatacept), they can inhibit interleukin actions (IL-1 receptor inhibitors – anakinra, canakinumab, riloncept and IL-6 receptor inhibitor - tocilizumab) or

they act via inhibiting inflammatory cascade through binding on B-lymphocyte surface protein CD20 (rituximab) (Jacobson et al. 2018).

3.2 MicroRNA

MicroRNAs are a group of small, non-coding RNA molecules involved in multiple biological processes and post-transcriptional regulations of cellular function, such as their development, differentiation and proliferation along with metabolism, apoptotic cell death, viral infection and cancer. These small RNAs are formed of approximately 22 nucleotides and they represent a relatively new but very promising field of study – the first miRNA was isolated in 1993 by Lee, Feinbaum and Ambros (Huang et al. 2011, Kumar Kingsley & Vishnu Bhat 2017).

3.2.1 Biogenesis of microRNAs

The procedure of miRNAs formation is a complex multi-step process taking part in both the nucleus and cytoplasm. In animals, genes for miRNAs biogenesis are transcribed to a primary miRNA (pri-miRNA). Pri-miRNA is characterized by a hairpin RNA structure which is therefore recognized by ribonuclease (RNase) III enzyme called Drosha and its cofactor - DiGeorge syndrome chromosomal/critical region 8 (DGCR8) and processed into miRNA precursor (pre-miRNA). The pre-miRNA is then transported from the nucleus to the cytoplasm by a protein named exportin-5. In the cytoplasm, pre-miRNA is cleaved by Dicer (RNase III-type endonuclease) and loaded onto the Argonaute protein to produce the effector RNA-induced silencing complex (RISC), which completes miRNA biogenesis (Bartel 2004).

Mature miRNA originates from either the 5' arm or the 3' arm of the hairpin precursor and, dependently on the arm from which it is derived, is denoted with a -5p or -3p suffix (Kozomara & Griffiths-Jones 2013).

The process of miRNAs biogenesis is illustratively displayed in Figure 3.

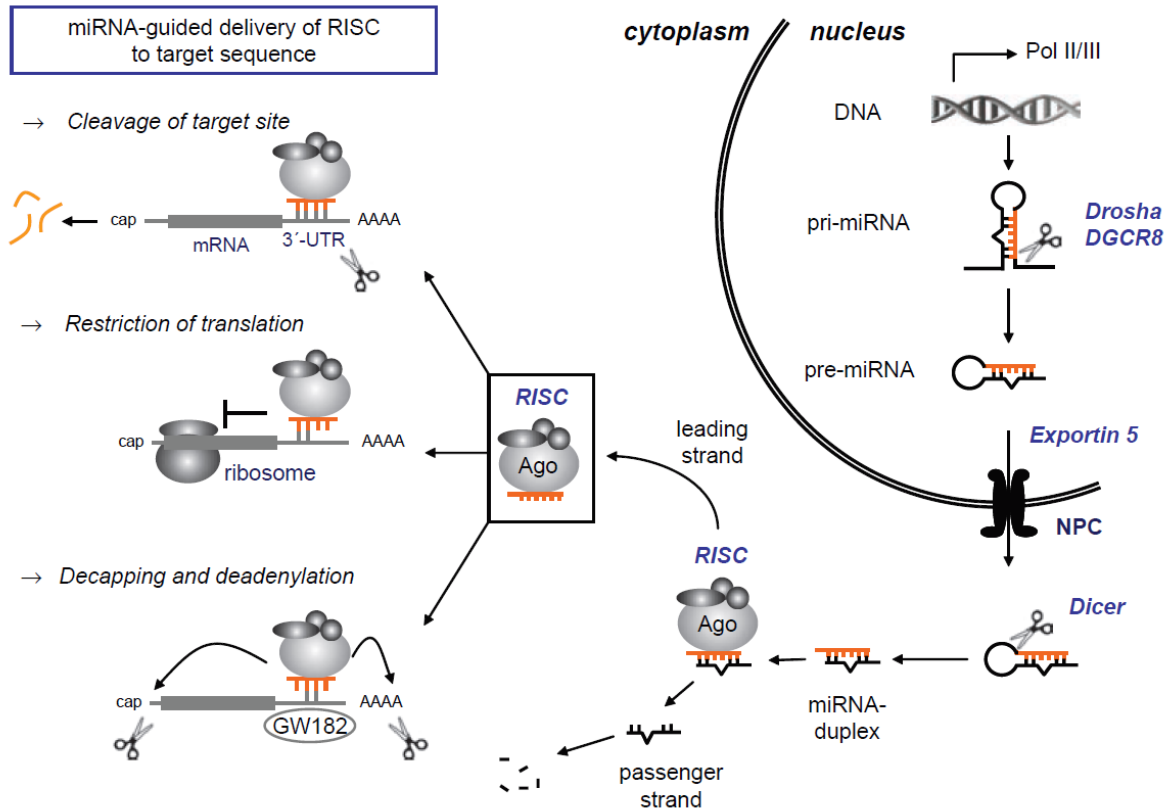


Figure 3 Biogenesis and mechanism of action of microRNAs (miRNAs) (Wittmann & Jack 2011)

The process in the nucleus consists of the genesis of pri-miRNA via the transcription by RNA polymerase II or III, the structure is then processed into pre-miRNA by RNase III enzyme Drosha and its cofactor DGCR8. The pre-miRNA is then exported into the cytoplasm by exportin 5 and processed into the mature double-stranded miRNA by the RNase III enzyme called Dicer. After associating with the RNA-induced silencing complex the miRNA strands are separated. Whilst the passenger strand is degraded, the leading strand and RISC bind predominantly to the 3'-UTR of mRNAs, which thereafter leads either to the cleavage of target sites, restriction of translation or decapping and deadenylation of the mRNA (Wittmann & Jack 2011).

3.2.2 Mechanism of action and biological functions of microRNAs

Once a single-stranded miRNA molecule is part of the RISC complex, it can bind to the messenger RNA (mRNA), with few exceptions, to the untranslated region (UTR) at the 3' end of mRNA. By linking to this complex, the mRNA is no longer to be translatable - either mRNA is cleaved and subsequently degraded or the mRNA remains undivided, but the RISC complex physically prevents ribosome to link to the mRNA, thereby inhibits the expression (Wittmann & Jack 2011, Furer et al. 2010, Carthew & Sontheimer 2009).

It is estimated that miRNA genes make up 1-2% of eukaryotic genomes and thus represents an important group of regulators involved in a broad spectrum of different physiological cellular processes. This means that up to one-third of all protein-encoding mRNAs could be regulated by miRNAs. miRNAs are known to function predominantly as inhibitors of the expression of target mRNAs either by repressing translation or via direct cleavage of mRNAs (Furer et al. 2010).

The interaction between every mature miRNA and a specific mRNA is typically realized through the pairing of nucleotide bases, the pairings do not have to be perfect across the whole mature miRNA sequences, in mammals, it is dominated by the so-called seed region consisting of 8 nucleotides. An important determinant of the regulatory mechanism is the extent of the complementarity of miRNA and its target gene. The seed region is alpha and omega for target recognition and silencing and is also among others, an important element in computational approaches of miRNA targets prediction tools (Furer et al. 2010, Kehl et al. 2017).

miRNAs play a key role in regulations of many biological processes including regulation of the immune system (Duroux-Richard et al. 2012; Vicente et al. 2016), and their abnormal expression is involved in the pathophysiology of immune-mediated inflammatory disorders (Huang et al. 2011). A restricted number of studies, which focused on the relationship of miRNAs and JIA suggest a link between these small RNAs and JIA pathophysiology (Kamiya et al. 2015, Li et al. 2016, Ma et al. 2013, Sun et al. 2016).

Based on the literature along with unpublished data obtained by the team of Prof. Apparailly showing high expression of miR-150 in the joints of patients with the oligoarticular type of JIA, a focus was drawn towards this miRNA.

3.2.3 miR-150

miR-150 is nowadays a frequent subject of study, several articles showed a link between this miRNA and many biological functions. It was shown that the expression of miR-150 is significantly increased in rheumatoid arthritis (RA)

patients with severe joint destruction, both in peripheral blood mononuclear cells (PBMCs) and synovial fluid (Niimoto et al. 2010). It was shown that miR-150 plays a role in hematopoietic cell differentiation (Vasilatou et al. 2010, He et al. 2014) and in inflammatory responses in macrophages (Zawada et al. 2017). It also regulates the mobilization and migration of bone marrow-derived mononuclear cells including monocytes into the circulation (Tano et al. 2011). Liu et al. (2015) suggested miR-150 as a potential target for therapeutic intervention in the setting of ischemic heart disease through the regulation of production of proinflammatory cytokines and monocyte migration. Vasilescu et al. (2009) proposed miR-150 as a potential marker of early sepsis, as the level of miRNA in leukocytes and plasma correlates with the severity of sepsis.

According to articles mentioned above, miR-150 is considered as pro-inflammatory (Liu et al. 2015, Niimoto et al. 2010, Vasilescu et al. 2009). Moreover, in a recent study, Selimoglu-Buet et al. (2018) showed a correlation between this miRNA and the differentiation of monocytes towards non-classical subtype. These and many other studies suggest miR-150 not just as a promising biomarker of various pathological conditions but also as a potential therapeutic target.

3.3 Monocytes

3.3.1 Characterization

Monocytes are by size one of the largest subtypes of the white blood cells spectrum. They are produced in the bone marrow from pluripotent stem cells and after their release, they make up approximately 5% of the circulating cell population, this amount deviates towards higher numbers during infection. They play a key role in the body defences, as they can process and present antigen to T lymphocytes, and moreover, through the secretion of biologically active substances, they can regulate many processes. They also have tumoricidal and antimicrobial abilities. The viability of circulating monocytes is around 24 hours, however, some of them migrate into tissues or to damaged locations where they eventually mature into macrophages (Delves & Roitt 1998, Monie 2017).

3.3.2 Monocyte subtypes

The classification approved by the Nomenclature Committee of the International Union of Immunological Societies divides human monocytes into 3 subtypes based on the expression of the cell-surface markers CD14 and CD16 (Ziegler-Heitbrock et al. 2010). These subgroups are thought to play different roles in inflammatory, repair and healing processes (Anbazhagan et al. 2014).

The biggest population typical with high CD14 but no CD16 expression ($CD14^{++}CD16^{-}$) is called **classical monocytes** and it accounts for 90% of total blood monocytes. These monocytes occur to be rather all-round, being able to respond to many external stimuli, mediating tissue repair or immune response. It is assumed that classical monocytes play a key role in antibacterial activity (Cros et al. 2010), inflammatory and atherosclerotic processes. They produce inflammatory cytokines such as $TNF-\alpha$, IL-1b and IL-6 and they take a big part in osteoclasts differentiation (Anbazhagan et al. 2014, Wong et al. 2011).

Non-classical monocytes ($CD14^{+}CD16^{++}$) are one of the two remaining minor groups, each covering around 5% of total blood monocytes. Non-classical monocytes are being associated with cytoskeletal dynamics, which allows them

to travel easily into the bloodstream, and give them the name of “patrolling monocytes”. They participate in repair and healing processes but there is also a correlation with inflammatory diseases as they are key actors of the viral surveillance (Cros et al. 2010). They are known for their high motility and patrolling behaviour and as the monocyte maturity is believed to be connected with CD16 expression along with proapoptotic functions, they are considered to be the most mature monocyte subset (Anbazhagan et al. 2014, Wong et al. 2011).

The last subtype called **intermediate monocytes** (CD14⁺⁺CD16⁺) is considered to be the representation of a direct intermediary link between the classical and nonclassical monocytes (Wong et al. 2011). In comparison with classical and non-classical monocytes, this subset has higher basal reactive oxygen species and inflammatory cytokines production when stimulated with lipopolysaccharide and therefore has significant proinflammatory potential. These monocytes also have a higher potential for antigen processing along with its presentation and have been described as predictors of cardiovascular events. Intermediate monocytes are also linked to angiogenesis (Zawada et al. 2017). Moreover, it has been shown that they are highly accumulated in the joints of RA patients (Smiljanovic et al. 2018).

4 Aim of the study

JIA is the most common form of the chronic rheumatic disease in childhood (Ravelli & Martini 2007), however, mechanisms involved in this pathology are still largely unknown. Preliminary results that were obtained in the laboratory revealed that JIA synovial fluid is enriched with intermediate monocytes and that coculture of classical monocytes isolated from healthy donors with JIA SF induces a switch of these monocytes from classical to intermediate. Moreover, the levels of miR-150 are highly elevated in SF of JIA patients in comparison with septic arthritis in children (unpublished data). The literature shows that miR-150 is involved in hematopoietic differentiation (Huang et al. 2011), as well as in monocytes mobility (Kumar Kingsley & Vishnu Bhat 2017, Wittmann & Jack 2011), and a study on RA also highlighted a correlation between the high expression of this miRNA and joint destruction (Niimoto et al. 2010). Therefore, the research team of Prof. Apparailly hypothesized that this miRNA might play a role in the pathogenesis of JIA. The goal of my project was to study the impact of miR-150 on monocytes that are involved in the inflammatory reaction located in children joints. Using *in silico* and *in vitro* functional analyses, I aimed at better understanding the mechanisms involved in JIA pathophysiology.

First, to investigate the effect of miR-150 on the monocytes, I performed several transfections of classical monocytes isolated from healthy patients and compared their phenotype and transcriptome with monocytes transfected with control miRNA. In parallel, *in silico* experiments were performed to identify genes potentially targeted by miR-150 and the list was compared with genes involved in monocyte differentiation and function. Finally, using real-time polymerase chain reaction (RT-qPCR), I analyzed the expression of the candidate genes in miR-150-modified monocytes.

5 Materials and methods

5.1 Materials

Lists of all reagents (Table 1), labware and disposables (Table 2), devices (Table 3) and software (Table 4) used in this work.

Table 1 List of reagents used in this work

Reagents	Manufacturer
Roswell Park Memorial Institute (RPMI) 1640 Medium, GlutaMAX™ Supplement	Gibco™
Fetal Bovine Serum, certified, heat inactivated	Gibco™
Streptomycin/Penicillin	Gibco™
Glutamine	Gibco™
Trypan blue solution	Sigma-Aldrich
PBS, pH 7,2,	Gibco™
Mirneasy minikit	QIAGEN
Chloroform RPE	Carlo Erba reagents
Tris-EDTA buffer	Sigma-Aldrich
hsa-miR-150 RT primer	Thermo Fisher Scientific
hsa-miR-150 TM primer	Thermo Fisher Scientific
RNU6b RT primer	Thermo Fisher Scientific
RNU6b TM primer	Thermo Fisher Scientific
High capacity microRNA Reverse Transcriptase kit	Applied Biosystems
High Capacity cDNA Reverse Transcriptase kit	Applied Biosystems
TaqMan Universal Master mix	Applied Biosystems
Ficoll® Paque Plus	GE Healthcare
DMSO HYBRI-MAX®	Sigma-Aldrich
autoMACS Running Buffer	Miltenyi Biotec
CD14 MicroBeads, human, MACS	Miltenyi Biotec
Opti-MEM™ I Reduced Serum Medium,	Gibco™
FuGENE® HD Transfection Reagent	Promega
hsa-miR 150 miRVana	Thermo Fisher Scientific
MirVana™ – miRNA inhibitor Negative Control #1 Ambion	Thermo Fisher Scientific
Zombie Violet™ Fixable Viability Kit (DMSO)	BioLegend
BD Pharmingen™ Human BD Fc Block	BD Biosciences
Mouse anti-human CD14 BD Pharmingen	BD Biosciences
Mouse anti-human CD16 BD Pharmingen	BD Biosciences
VERSYLENE® FRESENIUS – sterile water	Fresenius SE
TagMan® Custom Arrays 48 genes	Applied Biosystems

Table 2 List of labware and disposables used in this work

Labware and disposables	Manufacturer
TipOne 10/20 µl XL	Isogen Life Science
TipOne 200 µl	Isogen Life Science
TipOne 1000 µl	Isogen Life Science
Eppendorf Dualfilter 20 µl	Sigma-Aldrich
Countess™ cell counting chamber slide	Invitrogen
Corning flask 25 cm ²	Sigma-Aldrich
Rneasy MinElute Column	QIAGEN
Rotor adapters	QIAGEN
Disposable filter-tips 200 µl, 1000 µl	QIAGEN
MACS Smart Strainer, 30 µl	Miltenyi Biotec
LS column MACS	Miltenyi Biotec
QuadroMACS™ Starting Kit (LS)	Miltenyi Biotec
MACS MiltiStand	Miltenyi Biotec
Tissue Culture Plate, 24 well FALCON	Thermo Fisher Scientific
Elution tubes 1,5 ml	QIAGEN
Eppendorf safe-lock tubes 0,5 ml	Sigma-Aldrich
FALCON tube 50 ml	Thermo Fisher Scientific
CORNING tube 15 ml	Sigma-Aldrich
2ml, 5 ml, 10 ml, 25 ml, 50 ml – Falcon pipettes	Thermo Fisher Scientific
Strip tubes 0,1 ml	QIAGEN
Strip caps	QIAGEN

Table 3 List of devices used in this work

Devices	Manufacturer
Safety cabinet Class II	Thermo Fisher Scientific
Countess II FL	Life Technologies
PIPETBOY NI-MH 9V 180 mAh	Integra Biosciences
Multifuge X3 FR	Thermo Fisher Scientific
Eppendorf centrifuge 5415R	Sigma-Aldrich
Eppendorf centrifuge 5417C	Sigma-Aldrich
Jouan GR4i centrifugation	Thermo Electron Corporation
SANYO CO ₂ incubator MCO-19AIC (UV)	Marshall Scientific
TOP-MIX Bioblock scientific	Thermo Fisher Scientific
Microcentrifuge 3722L	Thermo Fisher Scientific
QiaCube	QIAGEN
C1000 Thermal Cycler	Bio-Rad
Rotor-Gene-Q	QIAGEN
NanoDrop One	Thermo Fisher Scientific
BD FACSCanto™ II Flow Cytometer	BD Biosciences
Fumehood LCCA ISO 9001	LCCA ISO 9001
ViiA 7 Real-Time PCR System	Thermo Fisher Scientific

Table 4 List of software used in this work

Software	Manufacturer
Microsoft Office	Microsoft Corporation
BDFACSDiva™	BD Biosciences
Via7 RUO software	Thermo Fisher Scientific
RotorGene Q series software	QIAGEN
Venny 2.1.0	BioInfoGP
GraphPad Prism 8	GraphPad Software

5.2 Purification of blood monocyte subsets

PBMCs (from the blood of 3 healthy donors) were obtained after Ficoll density centrifugation. The principle of this method is based on the separation of blood cells using a centrifugal force. During the centrifugation, each component moves through the medium based on their sedimentation coefficient differences and remains where the surrounding density of the medium corresponds to its own (Figure 4).

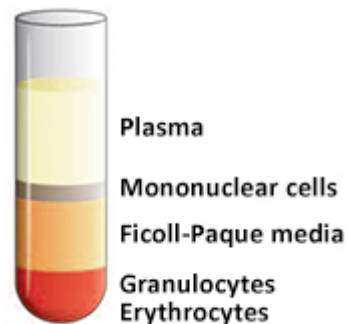


Figure 4 Principle of density centrifugation separation method

(Source: Sigma Aldrich [cit. 2019-03-12]. Available from: <https://www.sigmaaldrich.com/technical-documents/protocols/biology/isolation-of-mononuclear-cells/recommended-standard-method.html>)

The blood was diluted by Phosphate-buffered saline (PBS) in ratio 1:3 (8 ml of blood with 24 ml of PBS) and thereafter 32 ml carefully layered on 12 ml of Ficoll-Paque Plus and centrifuged (20 min at 400g, 20°C, with minimum brake and acceleration). The PBMCs were then recovered after removal of plasma layer, washed in PBS (centrifugation 10 min at 300g, 4°C), pellets were resuspended in PBS and pooled into one 50 ml tube adding PBS up to 45 ml and another washing took place with the same conditions. To remove the platelets,

the pellets were resuspended in 45 ml of PBS and centrifuged two times at 200g (lowering brakes to the minimum) first time for 20 minutes, the second time for 10 minutes. The cells were resuspended in PBS and underwent filtration using 30 μ m filter to remove cellular aggregates and then counted.

To isolate the CD14⁺ monocytes, the cells were centrifuged (10 min at 300g, 4°C) and the pellet was resuspended in running buffer containing PBS, bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), and 0.09% sodium azide (800 μ l per 1×10^8 cells). 200 μ l of CD14⁺ microbeads per 1×10^8 cells were added, mixed properly and incubated at 4°C for 15 minutes. After the incubation, another 10 ml of running buffer per 1×10^8 cells were added and the sample was centrifuged (10 min at 300g, 4°C). The supernatant was removed and the pellet resuspended in 500 μ l of running buffer per 1×10^8 cells. The cell suspension was put onto LS column after rinsing it by 3 ml of running buffer making sure that the column would not run dry. After that, the column was washed by adding 3 ml of running buffer three times, in order to remove cells that are not CD14⁺ and therefore not attached to the microbeads and to the column. Then another 5 ml of the buffer were put onto the column and the magnetically labeled cells were immediately flushed into a separate tube by firmly pushing the plunger into the column and the cell number was determined.

5.3 Transfection of CD14⁺ monocytes

4×10^5 of CD14⁺ monocytes were seeded in 24 well-plate with 400 μ l of an improved Minimal Essential Medium (Opti-MEM) reduced serum media containing 1% of Hi-fetal bovine serum (FBS) per one well, this medium allows for a reduction of FBS supplementation with no change in growth rate or morphology. The cells were transfected by human (hsa)-miR-150 inhibitor and MirVanaTM miRNA inhibitor Negative Control #1. For both conditions, we performed the transfection in 5 wells. The plate was put into an incubator at 37°C for 2 hours.

The transfection mix was prepared in 24 well plate to avoid the reaction of Fugene HD reagent with the plastic of test-tubes. For one well of CD14⁺ cells, the following mix was prepared:

MIX 1: 0.5 µl of Fugene HD with 49.5 µl of Opti-MEM serum-free medium

MIX 2: 2 µl of miRNA inhibitor (20 µM) with 48 µl of Opti-MEM serum-free medium

Those two mixes were put together and incubated for 10 minutes at room temperature. After that 100 µl of that mixture was put on the monocytes and incubated overnight at 37°C (min for 6 hours). At the end of the transfection 500 µl of RPMI medium, enriched of 20% of Hi-FBS, 1% of glutamine and 1% of penicillin/streptomycin, was added in each well and incubated for 24 hours at 37°C.

After the incubation, the cells from one condition were put together, washed in PBS and counted. A part of it was used for cytometry analysis (approximately 8×10^5 of cells) and the rest for the miRNA and mRNA quantification.

5.4 Cytometry analysis

Flow cytometry is a method which allows the profiling of large populations of cells, one by one, thanks to highly specific antibodies (Ab) tagged with fluorescent dyes. The mechanism is based on flow cell sorting according to fluorescence excitation at certain wavelengths. The cells are passed one after the other through a laser light beam and the light emitted by the cells is thereafter measured by a detection apparatus. The flow cytometer consists of three systems: fluidics, optics, and electronics, each playing a key role.

Before the analysis on the cytometer, cells were stained with 150 µl of zombie dye (Zombie Violet™ Fixable Viability Kit), to be able to detect dead cells, and incubated 15 minutes at room temperature and out of direct light. After washing in PBS, the cells were incubated at room temperature (10 minutes minimum) in 150 µl of Fc block 2% solution resulting in reducing the non-specific Ab bonds. The samples were transferred into 96 well-plate, centrifuged (240g, 3 minutes, 4°C) and stained with diluted CD14-FITC and CD16-APC antibodies (20 µl of 5% Ab in PBS solution per condition). The cells were incubated for 15 minutes at 4°C, washed in PBS and resuspended in 100 µl of PBS.

After that, the cytometry analysis was performed using BD FACSCanto™ II Flow Cytometer and BD FACSDiva™ Software.

5.5 RNA extraction

Manipulations with RNA samples were performed in an RNase free environment. After the samples were taken out from the freezer, they were incubated at room temperature for 5 minutes, 140 µl of chloroform was added to each sample, they were vortexed for 20 seconds and afterward kept 90 seconds at room temperature to allow the stabilization of the different phases. Centrifugation at 20 000g for 15 minutes at 20°C was performed to separate nucleic acids from proteins. After that, around 350 µl were taken from the aqueous (upper) part of the emulsion and transferred in 2 ml tubes. Then the RNAs were extracted using QIAcube and miRNeasy minikit.

QIAcube enables automation of RNA extraction. It does so by following the same steps (lysis and binding of the nucleic acids to the silicon membrane of the spin columns, which are then washed and thereafter eluted).

Concentration and quality of extracted RNA were measured using NanoDrop and the samples were put into a freezer at -20°C.

5.6 Reverse transcription

To be able to perform the real-time polymerase chain reaction, RNA samples first needed to be transcribed into complementary deoxyribonucleic acid (cDNA) by the process called reverse transcription. A key role in this procedure is exerted by an enzyme called reverse transcriptase that allows the RNA to be transcribed.

For the microRNA reverse transcriptions (RTs), mixes were prepared using TaqMan microRNA Reverse Transcriptase kit (Table 5). Primers were diluted in 1XTris EDTA buffer in ratio 1:99. Afterward, 12 µl of the mix and 3 µl of RNA (diluted with RNase-free water to get the final concentration 100 ng per 3 µl) were transferred into 0.2 ml tubes and put into thermocycler following this program: 30 min at 16°C/ 30 min at 42°C/ 5 min at 85°C/ 4°C.

For the mRNA RTs, mixes were prepared using the High Capacity cDNA Reverse Transcription kit (Table 5). 10 μ l of this mix was transferred into 0.2 ml tubes together with 10 μ l of RNA diluted with RNase-free water to get the final concentration of 100 ng per 10 μ l. Then the samples were put into thermocycler (10 min at 25°C/ 120 min at 37°C/ 5 min at 85°C/ 4°C).

Neither reverse transcriptase nor RNase inhibitor was vortexed to not disrupt their integrity and function.

Table 5 The composition of mixes for RT of microRNA (A) and mRNA (B) needed for one reaction (dNTP – deoxynucleotide)

A	N=1	B	N=1
RT primer pool	6 μ l	RT buffer	2 μ l
dNTP mix	0.3 μ l	dNTP mix	0.8 μ l
Reverse Transcriptase	3 μ l	Multiscribe reverse transcriptase	1 μ l
10X RT buffer	1.5 μ l	RT random primers	2 μ l
RNase inhibitor	0.19 μ l	Nuclease-free water	4.2 μ l
Nuclease-free water	1.01 μ l	total	10 μ l
total	12 μ l		

5.7 Real-time polymerase chain reaction

A polymerase chain reaction is a method that allows specific gene quantification after an amplification step. The extremities of the sequences are set by specific primers. The polymerase chain reaction consists of several steps: denaturation of cDNA, when due to the high temperature the hydrogen bonds are disrupted. After this step the temperature lowers, which allows primers to attach to specific parts of the strands, giving the enzyme called deoxyribonucleic acid (DNA)-polymerase a starting point for the synthesis of a new strand. The temperature rises again and the cDNA sequences are being polymerized in a direction from the 5' end to the 3' end. These steps are repeated several times.

RT-PCR is a technique that allows the quantification of RNA sequences over time. For the detection, we used the TaqMan Sequence Detection chemistry, which uses an oligonucleotide probe that contains a fluorescent dye (a reporter) on the 5' end, whereas on the 3' end a quencher dye is situated, which greatly reduces the fluorescence. The fluorescence is emitted while the probe is broken down in the process of synthesis of a new DNA strand and the quencher is no longer in the proximity of the reporter (Figure 5).

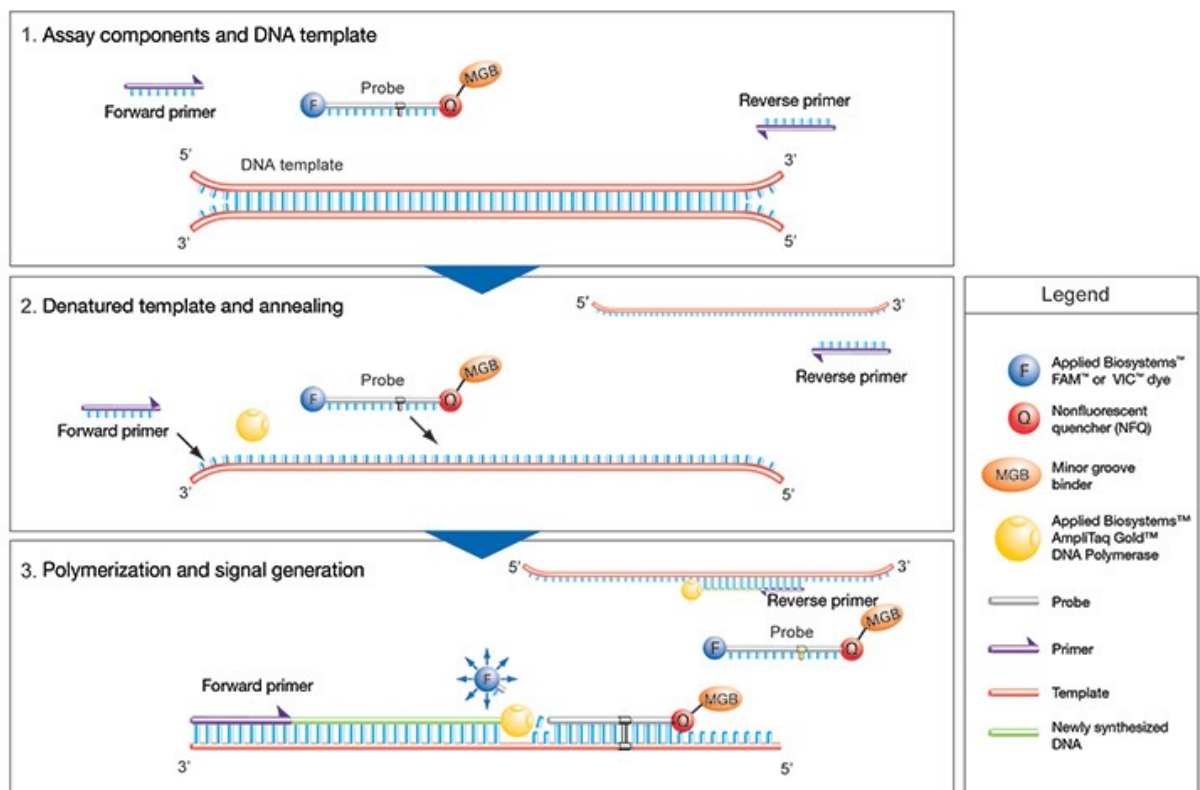


Figure 5 TaqMan chemistry probe mechanism – in the process of the synthesis of a new DNA strand, the polymerase cleaves the probe, which allows the fluorophore to be analyzed (Source: Thermo Fisher Scientific [cit. 2019-03-12]. Available from: <https://www.thermofisher.com/cz/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html>)

qPCR was carried out by RotorGene (miRNA analysis) or ViiA 7 (TaqMan Low-Density Array (TLDA) assay) using TaqMan Universal Master mix. The reaction mixes had different composition depending on the type of material that was quantified.

a) qPCR after the RT of miRNA

Reaction mix for each sample:

TaqMan Universal Master mix	5 μ l
RNase-free water	2 μ l
cDNA	1 μ l

Specific primers were diluted (primer/Tris EDTA buffer = 1/3 \rightarrow 10 μ l of primer diluted with 30 μ l of 1XTris EDTA) and then 2 μ l of this solution was transferred into strip tubes (0.1 ml) alongside with 8 μ l of the reaction mix listed above.

b) qPCR after the RT of mRNA

TaqMan Universal Master mix	50 μ l
cDNA	15 μ l
RNase-free water	35 μ l

Those volumes were used for one condition and had to be adapted according to the number of samples involved.

The program was unanimous for all quantifications regardless of the device used: 10 min at 95°C/ (15s at 95°C/ 60s at 60°C) for 40 cycles.

5.8 In silico analysis of putative targets of miR-150

We used four different databases (miRWalk, RNA22, miRanda and Targetscan) in order to find putative genes targeted by hsa-miR-150-5p with the three prime untranslated region (3'UTR) as a region of interest. And to more specify the search, the following input parameters were set: minimum seed length: 7 and/or p-value: 0.05.

Only targets, found by 3 or 4 of the databases mentioned above, were considered to have information value.

5.9 Statistical analysis

For data presentation, the arithmetic mean was used along with standard deviations. Two data sets were compared using a two-sample paired-t-test. Only those results with a p-value of less than 0.05 were considered as statistically significant.

6 Results

6.1 Transfection of CD14⁺ monocytes with miR-150 inhibitor and its impact on monocyte differentiation

To see the effect that miR-150 has on the monocytes, I performed several transfection experiments.

Seven transfections of CD14⁺ monocytes isolated from healthy patients were carried out independently of each other using a miR-150 inhibitor, following the protocol mentioned in methods (5.3). As a control, we used MirVana™ miRNA inhibitor Negative Control #1.

6.1.1 Transfection efficiency

To evaluate the transfection efficiency, I quantified the miR-150 expression of each sample using RT-PCR and RNU6b as a housekeeping gene. The results (displayed in Figure 6 as means of delta cycle thresholds (CTs) of RT-PCR duplicates \pm standard deviation (SD)) indicate that the transfections were successful, except for sample number 5, whose miR-150 expression was higher after the transfection with the inhibitor than with the control irrelevant sequence.

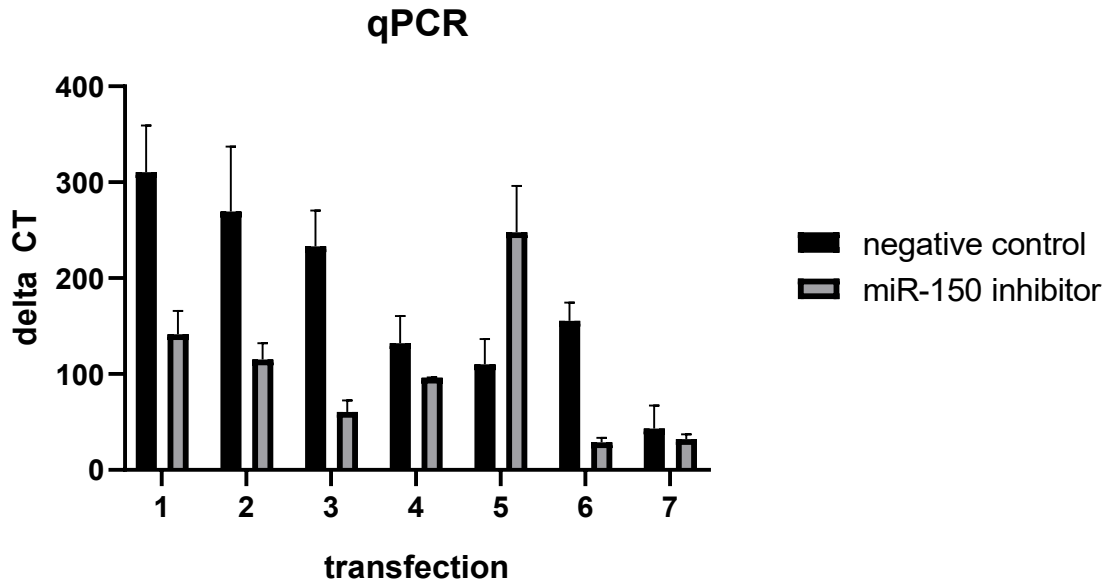


Figure 6 Expression of miR-150 analyzed by RT-PCR in 7 independent transfections of isolated human monocytes by miR-150 inhibitor. Expression analyses were performed employing RT-PCR using RNU6b as a housekeeping gene. Results are presented as means from duplicates \pm SD

6.1.2 Cytometry analysis of the impact of miR-150 on CD14 and CD16 expression on monocytes

To study the impact of miR-150 on monocyte phenotype, I labeled the cells that underwent the transfection, as mentioned in methods (5.12), and performed fluorescence-activated cell sorter (FACS) analysis. Dead cells were excluded from the analysis using the Zombie Violet™ Fixable Viability Kit. In order to reduce non-specific antibody bindings, we used the Human BD Fc Block™. The percentages of double positive monocytes (CD14⁺, CD16⁺) are shown in Figure 7 and Table 6. The respective proportion of double positive monocytes were not uniform, a decrease was seen after transfection of samples number 2, 3, 4 and 7, on the other hand after transfection of samples 1, 5 and 6 we detected increase instead. However, we must take into account that the transfection number 5 was not successful according to qPCR quantification of miR-150. In comparison with the previous transfections, I observed significantly lower relative expression of double positive cells in both mir-150 inhibitor and the negative control after transfection of sample number 7.

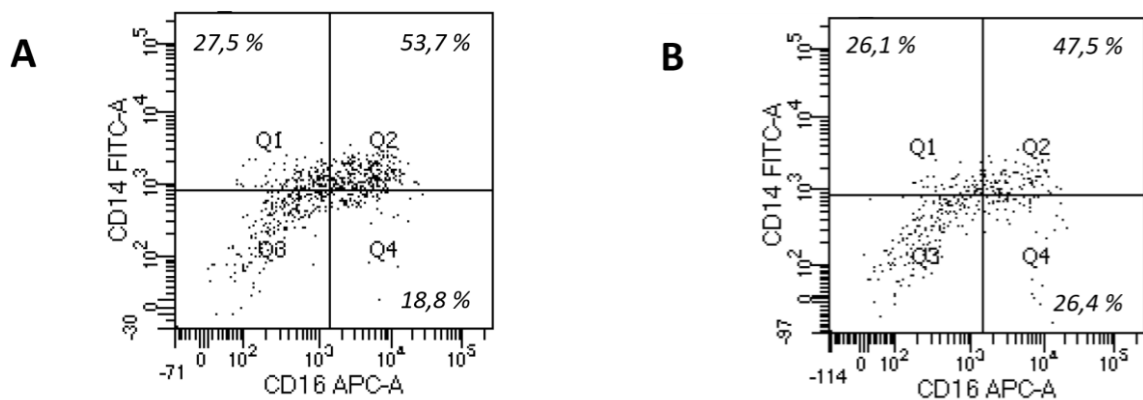


Figure 7 Flow cytometry analysis of samples from transfection number 4 - a relative decrease of double positive cells after transfection of CD14 monocytes by a miR-150 inhibitor (B) compared to the negative control (A). Samples were stained with CD14-FITC and CD16-APC antibodies.

(Q1 – CD14 positive monocytes, Q2 – double positive monocytes, Q3 – CD14 and CD16 negative monocytes, Q4 – CD16 positive monocytes). Results are displayed visually and by percentage representation.

Table 6 Percentage representation of double positive monocytes. Comparison of monocytes transfected by miR-150 inhibitor with those transfected by the negative control

transfection	sample	double positive cells percentage
1	negative control	61.8%
	miR-150 inhibitor	68.4%
2	negative control	59.8%
	miR-150 inhibitor	59.3%
3	negative control	58.5%
	miR-150 inhibitor	49.8%
4	negative control	53.7%
	miR-150 inhibitor	47.5%
5	negative control	61.6%
	miR-150 inhibitor	65.3%
6	negative control	54.5%
	miR-150 inhibitor	55.9%
7	negative control	18.9%
	miR-150 inhibitor	13.1%

Results from the FACS analysis of the samples, where I evidenced a decrease of double positive monocytes after transfection by miR-150 inhibitor and are shown in Figure 8.

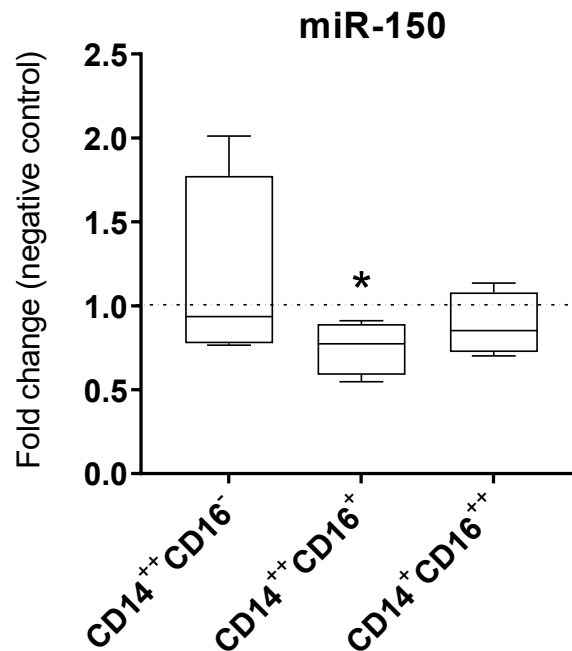


Figure 8 Impact of miR-150 inhibitor on monocyte differentiation. Results are shown from transfection experiments number 2,3,4 and 7, where a decrease of double-positive cells was observed. Results are displayed as a fold change comparing the effect of the miR-150 inhibitor and the negative control. (* $p < 0.05$)

6.2 In silico analysis of putative targets of miR-150-5p

As mentioned in methods (5.8) I used the miRWalk database to find putative target genes of human miR-150-5p. In total I obtained 8466 of potential targets, the list was right after reduced to 3318 by excluding targets with score 1 and 2 out of 4 possible, as I searched 4 databases.

Those 3318 hits were later compared with a list of 48 genes from TaqMan Custom Array plates (Table 7) constructed to study the function of monocytes, their activation and polarization. I found 3 overlapping genes, which means that 3 genes (CCR2 (C-C chemokine receptor type 2), CD68 and TNF) out of those genes contained in the array (Table 7) represented potential targets of miR-150. The results are illustrated in Figure 9.

Table 7 Genes contained in TaqMan Custom Array plate constructed to study the function of monocytes

ACTIN	CD1C	CXCL11	NFATC1
C-FOS	CD36	EBI3	NRG1
C-JUN	CD40	FCER1A	TNFRSF11A
C5R1	CD68	FCGR1A	REL-B
CCL13	CD79B	FCGR3B	SOCS3
CCL19	CD80	GAPDH	TLR1
CCR2	CD86	IL10	TLR2
CCR7	CDKN1C	IL12B	TLR4
CD14	CSF1R	IL1B	TNF
CD163	CTSK	ITGAM	TRAP
CD1A	CX3CR1	MMP9	VCAM
CD1B	CXCL10	MRC1	WNT5A

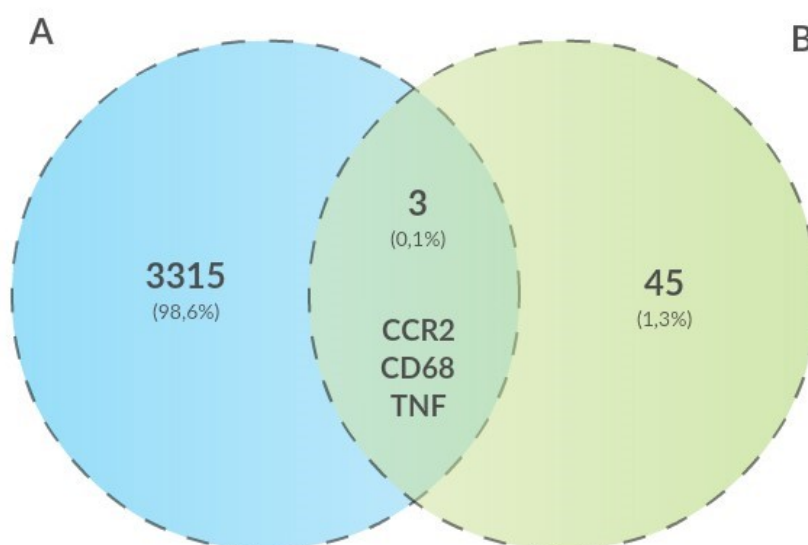


Figure 9 Overlap of putative genes obtained from the miRWalk database (A) and list of TLDA genes (B) – diagram created by online software Venny 2.1.0

6.3 Impact of miR-150 on mRNAs expression

The samples obtained after transfections were subjected to RT-PCR analysis using TaqMan® Custom Array composed to study the monocyte differentiation and their functioning. List of those genes is shown in Table 7.

Relative expressions were calculated from delta CTs using the mean of two housekeeping genes also contained in the TaqMan® Array (ACTB and GAPDH). Genes with the fold change higher than 1.2 and lower than 0.8 were considered as deregulated in monocytes transfected with miR-150 inhibitor. 27 genes were up-regulated, 7 of them with statistical significance ($p < 0.05$) as shown in Table 8. Moreover, 10 genes, which are listed in Table 9, were down-regulated.

Table 8 List of genes up-regulated in monocytes after transfection with a miR-150 inhibitor. As a control, we used MirVana™ miRNA inhibitor Negative Control #1

	Fold change	p-value
CD14	1.95	0.013
CCR2	1.54	0.014
MRC1	1.67	0.019
TLR1	2.35	0.025
IL10	1.82	0.025
C5AR1	1.49	0.029
TLR4	1.66	0.031
ACP5	1.43	0.062
FCGR1A	1.41	0.087
CD163	1.53	0.094
NFATC1	1.40	0.098
CD36	1.44	0.104
CX3CR1	1.61	0.125
CD86	1.43	0.129
WNT5A	1.51	0.220
RELB	1.35	0.260
JUN	1.25	0.269
CXCL10	2.16	0.274
CD68	1.26	0.292
CCR7	1.72	0.294
TLR2	1.26	0.301
VCAM1	2.16	0.400
CTSK	1.26	0.438
FCGR3B	1.37	0.486
IL12B	1.74	0.530
CD1A	1.42	0.531
CXCL11	1.45	0.620

Table 5 List of genes down-regulated in monocytes after transfection with miR-150 inhibitor, as a control we used MirVana™ miRNA inhibitor Negative Control #1

	Fold change	p-value
IL1B	0.34	0.196
CCL19	0.28	0.202
ITGAM	0.68	0.233
CD79B	0.54	0.246
CCL13	0.33	0.267
MMP9	0.65	0.312
NRG1	0.59	0.324
CD1C	0.47	0.340
EBI3	0.56	0.466
FCER1A	0.47	0.596

Among the 3 genes that were predicted to be direct miR-150 targets, only the expression of CCR2 was increased in the 7 separate transfection experiments when using a miR-150 inhibitor, as compared with negative control miRNA (Figure 10).

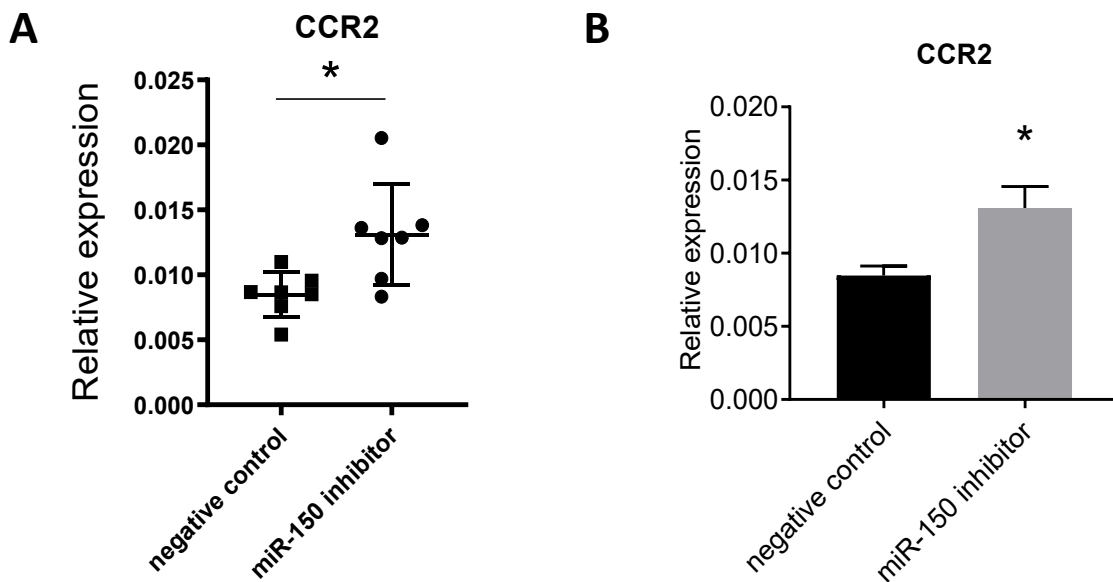


Figure 10 Relative expression of CCR2 up-regulated in monocytes transfected in 7 separate experiments with miR-150 inhibitor. Results displayed for each transfection separately (A) and as means of all 7 transfections \pm SD (B) (* $p < 0.05$)

The pairing of miR-150 and CCR2 sequences is displayed in Figure 11.

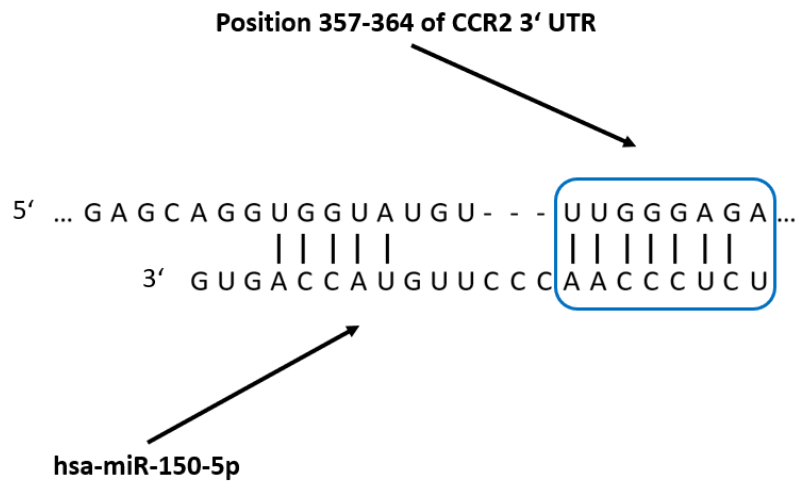


Figure 11 Predicted consequential pairing of CCR2 gene 3'UTR region (top) and miR-150 (bottom). Prediction obtained from TargetScanHuman database, release 7.2.

7 Discussion

Experimental work of this diploma thesis was focused on miR-150, a non-coding RNA, which was described to have a significant role in differentiation, mobilization and migration of bone marrow-derived mononuclear cells including monocytes (He et al. 2014, Liu et al. 2015, Tano et al. 2011). I mainly studied the impact of this miRNA on monocytes, an important component of the immune system that plays a critical role in the physiopathology of juvenile idiopathic arthritis (Gaur et al. 2017) and the severity of joint damages in RA patients (Niimoto et al. 2010).

Since preliminary data from the laboratory of Prof. Apparailly showed that miR-150-5p is over-expressed in the synovial fluid of patients with JIA and that the frequency of intermediate monocytes is higher than in children with septic arthritis, I investigated whether there is a link between these 2 observations using transfection experiments in CD14⁺⁺CD16⁻ monocytes. These monocytes were isolated from the blood of healthy donors.

My data evidenced a decrease in the percentage of intermediate monocytes (CD14⁺⁺CD16⁺) in four out of six successful transfection experiments when using miR-150 inhibitor. These results suggest that miR-150 might affect the switch of monocytes from the classical to the intermediate stage, revealing a new role for miR-150 in monocytes differentiation. This hypothesis has been recently validated by Selimoglu-Buet et al. (2018), who focused on both mouse and human monocyte subsets and suggested that miR-150 is involved in the generation of non-classical monocytes.

To further understand the function of miRNAs in biological mechanisms, molecular mechanisms have to be investigated through the analysis of their impact on gene expression. For this purpose, I performed in silico analysis searching several miRNA databases for miR-150 putative targets as mentioned in chapter 5.8. Based on the experience acquired by the team of Prof. Apparailly and based on literature, TaqMan Custom Array plates were designed, containing genes playing a certain role in monocyte functions. We found 7 genes (CD14,

CCR2, MRC1, TLR1, IL10, C5AR1, TLR4) up-regulated after transfection with miR-150 inhibitor with statistical significance.

Among them, our *in silico* analyses identified only 3 putative targets for miR-150-5p and *in vitro* experiments only found an inverse correlation between miR-150-5p and CCR2 expressions, suggesting that CCR2 might be indeed a direct target for miR-150-5p in human monocytes. Moreover, our conclusions correlate with the findings of a recently published study, which confirm a direct link between miR-150 and CCR2 (Hu et al. 2019).

CCR2 is a gene-encoding protein called C-C motif chemokine receptor 2, a receptor for monocyte chemoattractant protein 1 (CCL2, MCP1). CCR2 is highly expressed by classical monocytes, and much less expressed by non-classical monocytes, and plays a critical role in monocyte egress from the bone marrow and chemotaxis towards the inflamed tissues. CCR2 is also abundantly expressed in the RA-patients synovium mononuclear cells (Vergunst et al. 2008). Future study should unravel the importance of the connection between miR-150 and CCR2 with respect to the pathophysiology of juvenile idiopathic arthritis as well as to the whole immune system.

Further studies should also confirm the results from this work, including a more detailed analysis of the interaction between synovial fluid of JIA patients enriched with miR-150 and monocytic cells. For this purpose, a coculture of SF of JIA patients with classical monocytes should be considered and the comparison of their phenotype and transcriptome with results obtained in this work should be performed. Validating the CCR2 as a target of miR-150 via luciferase reporter gene assay would also help us to decipher the role of the epigenetics in the regulation of the immune system.

In addition, other miRNAs have been observed by several studies as deregulated in JIA patients and therefore linked to the pathophysiological processes of this disease and proposed as potential novel biomarkers. miR-16 and miR-146a were found over-expressed in plasma of children affected by JIA (Ma et al. 2013). Demir et al. (2018) similarly showed deregulations of miR-16, miR-204 and miR-155 in the plasma samples of patients with JIA. The elevation of miR-146a was seen also in the SF of joints affected by JIA, on the contrary, the

expression of miR-192-5p was reduced in the SF, which might be due to the suppression of proinflammatory signals (Derfalvi et al. 2016). Together with our results, these observations highlight a certain role of miRNAs in the pathological mechanisms of JIA and set a base for future, more detailed research.

8 Conclusion

In this work I showed that the inhibition by miR-150 in isolated human classical monocytes modifies their phenotype, suggesting that it prevents, at least partly, their differentiation into intermediate monocytes. Further studies need to be performed to confirm that miR-150-5p might play a role in the monocyte differentiation.

I also proposed CCR2 as a novel target of miR-150, which would indicate a connection between miR-150 and monocyte egress from the bone marrow into inflamed tissues.

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