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Medizinischen Fakultät Charité - Universitätsmedizin Berlin

DISSERTATION

Expression analysis of T-cell immune response cDNA 7 (TIRC7) in patients with rheumatoid arthritis based on quantitative real-time PCR

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

von

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Datum der Promotion: 25. 11. 2022

meiner Familie gewidmet

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Abbreviations / Abkürzungsverzeichnis

ACPA	anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ANA	anti-nuclear antibody
APC	antigen-presenting cell
API	Apicidin (class I HDAC inhibitor)
ATP	adenosine triphosphate
BCR	B-cell receptor
BLyS	soluble human B lymphocyte stimulator protein
BMI	body mass index
bp	base pair
BTK	Bruton's Tyrosin kinase
C5	complement component 5
ccl21	CC-chemokine ligand 21
CD	cluster of differentiation
CDAI	Clinical Disease Activity Index
cDNA	complementary deoxyribonucleic acid
CI	calcineurin inhibitor
CIA	collagen induced arthritis
CRP	C-reactive protein
cT	threshold cycle
CTLA-4	cytotoxic T-cell antigen 4
DAS	Disease Activity Score
DAS28	DAS including only 28 joints
DMARD	disease modifying anti-rheumatic drug
DNA	deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
EGA	evaluator global assessment
ERK	extracellular-signal regulated kinase
ESR	erythrocyte sedimentation rate

EULAR	European League Against Rheumatism
FcR	Fc-receptor
FLS	fibroblast-like synoviocyte
FK506	Tacrolimus
GH	global health assessment
GLP	Good Laboratory Practice
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA-DR	human leukocyte-antigen D related
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IL2RB	Interleukin 2 Receptor Subunit Beta
IRAK1	Interleukin-1 receptor-associated kinase 1
ITP	immune thrombocytopenia
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
mAb	monoclonal antibodies
MAPK	mitogen-activated protein kinase
mDC	myeloid dendritic cell
MLS	macrophage-like synoviocyte
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex
MS	multiple sclerosis
NRS	numeric rating scale
NSAID	non-steroidal anti-inflammatory drug
OC116	ovocleidin 116, long isoform of TIRC7
PADI4	peptidyl arginine deiminase, type IV
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell

PGA	patient global assessment
PI3k	phosphoinositid-3 kinase
PTPN22	protein tyrosine phosphatase, non-receptor type 22
RT-qPCR	real-time quantitative PCR
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor-κB
RF	rheumatoid factor
RNA	ribonucleic acid
SDAI	Simplified Disease Activity Index
SEM	standard error of mean
SJC	swollen joint count
TACI	transmembrane activator and CAML interactor
TCR	T-cell receptor
TFH	T follicular helper cell
Th	T-helper cell
TIRC7	T-cell immune response cDNA 7
TJC	tender joint count
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFAIP3	tumor necrosis factor, alpha-induced protein 3
TRAF1	TNF-Receptor Associated Factor 1
Treg	T-regulatory cell
TYK2	non-receptor tyrosine-protein kinase
VAS	visual analogue scale

Abstract

Background

T-cell immune response cDNA7 (TIRC7), a membrane-spanning protein on the surface of lymphocytes, is upregulated in expression after lymphocyte allostimulation, and in the course of transplant rejection, its expression is altered in different autoimmune processes, and its biological modification showed promising therapeutic results in animal models and human disease. Rheumatoid arthritis (RA) is a chronic auto-immune disease involving lymphocytes, especially T-cells, intra- and extracellular pathways leading to inflammation and destruction of joints and other tissues. Early diagnosis and therapy are vital for the life quality of patients and the prevention of irreparable damage. Diagnostics are based on the detection of autoantibodies in blood serum and imaging. The aim of this work is to develop a measuring method for TIRC7 expression. Alterations of TIRC7 expression in RA patients compared to healthy subjects and correlation with factors influencing immunologic processes (sex, age, body weight and smoking habits) and clinical disease activity measured by scores were hypothesized.

Materials and Methods

From 34 RA patients and 29 healthy volunteers, heparinized blood samples were drawn. By using the density gradient of Ficoll paque, peripheral blood mononuclear cells (PBMCs) were isolated. Employing the peqGold RNApure method, RNA was isolated from these cells, and reversely transcripted into cDNA. In these patient samples, TIRC7 expression was measured by quantitative real-time PCR, and for normalization the results were compared to the expression of β-actin as housekeeping gene. Statistical analyses of the TIRC7/β-actin ratio values were made using GraphPad Prism 5.

Results

TIRC7 expression, measured as TIRC7 / β-actin ratio values, is altered in PBMCs of patients suffering from severe RA compared to healthy volunteers, but also compared to only moderately affected RA patients. This study has also found a statistically significant correlation between the body mass index and TIRC7 expression in PBMCs of RA patients. Only in healthy subjects TIRC7 expression was statistically significantly higher in females than in males. The correlation of swollen joint count and TIRC7 expression in RA patients was statistically significant, whilst tender joint count, DAS28 scores, CRP and ESR showed no remarkable relation to TIRC7 expression.

Conclusions

Many questions regarding TIRC7 and RA remain unanswered and more research is needed, but the results presented here indicate the possibility of TIRC7 serving as new marker for diagnosis and monitoring of therapy. Furthermore, TIRC7 could be a candidate for a targeted therapy in RA.

Abstrakt

Hintergrund

T-cell immune response cDNA7 (TIRC7), ein membranumspannendes Protein auf der Oberfläche von Lymphozyten, ist erwiesenermaßen in seiner Expression hochreguliert nach Allostimulation von Lymphozyten und im Verlauf von Transplantatabstoßungen. Veränderungen in der TIRC7-Expression wurden in verschiedenen Autoimmunprozessen gefunden und biologische Modifikation von TIRC7 zeigte vielversprechende Ergebnisse in Tiermodellen und Krankheitsbildern. Rheumatoide Arthritis (RA) ist eine chronische Autoimmunerkrankung, deren Pathogenese Lymphozyten, besonders T-Zellen, sowie verschiedene intra- und extrazelluläre Signalwege involviert, was schließlich zu Entzündung und Zerstörung von Gelenken und anderen Geweben führt. Frühe Diagnosestellung und früher Therapiebeginn sind wichtig für den Erhalt von Lebensqualität und Prävention irreparabler Gewebeschäden. Die Diagnostik wird unterstützt durch Auto-Antikörper im Blutserum und Bildgebung. Ziel dieser Arbeit ist die Entwicklung einer Messmethode für TIRC7-Expression. Veränderungen der TIRC7-Expression in RA-Patient*innen im Vergleich zu Gesunden und durch Faktoren, welche immunologische Prozesse beeinflussen (biologisches Geschlecht, Alter, Körpergewicht und Zigarettenkonsum) sowie Korrelationen mit klinischen Krankheitsaktivitätsscores und -markern wurden als Hypothesen angenommen.

Materialien und Methoden

Von 34 RA-Patient*innen und 29 freiwilligen gesunden Teilnehmer*innen wurden heparinisierte Vollblutproben entnommen. Unter Ausnutzung des Dichtegradienten von Ficoll paque wurden hieraus periphere mononukleare Blutzellen (PBMCs) isoliert. Durch die peqGold RNA-pure-Methode wurde RNA aus diesen Zellen gewonnen, die revers transkribiert wurde und somit umgewandelt in cDNA. In diesen Proben wurde die TIRC7-Expression durch quantitative Real-Time-PCR gemessen. Zur Normalisierung wurden die Ergebnisse jeweils mit der Expression von β-Actin als Housekeeping-Gen verglichen. Statistische Analysen der TIRC7/β-Actin-Quotienten wurden ausgeführt mit GraphPad Prism 5.

Ergebnisse

TIRC7-Expression, gemessen als TIRC7 / β-Actin Quotienten, war erhöht bei schwer von RA betroffenen Patient*innen im Vergleich zu gesunden Freiwilligen, aber auch im Vergleich zu nur moderat betroffenen Patient*innen. Es wurde außerdem eine statistisch signifikante Correlation zwischen Body Mass Index (BMI) und TIRC7-Expression in RA-Patient*innen. Nur in gesunden Freiwilligen war die TIRC7-Expression signifikant höher in Frauen als in Männern. Andere Faktoren, die immunologische Prozesse beeinflussen, wie Alter und Zigarettenkonsum, wurden hingegen nicht in einer statistisch signifikanten Korrelation mit TIRC7-Expression gefunden. Die Korrelationen zwischen der Anzahl geschwollener Gelenke und der Expression von TIRC7 in RA-Patient*innen war statistisch signifikant, wohingegen die Anzahl schmerzhafter Gelenke und Blutsenkungsgeschwindigkeit (ESR) keine statistisch signifikanten Korrelationen mit TIRC7-Expression aufwiesen.

Schlussfolgerungen

Viele Fragen bezüglich TIRC7 und RA bleiben offen und mehr Forschung wird benötigt. Jedoch weisen die hier gezeigten Resultate auf die Möglichkeit hin, TIRC7 als Marker für Diagnose und Therapie einzusetzen. Weiterhin ist TIRC7 ein Kandidat für die Entwicklung gezielter Therapien für RA.

1 Introduction

1.1 T-cell immune response cDNA 7 (TIRC7)

1.1.1 Discovery, genetics and characteristics

T-cell immune response cDNA7 (TIRC7) was first described in 1998 by Utku et al. [1] as a T-cell mRNA transcript whose expression is upregulated early (within a few hours) after T-cell stimulation. Subsequently, the TIRC7 gene was described in 1999 by Heinemann et al. [2]. The highest expression was found at 24h after allostimulation of human lymphocytes. TIRC7 cDNA is 2488 bp long, whilst the protein has a predicted length of 614 amino acids, weighs 75 kDa, and contains seven hydrophobic, putatively membrane-spanning domains as well as an extracellular C-domain and an intracellular N-terminus (Fig. 1.1). There was no amino acid homology found with other proteins involved in T-cell activation, but homologies were found with different proteins serving as subunits of the vacuolar proton pump H+ ATPase in some species, e.g. chicken osteoclast plasma membranes and Archaebacteria [3, 4].
TIRC7 and OC116 (ovocleidin-116, also called a3 protein) [5] are both shown to be alternative transcripts of the same Atp6v0a3 gene lying on chromosome 11q13.4-13.5, but OC116 is solely expressed in human osteoclastoma tumor cells [2]. TIRC7 expression has been reported in immune tissues (spleen, lymph nodes, peripheral blood, appendix, bone marrow, fetal liver and thymus) as well as in CD4+ and CD8+ lymphocytes [1]. Interestingly, TIRC7 has also been demonstrated to be differentially expressed in different subsets of T-regulatory lymphocytes [6].

1.1.2 Physiology

As described by Utku et al. in 1998, antibody targeting of TIRC7 inhibits kidney transplant rejection in rats and TIRC7 is expressed early after T-cell alloactivation, leading to the conclusion that TIRC7 operates to regulate the T-cell activation process as a specific modulation target.
Furthermore, modulation of TIRC7 with anti-TIRC7 antibodies in vitro prevents human T-cell proliferation and IL-2 secretion and inhibits especially T-cell type 1 subset specific IFN-γ, but not T-cell type 2 specific IL-4 expression [1].

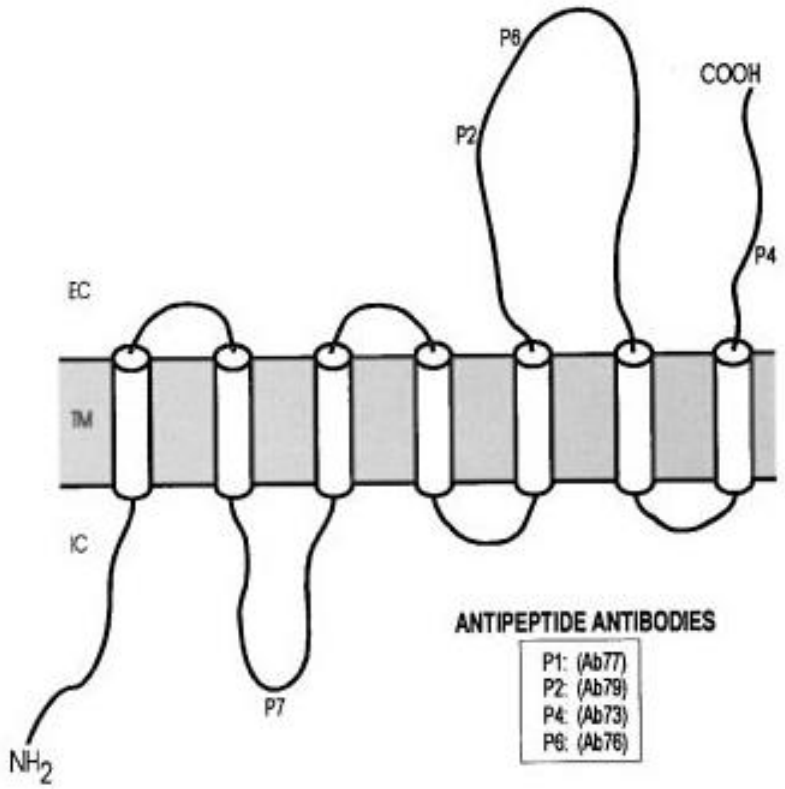


Figure 1.1: The secondary structure of TIRC7 protein with seven transmembrane domains (TM), peptides (P1-P7), the putative intracellular amino terminus (NH3), and the extracellular carboxy terminus (COOH). Anti-TIRC7 antibodies are listed in the box. EC = extracellular, IC = intracellular. *Image taken from [1].*

Antibody targeting of TIRC7 also induces early cell surface expression of cytotoxic T-cell antigen 4 (CTLA-4), which is co-expressed and co-localized with TIRC7. It has been demonstrated that TIRC7-mediated signals are dependent on CTLA-4 as CTLA-4-deficient mice do not respond to TIRC7 antibody targeting. However, a direct TIRC7 - CTLA-4 interaction has yet to be demonstrated [7].

In addition, it has been shown that the binding of TIRC7 to the alpha-2 domain of HLA-DR, which is known to activate lymphocytes via presentation of peptides to TCR-bearing CD4+ T-Cells [8], initiates an intracellular signalling cascade via activation of caspases 9 and 7, which mediates negative signaling and leads to anti-inflammatory and apoptotic effects in CD4+ and CD8+ T cells as well as to downregulation of the cytokine immune response [9].

A peptide identical to the C-terminus of TIRC7 protein was furthermore shown to cause an interference with differentiation pathways in monocytes [10].

In summary, TIRC7 expression is upregulated in - and an indication of - immune activation, while also being part of a self-limiting anti-inflammatory mechanism (Fig. 1.2).

1.1.3 Role in autoimmune processes

A leading cause for the interest in TIRC7 was its initial discovery as an upregulated peptide in several autoimmune processes and diseases. Since then, several clinical and pre-clinical trials have examined its role in autoimmunity and organ rejection:

The examination of intragraft and peripheral blood TIRC7 expression can serve as a diagnostic tool for acute cardiac allograft rejections in humans. Biopsies during or before allograft rejection showed higher TIRC7 mRNA expression levels than biopsies from patients without rejection. Moreover, TIRC7 mRNA expression levels in PBMC (peripheral blood mononuclear cells) of transplant recipients were lower than in healthy individuals and lower during graft rejection than in rejection-free periods [11].

The cause for these phenomena might be a T cell migration towards the graft inducing a decrease of these cells in peripheral blood [12].

TIRC7-deficient mice showed a disturbed activity of T and B cells as well as decreased levels of CTLA-4, indicating its immune inhibitory role [13].

The latest analyses have shown TIRC7 inhibiting TH1 cells by upregulating the expression of CTLA-4 and signal transducer and activator of transcription 3 (STAT3) in mice with acute graft-versus-host disease: Co-administration of monoclonal anti-TIRC7 and anti-CTLA4 anti-

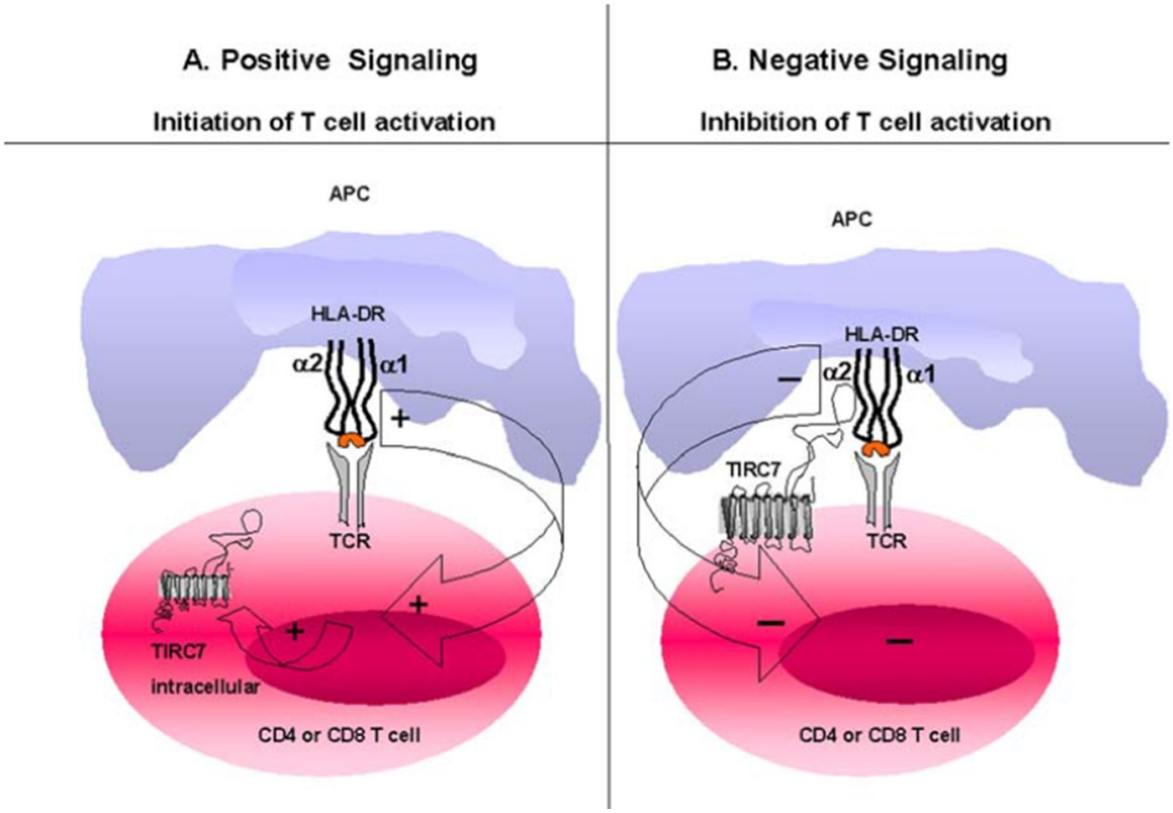


Figure 1.2: HLA-DR binding pathways. HLA-DR binding to T-cells initiates lymphocyte activation, but binding of the alpha 2 domain of HLA-DR to TIRC7 also mediates downregulation of immune response. APC = antigen-presenting cell. HLA-DR = human leukocyte-antigen D related. TCR = T-cell receptor. CD = cluster of differentiation. *Image modified from Bulwin et al. [9].*

bodies in mice led to a higher activation capacity of T lymphocytes, increased secretion levels of interferon-γ and other cytokines, and shortened recovery time [14].

Antibody targeting of TIRC7 led to decreased lymphocyte infiltration into cardiac allograft in mice, delayed graft damage and prolonged graft survival. Anti-TIRC7 mAb- (monoclonal antibodies) treated lymphocytes also showed hyporesponsivity against donor alloantigens indicating the induction of antigen-specific anergy [15].

TIRC7 expression was also shown to be induced in rejected human kidneys despite treatment with calcineurin inhibitors (CI), whereas anti-TIRC7 antibodies applied in combination with FK506 (Tacrolimus) prolonged the survival of kidney allografts in rats [16].

TIRC7 was upregulated in lymphocytes obtained from joint tissue of patients with rheumatoid arthritis (RA) and mice with collagen induced arthritis (CIA). Furthermore, the combination treatment of anti-TIRC7 mAb and soluble TNFα-receptor inhibited induction and progression as well as the according antibody response in mice suffering from CIA [17].

TIRC7 is also shown to be involved in inflammatory processes in tissues obtained from multiple sclerosis (MS) patients. Monoclonal antibodies (mAb) against TIRC7 prevent immune activation via selective inhibition of Th1- and Th17-associated cytokine expression, which might become a promising novel treatment option for MS [18].

In patients with immune thrombocytopenia (ITP), higher levels of TIRC7 and decreased levels of CTLA-4 (as a downstream molecule of TIRC7) were observed in both mRNA and plasma levels, leading to the conclusion that both molecules (TIRC7 and CTLA4) might be associated with the pathogenesis of ITP and could serve as an indicator for treatment efficacy [19, 20].

TIRC7 is also known to be involved in the pathogenesis of aplastic anemia. Antithymocyte globulin and cyclosporine A downregulate Th1 cells via modulation of TIRC7 [21].

In conclusion, both clinical and pre-clinical studies identified alterations of TIRC7 in a diverse set of autoimmune-related morbidities. Promisingly, biological modification of TIRC7 seems to have a beneficial effect in both humans and animal models. Thus, further investigation of TIRC7 could reveal new diagnostic and therapeutic options in transplantation and autoimmune diseases [22]. One clinically important autoimmune disease in which TIRC7 expression and effect has not yet been studied is rheumatoid arthritis (RA).

1.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting primarily the joints, from which approximately 0.5-1% of the population in industrialized countries suffer [23, 24]. Women are two to three times more likely to be affected by RA than men [25]. In women, RA mostly becomes symptomatic around the time of menopause, whilst men have a later disease onset [26]. A genetical component was demonstrated [27], as the HLA-antigen D related (DR) loci HLA-DRB1*01 and HLA-DRB1*04 are significantly associated with the risk of developing RA [28].

Additionally, epigenetic factors were found, e.g., alterations in DNA methylation and histone modification [29]. Another big risk factor is tobacco smoking [30, 31]. Silica exposure and smoke/dust inhalation also increases the risk of developing RA [32–35] as well as periodontal disease [36]. Furthermore, obesity (a BMI higher than 30 kg/m^2 compared to a BMI lower than 25 kg/m^2) is independently associated with a mild increase of risk for RA [37]. Low socioeconomic status and low educational level have been found to correlate with worse outcomes of RA [38].

1.2.1 Pathogenesis

The pathogenesis of RA involves autoantibody formations against immunoglobulin G (rheumatoid factor = RF) and citrullinated peptides (anti-citrullinated protein antibodies = ACPA) which lead to systemic inflammation and a variety of immunological events. The central and characteristic process is synovial inflammation beginning with expansion of the intimal lining and an increase and expansion of both synoviocyte types (macrophage-like synoviocyte = MLS; fibroblast-like synoviocyte = FLS) [39], leading to the release of cytokines (interleukines, tumor necrosis factors), matrix metalloproteinases (MMPs) and small molecule mediators (prostaglandins, leukotrienes) [40]. Activated FLS also express specific microRNA patterns which take part in their activation and tissue destruction [41–43].

This is followed by the infiltration of the synovial sublining by mononuclear, predominantly CD4+ cells, as well as stroma cells. Mature B-cells can proliferate and differentiate in the inflammation sites and produce antibodies. Plasma cells are also shown to be present and producing RF and ACPAs [44, 45].

Meanwhile, cytokines binding to receptors trigger several intracellular signal transduction cas-

cades, which connect extracellular events and gene activation. Acquiring different pathogenetic pathways (Fig. 1.3), these heterogenous processes lead to inflammation, joint destruction, osteoclast activation and finally also bone erosion [46].

1.2.1.1 Role of T-cells in RA

Regulation of immune inflammation depends on balances between the number and strength of different cell types. In the context of RA, T-cell mediated immune responses are considered as critical contributors in RA initiation and progression [48]. It has been suggested that autoreactive arthritogenic T-cells, which have escaped the negative selection in the thymus can recognize arthritogenic antigens, which in turn leads to tissue destruction [49]. On the other hand, in a rodent model of RA, a study demonstrated that infusion of T-cells can ameliorate arthritic pathology. Hence, depending on their activation history, T-cells can act as both arthritogenic and protective in RA [50]. T-cells mediate a variety of autoimmune diseases through the recognition of autoantigens [51]. In RA, the interplay between CD4+ T-cells and macrophages or monocytes is especially important [52].

One recently identified possible target of autoreactive-T cells (and autoantibodies) from RA patients is the protein RPL23A, a component of the 60S subunit of ribosomes, which is expressed in normal and arthritic joints, amongst others. Furthermore, it has been shown that it is able to stimulate CD4+ T-cells via RPL23A-derived peptide-MHC class II complexes [53]. On a molecular level, activation of T-cells is induced by different signaling pathways. In general, the process is initiated by peptides presented by MHC molecules on the surface of antigen-presenting cells (APCs) which can be detected by the T-cell receptor (TCR). However, for complete T-cell activation, additional co-stimulatory signals (receptor-ligand interactions between APC and T-cell membrane proteins) are necessary [54].

Such additional signals are provided by multiple receptor-ligand interactions mediated by other membrane proteins such as CD28, CTLA4, B7, CD40 (co-stimulatory signals). These proteins can regulate the T-cell activation and the immune response in positive and negative ways – either they interfere with the T-cell receptor or co-stimulatory signaling molecules directly, or they inhibit intracellular processes downstream from them. CTLA-4, for example, is a regulator of co-stimulation and inhibits the activation of T-cells by interfering with the interactions of APCs and CD28. It displays a directly inhibiting effect on the differentiation of osteoclasts which may contribute to the prevention of bone destruction in rheumatoid arthritis [54].

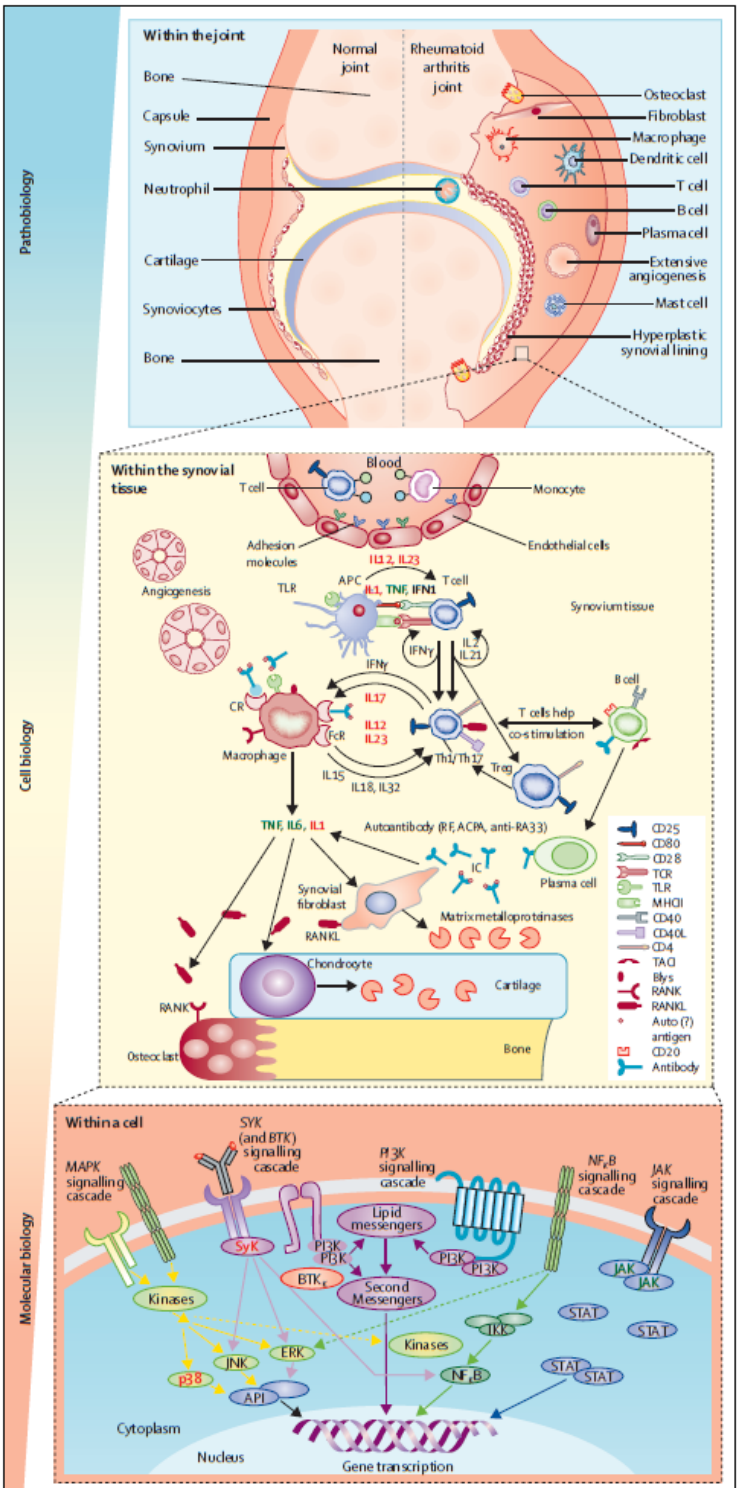


Figure 1.3: Pathogenetic pathways in rheumatoid arthritis, *image taken from Smolen et al. [47]*. Green = molecules or cells successfully targeted by therapies; red = molecules for which targeting was not effective. FcR = Fc-receptor. TACI = transmembrane activator and CAML interactor. TLR = toll-like receptor. BLyS = soluble human B lymphocyte stimulator protein. MAPK = mitogen-activated protein kinase. BTK = Bruton's Tyrosine kinase. PI3k = phophoinositid-3 kinase. JNK = c-Jun N-terminal kinase. ERK = extracellular-signal regulated kinase. API = Apicidin (class I HDAC inhibitor).

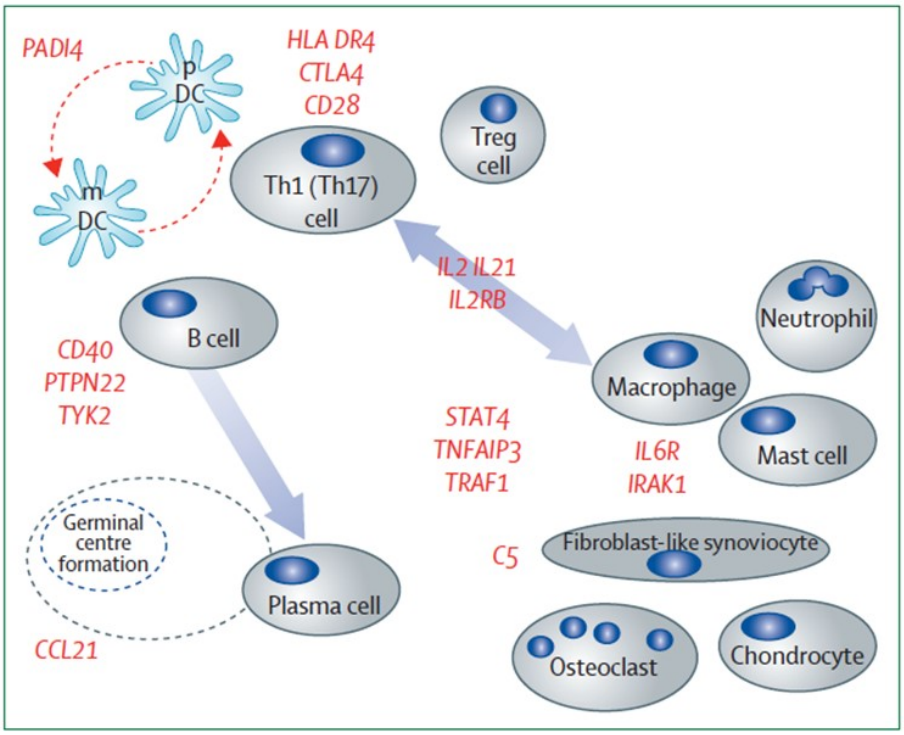


Figure 1.4: Key immune cells and loci associated with RA, *Image taken from Smolen et al. [47].*

TYK2 = non-receptor tyrosine-protein kinase 2. PTPN22 = protein tyrosine phosphatase, non-receptor type 22. PADI4 = Peptidyl arginine deiminase, type IV. C5 = complement component 5. CCL21 = CC-chemokine ligand 21. IL2RB = interleukin 2 receptor subunit beta. TNFAIP3 = tumor necrosis factor, alpha-induced protein 3. TRAF1 = TNF receptor-associated factor 1. IRAK1 = interleukin-1 receptor-associated kinase 1.

Fig.1.4 shows important cell types and their interaction in the pathogenesis of RA.

1.2.2 Symptoms

In many cases, RA progresses chronically and flare-wise, it can affect mobility and quality of life, and most patients suffer from it for their whole life [55, 56].

1.2.2.1 Joint manifestations

Cardinal symptoms of RA are joint swelling, tenderness and (morning) stiffness. In RA, the affecting of metacarpophalangeal and proximal interphalangeal joints of hands and feet, as well as wrist, ankle, elbow, shoulder, knee and hip joints is characteristic, but also manifestations in

other peripheral joints are involved. Conversely, involvement of distal interphalangeal or axial joints is atypical in RA, with the exception of the C1-C2 joint [57]. RA symptoms resemble many different diseases (important differential diagnoses: viral arthritis, Lyme arthritis, connective tissue disease, peripheral spondyloarthritis, psoriatic arthritis, osteoarthritis and other metabolic diseases) in its early stages, complicating early diagnosis and delaying therapy onset.

1.2.2.2 Extraarticular manifestations

RA is also associated with systemic acute-phase responses. Manifestations in the eyes, lungs, heart and other organs have been demonstrated to occur at different prevalences in RA patients [58–60]:

- Rheumatoid nodules and vasculitis can be found in severe RA.
- Cardiovascular disease is very common in RA.
- Interstitial lung disease, one of the most severe extraarticular manifestations in RA, with an average survival of 3 years, is reported to have an increasing incidence [61].
- RA is also associated to Sjögren's syndrome, which itself predisposes for cardiovascular disease, secondary amyloidosis and increased risk of developing a lymphoma.
- Fibromyalgia is also increased in prevalence in RA patients.

The incidence of these complications is known to be effectively reduced with treatment, underscoring the necessity of early diagnosis for patient outcome.

1.2.3 Diagnostics

Diagnosis of RA is challenged by intra- and interindividual heterogeneity, which has led to the development of complex classification criteria (most commonly the ACR/EULAR 2010 criteria). Due to their high specificity, these scores are appropriate to stratify patients for clinical trials, but less sensitive and therefore less eligible for clinical diagnostics [62, 63]. Clinically, early RA patients typically present mild swelling of the second and third metacarpophalangeal joints on both hands and sometimes the proximal interphalangeal joints. In established RA, joint subluxation, and Z- and swan-neck deformities of the fingers can be found. Rheumatoid nodules are typically found at the lateral side of fingers and arms.

Radiographically, bone erosions, joint space narrowing and mutilating changes (like pencil-in-cup phenomena and pannus formation) can be observed. Wrist and carpal bones can also be affected [48].

1.2.3.1 RA-specific seromarkers

Nonspecific systemic inflammation signs can be found, e.g. serologically. Moreover, several autoantibodies are demonstrated to play a role in the diagnostics of RA.
Most frequently, IgM antibodies against IgG (rheumatoid factors, RF) and against citrullinated peptides (ACPAs) can be found in RA patients. RF have been found in 50 - 66% of early RA patients and ACPAs in 41 - 48%. In the absence of disease, these antibodies are reported to be found in 7 - 13% and 3 - 9%, respectively [64–67].
ACPAs, RF, inflammatory cytokines and chemokines can be found 10 years before clinical disease onset, which means there are ongoing immune activation processes before symptoms occur. The presence of both antibodies is associated with a more severe disease course and therefore should be used as a diagnostic and prognostic marker [62, 68]. Through effective treatment, the levels of RF are demonstrated to decrease more strongly than ACPA levels, leading to the conclusion of differences in plasticity and/or cellular origin [69].
Several serologic markers such as anti-nuclear antibodies (ANA), microRNA and others are also known to be altered in some RA patients, but less specific and sensitive, as some of these antibodies can also occur in other (autoimmune) diseases.

1.2.3.2 Classification criteria

ACR/EULAR 2010 classification criteria
The American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) developed a classification including clinical and serological parameters. The criteria should only be applied to patients presenting at least one swollen joint:

- Joint involvement and distribution: 0 to 5 points.
- Symptom duration (less or more than 6 weeks): 0 to 1 point.
- Serology (RF and ACPA): 0 to 3 points.
- Acute-phase reactions - erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP): 0 to 1 point.

A score of ≥ 6 is required for the classification of rheumatoid arthritis [62].

CDAI and SDAI scores

Regarding the complexity of the pathogenesis of RA, there is no single marker to monitor the disease activity. Taken alone, neither acute-phase parameters as CRP or ESR nor autoantibodies as RF or ACPA correlate significantly with clinical disease course. Currently, the best known marker for measuring disease severity is clinical disease activity [70].

For assessing disease activity, the Clinical Disease Activity Index (CDAI) and the Simplified Disease Activity Index (SDAI) are commonly used [71, 72]. They total several clinical variables (see Fig. 1.5). Remission (no or minimal disease activity) is achieved by a CDAI score below ≤ 2.8 or an SDAI score below ≤ 3.3, which should be the therapy target in early RA. Remission is not always a realistic target in established RA, but alternatively at least low disease activity (CDAI score of 2.8 – 10 or SDAI score of 3.3 – 11) should be achieved. Assessing CDAI or SDAI in patients with active RA is recommended every 1-3 months until the desired treatment target is achieved; afterwards less frequent follow-up is requested.

DAS scores

There is also the Disease Activity Score (DAS) and a modification thereof including only 28 joints called DAS28 [73, 74] (for cut-offs see Fig. 1.5). These weigh different clinical variables (swollen joints, tender joints, erythrocyte sedimentation rate or C-reactive protein, respectively, and global health assessment) so that tender joint counts have a stronger influence than swollen joints. DAS can be assessed with or without patient global health assessment (with three or four items) and instead of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) can be used to calculate the DAS score, too [75]. Acute-phase reactants (ESR, CRP) have a great influence on the score, even within their normal reference ranges [76, 77].

ACR improvement criteria

The American College of Rheumatology (ACR) improvement criteria were developed to determine the improvement in disease activity achieved by therapy. The criteria compare improvements to baseline values in the following disease activity measures.

Two fixed measures:

- Swollen joint count.

Scoring system	Formula	Disease activity states			
		Remission	Low disease activity	Moderate disease activity	High disease activity
SDAI	SJC28+TJC28+PGA+EGA+CRP	≤3.3	>3.3–11	>11–26	>26
CDAI	SJC28+TJC28+PGA+EGA	≤2.8	>2.8–10	>10–22	>22
DAS	Complex formula including the Ritchie index, SJC44, ESR and GH	≤1.6	>1.6–2.4	>2.4–3.7	>3.7
DAS28	Complex formula including the TJC28, SJC28, ESR (or CRP) and GH	≤2.6	>2.6–3.2	>3.2–5.1	>5.1

Figure 1.5: Overview of diagnostic scores for RA, *image taken from Smolen et al. [48].* SDAJ = Simplified Disease Activity Index. CDAI = Clinical Disease Activity Index. DAS = Disease Activity Score. SJC28 = swollen joint count (of 28). TJC28 = tender joint count (of 28). PGA = patient's global assessment. EGA = examiner's global assessment. CRP = C-reactive protein. SJC44 = swollen joint count (of 44). ESR = Erythrocyte sedimentation rate. GH = global health.

- Tender joint count.

Plus three out of the next five variables:

- Patient global assessment.
- Physician's global assessment.
- Patient pain assessment.
- Physical function or quality of life score.
- Acute-phase reactant levels (ESR or CRP).

Improvements of 20, 50 or 70% (so called ACR20, ACR50 and ACR70 responses) are the corresponding reductions from baseline of the variables above [78].

1.2.4 Therapy

In addition to physiotherapy and life style changes (e.g. nutrition strategies reducing systemic inflammation in autoimmune diseases), there are four cardinal groups of drugs applied in rheumatoid arthritis.

- Non-steroidal anti-inflammatory drugs (NSAIDs).

- Glucocorticoids, mostly prednisolone.
- Synthetically produced disease-modifying anti-rheumatic drugs (DMARDs).
- Biological disease-modifying anti-rheumatic drugs (antibodies).

NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) reduce pain and stiffness and improve physical function in RA patients, but they do not interfere with tissue damage and are not disease modifying. The relief of pain and swelling is attributed to their inhibitory effects on prostaglandin synthesis [47].

Glucocorticoids (Prednisolone)

Application of glucocorticoids, especially when high-dosed, leads to fast symptomatic relief and disease modification; but is well known to be correlated to severe long-term complications and side effects [79]. Low doses of glucocorticoids ($<$7.5mg, better $<$5mg daily) as part of combination therapy show good treatment effects. If possible, their intake should be reduced in dosage and then stopped within six months after treatment has induced clinical relief [47, 80–82].

Synthetic disease-modifying anti-rheumatic drugs (DMARDs)

Synthetically produced disease-modifying antirheumatic drugs (DMARDs) are small chemical drugs. They are key therapeutic agents in RA as they reduce synovitis and systemic inflammation and improve joint function. The leading DMARD is methotrexate, which can be combined with other drugs of this type. Methotrexate is a conventional synthetic DMARD, whose modes of action are largely unknown. The newer generations of DMARDs were developed to target specific targets inside immune cells. According to current EULAR recommendations, a starting treatment with a combination of short-term glucocorticoids and methotrexate is suggested, rapidly escalating methotrexate to the optimal dose of 25mg once weekly, with folate substitution to prevent adverse events.

Biological disease-modifying anti-rheumatic drugs (= biologicals, antibodies)

Biological agents (mostly monoclonal antibodies, or less often receptor constructs) are used when arthritis is uncontrolled or toxic effects arise with synthetic DMARDs. They target sol-

uble extracellular or membrane-associated proteins involved in immune activation (see Fig. 1.6). Whether they should be used as a first line treatment instead of conventional synthetic DMARDs (plus glucocorticoids) is a topic of current debate. Data suggest that this might lead to an overtreatment [83–86]. An increased risk of infections and high costs restrict the prescription of biological agents [46].

In summary, modern treatment algorithms involve the use of conventional, biological, and new non-biological disease-modifying antirheumatic drugs. It has been shown that therapy effectiveness is improved if it was started directly after the diagnosis [87, 88]. Serious irreversible damage already can be induced in the early stages of RA. With current treatment paradigms, clinical remissions and relief of discomfort are possible. Nevertheless, RA is a severe chronic disease, which is commonly accompanied by disability, decreased life expectancy and a higher risk for several other diseases. After the treatment target of stringent remission (or at least low disease activity) is maintained, dose reduction should be attempted [47]. Long-term remission can and has to be induced by early, intensive, effective short-term treatment followed by optional maintenance therapy [46]. Not all questions regarding therapy paradigms have been answered conclusively.

Because best treatment effects can be achieved when RA is treated early, early diagnosis and intervention remain very important. It has been shown that synovial infiltration by inflammatory cells can occur years before symptoms appear [89, 90]. Therefore, preclinical diagnosis and preventive therapy of RA taking advantage of a "window of opportunity", has become a focus of current research [91, 92].
Timing of therapy is a critical factor [87] that cannot be resolved without a diagnostic marker that correlates with the stage of RA. Currently employed serological markers (RF, ACPA) and their respective assays are of good sensitivity and specificity, but are not sufficient for the definite diagnosis of RA. The commonly used clinical scores have an apropriate specificity for clinical trials, but their sensitivity is not sufficient to detect early RA reliably. Novel markers are necessary to specifically monitor disease activity and effects of RA therapy.
Rheumatoid arthritis is a chronic disease that carries a substantial burden for both the individual and society [93]. Although the prospects for most patients are now favorable, many still do not respond to current therapies and suffer from a significantly decreased quality of life [46, 55].

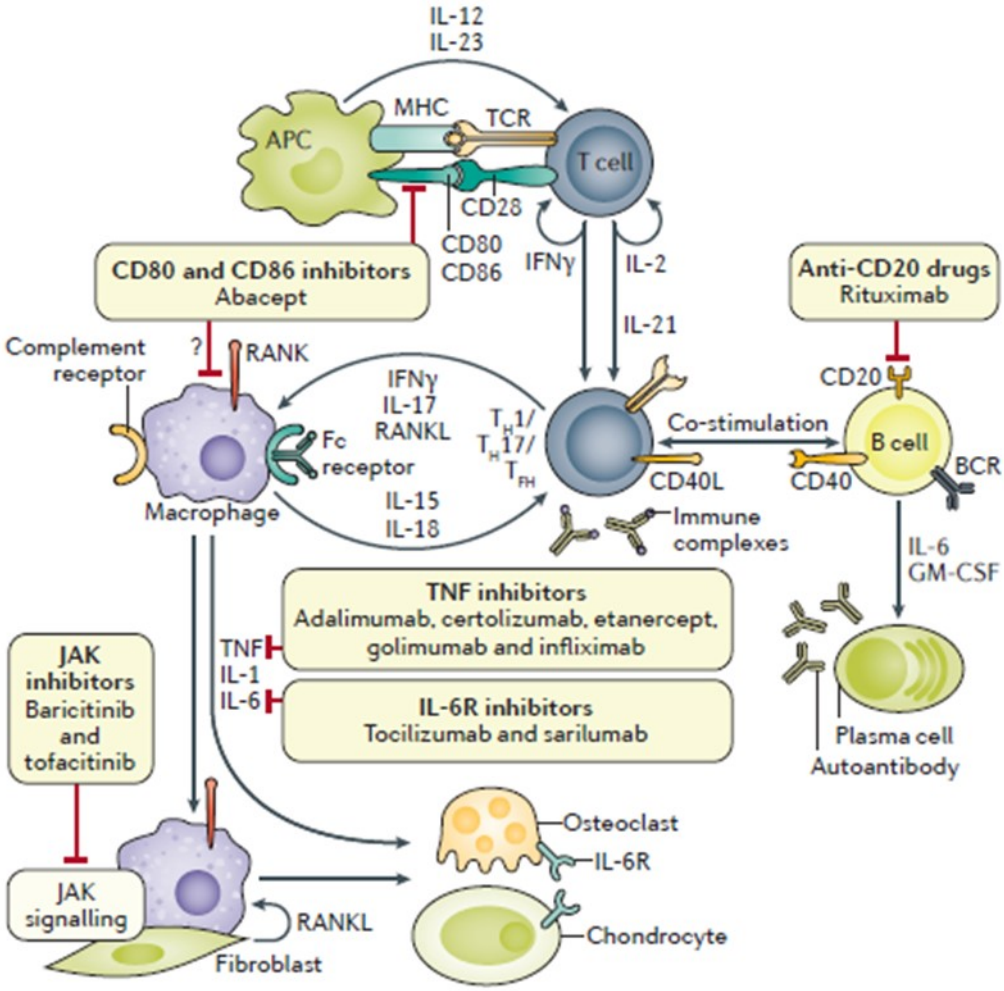


Figure 1.6: Overview of antibodies/biological agents used for therapy in RA and their respective targets, *Image taken from Smolen et al. [48].* Tumour necrosis factor (TNF) inhibitors, IL-6 receptor (IL-6R) inhibitors and Janus kinase (JAK) inhibitors inhibit pro-inflammatory cytokines. Abatacept or rituximab target upstream events, leading to a downregulation of expression of these pro-inflammatory cytokines.

Thus, more efforts are required to establish the diagnosis early, initiate treatment promptly, and design novel treatment strategies to control inflammation and reduce or prevent irreversible damage [47, 48]. At present, largely unfulfilled needs include Understanding the role of microbiome in disease development, as well as cunducting clinical trials with better characterized cohorts and older patients with comorbidities to better understand the safety and interactions of the disease and therapy[94].

1.3 Aims and hypotheses

Many questions regarding RA remain unanswered. The aim of this study is to develop a simple and exact measuring method for TIRC7 expression in peripheral blood and to perform pilot quantification studies in small groups of healthy individuals and RA patients. The results may contribute to facilitating the early diagnosis and therapy of RA.

In view of the preceding chapters, the following hypotheses are proposed:

1. TIRC7 expression is altered in peripheral blood mononuclear cells (PBMCs) in patients with rheumatoid arthritis (RA) compared to healthy subjects.

2. TIRC7 expression in PBMCs is altered by factors known to influence immunologic processes (age, sex, body weight, smoking habits), in line with its role as a marker of immune activation.

3. TIRC7 expression in PBMCs correlates with disease activity measured by disease activity scoring instruments in patients with RA.

2 Materials and methods

2.1 Chemicals, kits, materials and devices

All chemicals, reagents and solutions listed were obtained at maximal purity.

Name	Manufacturer
100bp DNA Ladder Extended	Roth
6x DNA Gel Loading Buffer	Novagen
Acetic Acid	Merck
Agarose	Serva
Ampuwa RNAse free Aqua dest.	Fresenius Kabi
Ampicillin	GIBCO
BglII	Thermo Scientific
Chloroform $\geq$99.5%	Sigma-Aldrich
Dulbecco's Phosphate-Buffered Saline (DPBS)	GIBCO
E. coli TOP10 competent cells	Thermo Fisher Scientific
ECORI	Thermo Scientific
EDTA	AppliChem
Electrophoresis chamber	Biorad
Ethanol absolute 'Baker Analyzed'	J.T.Baker
Ethidium bromide	Roth
Ficoll-Paque Plus	GE Healthcare
Heraeus Fresco 17 centrifuge	Thermo Scientific
Heraeus Megafuge 40R	Thermo Scientific
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Inverted microscope Wilovert 30	Hund Wetzlar

Name	Manufacturer
Isopropanol	Merck
LB Broth	GIBCO
MicroAmp™ Fast Optical 96-Well Reaction Plates	Thermo Fisher Scientific
MicroAmp™ Optical Adhesive Film	Thermo Fisher Scientific
Nanodrop spectrophotometer NanoVuePlus	GE Healthcare
Neubauer improved cell counting chamber	Optik Labor
pCR 2.1-TOPO	Invitrogen
peqGOLD plasmid miniprep Kit I	Peqlab
peqGOLD RNApure	Peqlab
Plasmid DNA purification Maxi Nucleobond	Macherey Nagel
puc19 DNA	Thermo Fisher Scientific
S.O.C. Medium	Invitrogen
StepOnePlus Real-Time PCR Systems	Thermo Fisher Scientific
Taq DNA Polymerase Recombinant Kit	Invitrogen
TaqMan Universal PCR Master Mix	Applied Biosystems
TaqMan Gene Expression Assay Hs00990751_m1 (TIRC7)	Thermo Fisher Scientific
TaqMan Gene Expression Assay Hs01060665_g1 (β-actin)	Thermo Fisher Scientific
Thermocycler T3000	Biometra
TOPO TA Cloning Kit with PCR2.1 TOPO	Thermo Fisher Scientific
Tris	AppliChem
Trypan Blue Solution 0.4%	Thermo Fisher Scientific

Table 2.1: Chemicals, kits, materials and devices

2.2 Sample materials

The patients participating in this study consisted of two different groups:

- 22 (19 female, 3 male) outpatients (average age: 60.8 $\pm$2.7, mean disease duration: 7.9 $\pm$2.3 years) who were moderately affected by RA, forming the 'RA-0' patient group included in July and August of 2017.

- 12 other patients (9 females, 3 males, average age: 57.5 $\pm$3.6, mean disease duration: 4.8 $\pm$1.2 years) suffering from more severe RA, who received antibody therapy with Abatacept (125 mg Orencia, BMS) and were examined before ('A-T0' group) and after 16 weeks of therapy ('A-T16' group). Samples from these patients were obtained from October 2014 to September 2016.

The control group consisted of 29 healthy volunteers ('HV') included between November 2015 and August 2017 (15 females, 14 males), with an average age of 28.6 $\pm$ 2.1 years, comprising mostly laboratory colleagues and fellow students.
Two venous blood samples in heparanized tubes (10 mL per tube) were drawn from each patient according to ethics committee votes EA1/138/14 and EA1/132/05.

2.3 Extraction of mononuclear cells from peripheral blood (PBMC)

Peripheral blood mononuclear cells (PBMCs) were isolated over a density gradient using the Ficoll Paque method, following the manufacturer's instructions. Heparinized whole blood was diluted with an equal volume of Gibco DPBS. 20 mL of Ficoll solution were carefully overlaid with 20 mL of diluted blood in a 50 mL conical tube and centrifuged at 900xg for 18 min at 20° C with the lowest possible deceleration. Next, the interphase layers containing lymphocytes were pipetted into a new 50 mL tube and washed with 50 mL Gibco DPBS two times.
The extracted cells were resuspended in DPBS and diluted in Trypan Blue in equal parts. Viable cells (cells that did not take up Trypan Blue) were counted using a Neubauer improved cell counting chamber under an inverted Hund Wetzlar microscope (15 - 50 $\times$ 10^6 cells/probe).

2.4 Isolation of RNA from PBMC

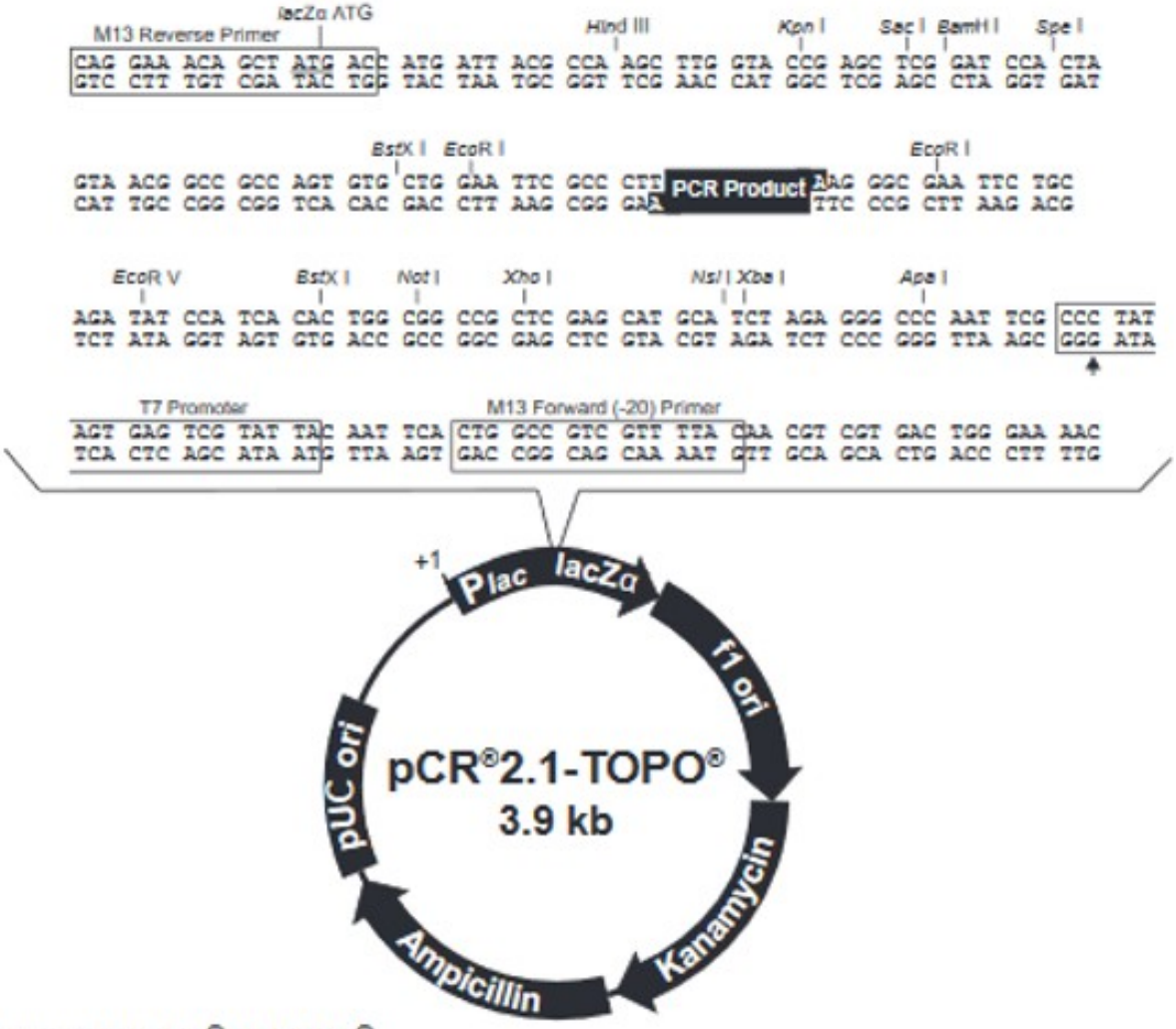
RNA was isolated from PBMC using peqGold RNAPure according to the manufacturer's protocol with 1mL RNAPure per 5 to 10×10^6 counted cells, which were washed again with DPBS and pelleted by centrifuging. After 5 minutes of incubation at room temperature, 0.2 mL chloroform per 1 mL RNAPure used were added. Samples were shaken vigorously and then incubated for 5 minutes on ice. Afterwards, they were centrifuged at 4° C and 12'000xg for 5 minutes. The clear, aqueous top layer (of three layers) containing RNA was placed into a new sterile tube. The RNA was precipitated by adding an equal volume of isopropanol and incubated for 15 minutes on ice. After centrifugation for 10 minutes, the precipitated RNA was washed two times with 75% ethanol. The RNA pellet was dried at 55° C (with an open tube cap), resuspended in Ampuwa endotoxin and RNAse free water and heated again for 5 minutes at 55° C with a closed cap.

The RNA pureness and quality was controlled by Nanodrop spectrophotometer. The RNA was subsequently reversely transcribed to cDNA via PCR (Tag Polymerase Kit, Taqman primer probe mix, Thermocycler T3000), following which cDNA pureness and quality was also controlled by Nanodrop spectrophotometer (for quality data, see 3.1).

2.5 Preparing DNA standards for TIRC7 and housekeeping gene β-actin

For normalization, β-actin was used, which is part of the cytoskeleton and usually expressed at the same levels in all cells, being a well-known housekeeping gene in RT-PCRs [95–99]. DNA fragments of TIRC7 and β-actin, respectively, was reproduced from the available cDNA of the PBMCs already obtained from healthy volunteers by performing standard PCR using target gene-specific Tagman primer probe mixes, the Taq Polymerase Kit (Life Technologies) and the Thermocycler T3000 (Biometra). As a control, a gel electrophoresis was performed with a 2% Agarose/TAE gel and a 100 bp Ladder Extended Marker, which showed the expected 90 and 66 bp long fragments for TIRC7 and β-actin, respectively. This PCR product was ligated into the vector pCR2.1-TOPO (Fig. 2.1) by using the TOPO TA cloning Topoisomerase, and then transferred into competent E. coli TOP10 bacteria, as well as puc19, which was used as a control for the subsequent cultures. The bacteria were cultivated on LB Agar plates with and without ampi-

cillin and left to incubate for 48 hours. Plasmid DNA was extracted from several clones of these cultures using the PeqGold Plasmid Mini Kit I. TIRC7 and β-actin DNA was cleaved from the plasmid rings using the restriction enzyme EcoRI, which cleaves pCR2.1 TOPO directly next to the DNA template. The cleavage was controlled by another gel electrophoresis. The DNA pureness and concentration were determined by using the Nanodrop spectrophotometer.



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure 2.1: Image of vector pCR2.1 TOPO.

http://tools.thermofisher.com/content/sfs/vectors/pcr2_1topo_map.pdf, October 2019.

Two of the TIRC7 and β-actin DNA samples, respectively, were sent to the LCG lab (LGC Genomics GmbH, Ostendstrasse 25, 12459 Berlin, Germany) for a forward and reverse sequence analysis. The analysis showed the expected base sequences of TIRC7 and β-actin, which was controlled by research in the NCBI database.
Following this proof of concept, this process was repeated on a larger scale for all PBMCs using the Plasmid DNA purification Maxi Nucleobond kit for plasmid DNA isolation. Plasmid DNA of both target genes was obtained as detailed above. Following another control gel electrophoresis, the rest of the plasmid DNA was linearized using restriction enzyme BglII, which cleaves pCR2.1 TOPO at base pair 1283. Afterwards, another concentration and quality measurement was performed using the Nanodrop spectrophotometer. Subsequently, the count of DNA template molecules per μL was calculated from the concentration value.

TIRC7 sequence:
GCCCTTCGTGGGCCGCTACCAGGAGGTCAACCCCGC
TCCCTACACCATCATCACCTTCCCCTTCCTGTTTGCT
GTGATGTTCGGGGATGTGGGCCACGGGCAAGGGC

β-actin sequence:
GGCTTCTGACCCATGCCCACCATCACGCCCTGGTGCC
TGGGGCGCCCCACGATGGAGGGGAAGACGGCAAGCC

2.6 qPCR

The quantitative Taq-PCRs (qPCR) were performed by using the StepOne Plus Real-Time PCR System, a 96-well PCR plate, Taqman Universal Master Mix and the target gene-specific Taqman primer probe mixes (FAM-labelled). In each PCR plate, a TIRC7 and a β-actin standard titration from 10^1 to 10^6 molecules and a negative control with aqua dest. were performed next to the healthy and patient sample set-ups. Every sample and standard titration was tested in duplicate. The qPCR reaction consisted of 5.0 μl TaqMan Universal PCR Master Mix (Thermo Fisher), 0.5 μL of the particular FAM-labeled Taqman probe primer mix, 3.5μL aqua dest. and 1 μL of cDNA in a total volume of 10 μL per well. The thermal cycling conditions for cDNA-amplification were: 2 minutes at 50° C and 10 minutes at 95° C, followed by 40 reaction cycles

(15 seconds at 95° C and 60 seconds at 60° C).

The obtained cT (threshold cycle) values were transferred into concentration values for both TIRC7 and β-Actin using a logarithmic formula derived from cT values of the standard dilution series for each PCR, respectively. The mean of this value from three PCRs for each probe results in the value with which all further calculations and graphs were made.

2.7 Statistical methods

Statistical calculations were performed in GraphPad Prism, version 5 (LaJolla, USA). Data are presented as mean $\pm$ SEM. P-values were calculated by using the Kruskal-Wallis test (KW) for overall group comparisons and the Mann-Whitney U Test (MWU) for independent samples or the Wilcoxo signed-rank test (WSR) for dependent samples, respectively. For correlation analysis, Spearman's rank correlation was applied. Statistical significance was set at $p < 0.05$. All data are presented as boxplots, outliers are defined by Tukey, crosses denote the mean.

3 Results

3.1 Robust quantification of TIRC7 expression by qPCR, spectrophotometry and gel electrophoresis

To quantify TIRC7 expression in the study population, a stable and reproducible protocol for measuring TIRC7 and β-actin expression on the cDNA level using the PCR method was established. For this analysis, cDNA of TIRC7 and housekeeping gene β-actin had to be amplified (see Chapter 2.5), serving as a control for the subsequent qPCRs. This process was controlled several times using gel electrophoresis.

The amplification of TIRC7 and β-actin cDNA from healthy volunteers' PBMCs using standard PCR showed the expected cDNA fragments of TIRC7 (90bp) and β-actin (66bp) cDNA in control gel electrophoresis (Figure 3.1A).

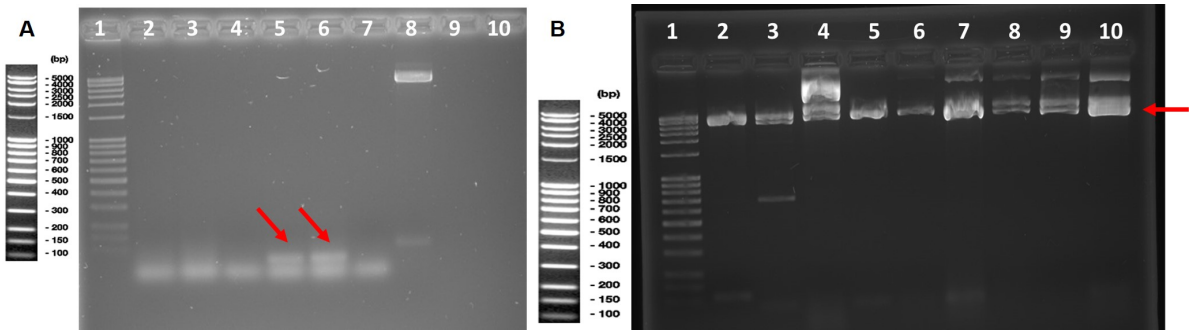


Figure 3.1: (A) Control gel electrophoresis for β-actin after standard PCR. Line 1 includes a 100bp Extended ladder marker (Carl Roth), lines 5 + 6 show the expected 66bp long fragment of β-actin DNA. Line 8 shows a 90bp TIRC7 fragment, already after enzymatic cleavage of pcr2.1 TOPO (see B). (Other lines: control samples after standard PCR with aqua dest. instead of cDNA.) (B) Control gel electrophoresis after enzymatic cleavage of pcr2.1 TOPO with TIRC7 and β-actin DNA by BglII. Pocket 1 = 100bp Extended ladder marker (Carl Roth), pockets 2-10 = expected 4kbp long fragment of linearized pcr2.1 TOPO with TIRC7 and beta-actin DNA, respectively.

After the enzymatic cleavage of the pcr2.1 TOPO plasmid rings by BglII, a control gel electrophoresis showed the circa 4kbp long linearized plasmids (3.1B).

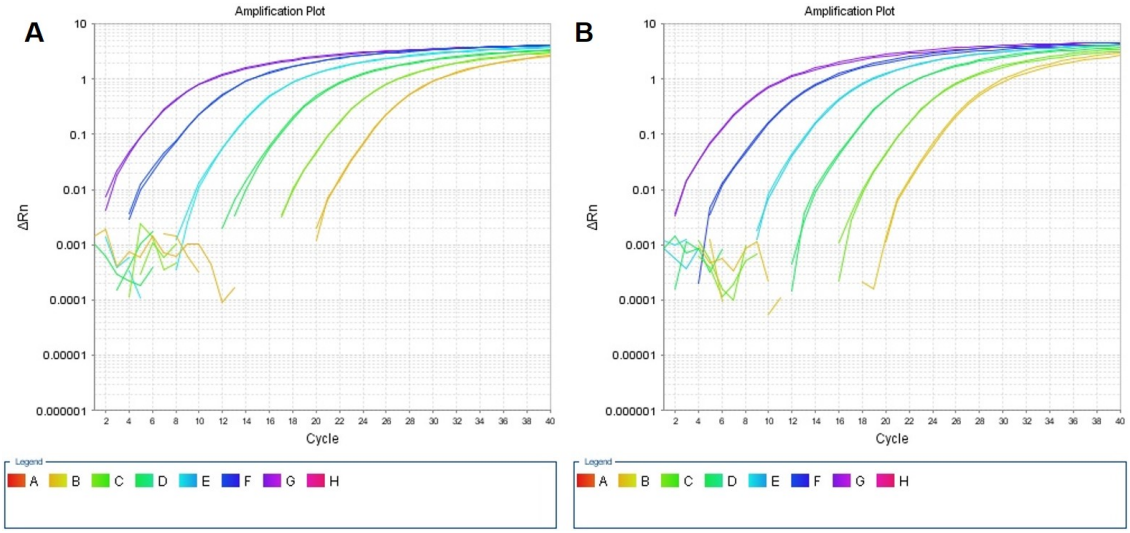


Figure 3.2: Example of a qPCR amplification plot of the standard dilution series (logarithmic titration) of TIRC7 cDNA (A) and the housekeeping gene β-actin (B). A = A. dest., B = 10 molecules/μL, C = 10^2 molecules/μL, ... , G = 10^6 molecules/μL. ΔRn (normalized fluorescence of reporter dye) plotted against PCR cycle number.

Total RNA quality was measured by Nanodrop spectrophotometer. The mean concentration was 608.93 ng/μL $\pm$ 6.36. The mean A260/A280 ratio, which was used to determine sample quality, was 1.83 $\pm$ 0.002. Only samples with sufficient quality (A260/A280 between 1.6 and 2.1) and concentration were used for further experiments.

Sample analysis was reproducible and within the standard range. Amplification occurred between PCR cycles 6 and 40 (Figure 3.2). Intra-run variability was expressed by a mean standard error of mean of 0.008.

Each sample was tested in three PCR plates, respectively, and in each plate, every sample was tested in duplicate to balance potential errors in pipette volume. For every PCR, the qualitative CT values of each well were transformed into concentration values to calculate the quotient of obtained values (TIRC7 / β-actin). Thus, a robust normalized value of TIRC7 expression could be determined and subsequently applied in the study populations.

3.2 Correlation of TIRC7 expression with characteristics and activation markers obtained from healthy volunteers and patients

3.2.1 Study participants

This study was designed to analyze TIRC7 expression in four groups:

1. Healthy volunteers (HV).
2. Moderately affected RA patients (RA-0).
3. Severely affected RA patients before Abatacept therapy (A-T0).
4. Severely affected RA patients (same patients as in 3), follow-up after 16 weeks of Abatacept therapy (A-T16).

Data for these groups are shown in Table 3.1. Participants in the HV group are younger than the participating RA patients. Differences in how the patient groups are affected are shown by clinical parameters.
In the following, statistically significant differences between the patient groups regarding clinical parameters will also be shown. For some parameters, few or no values were available across the different groups, restricting their comparability.

Group	HV	RA-0	A-T0	A-T16
n	29	22	12	12
TIRC7/β-actin ratio	0.026 ± 0.005	0.018 ± 0.004	0.072 ± 0.004	0.063 ± 0.006
Sex (female | male)	15 | 14	19 | 3	9 | 3	9 | 3
Age (years)	28.6 ± 2.1	60.8 ± 2.7	57.5 ± 3.6	57.5 ± 3.6
Smokers | non-smokers	2 | 21	3 | 6	2 | 6	n.a.
BMI (kg/m^2)	n.a.	26.4 ± 2.1	n.a.	n.a.
ACPA (+ | -)	n.a.	15 | 4	7 | 5	n.a.
ANA (+ | -)	n.a.	7 | 8	n.a.	n.a.
CRP (mg/L)	n.a.	3.8 ± 0.9	3.7 ± 1.1	2.9 ± 1.2
BSG (mm/h)	n.a.	22.5 ± 3.6	21.4 ± 6.8	14.5 ± 2.7
Tender joints	n.a.	4.3 ± 1.5	7.8 ± 1.9	3.0 ± 1.2
Swollen joints	n.a.	1.5 ± 0.5	4.2 ± 0.6	1.3 ± 0.7
DAS28-ESR	n.a.	3.34 ± 0.38	4.25 ± 0.34	2.76 ± 0.38
DAS28-CRP	n.a.	2.59 ± 0.30	4.03 ± 0.42	2.86 ± 0.86

Table 3.1: Clinical and biochemical characteristics of study participant groups - healthy volunteers (HV), moderately affected RA patients (RA-0) and severely affected RA patients (A-T0), re-examined after 16 weeks of Abatacept treatment (A_T16). n = number of participants, n.a. = data not available. Data shown as mean $\pm$ SEM.

3.2.2 Distribution of TIRC7 expression in study cohorts - characteristics of patients and healthy volunteers in relation to TIRC7 expression

Across groups, TIRC7 / β-actin ratio values ranged absolutely from a minimum of 0.001 to a maximum of 0.133. Kruskal-Wallis test shows a statistically significantly difference overall of $p < 0.0001$. The values differ statistically significantly ($p < 0.0001$) between the healthy volunteers (HV) group (mean 0.026 ± 0.005) and the Abatacept group before intervention (A-T0) (mean 0.072 ± 0.004) as well as between the moderately affected group of RA patients (RA-0) (mean 0.018 ± 0.004) and the A-T0 group. Patients in the Abatacept group after 16 weeks of Abatacept therapy (A-T16) showed a slight yet statistically insignificant ($p = 0.252$) decrease of TIRC7 / β-actin ratio values (mean 0.063 ± 0.006) compared to their initial values before the intervention. These data indicate that TIRC7 expression coincides with disease activity and might be modulated by targeted therapy (Figure 3.3). For concomitant changes in clinical aspects/parameters of Abatacept patients, see Chapter 3.2.4.

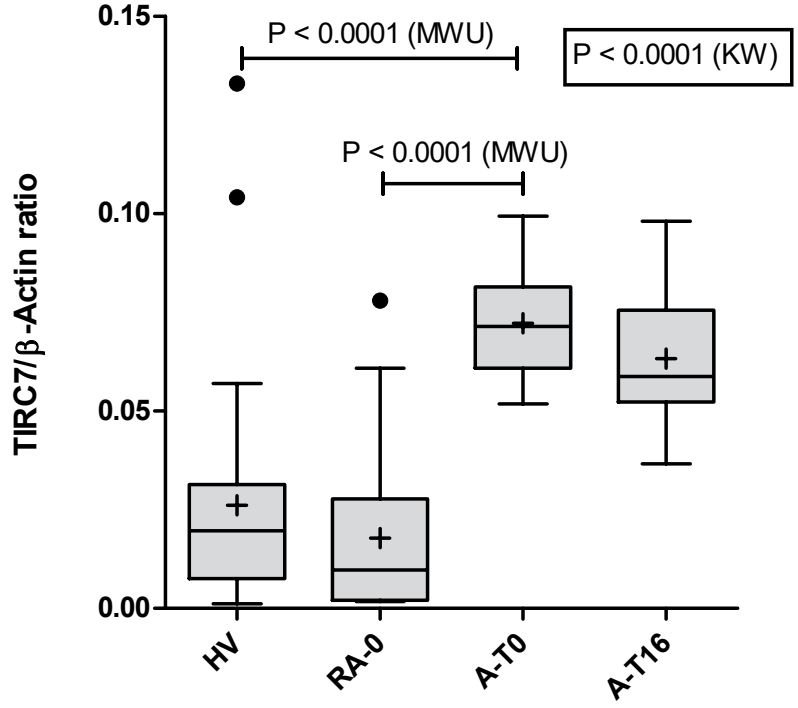


Figure 3.3: TIRC7 / β-actin ratio in healthy volunteers (HV), moderately affected RA patients (RA-0) and more severely affected RA patients participating in a study before (A-T0) and after (A-T16) 16 weeks of treatment with Abatacept. The Kruskal-Wallis test showed a statistically significant difference ($p < 0.0001$) between the groups. The differences between the HV and A-T0 groups examined by Mann-Whitney U test ($p < 0.0001$) as well as the RA-0 and A-T0 groups ($p < 0.0001$) are statistically significant, while the differences between the HV and RA-0 groups ($p = 0.162$) as well as between the A-T0 and A-T16 groups examined by Wilcoxon signed-rank test ($p = 0.339$) are not statistically significant.

3.2.3 Relationship of TIRC7 expression with predisposing factors

RA incidence and progression are known to be influenced by and correlated with variables such as sex, age, smoking habits and body mass index (BMI) (see Chapter 1.2). Comparing the TIRC7 / β-actin ratio to these variables revealed that, amongst healthy volunteers, the TIRC7 / β-actin ratio values were statistically significantly higher ($p = 0.025$) in females (0.037 ± 0.009) compared to males ($0.015, \pm 0.004$). However, no sex-specific difference in TIRC7 expression was observed in patient groups (p-values: RA-0 female vs. male: 0.702, A-T0 female vs. male: 0.6, A-T16 female vs. male: 0.482) (Fig. 3.4A). TIRC7/β-actin ratio levels did not change statistically significantly by age either in healthy volunteers (mean age: 28.6

years; Spearman's ρ = -0.004, p = 0.986) or in RA-0 (mean age 60.8 years; Spearman's ρ = 0.409, p = 0.059) or A-T0 patients (mean age: 57.5 years; Spearman's ρ = 0.476, p = 0,1176), although a downward trend with increasing age was observed for the HV group and upward trend for the patient groups (Fig. 3.4B). Also, no statistically significant difference between smokers and non-smokers was observed (Fig. 3.4C). However, in patients of the RA_0 group (n = 13), a higher body mass index (BMI) showed statistically significantly higher TIRC7 / β-actin ratio values indicating a direct correlation (Spearman's ρ = 0.67, p = 0.012) (Fig. 3.4D). For the other groups of study participants, BMI data were not available.

This study aims to explore possible relations between acute or chronic inflammation associated with autoimmunity and TIRC7 expression. Therefore, the duration of disease and its correlation with TIRC7 / β-actin ratio was also examined. The disease duration was documented for only 10 patients in the RA-0 patient group and did not show any statistically significant correlation with TIRC7 expression (r^2 = 0.109, p = 0.351; Spearman's ρ = 0.293, p = 0.412). In A-T0 patients, disease duration and TIRC7 / β-actin had a statistically significant negative correlation (r^2 = 0.378, p = 0.034; Spearman's ρ = -0.692, p = 0.013), as shown in Fig. 3.5A.

As the therapy paradigms of autoimmune diseases are known to influence immunological pathways in different ways, the relation of TIRC7 expression to various therapies was examined. Patients were split into groups by assessing the number of medicaments from the cardinal medication classes taken regularly (NSAIDs, corticoids, synthetic DMARDs and antibodies, see Chapter 1.2.4). These calculations were made for all patients from the RA-0, A-T0 and A-T16 groups, counting the latter ones as single data points because their medication paradigm had changed. The Kruskal-Wallis test did not reveal statistically significant differences between the groups (p = 0.388, see Fig. 3.5B).

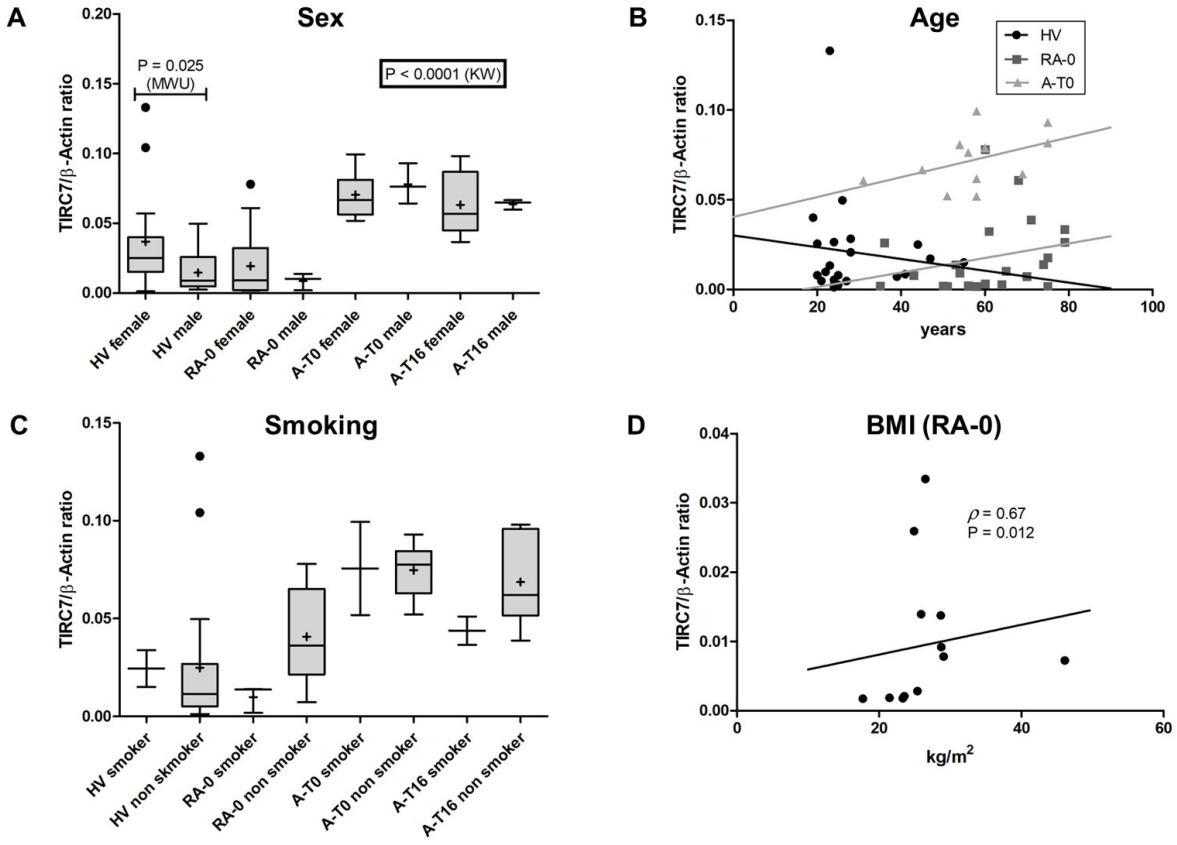


Figure 3.4: TIRC7 / β-actin ratio vs. different external variables. (A) Sex. TIRC7 / β-actin ratio in females and males, split into the groups of study participants (HV, RA-0, A-T0, A-T16). The results of the females and males within the healthy volunteers group (HV) differ statistically significantly ($p = 0.025$). (B) Age. TIRC7 / β-actin ratio vs. age in RA-0 (dark grey) and AT-0 patients (light grey) and all healthy volunteers (black). Neither in healthy volunteers nor in RA patients did the TIRC7 / β-actin ratio change statistically significantly during life. (C) Smoking. The TIRC7 / β-actin ratio in all RA patients and healthy volunteers divided as smokers and non-smokers. There are no statistically significant differences between the groups. (D) Body mass index (BMI). TIRC7 / β-actin ratio vs. body mass index (BMI) only in 13 patients of the RA-0 group. BMI and TIRC7 / β-actin ratio show a statistically significant correlation (Spearman's $\rho = 0.67$, $p = 0.012$).

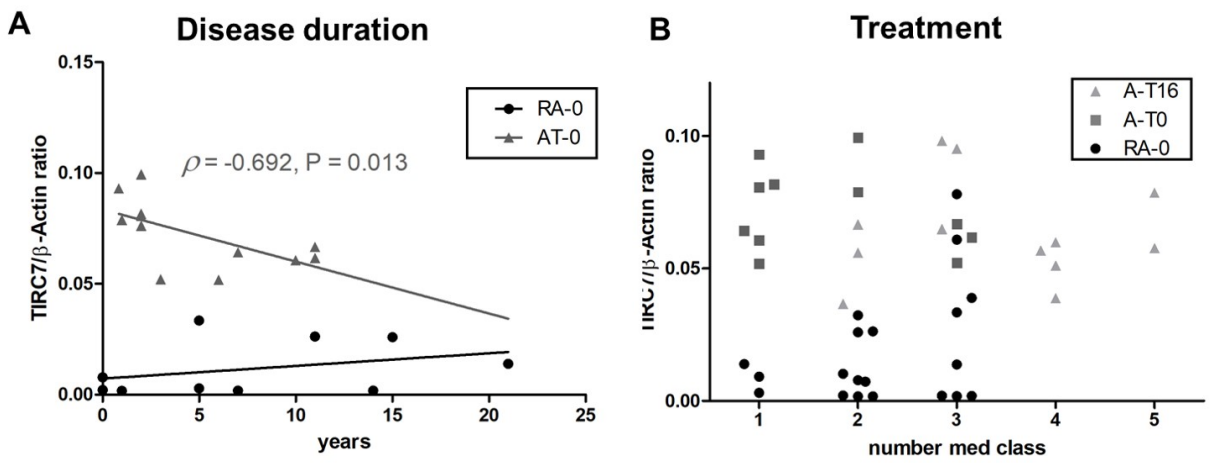


Figure 3.5: TIRC7 / β-actin ratio levels vs. duration of disease and treatment. (A) Disease duration. TIRC7 / β-actin ratio in RA-0 and A-T0 patients vs. time in years since first diagnosis. No statistically significant correlation was found between disease duration and TIRC7 / β-actin ratio in RA-0 patients, but in A-T0 patients a statistically significant negative correlation was shown (Spearman's ρ = -0.692, p = 0.013). (B) Treatment. TIRC7 / β-actin ratio in RA-0, A-T0 and A-T16 patients categorized into groups reflecting the number of medicaments from the cardinal rheumatoid arthritis medication classes taken regularly. No statistically significant difference was found between the groups, examined by Kruskal-Wallis test.

3.2.4 Correlation of TIRC7 expression with rheumatoid arthritis biomarkers and disease severity scoring systems

There are different seromarkers that are commonly examined in patients with (suspected) RA such as Rheumatoid factors (RF) and antibodies against cyclic citrullinated peptides (ACPA). As TIRC7 might be a marker for the early detection of disease flares and prognosis in RA patients, a comparison to the currently clinically used RA seromarkers seemed useful.

Rheumatoid factors were seropositive in all patients taking part in this study (where assessed). But as described in the previous chapters, there are more established seromarkers to be tested in (suspected) RA, e.g. antibodies against cyclic citrullinated peptides (ACPA). For differentiation between other autoimmune processes, the RA-0 patients were also partially examined for anti-nuclear-antibodies (ANA). TIRC7 expression differed statistically significantly ($p = 0.048$) in just the A-T0 group between ACPA-positive (0.065 ± 0.006) and -negative patients (0.082 ± 0.003), but not between ACPA-positive (0.021 ± 0.006) and -negative (0.014 ± 0.005) RA-0 patients ($p = 0.13$) (see Fig. 3.6A). Regarding ANA, no significant differences between seropositive ($0.0309 \pm 0{,}01$) and -negative in RA-0 patients (0.011 ± 0.004) were observed ($p = 0.96$) (Fig. 3.6B).

Different disease activity scores for RA are used in clinical practice and in trials, e.g. DAS28. For exploring the correlation of clinical manifestation and TIRC7 expression in RA patients, DAS28 and its components might be useful established parameters. In the majority of examined patients in this study, at least one DAS28 score was either assessed or could easily be calculated based on the collected data. The statistical calculations here were made with two alternative/reduced DAS28 formulae without global health assessment (usually VAS or NRS). These calculations contain the tender and swollen joint counts combined with either ESR (DAS28-ESR) or CRP (DAS28-CRP).

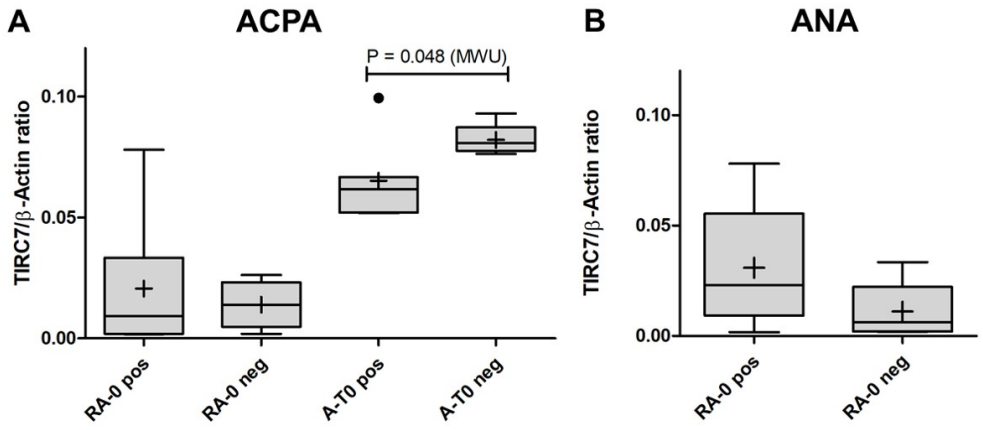


Figure 3.6: TIRC7 / β-actin ratio levels vs. seromarkers. (A) Antibodies against cyclic citrullinated peptides (ACPA). TIRC7 / β-actin ratio levels in RA-0 and A-T0 patients who are either ACPA positive or negative. There is a small, but significant difference (p = 0.048 in Mann-Whitney U test) between the ACPA positive and negative A-T0 patients. (B) Anti-nuclear antibodies (ANA). TIRC7 / β-actin ratio levels in RA-0 patients who are either ACPA positive or negative. No statistically significant difference between ANA positive and negative patients were shown.

The DAS28-ESR was newly calculated using only the three parameters - Tender Joints, Swollen Joints and ESR - as follows:

$$\text{DAS28-ESR} = 0.56 * (\text{tender joint count})^2 + 0.28 * (\text{swollen joint count})^2 + 0.70 * \ln(\text{ESR})$$

The DAS28-CRP was newly calculated using only the three parameters - Tender Joints, Swollen Joints and CRP - as follows:

$$\text{DAS28-CRP} = 0.56 * (\text{tender joint count})^2 + 0.28 * (\text{swollen joint count})^2 + 0.36 * \ln(\text{CRP}+1)$$

Both DAS28 scores were tested for differences between patient groups and for their relation with TIRC7 expression, the latter by either categorizing patients by their DAS28 levels or assessing linear correlation. DAS28-ESR values did not statistically significantly differ (p = 0.116) between RA-0 and A-T0 patient groups (3.335 $\pm$ 0.36 vs. 4.251 $\pm$ 0.341). However, in the latter group, values were statistically significantly (p = 0.02) reduced following Abatecept intervention (A-T16: 2.757 $\pm$ 0.384). DAS28-ESR values did not correlate with TIRC7-expression (see Fig. 3.7A-C). In contrast, DAS-CRP values were statistically significantly different (p = 0.024) between RA-0 and A-T0 patient groups (2.54 $\pm$ 0.293 vs. 4.030 $\pm$

0.415) with no statistically significant reduction observed following intervention (A-T16: 2.86 $\pm$ 0.86). In this case, no statistically significant correlation between DAS28-CRP values and TIRC7 expression was observed (see Fig. 3.7D-F).

Additionally, respective single components of DAS28 (tender and swollen joints, ESR, CRP) were examined for distribution in each patient group and correlation with TIRC7 expression. The evaluation of tender joints showed no statistically significant difference (p = 0.116) between the RA-0 (4.26 $\pm$ 1.488) and A-T0 (7.75 $\pm$ 1.875) groups, but identified a statistically significant difference (p = 0.014) between A-T0 and A-T16 patient groups (3.0 $\pm$ 1.181). No statistically significant correlation between tender joints and TIRC7 / β-actin ratio levels in patients was identified, whereas swollen joints counts statistically significantly differed between RA-0 (1.526 $\pm$ 0.4978) and A-T0 (4.167 $\pm$ 0.649) (p = 0.002) as well as between A-T0 and A-T16 (1.333 $\pm$ 0.6552) (p = 0.006) patient groups. Additionally, a statistically significant correlation of Spearman's ρ = 0.473 (p = 0.007) of TIRC7 / β-actin ratio levels and swollen joints was observed examining RA-0 and A-T0 patient data together. Swollen joints were shown to have a strong correlation with TIRC7 expression (Fig. 3.8).
No statistically significant differences between the patient groups or statistically significant correlations with TIRC7 expression could be shown for ESR and CRP (Fig. 3.9).

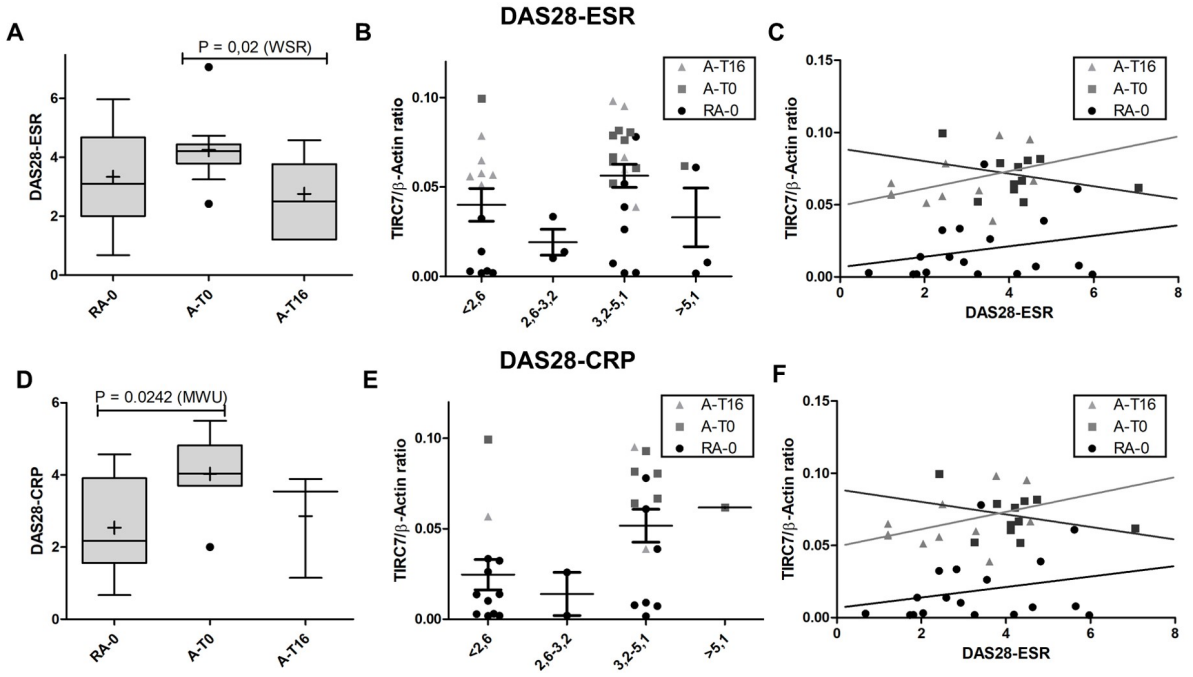


Figure 3.7: DAS28-ESR and DAS28-CRP in patients and vs. TIRC7. (A) DAS28- ESR scores in patient groups. There is a statistically significant difference (p = 0.02) between the DAS28-ESR levels of the A-T0 and the A-T16 groups in the Wilcoxon-signed rank test. (B) TIRC7 vs. DAS28-ESR in all patient groups. TIRC7 / β-actin ratio levels of RA-0, A-T0 and A-T16 patients categorized by their DAS28-ESR scores. There are no statistically significant differences between the groups examined by Kruskal-Wallis test. (C) TIRC7 vs. DAS28-ESR shown linearly in the RA-0, A-T0 and A-T16 patient groups. Despite a tendency, no statistically significant correlation was observed. (D) DAS28-CRP scores in all patient groups. The Mann-Whitney U test showed a statistically significant difference (p = 0.024) between the DAS28-CRP levels of the RA-0 and the A-T0 groups. (E) TIRC7 vs. DAS28-CRP in patient groups. TIRC7 / β-actin ratio levels of RA-0, A-T0 and A-T16 patients categorized by their DAS28-CRP scores. There are no significant differences between the groups examined by Kruskal-Wallis test. (F) TIRC7 vs. DAS28-CRP shown linearly in the RA-0, A-T0 and A-T16 patient groups. There are no statistically significant correlations.

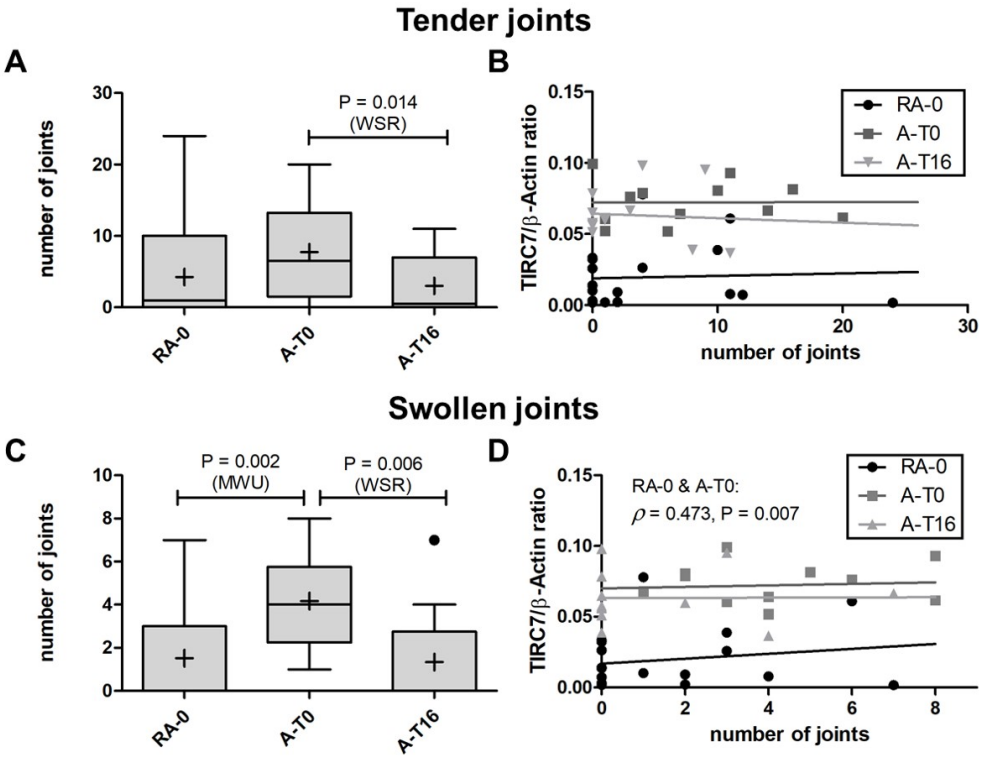


Figure 3.8: Tender and swollen joints in patients and vs. TIRC7. (A) Number of tender joints in all patient groups. There is a statistically significant difference (p = 0.014) between the number of tender joints in the A-T0 and the A-T16 groups. (B) TIRC7 vs. number of tender joints shown linearly in the RA-0, A-T0 and A-T16 patient groups. Despite a tendency, no statistically significant correlation could be shown. (C) Number of swollen joints in all patient groups. There is a statistically significant difference between the number of tender joints in the RA-0 and the A-T0 groups (p = 0.002) as well as between the A-T0 and the A-T16 groups (p = 0.006). (D) TIRC7 vs. number of swollen joints shown linearly in RA-0, A-T0 and A-T16 patient groups. A statistically significant correlation (Spearman's ρ = 0.473, p = 0.007) was observed examining RA-0 and A-T0 patient data together.

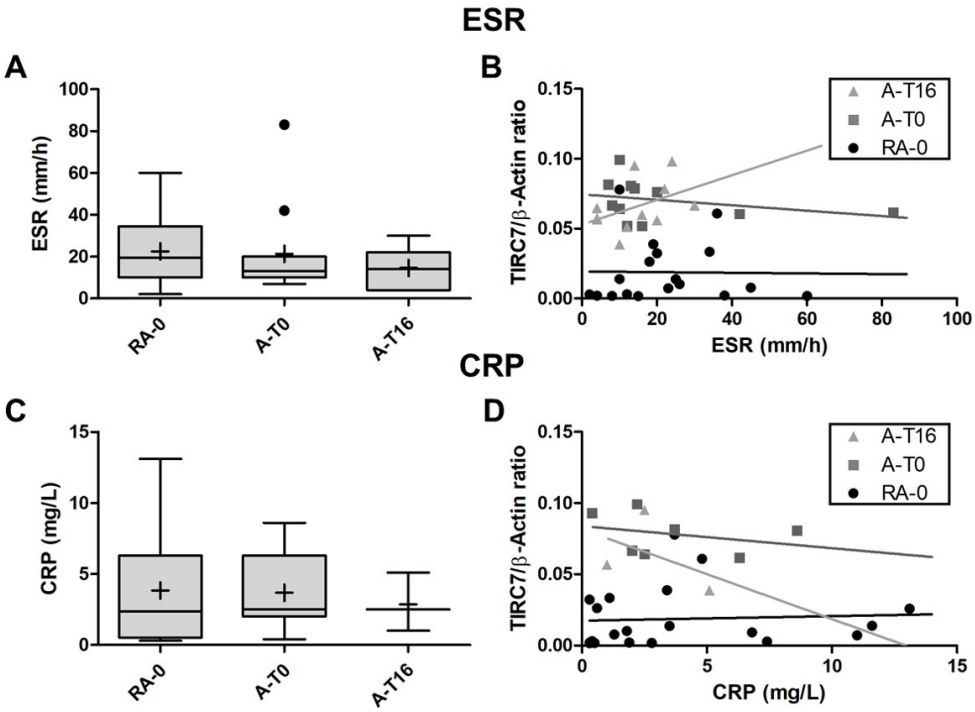


Figure 3.9: Erythocyte sedimentation rate (ESR) and C-reactive protein (CRP) in patients and vs. TIRC7. (A) ESR in mm/h in all patient groups. There is no statistically significant difference between the groups. (B) TIRC7 vs. ESR in mm/h shown linearly in the RA-0, A-T0 and A-T16 patient groups. No statistically significant correlation was shown. (C) CRP in mg/L in all patient groups. There is no statistically significant difference between the groups. (D) TIRC7 vs. CRP in mg/dl shown linearly in the RA-0, A-T0 and A-T16 patient groups. No statistically significant correlation was observed.

In the overall Spearman correlation analysis, a positive correlation between TIRC7 / β-actin ratio levels in patients and their swollen joints count can be found (Spearman's ρ = 0.473, p = 0.007). In contrast, no correlations between ESR or CRP and the numbers of tender and swollen joints were shown. Moreover, the known correlations between the particular DAS28 components (tender and swollen joints, ESR, CRP) and the respective DAS28 values themselves could be observed (Table 3.2).

	TIRC7/ beta-actin	ESR	CRP	Tender joints	Swollen joints	DAS28-ESR	DAS-CRP
TIRC7/ beta-actin		-0.142 (0.464)	0.233 (0.242)	0.309 (0.091)	**0.473 (0.007)**	0.297 (0.118)	0.355 (0.076)
ESR	-0.142 (0.464)		-0.048 (0.828)	0.142 (0.470)	0.264 (0.174)	0.430 (0.020)	0.059 (0.788)
CRP	0.233 (0.242)	-0.048 (0.828)		0.016 (0.940)	0.054 (0.799)	0.156 (0.476)	0.375 (0.059)
Tender joints	0.309 (0.091)	0.142 (0.470)	0.016 (0.940)		0.613 (<0.001)	0.910 (<0.001)	0.905 (<0.001)
Swollen joints	**0.473 (0.007)**	0.264 (0.174)	0.054 (0.799)	0.613 (<0.001)		0.752 (<0.001)	0.735 (<0.001)
DAS28-ESR	0.297 (0.118)	0.430 (0.020)	0.156 (0.476)	0.910 (<0.001)	0.752 (<0.001)		0.864 (<0.001)
DAS28-CRP	0.355 (0.076)	0.059 (0.788)	0.375 (0.059)	0.905 (<0.001)	0.735 (<0.001)	0.864 (<0.001)	

Table 3.2: Collective correlation analysis (Spearman's *rho*, two-sided) of the above singularly analyzed parameters, which shows a statistically significant correlation between TIRC7/β-actin ratio and swollen joints (Spearman's ρ = 0.473, p = 0.007). Also, all other known correlations between components of DAS28 (tender and swollen joints, ESR, CRP) and the according DAS28 values occurred as expected. No statistically significant correlations between ESR, CRP and tender or swollen joints were identified. Data are displayed as correlation coefficient (significance level).

4 Discussion

4.1 Discussion of results

This study is unique as the patients and methods employed in this study had not been utilized before to analyze TIRC7 in RA. The study revealed promising results showing statistically significant differences of TIRC7 expression in different cohorts of RA patients and healthy volunteers.

Affirming the hypothesis, based on the results, a statistically significant difference was observed in TIRC7 expression between patients suffering from severe RA and healthy volunteers, as well as between patients suffering from severe RA and only moderately affected patients.

Interestingly, the TIRC7 expression levels between healthy volunteers and moderately affected RA patients did not statistically significantly differ, indicating a decrease of TIRC7 expression under satisfactory treatment. These findings accord with former trials showing an increase of TIRC7 expression in peripheral blood cells in acute autoimmune processes [18–20]. In some autoimmune processes, a drift of TIRC7 expressing cells from peripheral blood to affected tissue has also been observed [12].

In particular, in joint tissue obtained from patients suffering from RA, an upregulation of TIRC7 expression in lymphocytes was previously demonstated [17]. Results of the present study are concordant to these findings. However, in the HV group there are several outliers that raise the mean. No methodic error could be identified for these samples, the standard error of mean for the three respective PCRs were within the normal range. No additional haemogram could be performed for the healthy volunteers, so acute inflammations cannot be excluded for the time of the blood withdrawal.

The question of whether the 6 out of 12 patients whose TIRC7 / β-actin ratio did not decrease after 16 weeks of Abatacept intervention did not respond to the therapy at all cannot be answered conclusively by the available data. Conversely, however, also in these patients e.g. the swollen and tender joint counts did indeed decrease, indicating at least a clinical improvement.

Interestingly, in the present study, an outstanding correlation of TIRC7 expression was observed with the body mass index (BMI). Fat tissue is widely known to be immunologically active and a higher BMI is known for increasing the risk of developing RA; both aaccord with these find-

ings. TIRC7 expression was only shown to be higher in females than in males in the HV group. Sex and smoking habits did not show conclusive results, which might be also explained by the low number of study participants in each group [37].

However, only 6 out of 34 RA patients are male, there is a large age difference between the HV and RA groups and the BMI was only available in the RA-0 group for 13 patients, hindering the drawing of valid conclusions. Additionally, smoking information was too limited in the RA groups to make meaningful assessments. The older patients in the RA group showed a statistically non-significant trend to higher TIRC7 expression (> 0.05). Zhe clinical characteristics are concordant, but no specific reason for this trend could be identified from the available data. In future studies, older healthy volunteers from 20 to 80 years of age should also be examined.

The disease duration and its relation to TIRC7 expression was examined, because a change of immunologic processes during the disease course has to be supposed [100]. In this study, a statistically significant negative correlation of disease duration and TIRC7 expression was only found in the A-T0 group of RA patients.

The number of drugs from the RA medication cardinal classes did not show a statistically signficant correlation with TIRC7 expression; however, these types of analyses require larger cohorts. Correlation of TIRC7 expression levels with RF, ACPAs or ANAs have to be examined further in a setting with more patients included and more information about the respective antibody titers. Titers of these RA specific seromarkers are known to be inconsistent over time. There can also be a seroconversion from seronegative to seropositive for one of these antibodies in one RA patient during the course of the disease. Thus, data on these markers has to be considered with caution.

DAS28 and its single components are appropriate for investigating the relation of TIRC7 to clinical disease activity. This data is easily assessed and is reproducible. Here, a version of disease activity score without global health assessment had to be chosen, because neither data for the numeric rating scale (NRS) nor for the visual analogue scale (VAS) were obtained from the patients. Because ESR and CRP were not available from each patient, two different formulae involving ESR or CRP and DAS28 were examined. The results promisingly point out significant differences of DAS28 between the patient cohorts. In particular, the swollen joint count correlated significantly with the assigned patient cohorts and with the TIRC7 expression, whereas the tender joint count, ESR and CRP did not.

Both inflammatory seromarkers (influencing the DAS28 scores strongly even at normal ranges)

are known to be interfered with by other acute inflammation processes, tumors and drugs, and are not always sensitive in rheumatoid arthritis, leading to DAS28 being not precisely predictive, and sometimes even leading to false-positive results for remission in actually non-remissive RA patients [101–103]. As TIRC7 expression in this study actually seems to relate better to the assigned patient group as well as to real-life clinical parameters like the swollen joints count in comparison to ESR or CRP, further studies might replace those parameters in disease activity scores with TIRC7 expression levels for therapeutic monitoring.

Nevertheless, the results of this study have to be supported by an additional larger trial of patients, as only a limited number of patients could be enrolled for the current analysis. Additionally, as some of the data was analyzed retrospectively, detailed information on specific parameters for some patients were missing, e.g., the body mass index was not known for most of the patients, and other additional diagnoses were not documented in the files of the majority of the participants, which would be important for the evaluation of the levels of inflammation seromarkers, amongst others.

4.2 Discussion of methods

The aim of this study was to quantify and compare expression of TIRC7 in healthy volunteers and patients suffering from rheumatoid arthritis. Due to RA being a systemic, inflammatory disease with multiple confounding and influencing parameters (e.g. age, weight, smoking habits, current medication), this study was designed to test TIRC7 expression in different patient cohorts of RA disease manifestation. Although any quantification measure benefits from larger samples, the samples provided here (> 10 per group) were sufficient to identify initial differences in TIRC7 expression. Some values were not available for every study participant, which might confound results, e.g., for age, smoking habits and BMI. Also, due to the natural distribution of sex in RA patients, very few male RA patients could be included in the study.

In this study, peripheral mononuclear blood cells were isolated from peripheral blood using Ficoll paque to measure their TIRC7 expression at the RNA level using qPCR. The combination of both experimental methods had only been used in one previous study on TIRC7 expression during cardiac allograft rejection in humans by Shulzenko et. al [11]. Other methods used to measure TIRC7 in peripheral blood monocytes have been mostly immunohistochemical or flow cytometric (compare e.g. compare [18]).

Isolating PBMCs from blood using the density gradient of Fiquoll paque is a cost effective, routinely used, and simple method. A disadvantage might be that only the entirety of mononuclear blood cells can be isolated. For exact differentiation between lymphocyte subsets, other methods like flow cytometry are required. Nevertheless, the Ficoll paque method was chosen here for data acquisition in order to assess a global measure of gene expression independent of individual cell type.

PCR and qPCR are very reliable and commonly used methods. Isolating RNA from PBMCs, then transcribing it into cDNA and measuring the TIRC7 cDNA in it is a complicated method and prone to inaccuracies. There are other ways to examine expression of TIRC7 in immune cells, that have been used in previous studies (e.g., immunohistochemistry and fluorescence microscopy, compare [18]). However, to investigate the upregulation of the genes directly, the method applied here was the most appropriate. To ensure the best possible accuracy, the samples from each patient were tested in three different PCR runs in duplicates, respectively. In further studies, a method has to be found to reduce the intra- and inter-run variability.

Here, for quantification, the TIRC7 mRNA concentration was only plotted against β-actin as a housekeeping gene. β-actin is a well-known housekeeping gene used for normalization in PCR, but regulation of this gene has been shown in activated immune cells [104, 105], which suggests that the results of this study should be considered with caution. Further studies will still have to compare different housekeeping genes when employing this method.

In summary, for evaluating the hypotheses, the method used in this study was appropriate; however, in case of use in clinical settings, this method has to be validated under GLP conditions.

5 Conclusions and future directions

5.1 Summary

In conclusion, even though there are uncertainties concerning the methods and results, a statistically significant correlation of TIRC7 expression and several factors influencing immune processes can be demonstrated in this study.

5.2 Evaluation of the hypotheses

1. *TIRC7 expression is altered in peripheral blood mononuclear cells (PBMCs) in patients with rheumatoid arthritis compared to healthy subjects.*
   TIRC7 expression is indeed altered in the PBMCs of patients suffering from severe RA compared to healthy volunteers, but also compared to only moderately affected RA patients.

2. *TIRC7 expression in PBMCs is altered by factors known to influence immunologic processes (age, sex, body weight, smoking habits), in line with its role as a marker of immune activation.*
   This study has found a statistically significant correlation between body mass index and TIRC7 expression in the PBMCs of RA patients. Only in healthy subjects was TIRC7 expression statistically significant higher in females than in males. Other factors influencing immunologic processes, such as sex and cigarette smoking habits, were not found to correlate clearly with TIRC7 expression.

3. *TIRC7 expression in PBMCs correlates with disease activity measured by disease activity scoring instruments in patients with RA.*
   A statistically negative correlation of TIRC7 expression and disease duration could only be found in A-T0 patients. The correlation of swollen joint count and TIRC7 expression in RA patients was shown to be statistically significant, whilst tender joint count, CRP and ESR showed no remarkable relation to TIRC7 expression here.

5.3 Future directions and opportunities

The role of TIRC7 as a marker of immune regulation still remains unclear, reflecting ambiguous findings for it either as a sign for autoimmunity or as a protective factor against autoimmunity in previous studies. Is it part of the cause for autoimmune processes or part of the body's response to them?

The question of cause and effect, however, might better be examined by a differently designed, prospective study.

A promising approach to follow in search of novel markers in RA might be the specifically expressed MicroRNA patterns of FLS [41–43].

Other approaches to be considered in future research about TIRC7 expression in RA were elaborated in detail in Chapter 4. There is still long-term research needed, until a new diagnostic marker, a marker for monitoring of therapy effectiveness or even a novel antibody or cell therapies for autoimmune diseases (like RA) might be developed with TIRC7.

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Appendix / Anhang

Statutory Declaration / Eidesstattliche Versicherung

"I, Luise Anne Lehmann, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic 'Expression analysis for T-cell immune response cDNA 7 (TIRC7) in patients with rheumatoid arthritis based on quantitative real-time PCR / Expressionsanalyse von T-cell immune response cDNA 7 (TIRC7) in Patient*innen mit Rheumatoider Arthritis mittels quantitativer Echtzeit-PCR', independently and without the support of third parties, and that I used no other sources and aids than those stated. All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility. Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons. My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice. I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty. The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date: _____

Signature: _____

Curriculum vitae / Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Acknowledgements / Danksagung

I would like to express my sincere appreciation to the following people:

- I'm extremely thankful to **PD Dr. Nalân Utku** who accepted my application in 2015 for this project and without whose constant support and steady positive reinforcement it could never have been accomplished.
- I would like to express my deep gratefulness to my first supervisor **Dr. Ulrike Erben**, who taught me patiently and thoroughly every method needed in the lab and who motivated me every day to keep on. I am glad to have met her.
- Profound thanks also go to **Dr. Anja Kühl** for her encouragement as well as technical help concerning my project and for the common participation in charity running events.
- I also wish to thank **Prof. Dr. Eugen Feist** for his helpful interventions.
- Many thanks go to all the people working in the Gastro Lab in Berlin Steglitz (Charité Campus Benjamin Franklin) and in the Rheumatologic ambulance in Charité Campus Mitte.
- My thanks also go to **Holger Jansen** for lecturing and valuable advices concerning statistics.
- Finally, I would like to express my special thanks to my family and friends for their ever-present support and love.