Novel marker for rheumatoid arthritis disease activity

by

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ABSTRACT

RATIONALE: Rheumatoid arthritis (RA) is a chronic autoimmune disease, characterized by pain in affected joints, stiffness, and symmetrical synovitis. Synovial membrane inflammation of diarthrodial joints is a distinctive feature of RA, leading to articular damage, decline in motility and eventually complications, such as cardiomyopathy, neurologic and metabolic disorders. Currently available biomarkers are not satisfactory in terms of monitoring disease activity of RA.

AIM: The objective of this study is to show Collagen triple helix repeat containing 1 (CTHRC1) protein`s potential in monitoring RA disease activity

HYPOTHESIS: CTHRC1 is a potential biomarker for assessing RA disease activity.

The diagnosis of RA depends primarily on clinical assessments. Serology tests routinely used in RA diagnosis are Anti-citrullinated peptide antibody (ACPA) and Rheumatoid factor (RF) level determination in serum/plasma. However, the value of RF for assessing RA remains debatable, because it is also detected in connective tissue diseases, chronic infections, malignancy and in healthy individuals. In comparison, ACPA's are present in the peripheral blood of almost 80% of RA patients with higher diagnostic specificity. However, in our study ACPA was not associated with disease activity in patients with established diagnosis of RA. Demand for quantitative assessment of disease activity in RA for the improvement of disease diagnosis, prognosis and management is still high. Here I had proposed that the CTHRC1 is a marker of differential diagnosis of RA from OA, ReA and showed high potential to be used to monitor disease activity.

METHODS: For this clinical cross sectional study in total 148 individuals with established diagnosis of RA (57), Osteoarthritis (OA-65), Reactive arthritis (ReA-12) and healthy volunteers (14) were recruited. All patients were undergoing treatment at the time of enrollment. Prior collecting and testing plasma samples of patients, they were clinically assessed, including current status, number of swollen and tender joints, tested for complete blood count parameters, current level of RF and ACPA, and also MRI and X-ray of knee joints were performed. Collected plasma samples were tested for the levels of CTHRC1, pro-inflammatory cytokines, such as interleukin 1 beta (IL-1b), interleukin 6 (IL-6), interleukin 8 (IL-8), and interferon gamma (IFN g). All collected data were analyzed including comparison among groups, correlation within each group and Receiver operating characteristic (ROC) analysis was further performed to assess the diagnostic value of CTHRC1.

CONCLUSION: This study showed high levels of CTHRC1 protein in RA plasma. These results indicate that CTHRC1 can be used as a novel plasma biomarker to evaluate disease activity in RA. Also it can be used for the differential diagnosis of RA from similar joint diseases, such as OA and ReA.

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Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

μCT	Micro-computed tomography
25(OH)2D3 :1	25-Dihydroxyvitamin D3
ACPA	Anti-citrullinated protein antibody
ADAMts12	A disintegrin and metallopeptidase with thrombospondin type 1 motif 12
AFA	Anti-filaggrin antibodies
AUC	Area under the curve
BA	Basophils
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
C1qtnf3	Complement C1q tumor necrosis factor-related protein 3
CamKII	Calmodulin kinase II
CD34	Hematopoietic progenitor cell antigen CD34
CDH11	Cadherin 11
CI	confidence interval
CRP	C-reactive protein
CSF-1	Colony stimulating factor 1
CTHRC1	Collagen triple helix repeat-containing 1 protein
DAS28	Disease activity score 28
DMARDs	Disease-modifying anti-rheumatic drugs
Dvl	Disheveled
EO	eosinophils
ESR	Erythrocyte sedimentation rate
FBS	Fetal Bovine Serum
Fzd	Frizzled
GC	glucocorticoids
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GSK3	Glycogen synthase kinase 3beta
GWAS	Genome-wide association studies
IFN	Interferon
IL-1	Interleukin 1
IL-11	Interleukin 11
IL-15	Interleukin 15
IL-1b	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IQR	interquartile range
LEF	Lymphoid enhancer binding factor
LRP	Lipoprotein receptor-related protein
LRP5	Low-density lipoprotein receptor-related protein 5
MBDA	Multi-Biomarker Disease Activity
MMP3	Matrix metalloprotease 3
MON	Monocytes

MTX	methotrexate
NE	neutrophils
NPV	negative predictive value
NSAIDs	non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OPG	Osteoprotegrin
OR	odds ratio
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PBS-TB	PBS containing 0.05% Tween 20, 0.1% BSA
PCP Pathway	Planar cell polarity pathway
PCR	Polymerase Chain Reaction
PKC	Protein kinase C
PLT	Platelets
PPV	Positive predictive value
PTH	Parathyroid hormone
RA	Rheumatoid arthritis
RA-FLS	Rheumatoid arthritis fibroblast like synoviocyte
RANKL	Receptor Activator of Nuclear factor-Kappa B ligand
ReA	Reactive arthritis
RF	Rheumatoid factor
rhCTHRC1	recombinant human CTHRC1
ROC	Receiver operating characteristic
ROR	Retinoic Acid Related Orphan Receptor
ROR2	Receptor tyrosine kinase-like orphan receptor 2
RPMI	Roswell Park Memorial Institute
RSPO2	R-spondin 2
SAA	Serum amyloid A
SDF1	Stromal cell-derived factor 1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Synovial fluid
SMAD2	Mothers against decapentaplegic homolog 2
SSZ	Sulfasalazine
TGF	Tumour Growth Factor
Th (cell)	T Helper (Cell)
THY1	Thymus cell antigen 1
TMB	3, 3', 5, 5'- tetramethylbenzidine chromogenic substrate
TNF	Tumor necrosis factor alpha
TRAP	Thrombin receptor activating peptide
VCAM	Vascular Cell Adhesion Molecule
WISP	Wnt inducible signaling pathway
WNT	Wingless/Integrated

List of Publications

Manuscripts arising from this thesis (3):

1. Shekhani MT, Forde TS, Adilbayeva A, Ramez M, **Myngbay A**, Bexeitov Y, Lindner V, Adarichev VA. Collagen triple helix repeat containing 1 is a new promigratory marker of arthritic pannus. *Arthritis Res Ther.* 2016. 18:171.
2. **Myngbay A,** Bexeitov Y, Adilbayeva A, Assylbekov Z, Yevstratenko BP, Aitzhanova RM, Matkarimov B, Adarichev VA and Kunz J (2019) CTHRC1: A New Candidate Biomarker for Improved Rheumatoid Arthritis Diagnosis. Front. Immunol. 10:1353. doi:10.3389/fimmu.2019.01353
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2. Bexeitov Y, **Myngbay A,** Adilbayeva A, Yevstratenko BP, Aitzhanova RM, Otarbayev NK, Adarichev VA. Circulating Collagen Triple Helix Repeat-Containing (CTHRC1) Level Correlates with Stem Cell Factor, Interferon Gamma and Rheumatoid Arthritis Severity. 18th Asia Pacific League of Associations for Rheumatology Congress (APLAR-2016). September 26-29. International Journal of Rheumatic Diseases. 2016.
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Chapter I

1. Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease of joints, resulting in destructive inflammatory disorders. Joint disorders are characterized by the formation of pannus, which arises from the lateral side of synovial cavities and causes inflammation, eventually leading to loss of function (1, 2). Some genetic and environmental factors increase the risk for RA, although the definitive pathogenesis is obscure and early diagnosis tools need further study (3). Usually for the diagnosis of RA clinicians assess clinical status, and the levels of antibodies against rheumatoid factor (RF) and Anti-citrullinated protein antibodies (ACPAs) in peripheral blood plasma.

Nowadays, RF is used as one of the main markers of RA, however is not considered to be specific marker, as it can be found in other inflammatory diseases and in 10-30% of healthy elderly population (4, 5). In comparison, ACPA is more specific for RA, and have major role in the progression of autoimmune responses during disease development (6).

Alongside with blood markers and clinical evaluations, radiography is used, and is indeed considered as most reliable tool in assessing disease status. However, radiography is not a good tool for early diagnosis of disease, as patients come to be tested, when there is already joint damage occurred (7). Early diagnostic markers for RA and established markers showing disease progression are still remains a challenge (8). Therefore, further studies are needed to find more specific and sensitive markers of RA, especially markers which show disease progression.

Many markers of different diseases have been identified using mass spectrometry approaches. And this is important, as synovial fluid content is in focus during disease progression, as analytes might correlate with disease severity (9). Also, mass spectrometry is used for differential diagnosis of RA and Osteoarthritis (OA) by performing quantitative analysis.

The progression of RA disease is characterized by flares of elevated disease activity caused by systemic immune responses and tissue-specific inflammation that can result in erosive joint and bone damage/impairment. (10, 11).

Effective RA diagnosis and treatment strategy for individual patients still require improvements. RA is a multifactorial disorder with major genetic and other factors (13-16) that render disease pathogenesis complex. Additionally, there are significant variations in the clinical symptoms of RA, such as degree and number of the swollen/tender joints as well as the levels of different

markers present in serum/plasma (10, 17, 18). The heterogeneity of the condition is further evident in the inadequate response of many patients to care (14, 19). Nonetheless, there is increasing evidence that RA's heterogeneity represents combination from both genetic and physiological factors, thus there may be molecularly distinct RA subtypes underlying the disease (20-22).

In the midst of this the growing understanding of the heterogeneity in disease, it has become apparent that there is a shortage of biomarkers for clinical diagnose of RA patients. Currently, RA diagnosis is mainly based on serum level of rheumatoid factor (RF) and anti-citrulline protein (ACPA) antibodies in peripheral blood. (23). Other RA classification criteria are based on the severity of the tenderness and stiffness of joints and the level of inflammatory markers, such as C-reactive protein (CRP) and Erythrocyte sedimentation rate (ESR). The most significant RF immunoglobulins, IgM and IgA RFs, are found in 60–70% of RA patients (24). Usually, patients with elevated level of RF develop more severe manifestations of the disease with greater impairment (23). In comparison, ACPA has high RA specificity (90–95 percent), but low sensitivity (60–75 percent) (23, 25, 26).

Studies have suggested that 12 plasma proteins could serve as a multi biomarker panel for assessing disease activity (27, 28). However, these markers failed to significantly correlate with disease severity and the level of joint/bone damage (27, 28). Such findings underscore the need for additional biomarkers or diagnosing criteria for better understanding RA pathogenesis, with biomarkers or cut-off levels adapted for specific populations of patients.

Bone damage primarily occurs due to synovial hyperplasia, starting from lateral sites of synovial cavity and eroding cartilage and bone. This formation is called pannus. (29). Pannus formation is the main cause of oedema, tenderness, cartilage/bone damage in patients with RA (29). Arthritic pannus is a multicellular vascular network comprising mesenchymal and hematopoietic cells, which lead to joint and cartilage degradation.

Recently it was reported that there is a strong correlation between increased levels of collagen triple helix repeat containing 1 protein (CTHRC1) and arthritis progression in mice models (6, 32-34). CTHRC1 was detected at the intimate layer of bone, and highly concentrated between the pannus and bone (35). Of note, CTHRC1 is reported as a Wnt signaling cofactor (36), promoting cell migration and proliferation by activating and stabilizing non-canonical signalling pathway (35). Hence CTHRC1 can activate FLS proliferation and migration, eventually forming pannus. These findings in mice lead to studies examining where CTHRC1 could be a biomarker of RA in humans.

1.1 Epidemiology

RA is one of the more common of all inflamed synovium joint diseases, leading to destruction of bone and cartilage (37). Around 1% of the population worldwide is affected by RA although its' activity remain obscure. Patients with established RA are susceptible to complications, such as cardiovascular disease leading to increased morbidity and mortality (38, 39). The incidence of RA is 2 times higher in females compared with males, according to the Global Burden of Disease 2010 study (40). Studies from United Kingdom (UK) and Finland have estimated the heritability of RA to be 53% and 65%, respectively. In this study, data collected from 2 previously published nationwide studies of twins with RA, conducted in Finland and UK (41). Genetic alterations in RA patients are linked to (HLA)-DRB1 alleles, mostly in rheumatoid factor (RF) and Anti-citrullinated protein antibody (ACPA) positive patients (42). Interestingly, breastfeeding women have lower risk of RA compared withof that never breastfeeded (43). Other environmental factors such as smoking, infectious diseases and their products are implicated as potential triggers of RA (44, 45).

1.2 Pathophysiology

Post translational modification of self-proteins that contain arginine residue results in conversion of arginine to citrulline residue by intracellular enzyme - peptidylarginine deiminase and loss of tolerance to these proteins leads to production of ACPAs against them (42). ACPA is collection of autoantibodies which recognize citrulline residue in proteins. ACPA is considered to be more specific marker of RA compared to RF, but neither of markers provide early diagnostics of RA. Further studies show that elevation of autoantibodies against ACPA and RF do not always indicate inflammation of synovium (46). Synovitis is not the result of cellular proliferation, rather leukocyte infiltration and accumulation in response to various pro-inflammatory cytokines (45). Proliferation of synovial cells (pannus), eventually leads to hypoxia, which in turn stimulates angiogenesis (47). Progression of synovial inflammation results in populations of various cell types causing joint destruction (45).

1.2.1 RANKL-OPG system in bone turnover

The chronic progressive autoimmune disease of synovial joints relies upon both systemic immune response and tissue-specific inflammatory events in the affected joints (48). Local inflammation of the synovial tissue ultimately leads to cartilage and bone erosion (49).
In healthy individuals, bone remodeling (formation and resorption) is a crucial component of old bone replacement to new bone tissue and maintains joint and bone strength. Receptor activator of the nuclear factor kB ligand (RANKL) and osteoprotegrin (OPG) pathways are for understanding bone formation/resorption mechanisms involved in the deterioration of rheumatoid arthritis patient status(50-53). RANK and RANKL belong to the tumor necrosis factor (TNF) superfamily members, first reported as products of dendritic cells, which activate T cells (54). Now we know that RANKL is produced by osteoblasts and stromal cells, and then binds to the RANK receptor leading to osteoclast differentiation and activation (55-57). On the other hand, OPG, member of TNF receptor family, was shown to be an inhibitor of osteoclastogenesis, it binds to RANKL and prevents osteoclast activation (58, 59).
In RA, bone erosion occurs at the site of pannus in the subchondral bone region, where osteoclasts are found and is associated with upregulation of RANKL expression (60).
It was previously shown that CTHRC1 expression was significantly higher at the pannus-bone region in inflamed joints in a model of collagen antibody induced arthritic mice. I demonstrated a high association between peripheral blood level of CTHRC1 and Disease Activity Score (DAS28), C-reactive protein (CRP) (35). Schett et al showed that osteoclasts penetrate subchondral bone and even invade the calcified compartment of cartilage. It became much clearer by development of Magnetic resonance tomography (MRI) (61). It was observed that RANKL/OPG production could also be regulated by T helper 17 (Th17) cells (62), synovial fibroblasts (35), macrophages, dendritic cells and activated B cells (63). It was shown that RANKL knockout mice as well as OPG treated mice were persistent for local inflammation of joints and bone damage (64, 65). Joint damage was observed when levels of Erythrocyte sedimentation rate (ESR) and RANKL/OPG ratio were high at baseline (66, 67). Patients with RA were studied during 6 month using denosumab, which is fully humanized antibody against RANKL. This study showed significant inhibition of bone erosion progression by MRI (68). precise and accurate diagnostic tool for the assessment of bone destruction and disease progression is considered to be MRI (11, 12, 69).

In non-inflammatory pathology, the amplified CTHRC1 expression was characteristic for metastatic solid cancers (70). Detailed immunohistochemistry analysis of a number of human cancers revealed that CTHRC1 expression is limited to tumor stromal cells (71, 72). I analyzed levels of CTHRC1, RANKL and OPG in the peripheral blood of RA and OA patients and found that while OA patients as well as healthy individuals were basically negative for these markers, RA patients' plasma exhibited a strong CTHRC1 and RANKL-positivity. CTHRC1 significantly correlated with RANKL and erosion depth, as well as with IFNγ, IL-6, and IL-1β levels (data is not shown).

1.3 Assessment of Disease Activity

Disease activity assessment is crucial for interpretation of clinical trials and treatment results. Sugimoto et al. suggested pannus volume measurement by MRI as an indicator of disease activity in RA. This study included 11 female patients with established diagnosis of RA, aged between 21-60 years of age. Average disease duration was 5 years at the time of enrollment. Patients were divided in 3 groups according to volume of enhancing pannus changes during 12-month follow-up: unchanged (n = 2), decreased (n = 6) and increased (n = 3). Volume of enhancing pannus (VEP) of both hands significantly differed between groups, suggesting that VEP can be used as an indicator of RA disease activity (73).

Other several disease activity indices include the Disease Activity Score DAS, the modified DAS in 28 joints (DAS 28), the Simplified Disease Activity Index (SDAI), and Routine Assessment of Patient Index Data 3 (RAPID3) (74-79). Among the laboratory tests Erythrocyte Sedimentation Rate (ESR) and C-reactive protein (CRP) levels were used as one of the approaches to assess disease activity, however none of them is considered to be specific markers of inflammation, since marker levels can change due to other factors such as age, anemia and presence of autoantibodies as RF (80-84). The multibiomarker disease activity (MBDA) test was suggested by Curtis et al., in which 12 biomarkers were used to establish the criterion and discriminant validity of MBDA as a measure of RA disease activity. In this study, Curtis and colleagues observed significant associations of their novel MBDA score with DAS28 using CRP levels in both seropositive and seronegative groups (27). But this study had limitations because levels of cytokines and mediators were difficult to detect due to low levels or inadequate test results in some patients meaning that this test cannot be applied to all patients.

Chapter II: Literature Review

2.1 The Role of Collagen Triple Helix Repeat-Containing 1 Protein (CTHRC1) in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease, which causes cartilage degradation and bone erosion within both small and larger joints including hand, wrist, knee and feet, leading to disability, systemic complications, and affects ~1% of the world population according to the World Health Organization (85). RA is a systemic autoimmune disease, and some genetic and environmental factors increase the risk for RA, although the definitive pathogenesis is obscure. Some breakthroughs, including the introduction of anti-Tumor necrosis factor alpha (anti-TNFα) in the treatment of RA, occurred in the mid-1990s, which lead to improvement of clinical outcomes in RA (86). Although some treatment strategies were developed using cytokine antagonists against TNFα, Interleukin-6 (IL-6) and Interleukin-1 (IL-1), the persistence of efficacious treatment remains a tremendous problem (86). Other therapeutic treatments have included B cell depletion and T cell co-stimulation blockers to some success (87). It has also been suggested that genetic background and environmental stimuli should be considered in treatment options to personalize response as patients can be TNFα-dominant, T cell-dominant, and B cell-dominant (37).

Significant work has been undertaken to identify biomarkers to diagnose RA. Autoantibodies to ACPA and RF are widely accepted cause of progressive autoimmune disease and are used in clinical practice as biologic markers of RA, although neither of these two markers have sufficient specificity. Interestingly, it was reported that ACPA and RF are detected in some patients' prior to development of arthritis, providing an opportunity for early intervention (88). CTHRC1 may represent a new biomarker in a wider panel, demonstrating elevated blood levels in RA compared to OA and healthy individuals.

CTHRC1 was first described by Pyagay et al. (2005) indicating a role in vascular remodeling during arterial injury (89). CTHRC1 has also been reported as an oncogenic protein in many cancer studies, together with significant associations in Hepatitis B virus replication, bone formation/resorption, antifibrotic effects in liver fibrosis, and high expression on atherosclerotic plaques (90-95). Finally, CTHRC1 has been reported as a pituitary hormone (72).

2.1.1 The CTHRC1 protein

Collagen triple helix repeat containing 1 (CTHRC1) is a secreted 30 kDa glycoprotein with 12 repeats of the GXY motif, which inhibits glycogen matrix deposition and promotes cell migration. Activity of CTHRC1 occurs following the cleavage at glutamic acid residue 46 (E46) and arginine residue 96 (R96) by plasmin, as it does not have a pro-peptide cleavage site. Initially, the expression of CTHRC1 was observed during murine embryonic development and upregulated expression was seen after injury of rat arteries on adventitial and intimal layer of smooth muscle. Additionally, aberrant expression of CTHRC1 was observed in cartilage and developing bones, myofibroblasts during wound healing, inner hair cells in murine models, fibroblasts and chondrocytic cells in response to the transforming growth factor β (TGFβ family members TGFβ, Bone morphogenetic protein 2 (BMP2) and Bone morphogenetic protein 4 (BMP4). Several reports showed overexpression during tumorigenesis and metastasis. It was shown that CTHRC1 inhibits TGFβ signaling by reducing phosphorylation of Smad2/Smad3, leading to reduction of Collagen Type I deposition, thereby representing a potential negative feedback loop for this pathway (96).

CTHRC1 is a protein which reduces collagen deposition and interacts with TGFβ signaling pathways. It has been shown that CTHRC1 gene transcription is activated by TGFβ signaling through Smad proteins (97), and CTHRC1 is highly expressed and associated with developing bone, especially in calcified tissues and cartilaginous matrix (8). Wound healing assays on the cells (PAC1 fibroblasts) overexpressing CTHRC1 showed a faster cell migration rate than control cells (97). In comparison, tumor cells migrated equally with the cells overexpressing CTHRC1, suggesting CTHRC1 as a mediator of cell migration and epithelial-mesenchymal transition in solid tumour cancer cells (70, 98, 99).

2.1.2 CTHRC1 Involvement in the Wnt signaling pathway

Among other signaling pathways, the Wnt signaling pathway is considered to be one of the major mechanisms of RA pathogenesis. In vitro studies showed upregulated expression of β-catenin in Rheumatoid Arthritis (RA) fibroblast-like synoviocytes (RA-FLS), caused by the canonical Wnt signaling pathway. It was further shown that β-catenin dependent pathway leads to stable activation of RA-FLS (100). Wnt signaling plays crucial roles in cell proliferation, differentiation, migration, cell adhesion and embryonic development. Wnt-glycoprotein ligands make interactions with the N-terminal extracellular cysteine-rich domain of a Frizzled (Fzd)

family receptor and co-receptors lipoprotein receptor-related protein (LRP) and receptor tyrosine kinase-like orphan receptor 2 (ROR2). This complex passes signals to intracellular regulators such as phosphoprotein disheveled (Dvl) and glycogen synthase kinase 3β (GSK3β) amongst others. There are two major divisions of the Wnt pathway, namely β-catenin dependent (canonical) and β-catenin independent (non-canonical) pathways.

The β-catenin dependent pathway causes an accumulation of β-catenin, which acts as a co-activator of transcription factors. Wnt proteins form a complex with Frizzled receptor (Fzd) and lipoprotein receptors (LRP 5/6) that activate Dvl, and the latter inactivates destruction complex protein GSK3β. Accumulated β-catenin interacts with lymphoid enhancer binding factors (LEFs) and T-cell factors (TCFs), eventually initiating transcription of target genes.

The β-catenin independent pathway (i.e. Wnt/Ca2+, Planar cell polarity) is involved in cell proliferation, adhesion, migration and differentiation. Wnt/Ca2+ is characterized by the release of Ca2+ and activation of kinases such as protein kinase C (PKC) and calcium-sensitive enzymes calmodulin kinase II (CamKII), eventually leading to actin polymerization and Cell Fate/cell migration, together with inhibition of the β-catenin dependent pathway. The planar cell polarity PCP pathway is responsible for organization of the cell cytoskeleton, and LRP are not involved in this pathway. Essentially, the Wnt/Fzd/co-receptor (i.e. ROR2) complex recruits Dvl, which activates small GTPases Rho A/Rac1, which leads to actin polymerization and modification of the cytoskeleton.

The Wnt/PCP pathway is selectively activated by the secreted glycoprotein CTHRC1. It was observed that CTHRC1 interacts with multiple Fzd receptors (Fzd3, Fzd5 and Fzd6), canonical and non-canonical Wnt proteins, non-canonical co-receptor Ror2, but not with LRP6, and PCP component Vangl2. Activation of both RhoA and Rac1 small GTPases by CTHRC1 co-expressed with Wnt3a, Wnt5a and Dvl2, demonstrated that CTHRC1 modulates both cascades of the Wnt/PCP pathway. It was also observed that Wnt3a interacts with Ror2 in the presence of CTHRC1, and the Wnt5a-Ror2 complex is enhanced (95).

In contrast, Kim et al demonstrated that Wnt5a is the main precursor of cytokine production during inflammation. Wnt5a expression was observed in RA-FLS, but not in normal tissue, and Wnt5a expression vector transfected RA-FLS showed enhanced production of pro-inflammatory cytokines and chemokines (101). These data suggest that Wnt5a mediated signaling leads to upregulated production of cytokines and chemokines and causes recruitment of leucocytes into the synovium to enhance RA disease progression. It was also demonstrated that

Wnt5a/Ror2 complex has a crucial role in Bone marrow derived Mesenchymal stem cell differentiation into osteoblasts (102).

These data suggest that CTHRC1 potentially has a crucial role in the Wnt signaling pathway in synovium, and might contribute to cell migration as well as cell differentiation by being part of both canonical and non-canonical Wnt signaling pathways. The role of CTHRC1 in RA-FLS is depicted in Figure 2-1.

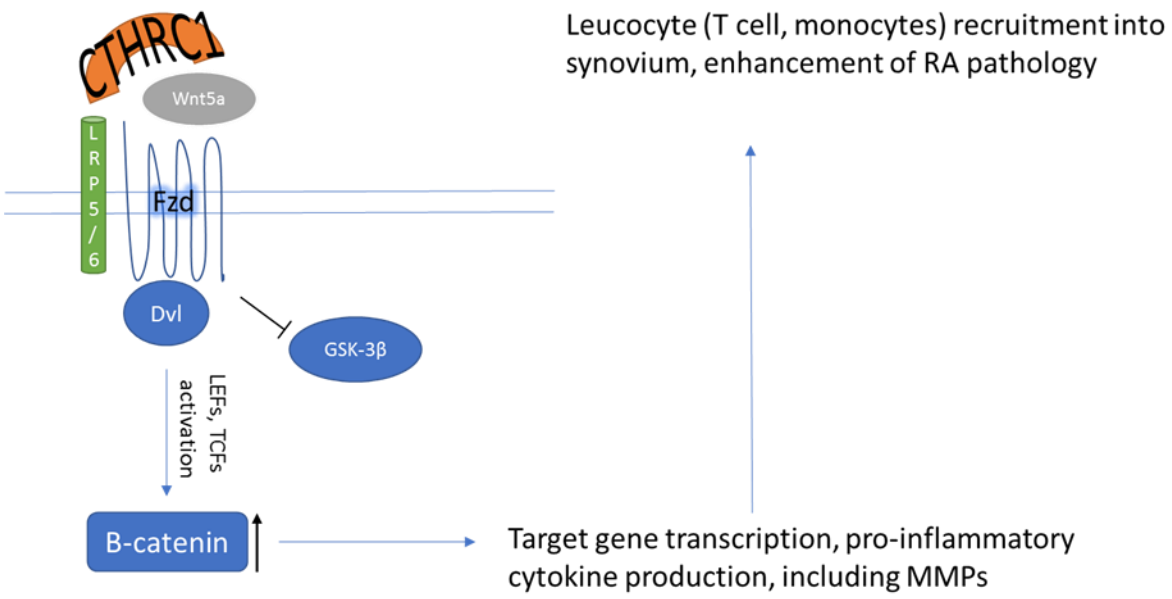

Figure 2-1. Wnt β-catenin dependent pathway in RA-FLS.

CTHRC1 stabilizes the Wnt5a/Fzd/LRP5/6 complex and leads to release of pro-inflammatory cytokines, MMPs, and recruits leucocytes into the synovium from circulation

2.1.3 Gender disparities in RA and inclusion of CTHRC1 as a potential link

Rheumatoid arthritis occurs more frequently in women (about 75%) and symptoms are more pronounced in this population (103, 104). Although there was no supporting data found in genome-wide association studies (GWAS) showing dependence of RA symptoms on sex chromosomes (105-107), it was reported that CTHRC1, metalloproteinase Adamts12, R-spondyn (Rspo2) and Syndecan (Sdc2) genes which are involved in Wnt signaling pathways and bone remodeling are highly associated with disease severity as well as linked to gender disparity in

RA (33). Estrogens are also one of the causes of female predominance in RA and highly linked to disease severity, similarly affecting bone remodeling by affecting the synovium (33). Wnt inducible signaling pathway (WISP) genes are members of the connective tissue family, up-regulated by Wnt signaling activation and contributing to cell adhesion, differentiation, cell polarity, and the establishment of cell fates. It was reported that estrogens upregulate the expression of Wnt1-inducible-signalling pathway 2 (WISP2) in RA-FLS and contribute to disease progression (108). Recent reports showed that CTHRC1 has a diverse effect on bone formation in males and females. Bone histomorphometry, microtomography (μCT) analysis and functional readouts of bone strength showed bone formation impairment of trabecular and cortical bone in male CTHRC1 null mice, whereas female CTHRC1 (-/-) knockout exhibited impairment only in trabecular bone. Deletion of CTHRC1 in female mouse models leads to a lower effect on bone morphology, whereas male CTHRC1 null mice showed a significant reduction in bone mass, and shorter femurs in comparison with wild-type mice (109). Interestingly, the CTHRC1 level in post-menopausal women is higher than in pre-menopausal women.

2.1.4 RA-FLS as one source of CTHRC1 production

Fibroblast-like synoviocytes (FLS) potentially have cellular effects in RA pathogenesis by locally producing inflammatory cytokines, mediators of inflammation and proteolytic enzymes. It is well established that joint inflammation is characterized by hyperplasia of cells lining the joints and destruction of cartilage and bone. Hyperplastic structures tend to invade cartilage and bone, which leads to major damages of intimal lining and sublining layers of synovial tissue. 30-50% of the sublining layer consists of CD4 + CD45RO+ T lymphocytes and ~5% of B lymphocytes. Histologic changes including B lymphocyte antigen-driven maturation, angiogenesis as well as local production of autoantibodies (RF, ACPA and anti-collagen antibodies) can be observed in the early onset of disease, prior to developing clinical symptoms (tenderness, swelling). Hypercellularity of the intimal lining is characterized by expansion of Type A (macrophage-like cells) and Type B (fibroblast-like cells) cells from 1-2 cells to a depth of up to 10-20 cells. The majority of reports suggest the predominance of Type A cells in the synovium due to migration from the bone marrow. Pro-inflammatory cytokines, chemokines and growth factors produced by activated Macrophage-like cells can induce the activation of Type B cells, which then start to produce their own cytokines, prostanoids and matrix

metalloproteinases. This type of network can lead to an influx of new cells into the synovium, destruction of extracellular matrix and increased severity of synovitis. This expansion of cells eventually results in the formation of a pannus at the cartilage-bone surface. The pannus is mainly composed of macrophages, osteoclasts and invasive FLS, and acts as a local invasive tumor. If osteoclasts are considered to erode bone, FLS tend to cause cartilage destruction due to their unique invasive properties and production of proteases (110).

Synovial inflammation leads to the formation of the pannus, which invades cartilage and bone, destructing the bone and causing deformities of the joint. Recently, we reported that CTHRC1 is a pro-migratory biomarker of RA, which promotes pannus formation, and it was shown that synovial CTHRC1 mRNA significantly associated with disease severity (35). Considering that CTHRC1 is inhibiting collagen deposition and promotes cell migration, it was hypothesized that CTHRC1 causes an influx of mesenchymal stem cells from circulation, which leads to the formation of pannus. While studying the proteoglycan induced arthritis 8 (Pgia8) locus of mouse chromosome 15, which controls proteoglycan-induced arthritis and collagen antibody-induced murine arthritis (CAIA) (33, 34) it was found that activated fibroblast-like synoviocytes of mice and humans (RA-FLS) express CTHRC1 (33). CTHRC1 expression in inflammatory conditions and positive correlation of CTHRC1 levels with arthritis severity is a novel property of this protein, which is normally expressed during embryonic development (8). In non-inflammatory pathology, the amplified CTHRC1 expression was characteristic for metastatic solid cancers (70) Detailed immunohistochemistry analysis of several human cancers revealed that CTHRC1 expression is limited to tumor stromal cells (71, 72). Recently we showed that one of the functions of CTHRC1 in synovium is promoting RA-FLS migration velocity and directness (35).

Adamts12, C1qtnf3 and CTHRC1 showed a significant correlation with arthritis severity (33, 112). Adamts12 is one of the enzymes triggering cartilage destruction by degrading cartilage oligomeric matrix protein (COMP), whereas C1qtnf3 and CTHRC1 are expressed in fibroblasts, both promoting cell migration (33, 113). CTHRC1 has been reported to stabilize the Wnt/Frizzled complex in the PCP-Wnt pathway, promoting prolonged cell motility as well as directionality (114). Blockade of Wnt5a/Fzd 5 signaling leads to downregulation of IL-6, IL-15 and RANKL, which inhibits synoviocyte activation (115). RA synoviocytes are suggested to have a crucial role in synovial hyperplasia, cartilage erosion and the formation of the pannus. It was shown that RA synoviocytes produce the cytokines IL-6, IL-8, IL-15 and SDF-1, which are

essential in lymphocyte penetration into the synovium (116). Also, RA synoviocytes enhances cartilage destruction by releasing precursors of metalloproteinases such as pro-MMP3 (117). RA synoviocytes initiate osteoclast differentiation by RANK-RANKL interactions, where RANKL is mainly expressed on Fibroblast like synoviocytes (FLS) and T cells and RANK on monocytes (118).

2.1.5 CTHRC1 in bone remodeling

Bone remodeling is a characteristic feature of RA (reviewed recently in (119-121)), appearing early in the disease, and characterized by bone erosions both within and around the affected joints, with additional widespread osteoporosis in some patients. Such erosions, and accompanying cartilage damage, within and around the affected joints can cause significant joint damage over time if effective treatment was not given.

The expression and/or role of CTHRC1 in bone biology is somewhat controversial. Bone formation/resorption is mainly maintained by osteoblast and osteoclasts. Osteoclasts are multinucleated cells, differentiating from the monocyte/macrophage under osteoblast/osteocyte control. Osteoblasts regulate osteoclast activity via colony stimulating factor-1 (CSF-1) and the receptor activator of nuclear factor kB ligand (RANKL). Wnt signaling pathways (canonical and noncanonical) play a crucial role in osteoblast differentiation, as well as in bone resorption. CSF-1 is constantly expressed, whereas RANKL expression depends on the bone resorption-stimulating factors (1α25(OH)2D3, PTH, IL-11). Osteoblasts also express osteoprotegrin (OPG), a soluble receptor for RANKL, which inhibits osteoclastogenesis by blocking the RANKL-RANK interaction. Studies showed that RANKL/RANK deficient mice develop osteopetrosis, whereas OPG deficient mice develop osteoporosis of trabecular and cortical bone (58, 122-124).

It was shown that deficiency of LRP5 (B-catenin dependent pathway co-receptor) leads to a reduction of the osteoblast count, eventually causing osteoporosis. Moreover, the Wnt antagonist Sclerostin (Sost)-deficient mice demonstrated an increase in bone mass, where Sost inhibits B-catenin signaling through binding to LRP5/6 (125, 126). As mentioned earlier, Wnt 5a is abundant in RA synovium and is involved in bone destruction. The stimulatory effect of Wnt5a on RANKL-induced osteoclast formation is inhibited by interacting with GST-sRor2, where the latter replaces RANKL. It was recently shown that Wnt5a is causing not only bone resorption, but also inflammation, by involvement in the chemotaxis of T cells and monocytes (102).

CTHRC1 is proposed to have role in osteogenic differentiation and regulation of bone remodeling. CTHRC1 is highly expressed on osteoblasts lining the trabecular and cortical bone surface of wild type mice and on some, but not all, osteocytes. In contrast, multinucleated osteoclasts do not express CTHRC1. This was confirmed by co-staining osteoclasts with thrombin receptor activating peptide (TRAP), where CTHRC1 expression was not revealed. It was also demonstrated that CTHRC1 expression was elevated as bone marrow stromal cells differentiate (109). And it was reported that CTHRC1 stimulates osteoblast proliferation and osteogenic differentiation. In contrast, Takeshita et al demonstrated that CTHRC1 is secreted by mature osteoclasts leading to osteoblastogenesis (94, 95). In comparison, Jin et al., demonstrated CTHRC1 as a secretory protein of osteoblasts, and inhibitor of osteoclastogenesis. Finally, it was suggested that CTHRC1 secreted by RA-FLS could have an anti-inflammatory effect in arthritis, by inhibiting arthritic joint destruction (109).

CTHRC1 was proposed to be secreted by mature osteoclasts and stimulate osteoclastogenesis. It was observed that CTHRC1 is overexpressed when osteoclasts were placed on dentin/hydroxyapatite and during high extracellular calcium levels. In vivo studies showed CTHRC1 elevation during RANKL injection, and downregulation with age and during treatment with alendronate. Deletion of CTHRC1 in mice leads to a deficiency in bone, whereas inhibited expression in osteoblasts showed no significant change. In comparison, Osteoclast-specific deletion of CTHRC1 leads to bone loss. Based on these data, Takeshita et al concluded that CTHRC1 is secreted by osteoclasts and has a major role in bone remodeling (95).

In contrast, Jin et al demonstrated that CTHRC1 protein is expressed by osteoblasts and osteocytes. In this study, bone resorption, osteoclast count and activity, as well as expression of osteoclastogenic genes (c-Fos, RANKL, Trap, Nfatc1) were elevated in CTHRC1 null mice. Furthermore, differentiation of bone marrow derived monocytes to osteoclasts were similar in both CTHRC1 null mice and wildtype, whilst in contrast monocyte-osteoclast differentiation and osteoclast driven bone resorption were inhibited in the presence of CTHRC1. Calvarial osteoblasts from CTHRC1 null mice downregulated osteogenic differentiation in comparison with wildtype mice. CTHRC1 inhibited the activation of NFkB by preventing IkBα degradation and ERK1/2 activation. This study suggests that CTHRC1 is secreted by osteoblasts/osteocytes but not by osteoclasts, and inhibits osteoclast differentiation. It was also suggested that CTHRC1 might have an anti-inflammatory potency and reduce bone impairment (109). Possible mechanisms within a joint are depicted in Figures 2 and 3. Drawing further conclusions from

these reported findings, and further linkage to RA pathology, will require additional studies beyond those summarized here in both cellular and in vivo systems.

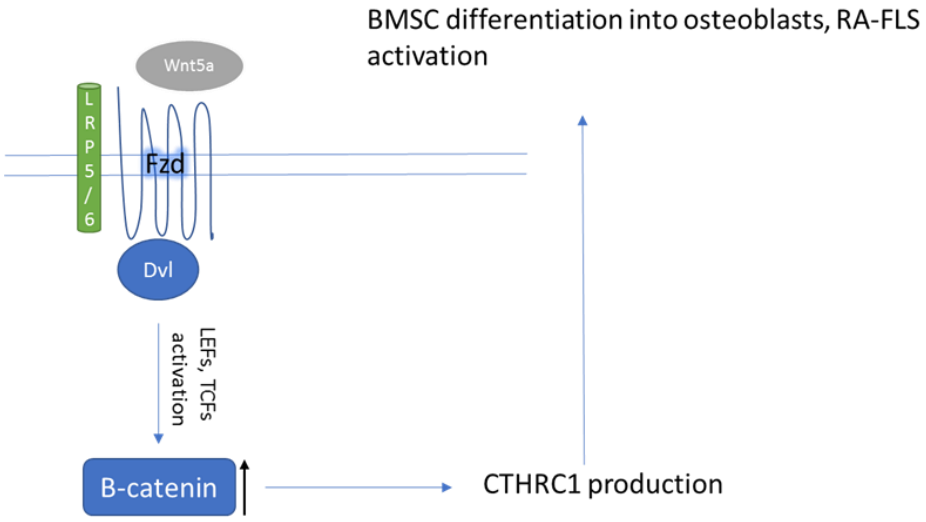

Figure 2-2. Wnt β-catenin dependent pathway in Osteoclasts.

Secreted CTHRC1 stabilizes the Wnt5a/Fzd/LRP5 complex in BMSC and leads to differentiation to osteoblasts and activation of RA-FLS

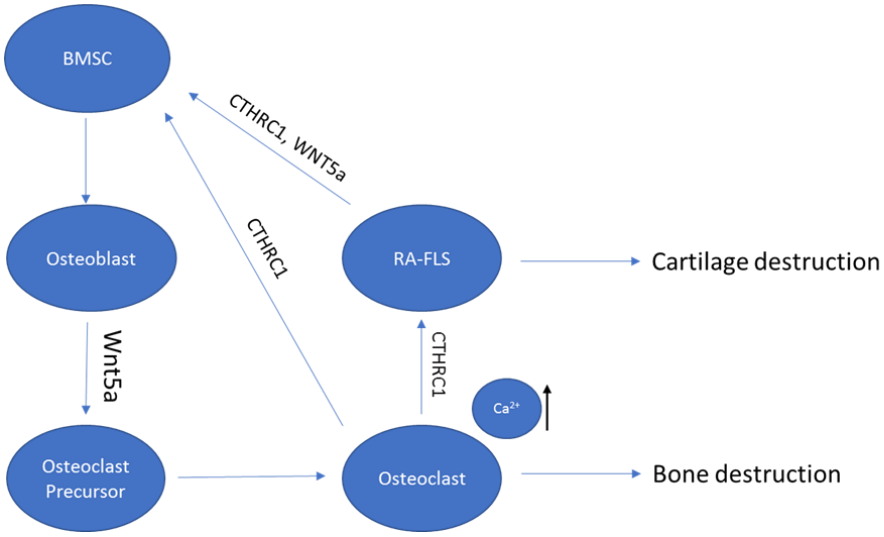

Figure 2-3. Cellular interactions within a joint.

Secreted CTHRC1 has suggested roles in RA-FLS activity and Osteoblast/Osteoclast balance, to potentially affect both cartilage destruction and bone remodeling

Cell type	Possible effect on cells	References
Rheumatoid Arthritis Fibroblast-like synoviocytes (RA-FLS)	Activation and initiation of cartilage destruction	(105)
Osteoblasts	Activation of osteoclastogenesis and bone destruction	(72)
Osteoclasts	Inhibition of monocyte-osteoclast differentiation and osteoclast driven bone resorption, inhibition of NFkB activation	(111)

Table 2-1. Potential sources of CTHRC1 within a joint

Chapter III: CTHRC1: A New Candidate Biomarker for Improved Rheumatoid Arthritis Diagnosis

3.1 ABSTRACT

The aim of this cross-sectional study was to identify the potential role of CTHRC1 protein a biomarker for enhanced diagnosis and surveillance of RA disease development in patients with RA. We tested CTHRC1 levels in the plasma of patients with established diagnosis of RA, osteoarthritis (OA), reactive arthritis (ReA), and in healthy volunteers. We then examined and observed significant association between CTHRC1 protein and a number of indices including DAS28, RF, CRP, ACPA, ESR, as well as proinflammatory cytokines, as interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), and interferon gamma (IFNγ).

CTHRC1 plasma levels in RA patients were considerably higher than in stable individuals, OA patients, and ReA patients. ROC curve and risk score results showed that plasma CTHRC1 can have practical importance for RA diagnosis.

Our studies indicate that CTHRC1 can be used to diferrentially diagnose RA from other similar diseases as OA and ReA, as well as from healthy volunteers. At the current level of understanding,

levels of plasma CTHRC1 can enhance RA diagnosis and these results require validation in a larger, more detailed population of patients.

3.2 METHODS AND MATERIALS

3.2.1 Study Population, Ethics Approval, and Consent to Participate

In the Republican Diagnostics Institute, Astana, Kazakhstan, 57 RA, 65 OA, 12 ReA patients and 14 healthy controls were recruited. Blood sampling for experiments was conducted during hospital consultation as part of the diagnostic process. At the time of blood sampling, demographic (age and sex) and disease-related criteria were identified in all patients. Complete blood count, biochemical analysis, disease duration, tender (68) and swollen (66) joint count, and a regional patient evaluation results were analyzed. Inclusion criterion: All patients with RA followed the 2010 ACR / EULAR RA (30) rating requirements, and ages range was between 30-75 years. A thorough review of the RA and OA cases was performed by an independent highly qualified rheumatologist. Patients` diagnosis was based on clinical status, CBC, biochemical parameters, DAS28 score and MRI of affected joints. Anti-rheumatic treatment was registered concurrently with any other concomitant therapy. OA cases were considered according to the professional decision of the practitioner and the lack of certain types of arthritis dependent on the clinical diagnosis of the main knee(s). Healthy individuals were recruited if they did not have a previous history of arthritis and showed negative results for inflammatory markers, including ESR, CRP, RF, ACPA and did not have any inflammatory diseases at the time of recruitment. Accredited central clinical laboratory at the Republican Diagnostic Center, Nur-Sultan, Kazakhstan carried out a study including measurement of RF, ACPA, CRP, and ESR in a blind fashion. Afterwards, the personal details were pseudonymised. The study was approved by the institutional review board at the Republican Diagnostic Center, and the Institutional Research Ethics Committee at Nazarbayev University, Astana, Kazakhstan (Protocol #N32) and complied with the International Ethical Guidelines for Biomedical Research Involving Human Subjects, Good Clinical Practice guidelines, the Declaration of Helsinki, and local laws. All patients gave written, informed consent to the analysis and reporting of the findings.

TABLE 3-1. Patient demographic and clinical characteristics.

Parameter*	RA (*n* = 57)	OA (*n* = 65)	ReA (*n* = 12)	Healthy (*n* = 14)	RA-OA	RA-ReA	RA-He
Age (years)	49.51 ± 13.65	56.78 ± 1 0.13	37.33 ± 9.58	34.5 ± 10.99	0.009	0.016	0.002[$]
Sex							
F	51 (89.47%)	56 (86.15%)	10 (62.50%)	12 (85.71%)			
M	6 (10.53%)	9 (13.85%)	6 (37.50%)	2 (14.29%)			
COMPLETE BLOOD COUNT WITH DIFFERENTIAL*							
Leukocytes (mil/mL)	6.7 ± 2.41	6.31 ± 1.37	5.59 ± 1.3	6.46 ± 1.2	>0.999	0.283	>0.999[$]
Hemoglobin (g/L)	124.49 ± 14.31	133.8 ± 12.67	139.8 ± 18.24	135.64 ± 7.47	<0.001	0.004	0.012[$]
Erythrocytes (mil/μL)	4.45 ± 0.48	4.63 ± 0.53	4.89 ± 0.45	4.5 ± 0.35	0.12	0.005	>0.999[$]
Platelets (mil/mL)	292.51 ± 85.31	253.2 ± 64.97	252.5 ± 63.84	260 ± 31.39	0.006	0.241	0.906[$]
Basophils (mil/mL)	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.026	0.04 ± 0.02	0.603	>0.999	<0.001[#]
Lymphocytes (mil/mL)	1.79 ± 0.69	2.04 ± 0.53	1.98 ± 0.5	2.01 ± 0.32	0.071	0.896	<0.001[$]
Monocytes (mil/mL)	0.54 ± 0.23	0.49 ± 0.15	0.47 ± 0.18	0.46 ± 0.12	0.912	>0.999	<0.001[$]
Neutrophils (mil/mL)	4.11 ± 1.92	3.53 ± 0.98	2.8 ± 0.73	3.78 ± 0.94	0.627	0.026	>0.999[$]
Eosinophils (mil/mL)	0.15 ± 0.19	0.19 ± 0.11	0.20 ± 0.12	0.14 ± 0.08	<0.001	0.029	0.874[#]
BLOOD ASSAYS*							
CRP (mg/L)	14.72 ± 17.45	4.9 ± 11.19	1.87 ± 1.6	0.83 ± 0.85	0.0003	0.001	<0.001[#]

ESR (mm/h)	20.6 ± 12.47	16.46 ± 9.56	8.58 ± 4.1	6.31 ± 2.14	0.483	0.001	<0.001[$]
RF (u/ml)	62.88 ± 66.11	10.42 ± 8.19	7.69 ± 3.9	n.a.	<0.001	<0.001	n.a.
ACPA (u/ml)	135.56 ± 212.33	0.38 ± 0.53	0.26 ± 0.4	n.a.	<0.001	<0.001	n.a.
CTHRC1 (ng/ml)							
Mean	20.39 ± 25.38	1.78 ± 4.49	2.15 ± 2.54	2.29 ± 3.73	<0.001	<0.001	<0.001[#]
Median [Q1–Q3]	8.28 (0–96)	0 (0–20.55)	3.27 (0–17.68)	0.05 (0–11.0)	<0.001	<0.001	<0.001[#]
CLINIC*							
DAS28-CRP	3.79 ± 0.85						

**Average values with standard deviation (±SD) are presented. p-values for statistical difference between indicated parameters were calculated using [$]ANOVA or [#]Kruskal-Wallis testing. n.a., —parameter not determined. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies; DAS28-CRP, disease activity score based on CRP.*

TABLE 3-2. RA Patient demographic and clinical characteristics.

	Total	**Men**	**Women**
COMPLETE BLOOD COUNT			
Leukocytes (mil/mL)	6.7 ± 2.4	9.27 ± 1.6	6.41 ± 2.28 (**)
Hemoglobin (g/L)	124.5 ± 14.31	136.5 ± 10.9	123.07 ± 13.84 (*)
Erythrocytes (mil/µL)	4.45 ± 0.47	4.77 ± 0.29	4.41 ± 0.47 (*)
Platelets (mil/mL)	292.5 ± 85.31	307.3 ± 96.48	290.76 ± 82.86
Basophils (mil/mL)	0.04 ± 0.024	0.05 ± 0.02	0.037 ± 0.03
Lymphocytes (mil/mL)	1.79 ± 0.69	2.22 ± 0.45	1.74 ± 0.68
Monocytes (mil/mL)	0.54 ± 0.23	0.85 ± 0.25	0.5 ± 0.19 (*)
Neutrophils (mil/mL)	4.1 ± 1.9	5.8 ± 1.18	3.91 ± 1.87 (*)
Eosinophils (mil/mL)	0.15 ± 0.19	0.46 ± 0.38	0.11 ± 0.09
BLOOD ASSAYS			
ESR (mm/h)	20.6 ± 12.47	18.83 ± 12.02	20.8 ± 12.38
CRP (mg/L)	14.72 ± 17.45	29.81 ± 19.07	12.94 ± 16.17

RF (U/ml)	62.88± 66.11	62.54 ± 50.19	62.91 ± 67.09
ACPA (U/ml)	135.6± 212.3	114.82 ± 118.5	138.01 ± 218.6
CTHRC1, ng/ml	20.39± 25.38	33.36 ± 31.72	18.86 ± 23.8
CLINIC			
N	57	6	51
Age	49.51± 13.65	47.83 ± 15.62	49.71 ± 13.24
DAS28-CRP	3.78± 0.85	3.94 ± 0.58	3.76 ± 0.87
Age of onset (years)	42.0 ± 14.58	36.66 ± 13.8	42.63 ± 14.54
Duration of disease (years)	8.04± 8.18	11.5 ± 10.07	7.67 ± 7.8
Duration of treatment (years)	5.05± 6.27	8.76 ± 9.39	4.41 ± 5.3
Tender joint count (0-28)	8.12± 3.07	8.16 ± 3.28	8.10 ± 3.04
Swollen joint count (0-28)	2.12± 2.27	1.16 ± 0.68	2.24 ± 2.38 (*)
TREATMENT			
Mtx	54.25%	n.a.	n.a.
MTX + GC	17.5%	n.a	n.a
Mtx + DMARDs	5.25%	n.a.	n.a.
Mtx + NSAIDs	1.75%	n.a	n.a
GC	3.5%	n.a.	n.a.
DMARDs	3.5%	n.a	n.a
Mtx+	3.5%	n.a.	n.a.
SSZ	1.75%	n.a	n.a
None	9.0%	n.a	n.a

**p-values for statistical difference between men and women parameters were calculated using Mann–Whitney U-test: *p < 0.05; **p < 0.01. Average values with standard deviation (±SD) are presented. n.a.—parameter not determined. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies; DAS28-CRP, disease activity score based on CRP. Mtx, methotrexate or metoject; Mtx + GC, methotrexate in combination with glucocorticoids; Mtx + DMARDs, methotrexate in combination with other synthetic DMARDs (leflunomide or hydroxychloroquine); Mtx + NSAIDs, methotrexate in combination with non-steroidal anti-inflammatory drugs; Mtx +, methotrexate in combination with NSAIDs and synthetic DMARDs, with or without ibandronate; GC, glucocorticoids (prednisolone or methylprednisolone); DMARDs, leflunomide or hydroxychloroquine alone or in combination; SSZ, sulfasalazine.*

3.2.2 CTHRC1 Immunodetection

Venous blood was collected into heparinized tubes, cells were removed by centrifugation at 1,000 × g for 10 min, plasma was stored at −70°C. A commercially available sandwich enzyme-linked immunosorbent assay (ELISA) for CTHRC1 quantification was performed in duplicates according to manufacturer protocol (www.mmcri.org/antibody, Maine Medical Center Research Institute, Scarborough, ME), as described previously (35). Briefly, 96-well Maxisorp plates (Nunc) were coated overnight at 4°C with 1.8 µg/ml capture antibodies 13E09 in a carbonate-bicarbonate buffer of pH 9.4. The next day, wells were washed with PBS-TB buffer (1 × PBS, 0.05% Tween 20, 0.1% BSA) and then blocked with the same buffer for 1 h. Human plasma rather than serum was assayed based on previous evaluation of the CTHRC1 ELISA assay showing superior sensitivity (35). Human plasma was diluted 1:5 to 1:100 in PBS-TB and incubated with absorbed capture antibodies for 2 h. Subsequently, the wells were washed and then incubated with the biotinylated detection antibody Vli10G07 diluted 1:500 in PBS-TB for 1 h. After washing, wells were incubated for 1 h with streptavidin conjugated with horseradish peroxidase (Thermo Fisher Scientific) diluted at 1:8,000 in PBS-TB. After a final wash, TMB 3,3′,5,5′-tetramethylbenzidine chromogenic substrate (Amresco) was added, and absorbance was measured at 450 nm. Absorbance was converted to absolute concentration using recombinant CTHRC1 protein (rhCTHRC1) provided in the kit as a reference. Sensitivity of the ELISA is better than 1 ng/ml level according to manufacturer's protocol.

3.2.3 Evaluation of CTHRC1 Protein Stability

To study CTHRC1 proteolysis, human plasma was spiked with rhCTHRC1 (5 ng/µL rhCTHRC1, 12% plasma or synovial fluid (SF), 1 x PBS) and incubated at 37°C. Samples were denatured at 97°C in a Laemmli sample buffer and separated on 12% SDS-PAGE. Polyvinylidene fluoride membranes (Merck Millipore) with transferred protein were blocked in 5% (w/v) dry milk for 1 h in PBST and then probed with rabbit antibodies to CTHRC1 (Vli55, www.mmcri.org/antibody) and were developed with secondary HRP–conjugated goat anti-rabbit (Sigma) antibodies. Enhanced chemiluminescent substrate (Thermo Fisher Scientific) and the BioSpectrum 800 Imaging System (UVP) were used to detect signal.

3.2.4 LegendplexTM Multiplex Analyte Microsphere-Based Immunoassay

LEGENDplex™ Multianalyte immunoassay for IL-1b, IL-6, IL-8, IFNγ, and SCF was performed in accordance with manufacturer's instructions (LEGENDplex™, BioLegend). In brief, human plasma samples were centrifuged to remove debris and diluted 1:5 to 1:100 in PBS-TB and added to wells containing beads conjugated with analyte-specific antibodies. Detection antibodies were subsequently added to each well. After incubation of the plate for 2 h at room temperature with shaking, streptavidin-phycoerythrin was added, and plates were shaken for an additional 30 min. Finally, beads were washed twice with PBS-T using centrifugation at 1,000 g for 5 min to collect beads after each washing step. Standard solutions containing eight different concentrations of analytes (from 0 to 50,000 pg/mL) were used on each plate for standard curve determination and were incubated the same way as assay samples. After incubation and washing, beads were analyzed using BD FACSAria SORP flow cytometer (BD Biosciences). PMT voltages for Allophycocyanin (APC) and Phycoerythrin (PE) channels were set up immediately before the analysis using the Setup Beads provided in the kit according to manufacturer's instructions. Data analysis and calculations of concentration for samples based on the obtained standard curves was performed using LEGENDplex™ data analysis software according to manufacturer's instructions.

3.2.5 Statistical Analysis

Patient data are summarized as the mean with standard deviation (SD) or as medians and interquartile ranges (IQR). Comparisons of patient group and gender was done with an ANOVA and *t*-test if the distribution was normal, if not with the Kruskal Wallis test and Mann-Whitney *U*-test, respectively. Pearson's Chi-square test was performed for qualitative variables. The Kruskal-Wallis with Dunn's *post-hoc* test with or without Bonferroni correction was used to test for differences between groups for CTHRC1. Non-parametric Spearman correlation coefficients were used to describe the association between two continuous variables. ROC Curves analysis and AUC estimation were also performed in order to determine the best threshold, which discriminates our group of interest thanks to the Youden index. Linear logistic regression analysis was performed to evaluate the association between plasma CTHRC1 levels and RA in terms of unadjusted odds ratio (OR). CTHRC1 levels followed a skewed distribution and were log transformed when used as continuous variables. Data were >95% complete. All reported *P*-values were two-tailed, with $P \leq 0.05$ being considered significant. Statistical analyses and graphic illustrations were performed

under GraphPad Prism version 6.03 for Windows (GraphPad Software, La Jolla California USA) and R (v3.5.1), using ggplot2 and pROC libraries.

3.3 RESULTS

3.3.1 Patient Demographics and Clinical Characteristics

The average age of subjects in the RA population was 49.5 years with a 42.0-year average age of disease onset. (Table 3-1). The research recruited more female patients to achieve gender preponderance in RA (89.5%, Table 3-2). At the time of blood and data collection, the 54.25% of RA patients were receiving methotrexate or metoject therapy (Mtx, Table 3-2). 17.5% were treated with methotrexate in conjunction with glucocorticoids (MTx+GC, Table 3-2), 5.25% methotrexate combined with DMARDs (MTx+DMARD, Table 3-2), and 3.5% of RA patients underwent methotrexate in combination with other therapies (NSAIDs and synthetic DMARDs, with or without ibandronate; MTx+, 3.5%; Table 3-2). And other subgroups were receiving different types of therapies as shown in table 3-2, also 9% of patients were not receiving any medications.

RF and ACPA were significantly higher (about 6-fold (RF) and > 350-fold (ACPA), $p<0.001$), in RA patients compared with OA and ReA. CRP levels were 3-fold and 8-fold higher in the RA in comparison with OA and ReA groups, respectively (Table 3-1). Women and men had similar clinical results among the RA population: average DAS28-CRP were 3.94 in men and 3.76 in women. There were no significant difference between males and females in age, CRP level, RF and ACPA values (Table 3-2).

3.3.2 CTHRC1 Plasma Levels Are Significantly Elevated in RA Patients

Peripheral blood from all populations were tested for CTHRC1 circulatory levels, by sandwich ELISA. CTHRC1 levels across patient groups and healthy controls were analyzed. Healthy controls showed on average 2.29 ng/ml and in median 0.05ng/ml levels of CTHRC1, indicating that CTHRC1 at low levels can be detected in population with no inflammatory sign. These results are in agreement with previously published data (72). Interestingly, there were no significant differences between CTHRC1 levels of OA, ReA and of the healthy control group. Average CTHRC1 level of OA patients was 1.78 ng/ml (Kruskal-Wallis test, $p<0.001$, Figure 3-1), whereas ReA patients had 2.15 ng/ml (Kruskal-Wallis test, $p<0.001$, Figure 3-1). CTHRC1 level was significantly different among RA subgroups receiving different medications, as Mtx and Mtx+GC (data not displayed). By comparing mean CTHRC1 plasma levels across samples,

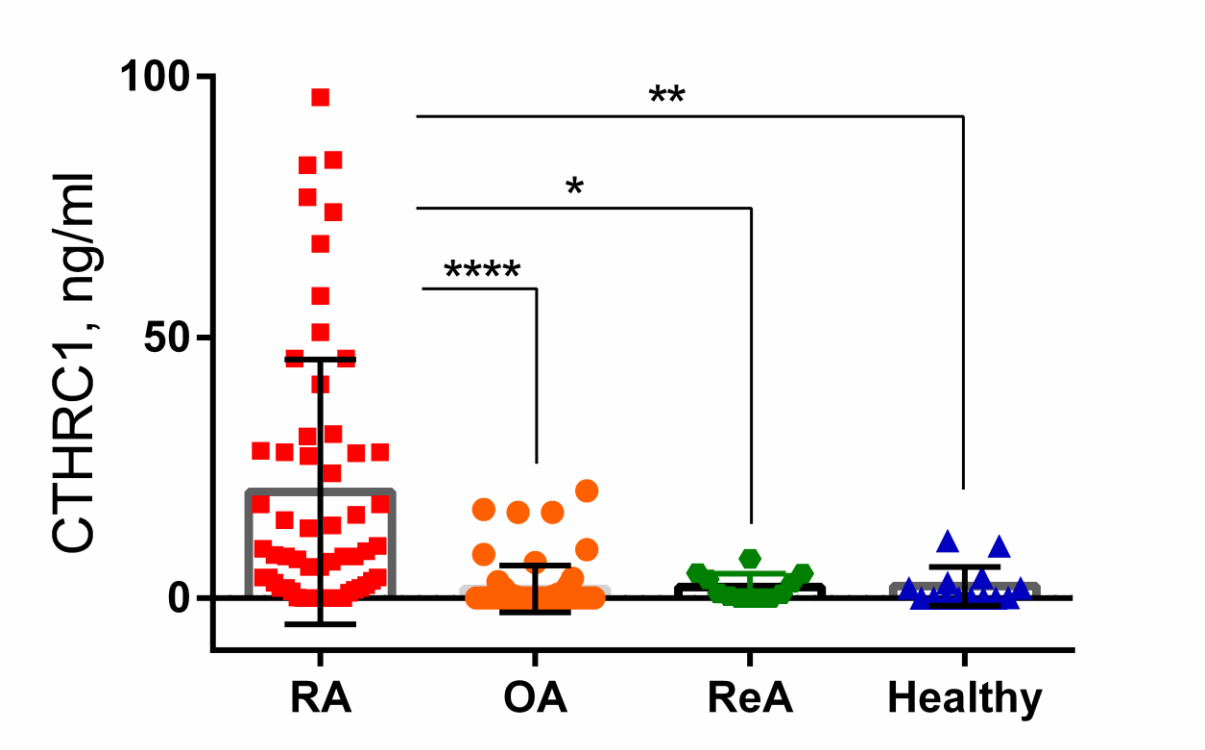

FIGURE 3-1. Blood plasma CTHRC1 levels in RA-patients and non-RA control groups. Plasma CTHRC1 concentration was measured using sandwich ELISA and recombinant CTHRC1 protein as a reference. Kruskal-Wallis with Dunn's *post-hoc* testing revealed a high statistical significance for the difference between RA (red squares) and healthy individuals' plasma (blue diamonds), as well as between RA and OA (orange circles), or ReA (green octagons) patients' plasma. Each diamond, octagon, square or circle corresponds to one patient. Box-and-whisker plot shows the median CTHRC1 levels within interquartile range and Tukey fences at 1.5 × IQR. The corresponding *p*-values are presented with asterisks: *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$; Kruskal-Wallis with Dunn's *post-hoc* test and Bonferroni adjustment.

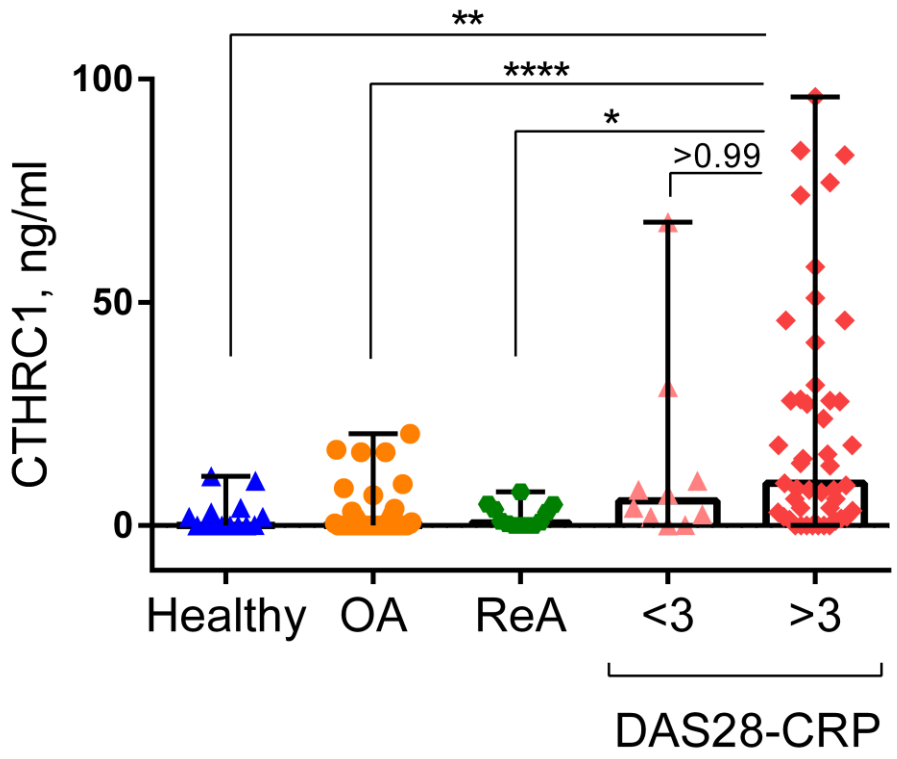

FIGURE 3-2. CTHRC1 plasma levels correlate with the arthritis severity score**.** CTHRC1 levels were calculated separately for healthy control (green circles) and OA (blue squares) groups, as well as for RA patients with a DAS28-CRP score <3 (<3, orange triangles), and >3 (>3, red diamonds). Each colored circle, square, triangle, or diamond corresponds to one patient in each group. Box-and-whisker plot shows the median CTHRC1 levels within interquartile range and Tukey fences at 1.5 × IQR. Statistically significant differences between RA groups vs. healthy controls and the OA patient cohort are indicated with asterisks: ** $p < 0.01$, **** $p < 0.0001$, Kruskal-Wallis with Dunn's *post-hoc* test and Bonferroni adjustment; * $p < 0.05$, Kruskal-Wallis test

3.3.3 Evaluation of CTHRC1 Protein Stability

To test the stability of CTHRC1 in plasma, recombinant CTHRC1 proteins (rhCTHRC1) were incubated in plasma for 2 hours at 37 C and checked protein degradation level by western blotting (Figure 3-3). Incubation of rhCTHRC1 with plasma or synovial fluid of RA patients did not show any significant degradation or loss of protein, similar effect was observed when protein was incubated with plasma/synovial fluid of OA, ReA and healthy controls (data not shown). These results indicate that circulating CTHRC1 levels are stable in plasma/synovial fluid at 37C and

obtained low levels of CTHRC1 in OA, ReA and healthy controls were not due to degradation in plasma and results are accurate.

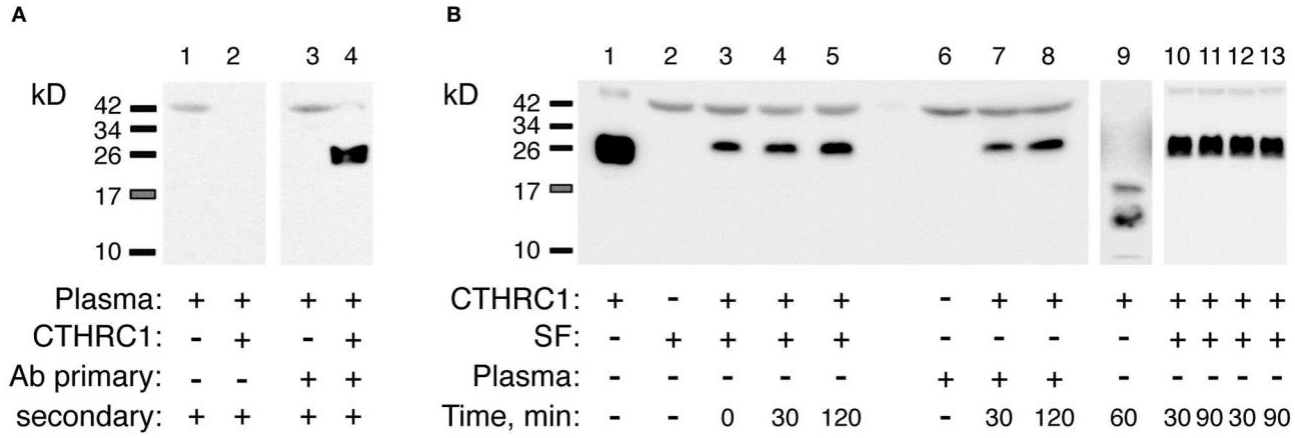

FIGURE 3-3. Detection and stability of plasma CTHRC1. **(A)** The specificity of the immunodetection was tested using 25 ng rhCTHRC1 spiked into the plasma (3 μL) of RA patients. Protein ladder bands (kD) are shown. **(B)** The resistance of the protein to proteolysis was tested with rhCTHRC1 spiked into synovial fluid (SF) or the plasma of RA patients. Final concentration of SF or plasma in the test was 12%, final amount of rhCTHRC1 loaded per lane was 15 ng. Lane 1 shows 50 ng of recombinant rhCTHRC1 protein as a reference. The Vli55 antibody was used for CTHRC1 immunodetection followed by appropriate secondary antibodies. As a positive control for digestion, incubation with trypsin was performed (lane 9). Lanes 10, 11: SF was heated for 30 min at 65°C (time, min) and then incubated with rhCTHRC1. Lanes 12, 13: SF was pre-heated for 30 min at 37°C.

3.3.4 CTHRC1 Diagnostic Value for RA Identification

We conducted ROC curve analysis to understand specificity and sensitivity levels of CTHRC1. The area under the curve (AUC) was 0.796 (95% CI: 0.681– 0.910) (Figure 3-4). According to Youden index, which showed 62% sensitivity and 86% specificity, threshold for CTHRC1 level was set to 5 ng/ml (Table 3-3). The positive predictive and negative values were 0.95 (95% CI: 0.88–1.00) and 0.36 (95% CI: 0.20–0.53, Table 3-3), respectively. Overall, CTHRC1 showed high sensitivity, as an indication for potential use as a marker for RA diagnosis.

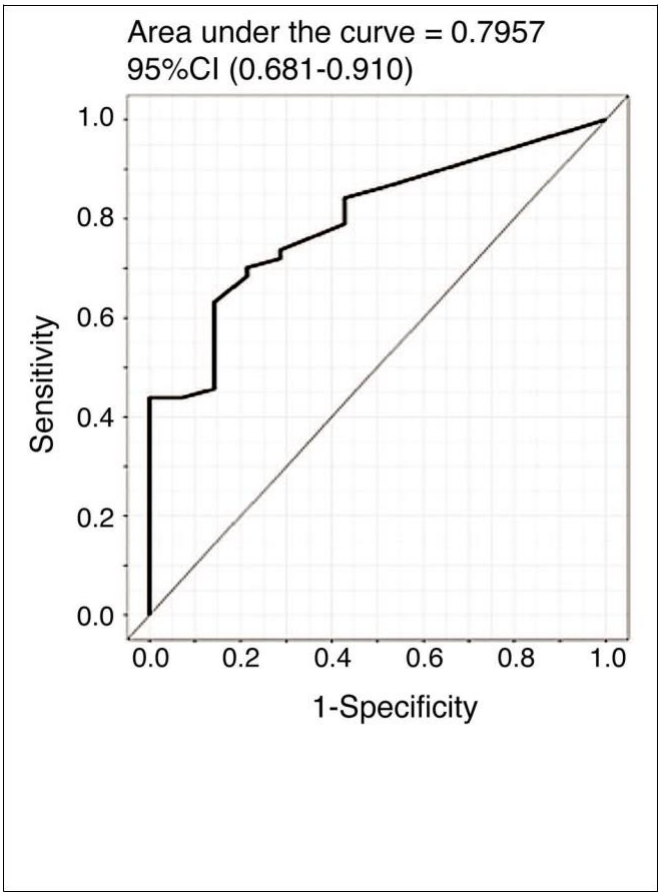

FIGURE 3-4. ROC curve for the risk prediction model. Receiver operating characteristic (ROC) curve analysis to assess the association of plasma CTHRC1 levels with RA vs. healthy status. The area under the curve was 0.796 for log(CTHRC1) (p = 0.032). Plots indicate individual protein abundances in patients.

TABLE 3-3. Receiver operating characteristic (ROC) analysis of CTHRC1 in RA.

ROC curve	
AUC 95% CI	0.796 (0.681–0.910)
Cutoff	5 ng/ml
P-value	0.004
Sensitivity, %	62
Specificity, %	86

Accuracy	68
PPV, %	95
NPV, %	36

AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic.

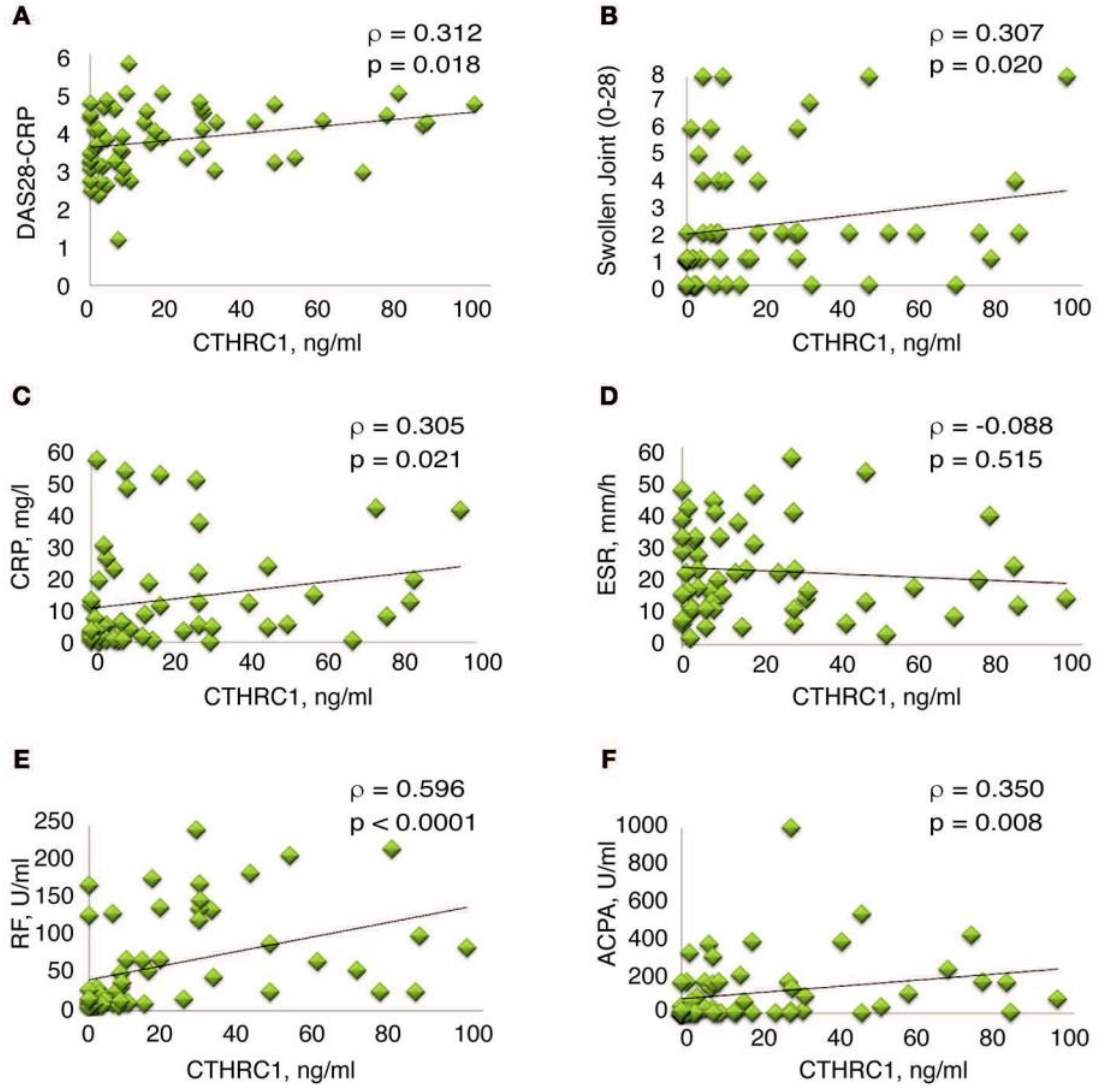

FIGURE 3-5. Correlation between clinical measures of RA and plasma levels of CTHRC1. **(A–E)** Correlation between plasma CTHRC1 and indicated clinical measures in the RA cohort. The y-axis reflects the plasma concentration of CTHRC1 in ng/ml. The x-axis reflects the DAS28-CRP score, the

swollen joint (0–28) score, the concentration of RF and ACPA (both in U/ml), the concentration of CRP in mg/L, and the measurement of ESR in mm/hr. Scatter plot graphs showing correlation between **(A)** CTHRC1 and DAS28-CRP, **(B)** CTHRC1 and Swollen joint count (0–28), **(C)** CTHRC1 and CRP, **(D)** CTHRC1 and ESR, **(E)** CTHRC1 and RF, and **(F)** CTHRC1 and ACPA. Each RA patient's sample is represented with a green diamond. The relationship between variables was evaluated using the Spearman rank correlation test. Trend lines indicate linear correlation. Spearman's coefficient of the correlation (ρ) and the corresponding *P*-value are shown on each plot.

3.3.5 CTHRC1 Levels Are Associated with RF and ACPA and May Correlate with Disease Activity

As DAS28 scores of recruited RA patients were from remission to moderate, population was divided into 2 groups, as DAS28-CRP < 3.0 (remission group) and DAS28-CRP < 3.0 (moderate group). This was done to whether CTHRC1 values show similar ranges among these groups. Although median CTHRC1 values of both groups differed, showing 5.5 ng/ml in remission and 9.5 ng/ml in the other group, the difference was not statistically significant ($p > 0.999$).

Median plasma levels of CTHRC1 between the moderate group and OA, healthy controls were significantly different (Bonferroni adjusted p<0.001, Figure 3-2). Whereas the significant difference between moderate group and ReA was achieved after applying the *post-hoc* Dunn's test ($p = 0.023$, Figure 3-2).

To check the correlation coeeficient Spearman's rank analysis was performed. Results showed significant correlation between CTHRC1 and DAS28 score ($\rho = 0.312$, $p = 0.018$, Figure 3-5A and Table 3-2S). Also, there was significant correlations between CTHRC1 levels and swollen joint count ($\rho = 0.307$, $p = 0.02$), CRP ($\rho = 0.305$, $p = 0.021$), RF ($\rho = 0.596$, $p < 0.0001$, Figure 3-5E) and ACPA ($\rho > 0.35$, $p = 0.008$, Figure 3-5F, Figure 3-5D).

. **TABLE 3-4.** Binary logistic regression results of CTHRC1 plasma levels for rheumatoid arthritis diagnosis.

Parameter	β	S.E.	Wald	OR	OR 95% CI	*P*-value
log(CTHRC1)	0.324	0.099	10.693	1.382	1.138-1.678	0.001

β, coefficient of logistic regression; S.E., standard error of β *value; Wald, Wald value of Wald tests; OR, odds ratio; OR 95% CI, 95% confidence interval of OR value.*

TABLE 3-5. Correlation of blood cell composition and blood analytes in RA patients.

	CTHRC1	Ne	Eo	Ba	MON	IL-1β	IL-6	IL-8
Ne	**0.57*§**							
Eo	0.19	0.24						
Ba	0.02	0.07	0.05					
MON	0.18	**0.54***	−0.29	0.03				
IL-1β	**0.88******	0.44	0.15	−0.06	0.16			
IL-6	**0.59******	0.24	0.26	−0.15	0.08	**0.45***		
IL-8	**0.86******	0.37	0.43	0.11	0.13	**0.81******	**0.45***	
IFNγ	**0.91******	0.43	0.25	−0.04	0.25	**0.86******	**0.65****	**0.83******

§*Statistically significant Spearman's correlation rank coefficients* ρ *are labeled in bold. The corresponding p-values for* ρ *coefficients are presented with asterisks as follows: *p < 0.05, **p < 0.01, ****p < 0.0001. Ba, basophil; Eo, eosinophil; IL-1*β *, interleukin 1 beta; IL-6, interleukin 6, IL-8 interleukin 8, IFN*γ*, interferon gamma; MON, monocyte; Ne, neutrophil.*

3.3.6 CTHRC1 Levels Correlate with Inflammatory Cytokines

In order to further explore the link between circulatory CTHRC1 and disease status, plasma samples were tested for inflammatory cytokines, as IL-1β, IL-6, IL-8, and IFNγ, by using multi analyte BioLegend immunoassays on a randomly selected subset of twenty RA patients (15, 16). Despite the possibility of affect of methotrexate on cytokines, we observed statistically significant association between these cytokines and CTHRC1 ($\rho = 0.59– 0.91$, $p < 0.0001$, Dunn's test; Table 3-5) (128). Additionally, IL-1β significantly correlated with IFNγ ($\rho = 0.88$ and 0.91, respectively, $p < 0.0001$, Dunn's test; Table 3-5) and with IL-6, IL-8 ($\rho = 0.59$ and 0.86, respectively, $p < 0.0001$, Dunn's test; Table 3-5).

Significant association of CTHRC1 with all 4 inflammatory cytokines, which have major role in RA pathogenesis, implies that this protein has major role as well and these findings support previously published paper data (33).

CHAPTER IV: DISCUSSION

Relevant and easy-to-measure biomarkers are unmet in diagnosing RA. And there is no currently available marker for monitoring disease activity, showing efficiency of used therapy strategy. This cross-sectional pilot study, demonstrated that CTHRC1 is a potential biomarker of RA. We initially showed high association of CTHRC1 with RA, analyzing genetic interactions in mice models (6, 32-34). Specifically, it was shown that CTHRC1 significantly correlates with IL1β and IL-6, as well as with disease severity in these mouse models. To expand this preliminary data, we conducted study using peripheral blood of patients with established diagnosis. Surprisingly, results were confirmed in human subjects, and showed significant association of CTHRC1 with main markers of RA, as RF, ACPA and CRP. Also, it was observed that, CTHRC1 level was under detectable range in all non-RA. Therefore, it was concluded that CTHRC1 not only the marker of RA, but can also be used for differential diagnosis. Although we showed significant association between CTHRC1 and disease severity, this phenomenon needs further studies with larger population including all types of disease severity groups.

Our data suggest that baseline CTHRC1 levels may help to identify RA patients at risk and might help in prescribing medicine for making treatment more effective. However, since ROC analysis was conducted using relatively limited sample size with limited range of patients with DAS28 score, that was used to construct the CTHRC1 association to disease diagnosis, it is warranted to validate our results in sufficiently larger number of cohorts in the future, including treatment-naïve patients, and more patients range from remission to severe disease activity groups.

Additional research should be conducted to analyze the exact role of CTHRC1 in RA pathogenesis and what function it has within synovial cavity. It was reported that CTHRC1 might have role in cartilage/bone erosion (129), thus further studies must include possible roles of CTHRC1 in osteoclastogenesis, as well as RANKL/OPG interactions. Beside in this study, we did not test, how treatment strategy can affect CTHRC1 level in peripheral blood.

In healthy volunteers, CTHRC1 protein is expressed during embryonic development (8). Also, it was found in wounded epithelium of arteries, bone remodeling cells, myocardium, etc which were undergoing regeneration of cells (72, 130, 131), which explains why we observed at low levels of this protein in healthy individuals (132). It was shown that CTHRC1 protein is highly expressed at intimal layer of the bone surface, and secreted by fibroblast like synoviocytes of pannus (35), hence pannus might be the one of the main sources of CTHRC1 elevation. This was observed while testing synovial fluid and primary cells isolated from it, showed high concentrations of CTHRC1 (unpublished observation). Based on current data, it is hard to

understand whether CTHRC1 has anti-inflammatory function or pro-inflammatory. Recent studies showed that CTHRC1 is not expressed in all FLS cells, but rather elevated in FLS subpopulation, which are CD34−THY1+CDH11+. These subpopulation cells only exists in RA and not in OA (133), and have major role in bone resorption by participating in osteoclastogenesis (129). Reported data implies possible role of CTHRC1 in bone erosion by interacting with FLS, eventually elevating disease severity.

In other reports, it was shown that CTHRC1 is secreted from osteoclasts and functions as a link between osteoclasts and osteoblasts, consequently acting as a regulator of bone resorption/formation process (72, 95, 109, 114, 131, 134). Thus, in CTHRC1 null mices showed bone mass loss, whereas overexpression of CTHRC1 demonstrated high bone mass (94, 95, 109). It was also reported that CTHRC1 inhibits osteoclastogenesis and differentiation of osteoclasts (109) which indicates that CTHRC1 may be part of an inflamed synovium defense repair system in response to joint and bone degradation.

Animal model studies of arthritis may contribute to develop and understand more insights of human genome studies, giving opportunity to elaborate mechanistically features of crucial components of joint inflammation, especially mechanisms of T and B cell responses in RA. Although, there were many studies conducted, none of those animal model studies convey full picture of RA pathogenesis. Ideal animal models of RA would be those that include genetically controlled inflammation of joints, showing association of major components of destruction of joint during autoimmune responses. One of the major contributions to this field was reported by Adarichev and his colleagues, where they studied cartilage proteoglycan (PG) aggrecan-induced arthritis (PGIA), as well as CAIA models were studied revealing PGIA locus. These studies gave some insights to disease and helped in understanding genetic and clinical models of the human RA, for example, it was identified that two important loci (Pgia26/Cia5 and Pgia2/Cia2/Cia3) in rodent models corresponds to PTPN22/CD2 and TRAF1/C5 loci in human (6, 32, 33). Such studies certainly would improve understanding of CTHRC1 role in joint inflammation of human RA.

This study has many limitations, as low sample size of control groups, lack of homogeneity of treatment, low range variations of disease severity. Treatment regimen could have impact on CTHRC1 level, thus treatment naïve-patients needs to be recruited in the future studies. Perhaps, some of the treatment regimens somehow effected CTHRC1, which could explain why there were RA patients with CTHRC1 level under detectable range. Also, CTHRC1 should be studied with more cytokines, as multi-biomarker panel markers, to understand possible role. In addition to

disease progression, activity, and diagnosis, a retrospective study will be needed to determine Cthrc1 expression and validate its potential link to synovitis and bone degradation.

CONCLUDING REMARKS

Here we provided evidence of the potential of CTHRC1 protein as a novel RA biomarker. CTHRC1 may be influential which might significantly improve RA treatment efficiency monitoring and to track disease progression/remission. It also can be used as a tool for differential diagnosis of RA from other similar joint diseases. Our findings confirm CTHRC1 and its physiological function in bone / cartilage degradation for future studies based on its potential as a marker for RA.

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