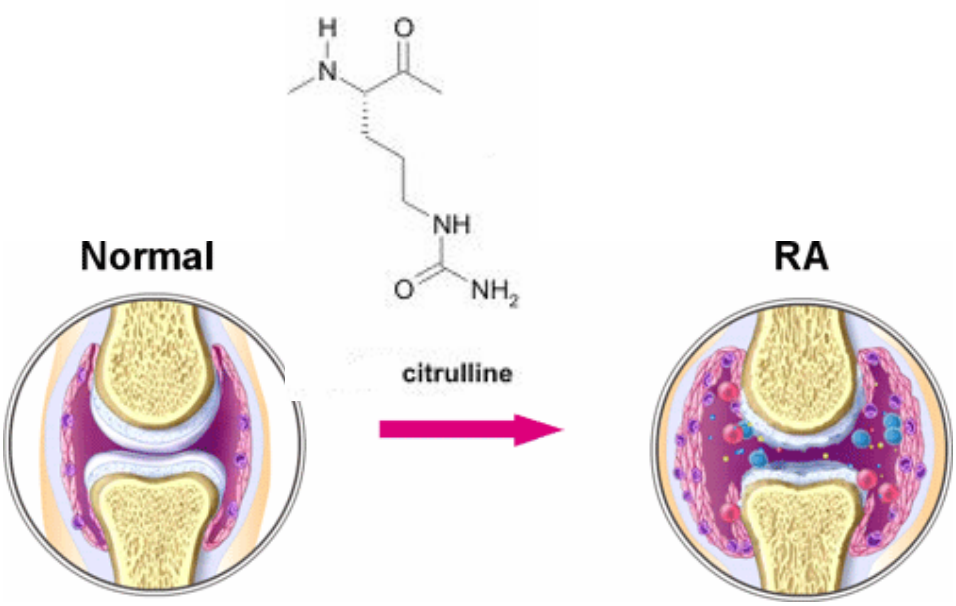
Identification of novel citrullinated proteins in Rheumatoid Arthritis patients



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Summary

Rheumatoid Arthritis (RA) is a common autoimmune disease. The disease is characterized by inflammation and swelling of the synovial joints. Specific for RA is the presence of autoantibodies against citrullinated proteins. The role of these citrullinated proteins in RA pathogenesis still remains unclear. Research on citrullinated proteins and their antigenic properties may give more insight in the molecular mechanism behind RA and may contribute to a better understanding of the cause and course of the disease. Furthermore, citrullinated proteins have proven to be useful in the detection of their autoantibodies in patients. Citrullinated peptides are used in the clinic nowadays for diagnosing RA. Newly identified citrullinated proteins can contribute to a better understanding of the disease and to diagnostic improvements. To identify new citrullinated proteins in RA patients, synovial fluids were analyzed by TANDEM mass spectrometry (MS). Synovial fluids of 33 RA patients were screened for the presence of citrullinated proteins by separation on a SDS-PAGE gel and detection on western blot with an anti-modified citrulline antibody. Thirteen synovial fluids containing citrullinated proteins were depleted for the abundant protein albumin. Albumin depleted samples were separated by size on a SDS-PAGE gel. The gel was stained with colloidal coomassie (CBB) staining and each sample lane was cut into bands. A matching western blot was made with anti-modified citrulline antibody to compare and determine which cut out bands from the CBB stained gel contained citrullinated proteins. From each citrullinated protein containing gel band a protein fragment mix was obtained by in-gel digestion with trypsin. The protein fragment mixes were analyzed by TANDEM MS. As a result 57 citrullinated proteins were identified. Five of the identified proteins are already known citrullinated proteins in RA patients. Eight citrullinated proteins were identified that contained multiple citrulline residues. Literature pointed out that these proteins may have functions involved in RA/ inflammatory related processes. Therefore these citrullinated proteins were marked as possible candidate auto-antigens.

Samenvatting

Rheumatoïde Arthritis (RA) is een veel voorkomende auto-immuunziekte. De ziekte wordt gekenmerkt door ontsteking en zwelling van de synoviale gewrichten. Specifiek voor RA is de aanwezigheid van autoantilichamen gericht tegen gecitrullineerde eiwitten. De rol van deze gecitrullineerde eiwitten in de pathophysiologie van RA is echter nog niet duidelijk. Onderzoek naar gecitrullineerde eiwitten en hun antigene eigenschappen kan meer inzicht geven in de moleculaire mechanismen achter RA en draagt bij aan kennis over het onstaan en verloop van de ziekte. Gecitrullineerde peptiden worden gebruikt om auto-antilichamen aan te tonen in RA patienten en zijn zodoende van belang in de diagnostiek van RA. Identificatie van meer en nieuwe gecitrullineerde eiwitten kan leiden tot een beter inzicht in de ziekte en kan bijdragen aan verbetering van RA diagnostiek. Om nieuwe gecitrullineerde eiwitten te identificeren bij RA patienten werdt gebruik gemaakt van TANDEM massa spectrometrie (MS). Synoviaal vloeistof van 33 RA patienten werdt gescreened voor de aanwezigheid van gecitrullineerde eiwitten door scheiding op een SDS-PAGE gel en detectie op western blot met anti-gemodificeerd citrulline antilichaam. Dertien synoviale vloeistoffen met gecitrullineerde eiwitten werden ontdaan van albumine, een dominant eiwit. Albumine gedepleteerde samples werden op grootte gescheiden met behulp van een SDS-PAGE gel. De gel werdt gekleurd met colloidaal coomassie (CBB) kleuring en elk sample “laantje” werd opgesneden in gel bandjes. Een western blot met anti-gemodificeerd citrulline antilichaam werd vergeleken met de gel om te bepalen welke uitgesneden bandjes gecitrullineerde eiwitten bevatten. Een peptide-mix werdt verkregen van elk bandje door een in-gel digestie met trypsine. De peptide-mix van elk bandje werd geanalyseerd doormiddel van TANDEM MS. De massa spectrometrie analyse resulteerde in de identificatie van 57 gecitrullineerde eiwitten. Vijf van deze geidentificeerde eiwitten zijn reeds identificeerde gecitrullineerde eiwitten in RA. Acht andere geidentificeerde eiwitten bevatte meerdere citrulline residuen. Literatuur wees uit dat deze eiwitten een functie zouden kunnen hebben in RA/ontstekings gerelateerde processen. Deze punten maakten de acht gecitrullineerde eiwitten interessant voor verder onderzoek om te verifiëren of het mogelijke auto-antigenen zijn in RA.

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Abbreviations

AA-BAA	Acrylamide-bisacrylamide
ACPA	Anti citrulline protein antibody
AFA	Anti fillagrin antibody
AKA	Anti keratin antibody
APS	Ammonium persulfate
BSA	Bovine serum albumin
CapZα-1	F-actin capping protein alpha-1 subunit
CBB	Coomassie brilliant blue
DAC	Depletion of albumin component
DAMO	Diacetylmonoxime
DTE	Dithioerythritol
EBVA-1	Epstein Barr virus nuclear antigen-1
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
eIF4G1	Eukaryotic translation initiation factor 4G1
eIF3S10	Eukaryotic translation initiation factor 3 subunit 10
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MALDI-TOF MS	Matrix-assisted laser desorption ionisation time of flight mass spectrometry
MHC	Major histocompatibility complex
MNDA	Myeloid cell nuclear differentiation antigen
mRNA	Messneger Ribonucleic acid
PAD	Peptidyl arginine deiminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
scFv	Single chain variable fragment
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STI	Soybean trypsin inhibitor
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid

1. Introduction

Rheumatoid Arthritis (RA) is a common autoimmune disease that affects approximately 1% of the total population, mainly women. The disease is characterized by inflammation and swelling of the synovial joints and can eventually lead to bone destruction. The etiology of the disease is still unknown despite extensive research, but it is likely that genetic and environmental factors play a role.

Specific for RA is the presence of autoantibodies. Endogenous proteins can undergo certain changes that result in recognition by the immune system as non-self. The presences of such proteins, autoantigens, result in the production of autoantibodies. Much research on the function of auto antigens and their autoantibodies in the cause and course of this disease is going on. One group of autoantibodies is of special interest in RA, the autoantibodies against citrullinated proteins.

The presence of citrullinated proteins can lead to the production of auto-antibodies and is therefore of importance in the pathophysiology of RA. However, the exact role of citrullinated proteins in the disease process is still unclear. But ongoing research on citrullinated proteins and their antigenic properties gives more insight in the molecular mechanism behind RA and may contribute to disease etiology. Furthermore, citrullinated proteins and especially citrullinated peptides have proven to be useful in the detection of their autoantibodies in patients. This makes them a suitable tool in RA diagnostics. Newly identified citrullinated proteins can contribute to diagnostic improvements.

1.1 Theory

1.2.1 Citrulline & PAD

Citrulline is an uncommon amino acid that can only be incorporated into a protein by a post translational modification. This type of post translational modification is called citrullination and is carried out by the enzyme Peptidylarginine deiminase (PAD). An arginine is converted into a citrulline by PAD in the presence of Ca^{2+} ions (Figure 1). The active site of the enzyme attacks the guanidine group of the arginine, which leads to the formation of a tetrahedral adduct. Ammonia is cleaved off after the nucleophilic attack of water, which leaves the keto-group that distinguishes arginine from citrulline [1]. This conversion leads to a change in charge from positive to neutral and a loss of 1kDa in mass for each converted arginine. The change in charge affects protein structure and protein-protein interactions, which can have a direct physiological and pathological effect.

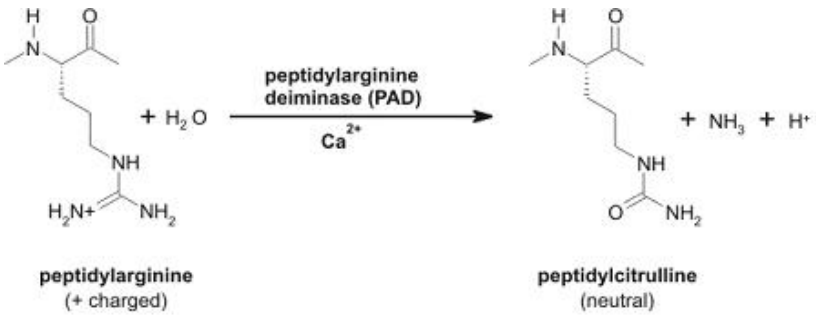

Figure 1. Conversion of peptidylarginine in peptidylcitrulline by peptidylarginine deiminase in the presence of Ca^{2+} ions.

PAD activity mainly depends on the calcium concentration and the accessibility of the substrate protein. Primary and secondary structure of the protein matters for accessibility of PAD and if citrullination can take place or not. Certain features make proteins more accessible for PAD such as β-turns and when arginine is flanked by another arginine or a glutamine or aspartic acid. For the calcium concentration it is established that levels must be in the range of 10^{-5} mol/l for PAD activation. This means that intracellular concentration of calcium, which range between 10^{-8}-10^{-6} mol/l, is not high enough and extracellular calcium is needed [3].

Five isotypes of PAD have been discovered, each with their own sites of expression (Table 1). PAD and citrullination are involved in a multitude of physiological processes as seen from the expression pattern of PAD. PAD1, expressed in the epidermis, citrullinates keratins and filagrins which are necessary for cornification of the epidermis. PAD3 is expressed in hair follicles and citrullinates trichohyalin, which is necessary for follicle formation. PAD is also involved in other processes such as cell differentiation, nervous growth, mammalian embryonic development, gene regulation and cell death.
Furthermore PAD is involved in several diseases. Citrullination is known to occur in Multiple sclerosis, Alzheimer's disease and multiple inflammatory diseases such as Crohn's, polymiositis and several types of arthritis. In RA PAD2 and PAD4 are likely to be responsible for citrullination. PAD2-expressing macrophages and PAD4 containing granulocytes can be found in inflamed RA synovium [4]. For PAD4 it is also suggested that it co-localized with citrullinated fibrin and other citrullinated proteins in RA synovial tissue [5].

Table 1. Expression sites of PAD isotypes. (adapted from [2]).

Isotype	Site of expression
PAD1	Epidermis, Uterus
PAD2	Skleletal muscle, brain, spinal cord, oligodendrocytes, uterus, páncreas, salivary gland, pituitary gland, sweat glands, spleen, macrophages, bone-marrow, yola-sac (leukocytes)
PAD3	Hair follicles
PAD4	Granulocytes, eosinophils, neutrophils, monocytes, macrophages
PAD6	Embryonic stem cells, oocytes

1.2.2 Auto-antibodies in RA

Several auto-antibodies are known in RA. Anti-RA33, anti-calpastatin, and anti-Bip are some of them. The best know RA auto-antibody is the rheumatoid factor (RF). RF is an antibody directed against the Fc part of IgG's and can be found in approximately in 75% of RA patients.

Some thirty years ago a RA antibody was discovered called anti-keratin antibody (AKA). Its was thought that the antibody recognized keratin but some ten years later the recognizing protein was found to be fillagrin and the antibody was renamed anti-fillagrin antibody (AFA). Fillagrin could however not be established as the target of AKA/ AFA. In 2001 Masson-Bessiere et al [6] identified the target of AKA/ AFA as citrullinated alpha- and beta-chains of Fibrinogen. In 1994 the anti-Sa antibody was discovered as a new RA-autoantibody [7]. The antigen of Sa was confirmed around 2000 to be the citrullinated form of vimentin [8]. And citrullination of the protein was proven to be of importance for the auto-antigenicity of its antibody [9].

The anti-fillagrin and anti-vimentin antibodies, both targeted to citrullinated proteins were named anti citrulline protein antibodies (ACPA's). ACPA's formed a new group of RA auto-antibodies comprising all antibodies targeted against citrullinated proteins. ACPA's are produced locally in the inflamed RA synovium by plasma cells as a result of a local antigen derived B-cell response [10]. This suggests that citrullinated proteins are formed in the

synovium and as antigen are responsible for ACPA production. Besides the inflamed synovium ACPA's are also detectable in synovial fluid and serum of RA patients.
Compared to other RA-autoantibodies, which also can be found in healthy patients and in other inflammatory diseases, ACPA's have a very high specificity up to 97%. Another feature of ACPA's, which distinguish them from other RA-autoantibodies is there presence in the very early stage of the disease. ACPA's can be found even before clinical onset of symptoms, and their titer also corresponds to disease severity in later stages. These findings suggested that these antibodies might play a role in the pathophysiology of the disease [11].

The high specificity of ACPA's and their presence in the early stage of RA make them very useful in RA diagnostics. Cyclic forms of citrullinated peptides derived from fillagrin were used as antigens in an ELISA test to detect presence of ACPA's in RA sera [12]. From this the anti-CCP (anti-cyclic citrullinated peptide)-test was developed, now using a mix of citrullinated peptides resulting in a diagnostic test with high specificity for RA.

1.2.3 Citrullinated proteins in RA
Fibrinogen and vimentin were the first antigens found in RA to be responsible for the induction of antibodies against citrullinated proteins. Because the antibody repertoire in RA is heterogeneous it was thought that probably more citrulline containing antigens are present in the inflamed synovium [13]

There are many citrullinated proteins present in RA, as well as in other (inflammatory) diseases. Not all of these citrullinated proteins are antigens, and are not capable of ACPA induction. This makes it likely that not the presence of citrullinated proteins but the presence of certain citrullinated epitopes are necessary to break the immune tolerance which lead to the development of RA.
In the last years many research is done to identify more citrullinated proteins that act as an antigen in RA. Several citrullinated proteins have been identified as possible candidate antigens (see table 2).

Table 2. Citrullinated proteins in RA.

Citrullinated proteins in RA	Refs
Fibrinogen/ Fibrin	[6]
Vimentin	[8]
Histones	[14]
α-Enolase	[15]
Nucleophosmin (B23)	[14]
Fibronectine	[16]
Anti thrombin	[17]
Eukaryotic translation initiation factor 4G1 (eIF4G1)	[18]
Collagen type I	[19]
Collagen type II	[20]
F-actin capping protein alpha-1 subunit (CapZα-1)	[21]
Epstein Barr virus nuclear antigen-1 (EBVA-1)	[22]

Most of these proteins have been found in in-vitro studies. The in-vitro citrullinated proteins reacted with RA serum in a specific manner, which marked them as candidate auto antigens. However, the proteins α-Enolase and eIF4G1 were not identified in citrullinated form in-vivo [15,18]. The CapZα-1 protein was first identified in an in-vivo study and was marked as a candidate auto antigen when the recombinant protein showed to react with antibodies RA serum [21].

Antithrombin, nucleophosmin and histones have been identified as citrullinated proteins in RA but are not confirmed to be a candidate auto antigen [14,17]. However, their cellular functions and their location in inflamed tissue makes them interesting proteins.

1.2.4 The hyopothesized role of citrullinated proteins in RA pathophysiology

Research on the role of citrullinated proteins in the development of and the chronicity of RA have lead to a hypothetical model (see figure 2). According to the model a certain trigger leads to inflammation. Inflammation is associated with the recruitment of inflammatory cells. In RA there is a build up of such cells in the synovial cavity of the joint. When these cells undergo apoptosis and inflammatory cells are not effectively removed by phagocytosis, PAD2 present in macrophages, and PAD4 present in macrophages and granulocytes, become active, as there is an influx of extra cellular Calcium. In this stage PAD can citrullinate every protein meeting substrate qualification in the synovial cavity. Some citrullinated proteins can elicit an immune response when they are phagocytosed by antigen presenting cells (APC) and via MHC II tetramers presented to T-cells. Active T-cells provoke B-cells to locally produce antibodies against the presented citrullinated protein. The citrullinated protein can form an immune complex with its antibody and via inflammatory cells lead to the production of cytokines. Cytokines, known to be a trigger for inflammation, stimulates recruitment of inflammatory cells in the synovial cavity again.

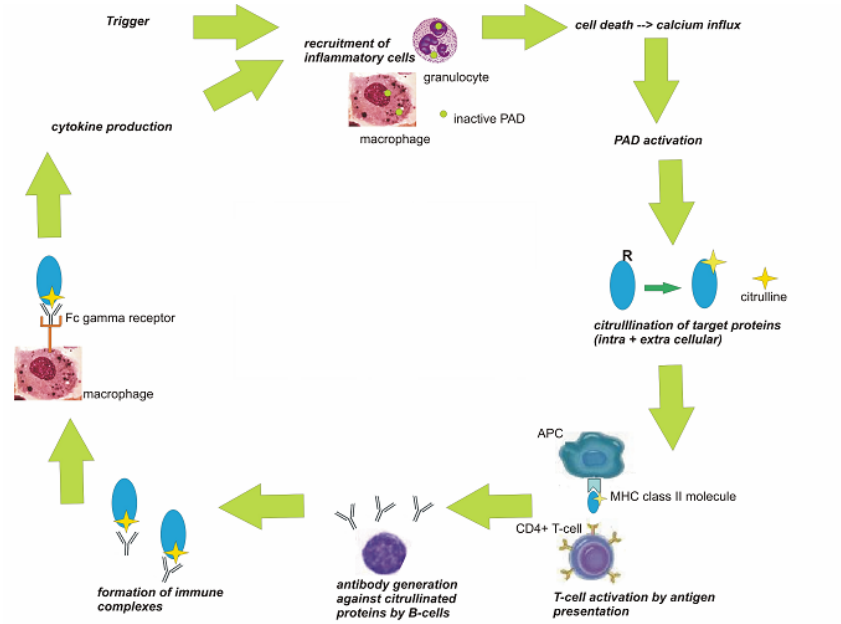

Figure 2. Hypothetical model of the role of citrullinated proteins in the development and chronicity of RA (adapted from [2]).

Inflammation, infiltration of PAD containing inflammatory cells and citrullination are processes that are not RA-specific. They also occur in healthy subjects and in other (inflammatory) disease. However, the immune response to the citrullinated proteins and antibody production is RA-specific. The switch that triggers the change-over from healthy to RA are probably distinct citrulline epitopes and is influenced by genetic and environmental factors.

Japanese gene linkage studies showed that in RA patients PAD4 mRNA is much more stable expressed [23]. A higher enzyme production, which could be its effect, will lead to enhanced citrullination. However this could not be found in RA population outside Asia. Other studies show a linkage between certain HLA molecules, the antigen presenting MHC II tetramers, and the occurrence of RA. Different HLA-DRB1 alleles with shared epitopes are associated

with the predisposition to develop RA [24] One of these alleles, HLA-DRB1*0401, showed that the interaction between a citrullinated peptide derived from vimentin and the shared epitope was able to elicit a T-cell response in mice [25]. Cytokines may play a role in initiation and chronicity of RA. Expression of certain IL-10 promotor polymorphisms may lead to an increased IL-10 production which may result in a higher antibody production by stimulating B-cell proliferation which leads to more antibodies, immune-complexes and new cytokines [26].

Among the environmental factors that play a possible role in the etiology of RA is smoking. Smoking is possibly involved in the increased presence of citrullinated proteins in the lungs. And smoking, combined with the HLA DRB1 shared epitopes, may be a trigger in RA-specific immune response to citrullinated proteins [27]. Other environmental factors such as vibration, injury, exposure to mineral dust and silica oil, are only associated with a higher rate of apoptosis [3]. More apoptotic cells can result in an increased amount of activated PAD and thus result in a greater amount of citrullinated proteins. Together with genetical factor this can also lead to a higher susceptibility to develop RA.

1.2 Objectives

The main objectives of this project are:

1. To identify novel citrullinated proteins in synovial fluid of RA-patients. Furthermore it would also be interesting to identify confirmed citrullinated proteins in own patient material.
2. To verify if newly found citrullinated proteins act as an antigen in RA.

2. Material and Methods

2.1 Identification of novel citrullinated proteins

An overview of the experimental outline for identification of novel citrullinated proteins is given in Figure 3.

2.1.1 Preparation of patient material
Synovial fluid material was obtained from 33 Rheumatiod Arthritis positive patients from the Sint Maartens Kliniek, Nijmegen, the Netherlands and Slovenia (Appendix A). Synovial fluids were divided in two fractions by centrifugation: a supernatant fraction containing soluble proteins, and a pellet fraction containing insoluble proteins. The two fractions were stored at - 70°C. The supernatant fraction of each patient and the pellet fractions of eight patients were prepared for further experiments.

One-hundred µl of each synovial fluid supernatant fraction was diluted in 400 µl standard lysis buffer (50mM Tris/HCl pH7.5, 100mM KCl, 1mM DTE (DTT), 10mM EDTA, 0.5mM PMSF). The diluted fractions were sonicated on ice for 30 seconds twice to disrupt any cells still present in the fluid and to bring most proteins into solution. After bringing the fractions up to room temperature they were vortexed, then boiled for 3 minutes, and vortexed again. After a spin down for 5 minutes at 13.000 rpm to remove insoluble matter, the prepared supernatant samples were stored at -20°C.

2.1.2. SDS-PAGE and western blotting
To verify the presence of citrullinated proteins in the patient material, each prepared supernatant and pellet sample was separated on a 13% SDS-PAGE gel. Running- and stacking-gel were made for the Biorad gel system (Appendix B). The supernatant and pellet samples were takes up in 4x sample buffer (50mM Tris-HCl; pH 6.8, 2% SDS, 10% Glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue) and boiled for 3 minutes, and centrifuged for 1 minute at 13.000rpm. Ten µl of STI (soybean trypsin inhibitor, 50ng/ µl), a positive control for the chemical modification procedure and antibody detection, was loaded together with the samples. Next, the proteins in the gels were transferred to hybond C-extra membranes by blotting the gel overnight in a blotting buffer (10% 10x secret buffer, 20% methanol, 70% demi water) at 25mA. The blots were dried and stained with Ponceau-S to see if the blotting procedure was successful. Ponceau-S staining was then removed by rinsing with demi-water.

To detect citrullinated proteins on the western blots, citrulline residues had to be chemically modified. During chemical modification diacetylmonoxime (DAMO) and antipyrin react with the ureido group of citrullines in a strong acidic environment in a two-step process. As a result an ureido group adduct is formed on citrulline. The modification occurs solely with citrullines and independent of any neighboring amino acids. This makes detection of every protein containing a citrulline possible. After blotting but before the actual chemical modification, the blots were blocked with 0.1% ovalbumin in PBS for 15 minutes and then shortly rinsed with demi-water. Then the blots were incubated for 15 minutes with 4% paraformaldehyde in PBS to cross-link the proteins with the blotted membranes. Two freshly made mixes for chemical modification (mixA: 0,75 ml acetic acid, 125 mg DAMO, 62,5 mg antipyrine, 17,8 ml MQ, mixB: 5,1 ml phosphoric acid, 6,35 ml sulphuric acid, 10 mg iron chloride, 20 ml MQ) were put together and added to the blots. Chemical modification of the blots took place overnight at 37°C while shaking. The blots were then washed two times with PBS.

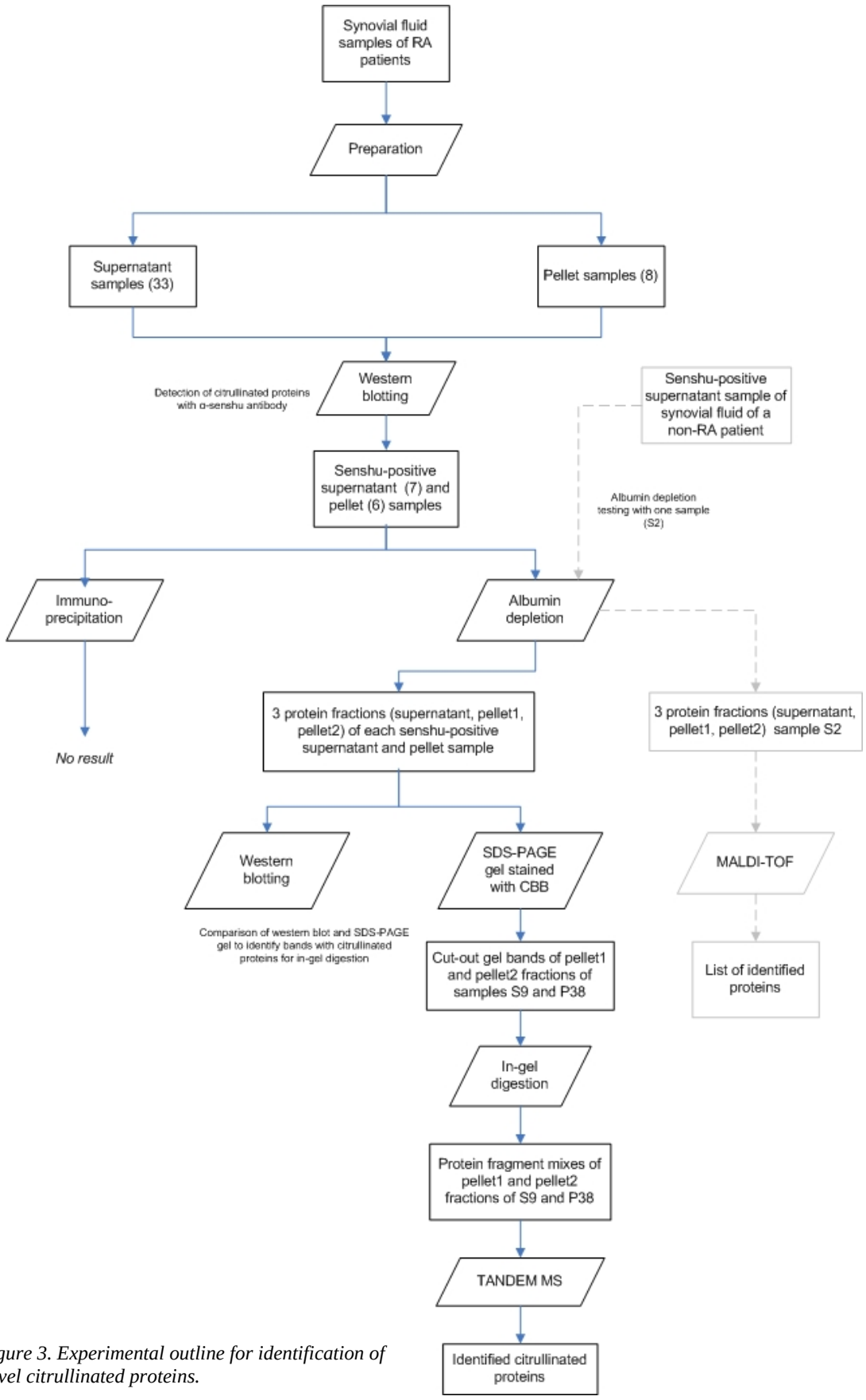

Figure 3. Experimental outline for identification of novel citrullinated proteins.

The next step was the detection of the (chemically modified) citrullinated proteins using anti-modified-citrulline[1] and Goat anti-Rabbit-HRP[2] antibodies. The anti-modified citrulline antibodies (senshu-antibody) recognizes and bind to the ureido group adduct on the modified citrullines. The blots were incubated for one hour in blocking buffer (5% Elk, PBS, 0.1% NP-40). Then the blots were incubated for three hours with anti-modified-citrulline antibody diluted 1:1600 in blocking buffer. After this incubation the blots were washed three times 10 minutes with blocking buffer. The blots were then incubated for one hour with Goat anti-Rabbit-HRP diluted 1:1000 in blocking buffer. Afterwards the blots were washed three times 10 minutes with wash buffer (PBS, 0.1% NP-40), and one time 10 minutes with PBS.

Enhanced chemiluminescence (ECL) antibody detection was used because the second antibody was horseradish-peroxidase conjugated. The two ECL-mixes (mix A: 8,85 ml MQ, 1 ml Tris/HCl pH8.5, 100 µl luminol solution, 44 µl coumaric acid, mix B: 9 ml MQ, 1 ml Tris/HCl pH8.5, 10 µl H_2O_2) were made separately and put together after the last washing step of the membrane with PBS. The blots were incubated with the ECL mix for one minute and then transferred to a film cassette.

2.1.3. Immunoprecipitation

Citrullinated proteins make up a small part of the total protein content in synovial fluid, which makes their identification difficult. To prevent the so-called "searching for a needle in a haystack", immunoprecipitation was used to separate the citrullinated proteins from the bulk of non-citrullinated proteins. Supernatant samples that contained citrullinated proteins (as identified by Western blotting) were used for immunoprecipitation.

Forty µl of Nickel NTA agarose beads were washed three times with IPP500 (10 mM Tris/Hcl pH 8, 500 mM NaCl, 0.1 % NP40, 0.1 % Tween20). Fifty µl of scFv RA3 antibody in 450µl IPP500 was coupled to the beads by a two hours incubation while rotating. Beads were washed 3 times with IPP500 and one time with IPP150 (10 mM Tris/Hcl pH 8, 150 mM NaCl, 0.1 % NP40, 0.1 % Tween20). Twenty-five µl supernatant sample in 475µl IPP150 was coupled to the beads by a 2 hour incubation at 4˚C while rotating. After washing the beads 3 times with IPP150, 4x sample buffer was added. By boiling in sample buffer for 3 minutes proteins were eluded from the beads and directly put on a 13% SDS-PAGE gel (Biorad gel system) and analyzed by western blot (section 2.1.2).

2.1.4 Albumin depletion

Because immunoprecipitation did not work to separate citrullinated proteins from non-citrullinated proteins in the supernatant samples, another way had to been found to lessen the amount of abundant non- citrullinated proteins in the samples.

Citrullinated proteins make up only a small portion of the total protein content in synovial fluid. When searching for these kinds of proteins there is a high probability that these are masked by highly abundant proteins when visualizing on protein gel or blot. This makes citrullinated proteins hard to identify. In synovial fluid the most abundant proteins are hyaluronic acid, albumin and immunoglobulin. Because of its size and abundance, Albumin (67 kDa) is seen as a great smear on SDS-PAGE gels, masking the presence of other proteins. Albumin can also hamper mass spectrometry when gel protein bands are processed for mass spectrometry analysis. To get rid of albumin in the synovial fluid samples, albumin depletion is performed according to the DAC protocol. This protocol states that the majority of albumin can be removed from samples with minimal loss of proteins [28].

Sodium Chloride stock solution was added to 200µl of synovial fluid supernatant sample to a final concentration of 0.1M. This was gently rotated for one hour at 4°C. Then cold Ethanol

[1] Anti-modified-citrulline antibody detection kit, Upstate Biotechnology
[2] Dako Cytomation

was added to a concentration of 42% and again rotated under the same conditions to precipitate the majority of proteins present except albumin. Precipitated proteins were pelleted by centrifugation for 90 minutes at 13.000rpm. To this pellet fraction (pellet 1) 10mM Tris buffer pH6.8 and 1M Urea was added before storage. The supernatant fraction containing the albumin was further processed to extract remaining proteins. The pH of the supernatant was lowered to pH 5.7 using cold 0.8M sodium acetate pH4.0 followed by gentle rotation for one hour at 4°C. This precipitates more proteins which were pelleted using centrifugation for 90 minutes at 13.000rpm. This pellet fraction (pellet 2) was treated the same way as pellet 1. Both pellet fractions were dissolved by vigorous pipetting and sonification in a water bath.

The pellet1, pellet2 and supernatant protein fractions of each albumin-depleted sample were then analyzed on SDS-PAGE gel and western blot. A large 13% SDS-PAGE gel was cast into a Horstmann gel system. All albumin depleted samples were taken up in 4x sample buffer, boiled for 3 minutes, and centrifuged for 1 minute at 13.000rpm. The samples were loaded together with a low molecular weight marker and STI, which is the positive control for the chemical modification method used during western blotting. Gels were run at 60mA until the frontline of the gel reached the bottom of the gel. The gels were then stained with colloidal coomassie brilliant blue overnight while shaking. Destaining was done by multiple washing steps with demi-water until sufficient destaining was observed. The gel was then photographed and cut into bands according to a grid pattern (Appendix C). The western blot procedure was performed in the same manner as described in section *2.1.2*.

2.1.5 In-gel digestion

A protein fragment mix was obtained from each cut-out SDS-PAGE gel band by in-gel digestion with trypsin. The protein fragments were extracted from the gel and brought in solution. This solution was used for mass spectrometry analysis.

Each cut-out band was washed twice with 100µl 25mM Ammoniumbicarbonate [NH4HCO3] and soaked in the same amount of ammoniumbicarbonate for 30 minutes at room temperature. Acetonitril was added to a final concentration of 30% and incubated for 10 minutes. Another volume of 100µl acetonitril was added after liquid removal from the gel band and incubated for 30 minutes. Again all liquid was removed and the tubes were placed in a 37°C stove for two hours for the bands to dry. When bands were completely dry, 20µl of trypsin solution (15 ng/0,3µl trypsin , 25mM NH4HCO3, 5mM n-octylpyranoglucoside) was added to the samples and incubated for one hour on ice. Twenty µl of 25mM NH4HCO3 / 5mM n-octylpyranoglucoside was added and incubated overnight at 37°C. The peptides were extracted by incubating the gel bands for 30 minutes in 10µl of 50% Acetonitril/ 0.5% TFA/ 5mM n-octylpyranoglucoside. Bands were sonified for two minutes in a cooled water bath (Bioruptor) to get all trypsin digested protein fragments in solution. All liquid was transferred to a new tube and stored at -20°C until further use.

2.1.6 Mass spectrometry

MALDI-TOF mass spectrometry was used to identify the total protein content in one albumin-depleted sample and to identify differences in protein content between the three different protein fractions of one sample.

The protein fragment mixes obtained via in-gel digestion were spotted on the target plate using the "sandwich method" which allows an even distribution of proteins with the matrix crystals. From each fragment mix 0.3µl was spotted on a MTP384 ground steel plate. Spots were topped by 0.3µl freshly made matrix solution (10 mg of 4-hydroxyl-a-cynocinnamic acid, 0.55 ml acetonitril, 0.55 ml 0,1% TFA) and air-dried. Together with the samples a calibration mix (0.5 µl P14R10, 0.5 µl angiotensin, 0.5 µl ACTH, 0.5 µl brad, 10µl matrix solution) was spotted to calibrate the measurements. Measurements were done on the Bruker Biflex III MALDI-TOF MS machine with the following settings:

- Reflectron mode (Ref_pos_600-4000)
- Positive ionization
- Calibration: peptide mix micro mono
- Detector voltage: 1.9kV
- Laser intensity: 70-80%.

Peaks from the mass spectrometry spectrum and their m/z values were automatically stored in the Bruker Daltronics X-tog program. The list of peaks representing the protein mass fingerprint was compared to a genomic database using the Matrix Science MASCOT search algorithm. This resulted in a list of proteins that were present in each spot and thus each gel band.

TANDEM mass spectrometry was used to identify proteins containing citrullinated peptides in albumin depleted samples. Three µl of in-gel digested samples (the protein mixes) were send to the FTMS facility, part of the Nijmegen Proteomics facility. Measurements were done with adjustments for finding protein modifications, in this case citrulline.

2.2 Antigenicity of new citrullinated proteins

The "eukaryotic translation initiation factor 3 subunit 10" (eIF3S10) protein was used in this study to see if this protein is a new candidate autoantigen in RA. Two different studies showed that this protein is citrullinated and contains multiple citrullinated peptides. This protein is also interesting because it is thought to be involved with eIF4G1 [29], an established candidate autoantigen. Furthermore, it was reported that it is involved in macrophage recruitment and is expressed ion the cell surface during apoptosis [30].

2.2.1. Primers

Primers were designed against the protein coding region of eIF3S10 (see table 3). Two sets of primers were designed because the PCR-product would be too large for direct cloning into the vectors that were available.

Table 3. Primers designed against eIF3S10 protein coding region.

		Primer	length	T_m (˚C)	Product size (bp)
A	Fwd	3'-ATGCCGGCCTATTTTCAG-5'	18	55.97	2165
	Rev	5'-CTTGTTGCTCCCACAGATCC-3'	20	57.58	
B	Fwd	3'-TTTGATAAAGAGCGCTTACG-5'	20	54.14	2076
	Rev	5'-GTGATCAAACCTATTTAAGACACC-3'	24	54.97	

2.2.2 PCR amplification

The eIF3S10 DNA product was amplified by PCR from a Terata carcinoma cDNA bank with the two primers sets A and B. A PCR-mix was made with 5µl PCR-buffer (10x), 1µl of 10µM dNTP, 3µl of 25mM $MgCl_2$, 2.5µl of 0.5µM forward primer, and the same amount of reverse primer. Finally 34.8µl of MQ was added to make a total amount of 48.8µl. To the mix 0.2µl Red Hot Polymerase[3] and 1µl Terata Carcinoma cDNA was added. The PCR reaction cycle was performed using the following conditions:

- - 2' 95°C
- - 30" 95°C ┐
- - 30" 55°C ┘ 35x
- - 1.30' 72°C
- - 10' 72°C.

[3] AbGene

PCR-product was analyzed on a 1% Agarose gel. Loading buffer was added to the samples and loaded on the gel together with a λHind marker. The gel was run at 80mV until the bands of the marker were well separated (visualized under UV light).

2.2.3 Cloning of primer set B eIF3S10 PCR-product in a pCR4-TOPO vector

The pCR4-TOPO vector[4] (Appendix D) allows insertion of all Taq amplified PCR-products and gives easy ligation. A 3'-A overhang was added to 3µl of the PCR product by addition of 1µl of 1U/µl Taq polymerase and incubated for 10 minutes at 72°C to give it the same 3'-end as a Taq amplified PCR product would have.

Four µl of the 3'-A-tailed product was added to 1µl salt solution and 1µl pCR4-TOPO vector and incubated for 15 minutes at room temperature for ligation. Six µl of the reaction mix was added to 50µl TOPO10 competent cells[4] and incubated for 30 minutes on ice. Transformation of the competent cells with the insert containing vector took place by a heat shock at 42°C for 30 seconds. LB medium (250µl) was added for the transformed cells to grow and incubated at 37°C while shaking 250rpm for a hour. Culture (150µl) was plated on pre warmed Agar plates containing ampiciline and kanamycin. Colonies were grown in a stove at 37°C, overnight.

2.2.4 Colony PCR

A colony PCR was performed on the eIF3S10 DNA containing pCR 4-TOPO colonies to see if the colonies really carried the vector plus insert. A PCR-mix was made with 5µl PCR-buffer (10x), 1µl of 10µM dNTP, 3µl of 25mM $MgCl_2$, 2.5µl of the M13 forward and reverse primer from the Invitrogen TOPO cloning kit, and 0.2µl Red Hot polymerase. Finally 34.8µl of MQ was added to make a total amount of 48.8µl. Twenty colonies were picked from a plate with a sterile toothpick (a small smear was made on a new plate) and placed into PCR-tubes containing the PCR-mix.

The PCR reaction cycle was performed using the following conditions:

- - 2' 95°C
- - 30" 95°C ┐
- - 30" 55°C ┘ 30x
- - 2' 72°C
- - 10' 72°C.

The PCR samples were analyzed on a 1% Agarose gel. One of the colonies that contained the pCR4-TOPO vector and the insert was grown in ampicilin (diluted 1:1000) containing LB medium overnight at 37°C. Three ml of this culture was pelleted for purification by miniprep with the Wizard Plus SV miniprep DNA purification system[5]. Three µl of the purified eIF3S10/ pCR4-TOPO with 0,6µl of M13 forward and reverse primer[4] was send for sequencing to verify eIF3S10 as the insert product.

2.2.5 Restriction enzyme digestion

The eIF3S10 insert was digested from the pCR4-TOPO vector with restriction enzymes XhoI and NotI for further cloning into the pGEX4T2 expression vector (Appendix D). Five µl NEB-3 buffer (10x), 0,5µl BSA (100x), and 29,5µl MQ was added to 10µl purified eIF3S10/ pCR4-TOPO. Finally 2,5µl of XhoI and NotI was added to the mixture, which was subsequently incubated for two and a half hours at 37°C. The digest was analyzed on a 1% Agarose gel to verify the digestion products.

[4] Invitrogen, TOPO cloning kit
[5] Promega

Ten µl pGEX4T2 expression vector was cut open with the same restriction enzymes using the same mixture of NEB-3 buffer, BSA and MQ. To prevent the open vector to ligate on itself, ends were dephosphorylated with alkaline phosphatase. Five µl dephosphorylation buffer (10x), 5µl calf intestine alkaline phophatase (10U/µl), and 40µl of MQ was added to the reaction mixture and incubated for 30 minutes at 37°C. The digested vector was then put on a 1% Agarose gel and purified by gel extraction using the Qiagen gel extraction kit.

2.2.6 Ligation of eIF3S10 insert in a pGEX4T2 expression vector

The eIF3S10 product digested from pcR4-TOPO vector was ligated into the pGEX4T2 expression vector. This vector contains an IPTG inducible promoter for protein expression and a glutathione S-transferase region, which is simultaneously expressed and serves as a tag for protein purification.

One µl of the digested vector and 6µl of the digested insert were mixed with 1µl of ligase buffer (10x), 1µl of T4 DNA ligase (200 NRB U/µl) and 1µl of MQ. Incubation took place overnight by 16°C and overnight by room temperature.

Two µl of ligation mix was transformed to 50µl TOP10 competent cells by a 42°C heat shock for 30 seconds after 30 minute incubation on ice. Transformed cells were grown in 250µl LB-medium for one hour and plated on pre-warmed ampicilin Agar-plates, all at 37°C. Colonies were grown overnight.

Verification of the ligation reaction was tested by restriction enzyme digestion with SalI and PstI. If the ligation was successful then two products of 2433 and 3868bp were expected. If no ligation of vector and insert took place then the digestion would have led to two products of 1032 and 3868bp. Colonies were transferred to 5ml ampicilin containing LB-medium and grown overnight at 37°C while shaking. 3ml of culture was used for purification by miniprep. To 4µl of miniprep DNA product 1µl NEB-3 buffer (10x), 1µl BSA (10x) and 3µl MQ were added. Finally 0.5µl of SalI and PstI were added to this mixture and incubated for 30 minutes at 37°C. The digestion was analyzed on a 1% Agarose gel.

3. Results

3.1 Identification of novel citrullinated proteins

3.1.1 RA patient material

The prepared synovial fluid materials were put on SDS-PAGE gel and western blot. The western blot was stained with an anti-modified citrulline antibody (anti-Senshu) to see which samples contained citrullinated protein.

From the 33 supernatant fractions and 8 pellet fractions, 13 samples of 9 different patients contained a detectable amount of citrullinated proteins (Figure 4, S7 not shown). Some of the positive samples came from the same RA patients (table 4). Four patients showed citrullinated proteins in their supernatant as well as the pellet fraction of their synovial fluid, although it is weak in two cases. All senshu positive samples were used in subsequent steps in preparation for mass spectrometry analysis.

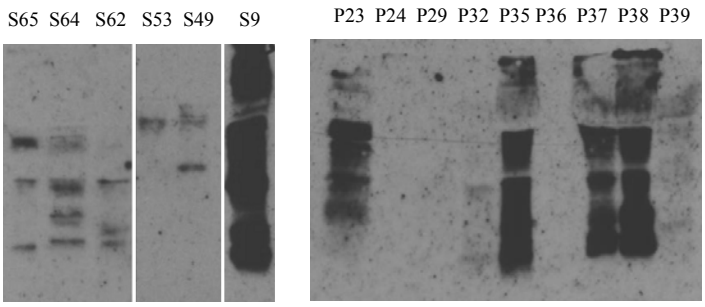

Figure 4. Detection of citrullinated proteins on western blot with an anti-Senshu antibody on supernatant and pellet synovial fluids.

Table 4. Senshu-positive samples per patient.

Fraction	Patient								
supernatant	S7	S9	S49		S53	S62		S64	S65
pellet				P23	P32	P35	P37	P38	P39

3.1.2 Immunoprecipitation

Immunoprecipitation of Senshu-positive samples did not give results. Histidine-tagged scFv RA_3-antibody, coupled to nickel NTA beads should bind citrullinated proteins. In this manner citrullinated proteins should have been separated from non-citrullinated proteins. However, it was observed that proteins could non-specifically bind to the beads themselves when beads without a coupled antibody were incubated with sample. This made proteins eluted from the RA_3-coupled beads non-usable as they still contained non-citrullinated proteins.

3.1.3 Albumin depletion

Testing of albumin depletion protocol on synovial fluid of a non-RA patient.

Colantonio et al [28], who reported the DAC protocol for albumin depletion, used human serum. Synovial fluid has partly the same composition as human serum but may behave different towards the chemicals used in the DAC protocol. For this reason the protocol was first tested on a Senshu positive synovial fluid sample from a non-RA patient.

After albumin depletion the three protein fractions (pellet1, pellet2 and supernatant) as well as a non-depleted input were put on SDS-PAGE gels. One gel was used for western blotting, the

other gel for in-gel digestion and preparation for MALDI-TOF MS. The western blot was incubated with anti-BSA antibody for albumin detection and, after stripping, incubated with anti-Senshu antibody to detect citrullinated proteins.

Figure 5 shows the western blot results and the CBB stained SDS-PAGE gel. It was expected that most of the Albumin would end up in the supernatant fraction. As can be seen on the CBB-gel and the anti-BSA blot the input contains large amount of albumin. After albumin depletion the amount of albumin decreases dramatically in pellet1 but still some is present. The majority of albumin ended up in the supernatant fraction. A reasonable amount of albumin is still left behind in pellet2, but this is still minimal when compared to the input. The anti-Senshu blot shows that the protocol does not interfere with the detection of citrullinated proteins. Most of the proteins precipitate in the first step of the depletion. Hardly any citrullinated proteins were left in pellet2 and none were left in the in the supernatant fraction.

When pellet1 is compared to the input some loss of protein is observed but some bands also appear to be more intense. One band, around the size of albumin, seems to be almost absent in the input and is very clear in pellet1 (arrow in Figure 5). Fibrinogen (alpha chain, 63,5kDa), a known citrullinated protein, has approximately the same size as albumin (67kDa) and could be the protein present in that band.

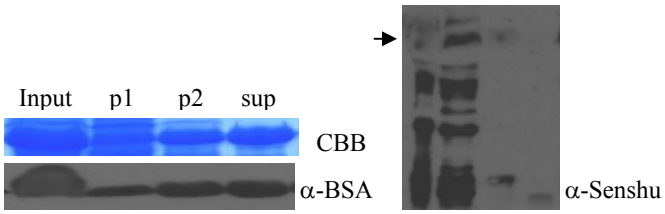

Figure 5. Albumin depleted samples of pellet1(p1), pellet2 (p2) and supernatant (sup) compared to the non-depleted input on western blot (α-BSA, α-Senshu) and CBB-stained SDS-PAGE gel (CBB).

Albumin depletion of synovial fluid samples of RA patients.
Two-hundred µl of the seven Senshu-positive supernatant and the six Senshu-positive pellet synovial fluid samples were used for albumin depletion. This resulted in three protein fractions: pellet1, pellet2 and a supernatant, for each of the 13 samples. With these 39 samples the second precipitation step in the protocol was slightly altered. After adding ethanol to the supernatant left after obtaining pellet1 incubation took place rotating overnight at 4°C instead of one hour at the same conditions.

The 39 albumin-depleted samples were put on two 13% SDS-PAGE gels. One gel was used for colloidal CBB staining, the other gel for western blot. The CBB stained gel was used to cut out protein bands for mass spectrometry analysis. The western blot was incubated with anti-Senshu-antibody to detect citrullinated proteins.

As can be seen in figure 6 the CBB stained gel shows many proteins in the input lanes as well as the albumin depleted fractions pellet1 and pellet2. The lanes containing supernatant fractions of the albumin depletion, which should contain albumin, contain much less protein. The western blot shows the citrullinated proteins present in pellet1 and pellet2 after albumin depletion. Samples S7, S9, P35, P37 and P38 showed a nice band pattern of citrullinated protein. Other samples, which were proven to be Senshu positive, did not show many citrullinated proteins except for large amounts of proteins with a size around 65kD, which could be Fibrinogen.

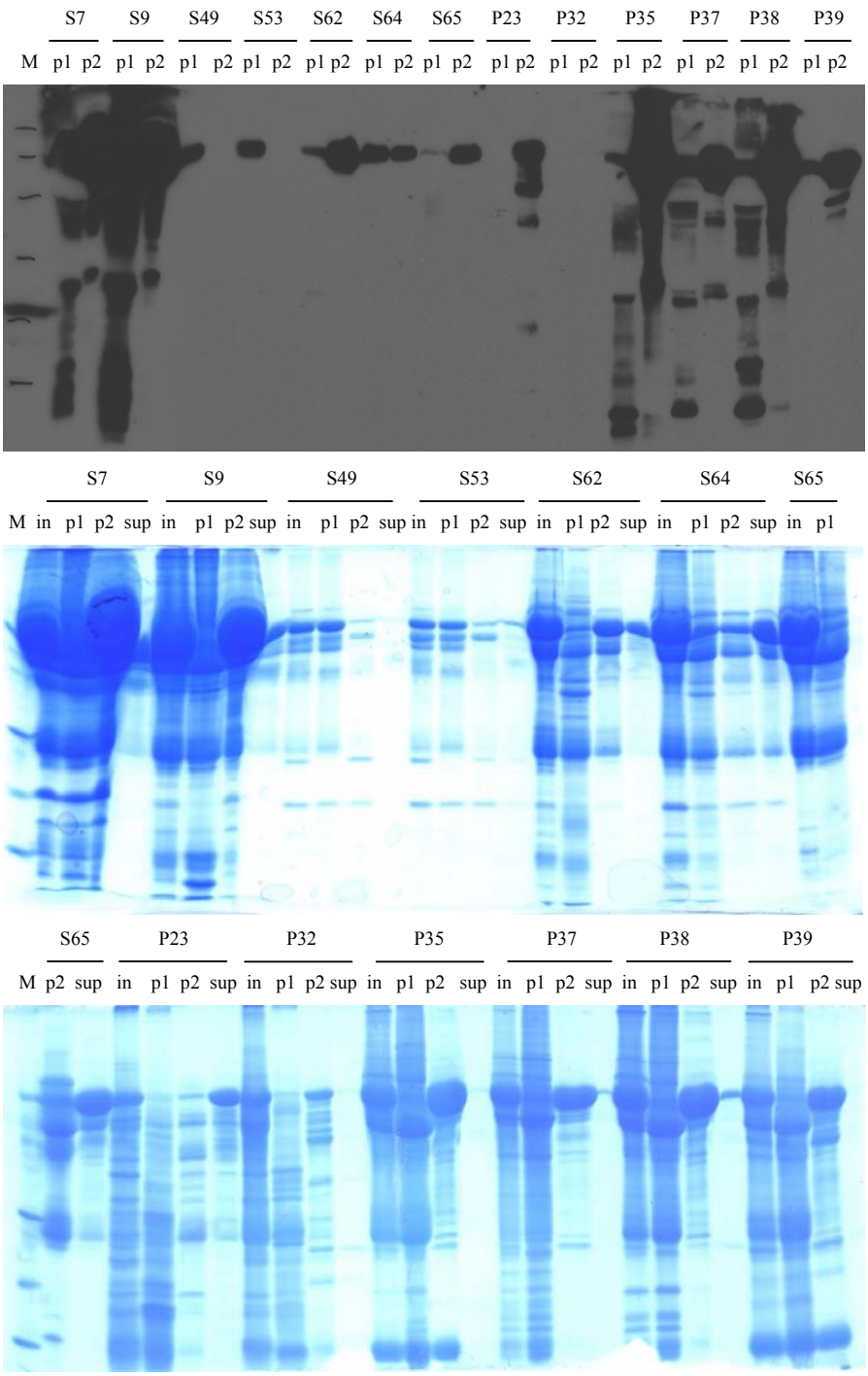

Figure 6. Western blot of Senshu positive albumin-depleted fractions pellet1(p1) and pellet2 (p2). CBB stained SDS-PAGE gels of Senshu positive albumin-depleted fractions p1, p2 and supernatant (sup) as well as a non-depleted input (in).

3.1.4 Mass spectrometry

MALDI-TOF MS

Senshu positive material from a non-RA patient was used to test the albumin depletion protocol. MALDI-TOF MS analysis was used to see how effectively albumin was depleted from the samples. Furthermore it was interesting if proteins could be identified in the pellet1 and pellet2 fractions which could not be found in the non albumin depleted input due to hindrance of albumin.

Excised bands from a CBB stained SDS-PAGE gel (Figure 7) were processed for and analyzed on the Bruker Biflex III MALDI-TOF MS. Mass spectrum data were analyzed with a computer program, which resulted in a list of identified proteins. Scores were given to each identified protein to indicate the significance of the finding.

Input, pellet1, pellet2 and supernatant bands of the same height were compared to each other. Protein lists for each band were searched for Albumin and Fibrinogen. The results of this search are listed in table 5. In bands around 67kDa albumin is found in the input and in all albumin depleted samples. However, in the input and pellet1 albumin was found among other proteins. In pellet2 and supernatant albumin was the dominant compound and few other proteins were found. The albumin found in pellet2 and supernatant also showed a higher significance score than albumin found in the input and pellet1. In bands below 43kDa fibrinogen beta chain was found in the input and pellet1 but the significance score of fibrinogen in pellet1 was much higher.

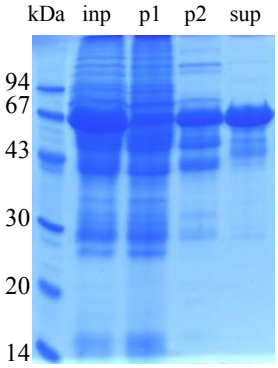

Figure 7. CBB stained SDS-PAGE gel of three protein fraction, pellet1 (p1), pellet2 (p2) and supernatant (sup) of albumin depleted senshu-positive non-RA synovial fluid. As well as an non-depleted input (inp).

Table 5. Result of MALDI-TOF MS analysis of in-gel digested bands from an albumin depleted S2 synovial fluid sample.

Band size (kDa)	input	Pellet1	Pellet2	Supernatant
67 – 94	Mix of Fibrinogen and Albumin	Fibrinogen	Albumin	Albumin
≤ 67	Albumin	Albumin	Albumin	Albumin
> 43	Albumin	Fibrinogen beta chain, fragments	X	X
< 43	Fibrinogen beta chain	Fibrinogen beta chain	X	X

TANDEM MS

TANDEM MS analysis was used to identify proteins containing citrullinated peptides in the albumin depleted samples of Senshu-positive synovial fluid material of RA patients. After comparing the CBB stained SDS-PAGE gel with the albumin-depleted samples to its western blot (Figure 6) it was decided that bands from pellet fraction 1 and 2 from S9 and P38 were

going to be analyzed because they contained the most citrullinated proteins. From the SDS-PAGE gels, which were already cut (Appendix C), bands were selected.

The selected bands (Appendix E) were processed by in-gel digestion with trypsin which resulted in a protein fragment mix from each band. Bands containing citrullined proteins on the same height in pellet1 as well as in pellet2 from one patient were pooled together to minimize the amount of samples.

An overview of proteins found with TANDEM MS analysis were delivered by the FTMS service of the Nijmegen proteomics facility in Mascot Daemon dataset files. All found peptides were listed with its matching protein found with a Mascot database search. All files were searched for proteins which contained citrullinated peptides. In the amino acid sequence of the peptides citrulline was depicted as an arginine (R) but was underlined (R).

Results were found to be significant when the score of the protein was above 50 and the score of the citrullinated protein was above 20 (Figure 8).

gi|4505227 **Mass:** 45807 **Score:** 170 **Queries matched:** 8
myeloid cell nuclear differentiation antigen [Homo sapiens]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
140	376.2188	750.4230	750.4276	-0.0045	0	26	0.21	1	K.ISQLYK.Q
298	424.7458	847.4770	847.4803	-0.0033	0	12	8.9	1	K.VFDINLK.E
523	485.7906	969.5667	969.5719	-0.0052	1	18	2.2	2	K.NLVNNLRK.E
1318	647.8627	1293.7108	1293.7180	-0.0072	0	88	2.3e-07	1	K.SLLAYDLGLTTK.M
1800	818.9299	1635.8453	1635.8580	-0.0127	0	60	0.00029	1	K.INQEEVGLAAPAPTAR.N
2039	940.4952	1878.9758	1878.9799	-0.0040	1	66	6.1e-05	1	K.INQEEVGLAAPAPTARNK.L
2090	970.4389	1938.8632	1938.8707	-0.0075	0	27	0.24	1	K.EASSVSDFNQNFEVPNR.I

Figure 8. Example of a protein from the Mascot search result list. The score of the protein indicates a significant finding. A citrullinated peptide with a significant score is present among the identified peptides for this protein.

The citrulline in the peptide was found to be significant when the majority of the b and y ions were indicated in bold red (Figure 9) in the query list of the peptide. This means that these amino acids were truly identified in the TANDEM analysis. Furthermore, the mass difference between citrulline and its neighboring amino acids should be 157 (m/z), indicated in the boxes in figure 9. Citrulline and arginine differ 1Da in mass. When an arginine would be present in the peptide a mass difference of 156 should have been observed. The mass of the protein should also correspond to the expected mass of the band in which the protein is found. Proteins of greater mass could be found in bands of lower mass due to fragmentation of the protein, small protein however can not be present in bands of higher expected mass.

Each sample lead to the identification of many proteins, up to 800 in some cases. The majority of the found proteins were immunoglobulin, varying from heavy- and light-chains to immunoglobulin fragments. None of the immunoglobulins, with one exception, were found to contain citrullinated peptides with significant scores. Albumin was also detected in some samples but did not make up the majority of the protein list as immunoglobulins. None of the albumin peptides contained a citrulline. Citrullinated proteins only made up a very small portion of all the identified proteins on the list. In total 57 proteins containing citrullinated peptides were found. Because some of these proteins belong to the same family there are 38 unique proteins. All identified proteins with citrullinated peptides that were found in S9 and P38 are listed in tables 6-9.

b	Seq.	y
114.0913	I	
228.1343	N	1766.9031
356.1928	Q	1652.8602
485.2354	E	1524.8016
614.2780	E	1395.7590
713.3464	V	1266.7164
770.3679	G	1167.6480
883.4520	L	1110.6266
954.4891	A	997.5425
1025.5262	A	926.5054
1122.5789	P	855.4683
1193.6160	A	758.4155
1290.6688	P	687.3784
1391.7165	T	590.3257
1462.7536	A	489.2780
1619.8387	R	418.2409
1733.8817	N	261.1557
	K	147.1128

Figure 9. Individual amino acids of a peptide are identified by the mass of their b and y ions. Bold red ions indicate that the identification of the amino acid is significant.

Table 6 lists all the proteins that contain citrullinated peptides in both S9 and P38. Table 7 lists all proteins that contain citrullinated proteins in S9 and which are present in its non-citrullinated form in P38. The same is true for P38 where citrullinated proteins were found that are present in S9 in the non-citrullinated form. Table 8 and 9 list all the citrullinated proteins that were only found in respectively S9 and P38.

Table 6. Citrullinated proteins identified with TANDEM MS in both S9 and P38 samples.

Protein	MW (kDa)	Size (aa)	Number of citrullinated peptides	Number of citrullines	Accession number
Fibronectin 1 isoform 5 protein	250	2296	5	6	gi | 47132553 |
Fibronectin precursor	256,5	2328	2	3	gi | 31397 |
Inter-alpha-trypsin inhibitor heavy chain H2 precursor	106	946	2	2	gi | 125000 |
Fibrinogen alpha chain preproprotein, isoform alpha	70,1	647	2	2	gi | 13591823 |
Fibrinogen alpha	49,3	462	1	1	gi | 223918 |
Alpha-fibrinogen precursor	69,8	644	8	7	gi | 182424 |
Beta Actin	41,7	375	10	10	gi | 4501885 |
Myeloid cell nuclear differentiation antigen	45,8	407	3	3	gi | 4505227 |
Apolipoprotein E	36,1	317	3	3	gi | 178853 |

Table 7. Citrullinated proteins found in S9 that are also present in their non-citrullinated form in P38, and vice versa.

Protein	MW (kDa)	Size (aa)	Number of citrullinated peptides	Number of citrullines	Accession number
S9					
Alpha-2-macroglobulin precursor	136	1474	2	2	gi | 112911 |
Apolipoprotein B-100 precursor	515	4563	1	1	gi | 178730 |
Complement component 3 precursor	187	1633	1	1	gi | 115298678 |
Vinculin	116,7	1066	1	1	gi | 24657579 |
IGHM protein	67,2	590	2	2	gi | 41388180 |
Pyruvate kinase	57,8	531	1	1	gi | 35505 |
Kininogen 1	47,9	427	1	1	gi | 4504893 |
Glyceraldehyde-3-phosphate dehydrogenase	36	335	2	2	gi | 7669492 |
Complement factor B	85,5	764	1	1	gi | 291922 |
Chain A, crystal structure of lipid free apolipoprotein A-I	28	243	4	3	gi | 90108664 |
Serum amyloid A4, constitutive	14,8	130	1	1	gi | 10835095 |
H3 Histone family, member T	15,5	136	1	1	gi | 4504299 |
H2A Histone family, member A	14,1	130	1	1	gi | 10645195 |
H2A Histone family, member L	14,1	130	2	2	gi | 4504245 |
H2A Histone family, member C	14,1	130	3	3	gi | 4504239 |
Histone 2A.2	13,9	126	3	3	gi | 31979 |
Beta fibrinogen precursor	54,7	483	1	1	gi |182430 |
P38					
Keratin, type II cytoskeletal 1	67	644	1	1	gi | 1346343 |
Keratin 9	62	623	1	1	gi | 55956899 |
Vimentin	53,7	466	4	4	gi | 62414289 |
Complement component C4A	193	1744	1	1	gi | 179674 |
Ribosomal protein S16	16,4	146	1	1	gi | 4506691 |
Chain A. Antithrombin Iii	49	432	2	2	gi | 999513 |

Table 8. Citrullinated proteins identified in S9.

Protein	MW (kDa)	Size (aa)	Number of citrullinated peptides	Number of citrullines	Accession number
Factor H	139	1231	2	2	gi | 31965 |
Megakaryocyte stimulating factor	151	1404	2	2	gi | 1572721 |
Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein), isoform CRA_b	101	914	3	4	gi | 119585669 |
MYC binding protein 2, isoform CRA_c	509,4	4637	1	1	gi | 119600972 |
Hexokinase 3	99	923	1	1	gi | 4504395 |
Periostin, osteoblast specific factor, isoform CRA_b	87	781	1	1	gi | 19628997 |
similar to Prostate, ovary, testis expressed protein on chromosome 2 isoform 2	121,4	1038	3	3	gi | 113413200 |
Apolipoprotein A-IV precursor	43,4	376	1	1	gi | 178779 |
Apolipoprotein L-I	42,4	383	1	1	gi | 12232634 |
Alpha 1 actin precursor	42	377	1	3	gi | 4501881 |
Chain H, Alpha-Thrombin Complex with hirulog 3 (prothrombin, coagulation factor II)	29,7	258	2	3	gi | 493792 |
Chain A, Human Cathepsin G	25,4	224	2	4	gi | 2392230 |
Glia maturation factor, gamma	16,8	142	1	1	gi | 4758440 |

Chain B, crystal structure of a Rac-Rhogdi Complex	20,5	180	1	1	gi | 9955206 |
Beta galactoside binding lectin precursor	14,7	135	1	1	gi | 4504981 |
Megakaryocyte stimulating factor; MSF	151	1404	1	1	gi | 1572721 |

Table 9. Citrullinated proteins identified in P38.

Protein	MW (kDa)	Size (aa)	Number of citrullinated peptides	Number of citrullines	Accession number
Myosin, heavy polypeptide 9, non-muscle	226,4	1960	1	1	gi | 12667788 |
Inter-alpha-trypsin inhibitor family heavy chain-related protein	103,3	960	2	2	gi | 4096840 |
Myeloperoxidase	83,8	745	1	1	gi | 34719 |
Coronin, actin binding protein, 1A	51	461	1	1	gi | 5902134 |
Gelsolin isoform a precursor	85,6	782	1	1	gi | 4504165 |
Chain A, Human Adp-Ribosylation Factor 1 Complexed With Gdp	20,6	180	1	1	gi | 1065361 |
Chain A, Cyclophilin A Complexed With Dipeptide Gly-Pro	17,9	164	1	1	gi | 1633054 |
Histone cluster 1, H1d	22,3	221	1	1	gi | 4885377 |

3.2 Antigenicity of new citrullinated proteins

To verify if the citrullinated protein eIF3S10 has antigenic properties in RA the protein was to be cloned and screened with RA sera .However the cloning of eIF3S10 was not successful and no recombinant protein was obtained. A part of eIF3S10 DNA was obtained via PCR-amplification and successfully cloned into the pCR-4TOPO vector. The PCR product was also send for sequencing. The outcome of the sequencing confirmed it to be a part of eIF3S10. The eIF3S10 product was cut from the pCR4-TOPO vector by restriction enzyme digestion to be ligated into the pGEX4T2 expression vector. This ligation could not be established after several attempts and adaptations of the protocol.

4. Discussion

4.1 Identification of novel citrullinated proteins

4.1.1 Immunoprecipitation

Pulling down of citrullinated proteins with scFv RA_3-antibody from the synovial fluid samples by immunoprecipitation did not work. Proteins were binding non-specifically to the NTA-Nickel agarose beads. This means that besides citrullinated proteins bound to the antibody also non citrullinated proteins bound to the beads were eluted from the beads. The immunoprecipitation protocol was adapted in an attempt to solve the problem of non-specific binding of proteins to the beads. Five adaptations of the protocol were tried.

- Different combinations of salt concentration of the IPP wash buffers were used. More stringent conditions could remove non-specific interactions. However, no difference in non-specific binding was seen.
- Imidazole was added to the IPP wash buffer after coupling of RA_3 to the beads. Imidazole should reduce non-specific binding. No non-specific binding could be seen on empty beads. No protein could be pulled down with the RA_3-coupled beads.
- Beads were incubated with the samples in IPP buffer containing 5%Elk. Elk should block all space on the beads not containing the RA_3-antibody and thus prevent non-specific binding. No non-specific binding could be seen on empty beads, but no protein could be pulled down with RA_3-coupled beads, meaning that Elk also blocked the antibody.
- Samples and scFvRA_3-antibody were dialyzed against the IPP500 wash buffer to bring the samples in the same condition.
- Protein A agarose beads instead of Nickel NTA beads were used. Protein A beads are known to give less non-specific binding than Nickel beads. The protein A beads were coupled to total RA_3 antibody (scFVRA_3 is His-tagged and only suitable for nickel beads). However, no non-specific binding could be seen on empty beads. Antibody coupled beads pulled down minimal protein and no citrullinated proteins were observed.

None of the adaptations to the protocol were successful. Non-specific binding of protein to the beads was still present or no citrullinated proteins could be pulled down at all.

Cell lysate containing citrullinated proteins was sometimes used as a control for the procedure. With cell lysate no or very little non-specific binding could be seen compared to synovial fluid. This indicates that synovial fluid, which has a complex composition, may influence protein binding.

4.1.2 Albumin depletion

Albumin depletion was performed to clear synovial fluid patient material of the highly abundant protein albumin. The question was two-fold. First, could albumin depletion clear the samples from albumin? Second, could other proteins be visualized by reducing the amount of albumin in the sample? Albumin might mask other proteins on the same height on gels and blots.

Figure 5 and 6 show that the amount of albumin decreases after depletion. That some albumin is still left behind can be because it is impossible to remove albumin for 100%. Inaccurate handling when the supernatant is separated from the pellets can also be a cause of albumin residues in an albumin-depleted sample.

The western blot of the non-RA albumin depleted synovial fluid shows that depletion does not have a negative effect on detection of citrullinated proteins. It seems that there is some loss of protein, but on the other hand some bands seem to be stronger.

4.1.3 Mass spectrometry

MALDI-TOF MS
The senshu-positive non-RA albumin depleted samples were analyzed by MALDI-TOF MS to see if albumin depletion had an effect on the detection of proteins. It was expected that albumin could be identified in all albumin-depleted protein fractions (α-BSA blot, figure 5). For pellet 1 it was expected that more other proteins could be identified because albumin is less dominant than in the non-depleted input. Fibrinogen could appear instead of albumin. For the supernatant fraction it was expected that albumin is the major component. Hardly any other proteins should present because they all precipitated in pellet1 and pellet2.

The results show a slight difference between the input and pellet1. Fibrinogen and albumin were present in both samples. However, in pellet1 fibrinogen was identified more often, and the significance scores were higher. Albumin on the other hand was less often identified and the significance was lower. This indicates that albumin depletion had an influence on the detection of other proteins as detection of fibrinogen became more significant when fewer albumin was present.

The result from the albumin depletion and the mass spectrometry analysis combined show that removal of albumin might give a better result when one wants to identify less abundant proteins. However, later it was found out that the laser of the MALDI-TOF apparatus did not function properly. This mend that small and less abundant proteins were not registered in the mass spectrum. The comparison of depleted and non-depleted sample and the difference in proteins detected is thus only based on albumin and fibrinogen.

TANDEM MS
S9 and P38 senshu positive albumin depleted samples were analyzed by TANDEM MS to identify new citrullinated proteins. Five of the fifty-five found proteins are known citrullinated protein in RA. Fibrinogen and Fibronectin were found in both S9 and P38. Histones were found in both S9 and P38 but citrullinated peptides were only detected in S9 samples. The same is true for antithrombin and vimentin but citrullinated peptides were only found in P38.

Two other proteins were found that where identified before in studies looking for new citrullinated proteins. Ten citrullinated peptides of beta actin were found in both S9 and P38 covering 48% of the total protein. Matsuo et al [21] reported this protein but only identified 2 peptides. Choong et al [31] reported a citrullinated Apolipoprotein A-I precursor which matches in amino acid sequence with Chain A, crystal structure of lipid free apolipoprotein A-I found citrullinated in S9 and present in P38. Therefore it can be worthwhile to test if these proteins are possible candidate auto-antigens in RA. For all other proteins identified it is difficult to say if these proteins are interesting for further research. In theory every citrulline and it neighboring amino acids can form an epitope. However, the better antigens often contain more citrullines and therefore more epitopes [2]. Therefore proteins were selected based the amount of identified citrulline residues. This resulted in eight interesting proteins (amino acid sequence in appendix F).

- Beta-Actin together with alpha 1 actin precursor, which has approximately the same amino acid sequence.
- The different apolipoproteins. Besides Chain A crystal structure of lipid free apolipoprotein A-I, Apolipoproteins E also contains more than one citrulline.
- Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
- Chain H, Alpha-Thrombin Complex with hirulog 3.
- Myeloid cell nuclear differentiation antigen (MNDA).
- Chain A, human cathepsin G.

This selection can also be justified by information from the literature. As named before actin and apolipoproteins were identified before. Their identification in another RA-patient population makes it more likely that they are citrullinated proteins specific for RA.

The other proteins are mainly interesting because of their function and location in inflammatory processes. For GAPDH is reported that accumulation in the nucleus is involved in the induction of apoptosis [32]. MNDA is a marker for monocytes and macrophages in chronic inflammation [33]. It is also reported that it binds to nucleophosmin, a known citrullinated protein in RA [34]. Cathepsin, a chemotractant for monocytes, is expressed by synovial lining cells [35]. Cathepsin G already has a connection with RA as it is a target for another type of auto-antibodies in RA, the anti-neutrophil cytoplasmic antibodies [36]. For alpha-thrombin it is reported that it may have an influence on inflammation and RA disease progression [37].

The results of the TANDEM analysis can not be backed up by results of a negative control. In an ideal situation synovial fluid of a healthy person should have been analyzed by TANDEM MS alongside the RA patient material. In this way naturally occurring citrullinated proteins could have been distinguished from citrullinated proteins specific for RA. However, synovial fluids of healthy persons are hard to come by.

4.1.4 General remarks on techniques used for citrullinated protein identification

The majority of the proteins identified with TANDEM MS in the S9 and P38 samples were not citrullinated. Most of these non-citrullinated proteins were immunoglobulins. Immunoglobulins are just as albumin highly abundant. Because immunoprecipitation did not work well for synovial fluid it was decided to remove albumin from the synovial fluid. Immunoglobulins, which can be easily depleted by incubating synovial fluid with protein G agarose beads, were not removed because of several reasons. In the first attempt to remove immunoglobulins from a synovial fluid sample no difference was observed between treated and non-treated sample. It seemed like no immunoglobulins were depleted. Furthermore citrullinated proteins can form an immune complex with their antibodies. It is therefore be possible that together with the immunoglobulin, a citrullinated protein is removed from the sample. With earlier experience from the immunoprecipitation and the non-specific binding of proteins to beads, it could be possible that citrullinated proteins could be lost in that manner.

To increase identification of citrullinated proteins with TANDEM MS in synovial fluid it is better to deplete samples of most non-citrullinated proteins. Immunoprecipitation is the best technique to accomplish this. Already many attempts have been made to immunoprecipitate synovial fluids, without success. But it may be worthwhile to make further attempts to pull down citrullinated proteins. The use of different beads and buffers are options to consider.

4.2 Antigenicity of new citrullinated proteins

Recombinant eIF3S10 was made to test if this protein is a possible candidate auto-antigen in RA. Cloning of a part of the protein coding region of eIF3S10 was successful. But ligation of the eIF3S10 product into an expression vector could not be established. No clear reason could be found why ligation was not successful. Insert and vector were both purified from gel with the Qiagen gel extraction kit which should result in clean samples without impurities that could affect the ligation process. Altering ratios between vector and insert resulted in more or in fewer colonies on the plate, but none of the colonies carried the ligated vector. Changing the incubation temperature from 16°C to room temperature also did not led to a successful ligation. Many more alterations could have been made in order to let the ligation succeed but because of time limitations this part of the project had to be abandoned.

5. Conclusions

The objectives of this project were to identify novel citrullinated proteins in synovial fluid of RA patients and to verify if newly found citrullinated proteins are auto-antigens in RA.

With TANDEM mass spectrometry fifty seven citrullinated proteins have been identified in synovial fluids of two RA-patients. Five of the identified citrullinated proteins (fibrinogen, fibronectin, antithrombin, vimentin and histones) were proteins known to be citrullinated in RA. Eight of identified citrullinated proteins were found to contain multiple citrullines (beta-actin, alpha actin 1 precursor, apolipoprotein E, Chain A crystal structure of lipid free apolipoprotein A-I, cathepsin G, Chain H Alpha-Thrombin Complex with hirulog 3, MNDA and GAPDH). These proteins display a high coverage of citrullinated peptides relative to their size. Literature points out that these proteins may have functions in RA/ inflammatory related processes. These features combined mark them as interesting proteins to verify them as candidate auto-antigens in RA. The verification of these proteins as auto-antigens remains for further investigation.

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Appendices

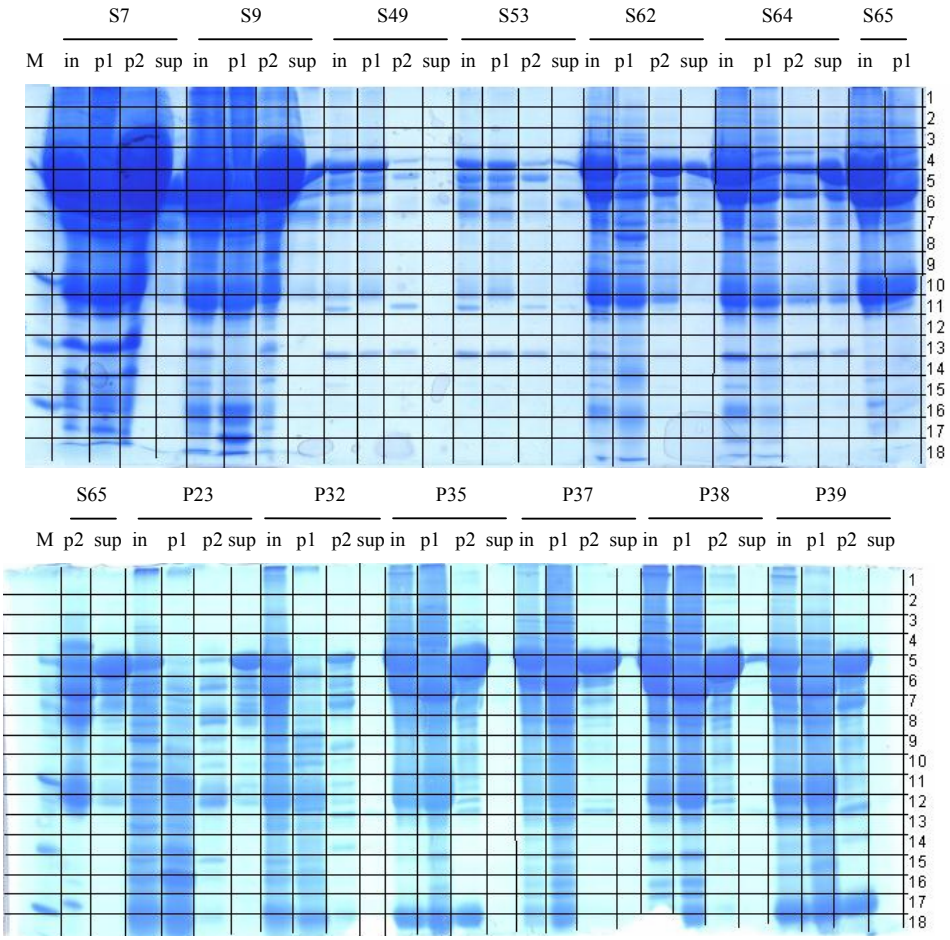
A. RA patient synovial fluids

	Patient										
Supernatant Pellet	S7	S8 P37	S9	S10	S11	S12	S13	S15	S21	S22	S24
Supernatant Pellet	S25	S26 P23	S28	S29 P24	S34	S35	S36	S38	S42	S43	S46
Supernatant Pellet	S47	S49	S53 P32	S58	S59	S60	S61	S62 P35	S64 P38	S65 P39	S66 P36

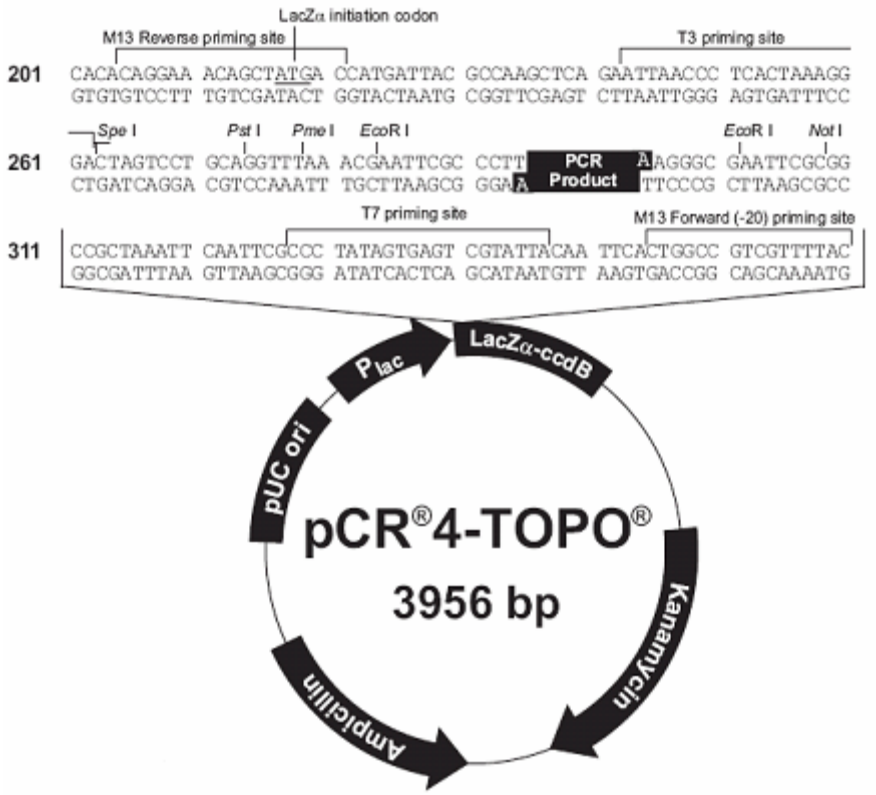
B. SDS-PAGE: Running- and Stacking-gel

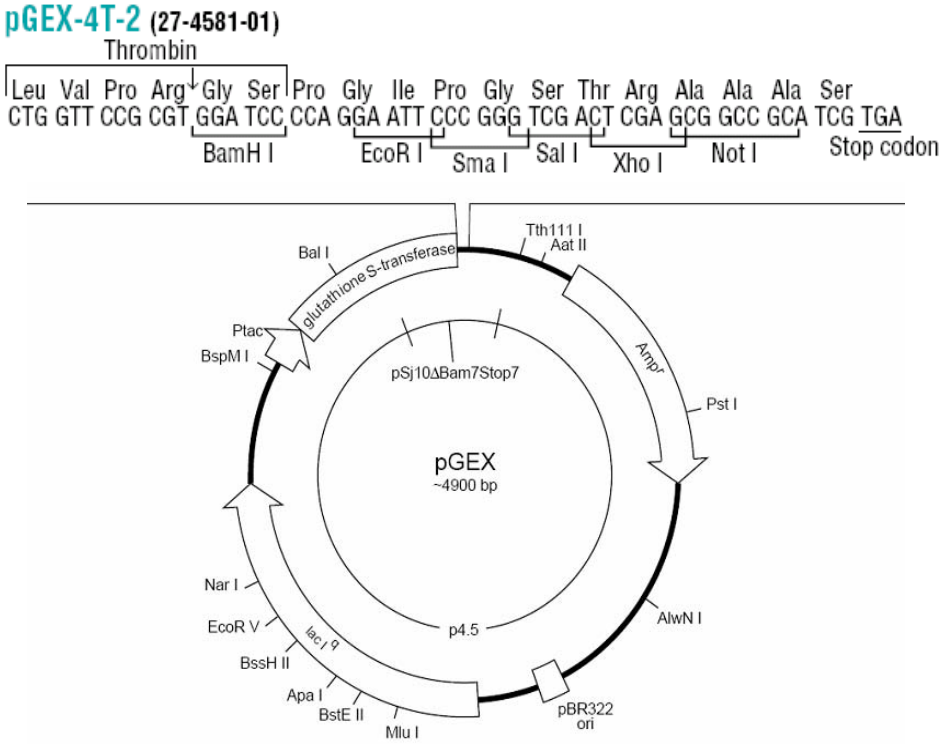
13% SDS-PAGE gel	Small (Biorad system)		Large (Horstmann gel system)	
	Running gel	Stacking gel	Running gel	Stacking gel
MilliQ	2,12 ml		5,3 ml	
1.5M Tris/HCl pH 8.8	3,33 ml		8,325 ml	
30% AA-BAA	4,33 ml		10,825 ml	
10% SDS	100 µl		250 µl	
10% APS	100 µl	25 µl	250 µl	62,5 µl
TEMED	10 µl	5 µl	25 µl	12,5 µl
Stacking Mix		2,5 ml		6,250 ml

C. SDS-PAGE of Albumin depleted samples; grid pattern



D. Vectors


LacZα initiation codon
M13 Reverse priming site
T3 priming site
201
CACACAGGAA ACAGCTATGA CCATGATTAC GCCAAGCTCA GAATTAACCC TCACTAAAGG
GTGTGTCCTT TGTCGATACT GGTACTAATG CGGTTCGAGT CTTAATTGGG AGTGATTTCC
Spe I
Pst I
Pme I
EcoR I
EcoR I
Not I
261
GACTAGTCCT GCAGGTTTAA ACGAATTCGC CCTT
PCR Product
AAGGGC GAATTCGCGG
CTGATCAGGA CGTCCAAATT TGCTTAAGCG GGAA
TTCCCG CTTAAGCGCC
T7 priming site
M13 Forward (-20) priming site
311
CCGCTAAATT CAATTCGCCC TATAGTGAGT CGTATTACAA TTCACTGGCC GTCGTTTTAC
GGCGATTTAA GTTAAGCGGG ATATCACTCA GCATAATGTT AAGTGACCGG CAGCAAAATG
P lac
LacZα-ccdB
pUC ori
pCR®4-TOPO®
3956 bp
Kanamycin
Ampicillin


pGEX-4T-2 (27-4581-01)
Thrombin
Leu Val Pro Arg Gly Ser Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
CTG GTT CCG CGT GGA TCC CCA GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG TGA
BamH I
EcoR I
Sma I
Sal I
Xho I
Not I
Stop codon
Bal I
glutathione S-transferase
Tth111 I
Aat II
Ptac
BspM I
pSj10ΔBam7Stop7
Amp^r
Pst I
pGEX
~4900 bp
Nar I
EcoR V
BssH II
Apa I
BstE II
Mlu I
lac I q
p4.5
AlwN I
pBR322 ori

E. Selected bands from S9 and P38 samples for mass spectrometry analysis.

Bands		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
S9	P1	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x
	P2				x	x	x	x	x	x	x	x		x	x	x			
P38	P1			x	x	x	x	x	x	x	x		x	x			x	x	x
	P2			x	x	x	x	x	x	x	x		x	x		x			x

F. Interesting citrullinated protein identified by TANDEM mass spectrometry analysis.

Beta Actin

```
LOCUS       NP_001092                 375 aa            linear
  1 mdddiaalvv dngsgmckag fagddaprav fpsivgprph qgvmvgmgqk dsyvgdeaqs
 61 krgiltlkyp iehgivtnwd dmekiwhhtf ynelrvapee hpvllteapl npkanrekmt
121 qimfetfntp amyvaiqavl slyasgrttg ivmdsgdgvt htvpiyegya lphailrldl
181 agrdltdylm kiltergysf tttaereivr dikeklcyva ldfeqemata asssskeksy
241 elpdgqviti gnerfrcpea lfqpsflgme scgihettfn simkcdvdir kdlyantvls
301 ggttmypgia drmqkeital apstmkikii apperkysvw iggsilasls tfqqmwiskq
361 eydesgpsiv hrkcf
```

Alpha 1 actin precursor

```
LOCUS       NP_001091                 377 aa            linear
  1 mcdedettal vcdngsglvk agfagddapr avfpsivgrp rhqgvmvgmg qkdsyvgdea
 61 qskrgiltlk ypiehgiitn wddmekiwhh tfynelrvap eehptlltea plnpkanrek
121 mtqimfetfn vpamyvaiqa vlslyasgrt tgivldsgdg vthnvpiyeg yalphaimrl
181 dlagrdltdy lmkiltergy sfvttaerei vrdikeklcy valdfenema taasssslek
241 syelpdgqvi tignerfrcp etlfqpsfig mesagihett ynsimkcdid irkdlyannv
301 msggttmypg iadrmqkeit alapstmkik iiapperkys vwiggsilas lstfqqmwit
361 kqeydeagps ivhrkcf
```

Chain A, crystal structure of lipid free apolipoprotein A-I

```
LOCUS       2A01_A                    243 aa            linear
  1 deppqspwdr vkdlatvyvd vlkdsgrdyv sqfegsalgk qlnlklldnw dsvtstfskl
 61 reqlgpvtqe fwdnlekete glrqemskdl eevkakvqpy lddfqkkwqe emelyrqkve
121 plraelqega rqklhelqek lsplgeemrd rarahvdalr thlapysdel rqrlaarlea
181 lkenggarla eyhakatehl stlsekakpa ledlrqgllp vlesfkvsfl saleeytkkl
241 ntq
```

Apolipoprotein E

```
LOCUS       AAB59397                  317 aa            linear
  1 mkvlwaallv tflagcqakv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt
 61 lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqaaqa
121 rlgadmedvr grlvqyrgev qamlgqstee lrvrlashlr klrkrllrda ddlqkrlavy
181 qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg
241 srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek
301 vqaavgtsaa pvpsdnh
```

Glyceraldehyde-3-phosphate dehydrogenase

```
LOCUS       NP_002037                 335 aa            linear
  1 mgkvkvgvng fgrigrlvtr aafnsgkvdi vaindpfidl nymvymfqyd sthgkfhgtv
 61 kaengklvin gnpitifqer dpskikwgda gaeyvvestg vfttmekaga hlqggakrvi
121 isapsadapm fvmgvnheky dnslkiisna scttnclapl akvihdnfgi veglmttvha
181 itatqktvdg psgklwrdgr galqniipas tgaakavgkv ipelngkltg mafrvptanv
241 svvdltcrle kpakyddikk vvkqasegpl kgilgytehq vvssdfnsdt hsstfdagag
301 ialndhfvkl iswydnefgy snrvvdlmah maske
```

Chain H, Alpha-Thrombin Complex with hirulog 3

LOCUS 1ABI_H 259 aa linear

```
  1 ivegsdaeig mspwqvmlfr kspqellcga slisdrwvlt aahcllyppw dknftendll
 61 vrigkhsrtr yernieksm lekiyihpry nwrenldrdi almklkkpva fsdyihpvcl
121 pdretaasll qagykgrvtg wgnlketwta nvgkgqpsvl qvvnlpiver pvckdstrir
181 itdnmfcagy kpdegkrgda cegdsggpfv mkspfnnrwy qmgivswgeg cdrdgkygfy
241 thvfrlkkwi qkvidqfge
```

Myeloid cell nuclear differentiation antigen

LOCUS NP_002423 407 aa linear

```
  1 mvneykkill lkgfelmddy hftsikslla ydlglttkmq eeynrikitd lmekkfqgva
 61 cldklielak dmpslknlvn nlrkekskva kkiktqekap vkkinqeevg laapaptarn
121 kltseargri pvaqkrktpn kekteakrnk vsqeqskppg psgastsaav dhpplpqtss
181 stpsntsftp nqetqaqrqv darnvpqnd pvtvvvlkat apfkyespen gkstmfhatv
241 asktqyfhvk vfdinlkekf vrkkvitisd yseckgvmei keassvsdfn qnfevpnrii
301 eianktpkis qlykqasgtm vyglfmlqkk svhkkntiye iqdntgsmdv vgsgkwhnik
361 cekgdklrlf clqlrtvdrk lklvcgshsf ikvikakknk egpmnvn
```

Chain A, Human Cathepsin G

1CGH_A 224 aa linear

```
  1 xiggresrph srpymaylqi qspagqsrcg gflvredfvl taahcwgsni nvtlgahniq
 61 rrentqqhit arrairhpqy nqrtiqndim llqlsrrvrr nrnvnpvalp raqeglrpgt
121 lctvagwgrv smrrgtdtlr evqlrvqrdr qclrifgsyd prrqicvgdr rerkaafkgd
181 sggpllcnnv ahgivsygks sgvppevftr vssflpwirt tmrs
```