A proteomic investigation of inflammatory status before and after treatment of osteoarthritis with gold microparticles, and correlation to rheumatoid arthritis

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Preface

Thanks to my supervisors Allan Stensballe, Lars Arenth-Nielsen, and Sten Rasmussen for giving guidance in the making of this project. A special thanks to my co-workers for making the many hours spent in the laboratory educational and interesting. Thanks for encouraging me and bringing great guidance patiently. Thanks to Aalborg University for providing the resources, facilities, and the opportunities to develop my skills in the laboratory through the making of this project.

List of abbreviations

ACPAs – Anticitrullinated protein antibodies

APC – Antigen presenting cell

bDMARD – Biological disease modifying anti rheumatic drugs

COX – Cyclooxygenase

DAMPs – Damage-associated molecular patterns

DMARDs – Disease modifying anti rheumatic drugs

ETN – Etanercept

FLS – Fibroblast-like synoviocyte

HLA – Human leukocyte antigen

IkB – Inhibitory molecule called inhibitor of NF-kB

IKK – IkB kinase

ILs – Interleukines

LtxA – Leucotxin A

LZ – Leuzine zipper

MMPs – Matrix metalloproteins

MTX – Methotrexate

NF-kB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NLS – Nuclear localization site

NSAIDs – Non-steroidal anti-inflammatory drugs

OA – Osteoarthritis

PAD – Peptidyl-arginine deaminase

PGs – Prostaglandins

PTM – Post translational modification

RA – Rheumatoid arthritis

RANK – Receptor activator of nuclear factor kappa-B

RANKL – Receptor activator of nuclear factor kappa-B ligand

RF – Rheumatoid factor

RHD – Rel homology domain

ROS – Reactive oxygen species

SRPs – Small leucine-rich repeat proteoglycans

TD – Transactivation domain

TLRs – Toll like receptors

TNF – Tumor necrosis factor

TRX – Thioredoxin

Abstract

Introduction: Arthritis is a broad clinical term that includes many different joint diseases of different etiology. The most common type is osteoarthritis, which is treated with NSAIDs and glucocorticoids to manage pain and dampen inflammation. However, most current treatment options are systemic with potential systemic adverse effects. A new possible treatment to overcome this is intra articular gold microparticle implants which is thought to inhibit articular inflammation in several ways, sch as inhibition of the NF-kB pathway. The most common autoimmune type of arthritis is rheumatoid arthritis, and despite a different pathogenesis than OA, inflammation also plays a major role in in RA, and the main goal of treatment is also here to decrease the inflammatory status and manage pain. Like in OA, NSAIDs and glucocorticoids are used in RA treatment along with DMARDs and bDMARDs which all have potential systemic adverse effects as well.

Aim: The aims of this study are to elucidate the supposed mode of action and anti-inflammatory potential of gold microparticles as a treatment of OA. Proteomic profiling of synovial fluid and serum samples before and after treatment were performed to investigate this novel treatment's impact on inflammation. Another aim is to search for biomarkers in gold treated patients to monitor treatment effect, and to enable the possibility for a personalized treatment. Furthermore, serum samples before and after RA treatment with etanercept will also be analyzed to look for similarities in inflammatory status that could suggest a possible effect of gold particles in the treatment of RA.

Methods: Paired biological samples from 29 OA patients were collected pre- and 8 weeks post treatment with gold particles. These were analyzed using quantitative proteomics, innate immune regulatory cfDNA-measurement, and cytokine profiling to evaluate inflammatory status before and after treatment. Paired biological samples from 18 RA patients before and after treatment with etanercept were also analyzed with proteomics and cfDNA-measurement as well and correlated to the results of OA gold treatment.

Results: 24 immunologically associated molecules identified by PEA were significantly downregulated following gold treatment of OA. Several of these have direct correlations to both OA, RA, inflammation, and the NF-kB pathway. MS found 22 proteins to be significantly regulated in OA, several of which can be correlated with OA, RA, inflammation, and NF-kB as well. cfDNA did not show a significant drop following eight weeks of OA gold treatment, but other studies and observations indicate that 8 weeks might not be enough time to elicit the full effect of a gold treatment.

Conclusions: The clinically observed effects of an OA gold treatment found basis in the observed proteomic changes identified in this study, in respect to both decreased inflammatory markers, modulation of NF-kB and connection to OA pathology. These effects coupled with the proteomic data and knowledge of an RA pathogenesis, opens the possibility of positive effects by gold microparticle treatment of RA as well.

Keywords: Osteoarthritis, rheumatoid arthritis, inflammation, gold microparticles, mass spectrometry, proteomics

Resume

Introduktion: Artrose er en bred klinisk betegnelse der inkluderer mange forskellige sygdomme med forskellig etologi. Den mest almindelige type er osteoartrose, sm behandles med NSAIDer og glukkokortikoider for at kontrollerer smerte og mindske inflammation. Men de fleste behandlingsmuligheder er systemiske med mulighed for svære systemiske bivirkninger. En ny mulig behandlingsform i stand til at undgå det er intraartikulære guld mikropartikel implantater, som formodes at inhiberer artikulær inflammation på flere måder, som inhibering af NF-kB signaleringsvejen. Den mest almindelige autoimmune type af artrose er rheumatoid artrit, men trods en forskellig patogenese fra OA spiler inflammation også er stor rolle i RA, og hovedformålet med behandlingen er også her at mindske den inflammatoriske status og kontrollere smerte. Som ved OA blive både NSAIDer og glukkokortikoider også brugt i behandlingen af RA, sammen med DMARDs og bDMARDs som alle har potentielle systemiske bivirkninger.

Formål: Formålet med dette studie er ar klarlægge den formodede virkningsmekanisme og antiinflammatoriske potentiale af guld mikropartikler som en behandling af OA. Proteomisk profilering af synovialvæske og serumprøver før og efter behandling blev udført for at undersøge denne nye behandlings effekt på inflammation. Et andet formål er at lede efter biomarkører i guldbehandlede patienter for at monitorere behandlingens effekt, for at muliggøre en personaliseret tilgang til guld behandling. Derudover er serumprøver, før og efter behandling af RA med etanercept, også analyseret for at lede efter fælles træk i inflammatorisk status der kunne foreslå en mulig positiv effekt af guldpartikler i behandlingen af RA.

Metode: Parrede biologiske prøver fra 29 OA-patienter blev indsamlet før- og 8 uger efter behandling med guldpartikler. Disse blev analyseret med kvantitativ proteomics, Innate immunregulatoriske cfDNA-målinger, og cytokinprofilering for at evaluerer inflammatorisk status før og efter behandling. Parrede biologiske prøver fra 18 RA-patienter før og efter behandling med etanercept blev også analyseret med proteomics og cfDNA-målinger og sammenlignet med resultaterne efter OA guld-behandling.

Resultater: 24 immunologiske molekyler blev identificeret med PEA til at være signifikant nedreguleret som følge af en OA-guldbehandling. Flere af disse har direkte forbindelse til både OA, RA, inflammation go NF-kB signaleringsvejen. MS identificerede 22 signifikant regulerede proteiner i OA, hvoraf flere også kan kædes sammen med OA, RA, inflammation og NF-kB. cfDNA viste sig ikke at blive signifikant ændret efter otte ugers guldbehandling, men andre studier og observationer indikerer at otte uger måske ikke er nok tid til at guldbehandlingen kan udøve sin fulde effekt.

Konklusion: De klinisk observerede effekter af OA guldbehandlingen fandt basis i de observerede proteomiske ændringer identificeret i dette studie, i forhold til både mindsket inflammatoriske markører, modulation af NF-kB og sammenhæng med OA-patologi. Disse effekter koblet med den proteomiske data og viden om RA patogenese, åbner for muligheden om en positiv effekt ved guld mikropartikel behandling af RA.

Nøgleord: Osteoartrose, rheumatoid artrit, inflammation, guld mikropartikler, massespektrometri, proteomics

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Introduction

Arthritis

Arthritis is a broad clinical term that includes more than 100 different joint conditions[1]. Arthritis refer to inflammation in one or more joints, but the pathogenesis varies between each subtype (*see table 1*). Accompanying the joint inflammation are symptoms such as pain and stiffness which are present in all arthridities[1]. Estimates suggest that the prevalence of arthritis is around 18% in adults between 18-65 years, and above 60% in adults over 65 years[2]. The incidence of arthritis is higher in women than in men and increases with age. Other risk factors for the development of arthritis are a family history, being overweight and previous joint injury[3]. The most common type of arthritis is osteoarthritis (OA) which affects about 15% of the global adult population[4]. The most common autoimmune form is rheumatoid arthritis (RA) with a prevalence in adults between 0.5% and 1%[5,6]. Inflammation plays a major role as a driver of the pathogenesis in both diseases, by recruiting leucocytes and increasing the immune response, which ultimately leads to irreversible joint damage and pain. There is currently no cure for either OA or RA, and treatment mainly focuses on relieving symptoms and decreasing inflammation to prevent further disease progression[6,7].

Type	Symptoms	Causes	Diagnosis	Risk factors	Treatment
Osteoarthritis (OA)	• Joint pain • Joint stiffness • Tenderness • Decreased range of motion	Progressive cartilage degeneration	Clinical presentation coupled with an X-ray and/or MRI	• Older age • Being female • Obesity • Joint injuries • Genetics • Joint deformities	• NSAIDs • Corticosteroids • Weight loss • Joint replacement
Rheumatoid arthritis (RA)	• Symmetrically affected joints • Joint pain • Swollen joints • Joint stiffness • Fever	Autoantibodies against joint associated proteins	Clinical presentation coupled with an X-ray and/or MRI and blood tests	•Older age • Being female • Obesity • Smoking • Genetics	• NSAIDS • Corticosteroids • DMARDs • bDMARDs
Gout	• Joint pain • Swollen, tender, red, and warm joints • Decreased range of motion	Urate crystal accumulation in the joint	Synovial fluid and blood tests coupled with an X-ray or ultrasound	• Being male • Diet • Obesity • Some medications • Recent surgery or trauma	• NSAIDs • Corticosteroids • Colchicine • Xanthine oxidase inhibitors • Uricosurics
Ankylosing spondylitis	• Pain and stiffness associated with the vertebrae	Fusing of the vertebrae	Clinical presentation coupled with an X-ray and/or MRI	• Being male • Age • Genetics	• NSAIDs • TNF blockers • IL-17 inhibitors
Septic arthritis	• Severe pain • Very limited joint movement • Swollen, red, warm, and tender joint • Fever	Bacterial, fungal, or viral joint infection	Synovial fluid analysis coupled with clinical presentation and an X-ray to assess joint damage	• Existing joint problems • Immune suppression • Infections • Injection and surgery	• Joint drainage and antibiotics

Table 1 *Five different types of arthridities, their symptoms, causes, diagnosis, risk factors, and treatment.*

Osteoarthritis

Osteoarthritis (OA) is a chronic degenerative disease of the synovial joints[4]. It is one of the leading causes of disability and inactivity in elderly people and is estimated to affect around 15% of the adult world population[4]. Osteoarthritis is characterized by degeneration of the articular cartilage over time accompanied by narrowing of the joint space and the formation of osteophytes (*see figure 1*)[7,8]. Symptoms of OA include pain, reduced movement, and morning joint stiffness[9]. OA mostly affects knee-, hip-, hand-, and spinal joints, but other joints can be affected as well. Previously OA was regarded as a non-inflammatory disease, but in recent years inflammation has been shown to play a crucial role in the pathogenesis, and evidence now suggest that the articular inflammation is one of the main drivers of the disease[4,10,11]. The onset of OA can be triggered in multiple ways like impact trauma, metabolic syndrome, ageing and genetic predisposition[11]. However, all of these will lead to an activation of inflammatory pathways in the affected joints, which then drives the further disease progression[11].

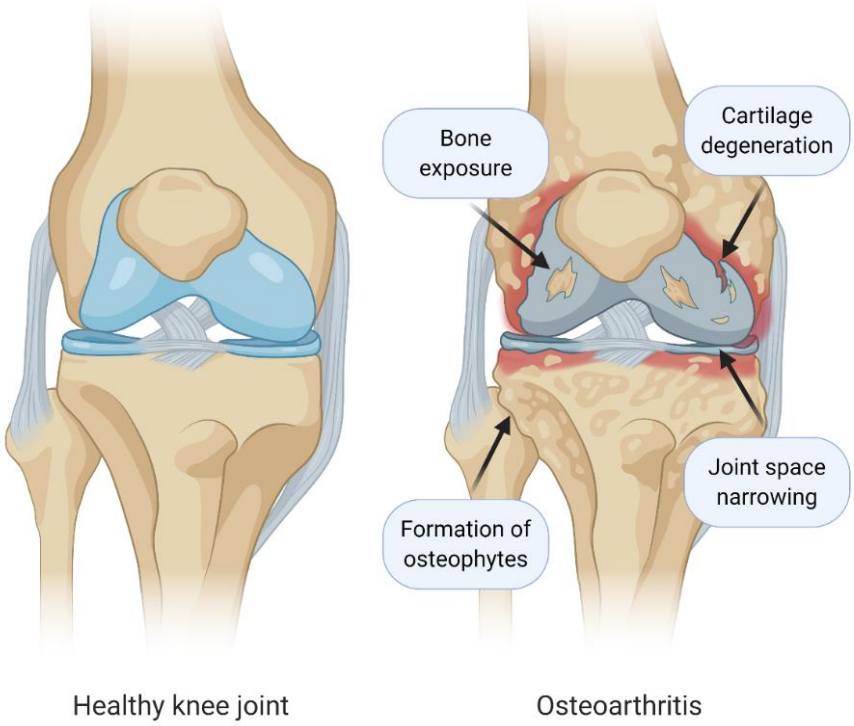

Figure 1 *Comparison of a healthy knee joint and one with OA, which presents with exposure of the subchondral bone, cartilage degeneration, formation of osteophytes and joint space narrowing.*

Pathogenesis and inflammation in osteoarthritis

In a healthy synovial joint the articular cartilage is mainly made up of water, collagen, and proteoglycans[4]. This matrix is continuously being synthesized and broken down by articular chondrocytes in a tightly regulated homeostasis between cartilage anabolism and catabolism[11,12]. The balance is controlled by different signaling molecules such as growth factors, cytokines, and breakdown products of the matrix itself[4,12].

When an individual ages, or as a result of genetic variations, metabolic syndrome and other factors, the composition of the articular cartilage changes. This decreases the overall integrity, leaving the cartilage more brittle and vulnerable to shock and pressure[12]. This can lead to an increased cartilage wear which the chondrocytes, at some point, will be unable to compensate for in terms of anabolism of new cartilage. The breakdown products and mechanically liberated cartilage fragments will initially be engulfed and cleared by articular macrophages (type A cells resident in the synovial membrane)[4]. But with increasing cartilage degradation the load from these particles, or "joint mice", overwhelms the macrophages ability to clear them from the synovial fluid and they become inducers of inflammation[4]. One way this occurs is by activation of the classical complement pathway[10]. This is done by small leucine-rich repeat proteoglycans (SLRPs) prom liberated cartilage proteins like osteomodulin and fibromodulin[13]. Another way articular debris induce inflammation is by acting as damage-associated molecular patterns (DAMPs), able to stimulate toll like receptors (TLRs)[4]. One of the main pathways that leads to inflammation in this way is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway[14]. NF-kB is a transcription factor from the NF-kB family of proteins[15]. This family consists of five different transcription factors, p50/p105, p52/p100, p65 (RelA), RelB, and c-Rel[15]. These five proteins share a common domain called the rel homology domain (RHD) which contains their DNA binding site (*see figure 2*)[15].

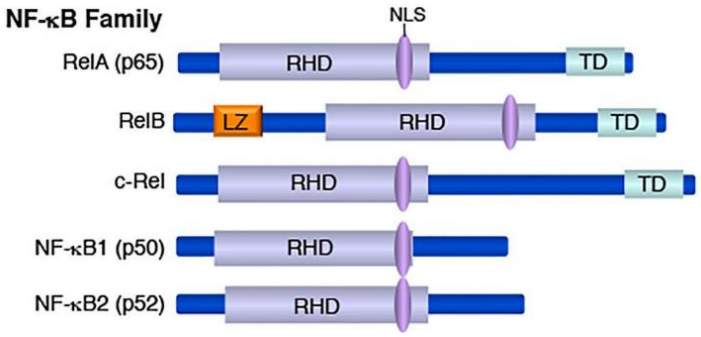

Figure 2 *The five members of the NF-kB family showing the rel homology domain (RHD), nuclear localization site (NLS), transactivation domain (TD) and leuzine zipper (LZ). Modified from*[16].

The RHD of NF-kB contains a highly conserved cysteine residue at position 61 (Cys61) which is essential for optimal DNA-binding of the transcription factor[15,17,18]. To achieve a successful binding, Cys61 must be at a reduced state[18]. If this cysteine residue becomes oxidized, DNA-binding is inhibited which results in decreased transcription of the NF-kB target proteins[19]. During inflammation the level of intracellular reactive oxygen species (ROS) rises and is able to oxidize the nuclear NF-kB[15]. To overcome this, the oxidized form of Cys61 gets reduced by the enzyme thioredoxin (TRX) which completely restores the DNA-binding activity of NF-kB[15]. The five Rel family members form homo- and heterodimers, with the heterodimer of p50 and p65 being the most common[15]. NF-kB transcription can be induced via a classic and an alternative pathway which both leads to the production of proinflammatory cytokines, chemokines, and other inflammatory mediators[20]. The alternative pathway is initiated by molecules like CD40L, BAFF, and RANKL[14]. The classic, or canonical pathway, is activated by interleukins (ILs), TNFα, LPS, toll like receptor ligands (TLRLs) and other molecules[15,21].

The main focus here will be the canonical pathway and the heterodimer of p50/p65 since these are most involved in the pathogenesis of OA and RA. Before stimulation, the inactive form of NF-kB is located in the cytosol bound to an inhibitory molecule called inhibitor of NF-kB (IkB)[15]. Upon receptor stimulation, the protein IkB kinase (IKK) gets activated and phosphorylates IkB which triggers IkB's proteasomal degradation[20]. The now unbound NF-kB translocate to the nucleus where it is able to bind to its target genes and initiate transcription. NF-kB controls around 180 genes, many of which play a role in inflammation (*see figure 3*)[18].

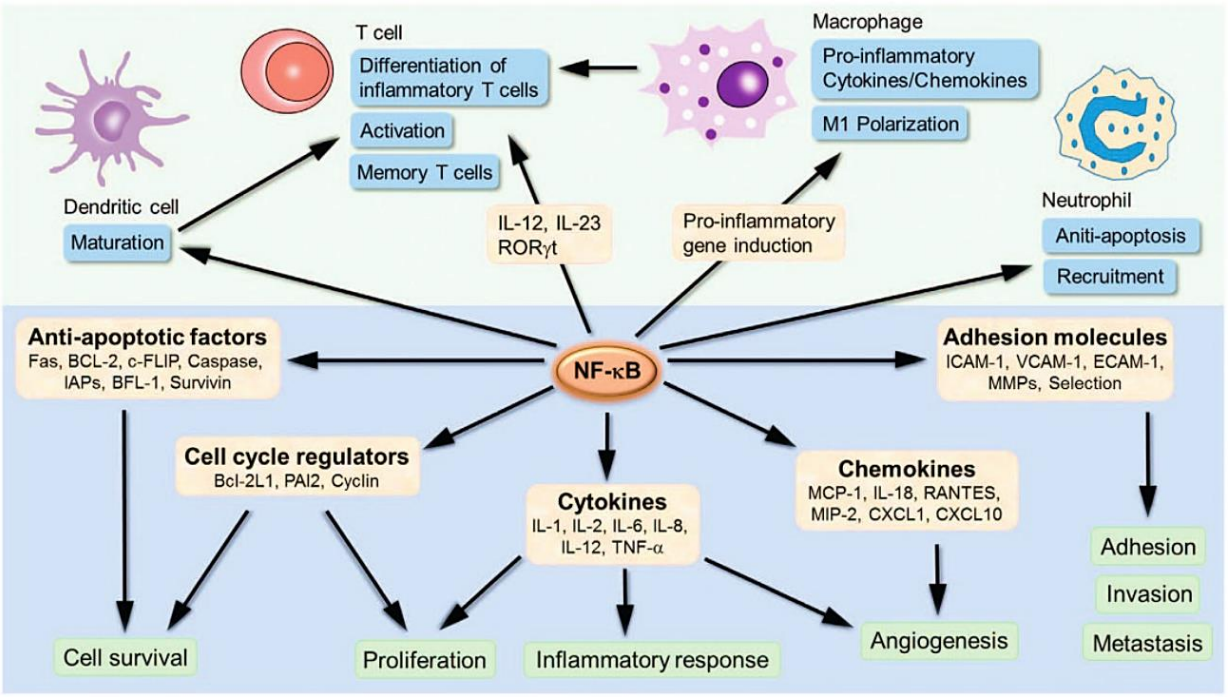

Figure 3 *Inflammation associated molecules produced via the NF-kB pathway and their effects on immune cells*[20].

One gene regulated by NF-kB is PTGS2 which codes for the enzyme prostaglandin G/H synthase 2, more commonly known as cyclooxygenase-2 (COX-2) [22]. The COX-2 enzyme contributes to inflammation, both in general and in OA[23,24]. This is achieved by the formation of prostaglandins (PGs) from the phospholipid arachidonic acid which is part of the cellular plasma membrane[23,24]. PGs are able to stimulate several receptors involved in inflammation, OA and RA as well as stimulate the production of interleukins and other cytokines, which further contribute to inflammation[23].

A key feature of OA is the progressive and active degradation of cartilage by matrix metalloproteinases (MMPs)[10]. These are produced in excess by the chondrocytes themselves as a response to cytokines produced by the stimulated macrophages[10]. This stimulation of chondrocytes induces a phenotypic shift towards a catabolic phenotype and apoptosis[10]. The change in phenotype also leads to a change in cytokine production by the chondrocytes. They start to produce larger amounts of IL-1 which further increases their production of MMPs[10].

The transcription factor AP-1 also regulates proinflammatory genes and works similar to NF-kB. AP-1 is responsible for the transcription of IL-1 and MMP genes, amongst others[25].

Treatment of osteoarthritis

The treatment of OA can be divided into surgical and non-surgical approaches and the non-surgical approach is further subdivided into pharmacological and non-pharmacological treatments[26]. Non-pharmacological treatment focuses on strengthening and stabilizing the affected joints as well as reduce load. This is done with patient information, weight loss, personal aids, exercise, and physical therapy[26]. Non-pharmacological treatment is usually accompanied by pharmacological treatment (*see table 2*) to further aid in pain relief and to decrease inflammation[26]. The first drug of choice to manage pain in OA is paracetamol, and even though the adverse effects are mostly of mild character, paracetamol is very weak and usually not enough to adequately manage pain on its own and is therefore often prescribed with NSAIDs[3,26].

	Paracetamol	**NSAIDs**	**Glucocorticoids**	**Opioids**
Clinical effect	• Week analgesic • Improves function • Reduces stiffness	• Week/moderate analgesic • Moderate anti-inflammatory effect	• Moderate analgesic • Improves function • Reduces stiffness • Strong anti-inflammatory effect	• Strong analgesic • Improves function
Most common adverse effects[27]	0.01%-0.1% experience allergic reactions	>10% experience abdominal pain, gastrointestinal bleeding, and dyspepsia	>10% experience edema, infections, osteoporosis, and adrenal insufficiency	1-10% experience nausea, dizziness, vomiting, drowsiness, and headaches
Other downsides	Very week effects, and usually not enough on its own	Treatment for over 6 weeks is not recommended	Articular injections only have short lasting effects	Risk of developing dependence

Table 2 *showing the most common pharmacological treatment options for OA, their clinical effects, adverse effects, and other downsides*[26]*.*

Due to their effects on pain as well as inflammation, NSAIDs are the most used drug for treating OA[3,28]. These drugs work by inhibiting the COX family enzymes, resulting in a decreased biosynthesis of prostaglandins which contribute to pain sensation as well as inflammation in the joint[28]. However, prostaglandins are also important in the gastric mucosa and aid in protection by reducing acid production, increasing $NaHCO_3$ secretion and stimulate mucus production[23,29]. The resulting gastrointestinal side effects is why long-term treatment with NSAIDs should be avoided[26].

If the effects of paracetamol and NAIDs are inadequate, supplementation with opioids is an option[26]. These drugs work by decreasing nociceptive neuronal excitability, thereby decreasing the sensation of pain[26]. But opioids are known to be strongly addictive as well as cause gastrointestinal problems like constipation and long-term use is therefore not recommended for opioids either[26].

If the pain and other symptoms are due to an acute flare of inflammation, an intraarticular injection of glucocorticoids will often be used[30]. The anti-inflammatory effects of glucocorticoids are due to the inhibition of several inflammatory mediators such as NF-kB and AP-1[30]. The injection of glucocorticoids thereby results in a decrease in cytokines, chemokines, and enzymes responsible for a big part of pain generation and inflammation[26]. However, the effect is short lived and due to its side effects, glucocorticoids should not be injected more than once every third month for a maximum of two years[31].

In the most extreme cases with severe joint degeneration and thereby decreased motion and severe pain, a total joint replacement is used[26].

Novel treatment with gold microparticle implants, effects on Inflammation

In 2018, a novel treatment of OA with solid intra articular gold microparticle implants (Berlock-Micro-Implants, BMI, Goldtreat APS), showed promising clinical results[32]. Patients given these implants reported significant improvements in both function, stiffness, and pain 8 weeks post treatment[32]. The mechanisms behind these findings are sparsely described but are thought to be due to the anti-inflammatory properties of gold[32]. The modulatory effects of gold on inflammation have been known for many years and is thought to be due to its affinity for thiols and selenocysteines[31]. Gold ions have been shown to inhibit the action of several proteins involved in inflammation like AP-1[24], TRX[19,31], COX-2[19,31] and NF-kB[33]. Compounds containing gold salts have been used as treatment in both cancer and rheumatoid arthritis (RA) for several years[31]. However, gold salts are rarely used anymore due to their severe side effects such as nephrotoxicity[31]. These compounds are based on gold ions (Au^{I} or Au^{III}) coupled to e.g. a thiol or a phosphine group[5,6]. Some of the gold salts still in use today are sodium aurothiomalate and tetraacetyl-b-D-thioglucose gold(I) triethyl- phosphine[31]. When a gold salt is ingested or injected, the gold ions will enter cells like macrophages, fibroblasts, and mast cells where they bind to the cysteine residues of several proteins[34]. This binding inhibits the proteins by blocking their active site and/or inducing conformational changes[31]. In NF-kB, gold ions bind to Cys61 which block its DNA-binding site, rendering the transcription factor inactive[31]. The inhibition of NF-kB in turn decreases the amount of TNF-α, IL-1, IL-6 and other inflammatory mediators and gold-inhibition of AP-1 lowers the amount of MMPs, and IL-2 produced[31]. However, the solid gold particles are too large (20-40 µm in diameter) to enter the cells like the free gold ions from the salts[32]. But macrophages recognize the particles as foreign and form a dissolucytosis membrane and liberate gold ions from the solid microparticle[32,34]. The liberated ions are then able to enter the cells and exert function like gold ions from gold salts (*see figure 4*).

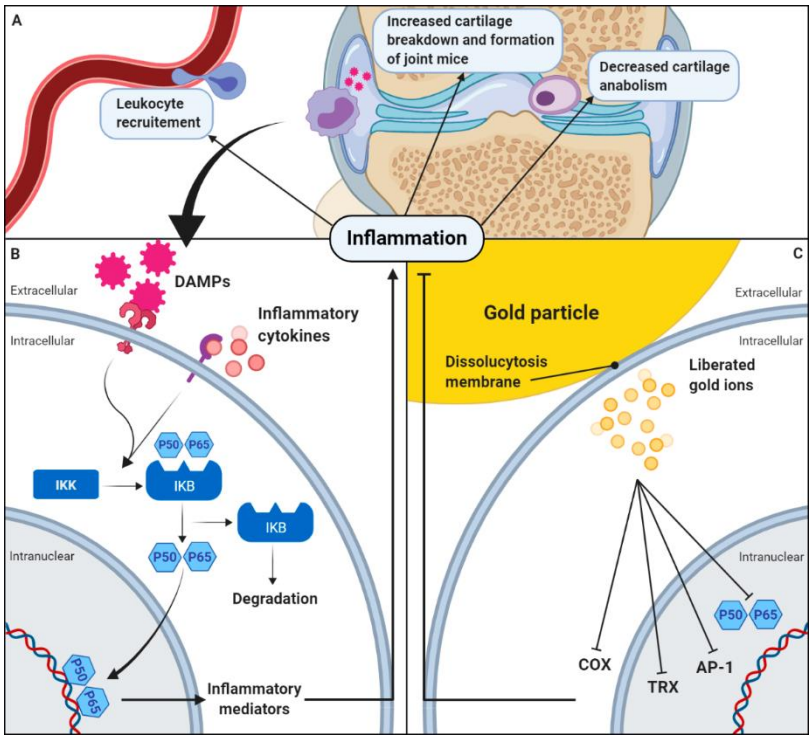

Figure 4 *Current knowledge of the therapeutic and anti-inflammatory effects of gold particles. A) pathologic effects of joint inflammation in OA. B) Activation of the NF-kB pathway in OA. C) Gold ions inhibition of inflammatory mediators.*

Rheumatoid arthritis

Rheumatoid arthritis is a systemic autoimmune disease that usually affects multiple joints by causing synovitis in a symmetrical manner, ultimately leading to permanent joint damage with bone erosion, cartilage damage (*see figure 5*) and systemic comorbidities[5,6]. As for other arthridities, women are more at risk of developing RA. Over twice as many women are affected than men at any age[5,6]. RA specific risk factors includes gingivitis caused by the bacteria *Porphyromonas gingivalis*, infections with *Aggregatibacter actinomycetemcomitans*, Epstein-Barr virus and expansion of Prevotella bacteria species in the gut[5]. Patients often presents with symmetrical joint associated pain, swelling and morning joint-stiffness for more than 30 minutes[5]. The wrist and finger joints are most commonly affected, but larger joints like the knee, elbow, ankle and shoulder are also frequently affected[6]. At the moment there is no curative treatment for RA[5]. Current treatment seeks to achieve remission or a low disease activity state (LDAS) within six months following diagnosis to minimize disease progression and permanent joint damage[6]. This is done by alleviating the inflammation in the joint capsule. The most common used drugs are non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and diseases modifying anti rheumatic drugs (DMARDs) like methotrexate[6,35,36].

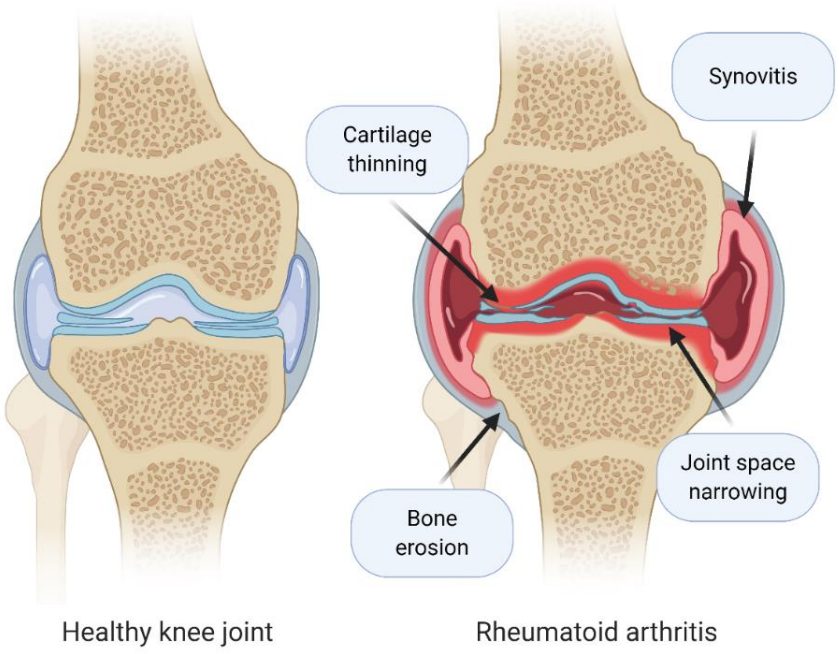

Figure 5 *Comparison of a healthy knee joint and one with RA, which presents with synovitis, bone erosion, cartilage thinning and joint space narrowing.*

Pathogenesis and Inflammation in rheumatoid arthritis

Rheumatoid arthritis is a progressive disease where both innate and adaptive immunity play a role in the pathogenesis, but adaptive B- and T-cells are thought to be the most important cellular components[6,37,38]. RA can be divided into two large subtypes depending on the presence or absence of anticitrullinated protein antibodies (ACPAs) and/or rheumatoid factor (RF), accordingly the seropositive and seronegative RA[6]. The vast majority, around 70% of RA patients, are seropositive and thereby have detectable levels of autoantibodies in their blood. The focus of this paper will therefore be the seropositive subtype.

ACPA and RF autoantibodies also serve as clinical phenotype predictors as the seropositive patients tend to have a more aggressive disease progression than seronegative patients[6,37]. The biggest risk factor for developing a seropositive pathogenesis is specific variants of the human leukocyte antigen (HLA) gene complex coding for MHC molecules, especially HLA-DR1 and HLA-DR4[37]. Several environmental factors have also been shown to contribute to the development of a seropositive phenotype, but not a seronegative phenotype[37]. This could be smoking, airway exposure to silica dust and other environmental factors[6,35]. The pathogenesis is typically described as three (sometimes four) distinct stages (*see figure 6*)[5,35]. The first stage is an asymptomatic pre-RA stage[5]. In this stage the cellular components of the immune system break tolerance and start to produce autoantibodies, primarily ACPAs and/or RF[5,35]. The pre-RA stage can last anywhere from a few months to over ten years[35]. Once symptoms are evident, patients will be in an early RA stage where no irreversible damaged has yet occurred[5,35]. If the inflammation is not resolved at this point, the patient enters the last and chronic stage of RA where irreversible joint damage starts to take place[35].

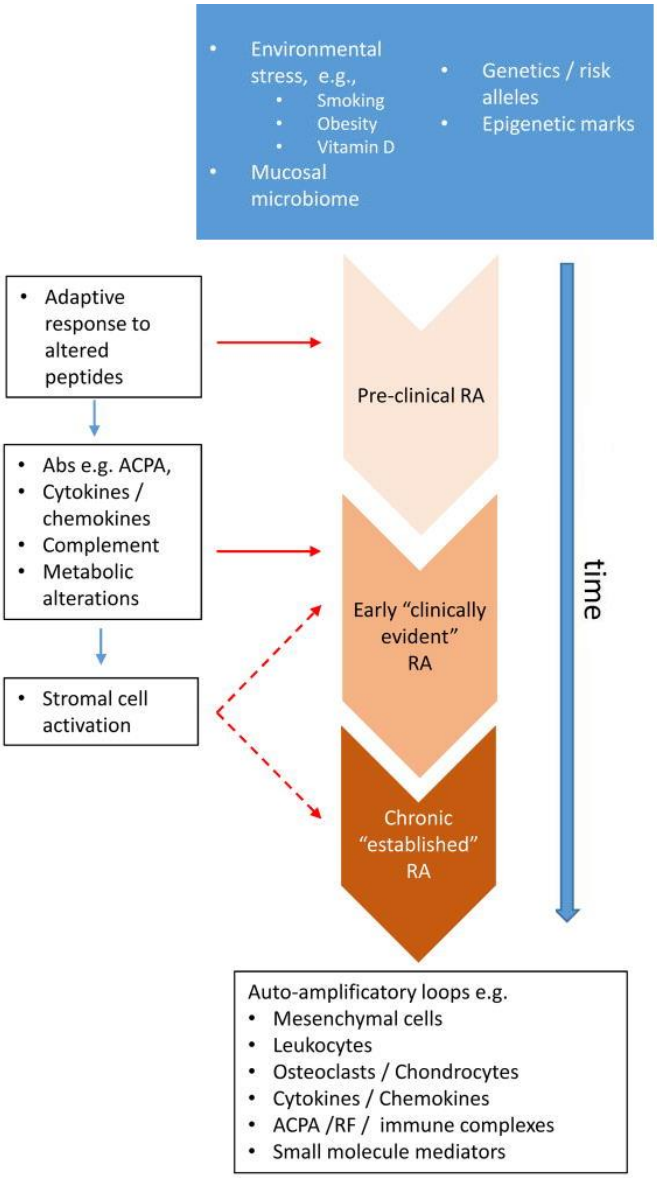

Figure 6 *Schematic representation of RA pathology progression showing a mostly adaptively driven pathogenesis, modified from*[35]

Pre-RA, triggering the autoantibody production

ACPAs and RF are autoantibodies with affinity for epitopes generated by citrullination, and the Fc part of misfolded IgG-antibodies, respectively. The most common targets for ACPAs are citrullinated collagen, fibrin, vimentin, and fibronectin[39]. Citrullination is a post translational modification (PTM) to genetically encoded arginine by peptidyl-arginine deaminases (PADs) which converts the amino acid arginine to citrulline[39]. This exchanges the positively charged arginine with the uncharged citrulline, resulting in structural changes and increased hydrophobicity of the citrullinated proteins. The high-risk HLA variants codes for MHC molecules with higher affinity for citrullinated peptides and is thus more prone to break tolerance and recognize the citrullinated peptide as foreign, initiate an immune response and trigger the production of autoantibodies[6,39]. Several processes have been shown to lead to a level of citrullination higher than the physiological level. One is smoking, which upregulates the expression of the PAD2 enzyme in antigen presenting cells (APCs) like B cells resulting in hypercitrullination[5,6]. Another trigger for autoantibody production is infection with certain bacteria and viruses, especially P. gingivalis, A. actinomycetemcomitans, Epstein-Barr virus and expansion of Prevotella species in the gut as mentioned above[39]. These pathogens have citrullinated proteins that might be similar enough to self-antigens to cause a break of tolerance and thereby initiate an immune response against these self-antigens[39]. In the case of A. actinomycetemcomitans, this bacterium is able to activate the body's own PAD enzymes by the release of a pore-forming toxin call leucotoxin A (LtxA)[39]. LtxA is able to bind to neutrophilic CD18 molecules resulting in an influx of calcium which in turn activate the cells own calcium dependent PAD enzymes leading to an increased citrullination of self-antigens[37]. Once citrullination has occurred, APCs will react with the neo-autoantigens, internalize them, and present them for T helper cells, converting them from naïve to activated. The activated Th cells are now able to activate naïve B cells to autoantibody producing plasma cells targeting citrullinated autoantigens[37]. All of this happens outside the joints, with the initial citrullination happening in e.g. the lungs as a consequence of exposure to cigarette smoke, or at the site of an infection with some of the mentioned pathogens[37].

Early RA, targeting the joints causing synovitis

Once B cell production of autoantibodies is activated, these will travel with the systemic circulation and be able to bind to their target proteins in the joints. Here the autoantibodies form immune complexes with the target proteins and/or each other[40]. Once deposited in the joint or attached to the synovial membrane the immune complexes activate the classical complement cascade[41]. Several components of this cascade, especially C3a and C5a, act as potent proinflammatory mediators and are able to recruit and activate leucocytes[35,40]. Some of these are neutrophils and monocytes, and together with tissue resident macrophages in the synovium these cells phagocytose the immune complexes via Fc-receptor binding[35]. This in turn stimulates the release of cytokines, chemokines, and matrix metalloproteinases (MMPs) which further increase inflammation[42]. ACPAs have also been shown to increase NF-kB activity by binding to the citrullinated surface protein, endoplasmic reticulum chaperone BiP on macrophages[6]. Inflammatory cytokine like IL-1, IL-6, and TNF-α is also able to activate articular fibroblast-like synoviocytes (FLSs). This induced proliferation of the FLSs as well as MMP production[43]. This proliferation of FLSs causes enlargement of the synovium, referred to as pannus, and is externally seen as swollen joints.

Chronic RA, fulminant disease with irreversible damage

The now established and self-reinforcing synovitis is what gives rise to the irreversible damage associated with fulminant RA. Inflammation stimulates the production of MMPs which are able to break down the articular cartilage such as in OA[6]. Furthermore, the inflammatory cytokines also create an apoptotic environment for the articular chondrocytes resulting in both increased cartilage catabolism and decreased cartilage anabolism[6]. The articular inflammation also stimulates an upregulation of receptor activator of nuclear factor kappa-B ligand (RANKL). This protein is the ligand for receptor activator of nuclear factor kappa-B (RANK) which stimulates the maturation of osteoclasts via the NF-kB pathway[6,44]. The osteoclasts are the cells responsible for the RA associated bone erosion[6,44]. This is achieved by osteoclast adhesion to the articular bones, forming a closed pocket between the cell and bone. The cell then secretes H+ ions via the enzyme H^+ATPase into the lumen which dissolves the calcified bone matrix. Under physiological conditions osteoblasts would deposit new bone matrix, but the inflammatory milieu in the joint suppresses osteoblast activity[6]. Other inducers of osteoclastogenesis is ACPA recognition of citrullinated surface proteins and binding of immunocomplexes to osteoclast Fc receptors[37]. Both leading to activation and bone resorption[37]. If the disease is allowed to progress, the pannus and proliferating FLSs are able to invade periarticular tissues and cause damage to tendons, cartilage, and bones surrounding the joint as well, further increasing joint damage. Upon binding of ACPAs to osteoclasts, the cell will produce the chemokine CXCL8 which is an autocrine stimulator of osteoclast activation[37]. However, CXCL8 is also able to stimulate nociceptive receptors causing pain, attract neutrophil granulocytes and induce NETosis[37]. Given enough time, the systemically present autoantibodies and migrating FLSs are able to affect multiple joints and cause symptoms and comorbidities outside the joints. The most commonly affected extra articular tissues to be affected in RA are the lungs, kidneys, heart, and skin [6].

Treatment of rheumatoid arthritis

The main focus of RA treatment is to manage pain and stop disease progression by reducing inflammation[45]. This is, like in, OA sometimes done with glucocorticoids and NSAIDs to manage pain. But, glucocorticoids, either orally or via injection, are mostly used in disease flares and should not be used for an extended period of time due to their side effects, and neither should NSAIDs[45]. Instead the inflammation is treated with drugs known as disease modifying anti-rheumatic drugs (DMARDs)[45]. DMARDs can either be synthetic or biological agents (bDMARDs) which vary in target an effect (*see table 3*).

Type	Name	Effect	Side effects
Old DMARD	Auranofin (gold salt)	NF-kB, TRX, and AP-1 inhibition	Nephrotoxicity
Conventional synthetic DMARD	Methotrexate	Antimetabolite	Pulmonary damage, oral ulcers, and increased liver enzymes
bDMARD	Etanercept	TNF-α inhibition	Infections, hypertension, and severe transfusion reaction
bDMARD	Tocilizumab	IL-6 inhibition	Infections and increased cholesterol
bDMARD	Denosumab	RANKL inhibition	Low blood Ca+ and phosphate levels and muscle camps

Table 3 *Five different treatment options for RA, their effects and side effects. Modified from*[6]

The drug of first choice, unless contradicted, is the conventional synthetic DMARD methotrexate (MTX)[45]. MTX is a folic acid analogue and inhibits the enzyme DHFR which results in cell cycle arrest and limited proliferation of T-lymphocytes[45]. MTX also downregulates the production of TNF-α and NF-kB related proteins, which further contributes to is effects in treating RA[45]. If MTX treatment is contradicted or unsuccessful, the next line of drugs are the bDMARDs. These can target a variety of the molecules and receptors described in the sections above, like TNF-α, IL-6 and RANKL. One example is the TNF inhibitor etanercept (ETN; Enbrel®).

RA treatment with Etanercept

TNF is a cytokine primarily produced by immune cells like macrophages and T-cells as a response to inflammation, DAMPs, and PAMPs. TNF is both produced via the NF-kB pathway and is also able to activate this pathway, along with other inflammatory pathways such as AP-1. ETN is a combination of the Fc portion of human IgG1 fused with the extracellular region of human TNF-receptor (TNFR)[46]. ETN is administered via subcutaneous injections of 50 mg once a week or 25 mg twice a week[46]. Upon injection, ETN enters the circulation and binds to extracellular TNF molecules[46]. This inhibits TNF´s ability to bind and stimulate the TNF receptor, thereby decreasing the pro-inflammatory signaling cascade by inhibiting the activation of NF-kB and others[46].

Hypothesis & Aims

A novel treatment of knee OA with intra articular gold microparticle implants have shown positive clinical results on both pain and movement[32]. But these findings are not indicative of the immunological mechanisms or pathophysiological effects of the gold treatment. The observed clinical effects are thought to be due to anti-inflammatory properties of liberated gold ions in the joints by macrophage induced dissuloctosis[47,48], but there is a lack of evidence and research regarding the effects of solid gold as a modulator of inflammation. Anti-inflammatory effects of gold salts have been known for many years and a few are still used to treat RA today, however due to severe systemic side effects like nephrotoxicity and hepatotoxicity, these are rarely used anymore[33,49]. Gold ions from these gold salt compounds have been shown to inhibit several inflammatory mediators, such as the NF-kB pathway[19,24,50]. Thus, there is both literary theoretical, and clinical basis for the effect of gold microparticles as a novel OA treatment.
This study therefore seeks to find proteomic evidence of the clinically observed effects in biological fluids from the same patient cohort of gold treated knee OA patients as used in the study by *Rasmussen et al*[32]. This leads to the following hypothesis and aim of study:

- ***The aim is to investigate the clinical effects of solid intra articular gold microparticle implants in the treatment of knee OA with proteomic strategies.***
- ***The hypothesis is that the treatment significantly decreases inflammation on a proteomic level, which in part can be contributed to NF-kB pathway modulations.***
- ***Furthermore, the findings will be correlated with the inflammatory pathogenesis and proteomic data of RA serum samples to search for evidence suggesting a possible future novel treatment option of RA with gold particles.***

Experimental strategy

To investigate this hypothesis and fulfil the aims of this study an analytical experimental research strategy was implemented. The strategy was carried out to elucidate proteomic changes in synovial fluid- and serum samples from the same patient cohort from the before mentioned study by *Rasmussen et al*[32], as well as serum samples from RA patients treated with etanercept. These changes will then be used to identify significantly regulated proteins in the samples following treatment and identify potential biomarkers to monitor gold treatment effect on a personalized proteomic level (*see figure 7*).

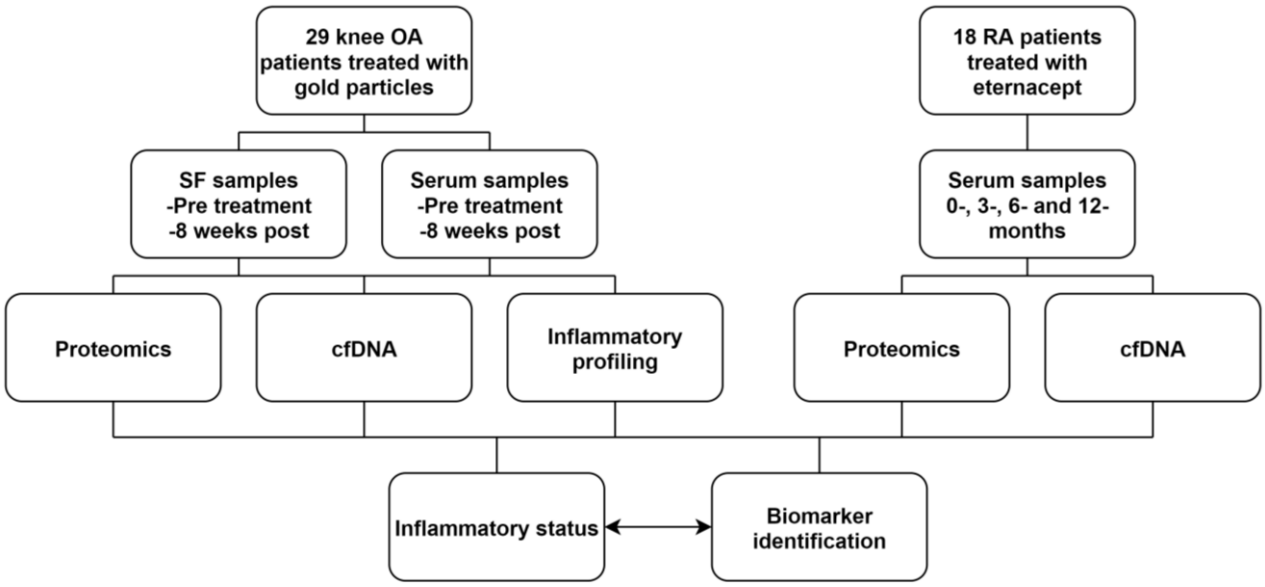

Figure 7 *Flowchart for project strategy and sample analysis for both OA and RA biological samples.*

Two types of biological samples are investigated in this project, namely synovial fluid, and serum. These biofluids will be analyzed in multiple ways to acquire a deeper insight into the proteome before and after treatment. A mass spectrometry driven quantitative proteomics approach will be used to gain knowledge about the most abundant peptides in the samples[51]. However, a regular LC-MS/MS setup is not capable of detecting low abundance signaling molecules, such as cytokines, or obtain a deep proteome coverage at the same time. Thus, a pre-MS pH-fractioning of selected samples is therefore carried out according to[52] to improve 'match between runs'- search outcome. This is done to decrease the sample complexity, and a few representatives from each sample set were chosen at random, half of the representatives being before- and the other half being after treatment. The pH-fractioning separates the digested sample peptides into 8 fractions depending on their pH associated elution from a hydrophobic resin. This is done by a step gradient with increasing amounts of acetonitrile (ACN) at high pH. The elutions can then be analyzed using LC-MS/MS where the high-performance liquid chromatograph (HPLC) further separates the digested peptides. This yields a 2D separation by initially separating the peptides at alkaline conditions due to the presence of triethylamine (pH 10.75) accompanied by high ACN concentrations and then, in the HPLC, at low pH due to TFA (pH 2) with low ACN concentrations.

Spreading out the sample complexity across multiple fractions yield more protein coverage and acts as a spectral library for the rest of the samples used to 'match from' during MaxQuant analysis of the raw MS data output. Three pH-fractioning's were carried out, one for each sample set, OA serum, OA synovial fluid, and RA serum. The sample digests and fractions were analyzed using label free quantification (LFQ). This quantifies precursor signal intensities as an LFQ intensity, which can then be used as a relative measure of abundance for each of the identified proteins. However, this is not able to be done for all peptides in complex biological samples like synovial fluid and serum despite pre-fractioning. The MS/MS setup is able to identify the top 15 most abundant peptides at any given scan time in the MS1 cell. These are then selected to be fragmented in MS2 and detected in the following orbitrap. This means that we only acquire information about the "tip of the peptide iceberg" at each scan time, and other methods must be utilized to gain deeper proteomic knowledge and coverage, which is why a second proteomics strategy was implemented in this study.

Olink is a 92 plex '*Proximity Extension Assay*' (PEA) technology, which was used to quantify the relative abundance of 92 manufacturer selected immunologically associated low abundance signaling proteins such as inflammatory-cytokines[53]. The PEA technology is based on a combination of antibody recognition and DNA multiplication with a quantitative polymerase chain reaction (qPCR). Specific antibodies are linked to small DNA fragments which act as primers for the following PCR polymerase. For each molecule of interest, there is both an antibody conjugated forward primer and an antibody conjugated reverse primer. When/if these bind to their target they will be in close enough proximity to be extended and amplified by qPCR (*see figure 8)*.

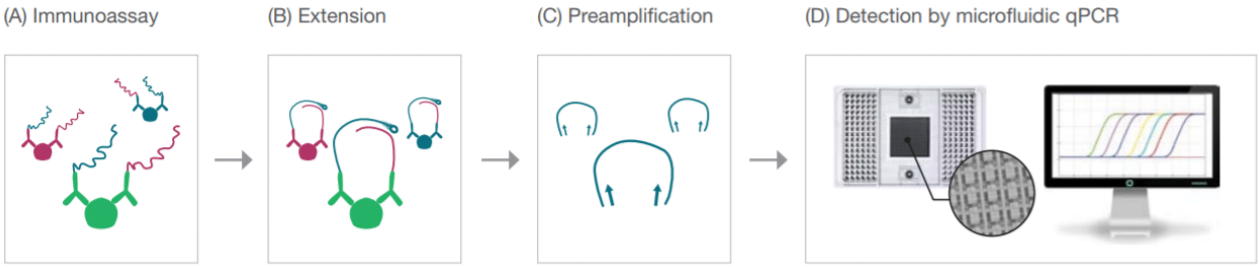

Figure 8 *Simplified overview of the olink PEA technology*[54].

The readout is therefore an amplified semiquantitative measure of the relative abundance of the targeted low abundance molecules, and cannot be used to determine the specific amount of the molecules.

Another immunological assay carried out on all samples was a double stranded DNA (dsDNA) assay, which uses a fluorescent signal, measured with an ELISA reader, to quantify the amount of dsDNA in the biofluids. This was done to determine the amount of cell free DNA (cfDNA) in each sample before and after treatment[55]. With increasing inflammation immune cells, namely neutrophil granulocytes will release their DNA in the form of neutrophil extracellular traps (NETs) via NETosis as a response to activation. The amount of cfDNA is therefore an indication of ongoing inflammation, because the cells responsible for the vast majority of the cfDNA are neutrophils, a large amount of cfDNA is a sign of an innately driven inflammation[55,56].

Materials and methods

Patient cohort for osteoarthritis

29 patients were selected according to strict inclusion and exclusion criteria[32]. These included a knee OA diagnosis based on the ACR criteria, Kellgren-Lawrence (KL) X-ray grade ≧ 2, pain for > 3 months, maximal pain intensity VAS ≧ 5 (0-10 scale) for the most painful knee during the last week, and no prior OA related treatment. From these patients 21 synovial fluid samples were collected before treatment and 23 after treatment. Along with 27 blood serum samples before treatment and 29 after treatment. The synovial fluid samples were centrifuged at 2200g for 10 minutes immediately after collection, to spin down cells and other debris. After centrifugation, the supernatant was removed by pipetting, transferred to a labeled cryotube and frozen at -80°C.
The blood samples were collected in 6 ml serum tubes with no additives. The tubes were rotated five times and left for 60 min. for sedimentation, followed by centrifugation at 3000 RPM for 15 min. The serum was then removed by pipetting and added to labeled cryotubes for storage at -80°C until needed.
All sample data were collected and kept according to Danish legislations on data handling at the Department of Biomedicine at Aalborg University Hospital. After the sampling period, all samples were brought to the department of Health Science and Technology (HST) at Aalborg university for analysis. Each patient sample was divided into aliquots to avoid unnecessary freezing and thawing cycles. The aliquots and remaining stock sample were kept at -80°C until needed.

Patient cohort for rheumatoid arthritis

A total of 18 patients diagnosed with RA according to ACR/EULAR recommendations about to undergo treatment with etanercept were included. Four blood samples were taken from each individual at baseline, 3-, 6- and 12-months following treatment start and handled the same way as above to extract and store serum from all blood samples. The samples were collected from the local branch Danish Rheumatic Biobank located at Department of Clinical Biochemistry, North Denmark Regional Hospital, Hjørring.
Sample collection was handled according to the Danish Reuma Biobank Laboratory Manual for Research Biobanks under the ethical approvals (H-2-2014-086; Tillægsprotokol 49419).

Cell-free DNA measurement

The amount of cell free DNA (cfDNA) in all patient samples was determined using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions using 96 well Microplates, PP, F-Bottom black chimney well design (Sigma Aldrich). In order to determine the cfDNA, a standard curve was made from a dsDNA dilution series of a known concentration before proceeding to the sample measurements of unknown concentration. A four-fold dilution series of DNA were included on all plates (1µg/mL, 250ng/mL, 62ng/mL, 15,6ng/mL, 3,9ng/mL, 970pg/mL, 243pg/mL, and 0pg/mL) to make the standard curve.

Firstly, a TE buffer containing 10 mM Tris-HCl, 1 mM EDTA, with a pH of 7.5 was made. This was used to make a PicoGreen® working reagent with a concentration of 1:200. Each unknown dsDNA sample was resuspended gently and diluted 1:1 with the PicoGreen® working reagent. These were then incubated in the dark for 5 minutes at room temperature. Next, plates were measured on an Enspire Multimode Plate Reader (Perkin Elmer, Waltham, MA) with excitation 480 nm and emission 520 nm. All measurements were subtracted the background noise from a blank measurement and related to the standard curve to yield the cfDNA concentration in ng/mL.

Inflammatory markers using the Proximity Extension Array (PEA)

An olink Proximity Extension Assay (PEA) was carried out on all OA synovial fluid samples to identify smaller and less abundant peptides affected by the gold treatment. After the samples had thawed, they were vortexed and centrifuged for 1 min at 400g. An incubation mix was prepared containing A- and B-probes, an incubation stabilizer, and an incubation solution. This was transferred to as many wells 96-well plate, as there were samples plus 3 negative controls. 1 ul of each sample was added to the incubation mix and the wells were sealed and spun down at 400g for 1 min followed by incubation overnight at around 5°C. The next day an extension mix was prepared containing high purity water, a PEA solution, PEA enzyme, and PCR polymerase. The extension mix was quickly added to all wells containing sample. The plate was vortexed thoroughly and centrifuged at 400g for 1 min. The well plate was then placed in a thermal cycler and a premade PEA program was run for approximately 1 h 40 min. A premade detection mix containing detection solution, high purity water, detection enzyme, and PCR polymerase, was added to a new 96-well plate (sample plate). Sample was added to the sample plate from the plate in the thermal cycler (incubation plate). The sample plate now containing both sample and detection mix was sealed, vortexed and centrifuged for 1 min at 400g. 5 uL from each primer plate well was then transferred to the left side of a primed 96.96 Dynamic Array IFC. Then 5 uL from each sample plate well was loaded into the right side of the 96.96 Dynamic Array IFC. The now ready plate containing both sample and primers were loaded in a Fluidigm IFC Controller HX and an Olink Protein Expression 96×96 Program was run to analyze the samples.

Protein quantification with BCA assay

To determine the protein concentration of each synovial fluid and serum sample, a BCA assay (Thermo Scientific Pierce™ BCA Protein Assay Kit) was carried out according to the manufacturer's instructions. Prior to the assay, a working reagent with a ratio of 50:1 reagent A:B was prepared. A bovine serum albumin (BSA) standard curve was prepared from a seven step 2-fold dilution series. To determine background noise, a blank test containing only Milli-Q water was made and subtracted from all results. Each sample was added to a well in a microwell plate and working reagent was added. The microwell plates were incubated for 30 min at 37°C and cooled to room temperature for 10 min to stop the reaction. The absorbance of each sample was measured in a microchannel plate reader (TECAN Sunrise) using a 570 nm filter. Afterwards, a linear model for the correlation of protein concentration and absorbance was made by linear regression of the BSA standard curve. This model was applied to the absorbance value for each sample to determine the protein concentration.

Filter aided sample preparation (FASP) of biological fluids

All samples were prepared for mass spectrometry with filter aided sample preparation (FASP). 100 ug of protein was transferred to a spin filter tube (VWR North America Filter, #FC9847) with 200 ul 0.5% SDC in 50mM TEAB (digestion buffer). The proteins were denatured in a heat block at 95°C for five minutes. Samples were then centrifuged at 14000g (Hettich Zentrifugen, Rotina 380R) for five minutes and the flow-through was discarded. The samples were rinsed again with 200 ul digestion buffer and centrifuged at 14000g for five min. Subsequently, the samples were reduced and alkylated by incubation with a mixture of digestion buffer, 10 mM TCEP and 50 mM chloroacetamide (CAA) for 30 minutes at 37°C. The samples were then centrifuged at 14000g for five minutes, and a digestion buffer wash followed by centrifugation was performed and the flow through was discarded thoroughly by pipetting. The spin filters were incubated overnight at 37°C with 1 ug trypsin (Thermo Scientific #815-968-0747) in digestion buffer.
The next day, the tubes were centrifuged at 14000g for five minutes, and digestion buffer was added to the filters, followed by centrifugation at 14000g for five minutes, leaving the digested proteins in the collection tubes. Remaining detergents were removed by phase separation with ethyl acetate (VWR, LOT#83621.320) and trifluoroacetic acid (TFA) (Thermo Fisher Scientific, LOT#a116-50). After adding the phase separation reagents to the samples, they were vortexed thoroughly and centrifuged for five minutes at 14000g which left an upper phase containing detergents and a lower phase containing the digested proteins. The upper phase was carefully removed by pipetting, and the phase separation was repeated with ethyl acetate without TFA. Finally, the samples were vacuum centrifuged until dry and kept at -80°C until further processing.

pH-fractioning for increased protein coverage

To expand the protein coverage of the MS analysis, a pH-fractioning of the enzymatically digested samples was carried out. This was done with a Pierce™ High pH Reversed-Phase Peptide Fractionation Kit from Thermo Scientific™ (catalog number 84868). 10-100 ug of the pre-digested proteins dissolved in 0.1% TFA were added to an equilibrated, high-pH, reversed-phase fractionation spin column. 8 independent elutions were then carried out using a step gradient with increasing amounts of acetonitrile (5-50%) and decreasing amounts of triethylamine 0.1% (95-50%). All elutions were made by centrifugation of the reversed-phase column at 3000g for 2 min between each gradient step. The samples were vacuum centrifuged until dry and kept at -80°C until further processing.

Sample loading prior to MS

Prior to loading the samples into well plates they were thawed and resuspended in MS loading buffer, containing ultrapure water, 2 % acetonitrile (ACN), 0.1 % trifluoroacetic acid (TFA), and 0.1 % formic acid. Following resuspension, the samples were sonicated for five minutes and centrifuged at 14000g for 10 minutes. The protein concentration of each sample was measured with a Nanodrop A280 to allow a precise and identical concentration in each well for MS analysis. The samples were randomized and loaded in technical duplicates into 96-well plates.

Processing and data acquisition of samples in LC-MS/MS

The samples were investigated in technical duplicates using a UPLC-nano ESI MS/MS setup consisting of a Dionex RSLC nano pump (Dionex RSLC 3500, Thermo Fisher Scientific) connected to a Q Exactive HF-X-Hybrid Quadrupole-Orbitrab mass spectrometer (Thermo Fisher Scientific). The peptide samples were loaded onto a C18 reversed-phase pre-column (µPAC, PharmaFluidics, Gent, Belgium) and separated on a 50-cm analytical C18 Micro pillar array column (µPAC, PharmaFluidics, Gent, Belgium) at 30 °C with a constant flow rate of 0.75 µL/min. The mobile phases were (A) water with 2% ACN and 0.1% FA and (B) ACN with 0.1% FA. The loading was done with 2% B over 5 min. The separation was performed by a linear gradient from 8% B to 30% B over 35 min. B was ramped up from 2% to 8% before separation on a linear gradient from 8% to 30%. A full MS scan in the mass range of 375 to 1200 m/z was acquired at a resolution of 120,000. The precursor ions were isolated using a quadrupole isolation window of 1.6 m/z and fragmented using higher-energy collision dissociation (HCD) with a normalized collision energy of 28. Fragmented ions were dynamically added to an exclusion list for 15 s.
The conversion of the raw MS/MS data output was conducted in MaxQuant (v. 1.6.12.0). The raw data were searched against a UniProt homo sapiens proteome database, and LFQ was used to quantify and allow for comparison of protein intensities between different samples. Further data analysis and statistical calculations were performed in Perseus (v. 1.6.12.0).

Results

To ensure more comparable results between pre- and post-treatment samples, only paired patient samples were used for the data analysis. All measurements were tested for normality with a histogram (*see appendix*) and screened for outliers with Pearson's correlations with all values being over 0.875 (*see appendix*), boxplots, and principal component analyses (PCAs) (*figures 9, 10, 16, and 18*). This allowed for the use of paired students t-tests to identify significantly regulated amounts of cfDNA, proteins, and molecules ($p<0.05$) between the two groups.

Indication of inflammatory status with cfDNA

The output from the dsDNA assay was a fluorescent absorbance measurement for each sample which was correlated to a standard curve to determine the amount of cfDNA in the samples in ng/mL.
To enroll higher confidence in the measurements and following analysis they were done in technical duplicates and only values varying less than 5% between these were used for further analysis. The average values of the duplicates were used to make boxplots for both OA synovial fluid- and OA serum samples before and after treatment with gold microparticles (*see figures 9 and 10*). The average amount of cfDNA decreased following treatment in both the synovial fluid and in the serum. However, this was not a significant drop ($p>0.05$) as determined with a paired student's t-test (*see tables 4 and 5*).

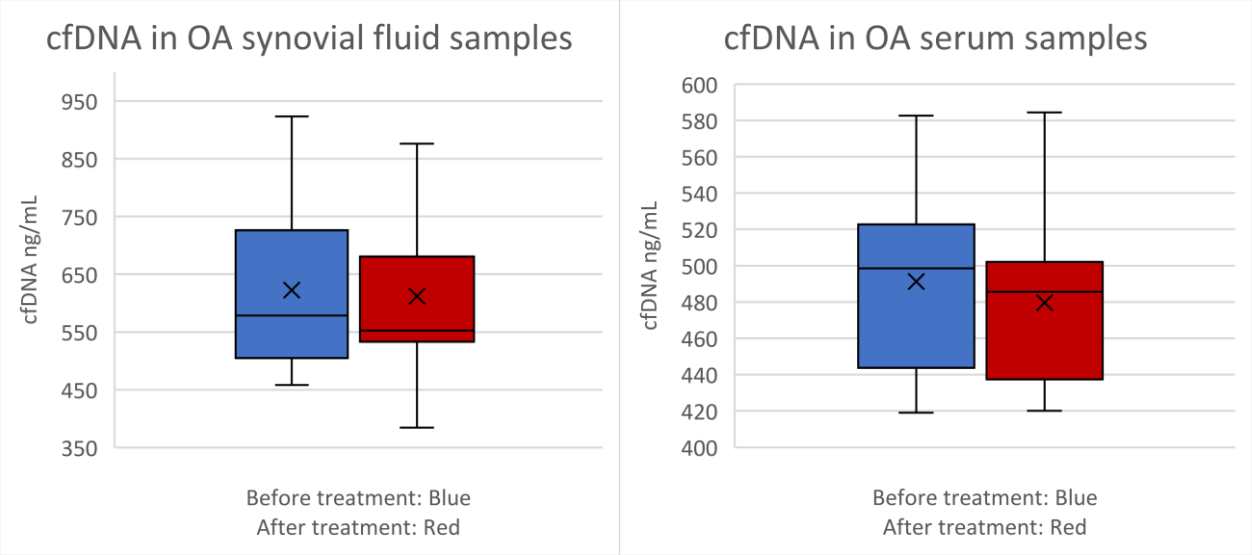

Figure 9 and 10 *Showing sample values expressed as boxplots with averages illustrated as an X. Blue= before treatment, Red= After treatment.*

Synovial fluid		Serum	
Average before	622,6 ng/mL	Average before	491,3 ng/mL
Average after	611,9 ng/mL	Average after	479,6 ng/mL
P-value	0,713	P-value	0,062

Tables 4 and 5 *Averages of all OA samples and their corresponding p-values.*

The same assay and workflow were utilized to make cfDNA measurements for each RA serum sample at both baseline, 3 months-, 6 months-, and 12 months post treatment. The baseline cfDNA measurements was tested against each post-treatment timepoints with paired student's t-tests. This showed that a significant drop ($p<0.05$) in cfDNA was not evident until six months post treatment of RA with etanercept, and that the drop continued to at least 12 months post treatment (*see figure 11 and table 6*).

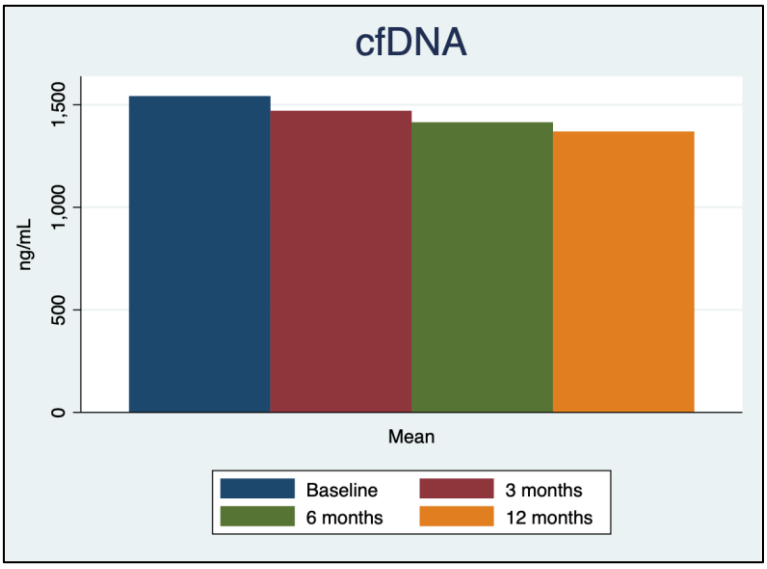

Figure 11 *Showing cfDNA measurements expressed as a bar chart illustrating the continuous fall in cfDNA following treatment of RA with etanercept.*

RA serum p-values	
Baseline vs 3 months	0,0745
Baseline vs 6 months	0,0064
Baseline vs 12 months	0,0126

Table 6 *Averages of all RA samples and their corresponding p-values.*

These results are seen as a slowly progressing decease of the immune response in the RA patients treated with etanercept. The slowly declining cfDNA concentrations suggest that 8 weeks of gold treatment might not be enough time to see a significant cfDNA drop in the OA samples. An observation to back this up is the fact that a slight tendency towards a drop was seen in both OA serum and synovial fluid, which emphasizes the need for a longer test period and further research.

Low abundance immunomodulatory molecules identified by PEA

Out of the 96 proteins tested for in the PEA, 24 molecules were significantly regulated ($p<0.05$) (all downregulated), following treatment of knee OA with gold microparticles (*see figure 12*). These results are made from paired synovial fluid samples before and after treatment, all results with values below the manufacturer stated limit of detection (LOD) have been filtered out along with results that did not pass PEA quality control. Represented in figure 8 below are 12 of the 24 significantly downregulated molecules. These were found to be directly involved in either inflammation, arthritis, and/or regulated by NF-kB. The rest are available in the appendix and are involved in chemotaxis, growth factors, immune modulators amongst others.

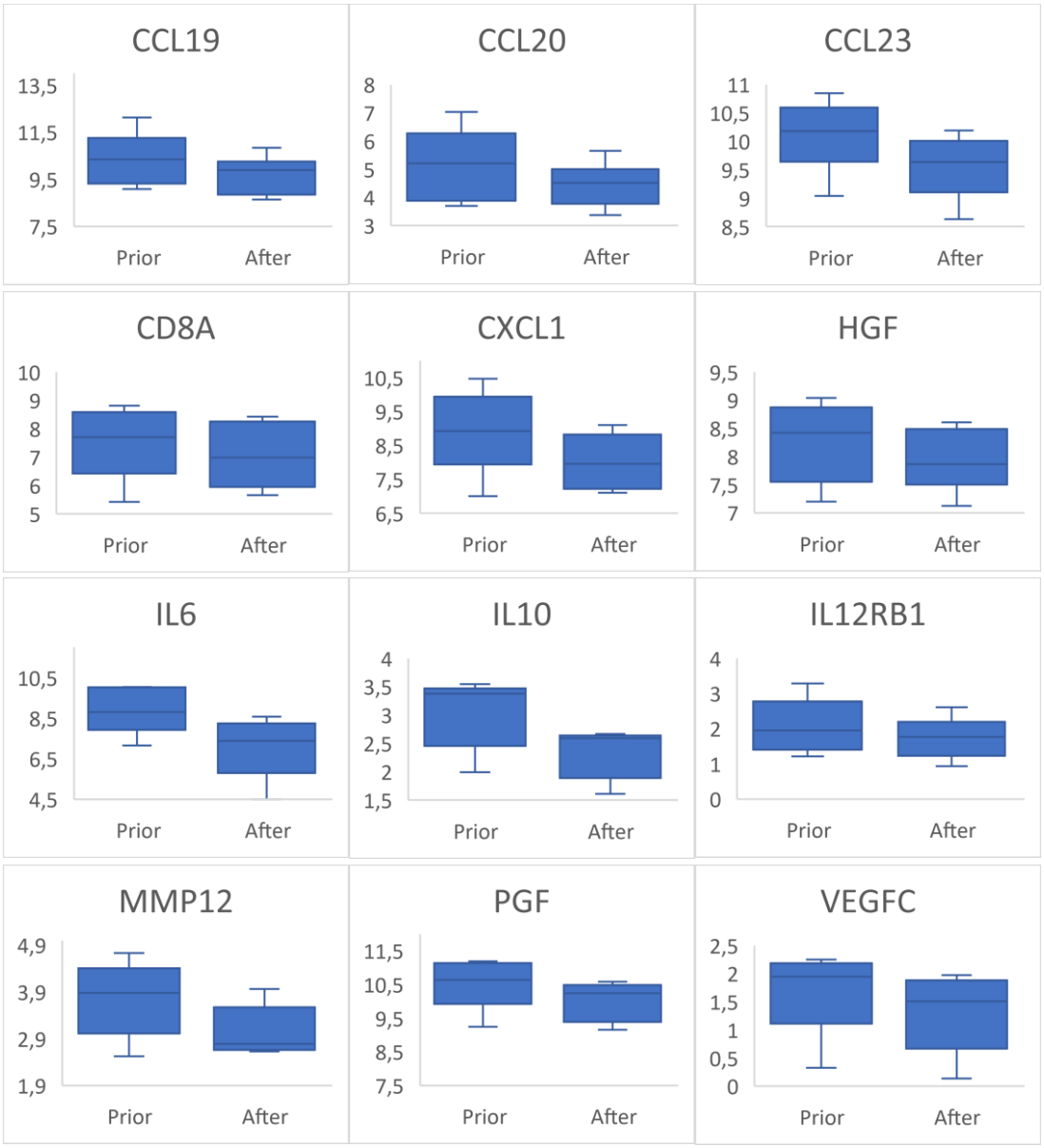

Figure 12 *Boxplots showing 12 of the 24 significantly regulated proteins found in synovial fluid following gold microparticle treatment of knee OA. The amounts on the Y-axis are relative and cannot directly be correlated to an absolute amount (figure continued in appendix).*

To determine if the regulated proteins have any known interactions with each other, a string analysis and diagram was made using string-db.org with a multiple protein entry (*see figure 13*). This showed a tight correlation and interaction between most of the 24 proteins with multiple connections to several NF-kB regulated proteins such as IL-6, IL-10, HGF, and CCL20.

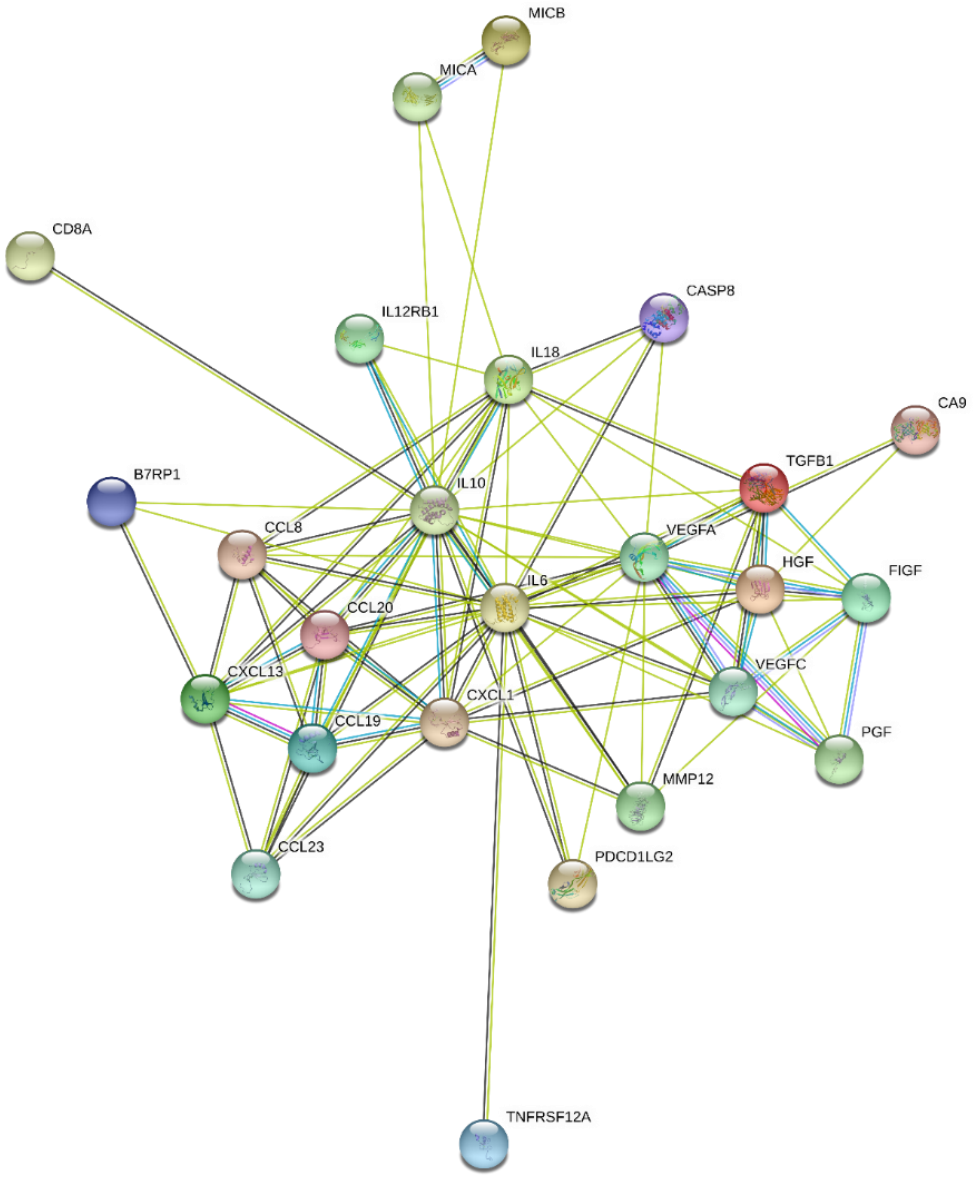

Figure 13 *String diagram showing tight correlation and interaction between most of the regulated proteins. The image illustrates that the identified molecules have very high correlations to IL6 and IL10, which are know o be some of the major inflammatory drivers in the OA pathogenesis.*

Furthermore, a functional enrichment analysis was made of the 24 proteins to determine their collected correlation to gene ontology (GO) biological processes, GO molecular function, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (*see table 7*). This revealed that 21 of the 24 proteins were involved in regulation of the immune system, 20 proteins functioned as receptor binding signaling molecules, and that 6 proteins were associated with RA. Many of the proteins also showed to be involved in cellular migration, especially of leukocytes, and display cytokine activity.

Biological Process (GO)			
GO-term	*description*	*count in gene set*	*false discovery rate*
GO:0002684	positive regulation of immune system process	19 of 882	9.08e-18
GO:0002682	regulation of immune system process	21 of 1391	9.08e-18
GO:0002687	positive regulation of leukocyte migration	11 of 127	3.96e-15
GO:0010469	regulation of signaling receptor activity	15 of 577	1.71e-14
GO:0032101	regulation of response to external stimulus	15 of 732	3.58e-13
Molecular Function (GO)			
GO-term	*description*	*count in gene set*	*false discovery rate*
GO:0005126	cytokine receptor binding	15 of 272	7.63e-20
GO:0048018	receptor ligand activity	15 of 458	7.26e-17
GO:0005102	signaling receptor binding	20 of 1513	9.98e-17
GO:0005125	cytokine activity	11 of 216	3.83e-14
GO:0008083	growth factor activity	9 of 160	8.07e-12
KEGG Pathways			
pathway	*description*	*count in gene set*	*false discovery rate*
hsa04060	Cytokine-cytokine receptor interaction	15 of 263	3.42e-20
hsa05323	Rheumatoid arthritis	6 of 84	5.96e-08
hsa05144	Malaria	5 of 47	1.67e-07
hsa05200	Pathways in cancer	9 of 515	2.08e-07
hsa05321	Inflammatory bowel disease (IBD)	5 of 62	3.69e-07

Table 7 *The five most significant association of the 24 proteins in respect to their GO biological processes, GO molecular function, and KEGG pathways.*

The finding of several downregulated proteins directly influenced by the NF-kB pathway (*see figure 12*) is a strong argument that treatment of knee OA with solid gold, in fact does inhibit this pathway like gold salts have been shown to. This is further supported by a tight interaction between the identified molecules and their close association to regulation of the immune system as seen in table 7 above.

Proteomic analysis by label-free quantitative discovery analysis

The raw orbitap data output was searched against a validated uniport human proteome database (5640_9606) in MaxQuant (1.6.12.0) to extract the identified proteins. These were then further processed and analyzed with the analytical software Perseus (1.6.12.0). The same general analytical filtration workflow was executed in the processing of all three datasets to allow comparison between sets (*see figure 14*). To ensure that only values and IDs of high confidence were used for further analysis a series of filtration steps was carried out. This eliminates peptides identified in reverse, only identified by site, proteins only identified by a single peptide, and proteins present in less than 50% of all samples.

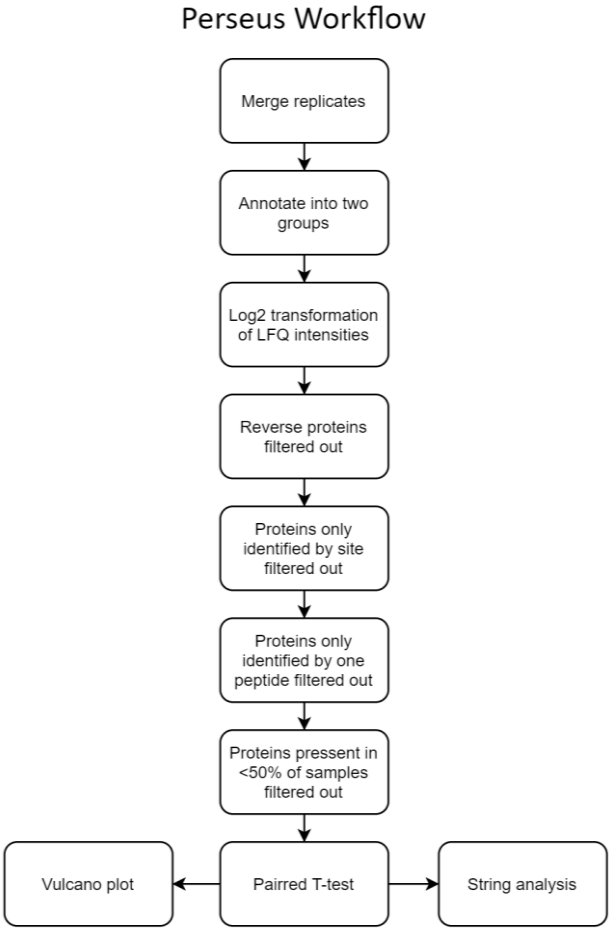

Figure 14 *Analytical filtration workflow for statistical analysis of the MaxQuant output in Perseus.*

A total of 449 different proteins were identified across all OA synovial fluid samples and fractions. After following the predetermined analytical filtration workflow (*see figure 14*) the total number of proteins used for further analysis were 217. Out of these, 16 were significantly regulated ($p<0.05$), 5 downregulated and 11 upregulated (*see table 8*), following eight weeks of gold treatment.

Protein name	Gene name	P-value	Log(diff)	Regulation	Fold change	Reg. In %
Cartilage oligomeric matrix protein	COMP	0.0369	0.1627	Up	1.1194	11.94
Catalase	CAT	0.0055	-0.8357	Down	0.5603	-43.97
Complement C1q subcomponent subunit B	C1QB	0.0004	0.3757	Up	1.2975	29.75
Complement C1q subcomponent subunit C	C1QC	0.0358	0.1771	Up	1.1306	13.06
Complement C3	C3	0.0362	0.0591	Up	1.0418	4.18
Complement C4-A	C4A	0.0491	-0.1394	Down	0.9079	-9.21
EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	0.0201	0.2122	Up	1.1585	15.85
Galectin-3-binding protein	LGALS3BP	0.0402	0.3003	Up	1.2314	23.14
Ig kappa chain V-I region Gal	IGKV1-17	0.0295	0.2465	Up	1.1863	18.63
Ig kappa chain V-III region B6	IGKV3-20	0.0181	0.1403	Up	1.1021	10.21
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	0.0138	0.2055	Up	1.1531	15.31
Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	0.0261	0.1062	Up	1.0764	7.64
Keratin, type II cytoskeletal 5	KRT5	0.0417	-0.9521	Down	0.5169	-48.31
Stromelysin-1	MMP3	0.0071	-1.1206	Down	0.4599	-54.01
Vimentin	VIM	0.0104	-0.3491	Down	0.7851	-21.49
Vitamin K-dependent protein S	PROS1	0.0033	0.1653	Up	1.1214	12.14

Table 8 *The 16 significantly regulated proteins eight weeks post treatment found in synovial fluid of knee OA patients.*

These proteins were regulated between -54,01% to 29,75% which corresponds to a fold change between 0,46 and 1,3 as seen in table 8.

In the significantly regulated protein from table 8, there are some indication of a locally decreased inflammation in the synovial joint, by the regulation of a few acute-phase proteins (APPs) in the form of complement factors and these will be further investigated in the *discussion* section below. But no clear picture is evident, as other common APPs like c-reactive protein were not identified by PEA to be significantly regulated. However, many APPs are produced as a response to some of the cytokines found to be significantly downregulated, especially IL-6. This could again suggest that not enough time have passed from collection of baseline and treatment samples. Some of the regulated proteins from table 8, does however suggest a positive effect on OA pathology, such as a downregulation of stromelysin (MMP3) by more than 50%. This could suggest a decrease of the catabolic environment in the knee joint as stromelysin in the synovial fluid have been found by other studies to be significantly higher in patients with both OA and RA and is associated with increased joint destruction[57,58]. Further investigation of the regulated proteins revealed that four proteins are directly controlled by the NF-kB pathway. These are C3, C4A, KRT5, and VIM and their role will also be further evaluated in the *discussion* section further down.

The identified proteins are visualized in figure 15 as a volcano plot. This spreads the most up- and downregulated proteins furthest form the middle of the X-axis and places regulated proteins with the lowest p-value highest on the Y-axis.

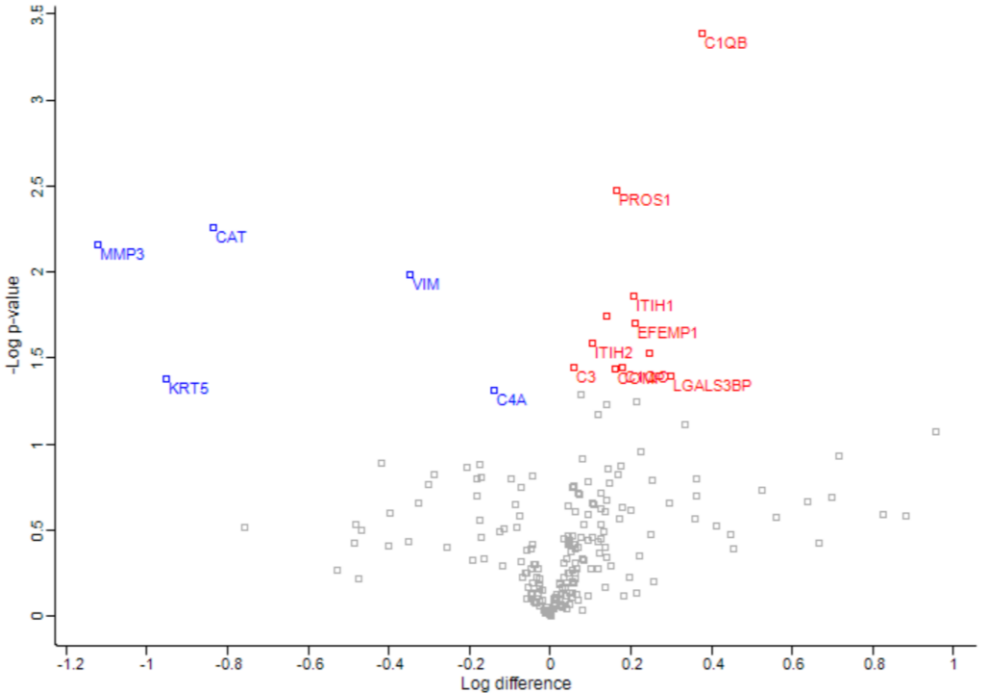

Figure 15 *Volcano plot showing significantly upregulated proteins, found in synovial fluid of knee OA patients, in red and significantly downregulated proteins in blue. X axis shows the logarithmic difference between the two groups and the Y axis is the negative logarithmic p-value calculated by a paired t-test. Each point shows gene name.*

To look for major proteomic changes between the sample populations, a PCA plot was made (*see figure 16*). This reduces the statistical complexity of the data, allowing for a simplified visualization of the individual sample's and their relation to each other. Both populations (before = blue, after = red) are represented as a homogenous mix and no clear differentiation happens following eight weeks of gold treatment. This indicates that the changes identified by MS are not large or specific enough to tell the two groups apart based on identified proteins and LFQ-intensities alone.

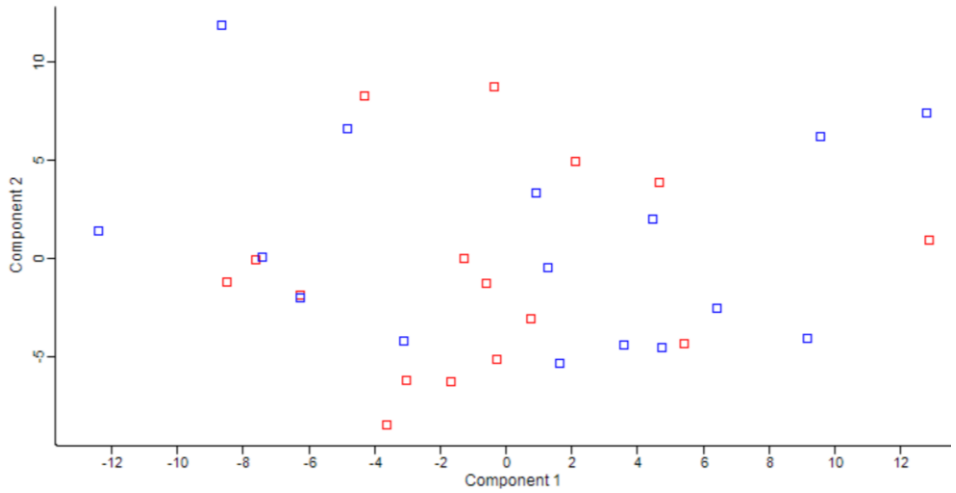

Figure 16 *PCA plot of synovial fluid samples. Blue=before treatment, red=after treatment. Illustrating that the gold treatment did not affect the sample population enough to separate treated and untreated individuals into two groups.*

As mentioned above, all OA serum data was treated using the same general statistical workflow as for OA synovial fluid.
A total of 352 proteins were identified across all OA serum samples and corresponding fractions. 227 of these were used for further analysis following the filtration steps from figure 14 above. A paired t-test reviled that six proteins had been significantly regulated following the eight weeks of gold treatment (*see table 9*). Out of these six proteins, five were downregulated and one was upregulated with fold changes between 0.6 and 1,18, correlating to a regulation between -40,34% and 17,65% as seen in table 9.

Protein name	Gene name	p-value	log(diff)	Regulation	Fold change	Reg. In %
Apolipoprotein C-III	APOC3	0,0317	0,2345	Up	1,1765	17,65
Centromere protein F	CENPF	0,0003	-0,7451	Down	0,5966	-40,34
Extracellular matrix protein 1	ECM1	0,0065	-0,5553	Down	0,6805	-31,95
Ig heavy chain V-II region ARH-77	IGHV4-61	0,0326	-0,2844	Down	0,8211	-17,89
Ig kappa chain V-II region Cum	IGKV2-40	0,0002	-0,4675	Down	0,7232	-27,68
Phospholipid transfer protein	PLTP	0,0225	-0,4028	Down	0,7564	-24,36

Table 9 *The 6 significantly regulated proteins eight weeks post treatment found in serum of knee OA patients.*

Out if the six significantly regulated proteins found in OA serum, apolipoprotein C-III, is another protein directly regulated by the NF-kB pathway. However, this protein was found to be downregulated 17.65%. This might be due to the fact that APOC3 mainly is a hepatically produced protein, and that the gold ions liberated from the particles in the knee is unable to exert their effects over long distance and mainly induces effects locally. This is further backed up by the small number of significantly regulated serum proteins following gold treatment. However, it is hard to draw any conclusions based on such a small number of regulated proteins, and this is just speculations.
To visualize the identified serum proteins in respect to magnitude of change and p-values, a volcano plot was made (*see figure 17*).

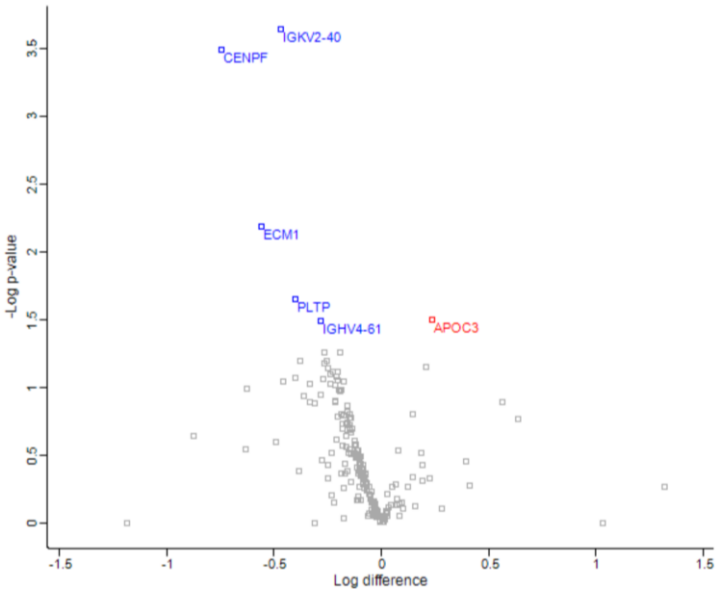

Figure 17 *Volcano plot showing significantly upregulated proteins, found in serum of knee OA patients, in red and significantly downregulated proteins in blue. X axis shows the logarithmic difference between the two groups and the Y axis is the negative logarithmic p-value calculated by a paired t-test. Each point shows gene name.*

A PCA was also made for the OA serum samples as shown in figure 18 below. But as for the synovial fluid, neither here was a separation evident for the two groups. This suggests, as before the synovial fluid samples, that the changes identified by MS are not large or specific enough to tell the two groups apart based on identified proteins and LFQ-intensities alone.

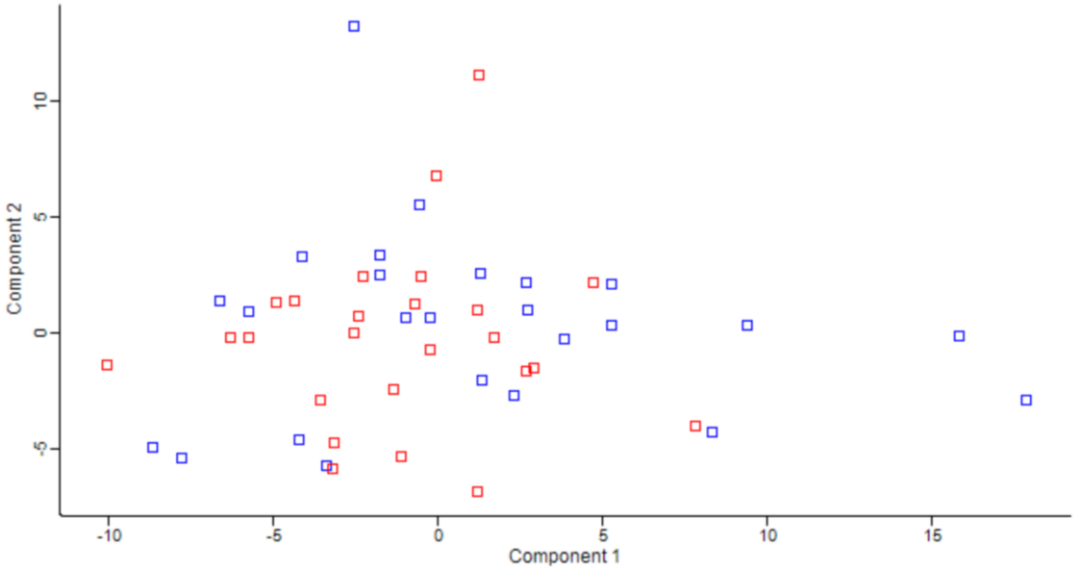

Figure 18 *PCA plot of OA serum samples. Blue=before treatment, red=after treatment. Illustrating that the gold treatment did not affect the sample population enough to separate treated and untreated individuals into two groups.*

Based on the collective MS data and analysis hereof, there is no signs of major proteomic changes following gold treatment as illustrated by the two PCA plots (*see figures 16 and 18*). However, several NF-kB associated proteins did show a significant decrease in the synovial fluid samples, but not in serum. That coupled with a regulation of a small number of APPs indicates that eight weeks of gold treatment did initiate some changes in respect to decrease NF-kB expression and inflammation. But more research is needed to determine how the effects will unfold following a longer treatment period.
To evaluate if gold induces any proteomic changes as similar to the anti-inflammatory treatment with etanercept of RA, 18 sets of 4 serum samples were analyzed following the same analytical strategy as for all OA samples (*see figure 14*). After these filtration steps and paired t-tests, 33 proteins were significantly regulated after 12 weeks of treatment and 40 proteins after 52 weeks (*see appendix*). These proteins and the RA cfDNA results, along with knowledge about RA pathology and the results from OA samples will be used to discuss the basis of a possible novel treatment option of RA with gold particles in the next section below. This will not be an analysis of etanercepts effect as a treatment for RA, but only used to draw parallels between the changes seen in OA and RA samples.

Discussion

Gold as a novel treatment option for OA

Treatment of OA is usually systemic, with possible severe systemic side effects such as gastrointestinal bleeding in more than 10% of long term NSAID treated patients[27], and a local treatment would therefore be to prefer. Local intraarticular injections with solid gold microparticle implants have shown positive and significant clinical effects on both pain, stiffness, and joint movement in more than 80% of the test subjects[32]. A few studies have shown that macrophages are able to release charged and biological active gold ions from the solid gold particles[34,47,48]. Following liberating these ions are taken up by tissue resident macrophages and fibroblasts and decrease inflammation via inhibition of several proteins and pathways like NF-kB, AP1, COX, and TRX[31,33,49,50]. This study found evidence of these changes in the decrease of several proteins and signaling molecules related to the before mentioned pathways. Furthermore, the bio-released gold ions are thought not to leave the periarticular environment due to the slow release and rapid uptake by nearby cells, and systemic effects should therefore be minimal. This is supported by the lack of proteomic change found in OA serum samples in this study. However, this is only based on 8 weeks of treatment, and gold ion measurements have not been made on either urine, or blood. The size of the implants and the rate of ion-release suggests that only a single injection is needed to supply intraarticular ant-inflammatory treatment for an extended period of time. The fact that the implants are solid and local also means that there is a possibility to remove them with an arthroscopic approach if any complications should arise.

Proteomic effects of gold

To interpret the results from this study, all identified significantly regulated proteins was systematically investigated for their relation to arthritis, inflammation, and NF-kB, if any. This was done to elucidate positive and negative proteomic changes in respect to gold treatment.

Proteomic evidence that supports inhibition of NF-kB was found in the downregulation of several NF-kB related molecules such as IL6, IL10, and some CCLs identified with PEA and mass spectrometry analysis further identified several regulated proteins with connection to NF-kB (*see table 10*). The significant downregulation of IL6 is especially interesting as this is thought to be one of the most important inflammatory molecules and are related to most parts of OA pathogenesis[58]. The lack of a clear separation between the pre- and post-treatment groups in a PCA plot, could be due to the fact that only eight weeks was allowed to pass before collecting the post samples. A study by *Yanni et al*[59] found that intramuscular gold decreased cytokine expression following 12 weeks, but not 2 weeks. Another study by *Lehman et al*[60] found that the effects of intramuscular gold therapy didn't reach a treatment plateau until after 24-48 weeks, and that the patients in the study would keep improving their American college of rheumatology (ACR) score far beyond 8 weeks post treatment. This suggests that the post-samples from this study might have been taken to early, and the full effect of the gold treatment might not yet have taken place. The short treatment span might also aid as an explanation on why only a few OA and/or inflammation associated regulations were seen in the most abundant peptides in the biological samples identified with MS. Out of the 22 significantly regulated protein from the OA biological samples, six were found to be directly regulated by the NF-kB pathway (*see table 10)*.

Protein name	Identified in	Regulation following gold treatment
Apolipoprotein C-III	Serum	Up
Complement C3	Synovial fluid	Up
Complement C4-A	Synovial fluid	Down
Keratin, type II cytoskeletal 5	Synovial fluid	Down
Vimentin	Synovial fluid	Down

Table 10 *Six different proteins directly influenced by NF-kB.*

Apolipoprotein C-III (APOC3) have been shown to be both anti-inflammatory and pro-inflammatory via NF-kB activation in several animal models[55,61]. This makes its role in this setting difficult to pinpoint and no conclusions are therefore made on the basis of an upregulated presence of APOC3. The two identified NF-kB related complement factors will be discussed further down in the biomarker section. Vimentin is an intermediate cytoskeletal associated protein contributing to cell stiffness, especially in mesenchymal cells such as chondrocytes[62]. Increased levels of vimentin have been shown to be released following joint injury[62], and the significant decrease of vimentin found in this study therefore suggests less joint and chondrocyte destruction following gold treatment. The last identified protein, keratin 5, is mostly found in the epidermis and is thought to be due to contamination and was also marked by MaxQuant as such. Other identified proteins of interest were MMP12 and MMP3 as these are able to enzymatically degrade the articular tissue and contribute to OA pathogenesis. Both of these were identified in the synovial fluid and both in decreased amounts following gold treatment.
One protein associated with a negative effect of the gold treatment is cartilage oligomeric matrix protein (COMP). This was upregulated by 11.94% and have been shown by several studies to be related to OA severity and linked to cartilage degeneration[58].

Gold as a novel treatment option for RA

As for OA one of the most important inflammatory pathways in RA pathogenesis is NF-kB and inhibition hereof is therefore the aim of several DMARDs[6]. IL6 is an aim for bDMARDs in RA because it plays an important role in the pathogenesis as it does in OA. The joint destruction in both diseases is caused by the presence og MMPs, which are produced in great extend due to the ongoing inflammations in the joint(s). This inflammation is thought to be one of the main causes of the symptoms shared b OA and RA, such as pain, stiffness, and decreased joint mobility. These similarities between the two pathogeneses arouses some interest into the potential of gold as a treatment for both diseases.
A support of this can be found in the fact that this study found gold to elicit many of the changes that DMARD and bDMARD treatment aims to do, such as decrease MMPs, ILs, and overall immune activity[6]. Another supporting find is a 50% decrease in vimentin expression following OA gold treatment. This could potentially be more significant in an RA setting than in OA as vimentin servers as a target for ACPAs in some seropositive RA patients[6]. A study by *Khan et al* found that the anti-inflammatory effects of gold and its ability to inhibit NF-kB and COX-2 was comparable to that of methotrexate in rats with collagen-induced arthritis[24]. Furthermore have gold salts been used to treat RA but have been replaced by other DMARDs due to their toxicity, but with a local treatment of the affected joint(s), gold could possibly be used to treat RA again. However, the extend, potency and full effect of solid gold is not known yet, and there could be long term side effects matching those of gold salts.

Biomarkers

A study done by *Ritter et al*[63] carried out a proteomic analysis of synovial fluid form knee OA patients and age-matched control subjects and identified differently expressed peptides between the two groups. The proteomic investigation of the knee OA samples from this current study identified some of the same peptides, but some were regulated in the opposite direction, suggesting a revers back towards a healthy expression of these proteins (*see table 11*).

Protein name	Regulation from healthy to OA	Regulation from OA to gold treated
Complement C1q subcomponent subunit C	Downregulated 1.49-fold	Upregulated 1.13-fold
Complement C4-A	Upregulated 1.46-fold	Downregulated 0.91-fold

Table 11 *Proteins regulated in opposite direction than from a healthy to an OA phenotype.*

This makes these two proteins possible candidates as biomarkers for the effect of an OA gold treatment on a personalized treatment level. However, a targeted search for these peptides and possibly a larger sample size would be needed to confirm this. Complement C1q subcomponent C is one of three subcomponents (A, B, C) that form the initiator of the classical component cascade, C1q. The fact that this protein is downregulated from healthy to OA can be seen as an activation of the cascade with more C1q molecules being bound to their targets and thereby detected in less abundance. Which means that an increase of C1qc could suggest less complement activation and more inactive C1q in the synovial fluid samples. This is further backed up by the second peptide identified to be regulated in the opposite direction from healthy to OA, complement C4-a. This is one of two products (C4a and C4b) from the enzymatic cleavage of complement component C4. A decreased amount of C4a following gold treatment could therefore also suggest a significantly lower complement activation as less C4 is enzymatically cleave. Stromelysin-1 (MMP3) was the synovial fluid protein with the biggest regulation with a decrease of over 50%. This protein is regulated by NF-kB and associated with joint damage which make it a strong candidate for a biomarker of gold treatment effect.

Conclusion

This study found proteomic evidence supporting the positive clinically observed effects of gold. Though no major changes were observed with MS and PCA, the fact that all identified molecules by PEA was significantly downregulated and several had direct associations with OA, inflammation, immune modulation, and NF-kB. This also supports the theory from the study by *Rasmussen et al*, that the mechanism behind these changes I due to gold ion inhibition of NF-kB. A theory that also finds support in the literature regarding the effects of other gold compounds, and macrophage interaction with gold microparticles. This novel treatment is likely local, as no major changes were observed outside the joint, which would strongly diminish the systemic side effects experienced with standard treatments for arthritis and gold salt compounds. However, 8 weeks is no enough time to see the full extend and effects of the gold treatment, and more research is therefore needed.

References

1. Arthritis Types | CDC. https://www.cdc.gov/arthritis/basics/types.html.
2. Jafarzadeh, S. R. & Felson, D. T. Updated estimates suggest a much higher prevalence of arthritis in US adults than previous ones HHS Public Access. *Arthritis Rheumatol* **70**, 185–192 (2018).
3. Arthritis Foundation. *Arthritis by the Numbers*. *Arthritis Foundation* https://www.arthritis.org/Documents/Sections/About-Arthritis/arthritis-facts-stats-figures.pdf (2017).
4. Man, G. S. & Mologhianu, G. Osteoarthritis pathogenesis - a complex process that involves the entire joint. *Journal of medicine and life* **7**, 37–41 (2014).
5. Aletaha, D. & Smolen, J. S. Diagnosis and Management of Rheumatoid Arthritis: A Review. *JAMA - Journal of the American Medical Association* vol. 320 1360–1372 (2018).
6. Guo, Q. *et al.* Rheumatoid arthritis: Pathological mechanisms and modern pharmacologic therapies. *Bone Research* vol. 6 (2018).
7. Xia, B. *et al.* Osteoarthritis pathogenesis: a review of molecular mechanisms. *Calcified tissue international* **95**, 495–505 (2014).
8. Kohn, M. D., Sassoon, A. A. & Fernando, N. D. Classifications in Brief: Kellgren-Lawrence Classification of Osteoarthritis. *Clinical orthopaedics and related research* **474**, 1886–93 (2016).
9. Heidari, B. Knee osteoarthritis diagnosis, treatment and associated factors of progression: part II. *Caspian journal of internal medicine* **2**, 249–55 (2011).
10. Goldring, M. B. & Otero, M. Inflammation in osteoarthritis. (2011) doi:10.1097/BOR.0b013e328349c2b1.
11. Houard, X., Goldring, M. B. & Berenbaum, F. Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. *Current rheumatology reports* **15**, 375 (2013).
12. Sophia Fox, A. J., Bedi, A. & Rodeo, S. A. The basic science of articular cartilage: structure, composition, and function. *Sports health* **1**, 461–8 (2009).
13. Silawal, S., Triebel, J., Bertsch, T. & Schulze-Tanzil, G. Osteoarthritis and the Complement Cascade. *Clinical medicine insights. Arthritis and musculoskeletal disorders* **11**, 1179544117751430 (2018).
14. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor perspectives in biology* vol. 1 (2009).
15. *NF-B STRUCTURE AND PRINCIPAL FEATURES*. www.liebertpub.com.
16. Zhang, H. & Sun, S. C. NF-KB in inflammation and renal diseases. *Cell and Bioscience* vol. 5 (2015).
17. Katsuyuki, M. *et al.* Two different cellular redox systems regulate the DNA-binding activity of the p50 subunit of NF-κB in vitro. *Gene* (1994) doi:10.1016/0378-1119(94)90005-1.

18. Gromer, S., Urig, S. & Becker, K. The thioredoxin system?From science to clinic. *Medicinal Research Reviews* **24**, 40–89 (2004).

19. Yang, J.-P. *et al.* Inhibition of the DNA-binding activity of NF-tcB by gold compounds in vitro. *FEBS 15229 FEBS Letters* **361**, 96 (1995).

20. Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy* vol. 2 (2017).

21. Clutterbuck, A. L. *et al.* High throughput proteomic analysis of the secretome in an explant model of articular cartilage inflammation. *Journal of Proteomics* **74**, 704–715 (2011).

22. Joo Weon Lim, Kim, H. & Kyung Hwan Kim. Nuclear factor-κB regulates cyclooxyoenase-2 expression and cell proliferation in human gastric cancer cells. *Laboratory Investigation* **81**, 349–360 (2001).

23. Ricciotti, E. & Fitzgerald, G. A. Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **31**, 986–1000 (2011).

24. Khan, M. A. & Khan, M. J. Nano-gold displayed anti-inflammatory property via NF-kB pathways by suppressing COX-2 activity. *Artificial Cells, Nanomedicine, and Biotechnology* **46**, 1149–1158 (2018).

25. Ye, N., Ding, Y., Wild, C., Shen, Q. & Zhou, J. Small molecule inhibitors targeting activator protein 1 (AP-1). *Journal of Medicinal Chemistry* vol. 57 6930–6948 (2014).

26. Brostrøm, S. *Knæartrose - nationale kliniske retningslinjer og faglige visitationsretningslinjer*. *Sundhedsstyrelsen* (2012).

27. pro.medicin.dk – information om medicin. https://pro.medicin.dk/.

28. Crofford, L. J. Use of NSAIDs in treating patients with arthritis. *Arthritis Research and Therapy* vol. 15 S2 (2013).

29. Cohen, M. M. Role of endogenous prostaglandins in gastric secretion and mucosal defense. *Clinical and Investigative Medicine* vol. 10 226–231 (1987).

30. van der Velden, V. H. J. *Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma*. (1998).

31. Berners-Price, S. J. & Filipovska, A. Gold compounds as therapeutic agents for human diseases. *Metallomics* **3**, 863 (2011).

32. Rasmussen, S. ;, Petersen, K., Kjaer, ; & Arendt-Nielsen, L. *Aalborg Universitet Intra-articular metallic gold micro particles relieve pain and enhance function in patients with knee osteoarthritis A pilot study*. https://clinicaltrials.gov/ct2/show/NCT03389906.

33. Larsen, A. *et al.* Gold ions bio-released from metallic gold particles reduce inflammation and apoptosis and increase the regenerative responses in focal brain injury. *Histochemistry and Cell Biology* **130**, 681–692 (2008).

34. Seifert, O., Matussek, A., Sjögren, F., Geffers, R. & Anderson, C. D. Gene expression profiling of macrophages: implications for an immunosuppressive effect of dissolucytotic gold ions. *Journal of inflammation (London, England)* **9**, 43 (2012).

35. Firestein, G. S. & McInnes, I. B. Immunopathogenesis of Rheumatoid Arthritis. *Immunity* vol. 46 183–196 (2017).

36. Takemura, S., Klimiuk, P. A., Braun, A., Goronzy, J. J. & Weyand, C. M. T Cell Activation in Rheumatoid Synovium Is B Cell Dependent. *The Journal of Immunology* **167**, 4710–4718 (2001).

37. Malmström, V., Catrina, A. I. & Klareskog, L. The immunopathogenesis of seropositive rheumatoid arthritis: From triggering to targeting. *Nature Reviews Immunology* vol. 17 60–75 (2017).

38. Rantapää Dahlqvist, S. & Andrade, F. Individuals at risk of seropositive rheumatoid arthritis: the evolving story. *Journal of Internal Medicine* vol. 286 627–643 (2019).

39. Derksen, V. F. A. M., Huizinga, T. W. J. & van der Woude, D. The role of autoantibodies in the pathophysiology of rheumatoid arthritis. *Seminars in Immunopathology* vol. 39 437–446 (2017).

40. Demoruelle, M. K., Deane, K. D. & Holers, V. M. When and Where Does Inflammation Begin in Rheumatoid Arthritis? doi:10.1097/BOR.0000000000000017.

41. Noris, M. & Remuzzi, G. Overview of complement activation and regulation. *Seminars in Nephrology* **33**, 479–492 (2013).

42. van Venrooij, W. J., van Beers, J. J. B. C. & Pruijn, G. J. M. Anti-CCP antibodies: The past, the present and the future. *Nature Reviews Rheumatology* vol. 7 391–398 (2011).

43. Qi, L., Zhang, X. & Wang, X. Heparin inhibits the inflammation and proliferation of human rheumatoid arthritis fibroblast-like synoviocytes through the NF-κB pathway. *Molecular Medicine Reports* **14**, 3743–3748 (2016).

44. Shiozawa, S. & Tsumiyama, K. Pathogenesis of rheumatoid arthritis and c-Fos/AP-1. *Cell Cycle* vol. 8 1539–1543 (2009).

45. Köhler, Günther, Kaudewitz & Lorenz. Current Therapeutic Options in the Treatment of Rheumatoid Arthritis. *Journal of Clinical Medicine* **8**, 938 (2019).

46. Haraoui, B. & Bykerk, V. Etanercept in the treatment of rheumatoid arthritis. *Therapeutics and Clinical Risk Management* vol. 3 99–105 (2007).

47. Danscher, G. In vivo liberation of gold ions from gold implants. Autometallographic tracing of gold in cells adjacent to metallic gold. *Histochemistry and Cell Biology* **117**, 447–452 (2002).

48. Larsen, A., Stoltenberg, M. & Danscher, G. In vitro liberation of charged gold atoms: autometallographic tracing of gold ions released by macrophages grown on metallic gold surfaces. *Histochemistry and Cell Biology* **128**, 1–6 (2007).

49. Faa, G., Gerosa, C., Fanni, D., Lachowicz, J. I. & Nurchi, V. M. Gold - Old Drug with New Potentials. *Current Medicinal Chemistry* **25**, 75–84 (2018).

50. Handel, M. L., Watts, C. K. W., Defazio, A., Dayt, R. O. & Sutherland, R. L. *Inhibition of AP-1 binding and transcription by gold and selenium involving conserved cysteine residues in Jun and Fos*. *Pharmacology* vol. 92 (1995).

51. Bennike, T. B. *et al.* A Cost-Effective High-Throughput Plasma and Serum Proteomics Workflow Enables Mapping of the Molecular Impact of Total Pancreatectomy with Islet Autotransplantation. *Journal of Proteome Research* **17**, 1983–1992 (2018).

52. Bekker-Jensen, D. B. *et al.* An Optimized Shotgun Strategy for the Rapid Generation of Comprehensive Human Proteomes Article An Optimized Shotgun Strategy for the Rapid Generation of Comprehensive Human Proteomes. *Cell Systems* **4**, 587-599.e4 (2017).

53. Giordano, R., Petersen, K. K., Andersen, H. H., Simonsen, O. & Arendt-Nielsen, L. Serum Inflammatory Markers in Patients With Knee Osteoarthritis. *The Clinical Journal of Pain* **36**, 229–237 (2020).

54. Proximity Extension Assay (PEA) Technology | Olink. https://www.olink.com/data-you-can-trust/technology/.

55. Nguyen, D. N. *et al.* Elevated levels of circulating cell-free DNA and neutrophil proteins are associated with neonatal sepsis and necrotizing enterocolitis in immature mice, pigs and infants. *Innate Immunity* **23**, 524–536 (2017).

56. van der Meer, A. J. *et al.* Systemic inflammation induces release of cell-free DNA from hematopoietic and parenchymal cells in mice and humans. *Blood Advances* **3**, 724–728 (2019).

57. Sasaki, S., Iwata, H., Ishiguro, N., Obata, K. & Miura, T. Detection of stromelysin in synovial fluid and serum from patients with rheumatoid arthritis and osteoarthritis. *Clinical Rheumatology* **13**, 228–233 (1994).

58. Saberi Hosnijeh, F., Bierma-Zeinstra, S. M. & Bay-Jensen, A. C. Osteoarthritis year in review 2018: biomarkers (biochemical markers). *Osteoarthritis and Cartilage* **27**, 412–423 (2019).

59. Yanni, G., Nabil, M., Farahat, M. R., Poston, R. N. & Panayi, G. S. Intramuscular gold decreases cytokine expression and macrophage numbers in the rheumatoid synovial membrane. *Annals of the rheumatic diseases* **53**, 315–22 (1994).

60. Lehman, A. J. *et al.* A 48-week, randomized, double-blind, double-observer, placebo-controlled multicenter trial of combination methotrexate and intramuscular gold therapy in rheumatoid arthritis: Results of the METGO study. *Arthritis & Rheumatism* **52**, 1360–1370 (2005).

61. Luo, M. & Peng, D. The emerging role of apolipoprotein C-III: Beyond effects on triglyceride metabolism. *Lipids in Health and Disease* vol. 15 (2016).

62. Haudenschild, D. R. *et al.* Vimentin contributes to changes in chondrocyte stiffness in osteoarthritis. *Journal of Orthopaedic Research* **29**, 20–25 (2011).

63. Ritter, S. Y. *et al.* Proteomic analysis of synovial fluid from the osteoarthritic knee: Comparison with transcriptome analyses of joint tissues. *Arthritis and Rheumatism* **65**, 981–992 (2013).