The Immunopathology of Rheumatoid Arthritis and Leishmania donovani Infection in Sudan

AMIR I. ELSHAFIE
Immune complexes (IC) and antibody production against self-antigens play a pathological key role in the development of autoimmunity that occurs in patients with parasitic infections and rheumatic diseases. My studies have targeted two groups of patients from Sudan; patients with visceral leishmaniasis (VL) and patients with rheumatoid arthritis (RA).

In VL patients I studied the functional role of IC and IC-induced cytokine production in the pathogenesis of the disease and their effect on kidney functions. For the Sudanese RA cohort, I performed a comparative study with Swedish RA patients. I also investigated the Sudanese RA cohort for the occurrence of RA-associated autoantibodies (rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA)) and the diagnostic and prognostic impact of these autoantibodies on the clinical outcome.

In the VL project, I demonstrated that Sudanese VL patients had elevated serum levels of IC and of IC-induced cytokine levels in vitro. GM-CSF levels were increased in acute VL patients and in VL patients with ongoing sodium stibogluconate treatment, and the only cytokine that correlated to a high degree with circulating IC levels. Cystatin C was shown to be a superior marker of glomerular function as compared to serum creatinine in VL patients. For the RA project, a comparative study was performed in collaboration with the rheumatology unit at Gävle hospital. We concluded that the clinical picture of RA in Sudan was more severe, with more widespread joint involvement and stronger laboratory signs of inflammation when compared to the Swedish RA patients.

ACPA and RF are both included in the new 2010 RA classification criteria. In many RA studies over the world the occurrence of ACPA and RF varies considerably, this may be due to both geographical differences and lack of standardization for RF and anti-CCP in the RA criteria. But in this study we aligned all antibodies to the same diagnostic specificity compared to Sudanese healthy controls. When doing so we determined that IgA RF had the highest diagnostic sensitivity, a finding that differs from Caucasian studies in which IgM RF predominates. IgG RF was also the autoantibody most strongly associated with early age of disease onset and hand deformities, a clinical picture that differs in most Caucasian studies in which ACPA are the strongest markers for bad prognosis. Thus data from this Sudanese RA cohort implies significant clinical and immunological differences compared to Caucasian RA patients.

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بسم الله الرحمن الرحيم

(وَقَلَ اعْمَلُوا فِيٌّ الَّذِيَ خَلَقْنِاهُ وَرَسَوْنِاهُ وَأَمْلِكُونَ) [النّبِيُّ: 105]

To the soul of my father Ibrahim Elshafie!
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*Authors contributed equally.

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Abbreviations

ACR  American College of Rheumatology
ACPA  Anti-citrullinated protein/peptide antibodies
ANOVA  Analysis of variance
Anti-TNFα  Antibody to tumor necrosis factor alpha
Anti-CCP  Anti-cyclic citrullinated peptide/protein
Anti-FcγRII, RIII  Anti-Fc gamma receptor II, III
AU  Arbitrary units
AUC  Area under the curve
BD  Boutonniere deformity
CD  Cluster of differentiation
DMARD  Disease-modifying anti-rheumatic drugs
ELISA  Enzyme-linked immunosorbent assay
EULAR  European League against Rheumatism
EDTA  Ethylenediaminetetraacetic acid
HEPES  Hydroxyethyl piperazineethanesulfonic acid
GFR  Glomerular filtration rate
GM-CSF  Granulocyte macrophage-colony stimulating factor
HLA DRB1 SE  Human leucocyte antigen DRB1 shared epitope
IC  Immune complexes
IU  International unit
IFN-γ  Interferon gamma
IL  Interleukin
IL-1ra  Interleukin-1 receptor antagonist
KD  Kilodalton
LD  Leishmania donovani
LPS  Lipopolysaccharide
PBMC  Peripheral blood mononuclear cells
PEG  Polyethylene glycol
PKDL  Post kala-azar dermal leishmaniasis
RA  Rheumatoid arthritis
RF  Rheumatoid factor
ROC  Receiver operator characteristics
RPMI  Roswell Park Memorial Institute
SND  Swan neck deformity
Th  T helper cell
TNFα  Tumor necrosis factor alpha
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>TNF-Rp75</td>
<td>Tumor necrosis factor receptor p75</td>
</tr>
<tr>
<td>UD</td>
<td>Ulnar deviation</td>
</tr>
<tr>
<td>VL</td>
<td>Visceral leishmaniasis</td>
</tr>
<tr>
<td>ZD</td>
<td>Z deformity</td>
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<tr>
<td>PEST</td>
<td>Penicillin-streptomycin</td>
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Introduction

*Leishmania donovani* infection

*Leishmania donovani* (LD) infection is a disease of public health importance in Sudan and many other tropical countries and is considered to be one of the main health foci in east Africa (1). LD infection is a disease caused by protozoa of the *Leishmania donovani* complex (*L. donovani, L. archibaldi, and L. infantum/chagasi*). *Leishmania*, which are introduced into their human hosts by sandflies, rapidly invade macrophages, where they multiply inside phagolysosomes. Human infections may be asymptomatic (sub-clinical) or may cause a severe visceral disease that is called visceral leishmaniasis (VL) or kala-azar (2-5). The geographical map of VL is gradually changing in Sudan. This may be partly related to the geopolitical and social mass movement of the Sudanese people and partly by the slow spread of the disease to occupy areas not previously known to harbor it (6).

The known endemic areas of VL are the south-eastern borders of Sudan covering an area of about 120 sq. kilometers along the Sudanese-Ethiopian border extending from Kassala in the Far East down to Kapoeta in the South. These areas are savannah and sub-Saharan, characterized by the presence of scattered shrubs of acacia trees punctuated with termite hills, a good habitat for the *Phlebotomus* fly (7).

*Leishmania* life cycle

*Leishmania* spp. are transmitted by the bite of female *Phlebotomus* sandflies. The sandflies inject the infective stage, metacyclic promastigotes, during blood meals (8). During its life cycle the *Leishmania* parasite alternates between two distinct developmental stages. In the mammalian host, the parasite exists as the non-motile amastigote form, which proliferates within the acidic and hydrolase-rich phagolysosomal compartment of host macrophages (9). Transmission of the parasite is mediated by the blood-sucking sandfly, of either the genus *Phlebotomus* (in the Old World) or the genus *Lutzomyia* (in the New World). When feeding on an infected mammal, the sandfly takes up amastigote-containing macrophages/monocytes. During digestion of the bloodmeal, amastigotes initiate their differentiation into the motile promastigote form, which will attach to the midgut epithelium to avoid being excreted together with the digested blood meal. Virulence is acquired during
metacyclogenesis, a process by which dividing, non-infective promastigotes (procyclic) transform into a non-dividing infective form (10). These metacyclic promastigotes detach from the gut epithelial cells and migrate towards the anterior end of the digestive tract. Upon the next blood meal, metacyclic promastigotes are inoculated into the mammalian host, where they must successfully evade and resist non-specific defense mechanisms such as complement-mediated lysis, to ultimately bind and enter mononuclear phagocytes by a receptor-mediated process. Once inside a parasitophorous vacuole or phagosome, metacyclic promastigotes avoid degradation and establish conditions favorable to their proliferation. The increased temperature and the decreased phagosomal pH provide the signals required for the differentiation from promastigotes to amastigotes (11). Ultimately, infected macrophages rupture, releasing the amastigotes into the surrounding environment where they can infect neighboring macrophages (Figure 1).

The socioeconomic situation is the most important factor governing the spread of this disease especially among nomadic cattle raisers, who search for water and grass for their cattle. Recently, the civil war has promoted the spread of the disease by forcing many tribes in the endemic area to move northward. This situation brought the disease with them to the vicinity of Khartoum state and the loss of cattle and other domestic animals upon which the sandflies feed has forced the Leishmania parasite to shift to humans instead (12, 13).

The clinical picture of VL
The clinical picture of VL includes recurrent fever, hepatosplenomegaly, general lymphadenopathy, pancytopenia, and anemia. Death occurs in the absence of appropriate chemotherapy (14). Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL and is characterized by a macular, maculopapular, and nodular rash in patients who have recovered from VL and who are otherwise well. The rash usually starts around the mouth, from where it spreads to other parts of the body depending on severity. PKDL is mainly evident in Sudan and India, where it appears in 50% and 5–10% of treated VL cases, respectively (15). The interval after therapy at which PKDL follows VL is 0–6 months in Sudan and 2–3 years in India (15). No firm evidence exists that predict which VL patients will develop PKDL, but an interesting finding is the high expression of interleukin (IL)-10 in VL patients subsequently developing PKDL after Leishmania therapy (16). This might be due to differences in the host immune response against the parasite itself or the response to treatment or both. Polymorphisms in genes controlling innate and adaptive immunity have been suggested as possible candidates for PKDL development (17).
Figure 1. Leishmaniasis is transmitted by the bite of infected female phlebotomine sandflies. The sandflies inject the infective stage (promastigotes) from their proboscis during blood meals (1). Promastigotes that reach the puncture wound are phagocytosed by macrophages (2) and other types of mononuclear phagocytic cells. Promastigotes transform in these cells into the tissue stage of the parasite (amastigotes) (3), which multiply by simple division and proceed to infect other mononuclear phagocytic cells (4). The parasite, the host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis ensues. Sandflies become infected by ingesting infected cells during blood meals (5, 6). In sandflies, amastigotes transform into promastigotes, develop in the gut (7) and migrate to the proboscis (8). Figure 1 reproduced with permission from www.dpd.cdc.gov.

IC and cytokines induction in VL:

*Leishmania* is an obligate intracellular parasite of macrophages. Studies have demonstrated that specific immunity in VL is mediated by CD4 T helper (Th) cells and that disease susceptibility is associated with the inability to produce a macrophage-stimulating cytokine profile (Th1 profile) including interferon-γ (IFN-γ), IL-2, and IL-12. Conversely, an elevated production of immunosuppressive cytokines such as IL-10 and IL-4 (Th2 profile), as well as high levels of tumor necrosis factor-α (TNFα), may be associated with susceptibility (18, 19).
The hemopoietic growth factor granulocyte macrophage-colony stimulating factor (GM-CSF) has many stimulatory effects on monocytes/macrophages that has beneficial effects during intracellular infections, such as enhancing phagocytic and metabolic functions and the release of other proinflammatory cytokines (20). Addition of GM-CSF to human monocytes in vitro increases their leishmanicidal effects (21). When mice infected with L. donovani were treated with murine GM-CSF they showed increased leishmanicidal activity, as well as leukocytosis and myelomonocytic accumulation of myelomonocytic cells in infected viscera (22). In a small placebo controlled study, GM-CSF-treated Leishmania chagasi-infected patients had a significantly reduced number of secondary infections, along with increased neutrophil counts (23). The production of GM-CSF was increased during infection in the liver of L. donovani-infected BALB/c mice (22) and in mouse bone marrow stromal macrophages (24), findings arguing for the potentially disease-limiting effect of Leishmania infection-induced GM-CSF production. This hypothesis is also sustained by the fact that Leishmania parasites genetically engineered to produce GM-CSF within their phagosomes exhibited poor survival within macrophages that had been activated by GM-CSF to produce an array of pro-inflammatory cytokines (25). Recently, L. donovani amastigote antigens have been reported to induce GM-CSF production in mouse peritoneal macrophages both in vitro and in vivo (26).

Leishmania-infected patients have increased levels of circulating Clq-binding immune complexes (IC) (27-32) that contain Leishmania antigens (33, 34).

Cystatin C

During infection with Leishmania spp. various host organs are affected, including the liver, spleen, and kidney. VL-related nephropathy is known both in humans and animals and was identified by the presence of hematuria and proteinuria, but also in some cases by rising serum creatinine or urea levels (35-40). As markers of kidney function, creatinine and urea levels are both confounded by several unrelated factors, which may seriously hamper the detection of any reduction in glomerular filtration rate. Serum creatinine levels are dependent on muscle mass (41) and urea is dependent on diet and diuresis (42). With the introduction of cystatin C, an accurate and sensitive way to estimate glomerular filtration rate (GFR) has become available, which is unaffected by factors that influence creatinine and urea levels (43-46).

Cystatin C is a low-molecular-weight protein of 13 kD produced by all nucleated cells. It is freely filtered by the glomerulus, reabsorbed, and catabolized, but it is not secreted in the tubules (47). The production of cystatin C is regulated genetically and it is independent of extra-renal factors such as
environmental changes or disease activity (48). Studies have demonstrated
the superiority of serum cystatin C compared to serum creatinine in detecting
minor GFR reductions (49). The main advantage of cystatin C compared to
serum creatinine as a GFR marker is that cystatin C is less dependent upon
the body composition than serum creatinine (41). Cystatin C is a promising,
easily measurable (50), effective and an earlier surrogate marker of de-
creased renal function than serum creatinine (51, 52).

The mechanisms underlying renal involvement in VL patients may be
many, but the involvement of IC and their deposition in the kidney has been
described to be involved in the pathogenesis of leishmaniasis (53, 54). IC
might also affect disease progression and disease outcome through induction
of pro- or anti-inflammatory cytokines like IL-10, GM-CSF and TNF-α (55,
56).

Rheumatoid Arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic multisystem inflammatory progres-
sive disease of unknown etiology that leads to pain, stiffness and physical
disability, and can result in joint destruction, especially if the diagnosis and
early treatment with disease-modifying anti-rheumatic drugs (DMARD) or
biologically engineered macromolecules are delayed (57-59).

The pathology of rheumatoid arthritis

RA is an autoimmune disease and until now the initial triggering mecha-
nisms underlying disease development are not well understood (60). It has
been demonstrated that several types of cells from both the innate and adap-
tive immune systems actively participate and form complex networks of
cell–cell interactions that contribute to the development and chronicity of
RA synovitis, and destruction of cartilage and bone destruction (61).

RA pathogenesis seems to involve a number of humoral and cellular im-
mune processes (62) as well as IC production and complement activation
(63, 64). The cellular mechanisms leading to RA pathology involves mono-
cyte-derived cytokines, lymphocytes (65), neutrophils and Th17 cells (66),
and the humoral mechanisms involve B cells and antibodies (67). Another
mechanism that could also worsen or lead to RA development is hypoxia,
due to its direct link to inflammation and angiogenesis in the affected joints
(68).

Nowadays, several lines of evidence stress to the role of B cells as a criti-
cal factor for RA development. In RA, B cells start to produce a variety of
autoantibodies such as rheumatoid factor (RF) and anti-citrullinated pro-
tein/peptide antibodies (ACPA), that subsequently form IC that deposit in the joints, resulting in complement activation and subsequent inflammation (67). In addition, patients with early RA were shown to have a disturbance in memory B cell circulation (69) with increased cytokine production in relation to B cell activation and survival (66, 70). B cells also play an important role in the process of antigen presentation and T cell activation, cytokine release, and ectopic lymphoid organogenesis (67).

Environmental factors, especially cigarette smoking and other pulmonary exposure (e.g. silica), in association with susceptibility genes are thought to drive the processes towards autoimmunity and inflammation apparent in RA (71, 72). Recent reports confirm the interplay between genetic and environmental factors and the process of protein citrullination as a key mechanism underlying RA development (60, 73).

The pathology of RA starts when the immune system of the host is triggered; cells of the immune system will produce autoantibodies and inflammatory cytokines, creating a cascade of inflammation resulting in pannus formation. Pannus is a soft tissue that expands on the cartilage surface and invades the underlying bone matrix. The leading edge of pannus is composed of fibroblast- and macrophage-like cells, which produce proteinases able to cause destruction of articular cartilage (74). Additional joint damage and systemic complications ensue, resulting from a complex process of inflammatory mediators being released in the affected joints (71).

The clinical features and classification criteria of RA

The clinical presentation of RA often starts insidiously but can also be episodic or acute. It is usually presented as a polyarthritis affecting small joints or a combination of small and large joints. Early disease is characterized by pain and other cardinal signs of inflammation (e.g. heat, swelling, functional loss due to inflammation, and possible erythema over the joints), but not by damage and deformity. If the disease remains active and uncontrolled the inflammation will usually spread to additional joints and gradual irreversible tissue damage will occur, causing deformity and instability of joints. One of the serious long-term disabilities is associated with damage to the larger weight bearing joints (75).

Inflammation of other synovial structures is common in RA and a similar process may occur in tendon sheaths, progressing to serious dysfunction and rupture. The typical RA deformities include ulnar deviation (UD) of the fingers, z deformity (ZD) of the thumb, swan neck deformity (SND) and boutonniere deformity (BD) that occur due to damage or displacement of tendons (75).

The standard for defining RA patients in the clinics is based on classification criteria. In 1987 the American Rheumatism Association, later named the American College of Rheumatology (ACR) developed classification
criteria (76) for RA diagnosis, but this set of criteria has some limitations, especially in describing patients with early RA. They are therefore not optimal in identifying early patients who would benefit from prompt effective treatment. Consequently, in 2010 a joint working group of the ACR and the European League Against Rheumatism (EULAR) developed new RA classification criteria (77) with specific emphasis on identifying patients with a relatively short duration of symptoms who may benefit from early institution of DMARD therapy that can prevent or delay the poor outcome of the disease. The 2010 ACR/EULAR classification criteria are specifically intended to be used before erosions become detectable on X-rays. They include four scored domains: symptom duration (< or >6 weeks), number and type of joints involved, biomarkers of inflammation (ESR and CRP), and biomarkers of specific autoimmunity (RF and ACPA). Only patients with at least 6 points (out of 10 possible) are classified as having RA (77).

Autoantibodies in RA

RA has been associated with several autoantibodies (78) and the ones most used in clinical diagnosis are RF and ACPA, with antibodies against cyclic citrullinated peptide (anti-CCP) version 2 being the most commonly used commercial test. After the discovery of ACPA both RF and ACPA were proposed to be used for the diagnosis of RA patients (79), and were both included as serological markers in the new ACR/EULAR classification criteria (77).

Rheumatoid factor (RF)

RF had been the best described RA-associated serological marker for a long time (80) and was included in the 1987 ACR classification criteria (76). RF was firstly described by Erik Waaler in 1940 (80) and was then hypothesized to be a pathogenic autoantibody with a key role in the physiopathology of RA (81). RF is an antibody that directly bind to the Fc portion of the normal human IgG and are locally produced by B cells in the lymphoid follicles and the germinal center-like structures that develop in the inflamed synovium (82). These autoantibodies are of different immunoglobulin isotypes (IgM, IgG, or IgA) and IgM RF is usually measured in RA patients and can be detected in 60–80 % of the patients (83). In Caucasian RA patients the occurrence of IgA and IgG RF was shown to be lower than of IgM RF (84, 85).

RF has been observed in many other autoimmune diseases, including systemic lupus erythematosus (SLE), as well as in non-autoimmune conditions, such as chronic infections, and also in healthy individuals (67). Nevertheless, RF from healthy subjects is of IgM class, polyreactive and with low affinity (86), whereas RF from RA patients belongs to all isotypes and exhibit affinity maturation (87).
Anti-cyclic citrulinated protein/peptide antibodies (ACPA)

ACPA were firstly described by Schellekens et al. (88) in 1998 and then confirmed by another group (89). According to a meta-analysis, these autoantibodies occur in 67% of RA patients (90), whereas another meta-analysis reports ACPA sensitivities between 39-94% (91). ACPA analyses have high disease specificity (90–98 %); this means that ACPA are rarely present in other diseases or in healthy individuals (92, 93). These autoantibodies recognize peptides or proteins containing citrulline, a non-standard amino acid. This peptide-bound citrulline is generated by the post-translational modification of arginine residues by peptidylarginine deiminase enzymes (94), in a process known as citrullination or deimination. The process of protein citrullination is a common phenomenon that occurs in normal situations like inflammation, apoptosis, or keratinization. In RA, ACPA are locally produced in RA joints, where proteins are citrullinated during the inflammatory process (95) and this citrullination can affect a number of proteins such as filamentin, fibrin and vimentin as well as nuclear and stress proteins (96). However, the major citrullinated protein in RA joints was determined to be fibrin (97).

Histologically, ACPA-positive patients have more infiltrating lymphocytes in synovial tissue, whereas ACPA negative patients have more fibrosis and increased thickness of the synovial lining layer (98). Importantly, ACPA not only have a better diagnostic value than RF in terms of specificity but also seem to be better predictors of poor prognostic features such as progressive joint destruction (90, 99, 100).

The role of ACPA/anti-CCP and RF in RA diagnosis

The diagnostic utility for RF was discovered to be suboptimal, since it has a variable sensitivity (54-88%) and lower specificity than ACPA, especially when compared with clinically relevant disease control groups (48-92%) (92, 101-104). ACPA are found to be highly specific for RA (88, 90, 104-106) and have the potential to predict development of RA in patients with early arthritis not yet fulfilling RA criteria (91, 107, 108).

The use of combined serological screening tests could help in the diagnosis of RA. A study by Raza et al. reported that in patients with early symptoms (<3 months) of synovitis, the combined occurrence of both RF and anti-CCP increased the specificity for RA development as compared to the occurrence of only one of the autoantibodies (109). In addition, Bas et al. showed that combining tests for anti-CCP, IgA and IgM RF would increase test specificity from 82% to 98% but with decreased sensitivity (41%) when compared to anti-CCP alone (68%) (110). Another report by Jonsson et al. showed that simultaneous elevation for both IgM and IgA RF has very high diagnostic specificity and positive predictive value for RA (111).
RF isotypes and treatment

IgG RF was determined to be a good predictive marker for treatment response when using B cell depleting therapy (112, 113). This is in agreement with the long-term conception that IgG RF is the main driver of autoimmune rheumatoid inflammation through production of TNFα and IL-1 (114) and its role in driving persistent RA inflammation (115). High pre-treatment levels of IgA RF were associated with poor clinical response to anti-TNFα drugs (116). It was also shown that efficient response to Rituximab treatment was only related to those patients with RF (117, 118); these studies looked only for IgM RF but not for the other RF isotypes.

RF isotypes and RA prognosis

In RA the presence of autoantibodies (RF and/or ACPA) are associated with poor prognosis such as the presence of bone erosions and extra-articular manifestations (119, 120). Patients with raised IgG RF and IgA RF in particular have progressive joint damage and extra-articular manifestations (121). The presence of IgA RF associates with poor clinical outcome and poor response to treatment. In a study performed by Can et al. increased levels of IgA RF associated with severe disease and extra-articular manifestations (122), and other reports have also shown that IgA RF may be strongly linked to more severe disease (123-125). Poor clinical responses to anti-TNFα drugs were also found to occur in patients with higher pretreatment levels of IgA RF (116).

Many studies had investigated the relationship between disease activity and RF isotypes. Pop et al. reported that only IgG RF levels but not IgM RF levels correlated well with disease activity (126), and Silvestris et al. reported that IgG RF and IgA RF levels correlated better with disease activity than did the level of IgM RF (127). In addition, an increased IgG RF level was reported to be significantly related to the occurrence of subcutaneous nodules (128). A study from Iceland also demonstrated that extra-articular manifestations were predominantly associated with raised IgA RF (129).

RF isotypes have a connection to RA mortality, patients with IgA and IgM RF isotypes having a higher risk for dying earlier than patients without these RF isotypes (130). No such association was determined for IgG RF.

Rheumatoid arthritis in Africa

To date the exact prevalence of RA in Africa is not well studied. One reason might be that reports from different parts of Africa have used different RA criteria for diagnosis (131, 132); another reason might be that the age structure differs considerably between different areas (133). Some papers have reported the disease prevalence in Africa to be lower than in Europe (132, 134, 135), others claim that the disease is increasing (136-140). Kalla et al.
postulated that this variation could be related to urbanization and the change in lifestyle in modern Africa (141). There are no scientific publications about the prevalence or the severity of RA in modern Sudan. I have only found one case report describing the evidence of RA lytic lesions in a Nubian female mummy from around 3000 years ago (142).

Reports from different parts of Africa have alluded to varying RA disease severity; however, none of these reports have actually been truly comparative between different parts of the continent (135-138, 140, 141, 143, 144). For example Malemba et al. argued that the disease severity (investigated as the occurrence of deformities, the presence of rheumatoid nodules and radiological erosions) in Congolese RA patients was less severe than in Western countries (137), whereas a study in Nigeria found that the disease was severe and more than 29% of their patients had extra-articular manifestations (136). Two old reports from Zimbabwe and Nigeria described that the disease was less severe in Africa when compared to Caucasian RA patients (134, 145). We have not found any recent reports that in a strict comparative way relate the clinical presentation of RA in any African population with a Western country.

In central and western Africa the disease severity has been reported to be relatively low (133, 146, 147). Some reports from the eastern (148, 149) and southern (150, 151) parts of Africa published during the last 40 years have claimed the disease severity to be increasing.

Comparison of the diagnostic and prognostic impact of anti-CCP and RF antibodies in RA patients has been performed more extensively in industrialized countries compared to in Africa (144, 152). In the industrialized countries the diagnostic utility of anti-CCP and RF isotypes was investigated in systematic reviews performed by Avouac et al. and Nishimura et al. These studies concluded that anti-CCP was a better marker than RF for RA diagnosis (90, 91), as well as a better prediction marker for bone erosions (90).

Rheumatoid arthritis treatment

RA significantly affects the quality of life and can result in functional impairment, disability and premature death due to cardiovascular complications (153). The burden of RA affects not only individual patients and their families but also cause reduction in the total social health, utilization of health care and loss in productivity (154). The current treatment goal in RA is to achieve persistent, total disease suppression that will result in remission or cure, with improvement of the quality of life (155).

RA treatment protocols have progressed substantially over the past two decades, and treatment strategies now include not only the traditional DMARDs but also the more recently introduced biologic therapies. The most commonly prescribed DMARDs are methotrexate (MTX), anti-malarials
including hydroxychloroquine (HCQ), sulfasalazine (SSZ) and leflunomide. Other DMARDs include gold salts, azathioprine (AZA), cyclosporine A and cyclophosphamide. The use of DMARD combinations is more effective than monotherapy at inducing remission in patients with early RA (156).

Environmental factors and Rheumatoid arthritis

Relatively little is known about environmental factors that may contribute to the development of RA except smoking, which is the main environmental factor that has been consistently related to an increased risk of RA (157-162). Silica exposure is also an independent risk factor for RA development (163). Other factors that have been associated with development of RA include lower socioeconomic status (164), dietary factors like vitamin D (165), anti-oxidants (166) and certain infections (167-170).

Several studies have reported that an increased RA risk was strongly associated with the cumulative smoking exposure, denoting the importance of extent to the exposure in risk of RA development (171, 172). In addition, the interaction between genes (HLA DRB1 SE) and an environmental factor (smoking) revealed that they conferred risk to the development of RA, especially in seropositive patients (i.e. RF positive and/or anti-CCP positive) (158, 173, 174). The interaction between smoking was found to occur with the HLA-DRB1*04 and HLA-DRB1*01/*10 alleles in RA patients from Sweden (161).
Materials and Methods

Study area, patients and establishment of VL diagnosis
The study was carried out at Tabarakalla rural hospital in Gadarif state, along the lower Atbara river in Gallabat Province, eastern Sudan. The area is located approximately about 70 kilometers southeast from Gadarif town. It is endemic for *Leishmania donovani* and its main vector in that area is *Phlebotomus orientalis* (175). Patients enrolled in the study come mainly from Tabarakalla and Barbar Elfogara villages, endemic areas with high prevalence of both VL and PKDL.

Figure 2. The outpatient clinic at Tabarakalla, Sudan.

A detailed clinical history was obtained. Particular emphasis was made regarding any previous form of leishmaniasis. Subjects were questioned about their tribe and geographic origin, and were examined for clinical mani-
festations of VL. A general clinical examination was conducted with particular reference to hepatosplenomegaly, enlargement of lymph nodes and presence of scars as signs of previous cutaneous leishmaniasis. Liver size was measured in the mid-clavicular line from the costal margin; the spleen size was assessed by measuring the distance between the costal margins in the anterior axillary line to the tip of the spleen. Lymphadenopathy was classified as localized if found only at one site and generalized if at two or more sites. The oral and nasal mucous membranes were examined for evidence of mucosal leishmaniasis. A thick and thin blood film for malaria parasites was examined from all individuals who either had fever, looked ill or had splenomegaly, and those with a positive blood films for malaria were excluded.

An inguinal lymph node aspiration was performed on those clinically suspected of having VL (i.e. all individuals with fever for more than 2 months, left upper quadrant pain, lymphadenopathy, splenomegaly or wasting). Those with a negative result underwent bone marrow aspiration from the superior posterior iliac crest. The smears were fixed with methanol, stained with Giemsa stain and examined under an oil-immersion lens using a non-electrical microscope. Severely ill VL patients from the study area were admitted to hospitals because of need for further medical care, and were classified as acute VL. Patients not severely ill and treated as outpatients with daily injections of sodium stibogluconate were classified as sub-acute VL. Patients on treatment all received daily intravenous injections of sodium stibogluconate, 20 mg/kg for 30 days, with a mean of four days between start of treatment and blood sampling. PKDL was diagnosed on clinical grounds, on the appearance and distribution of the rash after treatment in previously diagnosed VL patients. The interval between VL and the occurrence of PKDL and the duration of the rash was estimated from the patients' history.

Study area, patients and establishment of RA diagnosis

The RA project was conducted in two rheumatological outpatient units in Khartoum (at Alribat University Hospital and Omdurman Military Hospital, Khartoum). Blood samples and patient’s clinical records were collected between the first of December 2008 and until the end of September 2010. The RA diagnosis were established by rheumatology specialists according to the 1987 ACR criteria (76) and the patients were included at their first regular follow-up visit during the inclusion period. A total of 259 consecutive Sudanese RA patients were recruited. As controls, 167 healthy blood donors from the blood banks of Alribat University Hospital and Soba Teaching Hospital were recruited.
The clinical data included age, sex, disease duration, and the number of affected (tender and/or swollen) joints according to the EULAR 28 joint counts (176). Data on erythrocyte sedimentation rate (ESR), blood hemoglobin (Hb) level and X-rays of the hands including data on the occurrence of erosions and osteopenia was obtained from the patient records for 161, 169 and 84 of the patients, respectively. Information about hand and wrist deformities (ZD, SND, BD and UD) was recorded for all patients. Age at disease onset was calculated by subtracting disease duration from age at study inclusion.

Sample collection and transport to Sweden

The VL project:
Venous whole blood was drawn from the *Leishmania*-infected patients and their corresponding healthy controls from Tabarakalla rural hospital. The samples were drawn before treatment from newly diagnosed patients. Sera were separated by centrifugation within 2 h of collection and stored in liquid nitrogen in Tabarakalla, and thereafter stored at -70°C at Alribat University.
Hospital in Khartoum, until transported frozen on dry ice to Uppsala, Sweden. Samples were thawed the first time in conjunction to preparation of IC for cell culture stimulation.

The RA project:

*Sudan*
RA patient samples and their corresponding healthy controls were collected from Alribat University Hospital and Omdurman Military Hospital. Whole blood (2.5 ml) was left to coagulate, separated by centrifugation and frozen within 2 hours of sampling. Serum samples were stored at -70°C and transported frozen on dry ice to Uppsala, Sweden before analyses.

*Sweden*
The clinical data for the Swedish RA patients were collected from the national Swedish RA registry. Basic data had been interred by the patients themselves into the computerized RA registry and then completed by the responsible physician.

Polyethylene glycol precipitation of IC
Serum samples were thawed and immediately mixed with an equal volume of 5% polyethylene glycol (PEG) 6000 with 0.1M Ethylenediaminetetraacetic acid (EDTA) and left to stand at 4°C overnight before the precipitates were purified and washed in a single-step centrifugation procedure described previous (177). Briefly, 1 ml of phosphate buffer saline containing 5% human serum albumin (Baxter, Kista, Sweden) and 2.5 % PEG 6000 (PBS-HSA-PEG) was added to 1.5 ml autoclaved Eppendorf tubes. Plastic cylinders made out of 5 ml autoclaved pipette tips, by cutting of about 1.5 cm of the tips, were introduced in the Eppendorf tubes containing PBS-HSA-PEG. Plasma that had been precipitated overnight were diluted 1:3 in Roswell Park Memorial Institute (RPMI)-1640 containing 2.5 % PEG 6000 and then placed on top of the PBS-HSA-PEG in the pipette tips. An interface was then formed with the less dense, red RMPI-1640 solution on top. The tubes were centrifuged at 2100g at 4°C for 20 minutes, whereby the precipitates in the upper 2.5% PEG-RPMI solution were centrifuged down to the bottom of the Eppendorf tubes. The remaining PBS-HSA-PEG solution was removed and the pellet containing PEG-precipitated IC was resolved in ice-cold sterile PBS of the original plasma volume. The diluted PEG precipitates were placed on ice until used in cell culture experiments.
Preparation of mononuclear cells and cell cultures

Buffy coats were obtained from healthy blood donors in Uppsala, and diluted 1:4 in sterile PBS at room temperature, before separation on Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden). After two washes in PBS, peripheral blood mononuclear cells (PBMC) were diluted to $1 \times 10^6$ cells/mL in RPMI-1640 supplemented with glutamine, 1% hydroxyethyl piperazineethanesulfonic acid (HEPES), 1% penicillin-streptomycin (PEST) and 4% Ultroser G (Flow Laboratories, Irvine, Scotland, UK). This serum free system had been optimized for studies of IC-induced cytokine responses. We have earlier found that medium supplemented with 4% Ultroser G more efficiently sustain IC induced cytokine production as compared to other serum substitutes (55, 177, 178). PEG-precipitates (10% v/v) were added to the cell cultures directly after PBMC preparation and within two hours of finalizing PEG precipitation. Cell cultures were performed in 300 µl volumes in sterile flat-bottomed 96-well plates for 20 hours before harvest of the supernatants.

Our experience of different responder cell populations used for IC stimulation show that PBMC populations might either be good responders to IC or show generally low or generally activated cytokine production without substantial effects of added IC. Due to such variations two PBMC donors were used as responder cells in parallel in each experiment. The results presented are from the PBMC donor giving the strongest net response on IC stimulation, in agreement with earlier studies on IC function performed in our group (55, 178, 179).

Measurement of cytokines in supernatants

Measurement of TNFα, IL-1β, IL-10, TNF receptor 75 (TNF-Rp75), IL-10, GM-CSF and IL-1receptor antagonist (IL-1ra) in supernatants was performed using enzyme-linked immunosorbent assay (ELISA). All cytokine ELISAs had been established in the laboratory for the measurement of IC-induced cytokine responses in vitro and are described elsewhere (55, 180, 181), using biotinylated detection antibodies, streptavidin-horseradish peroxidase (R&D system, Abingdon, UK), and 3,3′,5′,5′-tetramethylbenzidine (DAKO AS, Glostrup, Denmark) as substrate. The antibodies and recombinant cytokine standards used in the assay were purchased from R&D Systems except for IL-10 (Pharmingen, San Diego, CA, USA) and TNF-Rp75 (Biosource, Nivelles, Belgium). As capture mouse monoclonal antibodies MAB610 (2.0µg/mL; for TNFα), MAB601 (2.0µg/mL; for IL-1β), 897C2G9 (4 µg/mL; for TNF-Rp75), MAB215 (2.0µg/mL; for GM-CSF) and MAB280 (5 µg/mL, for IL-1ra) were used. For detection biotinylated polyclonal goat antibodies BAF210 (0.1µg/mL; for TNFα), BAF201
(2.5 μg/mL; for IL-1β), BAF280 (25 ng/mL, for IL-1ra) and mouse monoclonal antibodies 911B3H10 (0.4 μg/mL; for TNF-Rp75) and MAB615 (2.0 μg/mL; for IL-1β) were employed. For IL-10, paired F(ab’)2 antibodies (Flexia™, Biosource) were used at concentrations recommended by the manufacturer. For IL-6 the capture (13A5) and the detecting antibodies (39C3) were obtained from Mabtech, Stockholm, Sweden and used at the concentrations 1 and 2 μg/mL, respectively.

**FcγR blocking experiments:**

Anti-FcγRII mAb (IV.3 [Fab fragment]; Medarex, Nutley, NY) or anti-FcγRIII (3G8 [Fab fragment]; Medarex) were added to the cells and left to stand at 4°C for 30 minutes before addition of dissolved PEG precipitates. The antibody concentration used was 1.5 μg/mL for both antibody fragments; preliminary experiments had shown equivalent blocking effect using either 1.5 or 4 μg/mL. Antibody IV.3 has earlier been shown to react specifically with FcγRIIa (182).

**C1q-binding assay for circulating IC**

Levels of circulating IC were measured by a solid-phase C1q-binding assay (Bindazyme C1q binding kit; Binding Site, Birmingham, UK). According to the manufacturer of the kit levels above 10.8 Eq/mL are regarded as positive. The range of the assay is 1.23-100 Eq/mL.

**Biochemical markers of kidney and liver functions**

Plasma levels of cystatin C, creatinine, uric acid, gamma-glutamyl transferase, albumin, lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, and conjugated bilirubin were all measured on the Architect instrument (Abbott Laboratories, Abbott Park, IL) at the routine department of clinical chemistry, University Hospital, Uppsala, Sweden, according to the instructions of the manufacturer.

**Autoantibody measurements**

RF isotypes (IgA, IgM and IgG) were measured by an enzyme immune assay (Phadia ImmunoCAP 250, Phadia, Uppsala, Sweden). Anti-CCP was also analyzed by the same technique. Anti-CCP was considered positive when concentration was > 7 IU/ml, in accordance with the reference range
unitized at Uppsala University Hospital. Using this cut-off, 4/167 Sudanese healthy controls were anti-CCP positive, corresponding to a diagnostic specificity of 97.6%. We then applied the same specificity level for the RF iso
types which are in accordance to the definition in the ACR criteria stating that <5% of healthy controls should be RF positive (76), a statement that has not been changed in the more recent RA classification criteria (77). Measurement ranges for anti-CCP was 0.4-340 arbitrary units (AU)/ml, and for IgA, IgM and IgG RF 0.4-214 international units (IU)/ml, 0.4-200 IU/ml and up to 600 µg/ml, respectively. For statistical reasons values above the reference range were noted as 400 AU/ml, 250 IU/ml and 250 IU/ml for anti-CCP, IgA RF and IgM RF, respectively. All values for IgG RF were within the measurement range. For a few patients, all serum had been used up in previous investigations, and therefore some patients lack serological results.

Ethical approvals

Sudan

VL project

Ethical approval for the VL project was obtained from the Ethical Commit-
tee of Sudan Health Ministry in Khartoum, from the Ministry of Health at Gadarif State, and from the Ethical Committee at Uppsala University. Oral approval was also obtained from the sheikh (the alderman) in Tabarakalla village. Informed consent was obtained from all of the adults who participated in the study. For young children, consent was obtained from their parents. Ethical approval for the RA project was obtained from the Ethical Committee of Alribat University Hospital and Omdurman Military Hospital prior to the study, and informed consent was obtained from all patients and controls before sampling.

Sweden

The approvals for the investigations including blood sampling for PBMC preparation from healthy donors, and for the use of RA registry data for the Gävle RA patients were obtained from the regional ethical board in Uppsala.
Statistics:

Paper 1
T test was used for comparisons between patient groups in unpaired design, and for investigating the effects of FcγR blockade in paired design. Pearson’s moment product correlation test was used to determine correlations, with Fisher’s R-to-S conversion to determine p values. The ratios between pro- and anti-inflammatory cytokines were determined individually for each patient or control, and the distributions of individual ratios were thereafter compared between the groups in unpaired design. Two-way analysis of variance (ANOVA) was used to investigate the combined effects of disease activity and ongoing sodium stibogluconate treatment on levels of circulating IC and IC-induced cytokines.

Papers 1, 2 and 3
For the comparison of more than two groups, we used ANOVA, and for the comparison between two groups, we used the non-parametric Mann-Whitney test for unpaired samples. To estimate correlation between variables, we used the Spearman non-parametric test. χ² test was used to test for differences between proportions, using Fisher’s exact test when appropriate. Swedish and Sudanese RA patients were compared both concerning the full cohorts as well as patients with early RA (< 12 months of disease duration) investigated separately. Receiver Operator Characteristics (ROC) curves were constructed and area under the curve (AUC) was measured using the Analyze-it software (Leeds, UK). Values of p < 0.05 were considered to be significant.
Aims of the study

Paper 1:
To investigate the presence of circulating IC in Leishmania-infected patients and their possible role in disease pathogenesis by means of cytokine induction as well as their association to disease severity and treatment.

Paper 2
To evaluate the effects of circulating IC on organ functions in patients infected with Leishmania donovani.

Paper 3
To investigate the appearance of RA in modern Sudan, in comparison with RA patients living in an industrialized western country (Sweden) investigated during the same time period.

Paper 4
To investigate the diagnostic and prognostic properties of conventionally used RA-associated autoantibodies (anti-CCP and IgM, IgA and IgG RF isotypes) in a Sudanese RA cohort.
Results and Discussions

Paper 1

In this study we observed that IC precipitated from serum of *Leishmania*-infected patients induced significantly higher levels of GM-CSF and IL-10, as well as of IL-6 and IL-1ra as compared with PEG precipitates from matched Sudanese controls. Data showed that induction of GM-CSF by PEG-precipitated IC was especially prominent in acute VL patients undergoing sodium stibogluconate therapy and that IC-induced levels of GM-CSF level also paralleled serum levels of circulating IC. No other investigated cytokine showed the same parallel to IC levels.

Numerous studies have described increased levels of circulating IC containing parasite antigens in *Leishmania* infection (16, 27, 29, 30, 32-34) and that GM-CSF induced by *Leishmania* antigens (183) might be either beneficial by activating macrophages to become leishmanicidal (21, 22) or detrimental by stimulating a Th1 response and possibly also facilitating *Leishmania*-associated kidney engagement (56, 184, 185). VL patients have shown decreasing levels of circulating IC after starting sodium stibogluconate treatment, whereas in our study the patients were sampled shortly after start of treatment. We assume that shortly after institution of antimony therapy, most likely massive numbers of parasites are being destroyed, releasing large amounts of antigen into the circulation. Then this will then result in an increasing load of circulating IC in the blood. This hypothesis concords with our finding of increased levels of circulating IC and IC-induced levels of GM-CSF in antimony-treated VL patients with active disease, and with the synergy between disease activity and ongoing activity as shown by two-way ANOVA. IC-induced levels of GM-CSF *in vitro* also correlated well with levels of circulating IC *in vivo*. Both measures were higher in VL patients with acute disease than in VL patients with subacute disease. The synergistic effects between circulating IC levels *in vivo* and GM-CSF production on response to IC *in vitro* on the one hand and disease activity and ongoing therapy on the other might also contribute to VL pathology. These findings indicate the importance of longitudinal studies of treated VL patients to the possible correlations between IC levels *in vivo*, levels of GM-CSF produced after IC stimulation *in vitro*, and the clinical symptoms like skin rash and signs of transient or permanent kidney involvement.
This study has shown that subjects with VL very often have impaired glomerular filtration and that serum cystatin C levels were significantly higher when compared to PKDL patients and Sudanese healthy controls.

To test the hypothesis that circulating IC might be involved in the impairment of kidney functions, we correlated the circulating IC levels with levels of cystatin C. The levels of circulating IC were significantly correlated with the levels of cystatin C in subjects with VL ($r=0.52$, $p<0.001$). A significant correlation was also seen in the subgroup of subjects with acute VL investigated separately ($r=0.52$, $p<0.05$), whereas no such relationships were found in the subgroups with sub-acute VL or with PKDL.

In previous studies of leishmaniasis, glomerular function has been estimated by serum creatinine and urea levels (36-38, 40). These reports have shown varying results and have mostly been based on case studies. Creatinine and urea levels are both confounded by several unrelated factors which seriously may hamper the detection of any reduction in glomerular filtration. It is well documented that serum creatinine levels are dependent on muscle mass, and therefore, different levels are found in adult men, women and in children. In addition, many of the subjects with VL were in poor condition, some with extensive muscle wasting, which would give rise to false-negative creatinine levels. The estimation of glomerular filtration by means of urea is even more complicated because urea, among other things, is dependent on diet and diuresis (42). With the introduction of cystatin C, an accurate and sensitive means of the estimation of GFR has become available, which is unaffected by these factors influencing creatinine and urea level (45, 186). Until now, only GFR has been shown to determine the circulating levels of cystatin C. Therefore, our findings of highly raised levels of cystatin C in the acute form of VL very clearly indicate that glomerular filtration is generally impaired in this condition as opposed to among patients with sub-acute VL or PKDL.

One aim of this report was to study of the mechanisms of organ impairment in VL. It was therefore of considerable interest that we determined very close correlations between the amount of circulating immune complexes and GFR as estimated by cystatin C, but not as estimated by creatinine levels. IC deposition in the glomeruli thus seems to be a likely cause of renal dysfunction in acute VL. Indeed, IC deposition has been previously reported in some cases with VL and also in animals infected by *Leishmania* (53, 54, 187-191).

In paper 1 we demonstrated elevated production of GM-CSF and some other cytokines by PBMC after incubation with serum PEG precipitates obtained from patients with acute VL. However, of the studied cytokines, only the production of GM-CSF was correlated with cystatin C. This was an interesting finding, because GM-CSF has recently been implicated in the pathogenesis of glomerulonephritis in animal studies (184). One putative chain
of reactions is that circulating IC stimulate the production of GM-CSF by monocytes/macrophages and that GM-CSF enhances the production and activation of neutrophil granulocytes, which in turn react to deposited IC in the glomeruli with ensuing destruction of the tissue. Another possibility is that IC deposition in the glomeruli directly induces GM-CSF production by the mesangial cells in the glomeruli with consequent activation of neutrophils (192).

By measuring the new and more sensitive and specific marker of glomerular filtration rate, cystatin C, our results re-emphasize the importance of renal dysfunction in VL and confirm earlier case reports in this matter (36-38, 40). Our study also suggests that the renal dysfunction may be directly related to the amount of circulating IC and to the induction of GM-CSF production.

We conclude that cystatin C is a superior marker of glomerular function in subjects with leishmaniasis and that IC deposition and GM-CSF are two functions which most likely are related and causally involved in the mechanisms leading to glomerular dysfunction in this disease. In leishmaniasis IC formation may be good for the host by the reduction of antigen deposition in certain organs, but also bad through deposition in vulnerable structures such as the glomeruli of the kidney.

**Paper 3**

To our knowledge this is the first ever study of the appearance of RA in Sudan. We organized it in a comparative manner, with RA patients being included during the same time period in Sudan and Sweden. We showed that Sudanese RA patients had higher ESR and more affected joints than Swedish patients, and also rather commonly developed classical RA-associated hand and finger deformities. Sudanese RA patients were significantly younger, had a significantly lower age of disease onset, disease duration and higher female preponderance compared to Swedish RA patients. This study also showed that fewer Sudanese RA patients were IgM RF seropositive.

The general age at RA symptom onset was significantly higher among the Swedish than the Sudanese RA patients. This finding of a difference between a poor and rich country concords with another recent study from Mexico reporting that Canadian RA patients had a higher age of onset when compared to RA patients from Mexico (193). We hypothesized that our finding might be secondary to the fact that Swedes have a 20 years longer life expectancy than in Sudan (194). We therefore divided our groups into five-year groups concerning age of inclusion. Contrary to our expectations we then found that for the age groups 41-45 and 46-50 years in opposite to the comparison between the full cohorts, Sudanese patients had significantly
higher age of disease onset. The trend was the same in all age groups above 30 years. One possible explanation for the higher age of onset among the Sudanese patients could be the higher parity among Sudanese than Swedish women (195), as high parity may lead to a delay of RA onset. Other authors showed that parity might have a protective effect on the development of RA (196-198). Another reason might be the difference in percentage IgM RF seropositivity. A Danish study reported younger RA onset age among IgM RF positive patients (199), and in our study less than half of the Sudanese patients were seropositive while the corresponding figure for Swedish patients was more than 75%. However, we could not reproduce the Danish findings in our two RA populations from Sudan and Sweden investigated separately.

In our study the proportion of females in the Sudanese cohort was statistically higher than among the Swedish patients. Our Swedish RA cohort with 72.5% females is representative for the full Swedish RA population that in 2008 included 58102 patients with 73% females (200). This lower female percentage in Sudan is also in agreement with corresponding figures for other countries in the Northeast of Europe: Finland (72.4%), Denmark (76.7%) and the Netherlands (66.3%) (201). On the other hand, the higher female preponderance in Sudan is in agreement with earlier African RA studies showing a high female preponderance, in Burkina Faso (81%), Egypt (85%), Cameroon (95%) and Senegal (90.6%) (144, 152, 202, 203). Internationally a high female preponderance was also described in North American native (80%) and Malaysia (86%) (204, 205). There thus seems to be a rather uniform finding of higher percentage of female RA patients in Africa and perhaps elsewhere as compared to Europe.

RF positivity in our study was significantly lower among Sudanese than among Swedish patients, agreeing with other studies from countries in central Africa: Nigeria (13% seropositive) (147), Congo (33.3%) (137), Uganda (28%) (206), Nigeria (38.7%) (136) and Zimbabwe (37%) (207) but not with other studies from western Africa: Senegal (84% in rural areas and 88% in urban areas) (202) and Morocco (78%) (208) or eastern Africa: Kenya (77%) (209). This discrepancy may hint that in Africa different RA subsets dominate in different parts of the continent and that the genetic and/or environmental triggering mechanisms (158) might differ between different parts of Africa. No comparison for ACPA reactivity was done between the two cohorts, as the Swedish RA registry do not contain information on anti-CCP or any other ACPA. We believe the difference in the distribution of RF reactivity in Africa would be accompanied with the same pattern of distribution for anti-CCP reactivity, because of the close association between the occurrence of RF and Anti-CCP both in earlier Swedish (210) and our present Sudanese cohort.

Relatively little is known about environmental factors which may contribute to the development of RA, except smoking, that is the main environ-
mental factor that has consistently been related to an increased risk of RA (157-162). Added to that, silica exposure is also an independent risk factor for RA development (163). Smoking among women is not socially acceptable in the Sudanese society. Earlier data on cigarette smoking in Sudan (211) show the prevalence of cigarette smoking among females to be much lower (0.7%) than among males (12.1%), data that very nicely match the occurrence of smoking in our present RA cohort (0% and 10.7%, respectively). So what could be the triggering mechanism behind the development of RA in Sudan? We hypothesize that Sudanese dukhan (smoke baths) used by woman for their beauty could be one triggering factor for the development of RA in Sudan. Dukhan is a universal custom in Sudan were married woman used to burn a special type of woods called talh in a hole in the ground, and to sit covered by a blanket on a low footstole over this hole. Dukhan is commonly not practiced by men. The women will then be exposed to the smoke of the burning wood, the inhaled smoke may produce lung inflammation leading to systemic inflammation (212). Unfortunately we have no data about dukhan habits in our cohort or in Sudan in general, but as we have a female majority (89.2%), we hypothesize that dukhan might be another environmental risk factor acting via the lungs like cigarette smoking (157-162), silica (213, 214) or traffic pollution (215) for RA development. One argument against such a hypothesis based on pulmonary exposure is the low prevalence of autoantibody positive RA in our cohort, as the strong association shown between smoking and RA is confined to the ACPA positive RA subset, at least among Caucasians (158) and in Malaysia (216).

The disease activity in Sudanese RA patients is higher than among Swedish RA patients, as reflected by significantly higher ESR levels and number of affected joints among Sudanese RA patients. In comparison to what had been written about RA in Africa (135, 136, 151) our Sudanese cohort represents a very active disease with high frequency of radiological erosions and many hand deformities. Report from Uganda describe that RA is severe, many of the patients (70%) with erosions and less than 30% of the patients with IgM RF (206). From our results and the Ugandan data there seem to be a clustering of highly active seronegative RA in central Africa.

The modalities of treatment used were similar in the two populations except for biologics that were used only in Sweden. Most probably this is a cost issue, as Sudanese patients besides having tighter budget than Swedish patients also pay their own drugs, whereas Swedish patients are part of a social system where society pays all prescribed drugs over a certain limit (currently approximate to 250 Euro/year). Yet there has also been an argument that TNF blockers might impose safety issues in developing countries where the proportion of inhabitants with manifest of latent tuberculosis is high (141, 217). Although proportions of patients treated with NSAID only were small in both populations, significantly more Sudanese patients had
such treatment regimen. Reasons might be both due to costs and to patients’ unwillingness to be treated with ‘cytotoxic drugs’.

Perhaps the most interesting finding concerning treatment differences was the difference in use of MTX, DMARD combinations and prednisolone. Generally Sudanese patients used lower doses of MTX and higher doses of prednisolone than Swedish patients. And especially among patients with short disease duration, the Sudanese patients were significantly less often treated with MTX or with DMARD combinations. Reasons might be fear of using cytotoxic agents and level of education. The somewhat higher doses of prednisolone in Khartoum might also be due to a wish to obtain a fast clinical response in patients with flares, in a health care context where intensive follow-up is difficult. The study is only cross-sectional concerning the Sudanese patients, however, and we have no longitudinal data on drug use. The time given for introduction of a newly diagnosed RA patient differs considerably between the two settings. Whereas a Swedish patient might attend a one-hour visit with the rheumatologist to get introduction to her/his disease and its treatment, the corresponding time in Khartoum is considerably less, mostly around 25 minutes.

We have found that Sudanese RA patients have strikingly more widespread joint involvement and stronger laboratory signs of inflammation as compared to Swedish RA patients during the same inclusion period. Sudanese patients also to a large extent have radiological erosions and RA-associated hand deformities, although only less than half of the patients are IgM RF seropositive. This finding of highly active seronegative RA in central Africa contrasts to the rather widespread belief that RA in Africa has a mild clinical course. The fact that Sudanese RA patients use lower methotrexate doses and higher prednisolone doses, and that fewer early RA patients use DMARD combinations or MTX monotherapy might be due to structural dissimilarities between health care systems, economic prerequisites, and different possibilities for clinical follow-up of RA patients in Sudan and Sweden.

**Paper 4**

According to our knowledge, this study is the first report that describes the diagnostic and prognostic properties of conventionally used RA-associated autoantibodies (anti-CCP and RF of the IgM, IgG and IgA isotypes) in a Sudanese RA cohort.

In our study we found that IgA RF was the most common autoantibody (56%) followed by anti-CCP and IgM RF (both 52%) and then by IgG RF (49.8%). The occurrence of anti-CCP was rather similar to what has been found among Swedish RA patients (56%) (210). The relative order of IgA and IgM RF isotypes are in agreement with a Cameroon study by Singwe-
Ngandeu et al, which reported a higher prevalence of IgA RF (84%) followed by IgM RF (77%); IgG RF was not investigated in that study (144). The frequency different RF isotypes does however differ from the IgM RF predominance that is commonly described in Caucasian RA patients. A study from Germany by Vallbracht et al. showed that IgM RF (66.4%) was the predominant type, closely followed by anti-CCP (64.4%), with lower frequencies of IgA RF (50.8%) and IgG RF (43.7%) (85). Another study from Sweden reported the same frequency order for RF isotypes, were IgM RF was found in (79%) of the RA cases, followed by IgA (78%) and IgG RF (68%) (84). Asian RA studies on the other hand report the lowest frequency for IgA RF. A study from India by Singh et al. showed a relative increase in impact for IgG RF, with 48% IgM RF positive RA patients, followed by IgG RF (42%) and IgA RF (37%)(218). A study in three ethnic groups (Malay, Chinese and Indian) living in Malaysia (219) also showed the same pattern of distribution for RF isotypes as in India (218). IgM RF (53.1%) was the most common autoantibody among these Malaysian patients followed by IgG RF (48.3%) and then by IgA RF (21.1%).

Thus there seem to be a relative difference in RF isotype distribution between RA patients from three continents, with IgA RF predominating in Africa [(144) and this study], IgM RF predominating in Europe (84, 85) and with a relative increase in IgG RF and decrease in IgA RF in Asia (218, 219). A report on the predominance of IgM RF (70%) over IgA RF (65%) among African American RA patients (220) show data corresponding to the isotype distribution among native American RA patient from the same continent (221) but differing from black patient in Africa as shown by Singwe-Ngandeu et al (144) and by us. This study of African American RA patients thus argues for divergent environmental triggers for RF isotype distribution in different continents. The fact that the same pattern is seen in three ethically distinct populations in Malaysia (219) indicates that this difference primarily is driven by environmental and not by genetic factors. Another fact also indicating that there might be environmental factors generally facilitating the production of IgA autoantibodies in Africa is the high prevalence of IgA anti-cardiolipin antibodies in African patients with SLE (222, 223), although IgA anti-phospholipid antibodies are no part of the presently used anti-phospholipid syndrome criteria (224).

When RF status is compared in different studies, a weakness is the lack of precision in the definition of RF cut-offs in the 1987 ACR classification criteria (76) and which have not been changed in the current 2010 ACR/EULAR classification criteria (77). The 1987 ACR criteria state that any technique can be used for RF determination, but that the diagnostic specificity should be >95% compared to healthy controls. There is however no definition of upper specificity limits, implying that e.g. a cut-off of 400 IU/ml for IgM RF is compatible with the RA criteria, although only a minority of RA patients normally regarded as IgM RF seropositive have such RF
levels. Today ACPA analyses are performed without any international standardization at all. A first international ACPA reference preparation was recently described, showing a >5 times difference in cut-off relative to the standard preparation (225). In our study we have very carefully aligned the specificity to be the same for all investigated antibodies, using national Sudanese healthy controls to define this diagnostic specificity, and to our knowledge this approach is seldom or never used, and we have not found any such studies from the African continent. Our finding could reflect that the diagnostic utility of IgA RF isotype may be more important than IgM RF and anti-CCP tests in African patients with RA.

For further comparisons of the diagnostic value of each assay, we undertook an ROC curve analysis and calculated the AUC. The ROC analysis displays pairs of sensitivity and specificity for different cut-off points of anti-CCP, IgG RF, IgA RF, and IgM RF concentrations. The AUC was highest for IgA RF. This fact also strengthens our perception that IgA RF is the best marker for detecting RA patients in central Africa, and that serological screening for RA might be performed primarily with IgA RF. Another recent RA report from central Africa (Congo) showed that the occurrence of IgM RF in their patients was infrequent (33%) (137). It would thus be interesting to see whether Congolese RA patients also have an IgA RF predominance as determined by us and in Cameroon (144). Use of the diagnostically most sensitive serological screening test will aid in early identification of more Sudanese RA patients, and early start of DMARD treatment to prevent joint destruction and disability as has been internationally recommended (226).

But even if IgA RF is superior to anti-CCP when the cut-off is set according to healthy controls, what really matters in real life health care is how the antibodies perform compared to disease controls. The clinical breakthrough for anti-CCP came when this antibody proved to have superior specificity compared to RF when patients with other rheumatic conditions and infections, i.e. relevant disease controls, were investigated (92). To really prove our hypothesis that IgA RF is the superior diagnostic marker, all antibodies should be investigated among relevant Sudanese disease controls; patients with other systemic inflammatory diseases or chronic infections.

When comparing the prognostic impact for all investigated autoantibodies, IgG RF isotype was shown to be the strongest marker for bad prognosis due to its association with younger age at inclusion, lower age of disease onset and hand deformities in our cohort. The striking finding in our study is that anti-CCP did not have the biggest prognostic impact on the clinical outcome for our patients, in contrast to what is commonly described (227, 228). Early studies have shown association between bone destruction and RF isotypes (229-231), and neither could we repeat this finding. Even if such an association between autoantibodies and radiological destruction would exist in Sudan, there are a number of reasons why we might lack this association:
few patient with X-ray data (32%), a cross-sectional design with different disease duration at the time of imaging and reporting only of qualitative X-ray data investigated by from different radiologists.

Intriguingly, when we assessed the correlation between anti-CCP and the different RF isotypes in our RA cohort, IgG RF correlated only weakly to anti-CCP levels, but more strongly with the other RF isotypes, indicating that any clinical associations to IgG RF in our cohort are not secondarily dependent on the simultaneous occurrence of anti-CCP. Our findings imply that the IgG RF isotype is the strongest marker of bad prognosis for Sudanese RA patients.

To conclude: the percentage of autoantibody-positive RA patients in Sudan is quite low, and IgA seem to be the most diagnostically sensitive marker, although crucial comparisons with national disease controls currently are lacking. IgG RF seem to be the strongest prognostic marker associated with young age of disease onset and with the occurrence of hand deformities. This is the first study for the role of autoantibodies in Sudanese RA patients, and will be followed by further studies.
Future perspective

Visceral Leishmaniasis in Sudan

In the literature there is a markedly divergent propensity to PKDL development between VL patients from Sudan and India (15). The tendency to develop PKDL probably depends on the single individuals’ unique response to sodium stibogluconate treatment, parasite load, genetic predisposition, environmentally acquired traits, or a combination of such factors. In our future we would like to pursue the following lines of investigation:

1. To investigate what factors could determine the development of PKDL. Gasim et al showed that plasma levels of IL-10 and keratinocyte expression of IL-10 in VL patients may predict PKDL development and that patients who later developed PKDL also had significantly larger spleens (16). This might reflect a heavier load of parasites or circulating IC containing parasite antigens sequestered in the spleen. In theory, strong IL-10 responses and as in our study GM-CSF responses in PKDL patients might reflect a larger parasite load.

2. To investigate the presence of genetic alterations (polymorphisms) that can exist in inflammatory cells such as eosinophils, neutrophils and monocytes and their association to the development of Leishmania donovani infection and especially VL. Especially I want to investigate polymorphism in proteins associated with eosinophil and neutrophil granulocytes: eosinophilic cationic protein, myeloperoxidase and eosinophil protein X.

3. Skin biopsy examinations for the presence of IC deposition and cytokine expression in diseased and non-diseased areas in patients with PKDL. The biopsies will be analyzed for parasite antigens, cellular markers for eosinophilic cationic protein and myeloperoxidase and infiltrating cells.

Rheumatic diseases in Sudan

Our autoimmunity group has a good knowledge and experiences in the field of rheumatic diseases with special focuses on patients with RA and systemic
lupus erythematosus (SLE) (210, 232-234). We established our current RA project since 2008 and in 2011 we started a new project were we targeted patients with SLE from three rheumatological units in Sudan. Sample collection in the SLE project will be ended in August 2013.

Rheumatoid arthritis

In future we are aiming to do the following sub-projects in RA patients:

1- To look for the association between autoantibodies, HLA and smoking in our cohort, compared with Swedish and Malaysian cohorts (158, 235).

2- The Sudanese RA cohort will be compared to Swedish RA patients concerning:
   i) Anti-CCP isotypes (IgG, IgA and IgM), using the same techniques described by our group (236, 237).
   ii) Anti-type II collagen antibodies, using the same techniques described by our group (238-240).
   iii) Anti-phospholipid antibodies (IgG/A/M anti-cardiolipin and anti-beta2GP1).

3- We need to investigate our cohort for the presence of RA-associated point mutations in the HLA region, PTPN22 and for other RA-associated susceptibility loci (241).

4- We will use the Phadia's ImmunoCAP ISAC microarray system for identification of a multiple RA-associated autoantibodies against the candidate autoantigens citrullinated fibrinogen, α-enolase, vimentin and collagen type II. We will compare our findings with the Swedish Epidemiological Investigation of Rheumatoid Arthritis case control data as recently described by others (242) as well as with the Malaysian RA cohort (Too Chun Lai, Rönnelid et al, to be published), to get an understanding on whether the same fine specificity of the anti-citrulline response is found in different parts of the world.

Systemic lupus erythematosus

Nothing has so far been published internationally about the clinical picture of SLE in Sudan and our current ongoing SLE project will represent the first one. When we will finish our sample collection (August, 2013) we are aiming to compare it with a cohort from Karolinska Hospital in collaboration with Iva Gunnarsson and Elisabet Svenungsson.
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