(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 August 2003 (21.08.2003)

PCT

(10) International Publication Number
WO 03/069349 A2

(51) International Patent Classification[7]: **G01N 33/68**

(21) International Application Number: PCT/EP03/01576

(22) International Filing Date: 17 February 2003 (17.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/077,486 15 February 2002 (15.02.2002) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR DIAGNOSIS OF INFLAMMATORY DISEASES USING MRP8/MRP14

(57) Abstract: The present invention is directed to a method for diagnosing inflammatory diseases based on the marker MRP8/MRP14, particularly for diagnosing specific stages of inflammatory diseases and/or for determining the risk of relapse and/or for discriminating between diseases with similar symptoms, said method comprising the steps of: (a) obtaining a biological sample of mammalian body fluid or tissue to be diagnosed; (b) determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acids encoding the polypeptide(s) present in said biological sample; and (c) comparing the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex determined in said biological sample with the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex as determined in a control sample and/or comparing the amount and/or concentration of nucleic acids encoding MRP8 and/or MRP14 polypeptide(s) determined in said biological sample with the amount and/or concentration of nucleic acids encoding MRP8 and/or MRP14 polypeptide(s) measured in a control sample, wherein the difference in the amount of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acids encoding the polypeptide(s) is indicative for the stages of the disease to be diagnosed.

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Method for diagnosis of inflammatory diseases using MRP8/MRP14

FIELD OF INVENTION

The present invention is directed to a method for diagnosing inflammatory diseases based on the marker MRP8/MRP14 particularly for diagnosing specific stages of inflammatory diseases and/or for determining the risk of relapse and/or for discriminating between diseases with similar symptoms.

BACKGROUND OF THE INVENTION

A lot of diseases are characterised by symptoms of inflammation (inflammatory diseases). An indication is the presence of inflammatory cells such as neutrophils and macrophages at local sites of inflammation. The inflammatory state can also be systemic, i.e. proteins secreted by inflammatory cells become detectable in the blood serum.

In spite of different or very often unknown pathogenic background, the early symptoms of inflammatory diseases may be very similar; e.g. fever is a very common symptom of acute inflammatory diseases. Known causes for inflammatory diseases are autoimmune reactions, bacterial, viral or parasite infections, genetic disorders, and allergies. In many cases, mixtures of these or other causes have been proposed, e.g. for the very common disease psoriasis, which is characterised by inflammation of the epidermis. In some cases of psoriasis patients, also the locomototive system may be affected resulting in psoriatic arthritis. Especially the joints are affected by strong inflammation in this disease eventually resulting in stiffness. This disease is characteristic in presumably being caused by multiple factors such as genetic predisposition, psychological stress or irritation of the skin.

Rheumatoid arthritis is an inflammatory disease which affects general mesenchymal tissues and which is very often associated with synovialitis. It is a clinically relevant disorder leading to severe destruction of joint tissue. Acute exacerbations are characteristic for this disease. Aetiology is largely unclear, but an autoimmune disease background is suggested.

In children, juvenile rheumatoid arthritis (JRA) is the most frequent rheumatic autoimmune disease. JRA, also called juvenile chronic arthritis (JCA) or juvenile idiopathic arthritis (JIA), is a group of chronic rheumatoid diseases which affects children up to 16 years. Among these, systemic onset juvenile rheumatoid arthritis (SOJRA) or Still's disease is the most severe and dangerous form of JRA. SOJRA is characterised by a systemic inflammatory reaction which involves several organ systems, e.g. spleen, liver, lymph nodes, bone marrow and skin. During the further course of this disease, patients develop a severe arthritis which often is refractory to anti-inflammatory therapy. The pathogenesis of this disorder is completely unknown. Patients with SOJRA show no characteristic immunological features at initial presentation but rather a general activation of their innate immune system, e. g. thrombocytosis, neutrophilia and activation of the complement system. This non-specific inflammatory pattern is responsible for the difficulties with regard to the early diagnosis, especially with regard to discrimination from bacterial infections. The fact that SOJRA resembles bacterial infections in early symptoms and that no reliable diagnosis marker exists, makes it in addition very difficult to choose the correct medication very early.

The exact regulation of treatment of the different forms of JRA by administration of anti-inflammatory substances can only be performed insufficiently to date. Pathogenesis of the different disease forms are largely unclear and hence, therapy cannot be directed to a specific target. Especially the endpoint of treatment represents a major problem in medication: About half of the patients have relapses or flares after discontinuation or reduction of methotrexat (MTX). Therefore, continuation of MTX for 12 to 24 months after induction of remission has been proposed (Ravelli *et al.*, 1995, *J Rheumatol* 22: 1574-1576; Gottlieb *et al.*, 1997, *Pediatrics* 100: 994-997). However, no controlled prospective studies have been performed to investigate the impact of early discontinuation of MTX-treatment on the duration of remission and the rate of relapses. An unsolved problem in JRA is to assess which patient is at risk for relapse and to adjust anti-inflammatory and immunosuppressive therapy to the children's actual disease activity especially in times of remission. Common inflammatory parameters such as C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) lack specificity and sensitivity (Giannini and Brewer, 1987, *Clin Rheumatol* 6: 197). Internationally accepted scores to determine disease activity mostly rely on clinical criteria (Giannini and Brewer, *supra*). This inadequate surveillance of disease activity results in steady treatment of the patients with immunosuppressant resulting in severe side effects (Giannini and Cassidy, 1993, *Drug Saf* 9: 325).

Patients can be subdivided into at least three major subgroups of oligoarticular, polyarticular and systemic JRA. The clinical course is characterized by changes in the degree of inflammation. The outcome is substantially variable, even within disease onset subtypes; some patients recover completely in adolescence, whereas others experience lifelong symptoms. Remission is achieved in up to two third of JRA patients (Gare & Fasth, 1995, *J Rheumatol* 22: 295-307). Thus, it is reasonable to strive for discontinuation of treatment once disease activity has been controlled, particularly for children in view of potential acute toxicity and chronic effects of long-term immunosuppression, e.g. by MTX-treatment.

It would be important to know when it is safe to withdraw immunosuppressive drugs like MTX or tumour necrosis factor (TNF) alpha-blocking agents such as anti-TNF-antibody, e.g. infliximab, because of their serious side effects. It would be harmful to continue these medications in patients who are in stable and potentially lifelong remission. The rate of relapses might well be influenced by residual synovial inflammation at the time of discontinuation of MTX. Unfortunately, reliable serum markers to detect such unapparent disease activity are lacking. To date, we have no means to identify those clinically inactive patients at special risk for relapses. On the other hand, there is a high rate of relapses in JRA after reduction of immunosuppressive therapy during clinical remission. Thus, it would be helpful to define more sensitive markers for determining the risk of remitting arthritis in JRA.

Kawasaki disease, on the other hand is an acute disease associated with fever and with multiple organs being affected. It is by far the most common systemic vasculitis in childhood. Children under the age of 1 year and boys are at special risk for fatal disease due to coronary artery abnormalities. However, the aetiology is largely unknown, although evidence points to an autoimmune disease in which neutrophils and endothelial cells are affected. Vasculitis, in particular Kawasaki disease, is a necrotising process predominantly affecting small and medium sized arteries. The aetiology and pathogenesis of vasculitis, in particular Kawasaki disease remains unclear. It may be best characterised by a generalised stimulation of inflammatory responses, possibly due to superantigens. The identification of a reliable marker for the diagnosis of the disease state and the identification of patients with an increased risk of heart complication would be advantageous for the adequate treatment of the patients.

Cystic fibrosis (CF) is a disease caused by genetic alterations with being the most common inherited lethal disease among whites with an estimated incidence of 1:3,400 live births. CF transmembrane conductance regulator (CFTR) mutations lead to defective Cl^- transport in respiratory epithelium, resulting in diminished mucus clearance. The consequence is enhanced production of mucus, chronic airway inflammation, recurrent infections and impaired host defense mechanisms. Chronic airway inflammation is the primary cause of morbidity and mortality. Pulmonary infections with a variety of Gram-positive and -negative bacteria including atypical strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* account to a large number of complications. Neutrophilic inflammation occurs early in life and contributes to progressive tissue changes. Acute exacerbations are a common reason for hospitalisation and antibiotic therapy. Due to the high level of chronic inflammation, it is very difficult to diagnose acute inflammatory excacerbations due to e. g. acquired bacterial infections. In order to ensure adequate treatment of this severe disease (only 80% of the patients get 19 years old or more), early diagnosis is a prerequisite.

One of the major problems lies in the diagnosis of acute exacerbations in patients suffering from chronic inflammatory diseases, in particular CF. One of the main tasks for physicians in CF is adjusting therapy to acute pulmonary complications of chronic inflammation. Identifying acute infectious exacerbations is based on clinical experience, rather depending on subjective impressions than using objective parameters. Consensus is lacking about criteria to define acute episodes. Conventional parameters normally used to identify acute infections, e.g. fever, leukocytosis, CRP, ESR, deterioration of lung function, and sputum cultures are not always helpful. The chronicity of pulmonary disease together with atypical presenting acute respiratory infections raise major problems for physicians dealing with CF. It would be helpful to have more reliable markers indicating infections to monitor disease and guide therapy. Ideal sensitive markers indicate local bronchial processes before systemic responses occur.

The attempt to find more reliable serum markers for exacerbations was repeatedly made in the past. CRP or ESR have failed to be generally useful in CF exacerbations (Watkin *et al.*,1994, *Pediatr Pulmonol* 17: 6-10). More sophisticated potential markers, such as interleukins or tumour necrosis factors, are not considered as useful tools by all investigators (see e.g. Wolter *et al.*, 1999, *Immunol* 6: 260-5). Eichler *et al.* proposed human neutrophilic lipocalin as a marker for CF exacerbations (1999, *Eur Respir J* 14: 1145-9). Sputum levels of various cyto-

kines are detectable, but analysing sputum is very critical (see e.g. Karpati *et al.*, 2000, *Scand J Infect Dis* 32: 75-9). Reliable examination often requires bronchioalveolar lavage (Smith et al., 1988, *J Pediatr* 112: 547-54). Exhaled nitric oxide has been shown to be not helpful in CF (Grasemann *et al.*, 1998, *Arch Dis Child* 78:49-53).

Consequently, there is a need for a reliable diagnostic marker especially in the early stages of an acute inflammatory excacerbation for determining the risk of relapse and/or to discriminate between diseases with similar symptoms in order to apply an appropriate medication.

As prototypic for the family of the calcium binding S100 proteins, myeloid-related protein 8 (MRP8) and MRP14 play a role in general inflammation. MRP8 is also designated S100A8 or Calgranulin A. MRP14 is also designated S100A9 or Calgranulin B (see for a review Hessian *et al.*, 1993, *J Leukoc Biol* 53: 197-204). Both proteins are about 100 amino acids in length, are expressed during myeloid differentiation and are predominantly found in early recruited granulocytes and monocytes (Roth *et al.*, 1993, *Biochem Biophys Res Commun* 191: 565-570; Roth *et al.*, 1992, *Immunobiology* 186: 304-314). Upon mixing, the polypeptides spontaneously form a heterodimeric MRP8/RP14 protein complex (e.g. Dale *et al.*, 1983, *Eur J Biochem* 134: 1-6; US 4,833,074) which is also designated Calprotectin, Cystic Fibrosis Antigen or L1 protein. In the presence of calcium, also tetramers of such heterodimers may be non-covalently formed.

S100 proteins accumulate at sites of inflammation, and high serum levels of MRP8 and MRP14 are found in inflammatory diseases like rheumatoid diseases including systemic lupus erythematodes (Haga *et al.*, 1993, *Lupus* 2: 47-50), reactive arthritis (Hammer *et al.*, 1995, *Clin Exp Rhematol* 13: 59-64), and rheumatoid arthritis (Brun *et al.*, 1992, *J Rheumatol* 19: 859-862), as well as in inflammatory bowel (or intestinal) disease and CF (Odink *et al.*, 1987, *Nature* 330: 80-82; Golden *et al.*, 1996, *Arch Dis Child* 74: 136-9; Frosch *et al.*, 2000, *Arthritis Rheum* 43: 628-37; Roth *et al.*, 2001, *Lancet* 357: 1041). MRP8/MRP14 are secreted by infiltrating phagocytes at sites of inflammation (Youssef *et al.*, 1999, *J Rheumatol* 26: 2523-2528). A strong correlation between concentrations of MRP8/MRP14 in synovial fluid and serum has been demonstrated, that is due to expression in inflamed tissue (Frosch *et al.*, 2000, *Arthritis Rheum* 43: 628-637). Thus, MRP8/MRP14 serum levels reflect local inflammation within the synovium. Furthermore, a significant correlation between MRP8/MRP14, CRP, ESR and several clinical parameters of disease activity was found in JRA (Frosch *et al.*, 2000,

Arthritis Rheum 43: 628-637; Berntzen *et al.*, 1991, *J Rheumatol* 18: 133-138). Overexpression of murine MRP8 was also detected in a mouse model of CF (Thomas *et al.*, 2000, *J Immunol* 164: 3870-3877).

However, specific diagnosis by discrimination from other diseases or clear prognosis of relapse risk of treated patients, especially by simple serum analysis, could not be achieved until now. In particular, the diagnosis of CF activity was not sufficiently promising to allow precise diagnosis (Golden *et al.*, 1996, *Arch Dis Child* 74: 136-139). Also, only simple analysis of MRP8/MRP14 in pauciarticular JRA before and after treatment is known (Frosch *et al.*, 2000, *Arthritis Rheum* 43: 628-637). However, the decisive diagnoses, especially of relapse risk after termination of treatment or identification of a specific disease similar in symptoms to other diseases, could not be achieved yet. A specific diagnosis of a disease is particularly important in those cases, where other diseases exist with similar symptoms, which, however, are treated completely different. Otherwise, wrong treatment might be initiated. This might be the case in SOJRA (Still's disease). The fact that SOJRA resembles bacterial infections in early symptoms and that no reliable diagnosis marker exist, in addition makes it very difficult to decide for the advisable medication at a very early stage.

Consequently, there is a need for diagnostic markers which are suitable for discriminating diseases with similar symptoms, especially SOJRA and bacterial infections, for monitoring disease activity, especially Kawasaki disease and CF, and for determining the risk of relapse of a certain disease, especially JRA. In particular, identifying acute excacerbations in chronic inflammatory diseases, especially CF acute excacerbations, and identifying subpopulations of patients, especially subpopulations of Kawasaki disease patients with coronary artery problems, would enable adequate treatment of these diseases.

It is therefore a major object of the present invention, to provide a new method for diagnosing inflammatory diseases by using a reliable marker of inflammation, particularly for diagnosing specific stages of inflammatory diseases and/or for determining the risk of relapse and/or for discriminating between diseases with similar symptoms in order to apply an appropriate medication.

It is a further object of the present invention, to provide a method of treatment of an inflammatory disease in a mammal in need thereof, which is based on a reliable marker of inflam-

mation. It is still a further object of the present invention, to provide a method of prevention of an inflammatory disease in a mammal in need thereof, which is based on a reliable marker of inflammation.

SUMMARY OF THE INVENTION

The present invention provides methods for the diagnosis of stages of inflammatory diseases and/or for determining the risk of relapse and/or for discriminating between diseases with similar symptoms which are based on the marker(s) MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex. Furthermore, the present invention provides methods for the treatment of diseases which comprise the inventive methods as an essential part for the treatments.

In one aspect of the invention, a method for the diagnosis of inflammatory diseases is provided, comprising the following steps:
First, a biological sample of mammalian body fluid or tissue to be diagnosed is obtained. The biological sample may include cell lines, biopsies, blood, sputum, stool, urine, synovial fluid, wound fluid, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, skin, heart, prostate, lung, breast, liver, muscle or connective tissue, histologic object slides, and all possible combinations thereof.

Next, the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acid(s) encoding the polypeptide(s) present in said biological sample is determined. This determination can be achieved via one of several techniques including but in no way limited to: (i) in situ hybridisation of the biological sample with probes detecting MRP8 and/or MRP14 mRNAs; (ii) immunohistochemistry of the biological sample utilising antibodies directed to MRP8 and/or MRP14 polypeptide(s)and/or MRP8/MRP14 protein complex; (iii) quantitative measurement of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex in the biological sample; (iv) measurement of the MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex in body fluids (e.g. whole blood, serum or synovial fluid); and (v) detecting MRP8 and/or MRP14 mRNA(s) using a PCR based method as an indicator, for example, of changes occurring in the biological sample.

In a preferred method according to the invention, a nucleic acid probe is used for determining the amount and/or concentration of MRP8 nucleic acid alone or together with MRP14 nucleic acid, wherein MRP8 nucleic acid encoding the MRP8 polypeptide is, more preferably, derived from the nucleic acid sequence depicted in SEQ ID NO:1. Vice versa, a nucleic acid probe is used for determining the amount and/or concentration of MRP14 nucleic acid alone or together with MRP8 nucleic acid, wherein MRP14 nucleic acid encoding the MRP14 polypeptide is, more preferably, derived from the nucleic acid sequence depicted in SEQ ID NO:3. Such probe is designed in a way to comprise, at least in part, nucleic acids hybridising to the nucleic acid sequence depicted in SEQ ID NO:1 and/or SEQ ID NO:3, and/or fragments thereof. The probe can thus contain mismatches and stretches of nucleic acid derivatives, like peptide nucleic acids, as long as the probe still hybridises with the nucleic acid sequence depicted in SEQ ID NO:1 and/or SEQ ID NO:3. Preferably, the probe can be used for PCR reactions or other template dependent elongation reactions involving a polymerase. Standard hybridisation conditions and assays are known to the person skilled in the art and can be found in the standard literature in this technical field. Furthermore, a PCR-based technique can be employed for the determination. Such techniques can comprise, but are not limited to, rtPCR and PCR involving labelled primer oligonucleotides.

In yet another preferred method according to the invention, a specific antibody is used for determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex. Preferably, said specific antibody recognises an epitope derived from the amino acid sequences depicted in SEQ ID NO:2 and/or SEQ ID NO:4. The generation of antibodies and determination of epitopes is well known to the person skilled in the art and can be found in the standard textbook literature in this technical field. Preferably, said antibody is selected from the group comprising polyclonal antiserum, polyclonal antibody, monoclonal antibody, antibody fragments, single chain antibodies and diabodies. Even more preferably, said antibody is used for performing an immunoassay, such as an enzyme immunoassay (EIA), e.g. ELISA, or an immunohistochemical method.

By "MRP8/MRP14" as used in the present specification, is meant a heterodimeric or heterotetrameric complex of non-covalently associated MRP8 and MRP14 polypeptides. Antibodies against MRP8/MRP14 as understood herein are directed against epitopes on the MRP8 and/or MRP14 polypeptide chain(s) and comprise antibodies recognising MRP8 and/or MRP14 as individual monomer(s) and/or as constituents of dimeric and/or polymeric complexes. Fur-

thermore, the MRP8 and/or MRP14 polypeptide(s) may naturally occur with modifications and/or may be modified *in vitro* whereby such modifications may comprise, for example, phosphorylation, acetylation, and the like.

In one particularly preferred method, the target MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex in the biological sample is/are exposed to a specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with an antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an EIA, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exists, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantified, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample.

Alternatively, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody absorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic wavelength visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength and the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescene and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

Finally, it is possible to perform an analysis of the expression of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein by proteolytic cleavage of the polypeptides, e. g. using a protease and subsequent analysis by mass spectroscopy, e.g. MALDI-TOF. Such methods are also known to the person skilled in the art.

As a next step, the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex determined in said biological sample is compared with the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex as determined in a control sample and/or the amount and/or concentration of nucleic acids encoding MRP8 and/or MRP14 polypeptide(s) determined in said biological sample is compared with the amount and/or concentration of nucleic acids encoding MRP8 and/or MRP14 polypeptides measured in a control sample. Such comparison will be based on the information obtained in the above determination of the amount and/or concentration of MRP8/MRP14. The data or information can be present in both written or electronic form, i.e. on a suitable storage medium. The comparison can either be performed manually and individually, i.e. visually by the attending physician or the scientist in the diagnostic facility, or done by a suited machine, like a computer equipped with a suitable software. Such equipment is preferred for routine screening, e.g. in an intensive care unit of a hospital. High-throughput environments (i.e. assemblies) for such methods are known to the person skilled in the art and also described in the standard literature.

As an optional step, the amounts and/or concentrations of at least one conventional inflammatory marker polypeptide and/or nucleic acids encoding the polypeptide present in said biological sample and in said control sample can be determined.

By "conventional marker" or "conventional inflammatory marker" as used in the present specification, is meant a marker other than MRP8/MRP14 that is induced in the course of an inflammatory disease. According to a preferred method according to the present invention, said conventional inflammatory marker is selected from the group consisting of CRP, human neutrophilic lipocalin, ESR, soluble receptors, e. g. Fas, and cytokines. Such conventional markers normally provide a simple "plus/minus" or "inflammation-yes/no" information with respect to an inflammation. For the purpose of the present invention, these markers provide both an internal control and fixed point in time, at which the inflammation is, for example, present and acute. The comparison of MRP8/MRP14 with the conventional marker and/or the expression in the control sample will thus provide additional viable information for the diagnosis, monitoring, treatment, and especially for the prevention of an inflammatory disease.

During the experiments performed in the course of completion of the present invention, the inventors found that MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex can be used as an early inflammatory marker, whose induction (or onset) occurs much earlier and to an extraordinary high extent in contrast to other conventional markers. This allows for a much earlier and thus more efficient diagnosis of stages of inflammatory diseases and, in turn, for a much earlier, efficient and less time consuming treatment of inflammatory diseases. The use of the inventive marker, and in particular in connection with a conventional inflammatory marker increases the comfort for the patients that experience the inflammation.

In addition, the high induction provides for a clear diagnosis and thus a very precise monitoring of the stages of inflammatory diseases. Preferred inflammatory diseases who can be diagnostically followed, comprise vasculitis, in particular Kawasaki disease, cystic fibrosis, chronic inflammatory intestinal diseases like, for example, ulcerative colitis or Crohn's disease, chronic bronchitis, inflammatory arthritis diseases like, for example, psoriatic arthritis, rheumatoid arthritis, JRA and SOJRA (Still's disease).

By "stages of inflammatory diseases" or "stages of diseases" as used in the present specification, is meant the different phases of the course of an inflammatory disease. Such phases include the early, acute, and regressive phase during the time period during which a patient experiences said disease. Stages of a disease include also an exacerbation of a present disease, secondary infections to an already existing disease, an acute inflammation above the background of a chronic inflammation, an acquired infection on the background of a chronic inflammatory disease, the risk of relapse, and/or discriminating between diseases with similar symptoms.

Thus in one aspect of the method according to present invention, the inflammatory disease is an acute inflammation above the background of a chronic inflammation. In another aspect of the method according to present invention, the inflammatory disease is an acquired infection on the background of a chronic inflammatory disease. In yet another aspect of the method according to present invention, the inflammatory disease is an exacerbation of an already present disease.

Preferably, the method according to present invention is used for diagnosing specific stages of inflammatory diseases and/or for determining the risk of relapse and/or for discriminating between diseases with similar symptoms. Preferably, the diagnosis according to the method of the present invention serves as a basis for prevention and/or monitoring of inflammatory diseases.

Stages of diseases in general, and in particular inflammatory diseases, are frequently diagnosed based on clinical symptoms that are observed by the attending physician. Based on the diagnosis, the stage (in most of the cases corresponding to the severity of the disease) is evaluated. Nevertheless, in addition to the "classical" diagnosis, which is usually based on visual inspection and conventional blood inflammation markers, in recent diagnosis, the analysis of inflammatory markers has become an additional tool for the analysis of the stages of inflammatory diseases. A prominent conventional marker of this family of diagnostically suitable markers is CRP. Nevertheless, this marker is quite slow in its response to an inflammation and not induced in all cases in a very high ratio, compared to its non-inflammation expression. For example, the stages of a disease can be designated as acute outbreak, exacerbation, relief, and include fever and other symptoms. Furthermore, the present invention allows the diagnosis of a disease even in patients showing a healthy appearance, but having a

risk of relapse for a disease. By the term "relapse" is meant that in contrast to a "naive" patient for the inflammation, the person already experienced at least one stage of the respective inflammatory disease. This includes also the distinction between diseases that were experienced and are newly acquired.

One example for the analysis and grading of stages of a disease is described here (in a not limited manner) in the case of rheumatoid arthritis. Rheumatoid arthritis can last for many years. The progression (i.e. stages or phases) of the disease is categorised by five different stages of development. Stage I: You will not experience any of the common signs or symptoms, although you may have a flu-like illness. Stage II: You experience mild pain and swelling in small joints such as your hands, wrists, knees and feet. You may also experience a general, continuing physical discomfort. X-rays of your joints will appear to be normal at this stage. Stage III: Your affected joints are warm and swollen. You also experience stiffness in the morning, a limitation of motion in affected joints, and general and ongoing physical discomfort and weakness. Stage IV: The symptoms you experienced in Stage III will become more pronounced. Stage V: Symptoms are more pronounced than in Stage IV. You will most likely experience the loss of function of the joints affected. Often deformity occurs. During this stage of the disease, the bone around the joint erodes and ligaments are stretched. Also, additional complications may occur such as tendon rupture, leg ulcers, Sjögren's syndrome and carpal tunnel syndrome.

In yet another aspect of the present invention, the method according to the present invention comprises determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acids encoding the polypeptide(s) involves determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MPR8/MPR14 protein complex and/or nucleic acids encoding the polypeptide as a local marker. By "local marker" as used in the present specification, is meant a marker that is produced directly at the site of the inflammatory disease. A local marker thus stands in contrast to conventional markers that are produced as a general response to an infection and/or inflammatory stimulus. Such markers include, amongst others, CRP, human neutrophilic lipocalin, ESR, soluble receptors, like Fas, and cytokines. Local markers have particular advantages in the analysis of a potential relapse of a disease, as could be shown in the present case with JRA patients that seemed to be healthy, yet having an increased risk of relapse for said disease. Nevertheless, the use of MRP8/MRP14 as marker shall not be limited

to localised inflammations, as this marker (although at a slightly later point in time) is present also in the, for example, serum of the patients.

As mentioned above, the method of the present invention can form the basis for a method of treatment of an inflammatory disease in a subject (i.e. a mammal) in need thereof. Thus, in yet another aspect of the present invention, the present invention provides a method of treatment of an inflammatory disease in a mammal in need thereof, comprising the steps of: a) Performing steps a) to c) according to the method of the present invention as indicated above; and b) medical treatment of the mammal in need of said treatment; wherein said medical treatment is based on the stage of the disease to be treated. By "medical treatment" or "medication" as used in the present specification, is meant the use of medicaments, therapeutics and/or exercises in order to support and accelerate the regression of the symptoms of the inflammation. Medical treatment is classically performed using drugs or combinations of drugs that are specifically prescribed by the skilled attending physician. Nevertheless, the term medication shall not be limited to the ingestion of drugs, but includes all possible ways of treatment that will show a benefit for the subject to be treated.

Due to the fact that the medication is based on the stage of the disease to be treated, the attending physician will usually alter the treatment scheme and/or the collection of drugs prescribed and used in order to treat the inflammatory disease. This alteration, which is based on the results of the diagnosis according to the method of the present invention, will allow for the treatment to be earlier, more specific, and thus more effective for the patient. Furthermore, an early medication will save costs, reduce the need to stay in clinics and allow for an ambulant treatment at home, which will increase the comfort of the patient even further. The alterations of the treatment scheme are based on the diagnosis according to the present invention, which, in this case, can be described by "monitoring" of the stages of the disease and the success of a medication. Furthermore, severe side effects that occur during treatment with chemotherapeutics, e.g., MTX, can be avoided in cases, in which the risk for the patients for a relapse was diagnosed as low or not present at all.

In a preferred method of treatment according to the present invention, the conventional inflammatory marker is selected from the group consisting of CRP, human neutrophilic lipocalin, ESR, soluble receptors, e. g. Fas, and cytokines. In most cases, such conventional markers provide a simple "plus/minus" or "inflammation-yes/no" information with respect to

an inflammation. For the purpose of the present invention, these markers provide both an internal control and fixed point in time, at which the inflammation is, for example, present and acute. The comparison of MRP8/MRP14 with the conventional marker and/or the expression in the control sample will thus provide additional viable information for the diagnosis, treatment, and especially for the prevention of an inflammatory disease.

In a preferred method of treatment according to the present invention, the inflammatory disease is a localised inflammatory disease. Such localised inflammations stand in contrast to systemic infections and/or inflammation, like, for example, sepsis or bacterial toxic shock syndrome.

In another preferred method of treatment according to the present invention the inflammatory disease is vasculitis, in particular Kawasaki disease. In yet another preferred method of treatment according to the present invention, the inflammatory disease is cystic fibrosis. In still another preferred method of treatment according to the present invention, the inflammatory disease is a chronic inflammatory intestinal disease like, for example, ulcerative colitis or Crohn's disease or chronic bronchitis. In yet another preferred method of treatment according to the present invention, the inflammatory disease is an inflammatory arthritis disease like, for example, psoriatic arthritis, rheumatoid arthritis or JRA. Particularly preferred is a method of treatment according to the present invention, wherein the inflammatory disease is SOJRA (Still's disease).

Thus, according to another aspect of the method of treatment according to the present invention, the inflammatory disease is an acute inflammation above the background of a chronic inflammation. In another aspect of the method according to the present invention, the inflammatory disease is an acquired infection on the background of a chronic inflammatory disease. In yet another aspect of the method according to the present invention, the inflammatory disease is an exacerbation of an already present disease.

As mentioned above, the method of the present invention can form the basis for a method of prevention of an inflammatory disease in a subject in need thereof. Thus, in yet another aspect of the present invention, the present invention provides a method of prevention of an inflammatory disease in a mammal in need thereof, comprising the steps of: a) Performing steps a) to c) according to claim 1; and b) medical treatment of the mammal in need of said treatment;

wherein said medical treatment is based on the stage of the disease to be prevented. In the context of the present invention, the term "prevention" is meant as a specific treatment of a disease that does not yet exhibit "classical" symptoms (like those mentioned above, e.g. induction of conventional markers), but can be diagnosed by the method according to the present invention above, e. g. relapse risk. Based on the information of the diagnosis according to the present invention, the attending physician will usually begin (e. g. "alter") with a treatment scheme and/or the collection of drugs prescribed and used in order to prevent (treat) the inflammatory disease. This "early onset"-treatment, which is based on the results of the diagnosis according to the method of the present invention, will allow for a more effective prevention than with conventional markers, thus allowing a more effective prevention for the patient. Furthermore, an early medication will save costs, reduce the need to stay in clinics and allow for an ambulant treatment at home, which will increase the comfort of the patient even further. Finally, the possibility to diagnose a risk for a relapse of a disease using the method of the invention allows for a treatment only in cases in which such treatment is necessary, thus avoiding and/or reducing side effects for patients that are treated, for example, treated with chemotherapeutics like, e.g. MTX.

In a preferred method of prevention according to the present invention, the conventional inflammatory marker is conventional in according to the present invention, the conventional inflammatory marker is selected from the group consisting of CRP, human neutrophilic lipocalin, ESR, soluble receptors, e. g. Fas, and cytokines. Such conventional markers provide a simple "plus/minus" or "inflammation-yes/no" information with respect to an inflammation. For the purpose of the present invention, these markers provide both an internal control and fixed point in time, at which the inflammation is, for example, present and acute. The comparison of MRP8/MRP14 with the conventional marker and/or the expression in the control sample will thus provide additional viable information for the diagnosis, treatment, and especially for the prevention of an inflammatory disease.

In a preferred method of prevention according to the present invention, the inflammatory disease is a localised inflammatory disease. Such localised inflammations stand in contrast to systemic infections and/or inflammations, like, for example, sepsis or bacterial toxic shock syndrome. In these cases, the prevention of inflammation will have the additional benefit, to prevent a spreading of the local infection and thus the development from a local towards a systemic (i.e. not localised) inflammation. Nevertheless, the use of MRP8/MRP14 as marker

shall not be limited to localised inflammations, as this marker (although at a slightly later time) is present also in the, for example, serum of the patients.

In another preferred method of prevention according to the present invention the inflammatory disease is vasculitis, in particular Kawasaki disease. In yet another preferred method of prevention according to the present invention, the inflammatory disease is cystic fibrosis. In still another preferred method of prevention according to the present invention, the inflammatory disease is chronic bronchitis or a chronic inflammatory intestinal disease like, for example, ulcerative colitis or Crohn's disease. In yet another preferred method of prevention according to the present invention, the inflammatory disease is an inflammatory arthritis disease like, for example, psoriatic arthritis, rheumatoid arthritis or JRA. Particularly preferred is a method of prevention according to the present invention, wherein the inflammatory disease is SOJRA (Still's disease).

Thus, according to another aspect of the method of prevention according to the present invention, the inflammatory disease is an acute inflammation above the background of a chronic inflammation. In another aspect of the method according to the present invention, the inflammatory disease is an acquired infection on the background of a chronic inflammatory disease. In yet another aspect of the method according to the present invention, the inflammatory disease is an exacerbation of an already present disease.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The invention shall now be further described by the following examples with respect to the attached figures. All examples are provided by way of example only, without any intended limitation of the scope of the invention. All cited references are incorporated herein by reference in their entireties. In the figures,

Figure 1: shows the MRP8/MRP14 serum concentration of SOJRA patients with active disease (n=20) compared to serum concentrations of healthy controls (n=30), patients suffering from active JRA (n=30) or JRA in remission (n=14), and patients with various kinds of bacterial infections (n=30). Data are presented as mean ± SEM. Asterisks indicate statistically significant differences ($p \leq 0.05$).

Figure 2: shows the MRP8/MRP14 serum concentrations of SOJRA patients at initial presentation (n=11), during relapse (n=9), in remission (n=14), and during active disease in the presence (n=8) or absence of fever (n=12). Data are presented as mean ± SEM. Asterisks indicate statistically significant differences ($p \leq 0.05$).

Figure 3: compares the ratio of MRP8/MRP14 and CRP serum concentrations in SOJRA patients to those of patients with bacterial infections. Data of individual patients are presented as ratio of MRP8/MRP14 and CRP concentrations [μg/mg]. Using a cut-off of 15 μg/mg, the MRP/CRP ratio discriminates with a specificity of 97.0% and a sensitivity of 87.5% between SOJRA and bacterial infections.

Figure 4: shows MRP8/MRP14 serum concentrations in SOJRA patients prior to and after ASCT. Data of each SOJRA patient (n=9) are presented individually (A) or patient data are presented as mean ± SEM (n=11) (A). Please notice the logarithmic scale of the y-axis in *Figure 4*A. Asterisks indicate statistically significant differences ($p \leq 0.003$).

Figure 5: shows a comparison of MRP8/MRP14 serum levels in JRA patients at different stages. Box plots show median (thin line), mean (bold line), 25th and 75 th percentile. Errors bars indicate 5th and 95th percentile, respectively. (A) Data on 29 patients with active disease (100 samples from 29 patients) or during remission (116 samples from 28 patients) and on 30 healthy controls. (B) MRP8/MRP14 serum concentrations of patients in remission but at relapse within the next months were significantly higher than MRP8/MRP14 levels of patients who continued to be in stable remission (*$p<0.05$; **$p<0.01$).

Figure 6: illustrates selected individual follow-ups of patients with changing disease activity. (A) Follow-up over seven years of a patient with oligoarticular JRA. (B) A patient with oligoarticular onset JRA who reached remission during the course of disease but relapsed after one year. (C) A patient with polyarticular JRA. Although levels of MRP8/MRP14 were in general lower in this patient, there was a striking elevation before a disease flare became apparent. The courses are representative for the study population. AJC = active joints count.

Figure 7: shows the MRP8/MRP14 serum concentration (A) in comparison with common inflammation parameters ESR (B) and CRP (C) of JRA patients after stopping MTX

administration. 1 = Patients with remission for 1-12 months (*Figure* 7A, n=7; *Figure* 7B and *Figure* 7C, n=8). 2 = Patients with remission for more than 12 months (n=6).

Figure 8: shows MRP8/MRP14 serum levels in JRA patients prior and after start of MTX treatment. MRP8/MRP14 concentrations were analysed in 22 patients with pauciarticular and polyarticular JRA. There was a significant difference between serum concentrations in active disease prior to starting treatment with MTX and in inactive disease after successful treatment, respectively. Box plots show median (thin line), mean (bold line), 25th and 75th percentile. Error bars indicate 5th and 95th percentile (** $p < 0.001$).

Figure 9: shows MRP8/MRP14 serum levels in JRA patients at the time when MTX was discontinued. MRP8/MRP14 concentrations were analysed in serum from patients in remission, obtained at the time when MTX treatment was stopped. Two groups of patients were compared according to their outcome within one year after withdrawal of MTX. Serum levels were significantly higher in patients who had a relapse within the following year compared to patients who stayed in stable remission. Data points show individual MRP8/MRP14 serum concentrations. Dotted line indicates a cut-off at 250 ng/ml with a sensitivity to detect risk for a relapse of 100%, while specificity was 70% (** $p < 0.01$).

Example 1: Identification of MRP8/MRP14 as a marker useful in monitoring SOJRA and in discriminating SOJRA from acquired bacterial infections

Preparation of antibodies against MRP8 and MRP14

Antisera against recombinant MRP8 (anti-MRP8) and MRP14 (anti-MRP14) were produced in rabbits as described previously (Odink *et al.*, 1987, *Nature* 330: 80, Teigelkamp et al., 1991, *J Biol Chem* 266: 13462, Roth *et al.*,1991, *Blood* 82: 1875).

Determination of MRP8/MRP14 concentrations by sandwich ELISA

Concentrations of MRP8 and MRP14 proteins in blood serum or cell lysates were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) system described previously (Roth *et al.*,1992, *Immunobiology* 186: 304, Rammes *et al.*, 1997, *J Biol Chem* 272: 9496). By this method, non-covalently associated complexes of MRP8 and MRP14 are detected (Roth *et al.*,1992, *Immunobiology* 186: 304). For calibration, different amounts (0.25-250 ng/ml) of the native complex of human MRP8 and MRP14, isolated from human granulocytes

(van den Bos *et al.*, 1998, *Protein Expression and Purification* 13: 313), were used. The assay has a high sensitivity with a detection limit of less than 0.5 ng/ml and a linear range between 1-30 ng/ml. Results are presented as ng/ml MRP8/MRP14.

Patients and healthy controls

88 serum samples from 20 patients (10 boys, 10 girls) with SOJRA, fulfilling the criteria for JRA established by the American College of Rheumatology, were analysed in a prospective manner over 5 years. The mean age of the patients at the time of entry into the study was 6.3 years (range 2.0-13.1 years), and the mean disease duration was 1,9 years (range 0.2-7.2 years). The mean follow-up was 30 months (range 6-59 months).

Patients were categorised as having active disease, with and without fever, or as being in remission according to the American College of Rheumatology criteria for clinical remission of rheumatoid arthritis after at least 3 consecutive months, including morning stiffness not exceeding 15 minutes, no fatigue, no active arthritis, and an ESR < 20 mm/hour. In addition to these variables, disease activity was further evaluated by documenting the number of joints with active disease, the number of joints with limited motion, fever of more than 38.5°C, leukocyte count (cells/µl), red blood cell count (g/dl), platelet count (cells/µl) and CRP (mg/l). Clinical disease activity was determined by two independent observers on the basis of medical history and physical examination.

30 healthy children with no history of inflammatory disorders or infections, and 30 patients with clinical (fever > 38.5°C) and laboratory signs (CRP > 50 mg/l) of bacterial infectious diseases, 30 JRA patients with active oligoarthritis and 14 JRA patients during remission served as controls.

Nine patients with SOJRA underwent autologous stem cell transplantation (ASCT) at the Wilhelmina Children Hospital (Utrecht, The Netherlands) after their disease had been refractory to conventional, immunosuppressive therapy. Serum samples were obtained prior to and after ASCT during clinical remission of SOJRA.

For determination of MRP8 and MRP14 concentration, blood samples of all patients were centrifuged within 1 hour after collection, and the sera were stored at -80°C until use in the ELISA system described above . This study was approved by the institutional ethics committee and written consent was obtained from patients and parents.

Statistical analysis

Statistically significant differences in serum concentrations of MRP8/MRP14 in different groups of patients and controls were calculated by Kruskal-Wallis tests and by the U-test according to Mann and Whitney (for values with non-parametric distribution). Values of $p>0.05$ were considered not to be significant. Correlation of different parameters are presented as Pearson's correlation coefficients.

Results of MRP8/MRP14 analysis

MRP8/MRP14 serum concentrations were found significantly elevated in SOJRA patients compared to that of JRA patients (up to 12 fold), patients suffering from various bacterial infections (12 fold) and of healthy controls (about 120 fold) (*Figure 1*). These data show MRP8/MRP14 as a serum marker particularly sensitive in SOJRA.

Furthermore, *Figure 2* demonstrates a correlation of MRP8/MRP14 serum concentrations of SOJRA patients during different stages and with different severity of the disease. Thus, MRP8/MRP14 serum concentration turned out as a suitable parameter for monitoring the course of SORJA.

In contrast to MRP8/MRP14, inflammatory markers routinely used in systemic diseases, e.g. CRP, were similarly high in sera of SOJRA patients and patients with bacterial infections. The ratios of MRP8/MRP14 and CRP concentrations calculated for SOJRA patients and patients with bacterial infections are compared in *Figure 3*. It is shown, that this ratio provides a very good parameter for differentiating severe bacterial infections from SOJRA (specificity 97.0%, sensitivity 87.5%; cf. *Figure 3*). This is an important clinical progress since differential diagnosis between systemic infection and SOJRA often represents a difficult dilemma in which conventional or novel experimental markers of inflammation, e.g. CRP, ESR, interleukin (IL)-1α, IL-6, IL-8, IL-12 or TNF alpha so far failed to allow correct diagnosis (Yilmaz *et al.*, 2001, *Clin Rheumatol* 20: 30).

Prior to treating SORJA patients by ASCT, MRP8/MRP14 serum concentrations were significantly increased compared to concentrations measured after ASCT (*Figure 4*B). Moreover, the decrease of MRP8/MRP14 serum concentrations following ASCT was observed in each individual patient examined (*Figure 4*A).

Example 2: Identification of MRP8/MRP14 as markers for clinically unapparent disease activity in JRA

Patients and healthy controls

In order to determine correlation of inflammatory parameters with changing disease activity in JRA we performed a follow-up analysis of 29 patients (20 girls and 9 boys) over a mean time of 2.9 years (range 1.2–7.0). Ten patients (8 girls, 2 boys) showed a polyarticular and 19 patients (12 girls, 7 boys) an oligoarticular onset. The mean age of the patients at the end of the study was 12.6 years (range 3.10–20.2 years) and the mean disease duration 7.4 years (2.3–14.1 years). Age at disease onset was on average 5.1 years (0.3-15.8 years). 3–15 samples per patient were analysed, all in all 216 serum samples. Patients were treated either with the disease modifying drug MTX or with non steroidal anti-rheumatic drugs or a combination of both.

Disease activity was documented by the physician's global assessment and patient's/parental assessment of disease activity, determination of functional ability, and the number of active joints (joint swelling or limitation of movement, with either pain on movement or tenderness). Patients were categorised as having active disease, or were considered to be in remission based on the ACR criteria (Pinals *et al.*, 1981, *Arthritis Rheum* 24; 1308-1315) for at least 3 consecutive months, including duration of morning stiffness not exceeding 15 minutes, no fatigue, no active arthritis and an ESR < 20mm/hour.

In order to investigate the predictive value of MRP8/MRP14 for relapses we divided the samples of those patients considered to be in remission in two groups. Patients who were in remission for at least nine further months were characterized as "non-relapsers", patients who relapsed within nine month (mean 3.7 months) were characterized as "relapsers". The last sample before relapse was included into the latter group.

Thirty healthy children (16 girls, 14 boys; mean age 14.1 years) who underwent blood sampling for other reasons, e.g. exclusion of growth hormone deficit, and who had no history of inflammatory disorders or infections served as controls. The study was approved by the local ethical committee.

Statistical analysis

Correlation was calculated using Pearson's Correlation. Student's t-Test as well as Mann-Withney-U test was used to analyse differences of means. Statistical analyses were performed using SPSS for Windows version 11.0.

Results of MRP8/MRP14 analysis

The blood samples were centrifuged within two hours and the serum was stored at –80°C until analysed for MRP8/MRP14. The concentrations of MRP8/MRP14 were determined by an ELISA as described above. Serum concentrations of MRP8/MRP14 are given as mean ± standard error of the mean if not mentioned otherwise. CRP in serum was analysed by nephelometry (mg/l) and ESR by the Westergren method (mm/h).

A good correlation between MRP8/MRP14 and disease activity was shown. MRP8/MRP14 serum concentrations of patients with active disease were significantly higher compared to patients with inactive disease (1,997 ± 217 ng/ml vs. 640 ± 61 ng/ml). Both groups showed serum levels that were elevated over the levels of healthy controls (360 ± 55 ng/ml) (*Figure* 5A). Mean MRP8/MRP14 serum concentration was 2,000 ng/ml in patients with active oligoarticular JRA and 1,930 ng/ml in active polyarticular JRA. In inactive disease MRP8/MRP14 serum levels were 720 ng/ml in oligoarticular JRA and 450 ng/ml in polyarticular JRA, respectively. MRP8/MRP14 serum levels correlated well with disease activity measured by MD global assessment ($r = 0.43$) and number of active joints ($r = 0.39$) in individual patients. Coefficients were statistically significant at the 0.01 level (2-tailed).

The patients were followed up over a mean period of 2.9 years (mean, range 1.2–7.0). Variable disease activity was reflected by variations in clinical and laboratory variables. Three courses that are representative for the relapsers of the study population are illustrated in *Figure 6*.

Table 1 summarises the demographic and clinical data of the two subgroups of our study population. The mean serum level of MRP8/MRP14 of the "non-relapsers" was within normal range (395 ± 60 ng/ml). The "relapsers" showed levels that were significantly higher than those of the "non-relapsers" (660 ± 70 ng/ml; $p<0.05$) (*Figure* 5B). No statistically significant differences between "relapsers" and "non-relapsers" were found for CRP (0.2 mg/dl vs. 0.1 mg/dl) or ESR (10.8 mm/hour vs. 8.6 mm/hour).

Table 1: Data for the subgroups of JRA patients in remission

	Relapsers	Non-Relapsers
No. of patients/samples	18/23	21/46
Sex, no. Male / no. female	5/13	5/16
No. of patients with oligoarthritis/ polyarthritis	11/7	14/7
Age at end of study, mean years	12.2	12.6
Disease duration, mean years	7.2	7.4

Receiver-operating characteristics (ROC) plot confirmed a diagnostic value of MRP8/MRP14 serum levels (area under curve 77%; p<0.01). MRP8/MRP14 levels over 450 ng/ml had a diagnostic sensitivity for a relapse of 65%, while specificity was 83% (likelihood ratio 3.7). Using a cut-off at 350 ng/ml, sensitivity was 78% with a specificity of 63%.

Here we confirm that the serum levels of MRP8/MRP14 correlate well with disease activity in children with oligoarticular and polyarticular JRA arthritis, that has also been found in previous studies (Frosch *et al.*, 2000, *Arthritis Rheum* 43: 628-637; Berntzen *et al.*, 1991, *J Rheumatol* 18: 133-138;). Moreover, it is shown that MRP8/MRP14 are reliable markers for clinically occult disease activity and have a predictive value for the risk of relapse. To our knowledge, a predictive marker for relapse in JRA has not been found so far. Routinely used inflammatory markers like CRP and ESR are not useful. Several adhesion molecules (Dolezalova *et al.*, 2002, *Clin Exp Rheumatol* 20: 249-254) as well as cytokines like interleukins (Gattorno *et al.*, 1996, *Ann Rheum Dis* 55: 243-247; Madson *et al.*, 1994, *J Rheumatol* 21: 2359-2363) and tumour necrosis factors (Spadaro *et al.*, 1996, *Rev Rheum Engl Ed* 63: 171-177) have been studied to find out if they could serve as markers of disease activity, but a predictive value has not yet been demonstrated.

In general, patients in remission showed higher MRP8/MRP14 serum levels compared to the healthy controls, while ESR, CRP and clinical assessment were in normal range. These results indicate that even patients considered to be in remission differ in basal disease activity, detected only by MRP8/MRP14. Comparing a subgroup of patients who relapsed with those who did not, we found significant lower serum levels of MRP8/MRP14 for the latter group. Patients with increased serum levels over 450 ng/ml have a high risk to relapse (positive like-

lihood ratio 3.7). With respect to the high pre-test probability for disease flares in JRA, the likelihood of a relapse is significantly elevated in patients with high levels of MRP8/MRP14 despite clinically inactive disease. As a consequence, in clinical practice reduction of therapy would not be reasonable for this group of patients. In contrast, in patients with serum levels under 355 ng/ml the risk to relapse is low (negative likelihood ratio 0.35), and anti-inflammatory therapy can probably be adjusted without provoking an early reactivation.

MRP8/MRP14 serum levels in individual disease courses can be even more reliable to determine an increase of disease activity. *Figure* 6B for example illustrates a patient who relapsed with serum levels under the chosen cut-off but with a significant increase before relapse (from 60 ng/ml to 290 ng/ml).

Although our results have to be interpreted carefully since the number of patients analysed in this study is relatively small, the data suggest that patients in remission showing a higher level of MRP8/MRP14 have an increased risk to relapse. This may indicate the need for careful follow-up for a subgroup of patients we consider to be in remission according to our clinical impression and routine diagnostic parameters. Withdrawal of medication might even be harmful for these patients. Our data indicate that local synovial inflammation might be present even months before a clinically apparent relapse is diagnosed.

In summary, serum levels of MRP8/MRP14 can indicate occult disease activity in the absence of clinical signs or symptoms and thus are reliable predictive markers for the risk of relapse in clinically inactive JRA. By this was, MRP8/MRP14 quantification in serum may help to adjust therapy in times of low or unapparent disease activity.

Example 3: Identification of MRP8/MRP14 as a marker useful in determining relapse risk of JRA after successful treatment

The MRP8/MRP14 concentrations in the serum of JRA patients in clinical remissions at the endpoint of the therapy with methotrexat (MTX) were measured as described in *Example 1* using the MRP8/MRP14 ELISA. CRP serum concentrations and ESR were also determined. Two groups were compared: relapse of the disease within one year (group 1), and no relapse within 1 year, i.e. long-term remission (group 2). It was found that only MRP8/MRP14 serum concentrations were significantly different between the two groups while the data for ESR

and CRP were not (*Figure 7*). Furthermore, CRP serum concentration was negative in all patients investigated with the exception of two; hence, sensitivity is highly inadequate. Thus, measurement of MRP8/MRP14 serum concentrations is suitable for prognosis in JRA and for ensuring adequate treatment.

With MRP8/MRP14 and a cut-off of 250 ng/ml protein, a sensitivity of 100% and a sensitivity of 85% is calculated. At a cut-off of 350 ng/ml a sensitivity of 50% and a specificity of 100% is calculated. This is highly superior to all known diagnostic parameters. Therefore, MRP8/MRP14 could be identified as the first marker for diagnosing the relapse risk.

Example 4: Determination of the right time to stop with MTX-treatment in JRA

Another objective was to investigate whether prolonged MTX-treatment after induction of remission influences the subsequent duration of remission in individual patients with JRA, and to analyse the usefulness of MRP8/MRP14 as predictive markers for the stability of remission at the time when MTX is withdrawn.

Patients

We performed a prospective study including 25 children who fulfilled the criteria for JRA with a pauciarticular or polyarticular course of the disease (Cassidy *et al.*, 1986, *Arthritis Rheum* 29: 274-281). At study entry all patients revealed clinical symptoms of active arthritis as defined previously (Fink *et al.*, 1995, *J Rheumatol* 22: 1566-1569). All patients received MTX (10 mg/m^2 body surface area) to induce remission. Patients were followed up over 2 - 3 years in intervals of 12 weeks. MTX therapy was stopped earlier than 6 months (group 1; 15 patients) or later than 6 months (group 2; 10 patients) after remission according to ACR criteria was documented. Characteristics of patients are summarised in *Table 2*.

Table 2: Characteristics of 25 MTX-treated patients with JRA

	Groups	
Discontinuation of MTX	(1) Early	(2) Late
Number of patients	15	10
Diagnosis (pauciarticular : polyarticular JRA)	7 : 8	5 : 5
Gender (female : male)	13 : 2	8 : 2
Age (years); mean (range)	6.6 (3-12)	6.0 (4-11)
MTX (mg/m^2); mean (range)	9.9 (5-15)	10.2 (5-15)
MTX continuation* (months); mean (range)	3.8 (0-6)	12.6 (7-18)
Tapering before stop; number (%)	11 (73)	4 (40)
Relapses; number (%)	7 (47)	6 (60)
Duration of remission (months); mean (range)	14,9 (3-36)	12,1 (1-32)
MRP active (ng/ml); mean (SEM)	2,970 (± 1,070)	2,920 (± 970)
MRP in remission (ng/ml); mean (SEM)	490 (± 80)	420 (± 80)
CRP active (mg/dl); mean (SEM)	1.9 (± 0.9)	2.3 (± 0,7)
CRP in remission (mg/dl); mean (SEM)	0.1 (± 0.1)	0.1 (± 0.1)
BSG active (mm/h); mean (SEM)	26.5 (± 4.5)	26.9 (± 5.6)
BSG in remission (mm/h); mean (SEM)	9.3 (± 1.1)	12.6 (± 2.6)
Active joints (maximally active); mean (range)	3.1 (1–8)	5 (2–13)

* Continuation of treatment with MTX after diagnosis of remission was documented according to ACR criteria.

Disease activity was documented by the physician's global assessment and patient's/parental assessment of disease activity, determination of functional ability, and the number of active joints (joint swelling or limitation of movement, with either pain on movement or tenderness). Patients were categorised as having active disease, or were considered to be in remission based on the ACR criteria (Pinals *et al.*, 1981, *Arthritis Rheum* 24: 1308-1315) for at least 3 consecutive months, including duration of morning stiffness not exceeding 15 minutes, no fatigue, no active arthritis and an ESR < 20mm/hour.

Statistical analysis

Mann-Whitney U-test was performed to analyse differences between the groups of patients. Data are given as mean ± standard error of the mean (SEM) except otherwise indicated. Receiver-operating curves were employed to analyse the diagnostic value of MRP8/MRP14 serum levels (American College of Rheumatology Ad Hoc Committee on Immunologic Testing Guidelines, 2002, *Arthritis Rheum* 47: 429-433).

Results of MRP8/MRP14 analysis

Serum concentrations of MRP8/MRP14 were determined by an ELISA as described above. Serum levels of MRP8/MRP14 were analysed in 22 patients at the time when treatment with MTX was stopped. Serum concentrations of MRP8/MRP14 are given as mean ± standard error of the mean if not mentioned otherwise. ESR (Westergren method) and CRP (nephelometry) were analysed at each time point of this study.

Thirteen (52%) patients had a relapse of JRA within one year after treatment with MTX was stopped. There was no difference between patients with pauciarticular or polyarticular JRA.

We found no difference in the duration of remission and the number of relapses between patients with prolonged or early discontinuated MTX-treatment. Seven patients had a relapse in group 1 (early withdrawal), while 6 patients relapsed in group 2 (late withdrawal). Mean duration of remission was 14.9 months in group 1 and 12.1 months in group 2.

There was a significant reduction of MRP8/MRP14 serum concentrations in response to MTX therapy (*Figure 8*). Mean MRP8/MRP14 serum levels in active disease prior to the start of MTX treatment were 2,970 (± 1,070) ng/ml in group 1 and 2,920 (± 970) ng/ml in group 2. Mean MRP8/MRP14 serum levels in inactive disease after successful MTX therapy were 490 (± 80) ng/ml in group 1 and 420 (± 80) ng/ml in group 2.

Patients who were in stable remission had significantly lower MRP levels when MTX was discontinuated than patients who had a relapse within one year (210 ± 30 ng/ml vs. 590 ± 90 ng/ml; $p < 0.01$). Data are shown in *Figure 9*. ESR and CRP showed no difference. With a cut-off for MRP8/MRP14 at 250 ng/ml, sensitivity and specificity were 100% and 70% for detecting a special risk for relapse, respectively. Using a cut-off at 270 ng/ml sensitivity was

92%, with a specificity of 80% (positive likelihood ratio 4.6). ROC-Analyses confirmed the diagnostic value for the risk of relapse (area under curve 0.946, $p < 0.001$).

Our data confirm the problem of a high relapse rate after withdrawal of MTX. Taken together, 13 of our 25 patients (52%) had a relapse after discontinuation of MTX therapy after clinical remission was achieved. A relapse may occur due to new developing synovial inflammation. On the other hand, the risk of relapse might be influenced by residual synovial inflammation even in the absence of clinical signs when MTX is discontinuated. So far, a reliable marker for such unapparent disease activity in JRA has not been analysed in a prospective follow-up approach. A higher sensitivity of a serum marker for detection of inflammatory changes compared to clinical parameters during follow-up would be a prerequisite for a prospective marker for the stability of remission of JRA.

MRP8/MRP14 serum concentration decreased significantly in response to effective treatment with MTX. However, differences in their serum levels point to the fact that a group of patients who are considered to be in remission due to clinical and routinely used laboratory criteria have undetected local disease activity. These patients are at special risk for a clinically apparent relapse of JRA as is frequently seen after withdrawal of MTX treatment. As a consequence, prolonged immunosuppressive therapy with MTX has been proposed for all patients (Ravelli *et al.*, 1995, *J Rheumatol* 22: 1574-1576; Gottlieb *et al.*, 1997, *Pediatrics* 100: 994-997).

Our results indicate that prolonged treatment with MTX does not improve the status of remission in general. Residual local disease activity is a possible factor influencing the rate of relapses after discontinuation of MTX-therapy. It is rather important to identify individual patients at special risk for relapse in whom withdrawal of MTX could be harmful. Even patients considered to be in remission differ in basal disease activity at the time point when MTX is withdrawn, detected only by MRP8/MRP14 in our patients. Comparing a subgroup of patients who relapsed with those who did not, we found significant lower serum levels of MRP8/MRP14 for the latter group. Thus, elevated MRP8/MRP14 serum levels point to patients with clinically undetectable local synovial inflammation for whom prolonged treatment with MTX will be beneficial.

Although our study population is relatively small, these data indicate a usefulness of MRP8/MRP14 as a marker of clinically unapparent disease activity to guide anti-inflammatory therapy in JRA. A controlled, randomised study will be necessary to analyse if the treatment with MTX can be safely stopped once individual patients have reached a status of remission without signs of residual synovial inflammation. Serum concentration of MRP8/MRP14 in clinical occult JRA may help to identify patients in whom MTX can be safely withdrawn after remission is achieved.

Example 5: Identification of MRP8/MRP14 as a marker useful in monitoring Kawasaki disease

Concentrations of MRP8/MRP14 in the serum of patients fulfilling the criteria of Kawasaki disease, who were treated with intravenous gammaglobulin (2 g/kg body weight), were analysed by the ELISA described in *Example 1*. Additionally, CRP serum levels were measured. Serum samples were taken at start of therapy, directly after treatment with gammaglobulin, 2 weeks after start of therapy, and in remission.

It turned out, that MRP8/MRP14 serum concentrations correlated to the stages of disease in individual patients. In particular, before and after gammaglobulin treatment, MRP8/MRP14 serum concentrations were different. Thus, MRP8/MRP14 represents a good parameter for monitoring the course of Kawasaki disease.

Some Kawasaki disease patients had coronary artery lesions (CAL) and were diagnosed with coronary aneurysms. Patients with CAL were characterized by longer duration of fever and increased counts of white blood cells and neutrophils. Furthermore, patients with coronary artery aneurysms showed higher initial and maximum MRP8/MRP14 and CRP serum concentrations compared to patients without cardiac complications. However, the difference of MRP8/MRP14 concentrations between the two groups of patients was clearly greater than that of CRP concentrations. Thus, MRP8/MRP14 is superior to CRP as a marker for identifying cases at high risk for coronary artery lesions and acute inflammation in the cascade of vasculitis.

Example 6: Diagnosing acute exacerbation in cystic fibrosis patients (CF) using MRP8/MRP14 as a marker

MRP8/MRP14 serum concentrations were analysed in CF in-patients who received intravenous antibiotic therapy on 21 occasions. Main reasons for hospitalisation were global deterioration of well-being, excessive production of viscous sputum, and increase of productive coughing. CF out-patients without acute exacerbations, who underwent blood samples at 20 occasions for other reasons, were investigated for the same inflammatory parameters.

Serum concentrations of MRP8/MRP14 were significantly elevated in CF patients with acute exacerbation compared to healthy controls. Two weeks after intravenous antibiotic therapy, MRP8/MRP14 serum concentrations in these patients were clearly decreased. The mean MRP8/MRP14 concentration in CF out-patients without exacerbations was comparable to the concentration of the in-patients after treatment.

Furthermore, serum levels correlated with disease activity in individual patients. In all of our patients, MRP8/MRP14 levels decreased during antibiotic therapy. Even in the cases with initial serum level inside the normal range, a decrease was detected, possibly indicating that personal profiles might be more useful than single serum tests. These results indicate that MRP8/MRP14 is a potent marker for acute CF exacerbations.

In more than half of the cases of acute exacerbations, CRP serum concentrations were elevated before initialisation of antibiotic therapy. There was a significant difference between mean levels of CRP in patients with acute exacerbations before and after antibiotic therapy. Nevertheless, mean differences between acute exacerbations and out-patients without acute infection were not significant. ESR was above the normal range in more than half of the cases. We found a significant difference for mean ESR between patients with acute exacerbations and out-patients. ESR of patients with acute exacerbations before and after antibiotic therapy did not differ significantly. In about half of the cases the leukocyte counts were above 10.000/µl. Leukocyte counts were significantly higher in acute exacerbations before than after antibiotic treatment, but no such difference was found between patients with acute exacerbations before treatment and out-patients.

In conclusion, MRP8/MRP14 is a more sensitive indicator for acute exacerbations than the conventional markers CRP, ESR and leukocyte count. It is the only parameter with highly significant differences between patients with acute exacerbations before treatment and after treatment, as well as between patients with acute exacerbations and CF out-patients, respectively. It is more reliable in detecting local processes than conventional markers of inflammation.

WHAT IS CLAIMED IS:

1. A method for the diagnosis of inflammatory diseases, comprising the steps of
   a) obtaining a biological sample of mammalian body fluid or tissue to be diagnosed;
   b) determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acids encoding the polypeptide(s) present in said biological sample; and
   c) comparing the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex determined in said biological sample with the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex as determined in a control sample and/or
   comparing the amount and/or concentration of nucleic acids encoding MRP8 and/or MRP14 polypeptide(s) determined in said biological sample with the amount and/or concentration of nucleic acids encoding MRP8 and/or /MRP14 polypeptide(s) measured in a control sample,
   wherein the difference in the amount of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acids encoding the polypeptide(s) is indicative for the stages of the disease to be diagnosed.

2. A method according to claim 1, wherein a nucleic acid probe is used for determining the amount and/or concentration of MRP8 and/or MRP14 nucleic acid(s) encoding MRP8 and/or MRP14 polypeptide(s).

3. A method according to claim 2, wherein said nucleic acid probe is derived from the nucleic acid sequence depicted in SEQ ID NO:1 and/or SEQ ID NO:3.

4. A method according to claim 2, wherein said nucleic acid probe comprises nucleic acids hybridising to the nucleic acid sequence depicted in SEQ ID NO:1 and/or SEQ ID NO:3 and/or fragments thereof.

5. A method according to claim 2, wherein a PCR-based technique is employed.

6. A method according to claim 1, wherein a specific antibody is used for determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex.

7. A method according to claim 6, wherein said specific antibody recognises an epitope derived from the amino acid sequence depicted in SEQ ID NO:2 and/or SEQ ID NO:4.

8. A method according to claim 6, wherein said antibody is selected from the group comprising polyclonal antiserum, polyclonal antibody, monoclonal antibody, antibody fragments, single chain antibodies and diabodies.

9. A method according to claim 6, wherein said antibody is used for performing an immunoassay such as an ELISA.

10. A method according to claim 1, wherein determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acids encoding the polypeptide(s) involves determining the amount and/or concentration of MRP8 and/or MRP14 polypeptide(s) and/or MRP8/MRP14 protein complex and/or nucleic acids encoding the polypeptide(s) as a local marker.

11. A method according to claim 1, wherein the inflammatory disease is vasculitis, in particular Kawasaki disease.

12. A method according to claim 1, wherein the inflammatory disease is cystic fibrosis.

13. A method according to claim 1, wherein the inflammatory disease is a chronic inflammatory intestinal disease like, for example, ulcerative colitis or Crohn's disease.

14. A method according to claim 1, wherein the inflammatory disease is chronic bronchitis.

15. A method according to claim 1, wherein the inflammatory disease is an inflammatory arthritis disease like, for example, psoriatic arthritis or juvenile rheumatoid arthritis.

16. A method according to claim 1, wherein the inflammatory disease is systemic onset juvenile rheumatoid arthritis (SOJRA, Still's disease).

17. A method according to claim 1, wherein the inflammatory disease is an acute inflammation above the background of a chronic inflammation.

18. A method according to claim 1, wherein the inflammatory disease is an acquired infection on the background of a chronic inflammatory disease.

19. A method according to claim 1, wherein the inflammatory disease is an exacerbation of an already present disease.

20. Use of a method according to claim 1 for diagnosing specific stages of inflammatory diseases and/or for determining the risk of relapse and/or for discriminating between diseases with similar symptoms.

21. Use of a method according to claim 1, wherein the diagnosis serves as a basis for prevention and/or monitoring of inflammatory diseases.

22. A method of treatment of an inflammatory disease in a mammal in need thereof, comprising the steps of
    a) Performing steps a) to c) according to claim 1; and
    b) medical treatment of the mammal in need of said treatment; wherein said medical treatment is based on the stage of the disease to be treated.

23. A method according to claim 22, wherein the inflammatory disease is a localised inflammatory disease.

24. A method according to claim 22, wherein the inflammatory disease is vasculitis, in particular Kawasaki disease.

25. A method according to claim 22, wherein the inflammatory disease is cystic fibrosis.

26. A method according to claim 22, wherein the inflammatory disease is a chronic inflammatory intestinal disease like, for example, ulcerative colitis or Crohn's disease.

27. A method according to claim 22, wherein the inflammatory disease is chronic bronchitis.

28. A method according to claim 22, wherein the inflammatory disease is an inflammatory arthritis disease like, for example, psoriatic arthritis or juvenile rheumatoid arthritis.

29. A method according to claim 22, wherein the inflammatory disease is systemic onset juvenile rheumatoid arthritis (SOJRA).

30. A method according to claim 22, wherein the inflammatory disease is an acute inflammation above the background of a chronic inflammation.

31. A method according to claim 22, wherein the inflammatory disease is an acquired infection on the background of a chronic inflammatory disease.

32. A method according to claim 22, wherein the inflammatory disease is an exacerbation of an already present disease.

33. A method of prevention of an inflammatory disease in a mammal in need thereof, comprising the steps of:
    a) Performing steps a) to c) according to claim 1; and
    b) medical treatment of the mammal in need of said treatment; wherein said medical treatment is based on the stage of the disease to be prevented.

34. A method according to claim 33, wherein the inflammatory disease is a localised inflammatory disease.

35. A method according to claim 33, wherein the inflammatory disease is vasculitis, in particular Kawasaki disease.

36. A method according to claim 33, wherein the inflammatory disease is cystic fibrosis.

37. A method according to claim 33, wherein the inflammatory disease is a chronic inflammatory intestinal disease like, for example, ulcerative colitis or Crohn's disease.

38. A method according to claim 33, wherein the inflammatory disease is chronic bronchitis.

39. A method according to claim 33, wherein the inflammatory disease is an inflammatory arthritis disease like, for example, psoriatic arthritis or juvenile rheumatoid arthritis.

40. A method according to claim 33, wherein the inflammatory disease is systemic onset juvenile rheumatoid arthritis (SOJRA).

41. A method according to claim 33, wherein the inflammatory disease is an acute inflammation above the background of a chronic inflammation.

42. A method according to claim 33, wherein the inflammatory disease is an acquired infection on the background of a chronic inflammatory disease.

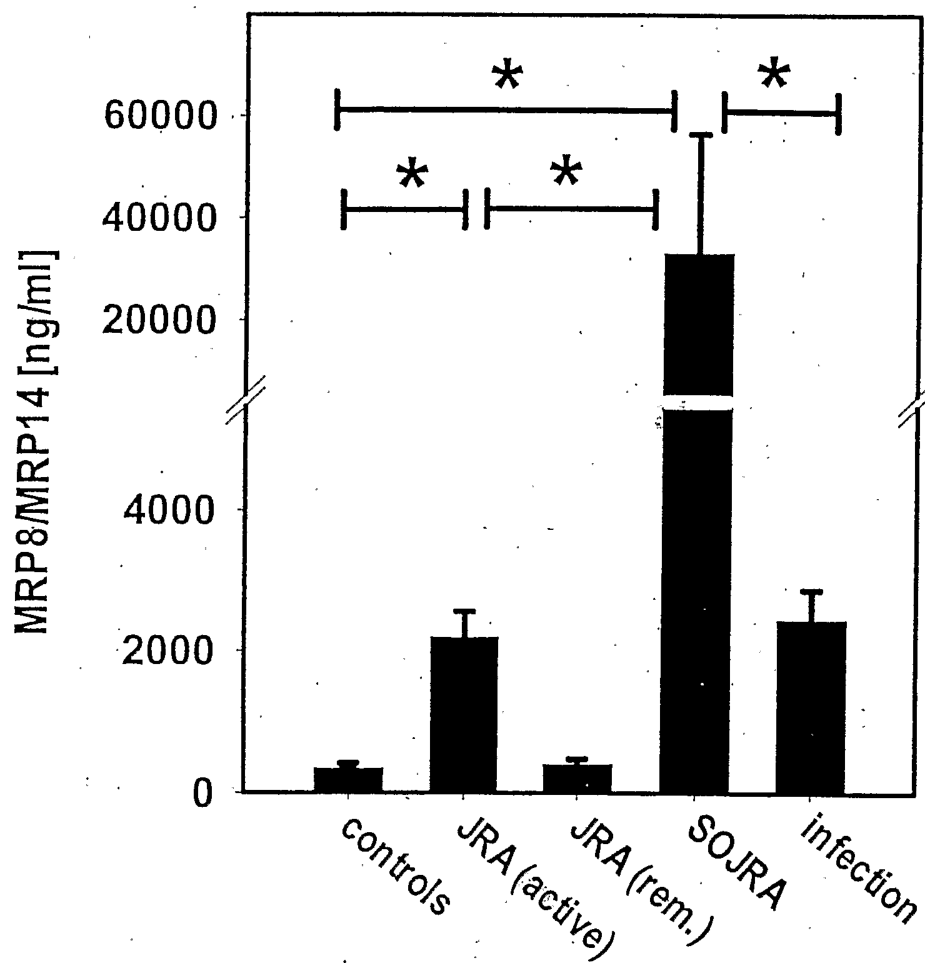
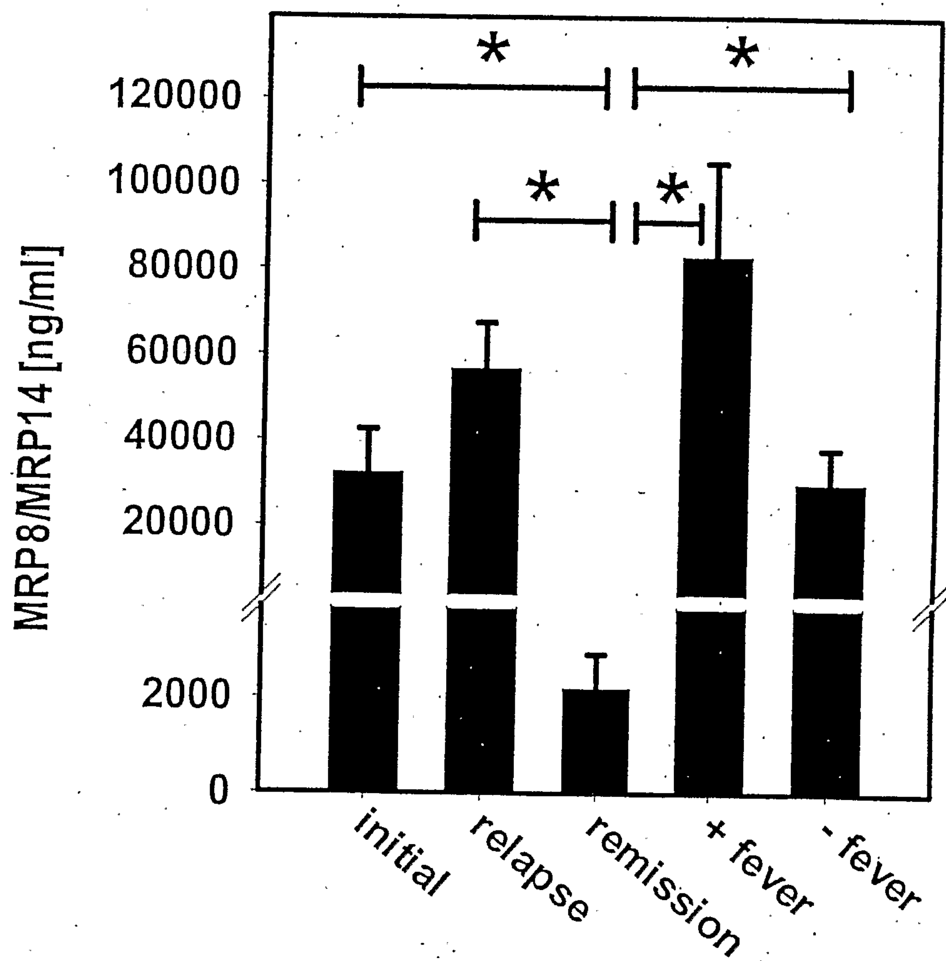


FIGURE 1



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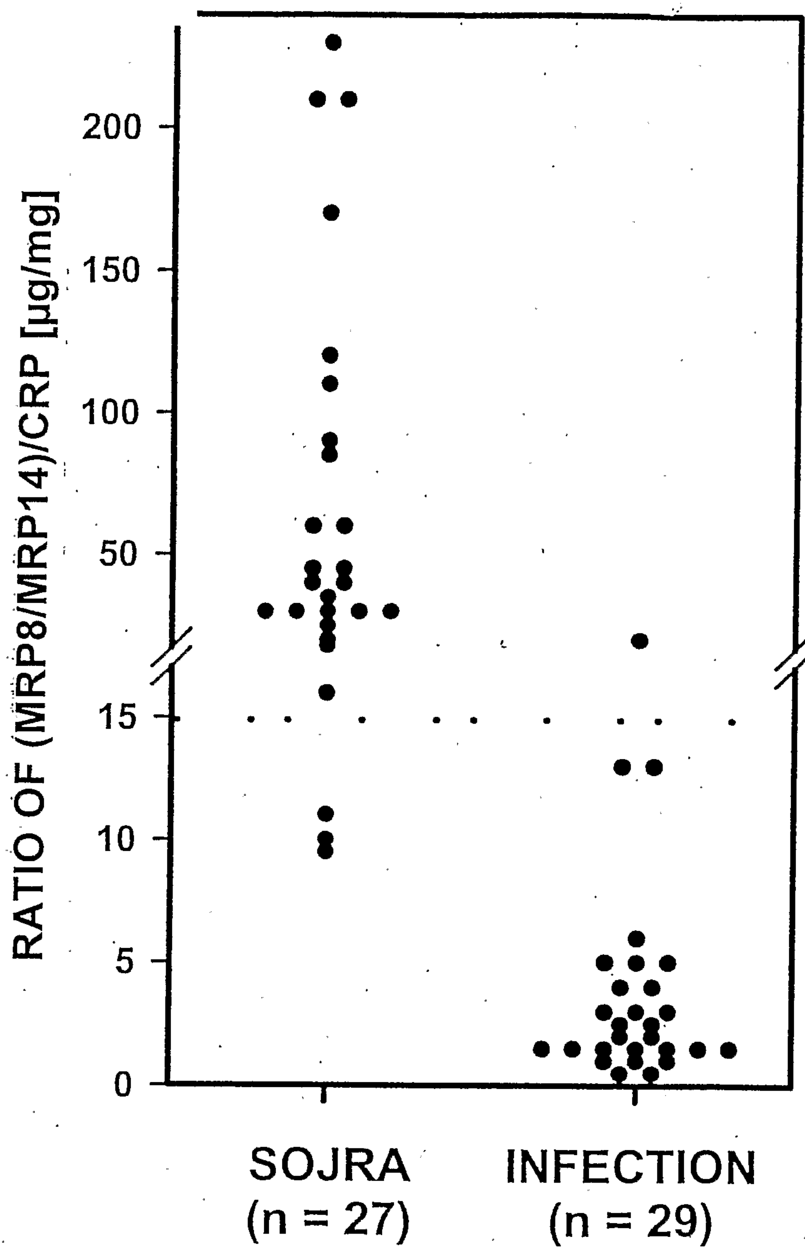


FIGURE 3

FIGURE 4B

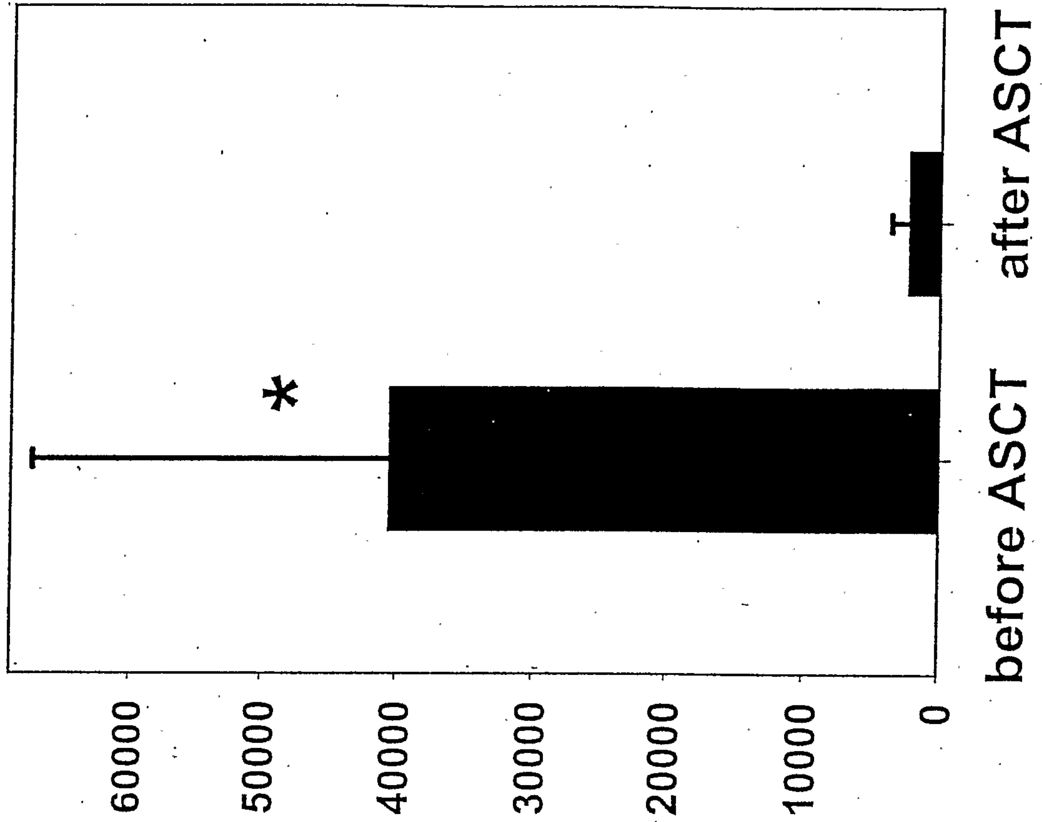


FIGURE 4A

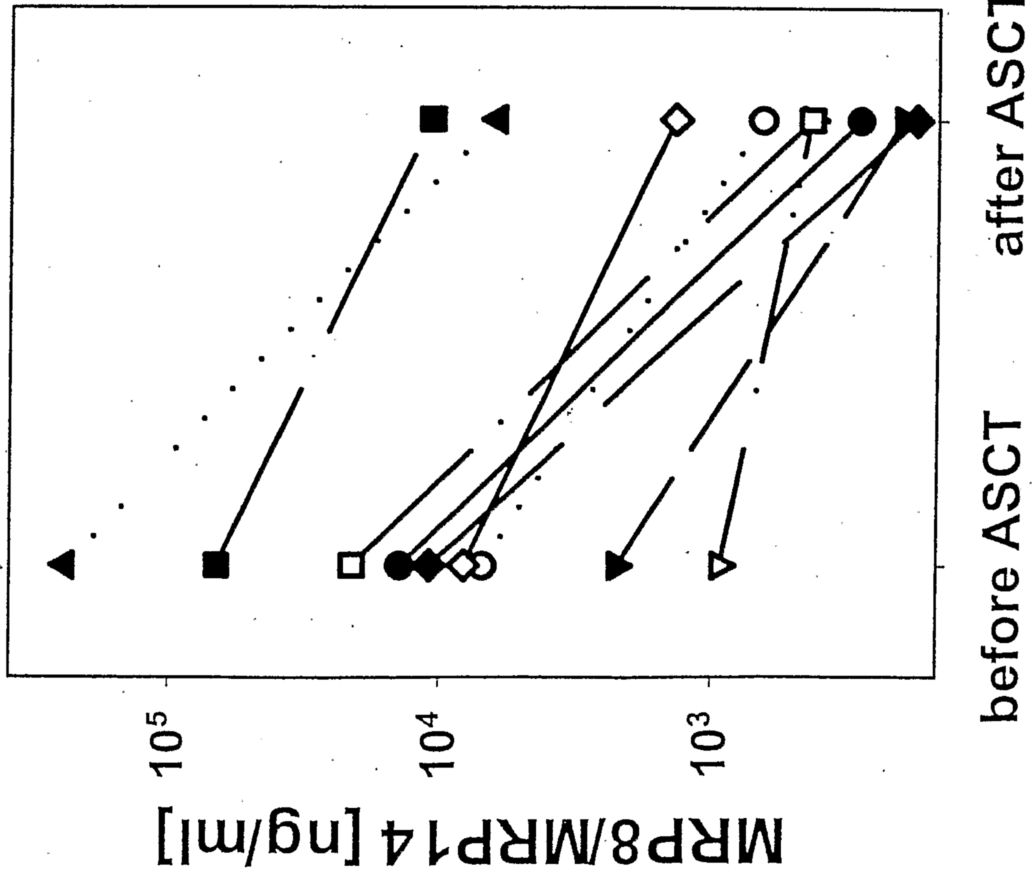


Figure 5

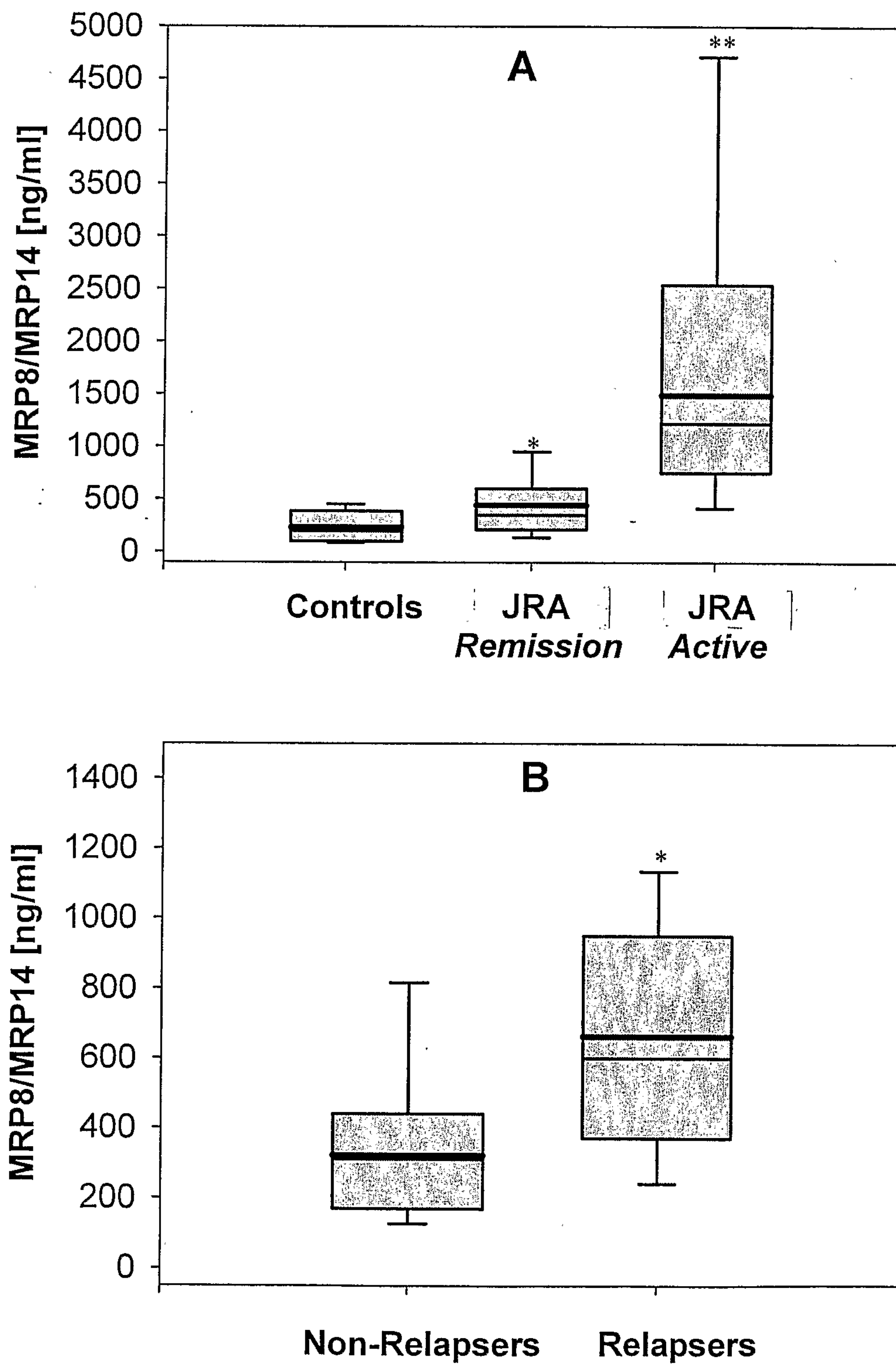
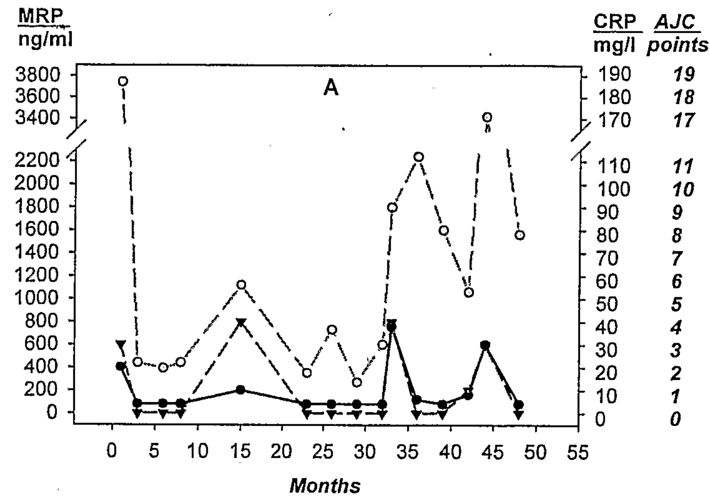
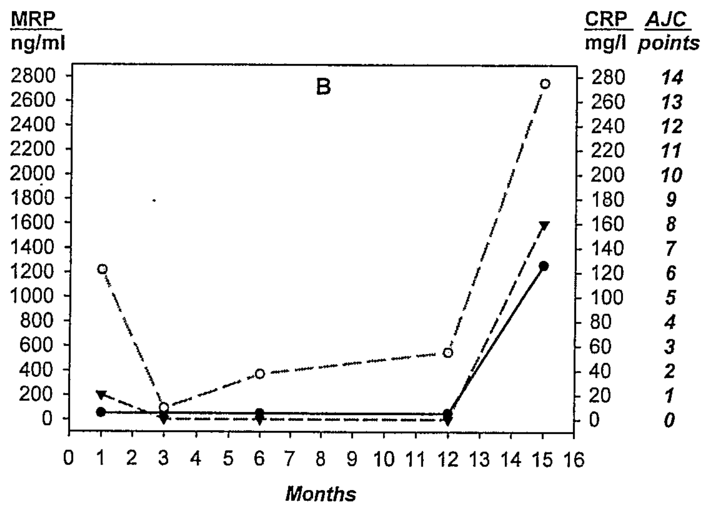
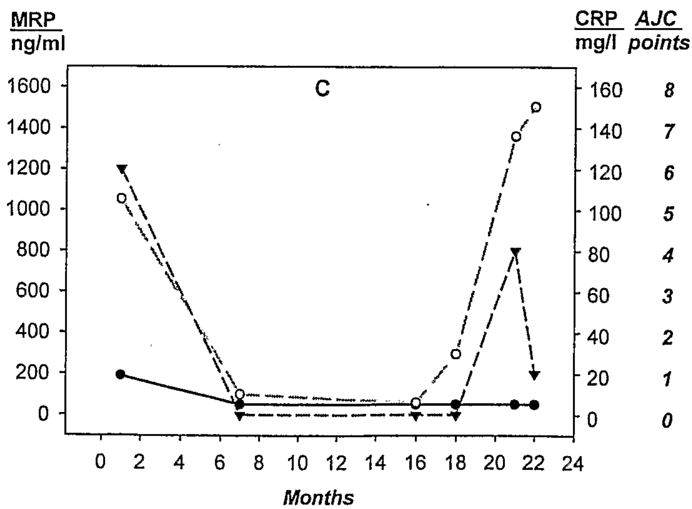


Figure 6







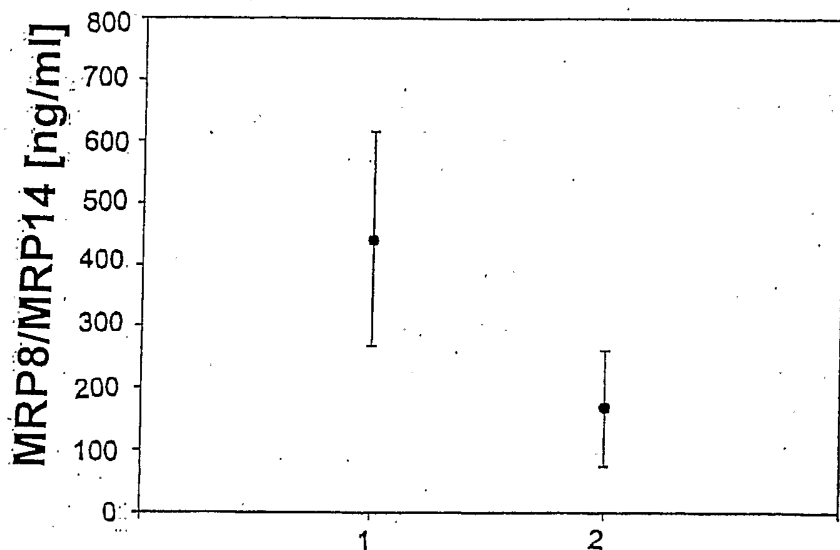


Figure 7A

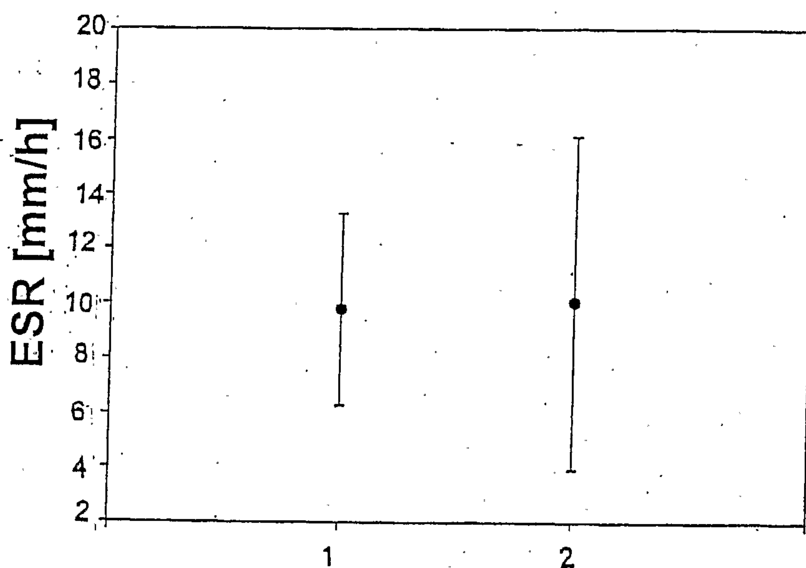


Figure 7B

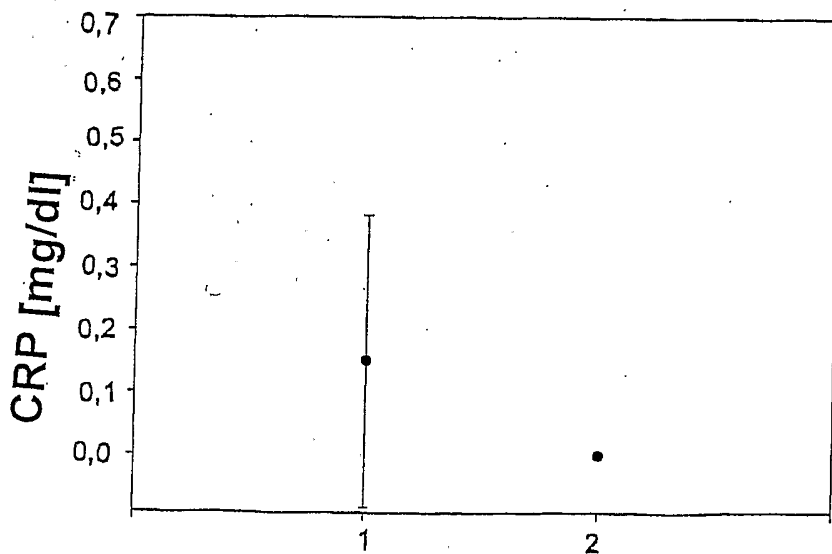


Figure 7C

Figure 8

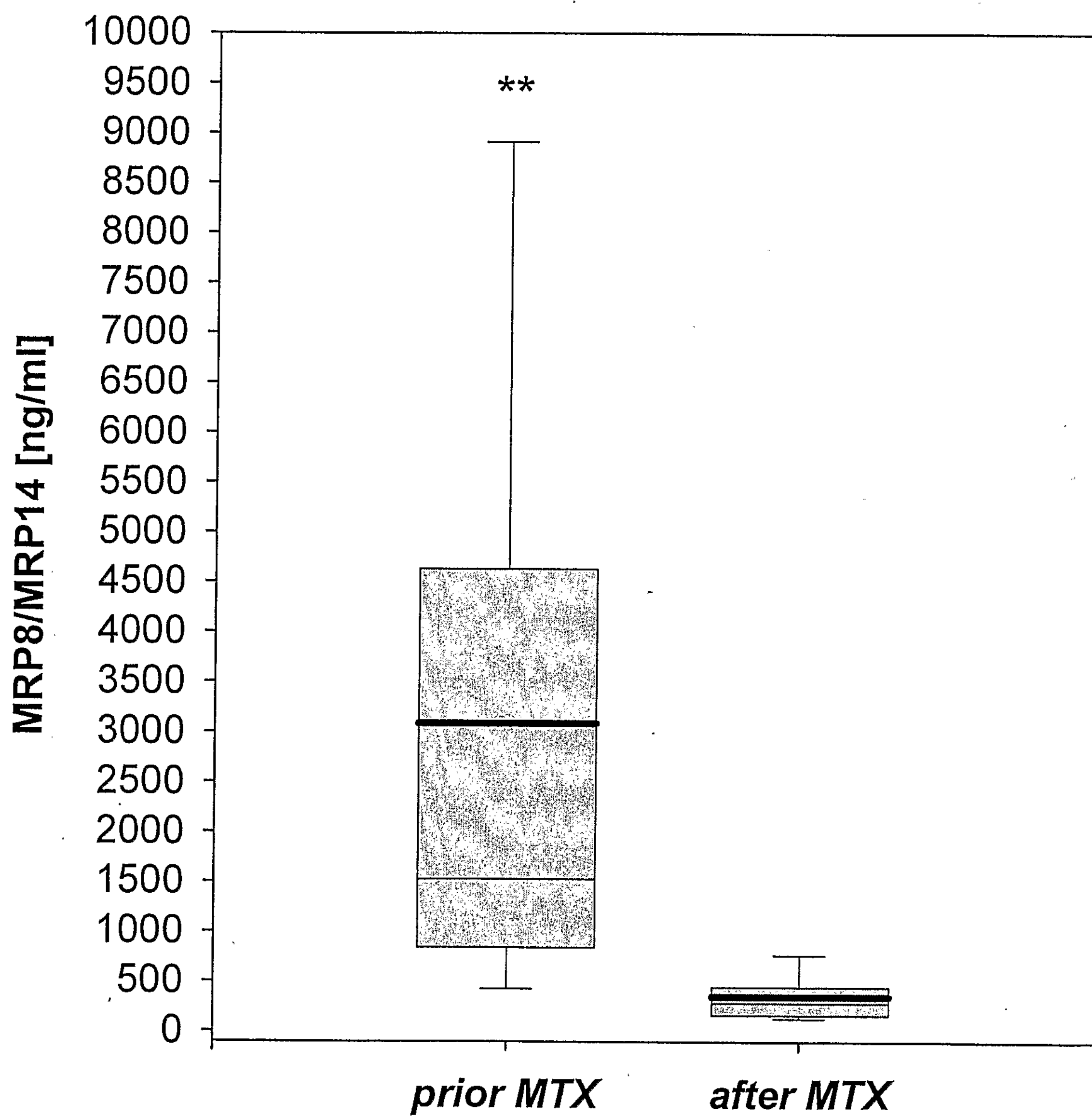
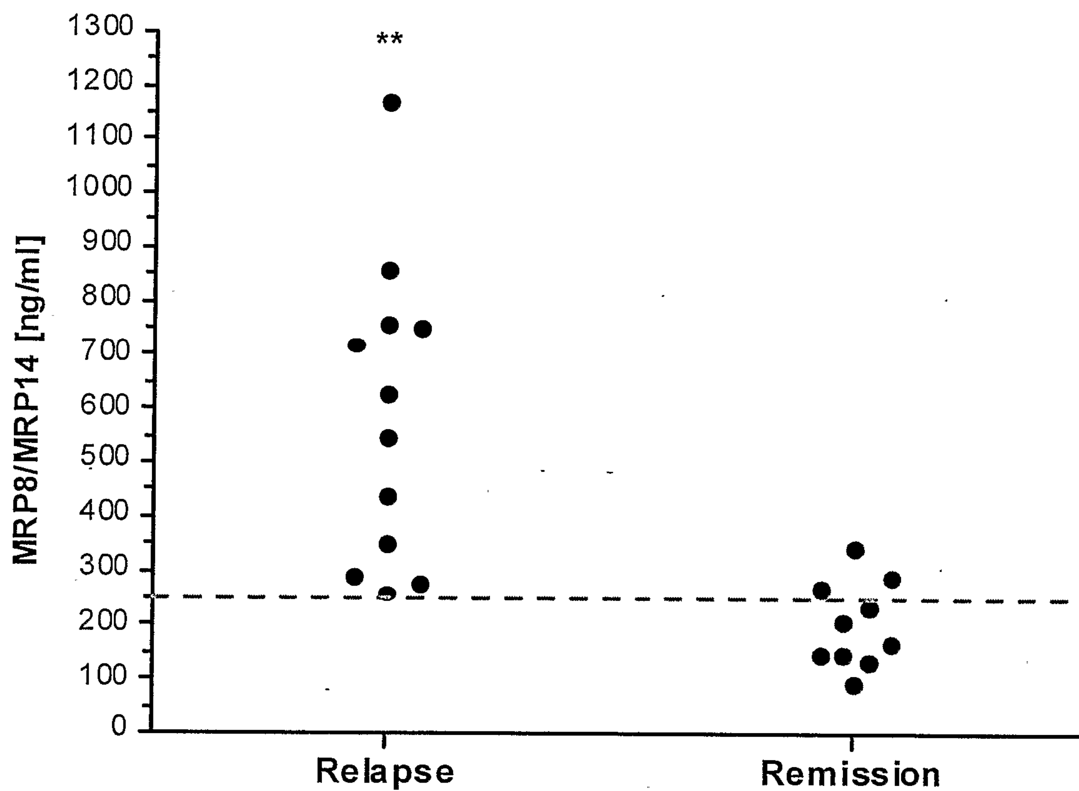


Figure 9



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