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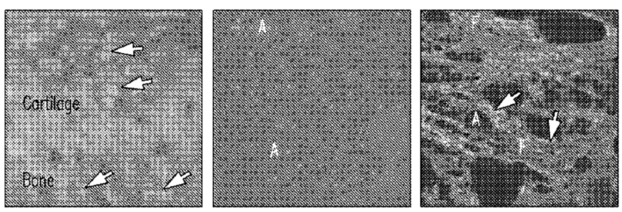

FIG. 1A FIG. 1B FIG. 1C

(57) Abstract: The present invention relates to methods and compositions for diagnosis of inflammatory disorders, and in non-limiting embodiments, of inflammatory disorders associated with elevated interleukin -1 β ("IL-Iβ"), based on increased levels of follistatin-like protein 1 ("FSTL-1"). In particular non-limiting embodiments, the invention further provides for methods of identifying subjects with systemic onset juvenile idiopathic arthritis ("SOJIA") who are at increased risk for developing macrophage activation syndrome ("MAS") comprising detecting, in said subjects, hyper-increased levels of FSTL-1 . In additional non-limiting embodiments, the invention provides for methods of identifying subjects with Kawasaki disease who are at increased risk of developing aortic aneurysms comprising detecting, in said subjects, hyper-increased levels of FSTL-1 .

FOLLISTATIN-LIKE PROTEIN-1 AS A BIOMARKER FOR INFLAMMATORY DISORDERS

PRIORITY CLAIM

This application claims priority to United States Provisional Application Serial Nos. 61/371,093 and 61/371,090 both filed August 5, 2010, the contents of which are hereby incorporated by reference herein in their entireties.

GRANT INFORMATION

This invention was made with government support under grants ROl AI073556, ROl AR056959, P30 AR047363, and AR052282 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention relates to methods and compositions for diagnosing inflammatory disorders, and particularly inflammatory disorders associated with elevated interleukin -1β ("IL-Iβ"), based on the serum and/or synovial fluid levels of follistatin-like protein- 1 ("FSTL-1").

2. BACKGROUND OF THE INVENTION

2.1 IL-lp-ASSOCIATED DISORDERS

Interleukin- 1β ("IL- 1β") is part of the eleven-member interleukin- 1 superfamily of cytokines that operate in immunity and inflammation. IL-Iβ has been found to be elevated in a number of inflammatory condtions, and in particular in a relatively recently recognized group of disorders termed "autoinflammatory disorders" characterized by recurrent bouts of fever and systemic and/or local inflammation, which are often responsive to IL- 1β blockade (see, for example, Dinarello, April 2011, Blood 117(14t:3720-3732V

2.2 JUVENILE IDIOPATHIC ARTHRITIS

Juvenile idiopathic arthritis ("JIA"), formerly known as juvenile rheumatoid arthritis (JRA), encompasses a heterogeneous group of diseases that are important causes of morbidity in children. JIA affects an estimated 250,000 children in the United States. The American College of Rheumatology (ACR) has classified JRAinto a number of subtypes, including systemic-onset, polyarthritis, and oligoarthritis (Cassidy et al., 1986, Arthritis Rheum. 29(2):274-281). Each of these subtypes has a different clinical presentation, prognosis, and response to specific therapies, suggesting that they differ in their pathogenesis and pathophysiology. For instance, polyarticular JIA responds well to anti-TNF therapy (Lovell et al., 2000, N. Engl. J. Med. 342(11):763-769; Lovell et al., 2003, Arthritis Rheum 48£1}:218-226) while systemic-onset JIA ("OJIA")does not (Horneff et al., 2004, Ann. Rheum. Dis. 63(12):1638-1644; Quartier et al., 2003, Arthritis Rheum. 48(4): 1093-1101). Systemic-onset JIA also differs from the other forms of JIA in that the arthritis is often accompanied by fever, rash, organomegaly, leukocytosis, and other systemic features in addition to arthritis. These systemic features can precede the development of arthritis by months or years, making the diagnosis at times difficult.

A number of biomarkers exist for aiding in the diagnoses and monitoring of rheumatoid arthritis (RA), including rheumatoid factor (Rose et al., 1948, Proc. Soc. Exp. Biol. Med. 680J:l-6) and anti-citrullinated proteins ("CCP"; Sebbag et al, 1995, J. Clin. Invest. 95(6):2672-2679; Young et al., 1979, Br. Med. J. 2(6182):97-99). However, these markers are usually not present in JIA. The most commonly used biomarkers used in JIA include elevation in erythrocyte sedimentation rate ("ESR"), C-reactive protein ("CRP"), and platelet count, but these are non-specific.

2.3 KAWASAKI DISEASE

Kawasaki disease (KD), an acute childhood vasculitis first described by Tomasaku Kawasaki in 1967 (Kawasaki, 1967, Arerugi 16(3):178-222). is the major cause of acquired cardiac disease in childhood (Taubert et al., 1991, J. Pediatr. 119(2):279-282). The etiology of KD remains unknown. It is believed that a possible undefined infectious agent triggers systemic inflammation and vasculitis in predisposed individuals (Galeotti et al., 2009, Autoimmun. Rev. 9(6):441-448; Rowley et al., 2007, Curr. Opin. Pediatr. 19(1):71-74). KD cases have been reported throughout the world, however there is a preponderance of expression of the disease among Asian populations and especially in Japan, where the incidence is increasing (Nakamura et al., 2010, J. Epidemiol. 20(4): 302-307).

The major complication of KD is the development of coronary artery aneurysms (CAA). The incidence of CAA has decreased due to treatment with IVIG, however up to 5% of treated patients still develop aneurysms, as compared to up to 25% of untreated patients (Newburger et al., 1986, N. Engl. J. Med. 315(6):341-347; Burns et al., 2004, Lancet 364f9433):533-544; Terai et al., 1997, J. Pediatr. 131(6):888-893; Kato et al., 1996, Circulation 94(6):1379-1385). Some cohort analyses have shown that Japanese male patients with known cardiovascular sequelae of childhood KD have a higher mortality ratio than other age-matched Japanese males (Gordon et al., 2009, J. Am. Coll. Cardio. 54(21): 191 1-1920), and incidents of very early onset acute coronary syndrome in young adults believed to be secondary to KD have been reported (Negoro et al., 2003, Circ. J. 67(4):362-365),

2.4 FSTL-1

In an effort to identify novel biomarkers for JIA (and other forms of arthritis) gene expression was analyzed in the mouse model of collagen-induced arthritis (CIA) and it was discovered that a poorly characterized gene, follistatin-like protein 1 (FSTL-1), originally cloned from an osteoblast cell line as a TGF-β inducible gene (Shibanuma et al., 1993, Eur. J. Biochem. 217(1}: 13-19), was highly-overexpressed in mouse paws during early arthritis, especially at the interface of synovial pannus and eroding bone (Thornton et al., 2002, Clin. Immunol. 105(2):155-168). FSTL-1 was recognized as a biomarker of inflammation, including in the context of JIA and Kawasaki Disease (see United States Patent Application Publication No. 201 10045507) and anti-FSTL-1 antibody has been shown to have beneficial effects in treating arthritis (see United States Patent No. 7,972,599).

FSTL-1 is highly conserved across mammalian species. Human and mouse FSTL-1 share 92% identity in their amino acid sequence. FSTL-1 is secreted by cells of the mesenchymal lineage, including cardiac myocytes, and suppression of FSTL-1 expression by siRNA treatment leads to increased cardiomyocyte apoptosis (Oshima et al., 2008, Circulation 117£24):3099-3 108). FSTL-1 has also been shown to have a role in promoting revascularization of skeletal muscle after ischemic injury (Ouchi et al., 2008, J. Biol. Chem. 283(47):32802-3281 1). FSTL-1 expression has been found to be elevated in patients with heart failure but returns to normal levels upon recovery (Lara-Pezzi et al., 2008, Endocrinol. 149(11):5822-5827).

FSTL-1 has been found in RA synovial tissue (Clutter et al, 2009, J. Immunol. 182(1):234-239; Tanaka et al., 1998, Int. Immunol. 10(9):1305-1314) and anti-FSTL-1 antibodies have been detected in the serum and synovial fluid of RA patients (Tanaka et al., 1998, Int. Immunol. 10(9):1305-1314). It was initially reported that administration of human FSTL-1 to Balb/c mice with antibody-induced arthritis ameliorated disease (Kawabata et al., 2004, Arthritis Rheum. 50(2):660-668), possibly by reducing synovial production of matrix metalloproteinases (Tanaka et al, 2003, Int. Immunol. 15(1):71-77). The effect was modest and it was subsequently demonstrated that FSTL-1 is a novel pro-inflammatory molecule with a previously unrecognized role in inflammation (Clutter et al., 2009, J. Immunol. 182(1):234-239; Myamae et al., 2006, J. Immunol. 177f7):4758-4762).

Transfection of FSTL-1 into macrophages and fibroblasts lead to up-regulation of proinflammatory cytokines felt to play central roles in chronic arthritis, including IL-Iβ and TNF-a. Induction of FSTL-1 requires NFKB (Clutter et al., 2009, J. Immunol. 182(l):234-239). Over-expression of FSTL-1 in mouse paws by gene transfer resulted in severe paw swelling and arthritis, while neutralization of FSTL-1 suppressed arthritis(Clutter et al., 2009, J. Immunol. 182(1):234-239). FSTL-1 was also found to be upregulated in the synovium of patients with RA, suggesting clinical relevance to our findings in the mouse model.

3. SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for diagnosis of inflammatory disorders, and in non-limiting embodiments, of inflammatory disorders associated with elevated interleukin -1β ("IL-Iβ"), based on increased levels of follistatin-like protein 1 ("FSTL-1"). In particular non-limiting embodiments, the invention further provides for methods of identifying subjects with systemic onset juvenile idiopathic arthritis ("SOJIA") who are at increased risk for developing macrophage activation syndrome ("MAS") comprising detecting, in said subjects, hyper-increased levels of FSTL-1. In additional non-limiting embodiments, the invention provides for methods of identifying subjects with Kawasaki disease who are at increased risk of developing aortic aneurysms comprising detecting, in said subjects, hyper-increased levels of FSTL-1.

4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1A-C. FSTL-1 is over-expressed in mesencbyme-derived tissues in CIA. FSTL-1 was visualized in knee joints from mice with CIA. (A) The bone-cartilage interface at high power, with the cartilage and bone visualized in phase contrast (red). Chondrocytes within amorphous matrix are seen in the upper part of the field; the cells label strongly for FSTL-1 (green; arrows). In the lower third of the field are osteocytes within the articular bone. These also label for FSTL-1 (arrows). The field shown is 100 μm square. (B) A high power field showing adipocytes (A) and fibroblast-like cells in synovial tissue. Anti-FSTL-1 antibody is labeled green; nuclei are visualized by Hoechst (blue fluorescence). The phase image is displayed in red with density inverted, so that the area of lipid, which is lost in processing, appear red. Field: 200 μm square. (C) FSTL-1 expression (green) in synovial fibroblasts (F) labeled with anti-CD90 (red); nuclei are blue. Note that many cells are labeled with both antibodies, and appear yellow (arrows). Adipocytes (A) appear as empty spaces; however, the phase image is inverted and merged as greyscale with the three colored fluorescent images. Field: 200 μm square.

FIGURE 2A-D. FSTL-1 is induced by pro-inflammatory cytokines. The osteoblast cell line, MC3T3 (A), the adipocyte cell line, 3T3-L1, (B) and 2 RA fibroblast-like synoviocyte (FLS) cell lines (C, D) were cultured for 3 days in the presence or absence of TGF-β, IL-Iβ, TNF-a, or IL-6 and supematants were assayed for FSTL-1. Each bar represents the mean and S.E.M. of 6 replicates. $*p < 0.05$.

FIGURE 3. FSTL-1 is elevated in sera of patients with active systemic-onset JRA. Sera from children with JRA, as well as pediatric control sera, were assayed for FSTL-1. Each circle represents an individual sample. Black circles indicate subjects with laboratory evidence of active disease (ESR ≥ 20 rnrn/hr or a platelet count ≥ 380×10^9/L). White circles indicate subjects with normal ESR and platelet count. Grey circles indicate subjects who did not have an ESR or platelet count. The horizontal line is drawn at a level of 18 ng/ml.

FIGURE 4. FSTL-1 is elevated in synovial fluids of patients with systemic-onset JRA. Synovial fluids from children with JRA, as well as fluids from control subjects, were assayed for FSTL-1 . Each bar represents the mean and S.E.M. of the indicated number of samples. $*p < 0.05$ compared to controls.

FIGURE 5A-B. (A) FSTL-1 serum levels in SJIA over time (unpaired samples). (B) FSTL-1 serum levels pre- and post-MAS over time in 3 patients (paired samples).

FIGURE 6. FSTL-1 plasma levels in KD. Each bar represents the mean ± SEM. n=number of patients.

FIGURE 7. Paired FSTL-1 plasma levels at presentation and after 6 months. Paired data was significantly different with p = 0.012.

FIGURE 8. Acute FSTL-1 plasma levels in patients with and without CAA. Each bar represents the mean ± SEM. n=number of patients.

FIGURE 9. Median, 25th and 75th percentiles values of plasma FSTL1. n=number of patients.

FIGURE 10. FSTL-1 mRNA copy number in mouse heart in response to in vivo administration of LPS. Hearts were collected from mice 7 hours following administration of PBS or LPS. mRNA was isolated and subjected to real-time PCR for mouse FSTL-1 transcript. Copy numbers were then normalized to 18s mRNA. Each bar represents the mean ± SEM of 3 mice.

FIGURE 11. Receiver Operator Curve analysis shows an area under the curve of 0.8223, (95% CI 0.6863, 0.9583).

5. DETAILED DESCRIPTION OF THE INVENTION

For clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) inflammatory disorders;

(ii) methods of measuring FSTL-1;

(iii) methods of diagnosis and

(iv) kits.

5.1 INFLAMMATORY DISORDERS

The present invention provides for the diagnosis of inflammatory disorders. In non-limiting embodiments, the present invention provides for the diagnosis of inflammatory disorders associated with elevated levels of IL-Iβ (meaning disorders having a laboratory finding in which serum levels of IL-Iβ are increased relative to control values and/or which may be treated by inhibiting IL-Iβ); in specific non-limiting embodiments the increase is by at least 20 percent or at least

30 percent or at least 50 percent over control values during at least a portion of the clinical course of the disorder). Examples of IL-lp-associated disorders that may be diagnosed according to the invention include but are not limited to juvenile idiopathic arthritis; systemic onset juvenile idiopathic arthritis; Kawasaki Disease; rheumatoid arthritis; periodic fever, aphthous stomatitis, pharyngitis, adenitis syndrome ("PFAPA"); urate crystal arthritis (gout); type 2 diabetes; smoldering multiple myeloma; postmyo cardial infarction heart failure; and osteoarthritis. Further IL-1β-associated disorders that are categorized as autoinfiammatory disorders or probable autoinflammatory disorders, and which may be diagnosed according to the invention, include but are not limited to familial Mediterranean fever ("FMF"); pyogenic arthritis, pyoderma gangrenosum, acne ("PAPA"); cryopyrin-associated periodic syndromes ("CAPS"); hyper-IgD syndrome ("HIDS"); adult and juvenile Still disease; Schnitzler syndrome; TNF receptor associated periodic syndrome ("TRAPS"); Blau syndrome; Sweet syndrome; deficiency in IL-1 receptor antagonist ("DIRA"); recurrent idiopathic pericarditis; macrophage activation syndrome; urticarial vasculitis; antisynthetase syndrome; relapsing chondritis; Behcet disease; Erdheim-Chester syndrome (histiocytosis); synovitis, acne, pustulosis, hyperostosis, osteitis ("SAPHO"), Muckle-Wells and neonatal-onset multisystem inflammatory disease (NOMID), and other disorders described in Dinarello, April 201 1, Blood U7(14) :3720-3732.

5.2 METHODS OF MEASURING FSTL-1

FSTL-1 may be measured by any method known in the art, including methods of measuring protein levels such as, but not limited to, enzyme-linked immunosorbent assay ("ELISA"); radioimmunoassay; polyacrylamide gel electrophoresis; or Western blot, etc. In certain non-limiting embodiments, levels of mRNA encoding FSTL-1 may be directly or indirectly measured and considered to reflect serum or synovial fluid levels of that protein; for example, mRNA may be prepared from a subject (e.g. patient or control) sample and then the amount of FSTL-1 encoding mRNA in the sample may be determined.

A sample may be collected from or sequestered from a patient. A sample may be, for example, but not by way of limitiation, a serum sample, a blood sample, a plasma sample, a synovial fluid sample, a cerebrospinal fluid sample, a peritoneal fluid sample, or a urine sample.

A subject may be a human or non-human subject, including but not limited to mammalian subjects.

In various non-limiting embodiments, FSTL-1 is measured by a method that comprises reacting FSTL-1 in a subject (e.g. patient or control) sample with a capture ligand to bind FSTL-1 to the capture ligand, and then directly or indirectly detecting the presence of FSTL-1 bound to the capture ligand.

In one set of non-limiting embodiments, FSTL-1 may be measured by the following method. For detection of human FSTL-1 in plasma, Nunc Immunomodule MaxiSorp F8 Framed ELISA plates may be coated with 5ug/ml polyclonal anti-FSTLl (AF1694; R&D Systems, Minneapolis, MN) in phosphate buffered saline (PBS) and incubated at 4° C overnight. Plates may then be washed with PBS/0.05%Tween 20 and blocked for one hour with bovine serum albumin (BSA) buffer (1% BSA and 5% sucrose in PBS). Plates may then be washed again, and human plasma samples diluted 1:10 may be added. After washing, 2.5ug/ml biotinylated monoclonal anti-FSTLl (MAB1694; R&D systems) may be added for 1 hour. Plates may then be washed again and incubated with Streptavidin-HRP conjugate at 0.25ug/ml for 20 minutes. BD OptEIA TMB Substrate Reagent may then be added, and plates may be incubated for an additional hour, following which development may be stopped with addition of 1M H_2S0_4. Plate absorbance may be read on a microplate reader with dual measurement of 450nm and 570nm reference level. A titration of purified FSTL-1 may be used to generate a standard curve from which plasma concentration of samples may be calculated.

In alternative specific, non-limiting examples of the invention, FSTL-1 levels may be measured by the following method. For detection of human FSTL-1 in sera and synovial fluids, standard bind plates (Meso Scale Discovery (MSD), Gaithersburg, Maryland) may be coated with 0.2 μg per well goat anti-human FSTL1 (AF1694; R&D Systems, Minneapolis, MN) in 0.03% Triton-XlOO overnight at 4° C. Plates may be washed with PBS/0.05% Tween-20 and blocked with MSD Human Serum Cytokine Assay Diluent for 1 hour. Human sera and synovial fluids, diluted 1:2 in MSD Human Serum Cytokine Assay Diluent, may be added overnight at 4° C. Plates may be washed and 0.5 μg/ml custom sulfo-tagged polyclonal rabbit anti-FSTL-1 may be added for 4 hours. Plates may be washed, 150 μl/well of 2x MSD

Read Buffer may be added, and plates then may be imaged in a MSD SECTOR Imager 2400.

5.3 METHODS OF DIAGNOSIS

The present invention relates to methods and compositions for diagnosis of inflammatory disorders, and, in non-limiting embodiments, of inflammatory disorders associated with elevated IL-1β, based on increased levels of FSTL-1.

In certain non-limiting embodiments, the present invention provides for a method of diagnosing an inflammatory disorder, or an inflammatory disorder associated with elevated IL-1β, or an autoinflammatory disorder, in a patient, comprising measuring the level of FSTL-1 in a sample of the patient and comparing that level to the level of FSTL-1 measured in a sample of a (healthy) control subject or an average level of FSTL-1 measured in samples from a plurality of control subjects, where a level in the patient that is about 20 -60 percent higher, or 30-60 percent higher, or 35-55 percent higher, or 30-50 percent higher, than the level or average level in the control subject or subjects is consistent with a diagnosis of an inflammatory disorder, or an inflammatory disorder associated with elevated IL-1β, or an autoinflammatory disorder, in the patient. In non-limiting examples, the sample may be a serum sample or a synovial fluid sample.

In certain non-limiting embodiments, the present invention provides for a method of diagnosing an inflammatory disorder, or an inflammatory disorder associated with elevated IL-1β, or an autoinflammatory disorder, in a human patient, comprising measuring the level of human FSTL-1 in a sample of the patient and comparing that level to the level of human FSTL-1 measured in a sample of a (healthy human) control subject or an average level of FSTL-1 measured in samples from a plurality of control subjects, where a level in the patient that is about 20 -60 percent higher, or 30-60 percent higher, or 35-55 percent higher, or 30-50 percent higher, than the level or average level in the control subject or subjects is consistent with a diagnosis of an inflammatory disorder, or an inflammatory disorder associated with elevated IL-1β, or an autoinflammatory disorder, in the patient. In non-limiting examples, the sample may be a serum sample or a synovial fluid sample.

In non-limiting embodiments, the present invention provides for a method of diagnosing an inflammatory disorder in a human patient comprising measuring the level of human FSTL-1 in a serum sample of the patient, where a level in the patient that is measured to be between about 200 and 300 ng/ml is consistent with a diagnosis of an inflammatory disorder in the patient. The inflammatory disorder, for example, may be an inflammatory disorder associated with elevated levels of interleukin-ï β or an autoinflammatory disoder as set forth above, for example, but not limited to, systemic onset juvenile idiopathic arthritis or Kawasaki disease. Said method may, for example, comprise comparing the serum level of human FSTL-1 measured in the patient to the level of human FSTL-1 measured in a serum sample of a healthy human control subject or an average level of human FSTL-1 measured in serum samples from a plurality of healthy human control subjects, where said level in a control subject, or said average level in control subjects, is measured to be between about 125-160 ng/ml.

In certain non-limiting embodiments, the present invention provides for a method of diagnosing SOJIA in a human patient comprising measuring the level of human FSTL-1 in a serum or synovial fluid sample of the patient and comparing that level to the level of human FSTL-1 measured in a serum or synovial fluid sample of a (healthy human) control subject or an average level of human FSTL-1 in serum or synovial fluid samples from a plurality of control subjects, where a level in the patient that is about 20-80 percent higher, or 20 -60 percent higher, or 30-60 percent higher, or 35-55 percent higher, or 30-50 percent higher, or 30-80 percent higher, than the level or average level in a control subject or subjects is consistent with a diagnosis of SOJIA for the patient. In a specific, non-limiting embodiment, where the control level in serum is measured to be about 15 ng/ml for a control subject or control subjects, a patient serum level measured to be between about 18 and 25 ng/ml is consistent with a diagnosis of SOJIA. In another specific, non-limiting embodiment, where the control level in synovial fluid is measured to be about 40 ng/ml for a control subject or control subjects, a patient synovial fluid level measured to be between about 60 and 70 ng/ml is consistent with a diagnosis of SOJIA. In yet another specific, non-limiting embodiment, where the control level in serum is measured to be about 125-160 ng/ml for a control subject or control subjects, a patient serum level measured to be between about 200 and 300 ng/ml, or between about 200 and 250 ng/ml, is consistent with a diagnosis of SOJIA. A diagnosis of SOJIA may be further corroborated by determining whether markers of inflammation, including, but not limited to, erythrocyte sedimentation rate ("ESR"), platelet count, and/or C reactive protein, are elevated, where an elevation in at least one of these markers more strongly indicates a diagnosis of SOJIA of active SOJIA in particular. In specific non-limiting examples of the invention, ESR ≥ 20 mm/hr or a platelet count ≥ 380x1 0^9/L would be corroborative of a diagnosis of SOJIA and of active SOJIA in particular.

In particular non-limiting embodiments, the invention further provides for methods of identifying a patient with SOJIA who is at increased risk for developing macrophage activation syndrome ("MAS") comprising detecting, in said patient, a level of FSTL-1 that is increased by 40 percent or more relative to control level(s). In particular non-limiting embodiments, the present invention provides for a method of identifying a patient with SOJIA who is at increased risk for developing or having MAS comprising measuring the level of human FSTL-1 in a serum or synovial fluid sample of the patient and comparing that level to the level of human FSTL-1 in a serum or synovial fluid sample of a (healthy human) control subject or an average level of human FSTL-1 in serum or synovial fluid samples from a plurality of control subjects, where a level in the patient that is 40 percent or greater, 50 percent or greater, and/or 75 percent or greater than the level or average level in a control subject or subjects is consistent with an increased risk that the patient will develop or has MAS. In a specific, non-limiting embodiment, where the control level in serum is measured to be about 125-160 ng/ml for a control subject or control subjects, a patient serum level measured to be between about 220 and 300 ng/ml, or greater than or equal to 230 ng/ml, or greater than or equal to 250 ng/ml, indicates that the subject is at increased risk of developing or having MAS. The increased risk of developing or having MAS may be further corroborated by detecting an elevation in one or more of the following MAS-associated biomarkers: IL-1 receptor, lipocalin 2, MMP8, MMP9, IL-18, and/or genes associated with TLR4/IL1 receptor signaling. Where a patient is deemed to be at increased risk for developing or having MAS, the diagnostic method of the invention may further comprise one or more of: performing a cytologic evaluation of the blood smear; determining the platelet count (where a decreased platelet count (e.g. less than 262 x 10^9/L) supports a diagnosis of MAS); determining the white blood cell count, where a decreased white blood cell count (e.g., less than 4 x 10^9) supports a diagnosis of MAS; and/or determining the level of serum fibrinogen, where a level less than 2.5 g/L is supports a diagnosis of MAS.

In certain non-limiting embodiments, the present invention provides for a method of diagnosing Kawasaki Disease in a human patient comprising measuring the level of human FSTL-1 in a serum sample of the patient and comparing that level to the level of human FSTL-1 measured in a serum sample of a (healthy human) control subject or an average level of human FSTL-1 measured in serum samples from a plurality of control subjects, where a level in the patient that is about 20-80 percent higher, or 20 -60 percent higher, or 30-60 percent higher, or 35-55 percent higher, or 30-50 percent higher, or 30-80 percent higher, than the level or average level in a control subject or subjects is consistent with a diagnosis of Kawasaki Disease for the patient. In a specific, non-limiting embodiment, where the control level in serum is measured to be about 120-140 ng/ml for a control subject or control subjects, a patient serum level measured to be between about 165-250 ng/ml is consistent with a diagnosis of Kawasaki Disease.

In additional non-limiting embodiments, the invention provides for methods of identifying a patient with Kawasaki disease who is at increased risk of developing an aortic aneurysm comprising detecting, in said patient, a level of FSTL-1 that is increased by 50 percent or more or by 60 percent or more relative to control level(s). In particular non-limiting embodiments, the present invention provides for a method of identifying a patient with Kawasaki Disease who is at increased risk for developing or having an aortic aneurysm comprising measuring the level of human FSTL-1 in a serum sample of the patient and comparing that level to the level of human FSTL-1 in a serum sample of a (healthy human) control subject or an average level of human FSTL-1 in serum samples from a plurality of control subjects, where a level in the patient that is 60 percent or greater and/or 75 percent or greater than the level or average level in a control subject or subjects is consistent with an increased risk that the patient will develop or has an aortic aneurysm. In a specific, non-limiting embodiment, where the control level in serum is measured to be about 125-160 ng/ml for a control subject or control subjects, a patient serum level measured to be between about 200 and 250 ng/ml, or greater than or equal to 200 ng/ml, indicates that the patient is at increased risk of developing or having an aortic aneurysm. Where a patient is deemed to be at increased risk of developing or having an aortic aneurysm, a further procedure may be recommended, for example but not limited to an angiogram, radiologic, CT or MRI to visualize the aorta.

5.4 KITS

In certain embodiments, the present invention provides for kits that may be used to practice the diagnostic methods of the invention. Said kits may comprise a sample of monoclonal or polyclonal antibody specific for human FSTL-1, where said antibody is optionally detectably labeled (for example, with an enzyme, fluorescent, or radioactive label), together with one or more, two or more, or three or more, or four or more, of the following: an antibody that specifically binds to the anti-human FSTL-1 antibody, an antibody that binds to human C-reactive protein; an antibody that binds to IL-1 receptor, an antibody that binds to lipocalin 2, an antibody that binds to MMP8, an antibody that binds to MMP9, an antibody that binds to IL-18, one or more antibody that binds to the product(s) of genes associated with TLR4/IL1 receptor signaling, and human FSTL-1, which, for example, may be used to create a standard curve of FSTL-1 dilutions.

6. EXAMPLE: FSTL-1 IS A MESENCHYME-DERIVED INFLAMMATORY PROTEIN AND MAY REPRESENT A BIOMARKER FOR SYSTEMIC ONSET JUVENILE RHEUMATOID ARTHRITIS

6.1 MATERIALS AND METHODS

Patient samples. Banked sera and synovial fluids were obtained from patients with JRA defined according to criteria established by the ACR (1 ; reference list at end of this section 6). Patient demographics are summarized in Table 1, below. The study patients were recruited from the rheumatology clinic at Children's Hospital of Pittsburgh. Banked synovial fluids were also obtained from the Cincinnati Children's Hospital Medical Center JRA Tissue Repository. Control synovial fluids were collected from children with no history of JRA or inflammatory disease who underwent an orthopedic procedure, such as ACL repair. The synovial fluid samples were placed on ice immediately after collection, centrifuged at 400 x g for 10 minutes to remove cells and debris and stored at-80°C. The sera were allowed to clot, centrifuged at 3,000 x g for 10 minutes to remove red blood cells, and stored at -80° C. The study was approved by the Institutional Review Board at the University of Pittsburgh. Informed consent was obtained from all guardians of patients and assent was obtained from the subjects when appropriate.

Table 1. Demographic and clinical characteristics of the study population*

Characteristic	Oligo (n = 54)	Poly (n = 26)	Systemic (n = 15)	Control (n = 15)
Age, years	9 ± 5.17	12 ± 6.17	13 ± 6.81	12 ± 8.31
Sex, no. (%)				
Male	18 (33)	9 (35)	8 (53)	6 (40)
Female	36 (67)	17 (65)	7 (47)	9 (60)
Disease Duration, years	3 ± 4.35	7 ± 5.99	7 ± 8.42	N/A

*Except where indicated otherwise, values are the mean ± SD. Oligo = oligoarticular JIA; Poly = polyarticular JIA; Systemic = systemic JIA

Mice. Male DBA/1 mice, 6-10 weeks of age, were purchased from Harlan (Indianapolis, IN). Mice were housed in the animal resource facility at the Children's Hospital of Pittsburgh Rangos Research Center (Pittsburgh, PA). The study was approved by the Children's Hospital of Pittsburgh's Animal Research and Care Committee. CIA was induced by intra-dermal immunization of DBA/ 1 mice with bovine collagen type II (Elastin Products, Owensville, MO), as previously described (16).

FSTL-1 immunoassay. For detection of human FSTL-1 in sera and synovial fluids, standard bind plates (Meso Scale Discovery (MSD), Gaithersburg, Maryland) were coated with 0.2 μg per well goat anti-human FSTL1 (AF1694; R&D Systems, Minneapolis, MN) in 0.03% Triton-XlOO overnight at 4° C. Plates were washed with PBS/0.05% Tween-20 and blocked with MSD Human Serum Cytokine Assay Diluent for 1 hour. Human sera and synovial fluids, diluted 1:2 in MSD Human Serum Cytokine Assay Diluent, were added overnight at 4° C. Plates were washed and 0.5 μg/ml custom sulfo-tagged polyclonal rabbit anti-FSTL-1 was added for 4 hours. Plates were washed, 150 μï/well of 2x MSD Read Buffer was added, and plates were imaged in a MSD SECTOR Imager 2400.

Immunohistochemistry. Knee joints from mice with CIA were frozen in liquid nitrogen-cooled isopentane. Seven micron sections were prepared with the Cryojane Tape Transfer System (Instrumedics, St. Louis, MO). Slides were fixed with 2% paraformaldehyde for 20 minutes and washed with PBS followed by BSA buffer (0.5% BSA and 0.15% glycine in PBS). Slides were blocked with a 1/20 dilution of normal donkey serum (Sigma-Aldrich, St. Louis, MO) in BSA buffer and washed three times with BSA buffer. Slides were incubated for 1 hour with 10 μg/ml affinity-purified polyclonal goat anti-mouse FSTL-1 (AF1738; R&D Systems, Minneapolis, MN). The slides were washed three times with BSA buffer, and bound antibody was visualized using 4 μg/ml Alexa Fluor 488-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA). For identification of fibroblasts, slides were reacted with 1.5 μg/ml rat anti-mouse CD90 (BD Pharmingen, Franklin Lakes, NJ) and bound antibody was visualized with 2μg/ml Alexa Fluor 594-conjugated donkey anti-rat IgG. Nuclei were stained with Hoechst 3343 blue (Invitrogen). A coverslip containing a drop of gelvatol was added, and slides were stored at 4° C until observation. Slides were imaged using an Olympus Fluroview 1000 (Olympus) confocal microscope or a Nikon TE2000 inverted phase-fluorescence microscope using a 12-bit 1600x1200 element CCD array to capture images (Spot, Diagnostic Instruments, Sterling Heights, MI); color, where shown, is assigned to the channel indicated and reflects approximate output fluorescence unless indicated. Filters for green fluorescence were: excitation 450-490 nm, 510 nm dichroic mirror, 500-570 nm barrier; for red fluorescence: excitation 536-556 nm, 580 nm dichroic mirror, 580-650 nm barrier. Photographs used a NA 0.70 long working distance 40 x phase contrast objective.

In vitro induction and detection of FSTL-1. MC3T3, 3T3-L1, and human fibroblast like synoviocytes derived from patients with rheumatoid arthritis undergoing joint replacement were cultured at a concentration of 3 x 10^4 cells/well in 96-well flat bottom plates for 3 days in triplicate with or without the addition of TGF-β (2 ng/ml), IL-1β (10 ng/ml) TNF-α (10 ng/ml), or IL-6 (50 ng/ml). Cell culture supernatants were assayed for mouse or human FSTL-1 by coating Nunc Immunomodule MaxiSorp ELISA plates (Nalgene, Rochester NY) with 5 μg/ml rat anti-mouse FSTL-1 (MAB1738; R&D Systems, Minneapolis, MN) or goat anti-human FSTL-1 (AF1694; R&D Systems) overnight at 4°C. Plates were washed with PBS/0.05% Tween-20 and blocked with 1% BSA/5% sucrose/0.05% Tween-20 for 1 hour. Cell culture supernatants were added at appropriate dilutions and held at room temperature for 1 hour. After washing, 5 μg/ml biotin-labeled goat anti-mouse FSTL-1 (AF1738; R&D Systems) or rat-anti-human FSTL-1 (MAB 1694; R&D Systems) was added for 1 hour. Plates were washed and incubated with streptavidin-HRP (Invitrogen, Carlsbad, CA), developed with Peroxidase Substrate System ABTS

(Kirkegaard & Perry, Gaithersburg, MD), and absorbance was read at 405 nm on a microplate reader.

6.2 RESULTS

FSTL-1 is produced in the joint space by cells of the mesenchymal lineage. Fluorescent antibody labeling in frozen sections of joints from mice with CIA was used to determine whether FSTL-1 is produced in joint tissues. FSTL-1 protein was found in cells of the mesenchymal lineage, including osteocytes and chondrocytes (FIGURE 1A), adipocytes (FIGURE IB) and fibroblasts (FIGURE 1C). No FSTL-1 expression was observed in cells of the hematopoietic lineage, such as macrophages, T cells, and B cells.

FSTL-1 secretion is induced in mesenchymal lineage cells by arthritis-promoting cytokines. The ability of various inflammatory cytokines to induce FSTL-1 secretion from mesenchymal cells was analyzed. For these experiments, the mouse osteoblast cell line, MC3T3, the mouse adipocyte cell line, 3T3L1, and two human fibroblast-like synoviocyte cell lines derived from hip joints of two patients with RA were utilized. Cells were stimulated with IL-1 β, TNF-α, or IL-6. TGF-β was used as a positive control, since FSTL-1 was originally described as a TGF-β inducible gene in MC3T3 cells (9). IL-1 β, TNF-α, and IL-6 all stimulated FSTL-1 secretion from osteoblasts (FIGURE 2A), while the adipocytes responded only to TGF-β (FIGURE 2B). One of the 2 fibroblast-like synoviocyte lines responded to IL-Iβ, TNF-a, and IL-6 (FIGURE 2C) while the other line responded to IL-Iβ, and TNF-a, but not IL-6 (FIGURE 2D). In both fibroblast-like synoviocyte cell lines, IL-Iβ induced a significantly greater secretion of FSTL-1 than did TNF-a. Monocytic cells (U937), T cells (Jurkat) and B cells (A20) failed to make any detectable FSTL-1, demonstrating that FSTL-1 is not produced by cells of the hematopoietic lineage. FSTL-1 was also not produced by hepatocytes.

FSTL-1 is elevated in sera and synovial fluids of patients with systemic-onset JRA. It has previously been shown that FSTL-1 is overexpressed in synovial tissues of mice with arthritis (10). To explore the possibility that FSTL-1 might play a role in JRA, FSTL-1 titers were measured in banked sera (n=55) and synovial fluids (n=74) from children with JRA. Patient demographics are summarized in Table 1.

FSTL-1 concentrations in sera. In sera, the mean FSTL-1 concentration of control subjects was 15.2 ng/ml and all controls had concentrations below 18 ng/ml (FIGURE 3). Mean FSTL-1 concentration of the oligoarthritis and polyarthritis JRA subtypes (both 15 ng/ml) did not differ significantly from that of controls. In marked contrast, the mean concentration of the systemic-onset JRA subtype (18 ng/ml) was significantly elevated ($p = 0.0007$). Furthermore, in systemic-onset JRA, a striking correlation was observed between elevated FSTL-1 concentration and laboratory markers of inflammation, defined here as either an ESR ≥ 20 mm/hr or a platelet count $\geq 380\text{x}10^{9}$/L (based on the upper limit of normal as reported by the laboratory). Four of the 7 sera from systemic-onset JRA subjects had concentrations above 18 ng/ml, with one as high as 23 ng/ml. All of these 4 subjects with elevated FSTL-1 concentrations had active disease, as defined above, while 2 of the 3 subjects with normal FSTL-1 concentrations had inactive disease. Thus, in all but 1 of the 7 samples from subjects with systemic-onset JRA, elevation of serum FSTL-1 correlated with laboratory evidence of active disease. In contrast, no correlation was observed between FSTL-1 concentrations and active disease in the oligoarthritis and polyarthritis subsets. These data suggest that elevation of serum FSTL-1 is a biomarker for systemic-onset JRA.

FSTL-1 concentrations in JRA synovial fluids. Only synovial fluids from systemic-onset JRA patients were significantly higher than controls (FIGURE 4), again suggesting that elevated FSTL-1 is a marker of the systemic-onset subtype of JRA. Synovial fluid FSTL-1 concentrations were 2-3 fold higher than those observed in serum, indicating that the joint is a source of FSTL-1.

6.3 DISCUSSION

The data presented here support the conclusion that FSTL- 1 is a major mediator of the inflammatory cascade that underlies arthritis. For instance, we have demonstrated that over-expression of FSTL-1 in mouse paws by gene transfer resulted in severe paw swelling and arthritis (15), while neutralization of FSTL-1 suppressed arthritis (11). Also, transfection of FSTL-1 into macrophages and fibroblasts lead to up-regulation of proinflammatory cytokines with central roles in chronic arthritis, including IL-1-β and TNF-α (11).

While expression of FSTL-1 in osteoblasts and fibroblasts has been previously reported, the finding that FSTL-1 can be produced by other mesenchymal cells, including adipoctyes and chondrocytes, is novel. These results, along with the observation that synovial fluid levels are 2-3 fold higher than serum levels, support the conclusion that the joint is a primary source of FSTL-1 . Furthermore, it supports the concept that the joint matrix, including bone, cartilage, and adipose tissue^ is not merely a passive target of destruction by blood-derived immune cells in arthritis; rather, this joint matrix plays an active role in perpetuating and amplifying the inflammatory response by releasing pro-inflammatory mediators, such as FSTL-1 .

The present study suggests that systemic-onset JRA is characterized by elevated concentrations of serum and synovial fluid FSTL-1 that are not observed in other forms of JRA. Elevation of serum FSTL-1 correlated closely with markers of disease activity in systemic-onset JRA , including elevated ESR and platelet count, but this correlation was not observed in oligoarthritis or polyarthritis. These data suggest that FSTL-1 may be useful as a biomarker of disease activity in this JRA subtype. An important caveat is that, although the results are statistically-significant, the number of systemic onset samples available to us was small so that these findings should be validated in a larger cohort of patients. Also, because banked samples were used, the clinical data available, other than ESR and platelet counts, was limited .

The specificity for systemic JRA is interesting in light of the finding that FSTL-1 secretion from human fibroblast-like synoviocytes was significantly greater following incubation with IL-1 β than with TNF-α. Systemic-onset JRA has recently been shown to have a strong IL-Iβ gene expression signature (17). Many patients with systemic-onset JRA respond well to the IL- 1 receptor antagonist, Anakinra, (17, 18) but less well to anti-TNF therapy (4, 5). However, it is as yet unclear why patients with polyarticular and oligoarticular arthritis did not have elevated FSTL-1 titers, since TNF-a also induced FSTL-1 secretion from fibroblast-like synoviocytes, albeit to a lesser degree, and TNF-a is a central cytokine in polyarticular disease (2, 3). It is possible that the preferential induction of FSTL-1 by IL-1 β is more pronounced in vivo than in vitro.

These findings suggest that FSTL-1 may be a useful biomarker in other disorders driven by IL-1β, such as the autoinflammatory syndromes, including Muckle-Wells and neonatal-onset multisystem inflammatory disease (NOMID) (19-22). None of the samples evaluated in the experiments described in this section were from subjects with macrophage activation syndrome (MAS), which is a serious complication of systemic-onset JRA that can lead to rapid deterioration and death if not treated aggressively.

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7. EXAMPLE: FSTL-1 PROMOTES ARTHRITIS BY ENHANCING CYTOKINE/CHEMOKINE GENE EXPRESSION

The aim of the experiments described in this section was to determine the mechanism by which FSTL-1 promotes arthritis, focusing on FSTL-1 as a putative mediator of pro-inflammatory cytokine and chemokine synthesis.

7.1 MATERIALS AND METHODS

CIA was induced in mice hypomorphic for FSTL-1 that were generated using a genetrap technique, resulting in a significant reduction of FSTL-1 protein expression.

Arthritis was assessed by measuring paw swelling and using a qualitative arthritic index.

Mesenchymal stromal cells (MSC) were isolated from the bone marrow of wild type and hypomorphic mice.

To suppress FSTL-1 expression, mouse stromal ST2 cells were transduced with a lentivirus encoding mouse FSTL-1 short hairpin RNA. MSC and ST2 cells were stimulated with IL-1β, TNF-α, or IL-6. Monocytic U937 cells, which do not normally express FSTL-1, were transfected with FSTL-1 and stimulated with phorbol myristate acetate (PMA) and lipopolysaccharide (LPS). The levels of FSTL-1, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) were assessed by ELISA.

7.2 RESULTS

In CIA, a significant correlation was found between serum FSTL-1 levels and both paw swelling and the arthritic index ($r=0.399$, $p<0.01$; $r=0.496$, $p<0.05$, respectively). FSTL-1 up-regulated IL-6, IL-8 and MCP-1 production in PMA- and LPS-stimulated U937 cells. Knockdown of endogenous FSTL-1 expression suppressed IL-6 and MCP-1 production by stimulated stromal ST2 cells and MSC. FSTL-1 protein could be induced in vivo after treatment of mice with LPS.

7.3 CONCLUSIONS

These findings demonstrate that FSTL-1 directly up-regulates pro-inflammatory mediators important in the pathogenesis of arthritis and that serum levels of FSTL-1 correlate with severity of arthritis.

8. EXAMPLE: SERUM FSTL-1 IS ELEVATED IN SYSTEMIC JUVENILE IDIOPATHIC ARTHRITIS AND IS A BIOMARKER FOR MACROPHAGE ACTIVATION SYNDROME

8.1 MATERIALS AND METHODS

FSTL-1 serum levels were measured by ELISA in 27 patients with sJIA, including 6 patients who developed MAS, as well as in 15 normal controls. Levels were correlated with CD163 and sIL2Ra expression. Peripheral blood mononuclear cells (PBMC) were separated on Ficoll gradients, and RNA was analyzed to evaluate differential gene expression.

8.2 RESULTS

FSTL-1 serum levels are elevated at the time of initial diagnosis of sJIA, as compared to controls (mean of 216.3 ng/ml vs. 156.1 ng/ml, p=0.01). FSTL-1 levels decreased during the course of treatment (FIGURE 5A; mean of 132.5 ng/ml after 24 months, p = 0.001). Especially high levels of FSTL-1 were present in patients during acute MAS (mean of 231.5 ng/ml). In 3 patients for whom paired samples were available pre- and post-treatment for MAS, FSTL-1 levels changed from a mean of 289.5 ng/ml to a mean of 124.0 ng/ml, p=0.08 (FIGURE 5B). Patients with elevated FSTL-1 levels showed increased expression of markers previously associated with MAS, including CD163. PBMC from these patients also showed a 2-fold or greater increase in expression levels of IL-1 receptor, Lipocalin 2, MMP-8, MMP-9, IL-18, as well as other genes associated with TLR4/IL1R signaling (p O.05).

8.3 DISCUSSION

Systemic juvenile idiopathic arthritis (sJIA) can be complicated by macrophage activation syndrome (MAS), an often fatal disorder characterized by multisystem organ failure. IL-1β and IL-6 are key inflammatory mediators in SJIA and blockade of these cytokines can ameliorate disease activity in a subset of patients. As discussed in section 6, above, FSTL-1 can increase secretion of IL-1β and IL-6 from monocytes and mesenchymal stromal cells (MSC). The experiments described in this section were performed to determine how FSTL-1 levels correlate with measures of clinical disease activity and development of MAS. It was demonstrated that serum FSTL-1 is elevated in clinically active sJIA and increased FSTL-1 correlates with the development of MAS. Patients with elevated levels of FSTL-1 had increased expression of Interleukin-1 and TLR4 related genes, and may represent a subgroup with more severe disease.

9. EXAMPLE: FSTL-1 IS ELEVATED IN KAWASAKI DISEASE AND ASSOCIATED WITH CORONARY ANEURYSM FORMATION

9.1 MATERIALS AND METHODS

Patient samples. Banked plasma samples were obtained from patients with KD whose diagnosis was made through established clinical criteria (19). Banked samples were obtained through the pediatric cardiology clinics and inpatient services at Cincinnati Children's Hospital Medical Center and from Northwestern University Children's Memorial Hospital. Serial samples from 41 individual patients were obtained at acute presentation (prior to IVIG), and, when available, at 2 weeks, 6 weeks, and 6 months following presentation. An additional 6 patients did not have an acute sample but had samples at later time points. Seven additional samples were obtained from patients with acute KD who subsequently developed CAA. Control plasma samples were obtained from 23 children with no history of inflammatory disease who underwent surgical procedures including hernia repair, tonsillectomy and adenoidectomy, and simple ophthalmologic procedures. Use of samples was approved by the Institutional Review Board at the University of Pittsburgh. Patient demographics are summarized in Table 2, which is at the end of this section.

FSTL-1 ELISA. For detection of human FSTL-1 in plasma, Nunc Immunomodule MaxiSorp F8 Framed ELISA plates were coated with 5ug/ml polyclonal anti-FSTLl (AF1694; R&D Systems, Minneapolis, MN) in phosphate buffered saline (PBS) and incubated at 4° C overnight. Plates were then washed with PBS/0.05%Tween 20 and blocked for one hour with bovine serum albumin (BSA) buffer (1% BSA and 5% sucrose in PBS). Plates were washed again, and human plasma samples diluted 1:10 were added. After washing, 2.5ug/ml biotinylated monoclonal anti-FSTLl (MAB1694; R&D systems) was added for 1 hour. Plates were washed again and incubated with Streptavidin-HRP conjugate at 0.25ug/ml for 20 minutes. BD OptEIA TMB Substrate Reagent was added, and plates were incubated for an additional hour, following which development was stopped with addition of 1M H2S04. Plate absorbance was read on a microplate reader with dual measurement of 450nm and 570nm reference level. A titration of purified FSTL-1 was used to generate a standard curve from which plasma concentration of samples was calculated.

Mice. Male DBA/1 mice, 6-9 weeks of age, were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in the animal resource facility at the Children's Hospital of Pittsburgh Rangos Research Center (Pittsburgh, PA). Mice were intraperitoneally injected with 200ul sterile phosphate buffered saline (PBS) containing 50□g lipopolysaccharide (LPS). Controls were intraperitoneally injected with PBS only. After 7 hours, mice were sacrificed and perfused with 10ml PBS. The hearts were harvested and ground into a powder using a mortar and pestle while on dry ice. Samples were stored at -80C freezer.

Quantitative RT-PCR. Total RNA was isolated from mouse heart tissue using Invitrogen's RNA TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. cDNA was synthesized with random hexamer oligonucleotides using 1 ug of RNA and Invitrogen's Superscript II Reverse Transcriptase Kit. PCR was performed in a Light- Cycler (Mx3000P; Stratagene) using Brilliant SYBR Green QPCR Master Mix (Stratagene) according to the protocol (95°C hot start for 10 min followed by 40 amplification cycles, denaturation at 95°C, primer annealing at 59°C, and amplicon extension at 72°C) using oligonucleotide primer sets for mouse FSTL 1 (forward 5' AACAGCCATCAACATCACCA-3 ' (SEQ ID NO:l); reverse 5'-GGCACTTGAGGAACTCTTGG-3 ' (SEQ ID NO:2)) The copy number (number of transcripts) of amplified products was calculated from a standard curve obtained by plotting known input concentrations of plasmid DNA and normalized to total RNA content using 18s RNA as a housekeeping gene.

Statistical Analysis. Statistical analysis was performed using STATA version 11. Sensitivity and specificity for aneurysm development by FSTL-1 concentration cutpoint was calculated, as well as positive predictive values. A Receiver Operating Curve (ROC) of FSTL-1 levels at non-aneurysm and aneurysm status was constructed. Mean values were compared by use of Student's t test to determine statistical differences between experimental groups. All reported p values are 2-sided and considered significant at $p < 0.05$.

9.2 RESULTS

FSTL-1 is elevated in the blood of patients with acute KD. In order to determine whether patients with acute KD had elevation in FSTL-1 levels, plasma samples from 54 patients with KD were assayed. Of these patients, 7 were known to have developed coronary artery aneurysms. Patients had an initial sample collected during acute disease (prior to IVIG). When possible, additional samples were collected at 2 weeks, 6 weeks, and 6 months post presentation. As shown in FIGURE 6, the mean and standard error of the mean (SEM) FSTL-1 level in acute KD was 169.7 ± 6.9 ng/ml, compared to 128.3 ± 6.1 ng/ml in controls ($p < 0.001$). Mean FSTL-1 levels declined to l50.3 ± 5.6 ng/ml at 2 weeks (p value not significantly different from acute time point) and 141.8 ± 4.9 ng/ml at 6 weeks ($p = 0.03$ compared to acute time point). FSTL-1 levels declined further to a level of 119.6 ± 5.2 ng/ml at 6 months, which was not statistically different from normal controls, but significantly different from the acute time point ($p <0.001$). Paired data analysis by paired t-test of all patients with an initial (acute) time point and a recovery (6 month post presentation) time point also showed statistical significance, $p=0.012$ (FIGURE 7).

Patients developing CAA have higher FSTL-1 levels in the acute phase than patients who do not. It was then determined whether FSTL-1 level during acute disease might be a useful predictor for the risk of developing CAA. The acute FSTL-I levels in the 7 patients who went on to develop aneurysms were compared to the FSTL-1 levels in the 47 patients who did not develop aneurysms. As shown in FIGURE 8, the mean FSTL-1 level in patients who developed aneurysms was significantly higher than patients who did not (219.2 ± 20.9 ng/ml versus 161 .2 ± 7.2 ng/ml; $p = 0.024$). As shown in FIGURE 9, patients who developed CAA had substantially higher levels of FSTL-1 at presentation than did patients without CAA at all stages of disease. The median, 25th and 75th percentiles FSTL-1 values at presentation in patients who did not develop CAA was 148.5 ng/ml, 130.4 ng/ml and 183.6 ng/ml. Median, 25th and 75th percentiles FSTL-1 values at presenation in patients who did develop CAA was 195.5 ng/ml, 177.8 ng/ml and 268.7 ng/ml.

Transcription of FSTL-1 gene is increased in the mouse heart in response to LPS stimulation. FSTL-1 production is known to be induced in the mouse heart by ischemic injury (13). It was queried whether FSTL-1 is induced in the heart and the coronary arteries in response to an acute systemic inflammatory condition such as KD. LPS was administered to mice as a surrogate of an acute systemic inflammatory insult. Mice were sacrificed 7 hours later and the hearts were collected and assayed by QT-PCR for FSTL-1 transcript. FSTL-1 transcript number was significantly increased approximately 2-fold, compared to control mice injected with PBS (FIGURE 10).

FSTL-1 concentration has strong sensitivity and specificity as a biomarker for aneurysm development. The ROC analysis for FSTL-1 levels at non-aneurysm and aneurysm status had an area under the curve of 0.8223, (95% CI 0.6863, 0.9583), corresponding to good accuracy of FSTL-1 plasma concentration for aneurysm status (FIGURE 11). Using the ROC, a threshold of 178 ng/ml yielded a sensitivity of 85%, with specificity of 71% (Table 3. at the end of this section).

9.3 DISCUSSION

As described above, FSTL-1 induces inflammation in mice and is associated with elevated pro-inflammatory cytokines such as IL-1 and IL-6 (12, 16, 18). FSTL-1 was also found to be elevated in the blood and synovial fluids of children with active systemic Juvenile Idiopathic Arthritis (18), an illness with clinical features that resemble KD. The results of the study described in this section suggest that high levels of FSTL-1, which is produced by cardiac myocytes, might be a biomarker for development of CAA in KD.

Development of CAA is the major cause of morbidity and mortality associated with KD. Efforts to define clinical or serological risk factors for the development of CAA have been described over the last 30 years. However, none of these methods are widely used, and some have not been independently validated. These risk factors have included non-coronary cardiac abnormalities (20), incomplete clinical presentation at very young ages, and resistance to IVIG therapy at older ages (21, 22). Clinical scoring mechanisms to predict development of CAA have been developed that utilize neutrophil and band counts, hemoglobin concentrations, platelet counts, and temperature on the day after infusion of IVIg(23). Nakano et al. (24) and Iwasa et al. (25) identify, among other findings, lab markers such as high white blood cell count, thrombocytopenia, anemia, C-reactive protein levels, age of presentation and male sex as risk factors for development of aneurysm formation. Koren et al. (26) noted that children with CAA had significantly higher temperature during days 10 to 13 of the disease.

Lin et al. investigated other serologic markers for development of CAA including IL-6, TNF-a and soluble IL-2 receptor (27). This evaluation included multiple monthly blood samples and showed that elevated levels of IL-6 and IL-8 in the first week of illness could predict development of CAA. The findings reported here indicate that elevated FSTL-1 levels correlate with increased risk of development of CAA in KD, suggesting that a simple blood test at the time of diagnosis might provide high sensitivity and specificity in identifying patients at high risk. Limitations to this study include the relatively small numbers of patients in the CAA group, as well as the overrepresentation of Hispanic patients in this group. Additionally, all of the patients included met the criteria for complete KD, and we cannot generalize the findings to patients with atypical or incomplete KD. Despite these limitations, the results herein are consistent with reports that FSTL-1 expression has been shown to be increased in the heart in the setting of cardiac injury and may play a protective role against hypoxic injury to myocytes(13). FSTL-1 was also found to be elevated in patients with heart failure, and FSTL-1 staining was seen in myocytes and vascular endothelial cells of capillaries, small vessels, and smooth muscle cells of larger vessels in these patients (15).

Alterations in eNOS expression have been found to be associated with aneurysm formation both in mouse and human models of aneurysmal disease . Aged eNOS knockout mice have decreased aneurysm formation in comparison to wild type mice (28) suggesting that eNOS activity contributes to aneurysm formation. Polymorphisms in the eNOS gene have been shown to predispose individuals to develop abdominal aortic aneurysms (29), and alterations in nitric oxide production has been shown to cause development of cerebral aneurysms in rats (30). Finally, histopathologic analysis of coronary artery aneurysms in KD demonstrates decreased eNOS staining, among other features of coronary artery senescence (31). FSTL-1 overexpression has recently been shown to improve the revascularization of ischemic limbs in wild type mice, enhance endothelial cell differentiation and migration, and lead to phosphorylation and activation of endothelial nitric oxide synthase (eNOS) (14). Furthermore, FSTL-1 overexpression did not lead to revascularization in mice deficient in eNOS. These actions of FSTL-1 on vascular endothelium through the Akt-eNOS signaling pathway thus suggests a mechanism by which elevated expression of FSTL-1 might contribute to aneurysm formation in KD.

9.4 REFERENCES

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TABLE 2

Patient Characteristic	KD without aneurysm (n=47)	KD with aneurysm (n=7)	Control (n=23)
Age,months*	42.09	41.7	43.22
Sex†			
Male	29(64%)	5	17 (74%)
Female	17 (36%)	2	6 (26%)
Unknown	1		
Race!			
White	25	2	17
Black	17	1	6
Other/unknown	5	4 (1 Asian, 3 Hispanic)	-

*p value age: KD w/o aneurysm vs. control = 0.87 (t test), KD w/o aneurysm vs. KD with aneurysm = 0.96 (t test)

TABLE 3

FSTL-1 Cutpoint (ng/ml)	Sensitivity	Specificity	Likelihood Ratio	Positive Predictive Value (5% prevalence)	Positive Predictive Value (25% prevalence)
130	100%	24%	1.3	6.5%	30%
170	100%	63%	2.7	12.6%	47%
178	85%	71%	2.9	13.4%	49%
214	43%	85%	2.9	13.4%	49%
250	29%>	92%	3.9	17%	56%

Sensitivity, Specificity, Likelihood ratio and Positive Predictive Values (for 5% and 25% prevalence of coronary artery aneurysms in Kawasaki disease)

Various references are cited herein, the contents of which are hereby incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method of diagnosing an inflammatory disorder in a human patient comprising measuring the level of human FSTL-1 in a sample collected from the patient, where a level in the patient that is measured to be between about 200 and 300 ng/ml is consistent with a diagnosis of an inflammatory disorder in the patient.

2. The method of claim 1, comprising comparing the serum level of human FSTL-1 measured in the patient to the level of human FSTL-1 measured in a sample of a healthy human control subject or an average level of human FSTL-1 measured in samples from a plurality of healthy human control subjects, where said level in a control subject, or said average level in control subjects, is measured to be between about 125-160 ng/ml.

3. The method of claim 1 or 2, where the sample is a serum sample.

4. The method of claim 1 or 2, where the inflammatory disorder is associated with elevated levels of interleukin-ï β.

5. The method of claim 1 or 2, where the inflammatory disorder is an autoinflammatory disorder.

6. The method of claim 1 or 2, where the inflammatory disorder is systemic onset juvenile idiopathic arthritis.

7. The method of claim 1 or 2, where the inflammatory disorder is Kawasaki disease.

8. A method of identifying a patient with systemic onset juvenile idiopathic artritis who is at increased risk for developing macrophage activation syndrome comprising detecting, in a sample collected from said patient, a level of FSTL-1 that is increased by 40 percent or more relative to control levels.

9. The method of claim 8, where the FSTL-1 in a serum sample of the patient is measured to be about 230 ng/ml or greater.

10. A method of identifying a patient with Kawasaki Disease who is at increased risk for developing macrophage activation syndrome comprising detecting, in a sample collected from said patient, a level of FSTL-1 that is increased by 50 percent or more relative to control levels.

11. The method of claim 7, where the FSTL-1 in a serum sample of the patient is measured to be about 200 ng/ml or greater.

12. The method of any of claims 1-1 1, where FSTL-1 is measured by a method that comprises reacting FSTL-1 in a subject sample with a capture ligand to bind

FSTL-1 to the capture ligand, and then directly or indirectly detecting the presence of FSTL-1 bound to the capture ligand.

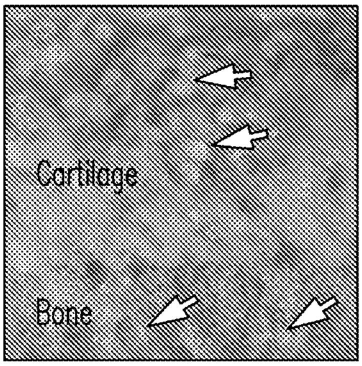


FIG. 1A

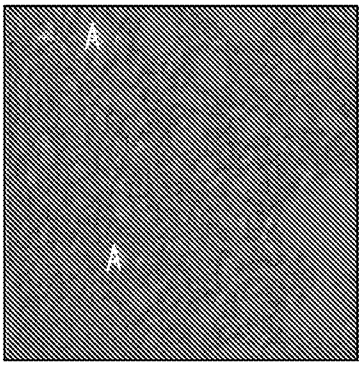


FIG. 1B

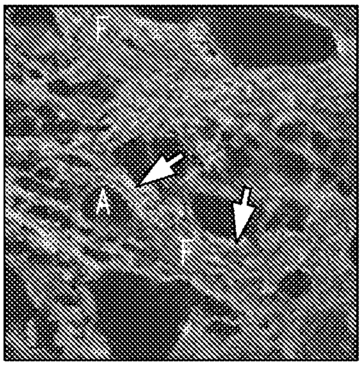


FIG. 1C

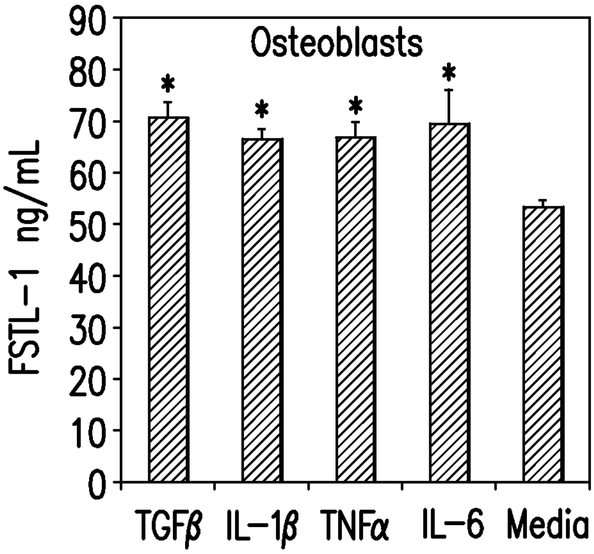


FIG. 2A

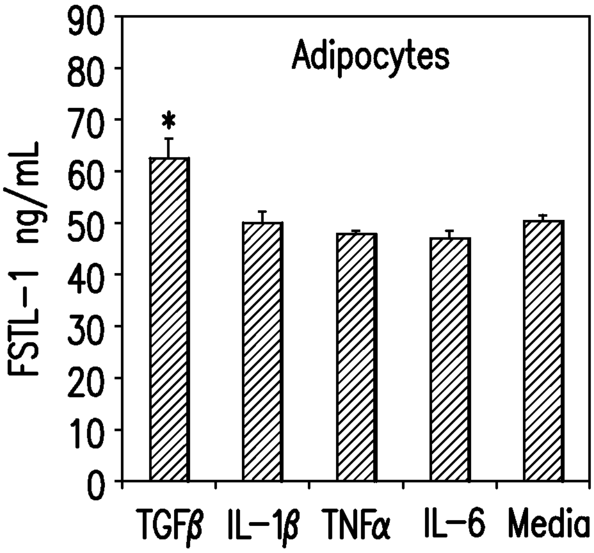


FIG. 2B

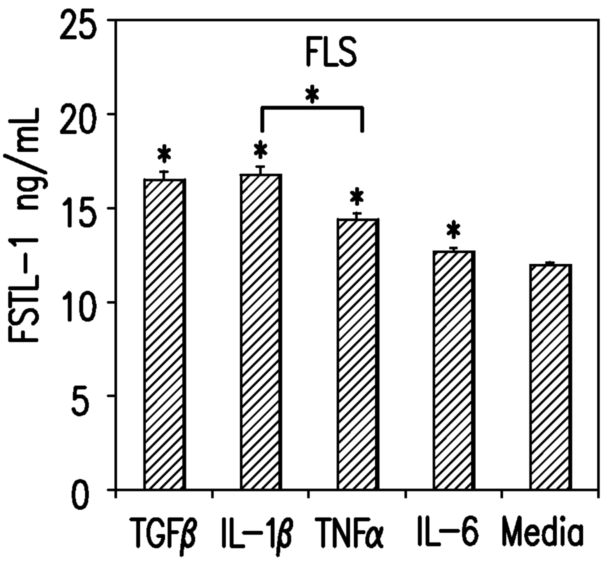


FIG. 2C

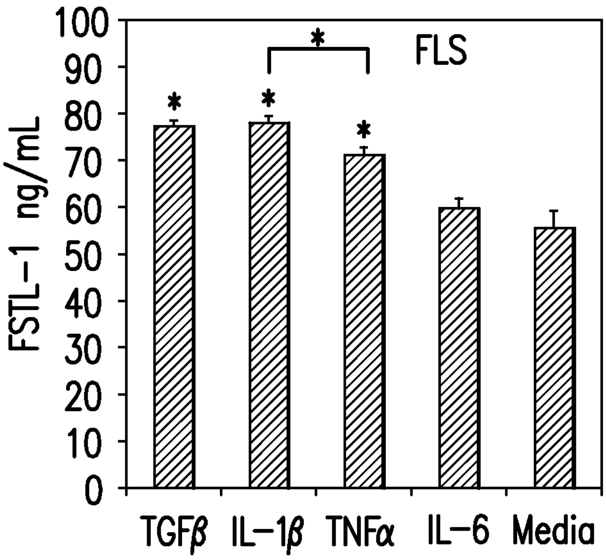


FIG. 2D

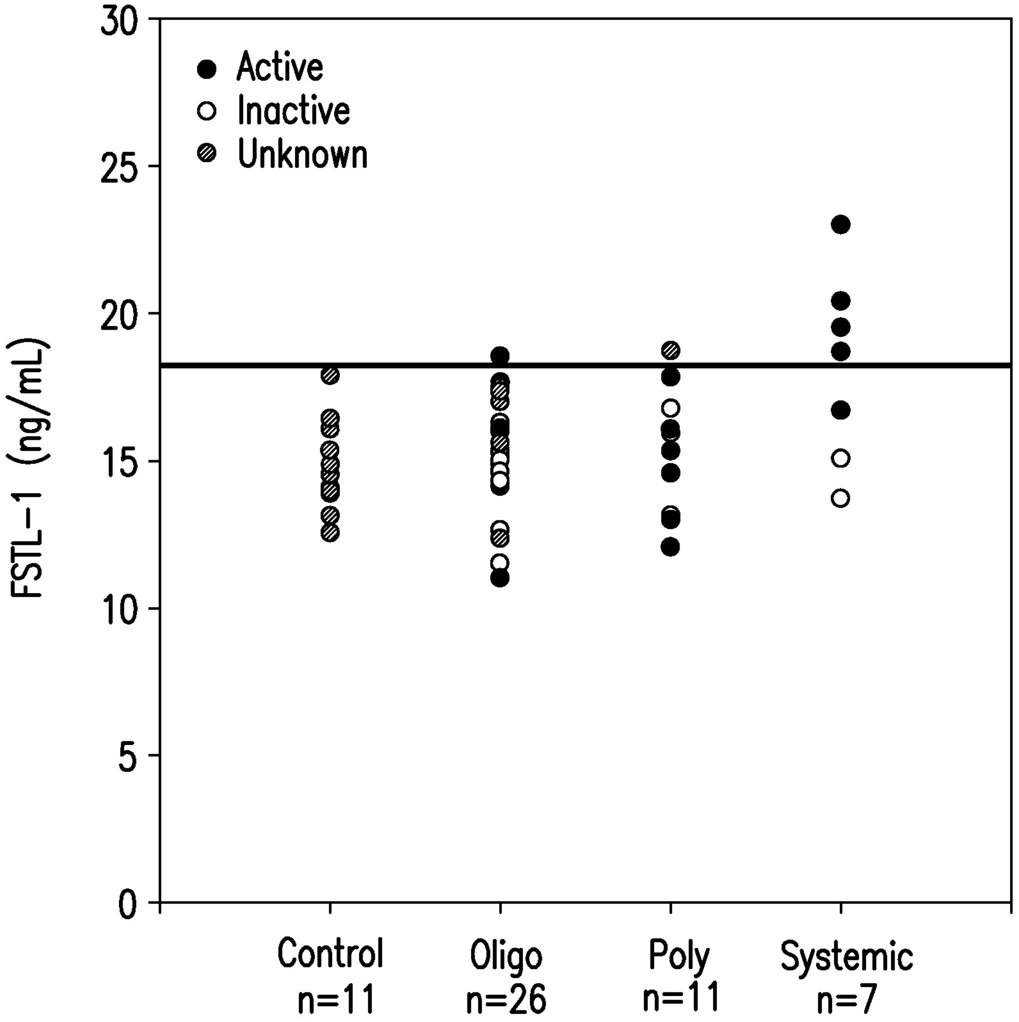

30
25
20
15
10
5
0
Active
Inactive
Unknown
FSTL-1 (ng/mL)
Control
n=11
Oligo
n=26
Poly
n=11
Systemic
n=7

FIG. 3

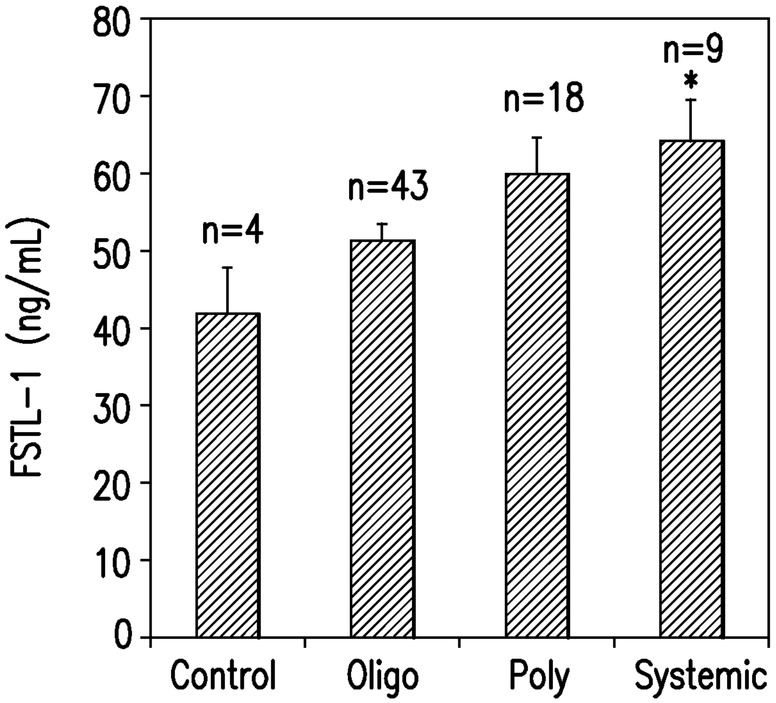

80
70
60
50
40
30
20
10
0
FSTL-1 (ng/mL)
n=4
n=43
n=18
n=9
*
Control
Oligo
Poly
Systemic

FIG. 4

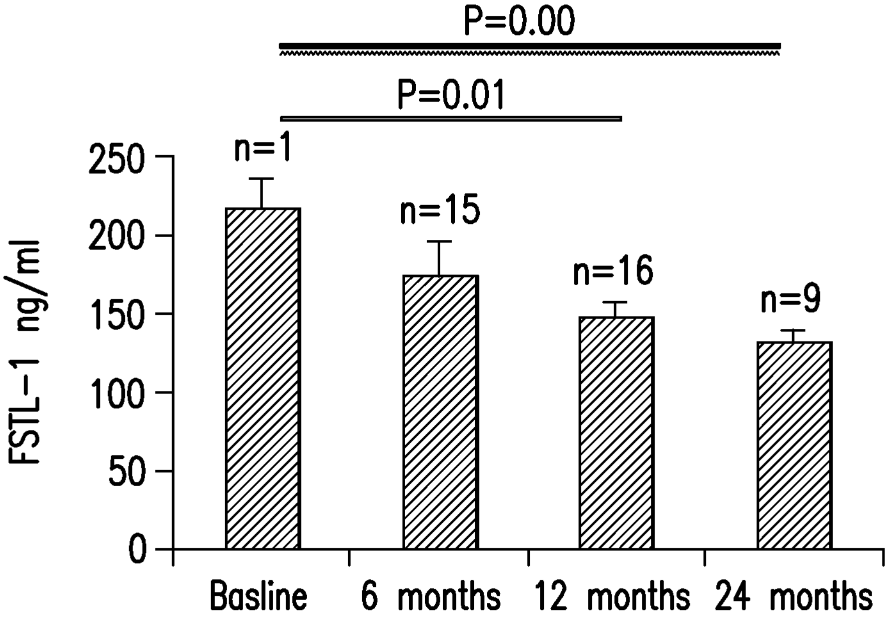

P=0.00
P=0.01
n=1
n=15
n=16
n=9
250
200
150
100
50
0
FSTL-1 ng/ml
Basline
6 months
12 months
24 months

FIG. 5A

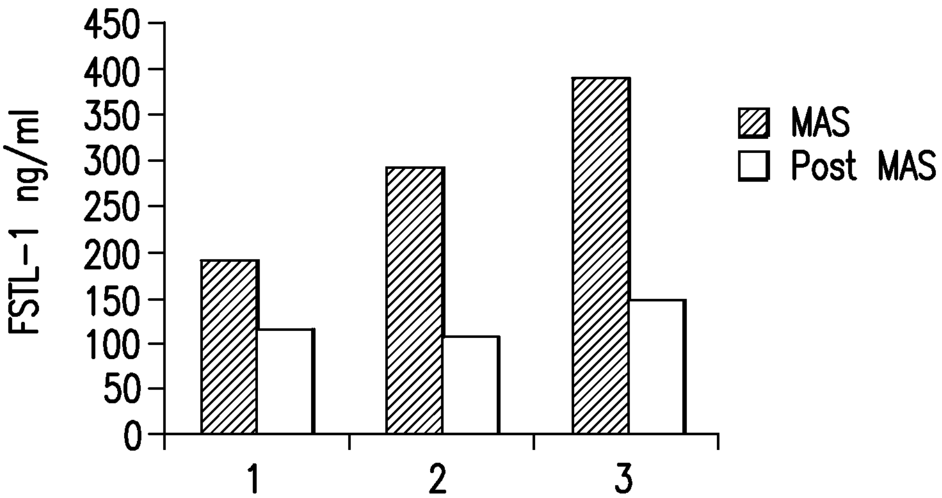

450
400
350
300
250
200
150
100
50
0
FSTL-1 ng/ml
MAS
Post MAS
1
2
3

FIG. 5B

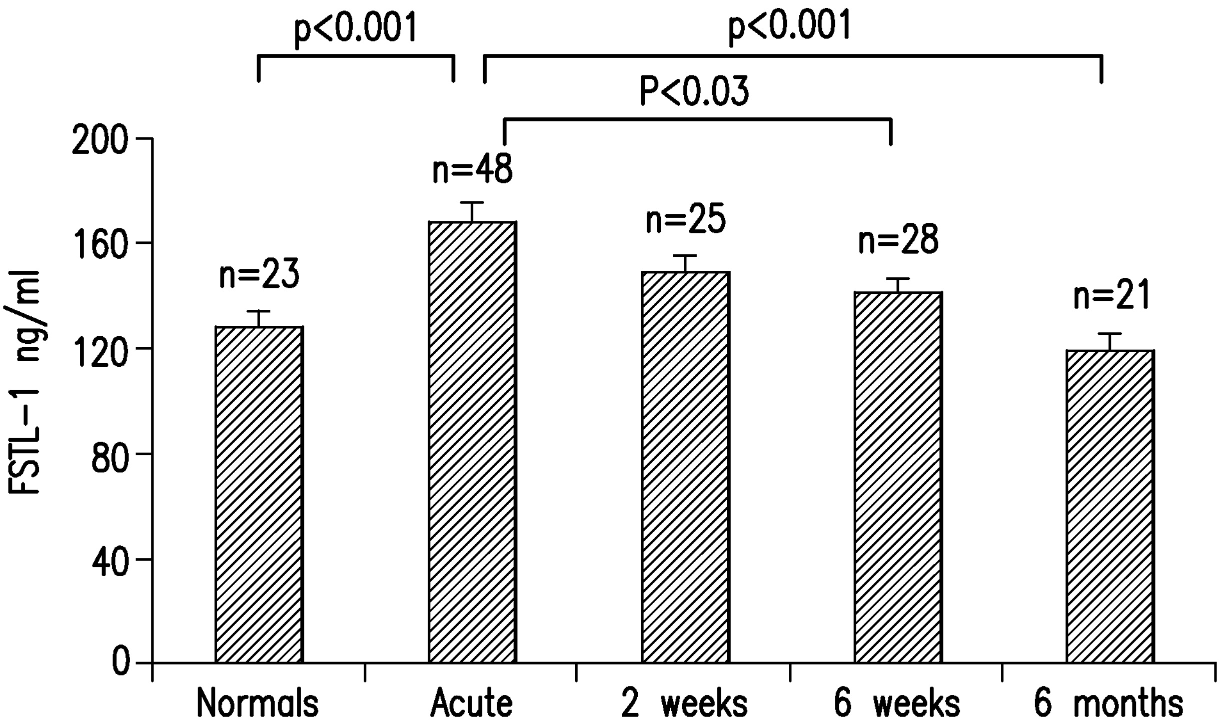

p<0.001
p<0.001
P<0.03
FSTL-1 ng/ml
200
160
120
80
40
0
n=23
n=48
n=25
n=28
n=21
Normals
Acute
2 weeks
6 weeks
6 months

FIG. 6

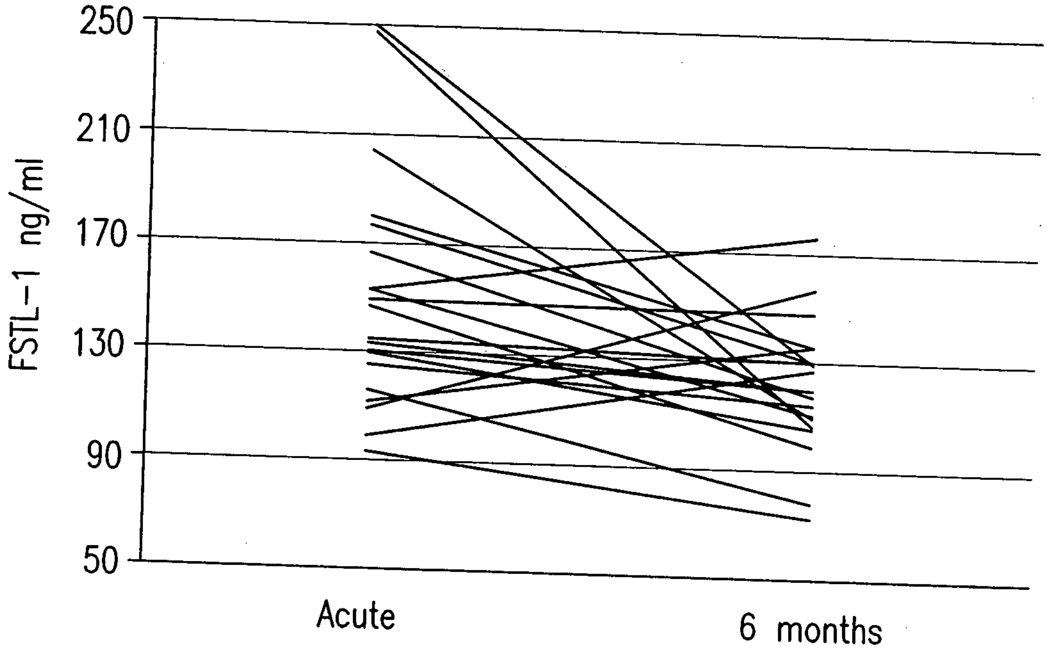


FIG. 7

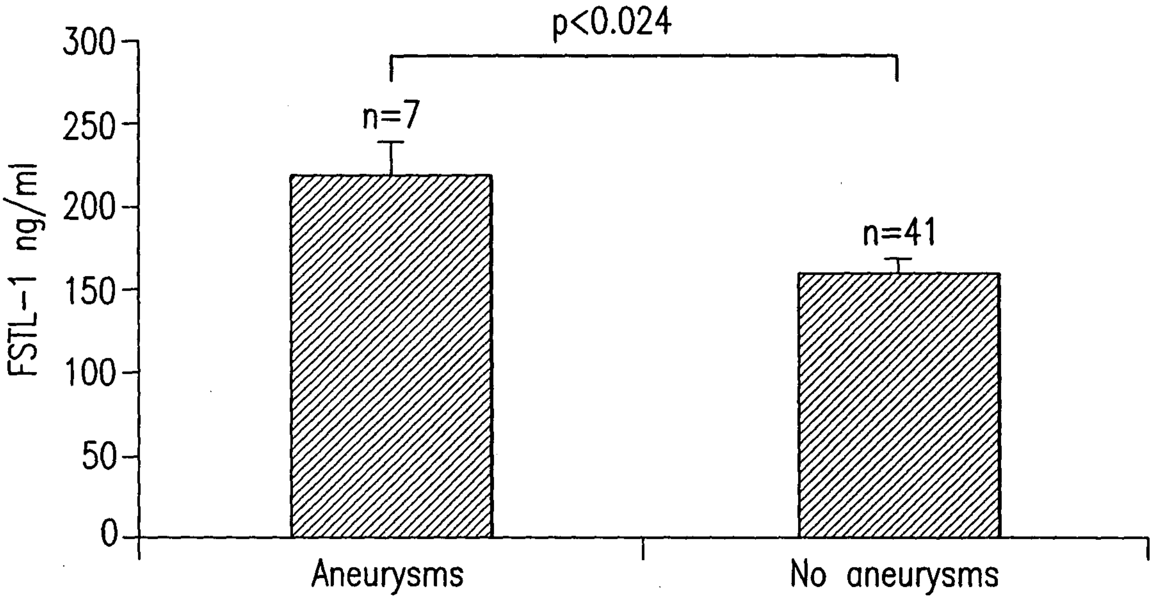

p<0.024
300
250
200
150
100
50
0
FSTL–1 ng/ml
n=7
n=41
Aneurysms
No aneurysms

FIG. 8

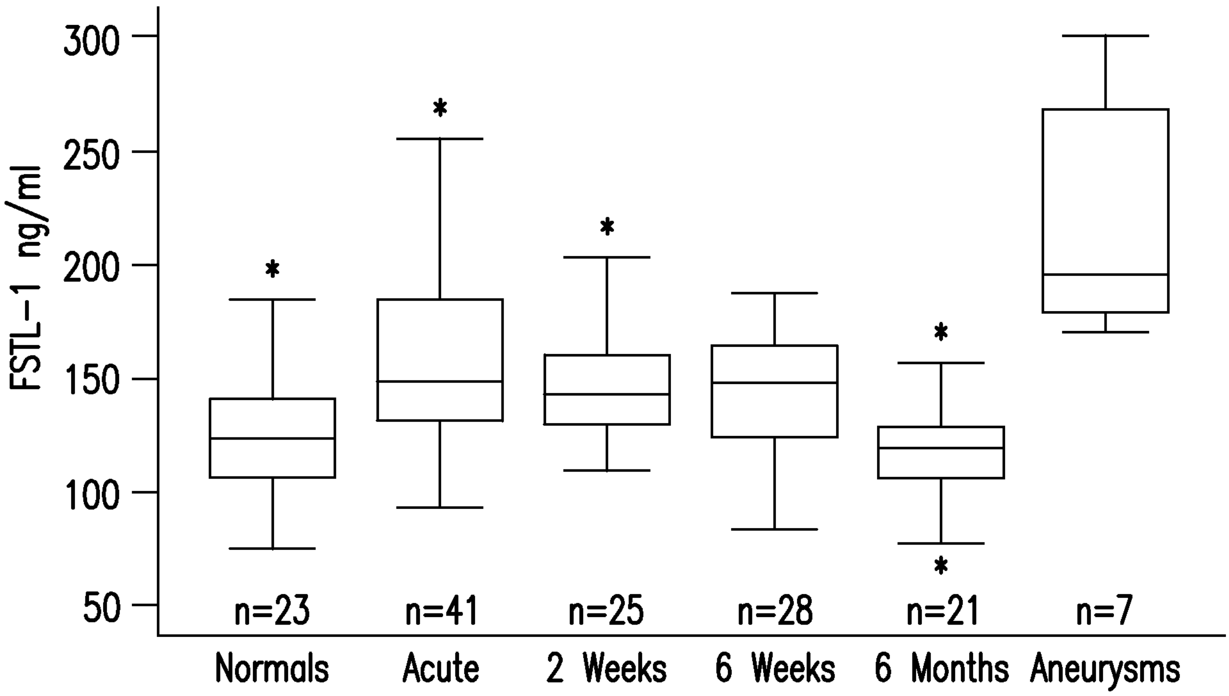
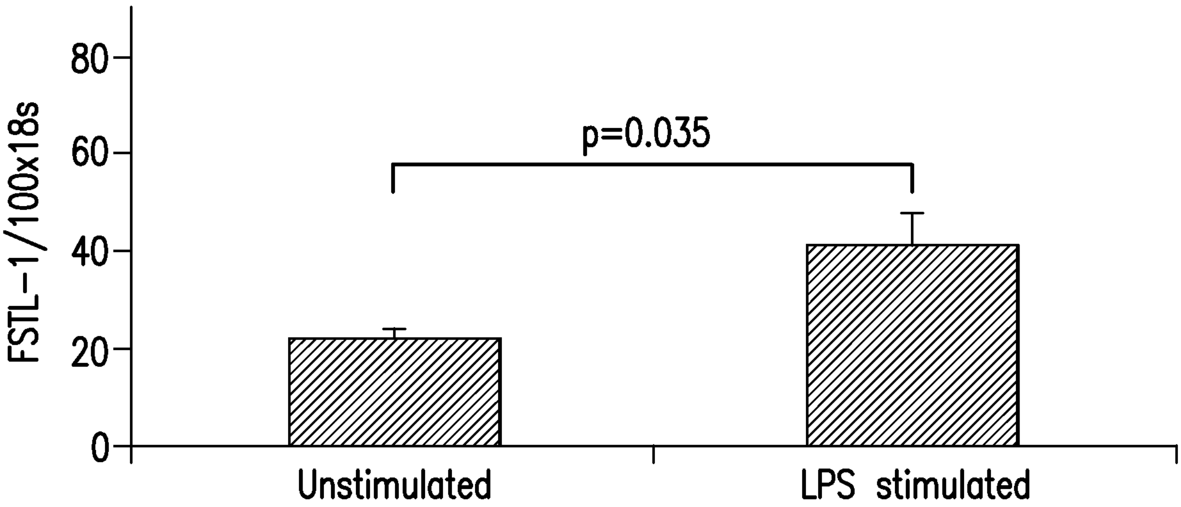

300
250
200
150
100
50
FSTL-1 ng/ml
*
*
*
*
*
n=23
n=41
n=25
n=28
n=21
n=7
Normals
Acute
2 Weeks
6 Weeks
6 Months
Aneurysms

FIG. 9



FSTL-1/100x18s
80
60
40
20
0
p=0.035
Unstimulated
LPS stimulated

FIG. 10

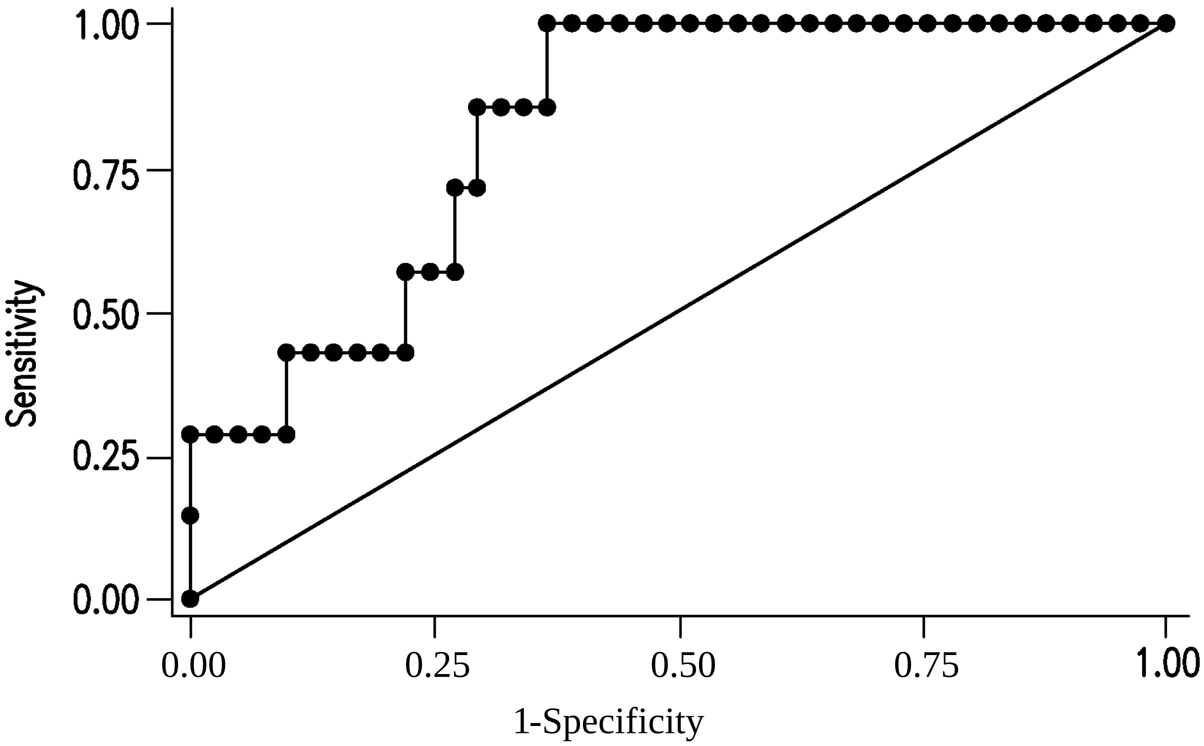

1.00
0.75
0.50
0.25
0.00
Sensitivity
0.00
0.25
0.50
0.75
1.00
1-Specificity

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US20 11/046742

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-7,11,12
because they relate to subject matter not required to be searched by this Authority, namely:

The subject matter of claims 1-7,1 1,12 relates to a diagnostic method which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/046742

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68(2006.01)i, G01N 33/53(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N 33/68; G01N 33/53; G01N 33/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: FSTL-1, juvenilie idiopathic artritis, macrophage activation syndrome, kawasaki disease

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2009-097424 A1 (UNIVERSITY OF PITTSBURGH-OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION et al.) 06 August 2009 See Abstract; Pages 6,23,24,30-34; Claims 1-4	8-10
Y	KELLY, A and RAMANAN, A.V., `Recognition and Management of Macrophage Activation Syndrome in Juvenile Arthritis`, Curr Opin Rheumatol., vol.19, no.5, pp.477-481, September 2007 See Abstract; Page 477	8-10
Y	RAVELLI, A. et al. `Preliminary Diagnostic Guidelines for Macrophage Activation Syndrome Complicating Systemic Juvenile Idiopathic Arthritis", The Journal of Pediatrics, vol.146, pp.598-604, May 2005 See Abstract	8-10
Y	BLEESING, J. et al. "The Diagnostic Significance of Soluble CD163 and Soluble Interleukin-2 Receptor a-Chain in Macrophage Activation Syndrome and Untreated New-Onset Systemic Juvenile Idiopathic Arthritis", Arthritis and Rheumatism, vol.56, no.3, pp.965-971, March 2007 See Abstract	8-10

☒ Further documents are listed in the continuation of Box C.　　☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 MARCH 2012 (28.03.2012)	**06 APRIL 2012 (06.04.2012)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/046742

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SURESH, N. and SANKAR, J., 'Macrophage Activation Syndrome: A Rare Complication of Incomplete Kawasaki Disease', Annals of Tropical Paediatrics, vol.30, pp.61-64, March 2010 See Abstract	8-10
Y	SIMONINI, G. et. al., 'Macrophage Activation Syndrome/Hemophagocytic Lymphohistiocytosis and Kawasaki Disease', Pediatr Blood Cancer, vol.55, pp.591, 7 June 2010 See Abstract	8-10

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2011/046742

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009-097424 A1	06.08.2009	US 2011-0045507 A1	24.02.2011