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(54) **Title:** USE OF NEW MARKERS IN A DIAGNOSTIC ASSAY FOR DETERMINING SEVERITY OF RSV INFECTION

(57) **Abstract:** The invention comprises a method for the prediction of the severity of a disease developing from an infection with human respiratory syncytial virus (RSV) in a subject, comprising detection of overexpression of OLFM4, CD177, MMP8, MMP9, PTX3 or a combination of one or more of these with one or more chosen from the group of IL8, RANTES and CD4 count. The overexpression can be detected in a blood sample by a nucleic acid assay or through an immunoassay.



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Title: Use of new markers in a diagnostic assay for determining severity of RSV infection

5 FIELD OF THE INVENTION

The invention relates to the field of diagnostics, more particularly diagnostics for viral diseases, especially diseases caused by Respiratory Syncytial Virus (RSV).

10 BACKGROUND

Human respiratory syncytial virus (RSV) is a virus that causes respiratory tract infections. It is the major cause of lower respiratory tract infection and hospital visits during infancy and childhood. For most people, RSV produces only mild symptoms, often indistinguishable from common colds and minor illnesses. The Centers for Disease Control consider RSV to be the "most common cause of bronchiolitis (inflammation of the small airways in the lung) and pneumonia in children under 1 year of age in the United States". For some children, RSV can cause bronchiolitis, leading to severe respiratory illness requiring hospitalization and, rarely, causing death. This is more likely to occur in patients that are immunocompromised or infants born prematurely. Other RSV symptoms common among infants include listlessness, poor or diminished appetite, and fever.

Recurrent wheezing and asthma are more common among individuals who suffered severe RSV infection during the first few months of life than among controls; whether RSV infection sets up a process that leads to recurrent wheezing or whether those already predisposed to asthma are more likely to become severely ill with RSV has yet to be determined.

One of the challenges for clinicians encountering children with RSV infections is to differentiate children who require hospitalization for supportive interventions from those who can be safely sent home. For instance, 35% of the children hospitalized with bronchiolitis do not receive any supportive

intervention. On the other hand, clinicians do not want to discharge those children who may experience clinical deterioration. For about 4.6-6.8% of the children sent home with the diagnosis bronchiolitis it appeared that hospitalization was required later on during infection. There is thus need of
5 diagnostic tools that can help to predict the severity of the disease, in order to help clinicians in the decision to hospitalize or not.

Nowadays the risk for severe disease upon RSV infection in children is estimated by the physician based on demographic data, medical history and clinical assessment. A disadvantage of current clinical prediction models for
10 RSV infection is that they are mainly based on clinical signs that will be present in a late stage of clinical deterioration. Ideally, a clinical prediction model should be able to identify children before severe symptoms are clinically displayed.

Several risk factors are associated with an increased severity of
15 RSV-infection. Infants with bronchopulmonary dysplasia, congenital heart disease or prematurity are at risk for severe RSV infection. The risk of severe RSV infection increases if multiple factors are apparent. These well known risk factors do not explain all severe RSV infections and also apparently healthy children can develop bronchiolitis.

20 One of the well known demographic risk factors, as also confirmed in the experimental part of the description, is young age. An immature immune system in combination with the lack of *in utero* sensitization to RSV and small airways makes that clinically most severe RSV infections are predominantly observed in young infants (age 1-3 months). The immaturity of the immune
25 system in young children is reflected by a delay in recruitment of neutrophils and monocytes to infected tissues, less efficient antigen presentation by macrophages and dendritic cells and defects in neonatal adaptive immunity, such as an impaired production of Th1 associated cytokines (IFN- γ) and diminished NK cell cytotoxicity reflect. The developing immune system and
30 the resulting changing values of concentrations of T-cells, cytokines and other

inflammatory markers over age, impair the realization of a reliable assay for diagnosing and/or predicting the severity of an infectious disease.

As said above, mainly clinical parameters have been used to predict severity of disease in RSV infection, but the use of clinical prediction models is limited. Several studies have associated severity of RSV disease with particular cytokines, such as IL-8, IL-6 and Th1 and Th2 related cytokines such as IL-4 and IFN- γ (Bont, L. et al., 1999, Eur. Respir. J. 14:144-149; Hornsleth, A. et al., 1998, Pediatr. Infect. Dis. J. 17:1114-1121; Brandenburg, A.H. et al., 2000, J. Med. Virol. 62:267-277; Bermejo-Martin, J.F. et al., 2007, Eur. Cytokine Netw. 18:162-167). Hornsleth, A. et al. (2001, J. Clin. Virol. 21:163-170) described the relation between ratios of inflammatory mediators like cytokines, chemokines and receptors, and the severity of RSV infection and they found that the ratios of IL-1/RANTES, IL-8/RANTES, RANTES/IL-10 and TNF-R1/RANTES were correlated with severity.

Many, if not all, of these studies, however, have been conducted on nasopharyngeal secretions. Taking a nasopharyngeal aspirate from young children is cumbersome and unpleasant for the patient. More importantly, quantitative diagnosis on nasopharyngeal lavage fluid is practically impossible: the amount of fluid used for the lavage and the pressure with which it is applied is variable as is the amount of biological material that is washed out with the fluid. It would therefore be advantageous if there would be an assay that would be feasible on blood or plasma, since this can be obtained more standardized manner and is a common diagnostic procedure of infectious disease. It has appeared, however, that the concentrations of the various inflammatory mediators are different between mucosal tissue, such as the nasopharyngeal tissue, and the blood (see Bermejo-Martin et al., *supra*). Also, it has been discussed that mechanical ventilation, as is often applied in children with bronchiolitis, influences the mucosa and plasma levels of chemokines and cytokines (Schultz, C. et al., 2001, Eur. Respir. J. 17:321-324). Recently, in a co-pending application (PCT/NL2010/050765), we have shown

that the combination of IL-8, RANTES and CD4 count could give a high and clinically useful prediction of severity of an RSV infection.

Nevertheless, there is still need for other biological markers that are associated with the severity of an RSV infection, especially if these markers
5 can be detected in a blood or plasma sample.

SUMMARY OF THE INVENTION

The invention now relates to a method for the prediction of the severity of a
10 disease developing from an infection with human respiratory syncytial virus (RSV) in a subject, comprising the steps of:

- a. determining overexpression of OLFM4, CD177, MMP8, MMP9, PTX3 or a combination of one or more of these with one or more chosen from the group of IL8, RANTES and CD4 count;
- 15 b. making a prediction on basis of the amount of overexpression detected in step a).

In such a method said overexpression is preferably detected by a nucleic
20 acid assay or by an immunoassay.

In another embodiment, the invention relates to a method, wherein the subject is a child, preferably a child having an age of less than 6 months, more preferably a child having an age of less than three months.

Also, the invention relates to a method as described above where the
25 detection is performed on a blood sample of said subject. Preferably in such a method the detection of overexpression of OLFM4 is performed on a cell component derived from peripheral blood mononuclear cells, the detection of overexpression of CD177 is performed on a cell component derived from neutrophils, the detection of overexpression of MMP8 is performed on a cell
30 component derived from peripheral blood mononuclear cells, the detection

of overexpression of MMP9 is performed on a cell component derived from peripheral blood mononuclear cells or from neutrophils, and/or the detection of overexpression of PTX3 is performed on a cell component derived from peripheral blood mononuclear cells.

5

The invention in a further embodiment relates to a method according to the invention, wherein a subject is predicted to develop a severe disease state if in a sample of said subject OLFM4, CD177, MMP8, MMP9 or PTX3 or combinations thereof are found to be overexpressed by a factor of 1.5, more preferably by a factor of 2, most preferably by a factor of 3 or more.

10

Also, in a method according to the invention the cell component which is assayed is a nucleic acid, more preferably mRNA. Preferably the assay is a PCR assay. Alternatively, the cell component which is assayed is a peptide in which case the assay is preferably an ELISA assay.

15

In another aspect, the invention comprises a detection kit for performing a method according to any of the preceding claims, comprising means for detection of RSV particles and means for the detection of overexpression of OLFM4, CD177, MMP8, MMP9 or PTX3 or combinations thereof. Preferably in such a kit the means for detection overexpression comprise primers, probes or antibodies. More preferably, in such a kit a pair of bidirectional primers for detection of overexpression of OLFM4 comprises the primers 5'-atcaaacacccctgtcgtc-3' and 5'-gctgatgttcaccacaccac-3', a pair of bidirectional primers for detection of overexpression of CD177 comprises the primers 5'-gggcaggtgtgtcaggag-3' and 5'-ccaccagggttgatgtgagt-3', a pair of bidirectional primers for detection of overexpression of MMP8 comprises the primers 5'-CCAGTTTGACATTTGATGCTATCAC-3' and 5'-CTGAGGATGCCTTCTCCAGAA-3, and/or a pair of bidirectional primers for detection of overexpression of MMP9 comprises the primers 5'-GCCCCCCTTGCATAAGGA-3' and 5'-CAGGGCGAGGACCATAGAG-3.

30

Also part of the invention is the use of a kit according to the invention for predicting the severity disease state upon RSV infection in a subject.

5 LEGENDS TO THE FIGURES

Fig. 1 Relative gene expressions in cell fractions of children with RSV infection during acute infection compared with recovery. OLFM4 PBMC $p < 0.001$, CD177 PBMC $p < 0.001$, CD177 neutrophils $p < 0.001$

Fig 2. Relative gene expression in different cell fractions in blood
10 from children with a RSV infection categorized by disease severity. *OLFM4 PBMC mild vs sev $p < 0.001$, mod vs sev $p = 0.002$. Olfm neutrophils mild vs sev $p = 0.002$, mod vs sev $p = 0.01$. CD117 PBMC mild vs sev $p < 0.001$, mod vs sev $p = 0.01$, mild vs mod $p = 0.03$. CD177 neutrophils mild vs sev $p = 0.02$, mod vs sev $p = 0.006$

15 **Fig 3:** ratio's of OLFM4 transcription after stimulation

Fig 4. Plasma concentrations of OLFM4 in acute and recovery samples for moderate and severe disease

Fig 5. Plasma concentrations of OLFM4 in acute samples for different severities

20 **Fig 6:** Plasma olf4 concentrations after 24 hours stimulation with different stimuli

Fig. 7: **Gene expression levels of MMP-8 and MMP-9 in granulocytes from children with viral lower respiratory tract infections.** Relative gene expression levels of MMP-8 (A) and MMP-9 (B) in granulocytes from children during acute RSV positive (RSV+) and
25 RSV negative (RSV-) viral lower respiratory tract infections and after recovery are given. For RSV positive children, relative gene expression levels of MMP-8 (C) and MMP-9 (D) in mild, moderate and severe disease are given. * $p < 0.05$, ** $p < 0.01$

Fig. 8: **Gene expression levels of MMP-8 and MMP-9 in PBMCs from children with viral lower respiratory tract infections.** Relative gene expression levels of MMP-8 (A) and MMP-9 (B) in PBMCs from children during acute RSV positive (RSV+) and RSV negative (RSV-) viral lower respiratory tract infections and after recovery are given. For RSV positive children, relative gene expression levels of MMP-8 (C) and MMP-9 (D) in mild, moderate and severe disease are given. * $p < 0.05$, ** $p < 0.01$

Fig. 9: **Stimulation of PBMCs and neutrophils by LPS and RSV *in vitro*.** Human PBMC of healthy volunteers (n=4) were stimulated with LPS (1 ng/ml) or RSV A2 (MOI 1) and MMP-8 and MMP-9 concentrations in supernatant were measured after 24 by ELISA (panel A) or at transcriptional level by q-PCR (Panel B). Neutrophils were stimulated for 4 hours and MMP levels were determined in the supernatant (panel C).

Fig. 10: Relative gene expression of OLFM4 in children with viral lower respiratory infection during the acute phase of infection compared with recovery. PBMCs $P = 0,0013$, granulocytes $P = 0,0053$

Fig. 11: Relative gene expression of OLFM4 in children with respiratory infection categorized by disease severity. PBMCs mild vs. moderate $P = 0,1554$, mild vs. severe $P = 0,2031$, moderate vs. severe $P = < 0,0001$. Granulocytes mild vs. moderate $P = 0,0181$, mild vs. severe $P = 0,580$, moderate vs. severe $P = 0,0002$

Fig. 12: Relative gene expression of OLFM4 in PBMCs from children < 3 months and > 3 months of age. $P = 0,0043$. The red symbols indicate severe disease. Difference between OLFM4 gene expression levels from severe disease in the group < 3 months vs. > 3 months $p = 0,0233$.

Fig. 13: Distribution of mild, moderate and severe disease samples in the groups < 3 months and > 3 months of age. For < 3 months: 14,9%, 40,4% and 44,7% for mild, moderate and

severe disease respectively. In the >3 months group de distribution is 5,1%, 74,4% and 20,5%.

5 DETAILED DESCRIPTION

In the following description and examples a number of terms are used. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given to such terms, the following definitions are provided. Unless otherwise defined herein, all
10 technical and scientific terms used have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

“Plasma” as used in the present application is used in the normal meaning of the word, i.e. the liquid component of the blood. However, for assaying plasma levels, the assays may be performed on (full) blood, blood
15 serum or plasma.

RSV infection is defined as resulting in a “severe disease” state if the patient requires hospitalization, where supportive intervention can be given. This supportive intervention can be mechanical ventilation, treatment with antiviral medicine, and measures to prevent secondary clinical effects and co-
20 infections with other pathogens affecting the respiratory pathways. Children without hypoxia or severe feeding problems are considered to be only mildly affected, those requiring hospitalization for supplemental oxygen (oxygen saturations <93%) and/or nasogastric feeding are considered to be moderately affected and children requiring mechanical ventilation are considered to be
25 severely affected (see: Gern J.E. et al., 2002, *Pediatr. Allergy Immunol.* 13:386-393; and Wan et al., 1992, *Am. Rev. Respir. Dis.* 145:106-109).

The term "capable of specifically hybridizing to" refers to a nucleic acid sequence capable of specific base-pairing with a complementary nucleic acid sequence and binding thereto to form a nucleic acid duplex.

A "complement" or "complementary sequence" is a sequence of nucleotides which forms a hydrogen-bonded duplex with another sequence of nucleotides according to Watson-Crick base-pairing rules. For example, the complementary base sequence for 5'-AAGGCT-3' is 3'-TTCCGA-5'.

5 The term "stringent hybridization conditions" refers to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH, formamide concentration and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of the primer or the probe to its target nucleic acid sequence. The

10 terms as used includes reference to conditions under which a probe or primer will hybridise to its target sequence, to a detectably greater degree than other sequences (e.g. at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent

15 conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridises to a perfectly matched probe or primer. Typically, stringent conditions will be those in which the salt

20 concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing

25 agents such as formamide. Exemplary low stringent conditions or "conditions of reduced stringency" include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37°C and a wash in 2x SSC at 40°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1x SSC at 60°C.

30 Hybridization procedures are well known in the art and are described in e.g.

Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons Inc., 1994.

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target allowing a DNA polymerase to attach thereby serving as a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase, and at a suitable temperature and pH. The (amplification) primer is preferably single stranded for maximum efficiency in amplification. Preferably, the primer is an oligodeoxy ribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. A "pair of bi-directional primers" as used herein refers to one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

The term "probe" refers to a single-stranded oligonucleotide sequence that will recognize and form a hydrogen-bonded duplex with a complementary sequence in a target nucleic acid sequence analyte or its cDNA derivative. Such a probe will often be labelled to enable easy recognition.

"Expression" refers to the transcription of a gene into structural RNA (rRNA, tRNA) or messenger RNA (mRNA) and, if applicable, subsequent translation into a protein.

Polynucleotides have "homologous" sequences if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence as described herein. Sequence comparison between two or more polynucleotides is generally performed by comparing portions of the two sequences over a comparison window to identify and compare local regions of sequence similarity. The comparison window is generally from about 20 to 200 contiguous nucleotides. The "percentage of sequence homology" for

polynucleotides, such as 50, 60, 70, 80, 90, 95, 98, 99 or 100 percent sequence homology may be determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may include additions or deletions (i.e. gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by: (a) determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions; (b) dividing the number of matched positions by the total number of positions in the window of comparison; and (c) multiplying the result by 100 to yield the percentage of sequence homology. Optimal alignment of sequences for comparison may be conducted by computerized implementations of known algorithms, or by inspection. Readily available sequence comparison and multiple sequence alignment algorithms are, respectively, the Basic Local Alignment Search Tool (BLAST) (Altschul, S.F. et al. 1990. J. Mol. Biol. 215:403; Altschul, S.F. et al. 1997. Nucleic Acid Res. 25:3389-3402) and ClustalW programs both available on the internet. Other suitable programs include GAP, BESTFIT and FASTA in the Wisconsin Genetics Software Package (Genetics Computer Group (GCG), Madison, WI, USA).

As used herein, "substantially complementary" means that two nucleic acid sequences have at least about 65%, preferably about 70%, more preferably about 80%, even more preferably 90%, and most preferably about 98%, sequence complementarity to each other. This means that the primers and probes must exhibit sufficient complementarity to their template and target nucleic acid, respectively, to hybridise under stringent conditions. Therefore, the primer sequences as disclosed in this specification need not reflect the exact sequence of the binding region on the template and degenerate primers could be used. A substantially complementary primer sequence is one that has sufficient sequence complementarity to the

amplification template to result in primer binding and second-strand synthesis.

The term "hybrid" refers to a double-stranded nucleic acid molecule, or duplex, formed by hydrogen bonding between complementary nucleotides.

5 The terms "hybridise" or "anneal" refer to the process by which single strands of nucleic acid sequences form double-helical segments through hydrogen bonding between complementary nucleotides.

"Overexpression" or "upregulation" of a gene in a particular cell or sample means that more mRNA is transcribed from the gene in a particular
10 cell or sample than in control cells or samples. Alternatively, said increase in expression can be measured from the concentration of the gene product (protein) in a cell. The increase in expression should amount to at least 1.5 times the expression in controls, preferably at least 2 times, more preferably at least 3 times or more. This increase in expression can be measured against the
15 expression of that gene in a control sample, or, within the same sample against the expression of a control gene.

"Underexpression" or "downregulation" of a gene in a particular cell or sample means that less mRNA is transcribed from the gene in a particular cell or sample than in control cells or samples. Alternatively, said decrease in
20 expression can be measured from the concentration of the gene product (protein) in a cell. The decrease in expression should amount to at least 0.5 times the expression in controls, preferably at least 0.25 times, more preferably at least 0.1 times or less. This decrease in expression can be measured against the expression of that gene in a control sample, or, within
25 the same sample against the expression of a control gene.

Many factors have been identified that play a role in RSV infection and the development of bronchiolitis. Among these are the not exhaustive list of Th1 cytokines, like IL-1 β , IL-2, IL-12p70, IFN γ , TNF α , Th2 cytokines, like
30 IL-13, IL-4, IL-6, IL-10, chemokines, like IP-10, IL-8, MIP1 α (CCL3), MIP-1 β

(CCL4), RANTES, growth factors, like FGFb, PDGFbb, GCSF, VEGF, and IL-1, IL-1RA and IL-17 (see e.g. Bermejo-Martin, *supra*).

The inventors now have found that tOLFM4 and CD177 gene expression is upregulated in circulating leukocytes of patients that will develop a severe RSV infection. It has also been found that gene expression of MMP-8 and MMP-9 was upregulated in circulating leukocytes of patients that will develop a severe RSV infection For MMP-8 this also resulted in an increase of the level of the protein in plasma. It was further found that the expression of pentraxin genes, more specifically pentraxin 3 (PTX3), was upregulated in patients that will develop a severe RSV infection. Also here an increase of the PTX3 protein in plasma was found. As such, an assay for the expression of these genes in circulating leukocytes and/or for the level of the protein concentration in plasma can be used to predict the severity of the disease.

OLFM4 (olfactomedin 4, also known as GC1, hGC-1 or GW112) is a gene that was originally cloned from human myeloblasts and found to be selectively expressed in inflamed colonic epithelium. The protein encoded is a member of the olfactomedin-related protein family. The exact function of this gene has not yet been determined.

It was found to be a marker for colon, breast and lung cancer (Koshida, S. et al., 2007, Cancer Sci. 98:315-320), and it was found to be highly expressed in pancreatic cancer tissues (Kobayashi, D. et al., 2007, Cancer Sci. 98:334-340). Recently a connection with drug responsiveness in leukemia was established (Liu, W. et al., 2010, Blood 116:4938-4947), while it was also reported that it had a beneficial effect on prostate cancer (Chen, L. et al., 2011, Carcinogenesis, doi: 10.1093/carcin/bgr065). OLFM4 has been indicated as a marker for intestinal stem cells (Van der Flier, L. et al., 2009, Gastroenterol. 137:15-17).

CD177 is an important neutrophil gene that encodes the neutrophil membrane glycoprotein (gp) NB1. NB1 gp has been studied for more than 20 years and during that time several different names have been used to describe this gp, its antigens, and the gene that encodes this molecule. NB1 was first described by

5 Lalezari and colleagues while investigating a case of neonatal alloimmune neutropenia (Lalezari P, et al., 1971, J Clin Invest, 50:1108-1115). Occasionally, during pregnancy, a mother produces alloantibodies to neutrophil antigens that cross the placenta, react with neutrophils in the foetus, and cause the neonate to become neutropenic. One antigen recognized

10 by such antibodies was described as "NB1" by Lalezari. Later, this antigen was renamed as Human Neutrophil Antigen-2a (HNA-2a) and the gp carrying this antigen was called NB1 gp (Bux, J. et al., 1999, Vox Sang. 77:251). Monoclonal antibodies specific for NB1 gp have been produced and clustered as CD177 (Mason, D. et al., 2002, Blood 99:3877-3880). In 2001 Kissel and colleagues

15 sequenced the gene encoding NB1 gp and called the gene *NB1* (Kissel, K. et al., 2001, Eur. J. Immunol. 31:1301-1309). However, this gene was highly homologous to a gene called *PRV-1* that had been sequenced the year before. Temerinac and colleagues identified and sequenced *PRV-1* in 2000 while searching for genes overexpressed in neutrophils from patients with

20 polycythemia vera (Temerinac, S. et al., 2000, Blood, 95:2569-2576). The coding regions of *NB1* and *PRV-1* differ at only 4 nucleotides that result in amino acid changes and Caruccio, Bettinotti, and colleagues have shown that *PRV-1* and *NB1* are alleles of a single gene which herein is referred to as *CD177*. (Caruccio, L. et al., 2004, Transfusion 44:77-82). Except for the

25 mentioned effects, the clinical importance of under- or overexpression of CD177 is unknown.

Matrix metalloproteinases (MMPs) are family of zinc endopeptidases capable of degrading components of the cellular matrix, and consequently, are suggested to be important in several diseases associated with tissue

30 remodelling. Pronounced increase in their expression is thought to be

associated with a variety of inflammatory disease, including respiratory diseases. (Greenlee *et al.*, 2007, *Physiol Rev* **87**, 69-98).

MMPs play a role in cellular migration of neutrophils, lymphocytes and other immune cells to the lungs by degrading extracellular matrix, but also have pro- and anti-inflammatory properties. An imbalance in production, activation, or inactivation of MMPs might augment airway inflammation through direct or indirect effects upon signaling pathways that influence migration of leukocytes through the tissues. (Greenlee *et al.*, 2007,) Increased amounts of MMP-8 and MMP-9 have been observed in respiratory samples and blood obtained from adults and children with acute lung injury and pneumonia as well as in chronic lung diseases such as asthma. (Fligiel *et al.*, 2006, *Hum Pathol* **37**, 422-430.; Hartog *et al.*, 2003, *Am J Respir Crit Care Med* **167**, 593-598; Kong *et al.*, 2009, *Int J Med Sci* **6**, 9-17; Obase *et al.*, 2010, *Int Arch Allergy Immunol* **151**, 247-254; Prikk *et al.*, 2002, *Lab Invest* **82**, 1535-1545; Schaaf *et al.*, 2008, *BMC Pulm Med* **8**, 12) In addition, a relation between MMP concentrations and disease severity of pneumonia and asthma has been described. (Belleguic *et al.*, 2002, *Clin Exp Allergy* **32**, 217-223; Hartog *et al.*, 2003, *supra*; Mattos *et al.*, 2002, *Chest* **122**, 1543-1552; Schaaf *et al.*, 2008, *supra*)

Yeo and co-workers have reported that MMP-9 protein expression is increased in human airway epithelial cell lines infected with RSV. (Yeo *et al.*, 2002, *Arch Virol* **147**, 229-242) In addition, MMP-9 gene expression is increased in the lungs of RSV-infected mice. (Li & Shen, 2007, *Chin Med J (Engl)* **120**, 5-11) Another study demonstrated that nasopharyngeal samples from infants infected with RSV and PIV contain increased MMP-9 and tissue inhibitor of metalloproteinases-1 (TIMP-1) concentrations. (Elliott *et al.*, 2007, *J Med Virol* **79**, 447-456).

Pentraxin-related protein PTX3 also known as TNF-inducible gene 14 protein (TSG-14) is a protein that in humans is encoded by the *PTX3* gene.

Pentraxin 3 (ptx3) is a member of the pentraxin superfamily. This super

family characterized by cyclic multimeric structure. PTX3 is rapidly produced and released by several cell types, in particular by mononuclear phagocytes, dendritic cells (DCs), fibroblasts and endothelial cells in response to primary inflammatory signals [e.g., toll-like receptor (TLR) engagement, TNF α , IL-1 β].

5 PTX3 binds with high affinity to the complement component C1q, the extracellular matrix component TNF α induced protein 6 (TNAIP6; also called TNF-stimulated gene 6, TSG-6) and selected microorganisms, including *Aspergillus fumigatus* and *Pseudomonas aeruginosa*. PTX3 activates the classical pathway of complement activation and facilitates pathogen

10 recognition by macrophages and DCs.

Since no correlation with viral infection for OLFM4 and CD177 is reported, it is thus very surprising that overexpression of these two genes

15 appears to be related to the development of severe symptoms upon infection with RSV. For the MMPs and PTX3 the relationship with infection is more pronounced, but it has up till now not been shown or suggested that these markers would be predictive for the severity of an RSV infection.

First of all, it is surprising that such a prediction can be made by

20 assaying the gene expression and/or the plasma levels of the listed compoundsThe RSV infection localizes in the upper respiratory tract of the body and predominantly affects the mucosal tissues that are present there. The immune reaction that is triggered by the virus thus has a strong local, mucosal character of which the local inflammation (bronchiolitis) is one of the

25 most characteristic phenomena. This thus means that most of the components that are involved in the immune reaction (such as the above listed cytokines and chemokines) will be formed and will act locally. It is thus only logical that the local levels of those compounds will be predominantly influenced by the viral infection, while plasma levels may react more slowly or even remain

30 virtually unaltered. It has also been discussed in the scientific literature that

there is a difference between mucosal levels and plasma levels of these compounds. Pitrez et al. (Ann. Allergy Asthma Immunol., 92:659-662, 2004) found that cytokines produced by *in vitro* PBMCs may not necessarily reflect the concurrent cytokine pattern production at the mucosal surface in the respiratory tract of infants with acute bronchiolitis. Vieira et al. (J. Bras. Pneumol., 36:59-66, 2010) found a positive correlation of disease severity with mucosal (nasopharyngeal) increased levels of sICAM-1 and IL-10, while in the serum IL-6 levels were found to be predictive. There is thus a recognized difference in cytokine response between mucosal tissue and serum levels.

Secondly, it is surprising that expression of OLFM4 and/or CD177 is involved, since these genes seem to be unrelated to viral immunity. A possible link between OLFM4 and inflammation and immunity has been suggested on basis of the finding that G-CSF and NF- κ B modulate expression of OLFM4 (Chin, K. et al., 2008, Br. J. Hematol. 143:421-432), but no links with the effects of viral infection have been mentioned. CD177 even seems further removed from immunity phenomena, since this gene is only reported to play a role in neutrophils. Also for MMP8, MMP9 and PTX3 no clear link to viral infection has been established. For MMP8 and MMP the experiments reported herein that have been performed show that even a direct stimulation of PBMCs with RSV virus did not result in an increased gene expression of MMP8 and only a moderate increase of MMP9 expression.

As can be seen from the experimental part of the present application, the increased expression of the genes coding for the above-mentioned proteins can be detected by nucleic acid based detection methods and by protein based detection methods. To establish the presence of overexpression, needed for identifying the prediction for severe conditions upon RSV infection according to the present invention, both types of detection may be used.

The cell component used for the assay can thus be nucleic acid, such as RNA, preferably mRNA, or protein. When a cell component is protein, the reagent is typically an antibody against the protein produced by the gene. When the component is nucleic acid, the reagent is typically a nucleic acid
5 (DNA or RNA) probe or (PCR) primer. By using such probes or primers, gene expression products, such as mRNA may for example be detected.

The test cell component may be detected directly *in situ* or it may be isolated from other cell components by common methods known to those of skill in the art before contacting with the reagent (see for example, "Current
10 Protocols in Molecular Biology", Ausubel et al. 1995. 4th edition, John Wiley and Sons; "A Laboratory Guide to RNA: Isolation, analysis, and synthesis", Krieg (ed.), 1996, Wiley-Liss; "Molecular Cloning: A laboratory manual", J. Sambrook, E.F. Fritsch. 1989. 3 Vols, 2nd edition, Cold Spring Harbor Laboratory Press)

15 Detection methods include such analyses as Northern blot analysis, RNase protection, immunoassays, in situ hybridization, PCR (Mullis 1987, U.S. Pat. No. 4,683,195, 4,683,202, en 4,800,159), LCR (Barany 1991, Proc. Natl. Acad. Sci. USA 88:189-193; EP Application No., 320,308), 3SR (Guatelli *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), SDA (U.S. Pat. Nos.
20 5,270,184, en 5,455,166), TAS (Kwoh *et al.*, Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi *et al.*, 1988, Bio/Technology 6:1197), Rolling Circle Amplication (RCA) or other methods for the amplification of cDNA/DNA. In an alternative method RNA may be detected by such methods as NASBA (L. Malek *et al.*, 1994, Meth. Molec. Biol. 28, Ch. 36, Isaac PG, ed.,
25 Humana Press, Inc., Totowa, N.J.) or TMA. These include PCR analyses on microfluid array platforms, allowing simultaneous detection of multiple targets in one sample using limited amounts of input material. Nucleic acid probes, primers and antibodies can be detectably labeled, for instance, with a radioisotope, a fluorescent compound, a bioluminescent compound, a
30 chemiluminescent compound, a metal chelator, an enzyme or a biologically

relevant binding structure such as biotin or digoxigenin. Those of ordinary skill in the art will know of other suitable labels for binding to the reagents or will be able to ascertain such, using routine experimentation.

Other methods for detection include such analyses as can be
5 performed with nucleic acid arrays (See i.a. Chee *et al.*, 1996, Science 274(5287):610-614). Such arrays comprise oligonucleotides with sequences capable of hybridizing under stringent conditions to the nucleic acid cell component of which the level is to be detected in a method of the present invention.

10

The invention now provides a nucleic acid assay or immuno assay for the prediction of the severity of an RSV infection and targeting either OLFM4, CD177, MMP8, MMP9, PTX3 or a combination of one or more of these with one or more chosen from the group of IL8, RANTES and detection of CD4 count
15 (see for a discussion on the relevance of IL-8, RANTES and CD4 count the description of co-pending application PCT/NL2010/050765).

Such an assay preferably comprises the following steps:

- a. taking a blood sample;
 - b. optionally isolating the nucleic acid and/or protein from the
20 sample or enriching the sample for the presence of the nucleic acid or protein;
 - c1. analyse the gene expression profile of said nucleic acid by assaying it with a nucleic acid assay according to the invention; or
 - c2. analyse gene expression of said protein by assaying the sample with an immuno assay according to the invention; and
 - 25 d. identifying the expression of one or more of the above mentioned proteins; and
 - e. assessing the prediction on basis of the expression found in step d.
- Preferably, the samples in the above methods are fresh samples.

Gene expression analysis is preferably done using a nucleic acid assay or immunoassay.

To investigate gene expression the assay should target polynucleotide molecules or proteins from a clinically relevant source, in this case e.g. a sample from a patient suspected of RSV infection. Presence of RSV infection may be proven by concomitant assaying for the presence of RSV particles, which can also be done in a nucleic acid assay or immunoassay. The skilled person will be capable of performing these assays, since many of them are commercially available (e.g the Quidel™ Quick View RSV test, the ClearView™ RSV rapid test kit, the BINAX NOW® RSV testkit, the Directigen™ EZ RSV kit, the ProFlu+™ Assay, the 3M™ Rapid Detection RSV testkit, Norgen's RSV-A-RT-PCR Detection Kit, and many others). Preferably, the test is performed on a fresh sample. Said target polynucleotide molecules should be expressed RNA or a nucleic acid derived therefrom (e.g., cDNA or amplified RNA derived from cDNA that incorporates an RNA polymerase promoter). If the target molecules consist of RNA, it may be total cellular RNA, poly(A)+ messenger RNA (mRNA) or fraction thereof, cytoplasmic mRNA, or RNA transcribed from cDNA (cRNA). Methods for preparing total and poly(A)+ messenger RNA are well known in the art, and are described e.g. in Sambrook et al., (1989) *Molecular Cloning- A Laboratory Manual* (2nd Ed.) Vols. 1-3, Cold Spring Harbor, New York. In one embodiment, RNA is extracted from cells using guanidinium thiocyanate lysis followed by CsCl centrifugation (Chrigwin et al., (1979) *Biochem.* 18:5294-5299). In another embodiment, total RNA is extracted using a silica-gel based column, commercially available examples of which include RNeasy (Qiagen, Valencia, CA, USA) and StrataPrep (Stratagene, La Jolla, CA, USA). In another embodiment, total RNA is extracted using commercially available RNA isolation reagents examples of which include Trizol (Life Technologies, Breda, The Netherlands) and RNAbee (Tel-Test Inc., Friendswood, Texas, USA). In another embodiment, total RNA is extracted using automated nucleic acid extraction

platforms an example of which is the Nuclisens EasyMAG (BioMerieux, Durham, NC, USA). Poly(A)+ messenger RNA can be selected, e.g. by selection with oligo-dT cellulose or, alternatively, by oligo-dT or hexamer primed reverse transcription of total cellular RNA. In another embodiment, the polynucleotide
5 molecules analyzed by the invention comprise cDNA, or PCR products of amplified RNA or cDNA.

When desiring to predict the development of severe symptoms upon RSV infection of a subject, the practitioner should take a sample from that
10 subject, and after isolation of the RNA the expression of one or more of IL-8, RANTES, OLFM4, CD177, MMP8, MMP9 and PTX3 be determined. To normalize these expression data it is possible to correct the data for variations with the help of expression data of a control gene or element which is not affected by the infection (such as a housekeeping gene), which is present in the
15 nucleic acid assay that has been used to determine the expression profile of the subject to be assessed. Instead of one control gene, also the mean value of a pool of control genes can be taken. This correction can, for instance, be done by dividing the expression level of each of the tested genes by the expression level of the control gene(s)/element(s). An example of such a housekeeping gene,
20 which has been used in the experiments reported herein, is actin.

For purposes of the invention, probes specific for polynucleotides of IL-8, RANTES, OLFM4, CD177, MMP8, MMP9 or PTX3 may be used to detect and quantify the polynucleotide of the gene in biological fluids or tissue samples.

25 Any specimen, i.e. blood sample, containing a detectable amount of polynucleotide or encoded polypeptide of the IL-8, RANTES, OLFM4, CD177, MMP8 or PTX3 genes can be used. Preferably, for detection of OLFM4, MMP8, MMP9 or PTX3 the peripheral blood mononuclear cells (PBMCs) are isolated from the blood sample. For detection of CD177 preferably the neutrophils from
30 the blood sample are taken. For detection of MMP9 both PBMCs and

neutrophils could be used. For preferred methods for detection of IL-8, RANTES and the CD4 count, see PCT/NL2010/050765.

Nucleic acid can also be analyzed by RNA *in situ* methods that are known to those of skill in the art such as by *in situ* hybridization. Although the
 5 subject can be any mammal, preferably the subject is human and even more preferably, the subject is a child of young age, including prematurely born infants.

For purposes of the invention, an antibody specific for the IL-8,
 10 RANTES, OLFM4, CD177, MMP8, MMP9 or PTX3 protein may be used to detect and quantify the presence of the polypeptide produced by the gene in the samples.

The invention methods can utilize antibodies immunoreactive with polypeptide encoded by the IL-8, RANTES, OLFM4, CD177, MMP8, MMP9 or
 15 PTX3 genes, the predicted amino acid sequences of which are available from public databases, or immunoreactive fragments thereof. For OLFM4 sequence information can be found in the EMBL database under accession numbers, CAH71311.2, AAH47740.1, AAI17330.1, AAQ88930.1 and EAW52052.1, and in the UniProt database under accession no. Q6UX06. Preferably, the protein has
 20 the following sequence:

```
MRPGLSFLLA LLFFLGQAAG DLGDVGPPIP SPGFSSFPGV DSSSSFSSSS
RSGSSSSRSL GSGGSVSQLF SNFTGSVDDR GTCQCSVSLP DTTFPVDRVE
RLEFTAHVLS QKFEKELSKV REYVQLISVY EKKLLNLTVR IDIMEKDTIS
YTELDFELIK VEVKEMEKLIV IQLKESFGGS SEIVDQLEVE IRNMTLLVEK
25 LETLDKNNVL AIRREIVALK TKLKECEASK DQNTFPVHPP PTPGSCGHGG
VVNISKPSVV QLNWRGFSYL YGAWGRDYSP QHPNKGLYWV APLNTDGRLL
EYYRLYNTLD DLLLYINARE LRITYGQSG TAVYNNMYV NMYNTGNIAR
VNLTNTTIAV QTLPNAAYN NRFSYANVAW QDIDFAVDEN GLWVIYSTEA
STGNMVISKL NDTTLQVLNT WYTKQYKPSA SNAFMVCGVL YATRTMNTRT
30 EEIFYYYDTN TGKEGKLDIV MHKMQEKVQS INYNPFDQKL YVYNDGYLLN
YDLSVLQKPQ
```

For CD177 the sequence can be found in UniProt accession no. Q8N6Q3 or GenBank NP_065139.2. Preferably this sequence is:

```

MSAVLLLALL GFILPLPGVQ ALLCQFGTVQ HVWKVSDLPR QWTPKNTSCD
SGLGCQDTLM LIESGPQVSL VLSKGCTEAK DQEPRVTEHR MGPGLSLISY
5 TFVCRQEDFC NNLVNSLPLW APQPPADPGS LRCPVCLSME GCLEGTTEEI
CPKGTTHCYD GLLRLRGGGI FSNLRVQGCM PQPGCNLLNG TQEIGPVGMT
ENCNRKDFLT CHRGTTIMTH GNLAQEPTDW TTSNTEMCEV GQVCQETLLL
LDVGLTSTLV GTKGCSTVGA QNSQKTTIHS APPGVLVASY THFCSSDLN
SASSSSVLLN SLPPQAAPVP GDRQCPTCVQ PLGTCSSGSP RMTCPRGATH
10 CYDGYIHL SG GGLSTKMSIQ GCVAQPSSFL LNHTRQIGIF SAREKRDVQP
PASQHEGGGA EGLESLTWGV GLALAPALWW GVVCPSC

```

For MMP8, the sequence can be found under NCBI Reference Sequence: NP_002415.1. Preferably, this sequence is:

```

15 MFSLKTLPLF LLLHVQISKA FPVSSKEKNT KTVQDYLEKF YQLPSNQYQS
TRKNGTNVIV EKLKEMQRFF GLNVTGKPNE ETLDMMKKPR CGVPDSSGFM
LTPGNPKWER TNLTyrIRNY TPQLSEAEVE RAIKDAFELW SVASPLIFTR
ISQGEADINI AFYQRDHGDN SPFDGPNIGL AHAFQPGQGI GGDAHFDAEE
TWTNTSANYN LFLVAAHEFG HSLGLAHSSD PGALMYPNYA FRETSNYSLP
20 QDDIDGIIQAI YGLSSNPIQP TGPSTPKPCD PSLTFDAITT LRGEILFFKD
RYFWRRHPQL QRVEMNFISL FWPSLPTGIQ AAYEDFDRDL IFLEKGNQYW
ALSGYDILQG YPKDISNYGF PSSVQAIDAA VFYRSKTYFF VNDQFWRYDN
QRQFMPEGYP KSISGAFFGI ESKVDAVFQQ EHFFHVFSGP RYYAFDLIAQ
RVTRVARGNK WLNCRYG

```

25

For MMP9, the sequence can be found under NCBI Reference Sequence: NP_004985.2. Preferably, this sequence is:

```

MSLWQPLVLV LLVLGCCFAA PRQRQSTLVL FPGDLRTNLT DRQLAEEYLY
RYGYTRVAEM RGEKSLGPA LLLLQKQLSL PETGELDSAT LKAMRTPRCG
30 VPDLGRFQTF EGDWKWHHHN ITYWIQNYSE DLPRVIDDA FARAFALWSA
VTPLTFTRVY SRDADIVIQF GVAEHGDGYP FDGKDGLLAH AFPPPGPIQG
DAHFDDDELW SLGKGVVPT RFGNADGAAC HFPFIFEGRS YSACTTDGRS
DGLPWCSTTA NYDTDDRFGF CPSERLYTQD GNADGKPCQF PFIFQGQSYS
ACTTDGRSDG YRWCATTANY DRDKLFGFCP TRADSTVMGG NSAGELCVFP

```


FTFLGKEYST CTSEGRGDGR LWCATTSNFD SDKKWGFCPD QGYSLFLVAA
 HEFGHALGLD HSSVPEALMY PMYRFTEGPP LHKDDVNGIR HLYGPRPEPE
 PRPPTTTTPQ PTAPPTVCPT GPPTVHPSER PTAGPTGPPS AGPTGPPTAG
 PSTATTVPLS PVDDACNVNI FDAIAEIGNQ LYLFKDGKYW RFSEGRGSRP
 5 QGPFLIADKW PALPRKLDSV FEERLSKKLF FFSGRQVWVY TGASVLGPRR
 LDKLGLGADV AQVTGALRSG RGKMLLFSGR RLWRFDVKAQ MVDPRSASEV
 DRMFPGVPLD THDVFQYREK AYFCQDRFYW RVSSRSELNQ VDQVGIVTYD
 ILQCPED

10

For PTX3 the sequence can be found under NCBI Reference
 Sequence: NP_002843.2. Preferably, this sequence is:

MHLAILFCA LWSAVLAENS DDYDLMYVNL DNEIDNGLHP TEDPTPCACG
 15 QEHSEWDKLF IMLENSQMRE RMLLQATDDV LRGELOQLRE ELGRLAESLA
 RPCAPGAPAE ARLTSALDEL LQATRDAGR LARMEGAEAQ RPEEAGRALA
 AVLEELRQTR ADLHAVQGWA ARSWLPAGCE TAILFPMRSK KIFGSVHPVR
 PMRLESFSAC IWVKATDVLN KTILFSYGTK RNPYEIQLYL SYQSIVFVVG
 GEENKLVAEA MVSLGRWTHL CGTWNSEEGT TSLWVNGELA ATTVEMATGH
 20 IVPEGGILQI GQEKNGCCVG GGFDETLAFS GRLTGFNIWD SVLSNEEIRE
 TGAESCHIR GNIVGWGVTE IQPHGGAQYV S

An antibody preparation that consists essentially of pooled
 25 monoclonal antibodies with different epitopic specificities, as well as distinct
 monoclonal antibody preparations can be used. Monoclonal antibodies are
 made against antigen containing fragments of the protein by methods well
 known to those skilled in the art (Kohler, et al., Nature, 256: 495,1975). Both
 monoclonal and polyclonal antibodies are commercially available, e.g. from
 30 AbD Serotec, Abnova Corporation, Bachem, BioLegend, BioByte, eBioscience,
 EXBIO, GeneTex, GenWay Biotech Inc., LifeSpan Biosciences, MBL Int.,
 Novus Biologicals, ProSci Inc, Raybiotech Inc., Sigma-Aldrich, and many other
 companies.

The term antibody as used in this invention is meant to include intact molecules as well as fragments thereof, such as Fab and F(ab')₂, which are capable of binding an epitopic determinant on genes listed in Tables 1 (and 2). Antibody as used herein shall also refer to other protein or non-protein
5 molecules with antigen binding specificity such as miniantibodies, peptidomimetics, anticalins etc.

Monoclonal antibodies can be used in the diagnostic methods of the invention, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal
10 antibodies in these immunoassays can be detectably labelled in various ways. Examples of types of immunoassays that can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection
15 of the antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays that are run in either the forward, reverse, or simultaneous modes, including immunohistochemical or immunocytochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

20 Monoclonal and polyclonal antibodies can be bound to many different carriers to be used to detect the presence of the gene products of the genes of Table 1 and 2. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature
25 of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

In performing the assays it may be desirable to include certain
30 "blockers" in the incubation medium (usually added with the labelled soluble

antibody). The "blockers" are added to assure that non-specific proteins, proteases, or antiheterophilic immunoglobulins to the immunoglobulins present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabelled indicator antibody, to yield
5 false positive or false negative results. The selection of "blockers" therefore may add substantially to the specificity of the assays described in the present invention. A number of nonrelevant (i. e., nonspecific) antibodies of the same class or subclass (isotype) as those used in the assays (e. g., IgG1, IgG2a, IgM, etc.) can be used as "blockers". The concentration of the "blockers" (normally 1-
10 100 µg/µL) may be important, in order to maintain the proper sensitivity yet to inhibit any unwanted interference by mutually occurring cross-reactive proteins in the specimen.

As indicated above, it is advantageous to detect a combination of
15 markers, if only to make the prediction of the severity of RSV infection on basis of the expression data of these markers more accurate.

Also part of the invention is a detection kit comprising means for the detection of OLFM4, CD177, MMP8, MMP9 or PTX3 and/or combinations
20 thereof or combinations with IL-8 and RANTES. Such a detection kit can be used by the pediatrician to predict the severity status of the disease that the child will develop after RSV infection. In order to speed up the clinical diagnosis, it is preferred that the detection kit also comprises means to detect viral particles of the virus. Normally, the test for identifying the viral cause of
25 the disease is done separately, but of course it is more efficient to confirm the diagnosis of RSV infection concomitantly with the assay for predicting the severity of the disease that will develop. However, to be able to accurately predict the severity of the disease, the infection should be well established and local and systemic immune responses should already have developed. Again,
30 for the detection of RSV particles in samples from the patient, many tests are

commercially available, such as the Sure-Vue® test kit of Fisher Healthcare, the NOW RSV assay of Meridian Bioscience, the SimulFluor® RSV/Para 3 assay of Millipore, and many others. The tests to detect the presence of RSV can be based on immunological techniques, but may also be based on nucleic acid detection, such as (RT-)PCR.

The invention will be illustrated in the following Example(s), which is for illustrative purpose and not deemed to be limiting the invention as claimed.

EXAMPLES

EXAMPLE 1 – Involvement of OLFM4 and CD177

Methods

Patients Children from 0-5 years of age with clinical symptoms of an acute lower respiratory infection were included during three consecutive winter seasons (2006-2009). From all children clinical data, a nasopharyngeal aspirate (NPA) and a 5 ml blood sample (identified as ‘acute sample’) were obtained. Patients who were hospitalized were asked to return 3-4 weeks later at the outpatient clinic for evaluation, a recovery NPA and blood sample (identified as ‘recovery sample’) were obtained. The included subjects were subdivided into 3 groups based on severity of disease; mild (no supportive treatment), moderate (supplemental oxygen and/or nasogastric feeding) and severe (mechanical ventilation).

RSV detection

From the obtained NPA 300 µl was taken for virus detection with multiplex PCR as previously described. The PCR had the capacity of simultaneously detection of fifteen viruses: RSV, hMPV, hBoV, Parechovirus, Adenovirus, Rhinovirus, Enterovirus, Parainfluenza 1-4, Influenza A and B

and Coronaviruses OC43 and 229E. For this study only RSV mono-infections were further analyzed.

RNA extraction and cDNA synthesis

The acute blood samples were obtained in a sodium heparin tube.

5 Cell differentiation with lymphoprep was performed to separate the cell fractions: PBMCs and neutrophils. The different cell fractions were stored in Trizol until further processing. The recovery samples were handled in the same way. At the end of this process four tubes per child were available for analysis.

10 RNA was extracted from the Trizol-cell samples using the Trizol and RNA cleanup protocol with the RNeasy Kit (Qiagen). 1 µg of the total RNA was converted to cDNA using the RT-PCR protocol: first-strand cDNA synthesis using Superscript III (Invitrogen).

qPCR on clinical samples

15 For each gene two primers were developed. The region containing the OLFM4 gene was amplified using the forward 5'- atcaaacacccctgtcgtc-3' and reverse 5'- gctgatgttcaccacaccac-3' primer.

The CD177 gene was amplified using the forward 5'- gggcaggtgtgtcaggag -3' and reverse 5'- ccaccaggttgatgtgagt -3' primer.

20 For actin the forward primer 5'- cgtcacacttcgatgatggagtg-3' and the reverse primer 5'-cttccttctctgggcatgga - 3' were used. The expression of the different genes was measured by quantitative Sybergreen® real time PCR with the ABI 7500 Fast Real-Time PCR system and software. All samples were run for 40 cycles in duplicate, taking the mean of the cycle threshold (Ct) values as measured value. Ct values of OLFM4 en CD177 were normalized against the reference gene actin. Relative quantities of mRNA were calculated using a (2- $\Delta\Delta$ Ct) transformation (Livak, K.J. et al., 2001, Methods 25:402-408)

PBMCs isolation and stimulation

30 From healthy volunteers forty ml of venous blood was drawn into EDTA tubes. The blood was diluted with PBS (1:2). The diluted blood was

carefully added to 15 ml Lymphoprep and centrifuged at 750*G for 20 minutes. The inter-phase containing the PBMCs was carefully removed and washed two times with PBS. The PBMCs were resuspended in RPMI-glutamax at a density of 5 x 10⁶ /ml. In a 96-wells plate a volume of 100 µl was added per well and
5 100 µl of stimuli was added. The stimuli were RSV-A2, muramyldipeptide (MDP), Pam3Cys (P3C), *E.coli* endotoxin (LPS), R848, G-CSF and prednisolon and R848. The plates were incubated at 37°C for respectively 4 and 24 hours.

After incubation the plates were centrifuged at 400*G. 180 µl of supernatant was transferred to a new 96 wells plated. The pellet was
10 resuspended with RLT buffer (RNeasy Kit, Qiagen).

qPCR in vitro samples From the resuspended pellet in RLT buffer RNA was isolated and conversion the cDNA was performed using the previously described protocols. A qPCR for OLFM4 and Actin was performed. Ratio's were calculated using the Pfaffl method (Pfaffl, M.W., 2001, Nucl. Acids
15 Res. 29:e45).

ELISA For the measurement of OLFM4 concentration in the plasma of RSV patients and in the supernatant of the in vitro assay an ELISA (USCNK, Life Science Inc, Wuhan, China) was performed according to the manufacturer's recommendations.

20 *Statistical analysis*

The association between the expression of OLFM4 and CD177 and the severity of RSV was analyzed by the Mann-Whitney U- test for statistical significance. The concentration of OLFM4 measured by ELISA was compared by the Mann-Whitney U-test.

25 A p-value of less than 0.05 was considered statistically significant. All statistical tests were performed by SPSS 16.0 for Windows.

Results

Demographics of RSV infected children

A total of sixty children with a RSV mono-infection were included in this study. Of which 13 infants were included into the mild, 25 into to moderate and 22 into the severe category. Table 1 shows the characteristics of the patients. No patients with chronic lung disease, immune deficiency or immunosuppressive medication were included. The children in the severe group were significantly younger than those in the moderate groups ($p=0.003$). Prematurely born was more present in severe compared to the moderate group ($p=0.002$). Children in the severe and moderate groups had significantly more siblings than those in the mild ($p=0.009$ and $p<0.001$, respectively).

No other significant differences in clinical parameters were found between the patients groups.

Table 1. Patient characteristics

	Total N=60	Mild N=13	Moderate N=25	Severe N=22	P-value
Age in days	45 (27-102)	60 (47-203)	47 (28-135)	28 (18-54)	0,003*
Median (25-75)					
Male (%)	40 (67)	9 (69)	16 (64)	15 (68)	NS
Birth weight (2SD)	3233 $\pm$ 773	3295 $\pm$ 814	3388 $\pm$ 747	3027 $\pm$ 773	NS
Prematurity <35 wkn (%)	8 (13)	1 (8)	0	7 (32)	0,002*
Smoking during pregnancy	12 (20)	2 (15)	5 (20)	5 (23)	NS
Breast feeding	30 (50)	9 (69)	11 (44)	10 (45)	NS
Astma	2 (3)	0	1 (4)	1 (5)	NS
Eczema	3 (5)	2 (13)	0	1 (5)	NS
Allergies	3 (5)	0	2 (8)	1 (5)	NS
Pulmonary	0	0	0	0	
Hart	2 (3)	0	0	2 (9)	NS
Immunologic	0	0	0	0	
Family history of atopy	35 (58)	9 (69)	13 (52)	13 (59)	NS
Siblings	46 (78)	5 (38)	21 (84)	21 (95)	0,001*
					**
Day care	3 (5)	1 (8)	1 (4)	1 (5)	NS
Duration of symptoms (median, 25-75)	4 (3-6)	4 (2,5-7)	4 (3-6)	4,5 (2,75,25)	NS

15 *qPCR*

A total of 166 samples were analyzed, subdivided in four categories: 55 acute PBMCs, 26 recovery PBMCs, 56 acute neutrophil and 29 recovery

neutrophil samples. To analyze whether the OLFM4 and CD177 genes could be a marker for infection, the paired acute and recovery samples were compared. The distribution of the transcription of OLFM4 and CD177 in acute and recovery samples is shown in figure 1.

5 The expression of OLFM4 in PBMCs in the acute samples was significantly higher compared to the recovery samples ($p < 0.001$), whereas in the neutrophils no significant upregulation was found. The CD177 transcripts in both the neutrophils and the PBMCs were significantly upregulated in the acute compared with the recovery samples, ($p < 0.001$ and $p < 0.001$,
10 respectively).

To identify genes differentially expressed between patients with a different severity, the samples from mild, moderate and severe infections were compared. There was a significant upregulation of OLFM4 expression in the
15 severe compared to moderate and mild group for both cell fractions. For the PBMCs $p = 0.002$ and $p < 0.001$, for the neutrophils $p = 0.013$ and $p = 0.002$ respectively.

The gene expression of CD177 in neutrophils was significantly upregulated in the severe compared to the moderate and mild infection
20 ($p = 0.006$ and $p = 0.02$ respectively). In the PBMCs the gene expression of CD177 was significantly upregulated in the severe compared to the moderate and mild infection $p = 0.01$ and $p < 0.001$ and in the moderate compared to the mild infection $p = 0.031$, see figure 2. These results indicate that OLFM4 and CD177 transcripts are a good marker for RSV-infection and severity.

25

PBMCs stimulation

To identify a possible regulatory pathway for OLFM4 transcription, the effects of 8 different stimuli on the PBMCs obtained from healthy volunteers were examined. Using qPCR we evaluated changes in transcription
30 levels of OLFM4 in response to the different stimuli. The results are shown in

figure 3. Compared to unstimulated cells RSV induced an upregulation of OLFM4 (2.8 times $\pm$ SEM). Down regulation was seen in G-CSF and prednisolon. Stimulation of PBMCs with NOD2 ligand MDP, TLR2 ligand P3C, TLR4 ligand LPS and the TLR7/8 ligand R848 resulted in a weak
5 upregulation of OLFM4. These results were not statistically significant.

ELISA

To examine if the up regulated transcription of OLFM4 in RSV infection also lead to translation, quantification of the protein with ELISA was
10 performed. 26 acute and 19 recovery samples of clinical patients with different disease severities were analyzed. The concentration OLFM4 in acute plasma samples of patients with a severe infection was 101.9 ± 27.4 ng/ml (mean $\pm$ SEM), and 65.8 ± 20.9 ng/ml in the recovery samples of these patients. In the plasma of patients with a moderate infection higher concentrations of OLFM4
15 102.9 ± 32.7 ng/ml were measured in the acute and 79.9 ± 25.2 ng/ml in the recovery samples. None of these results were statistically significant. These results are shown in figure 4. Also no significant difference was found between the acute samples of patients with a different severity, see figure 5.

We also examined whether OLFM4 could be detected by ELISA in
20 the supernatant after 24 hours incubation with different stimuli. The concentration of OLFM4 protein was higher after stimulation with RSV, MDP and R848 in comparison with RPMI. Low concentrations were detected after stimulation with G-CSF and prednisolon. In P3C and LPS a higher concentration of OLFM4 with a broad SEM was detected. Results are shown in
25 figure 6. No significant results were obtained.

Discussion

Our qPCR results showed that the expression of OLFM4 transcripts is upregulated during RSV-infection. Also statistical differences were found

between severe and moderate or mild infection. These results validate our hypothesis that OLFM4 is a biomarker for RSV severity.

We are the first to describe the association between OLFM4 and RSV disease. Several studies associated OLFM4 expression with gastro-
5 intestinal tumors and cervical neoplasia (Chin, K.L. et al., 2008, Br. J. Haematol., 143:421-432). At present time little is known about the transcriptional regulation of OLFM4 and its signalling pathways. Gover et al. (2010, Cancer Metastasis 29:761-775) showed that NF- κ B plays an important role in the induction of OLFM4. They also showed that G-CSF induces OLFM4
10 transcription. Surprisingly our experiments showed that stimulation with G-CSF leads to down regulation of OLFM4 expression. After stimulation with prednisolon to mimic a general stress response also a downregulation was observed. The role of the NF- κ B pathway was confirmed by the upregulation after stimulation with specific TLR ligands known to be activated by
15 respiratory bacteria and RSV. Interestingly direct stimulation of PBMCs with RSV resulted in upregulation. This might indicate a possible direct interaction between RSV and circulation leukocytes.

Another possible influential factor on OLFM4 transcription could be low oxygen levels. Children with a severe infection have lower oxygen levels in
20 comparison with children with mild and moderate infections.

The OLFM4 plasma concentrations were not significantly associated with RSV-infection or severity. This is likely to be caused by the small sample size. It is submitted that plasma OLFM4 will appear to be significantly upregulated in plasma in severe infections, which will enable the development
25 of a possible rapid test to predict RSV severity.

The association between CD177 expression levels and RSV disease was also investigated. CD177 is known to be exclusively expressed on neutrophils and is reported to be increased in severe bacterial infections and myeloproliferative disorders (Stroncek, D.F., 2007, Curr. Opin. Hematol.
30 14:688-693; Sachs, U.J. et al., 2007, J. Biol. Chem. 282:23603-23612; Gohring,

K. et al., 2004, Br. J. Haematol. 126:252-254). Neutrophils play an important role in the inflammation process in children with RSV (Bataki, E.L. et al., 2005, Clin. Exp. Immunol. 140:470-477).

In our experiments the CD177 expression in neutrophils and PBMCs was significantly upregulated in acute RSV-infection. In both cell types we found significantly higher expression of CD177 in severe compared with mild and moderate infection. In PBMCs there also was a significant upregulation between the moderate and the mild infection. In our study we found CD177 expression in the PBMC s, at lower base-line levels than the neutrophils, which may be caused by contamination by neutrophils after lymphoprep separation.

In this study younger age, prematurely born and having siblings were associated with severe RSV-infection. Young age, prematurity and having siblings are known risk factors for severe RSV-infection. Young age is associated with an immature immune system and small airways. An insufficient lung development and an immature immune system are associated with prematurity (Boyce, T.G. et al., 2000, J. Pediatr. 137:865-870; Welliver, R.C., 2003, J. Pediatr. 155:S112-S117). Having siblings leads to exposure to viral pathogens at a younger age.

However these factors cannot explain the differences we found in transcription levels of the markers of the invention, since differences in OLFM4 and CD177 transcription were found in the paired acute and the recovery samples.

To conclude, in this study we are the first to show that OLFM4 transcription is associated with RSV-infection and severity. Also CD177 expression in neutrophils is associated with RSV-infection and disease severity. These results are useful for the development of a diagnostic tool for predicting RSV disease severity.

EXAMPLE 2 – Involvement of MMP8 and MMP9

Methods

Study design

Children younger than 5 years of age with laboratory confirmed viral LRTI were prospectively included during three consecutive winter seasons (from November to April in the years 2006-2009). Viral LRTI was defined as an acute infection of the lower airways, characterized by increased respiratory effort (tachypnea and/or use of accessory respiratory muscles) and/or expiratory wheezing and/or crackles and/or apnea) in combination with a laboratory confirmed viral etiology. Written informed consent was obtained from all parents and the study was approved by the Committee on Research involving Human Subjects of the University Nijmegen Medical Centre. Within 24 hours after admission a blood sample and nasopharyngeal aspirate was collected and parents from hospitalized children were asked permission to draw a second blood sample and nasopharyngeal aspirate 4 to 6 weeks after admission. Medical history, demographics and clinical parameters were collected from questionnaires and medical records. Patients were classified into three different groups based on severity of disease. Children without hypoxia or severe feeding problems were allocated in the mild group, those requiring hospitalization for supplemental oxygen (oxygen saturations <93%) and/or nasogastric feeding in the moderate group and children requiring mechanical ventilation in the severe group.

Sample collection

A nasopharyngeal aspirate was collected by introducing a catheter, connected to a collection tube and an aspiration system, into the nasopharyngeal cavity. Then, 1.5 ml of saline was instilled into the catheter and, while slowly retracting the catheter, the nasopharyngeal fluid was aspirated in a collection tube. Afterwards the catheter was flushed with 1 ml of saline and added to the collection fluid. The samples were kept cold and immediately transferred to the laboratory. The nasopharyngeal aspirate was

centrifuged at 500 g for 10 minutes at 4°C and the supernatant was frozen at -80°C.

Five ml blood was collected into sodium heparin tubes and directly transferred to the laboratory. A thin blood smear was prepared and stained
5 with (May-Grunwald-)Giemsa to determine the percentages of granulocytes and PBMCs. PBMCs were obtained by density gradient centrifugation (Lymphoprep®, Axis Shield, Norway) and stored in Trizol at -80°C for RNA isolation. Plasma samples were stored at -80°C for ELISAs.

**Quantitative mRNA expression of MMP-8 and MMP-9 in
10 PBMCs and granulocytes**

RNA from PBMC and granulocytes was extracted using Trizol (Invitrogen Life Technologies) according to the manufacturers' protocol. Subsequently, a clean-up was performed on total RNA with the RNeasy Minikit (Qiagen) according to the manufacturers' instructions. Total RNA (2
15 ug, measured with spectrophotometry, Nanodrop) was reverse transcribed using a high-capacity cDNA reverse transcription kit according to the manufacturers' instructions (Applied Biosystems, Foster City, CA) and cDNA was stored at -20 degrees. The relative gene expression was measured with SYBR Green PCR Mastermix (Applied Biosystems; P/N 4367659) on the ABI
20 7500 Fast Real Time PCR system using standard program and software. After 40 repetitions a dissociation curve was performed as control for the specificity of the PCR reaction. The following primers were used: hActin FW: CGTCACACTTCATGATGGAGTTG, hActin RV: CTCCTTCCTGGGCATGGA, hMMP-9 FW: GCCCCCCTTGCATAAGGA, hMMP-9 RV:
25 CAGGGCGAGGACCATAGAG, hMMP-8 FW: CCAGTTTGACATTTGATGCTATCAC and hMMP-8 RV: CTGAGGATGCCTTCTCCAGAA. All reactions were performed in duplo. Actin was used as reference gene. After a quality check (melting temp, curve of reaction and standard deviation Ct) the Δ Ct of the MMP-8 and MMP-9 to actin
30 was calculated and expressed as relative expression.

MMP-8, MMP-9 and TIMP-1 concentrations in plasma and nasopharyngeal aspirates

Concentrations of total MMP-8 and MMP-9 in plasma, nasopharyngeal aspirate and supernatants of cell stimulation assay were measured by ELISA according to the manufacturers' protocol (DuoSet, R&D systems). In addition, TIMP-1 concentrations in plasma were determined as described above.

In vitro stimulation of PBMC and neutrophils from healthy volunteers

After informed consent, blood was drawn from healthy volunteers and collected in EDTA tubes. Blood was diluted 1:1 with pyrogene free PBS (Lonza, Basel, Switzerland). PBMCs and granulocytes were obtained by density gradient centrifugation (Lymphoprep®, Axis Shield, Norway). After washing, PBMCs were brought at a concentration of 5×10^6 cells/ml in serum free RPMI (Gibco, Invitrogen, Paisley, United Kingdom) with 100 U/ml of penicillin/streptavidin (Gibco, Invitrogen, Paisley, United Kingdom). Granulocytes were purified by lysing the red blood cells (0.155 M NH_4Cl , 0.0001 M Na_2EDTA and 0.01 M KHCO_3) and, after washing, granulocytes were suspended at a concentration of 5×10^6 cells/ml in RPMI supplemented with 0.5% Human Serum Albumin (Sanquin, Amsterdam, The Netherlands). Mononuclear cells (5×10^5 in 100 μl) were added to round-bottom 96-well plates and stimulated with either 100 μl culture medium (negative control), 1 ng/ml LPS (*Escherichia coli* serotype 055:B5, Sigma Aldrich, purified as described previously (Hirschfeld *et al.*, 2000) or MOI 1 of RSV A2 (kindly provided by Dr. R. de Swart, Erasmus MC, Rotterdam, The Netherlands). RSV A2 was cultured in HeLa cells and purified by ultra-centrifuge over a sucrose 30% gradient. After incubation for 24 hours at 37 °C and 5% CO_2 , supernatant was collected and stored at -80 °C. Neutrophils (5×10^5 in 100 μl) were stimulated and incubated in the same way for 4 hours and supernatant was stored at -80 °C. Apoptosis was determined on the

FACScalibur by Annexin V apoptosis detection kit (BD) according to the manufacturers' instructions and no differences between stimuli were founds after 4 hours.

Statistics

5 Values are expressed as percentages for categorical variables and as mean and standard error (SE) or median and interquartile range (IQR) for continuous variables. For variables that were not normally distributed, Kruskal-Wallis test was performed to compare continuous variables followed by Mann-Whitney U tests for individual comparisons. Chi-squared tests were
10 performed to compare categorical data. A two sided value of $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

15 In total, 153 patients were included. In 109 patients (71%) RSV was detected. RSV positive children were significantly younger than RSV negative children. No other significant differences were observed between these groups (Table 2).

A total of 54, 60 and 39 children were classified as having mild,
20 moderate and severe disease, respectively. Patients with severe disease were significantly younger compared to those with mild disease (105 days vs 278 days; $p < 0.05$). More prematurely born children were observed in the severe group compared to the mild and moderate group. No other significant differences in clinical parameters were found between the different severity
25 groups (Table 3). In addition, total leukocytes and neutrophil counts were comparable between all groups.

Table 2. Patient characteristics

	Total	RSV+	RSV-	p-value
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	(N=153)	(N=109)	(N=44)	
Age (days±SE)	206 ±26	149 ±20	347 ±72	p=0.00
Male	95 (62%)	70 (64%)	25 (57%)	NS
Prematurity	21 (14%)	16 (15%)	5 (11%)	NS
Family history of atopy	82 (57%)	56 (54%)	26 (63%)	NS
Symptomatic days before presentation (days±SE)	5.5 ±0.4	5.2 ±0.4	6.3 ±1.1	NS

Data are presented as number (%), unless otherwise specified. NS= not significant SE= standard error

Table 3. Patient characteristics for mild, moderate and severe infections

	Total (N=153)	Mild (N=54)	Moderate (N=60)	Severe (N=39)	p- value
Age (days)	206 ±26	278 ±52	206 ±38	105 ±36	p=0.01
Male (%)	95 (62%)	32 (59%)	36 (60%)	27 (69%)	NS
Prematurity (%)	21 (14%)	5 (9%)	6 (10%)	10 (26%)	p=0.04
Family history of atopy (%)	82 (57%)	27 (52%)	36 (62%)	19 (55%)	NS
Symptomatic days before presentation	5.5±0.4	7.0±1.1	4.8±0.3	4.6±0.4	NS
RSV (%)	109 (71%)	35 (65%)	45 (75%)	29 (74%)	NS
Leukocytes counts	8.7±0.7	9.8±1.1	8.6±1.0	8.2±1.4	NS
Neutrophil counts	3.5±0.5	3.1±0.7	2.9±0.7	4.3±1.1	NS

Data are presented as percentages or mean $\pm$ standard error (SE). Cell counts are given as $\times 10^6$ cells/ml $\pm$ SE. Kruskal-Wallis tests were performed, followed by Mann Whitney U test for one to one comparisons, $p < 0.05$ was considered to be statistically significant.

5

Disease severity is associated with increased gene expression levels of MMP-8 and MMP-9 in both granulocytes and PBMCs

During acute viral infection we observed increased MMP-8 and
10 MMP-9 gene expression in both PBMCs and granulocytes compared to recovery. No differences in gene expression of MMP-8 and MMP-9 in both PBMCs and granulocytes were found between RSV positive and RSV negative children during acute infection. In general, MMP-9 gene expression levels were higher in granulocytes than in PBMCs. For MMP-8, the same trend was
15 noticed (Figures 7 and 8).

Increased disease severity was associated with higher MMP-8 and MMP-9 gene expression levels in both PBMCs and granulocytes (Figures 7 and 8). To determine whether this association was dependent on the type of virus, we also analysed RSV positive and RSV negative children separately (data not
20 shown). For RSV positive patients, the same association was found between disease severity and gene expression levels. For RSV negative patients, MMP-8 and MMP-9 gene expression was higher in children with severe disease compared to those with mild. In addition, in RSV negative children with severe disease higher MMP-8 expression in both PBMCs and granulocytes were
25 observed compared to those with moderate disease.

Disease severity is associated with increased MMP-8 plasma levels

In plasma, both MMP-8 and TIMP-1 concentrations were increased during acute RSV infection compared to those after recovery (Table 4). Higher

MMP-8 plasma concentrations were found in children with severe and moderate disease compared to those with mild disease. Increased TIMP-1 levels were observed in children with severe disease than in those with mild disease (Table 4). For RSV positive patients, the same associations were found between plasma concentrations and disease severity. In RSV negative patients, higher MMP-8 plasma levels were observed in children with moderate and severe disease compared to those with mild disease. In nasopharyngeal samples, MMP-8 and MMP-9 concentrations were increased during acute infection compared to recovery samples. No differences in MMP-8 levels were observed between the different severity groups (Table 4). In children with moderate disease, increased MMP-9 concentrations in nasopharyngeal fluid were measured compared to those with mild disease (Table 5). However, no significant differences in MMP-9 concentrations in children with severe disease compared to those with mild and moderate disease were observed.

No differences in plasma or nasopharyngeal levels of MMP-8, MMP-9 and TIMP-1 plasma levels were observed between RSV positive and RSV negative children during acute viral respiratory infection (Table 4). In general, MMP-8 and MMP-9 concentrations were higher in nasopharyngeal samples compared to plasma.

Table 4. MMP-8 and MMP-9 concentrations (ng/ml) in plasma and nasopharyngeal samples from children with LRTI caused by RSV or other viruses during acute infection and after recovery.

	RSV			Other		
	acute (N=102)	rec (N=45)	p-value	acute (N=43)	viruses rec (N=10)	P- value
MMP-8	33.4 ±7.9	12.3 ±2.4		27.6 ±6.1	10.6±3.0	NS

plasma	p=0.000					
MMP-9	135.7 ±13.2	185.1 ±22.6	NS	170.3 ±19.8	141.5±41.6	NS
plasma						
TIMP-1	170.4 ±7.7	158.3 ±12.6	p=0.08	176.5 ±10.3	136.3±27.8	NS
plasma						
MMP9/TIMP-1	0.91 ±0.95	1.35 ±0.17	NS	1.04 ±0.14	1.68±0.72	p=0.04
MMP-8 NPA	978.1 ±133.9	419.6±97.4	p=0.003	947.9 ±173.7	639.5±252.1	NS
MMP-9 NPA	947.4 ±65.3	484.7±45.2	p=0.000	985.7 ±117.5	638.7±91.7	p=0.04

Concentrations are given in mean ± standard error. Mann Whitney U test were performed to compare children infected by RSV and other viruses. Paired analyses (Wilcoxon) were performed to compare acute and recovery samples. NS=not significant

5 **Table 5. MMP-8 and MMP-9 concentrations (ng/ml) in plasma and nasopharyngeal samples from children with mild, moderate and severe viral respiratory infection**

	mild (N=50)	moderate (N=57)	severe (N=38)	p-value
MMP-8	15.4 ±4.1*§	45.9 ±13.9§	31.6 ±4.8*	*p=0.001,
plasma				§p=0.001
MMP-9	128.8 ±15.1	170.3 ±21.0	132.1 ±19.0	NS
plasma				
TIMP-1	164.9 ±9.8*	165.8 ±10.4	191.7 ±12.1*	* p=0.057
plasma				
MMP9/TIMP-1	0.90 ±0.14	1.05± 0.13	0.82 ±0.13	NS

MMP-8 NPA	664.2 ±120.2	1033.6 ±168.8	1296.8 ±282.5	NS
MMP-9 NPA	760.1 ±75.1*	1121.4 ±105.5*	978.9 ±104.7	* p=0.009

Concentrations are given in ng/ml (mean ± standard error), NS=not significant

5 **MMP-9 plasma concentrations are correlated with the number of granulocytes**

MMP-9 plasma concentrations correlated with the number of granulocytes measured during acute RSV infection (Pearson correlation coefficient 0.33; p=0.019). No correlation was found between the number of granulocytes and MMP-8 plasma and nasopharyngeal concentrations and
 10 MMP-9 nasopharyngeal concentrations (data not shown).

MMP-8 and MMP-9 mRNA and protein expression by PBMCs and neutrophils is not induced by RSV in vitro

To investigate whether the source of plasma MMP-8 and MMP-9
 15 during RSV infection was the result of direct interaction of PBMCs or neutrophils with RSV, we stimulated PBMCs and neutrophils with RSV *in vitro*. Stimulation of PBMCs with LPS (TLR4 agonist) induced MMP-9 secretion, whereas stimulation with RSV had no effect. None of the stimuli induced MMP-8 secretion by PBMC (Figure 9A). Stimulation of PBMCs with
 20 RSV did not result in increased gene expression of MMP-8 and only a moderate increase of MMP-9 expression was observed (Figure 9B). Unstimulated neutrophils secreted high levels of MMP-8 and MMP-9. Stimulation with LPS and RSV had no effect on the release of MMP-8 and MMP-9 by neutrophils (Figure 9C).

25

Discussion

This study demonstrates that disease severity of viral LRTI in children is associated with increased gene expression levels of MMP-8 and MMP-9 in both PBMCs and granulocytes and with elevated MMP-8 plasma concentrations. These associations were observed in children with LRTI caused by either RSV or other viruses. The in vitro experiments in this study show that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes is not induced by stimulation with RSV. Consequently, other factors than direct viral interaction induce gene expression in PBMCs and granulocytes.

To our knowledge, this is the first study that describes an association between MMP-8 and MMP-9 gene expression and disease severity of viral lower respiratory infections in children.

In an experimental model of viral infection of the upper respiratory tract in adults with RSV, influenza and rhinovirus, no up-regulation of MMP-8 and MMP-9 was detected in whole blood transcriptional profiles. (Zaas *et al.*, 2009, *Cell Host Microbe* **6**, 207-217) However, these infections were all mild with consequently low levels of inflammatory markers.

TIMP-1 is an inhibitor of the protease activity of all known MMPs. (Gomez *et al.*, 1997, *Eur J Cell Biol* **74**, 111-122) Previous studies have described an association between an imbalance between MMP-9 and TIMP-1 and tissue degradation and airflow obstruction in asthma and chronic bronchitis. (Mautino *et al.*, 1999, *J Allergy Clin Immunol* **104**, 530-533; Vignola *et al.*, 1998, *Am J Respir Crit Care Med* **158**, 1945-1950) In addition, elevated MMP-9/TIMP-1 ratios have been observed in plasma from patients with status asthmatics. (Belleguic *et al.*, 2002, *supra*) Elliot *et al.* have shown that increased TIMP-1 concentrations, but not MMP-9, in nasopharyngeal aspirates of RSV-infected children correlated with disease severity and suggested that a disturbed MMP-9/TIMP-1 homeostasis contributes to disease severity. (Elliott *et al.*, 2007, *J Med Virol* **79**, 447-456) Although, in this study,

higher TIMP-1 concentrations were measured in plasma in children with severe viral respiratory infection compared to those with mild infection, no differences in MMP-9/TIMP-1 ratios were noticed.

Although both MMP-8 and MMP-9 concentrations in
5 nasopharyngeal samples were increased during acute infection compared to recovery samples no association with disease severity was observed. The wide range of nasopharyngeal concentrations between individuals or the fact that upper respiratory samples do not necessarily represent the situation in the lower airways may explain this observation.

10 The in vitro experiments in this study show that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes were not induced by stimulation with RSV. Other factors than direct interaction between RSV and host cells could explain the increased gene expression levels of MMP-8 and MMP-9 in children with viral LRTI. Influx of bone marrow-derived neutrophil
15 precursors in blood from children with severe RSV infections can result in higher MMP-9 expression due to granule protein production, such as MMP-8 and MMP-9, during immature stages of neutrophil development. (Lukens *et al.*, 2010, *J Virol* **84**, 2374-2383) Also inflammatory mediators such as growth factors, pro-inflammatory cytokines, oxidative stress upon viral infection can
20 induce elevated gene expression levels of MMPs (Greenlee *et al.*, 2007, *Physiol Rev* **87**, 69-98). It has also been shown that lung injury caused by mechanical ventilation has resulted in increased MMP-8 and MMP-9 expression. (Albaiceta *et al.*, 2010, *Am J Respir Cell Mol Biol* **43**, 555-563) However, in this study, the last mentioned cannot completely explain the differences in gene
25 expression since also differences in gene expression between patients with mild and moderate disease were observed, all non-ventilated patients.

The results of this study indicate that neutrophils are the major source of MMP-9 production. The higher MMP-8 and MMP-9 concentrations in nasopharyngeal samples compared to plasma may therefore reflect the influx
30 and degranulation of neutrophils in the airways during infection.

This is in contrast to observations made by others that suggest that airway epithelial cells are the primary source of MMP-s. Yeo et al showed that MMP-9 gene expression is increased in human airway epithelial cell lines infected with RSV (Elliott *et al.*, 2007, *supra*; Yeo *et al.*, 2002, *supra*) and Wen et al. demonstrated that gene expression of MMP-9 is elevated in the lungs of RSV-infected mice. (Li & Shen, 2007, *supra*) However, another study indicated that infected human airway epithelial cells are not the primary source of MMPs and TIMP-1 and that infiltrating leukocytes are responsible for MMP-9 in airway samples. (Elliott *et al.*, 2007, *supra*) For MMP-8, no correlation with neutrophil counts was observed and gene expression levels in granulocytes and PBMCs were comparable indicating that MMP-8 transcription and secretion is different regulated than MMP-9. This is supported by differences in the degranulation of subcellular neutrophilic granules, in which MMP-8 and MMP-9 are stored (Faurschou & Borregaard, 2003, *Microbes Infect* **5**, 1317-1327) and differences in transcriptional events that induce MMP-8 and MMP-9 mRNA expression. For example, it has been shown that pro-inflammatory cytokines, particularly IL-1 β , play a central role in the modulation of MMP-8 expression. (Abe *et al.*, 2001, *J Periodontal Res* **36**, 153-159; Knauper *et al.*, 1993, *Biochem J* **291** (Pt 3), 847-854).

20

EXAMPLE 3 – Follow-up study OLFM4

Study design

Children from 0-3 years of age with clinical symptoms of an acute viral lower respiratory infection (vLRI) who were attended to the University Nijmegen Medical Centre or Canisius Wilhelmina Hospital in Nijmegen were included during two winter seasons (2010-2011 and 2011-2012). Viral LRI was defined as an acute infection of the lower airways, characterized by increased respiratory effort (tachypnea and/or use of accessory respiratory muscles and/or expiratory wheezing and/or crackles and/or apnea). Written informed consent was obtained from all parents and the study was approved by the Committee on Research involving Human Subjects of the University Nijmegen Medical Centre. From these children a 3 ml blood sample, a nasopharyngeal aspirate (for virology), medical history, demographics and clinical parameters were obtained in the acute stage of the disease. They were asked to return 4-6 weeks later for a recovery sample. The included patients were subdivided into three groups based on the severity of the respiratory

infection: mild (no supportive treatment), moderate (supplemental oxygen and/or nasogastric feeding) and severe disease for those who needed mechanical ventilation.

Sample collection

5 Within 24 hours after admission 3 ml blood was collected into a sodium heparin tube and directly transferred to the laboratory. Cell fractions were separated into PBMCs and neutrophils. The samples were stored in Trizol at -80°C for RNA isolation.

10 A nasopharyngeal aspirate was collected by introducing a catheter, connected to a collection tube and an aspiration system, into the nasopharyngeal cavity. Then, 0.5 ml of saline was instilled into the catheter and, while slowly retracting the catheter, the nasopharyngeal fluid was aspirated in a collection tube. Afterwards the catheter was flushed with 1 ml of saline and added to the collection fluid. The samples were kept cold and immediately transferred to the laboratory. The nasopharyngeal aspirates were processed according to the protocol (see appendix).

RNA isolation and cleanup

15 RNA from PBMCs and granulocytes was extracted using Trizol (Invitrogen Life Technologies) according to the manufacturers' protocol. RNA cleanup was performed with the RNeasy Minikit (Qiagen). See also the protocols in the appendix. Total RNA was measured with the Nanodrop spectrophotometer.

cDNA synthesis

20 According to protocol 'cDNA synthesis', 2 µg RNA was reversed transcribed into cDNA using Superscript III (Invitrogen). When the amount of RNA in a sample was very low, 22 µl of the sample was used. Large amounts of total RNA were diluted with dH₂O to get a solution of 2 µg RNA in 22 µl. cDNA was stored at -20°C.

qPCR

25 The relative gene expression was measured with SYBR Green PCR Mastermix (Applied Biosystems; P/N 4367659) on the ABI 7500 Fast Real Time PCR system using standard program and software. The following primers were used: hActin FW: CGTCACACTTCATGATGGAGTTG, hActin RV: CTTCTTCTCTGGGCATGGA, OLFM4 FW: 5'- ATCAAAACACCCCTGTCGTC-3' and RV: 5'- CCACCAGGGTTGATGTGAGT-3'

30 All reactions were performed in duplo. Actin was used as reference gene. After 40 repetitions a dissociation curve was performed as control for the specificity of the PCR reaction. The mean of the cycle threshold (Ct) values were taken as measured value. Ct values of OLFM4 were normalized against actin.

Microarray literature study

A literature search was performed in GEO and in arrayexpress, online databases with datasets and profiles of performed microarray studies to validate our results. Terms for searching were:

5 *OLFM4, affymetrix, whole blood children, RSV and/or homo sapiens*. More than 90 microarray studies were found. 18 studies were selected based on the population (children/infants), sample size and type of the disease (viral/bacterial, infection/ auto-immune disease, lung diseases). From the series matrix files the results on OLFM4 gene expression were selected and analyzed to look for gene expression up or down regulation to gain insight in regulatory pathways for OLFM4 and specificity in viral infections. All data were log transformed and statistically analyzed.

10 Statistics

The Chi square test and the Kruskal Wallis test were performed to analyze patient characteristics in the different groups. To analyze the association between the relative gene expression of OLFM4 and disease severity the Mann-Whitney U-test was used. For the other results the Mann-Whitney U-test, the unpaired T-test and the Wilcoxon matched-pairs signed rank test were used.

15 The microarray data were analyzed with the paired and unpaired T-test (normal distributed data) or the Mann-Whitney U test for not normally distributed data (tested with the Shapiro Wilk Normality test). A value of $P < 0.05$ is considered to be statistically significant.

RESULTS

20 Patient characteristics

A total of 105 children with a viral lower respiratory infection were included in this study. 8 patients were classified into the mild, 66 into the moderate and 31 into the severe group. Table 6 shows the results. The children in the moderate group were significantly older than the children in the mild and severe groups ($P = 0.0048$ and $P = 0.0003$). This can be due to the fact that younger children are more susceptible to severe disease and also that they are more often admitted 'just in case'. The duration of hospitalization was higher as severity increased. Another significant difference was found in RSV positive and RSV negative samples between the severity groups. Siblings, a known risk factor for severe disease was significant different between the mild and moderate group. No other significant differences were found.

30

OLFM4 gene expression is a marker for infection

A total of 284 samples were analyzed. The samples were subdivided into four categories: 90 acute PBMCs, 45 recovery PBMCs, 99 acute granulocyte and 50 recovery granulocyte samples. First the difference between the relative gene expression of OLFM4 in acute and recovery samples was analyzed to see if OLFM4 is a marker for viral lower respiratory infection in children. Figure 10 shows the differences in relative gene expression.

35

The expression of OLFM4 in PBMCs and granulocytes in the acute samples is significantly higher compared to the recovery samples ($P = 0.0013$ and $P = 0.0053$ respectively). This makes OLFM4 a marker for viral lower respiratory infection in children.

40

Severity marker

To determine whether the level of OLFM4 gene up regulation is correlated with disease severity, the mild, moderate and severe infection samples were compared. No statistical significant differences were found between mild/moderate and mild/severe disease in PBMCs. There was a significant difference between moderate and severe disease ($P < 0.0001$). In the granulocyte samples there was a significant difference between the mild and moderate group and between the moderate and severe disease group ($P = 0.0181$ and $P = 0.0002$ respectively). The relative gene expression in the moderate group was higher than in the severe group. No differences in gene expression between mild and moderate disease were found (figure 11).

45

Table 6. Patient characteristics

	Total N= 105	Mild N= 8	Moderate N= 66	Severe N= 31	P-value
Male	57 (54)	3 (38)	35 (53)	19 (61)	NS
Age in days, median (25-75)	84 (38-285)	39 (22-82)	134 (63-441)	39 (22-202)	<0,0001*
Duration of symptoms, median (25-75)	3 (2-4)	3 (2-4)	3 (2-4)	4 (2-5)	NS
Duration of hospitalization	6 (3-10)	3 (2-4)	5 (3-7)	11 (8-13)	<0,0001**
RSV + (6 missing)	64 (61)	0	47 (71)	17 (55)	<0,0001***
Prematurity < 36 weeks	15 (14)	0	11 (17)	4 (13)	NS
Co morbidity	23 (24)	0	19 (13)	4 (13)	NS
Asthma	10 (11)	0	8 (12)	2 (7)	NS
Eczema	12 (13)	0	10 (15)	2 (7)	NS
Pulmonary disease	1 (1)	0	1 (1,5)	0	NS
Cong. heart disease	2 (2)	0	1 (1,5)	1 (3)	NS
Family history of atopy	64 (61)	2 (25)	44 (67)	18 (58)	NS
Siblings	80 (76)	8 (100)	43 (65)	29 (93)	0,002****
Smoking at home	12 (11)	0	9 (14)	3 (10)	NS

Data are presented as number (%), or otherwise specified. Co morbidity= asthma, eczema, bronchopulmonary dysplasia, congenital lung disease, congenital heart disease, milk allergy, immunodeficiency.

5

Objective markers

Disease severity was determined retrospectively by the therapy given: nasogastric feeding OR oxygen therapy in moderate disease and mechanical ventilation in severe disease. More objective markers for disease severity are probably the duration of hospitalization or the need for supplemental oxygen during admission (71% of the children in the moderate group received supplemental oxygen). For this the relation between the gene expression of OLFM4 and the number of days patients were admitted and the need for oxygen therapy during admission was compared. There is a trend but no significant difference in OLFM4 gene expression between children who need oxygen therapy vs. children who don't need supplemental oxygen ($P=0,0617$, data not shown). The duration of hospitalization is correlated with relative gene expression rates of OLFM4 (correlation 0,333, $p=0,0022$), data not shown. To conclude, a high OLFM4 gene expression is not a good predictor for the need of oxygen therapy, however it is a predictor of duration of hospitalization.

20

Age

Young age is associated with higher relative gene expression levels of OLFM4 (figure 12, $P=0,0043$). There are different explanations for this: 1) young children always have higher (also baseline) gene expression levels of OLFM4 due to unknown factors, 2) younger children more often have severe infections and therefore a higher OLFM4 gene expression. 3) OLFM4 is more up regulated in young children during infections, due to unknown factors. The red symbols in figure 6 indicate severe disease. In the youngest group the OLFM4 gene expression from severe diseased children is significantly higher ($p=0,0233$) compared to the severe disease samples from children in the group >3 months. There are more than twice as many severe diseased children in the group < 3 months of age compared to the older group (figure 13). When age is corrected for gestational

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age (to a duration of 40 weeks) there is still a significant difference between children <3 months and >3 months of age ($P=0,0051$).

Other (risk)factors

- 5 Besides young age there are more risk factors for severe disease in viral lower respiratory infections, like prematurity. Premature children have no higher OLFM4 gene expression levels than a term born children ($p=0,3607$). There was also no correlation found between the duration of symptoms before hospitalization and OLFM4 gene expression ($p=0,9307$). Data not shown.

10 Discussion

- It is difficult to predict disease severity in children with a viral lower respiratory infection, therefore this study aimed to investigate if Olfactomedin 4 gene expression is a marker for disease severity. The results showed that OLFM4 gene expression is a marker for viral lower respiratory infection because both in PBMCs as well as in granulocytes OLFM4 is significantly
15 higher in the acute samples compared to the recovery samples. Also statistical differences were found between moderate and severe disease in the PBMC samples and between moderate and severe and moderate versus mild disease in granulocytes. This makes OLFM4 gene expression a good biomarker for disease severity.

Claims

1. Method for the prediction of the severity of a disease developing from an infection with human respiratory syncytial virus (RSV) in a subject, comprising the steps of:
 - a. determining overexpression of OLFM4, CD177, MMP8, MMP9,
5 PTX3 or a combination of one or more of these with one or more
chosen from the group of IL8, RANTES and CD4 count;
 - b. making a prediction on basis of the amount of overexpression
detected in step a).
- 10 2. Method according to claim 1, wherein said overexpression is detected by a nucleic acid assay or by an immunoassay.
3. Method according to any of claims 1 – 2, wherein the subject is a
child, preferably a child having an age of less than 6 months, more
15 preferably a child having an age of less than three months.
4. Method according to any of the preceding claims, where the
detection is performed on a blood sample of said subject.
- 20 5. Method according to claim 4, wherein the detection of overexpression of OLFM4 is performed on a cell component derived from peripheral blood mononuclear cells.
6. Method according to claim 4, wherein the detection of overexpression
25 of CD177 is performed on a cell component derived from neutrophils.

7. Method according to claim 4, wherein the detection of overexpression of MMP8 is performed on a cell component derived from peripheral blood mononuclear cells.
- 5 8. Method according to claim 4, wherein the detection of overexpression of MMP9 is performed on a cell component derived from peripheral blood mononuclear cells or from neutrophils.
9. Method according to claim 4, wherein the detection of overexpression
10 of PTX3 is performed on a cell component derived from peripheral blood mononuclear cells.
10. Method according to any of the preceding claims, wherein a subject is predicted to develop a severe disease state if in a sample of said subject
15 OLFM4, CD177, MMP8, MMP9 or PTX3 or combinations thereof are found to be overexpressed by a factor of 1.5, more preferably by a factor of 2, most preferably by a factor of 3 or more.
11. Method according to any of the preceding claims, wherein the cell
20 component which is assayed is a nucleic acid, more preferably mRNA.
12. Method according to claim 8, wherein the assay is a PCR assay.
13. Method according to any of claims 1 - 10, wherein the cell component
25 which is assayed is a peptide
14. Method according to claim 13, wherein the assay is an ELISA assay.
15. Detection kit for performing a method according to any of the
30 preceding claims, comprising means for detection of RSV particles and

means for the detection of overexpression of OLFM4, CD177, MMP8, MMP9 or PTX3 or combinations thereof.

16. Detection kit according to claim 15, wherein the means for detection
5 overexpression comprise primers, probes or antibodies.

17. Detection kit according to claim 16, wherein a pair of bidirectional
primers for detection of overexpression of OLFM4 comprises the primers 5`-
atcaaaacacccctgtcgtc- 3`and 5`- gctgatgttcaccacaccac-3`.
10

18. Detection kit according to claim 16, wherein a pair of bidirectional
primers for detection of overexpression of CD177 comprises the primers 5`-
gggcaggtgtgtcaggag -3`and 5`- ccaccaggggtgatgtgagt -3`.

15 19. Detection kit according to claim 16, wherein a pair of bidirectional
primers for detection of overexpression of MMP8 comprises the primers 5`-
CCAGTTTGACATTTGATGCTATCAC -3`and 5`-
CTGAGGATGCCTTCTCCAGAA -3.

20 20. Detection kit according to claim 16, wherein a pair of bidirectional
primers for detection of overexpression of MMP9 comprises the primers 5`-
GCCCCCCTTGCATAAGGA -3`and 5`- CAGGGCGAGGACCATAGAG -3.

21. Use of a kit according to any of claims 15 - 20 for predicting the
25 severity disease state upon RSV infection in a subject.

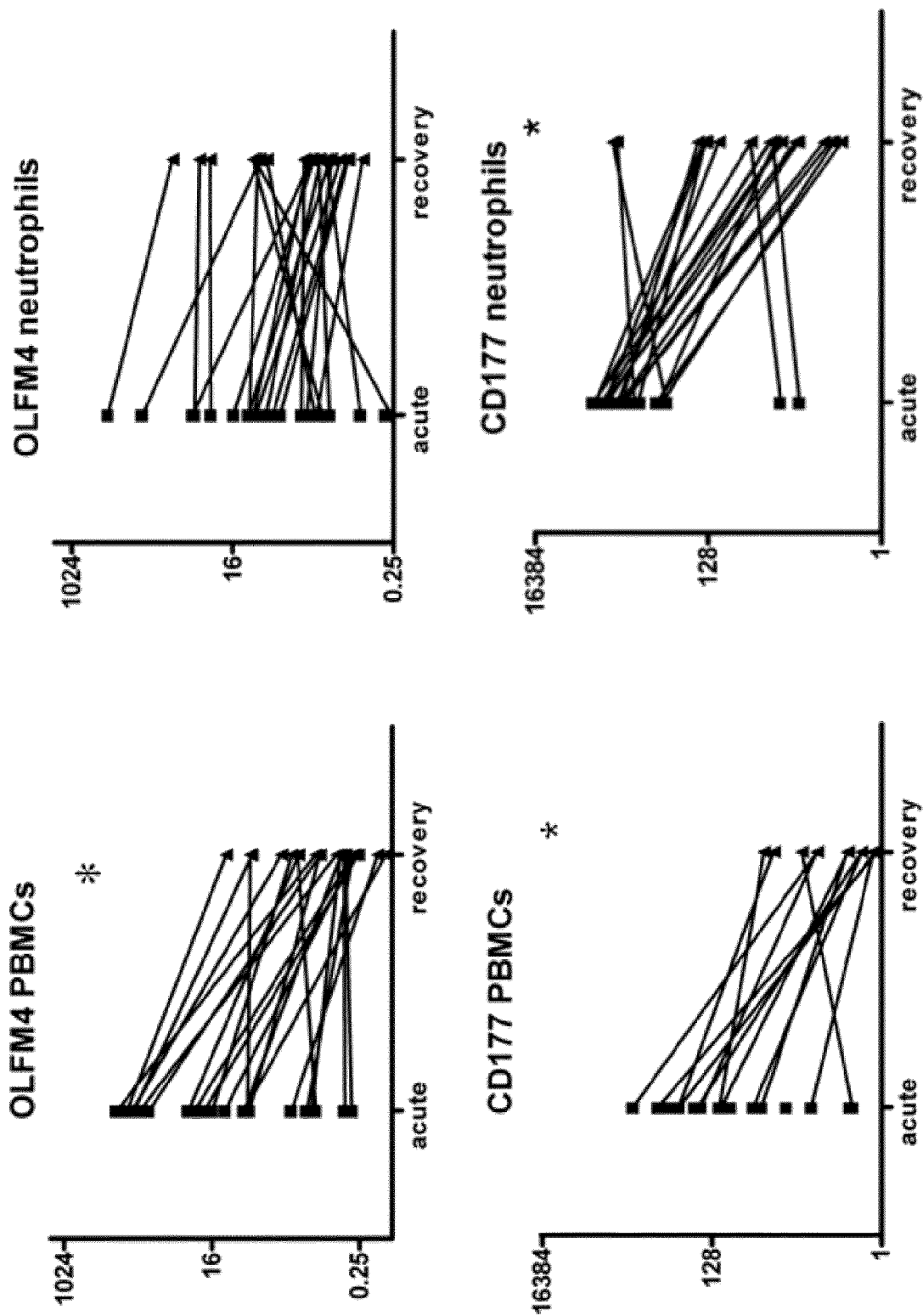


FIG. 1

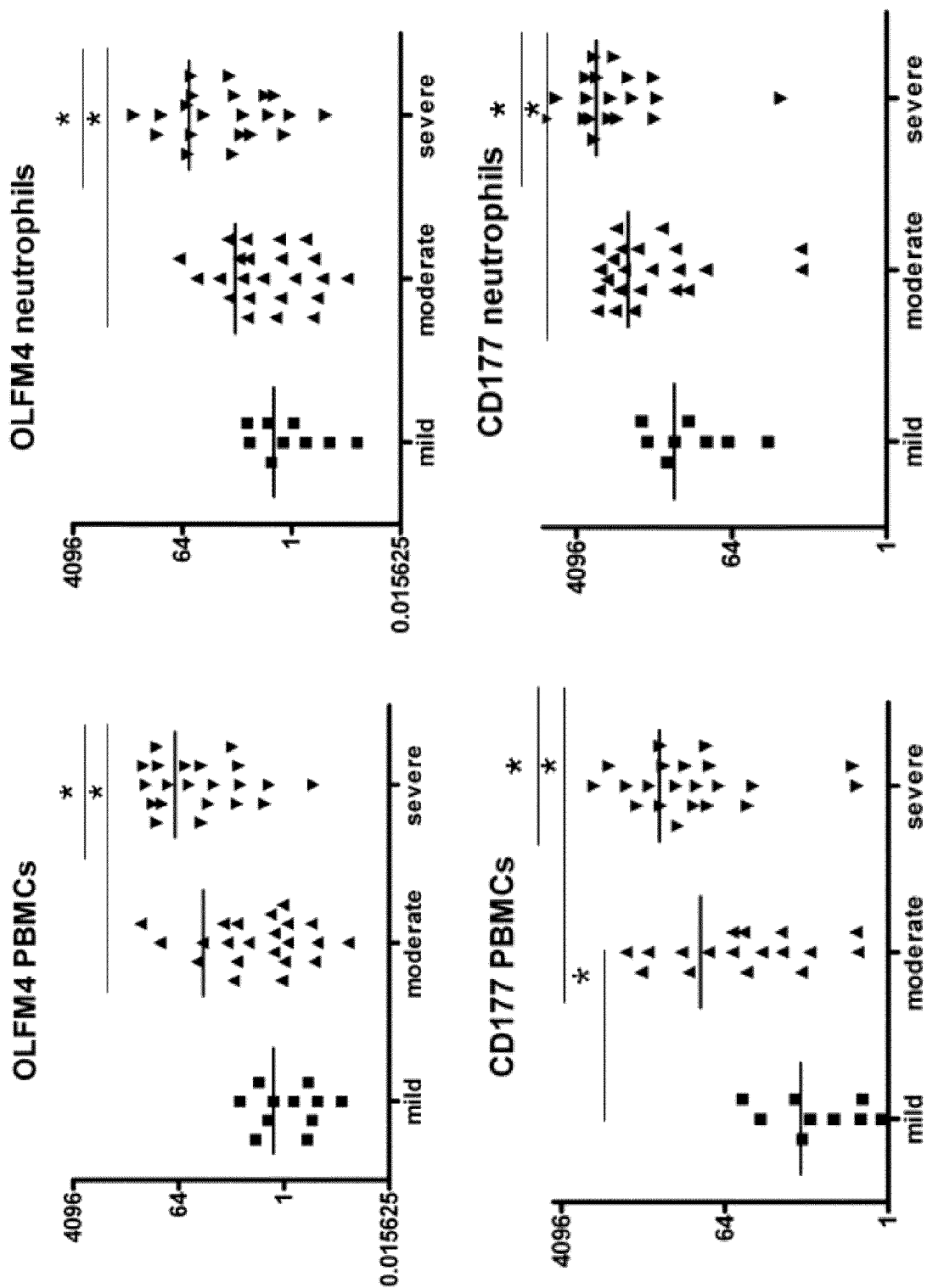


FIG. 2

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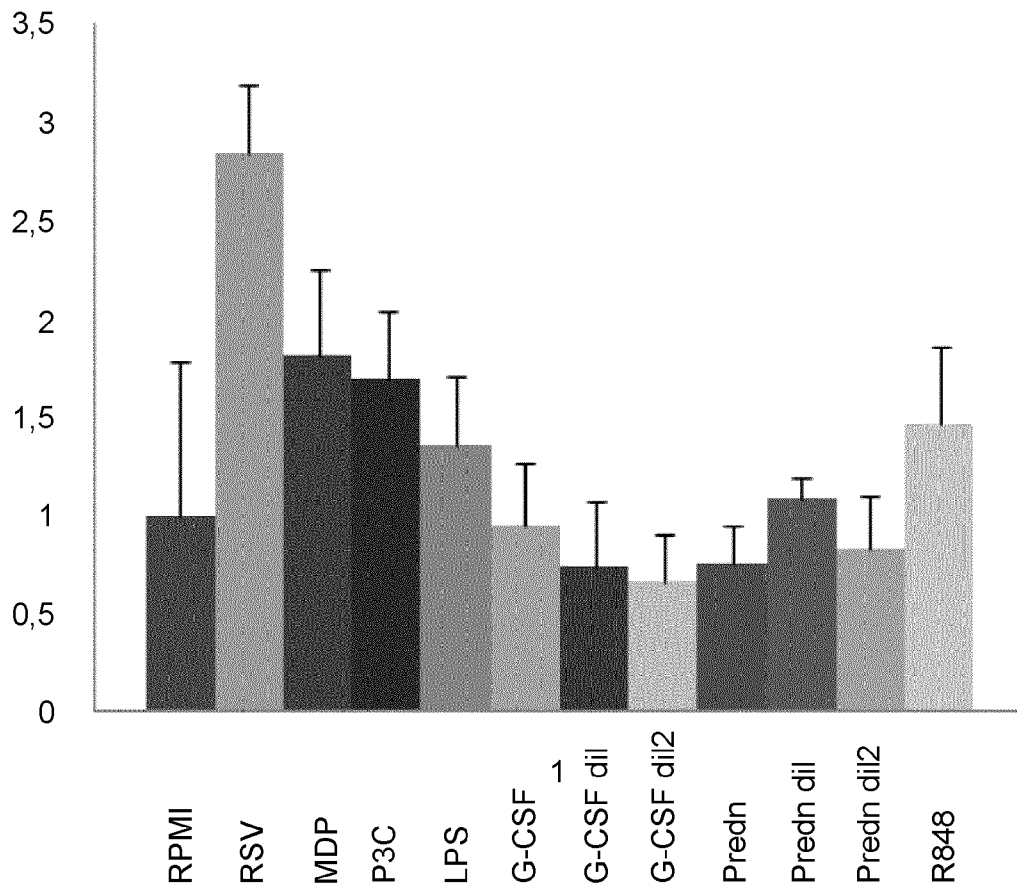


FIG. 3

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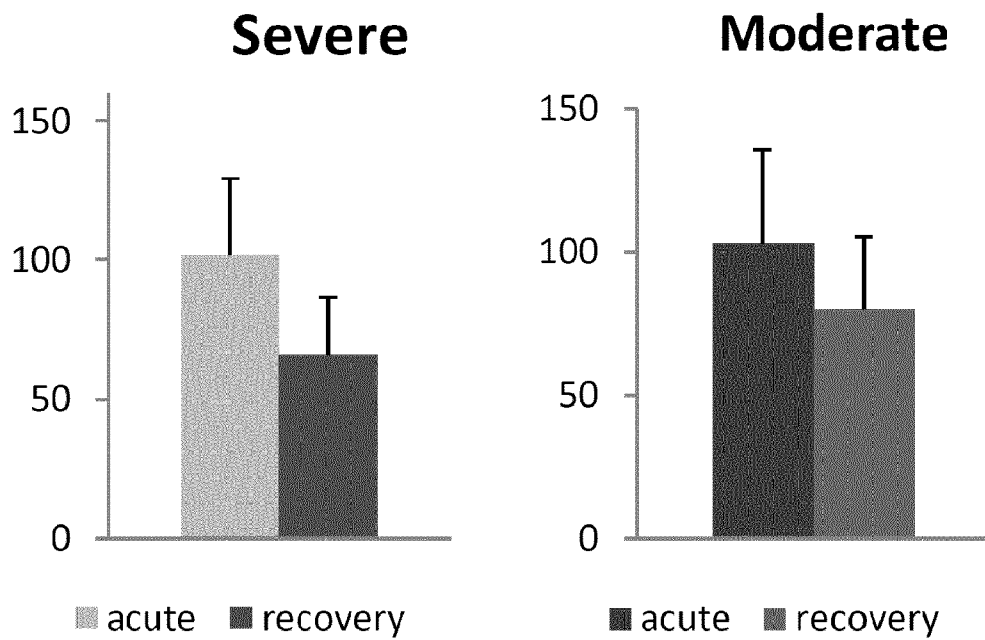


FIG. 4

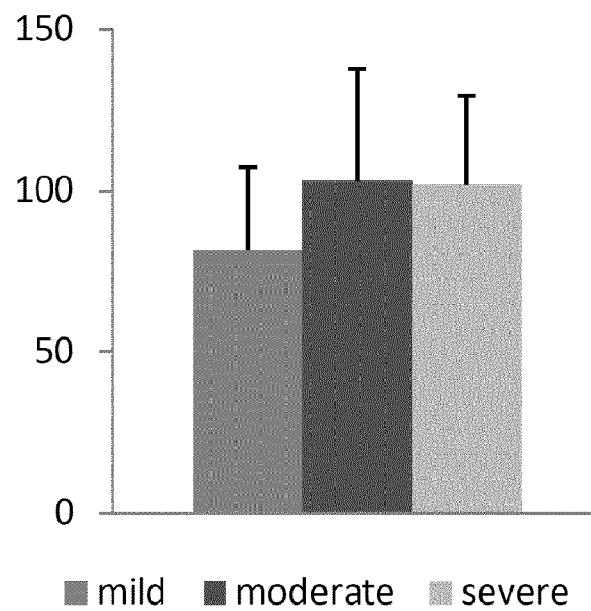


FIG. 5

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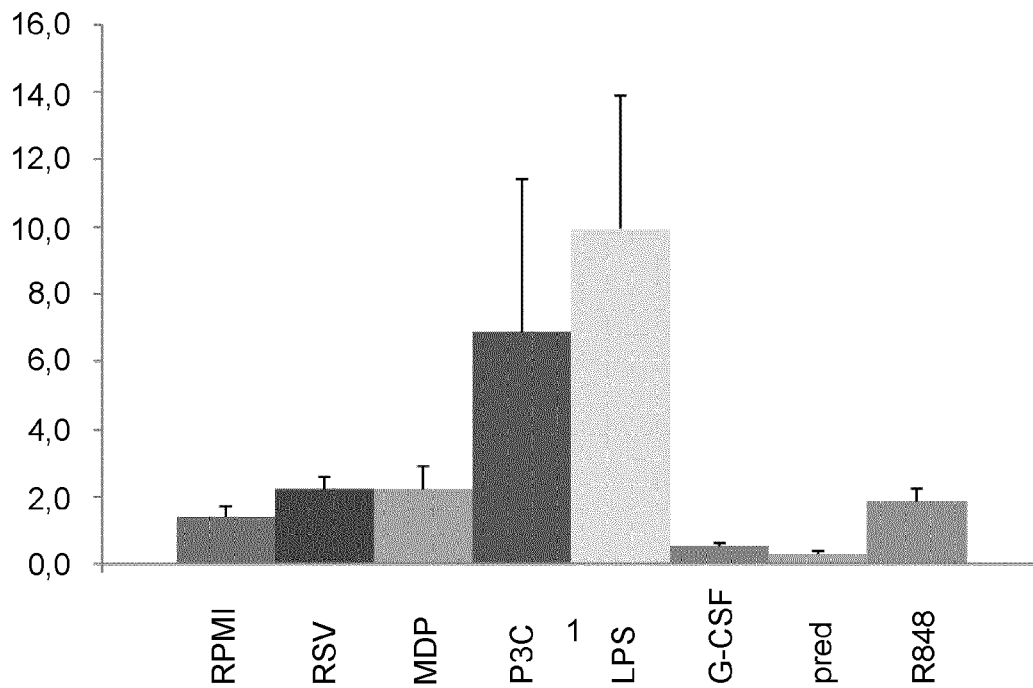


FIG. 6

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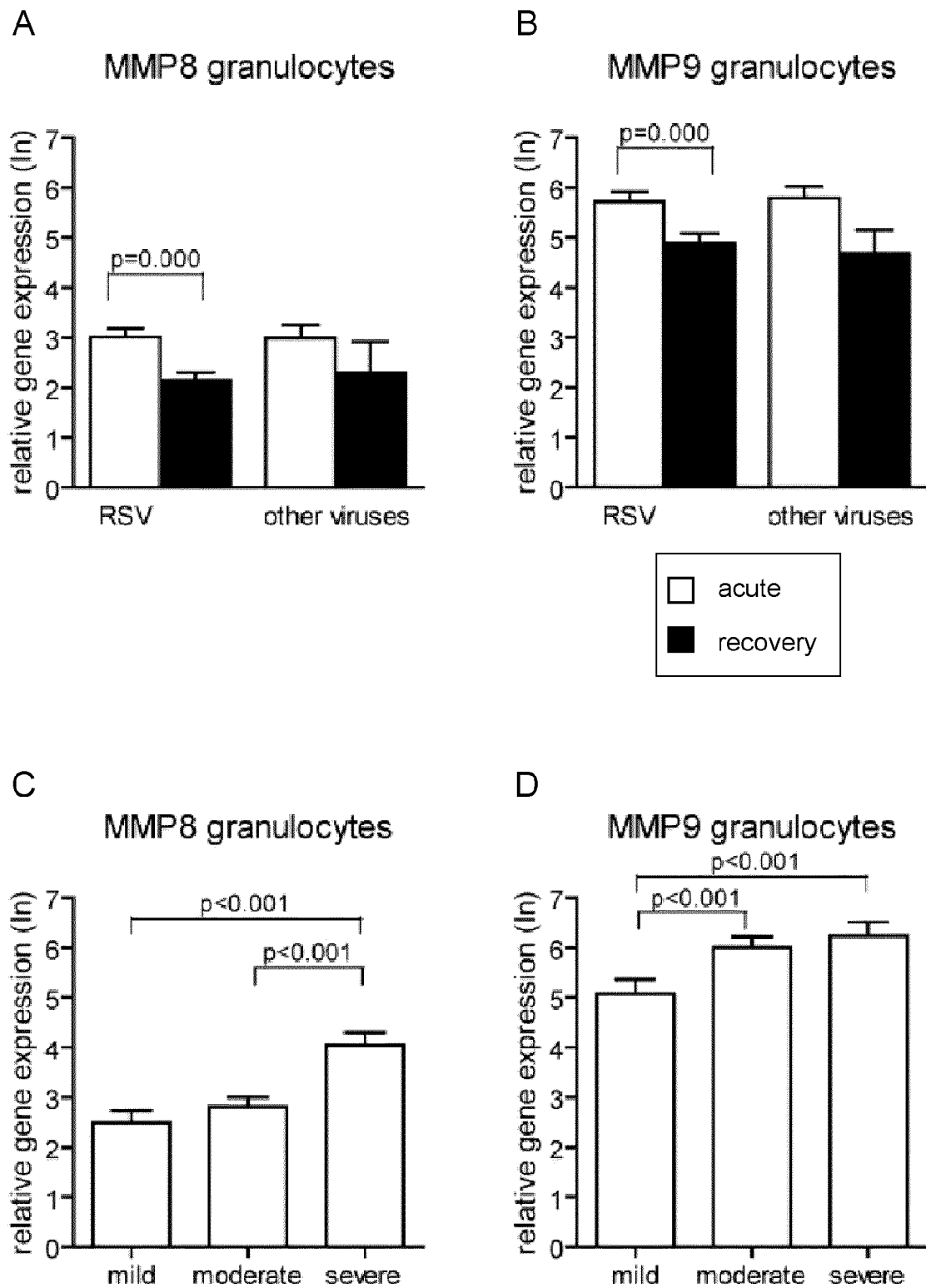


FIG. 7

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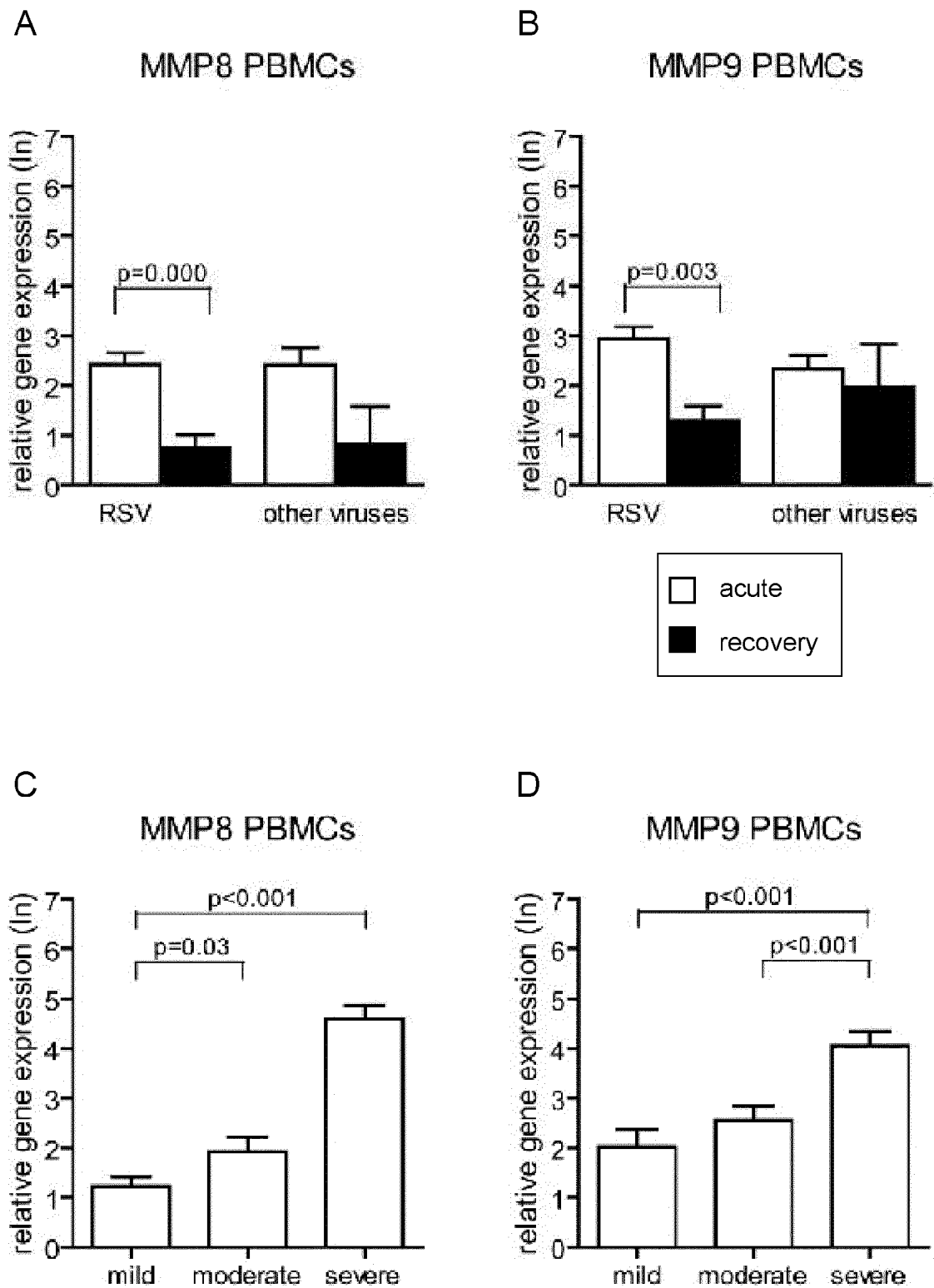


FIG. 8

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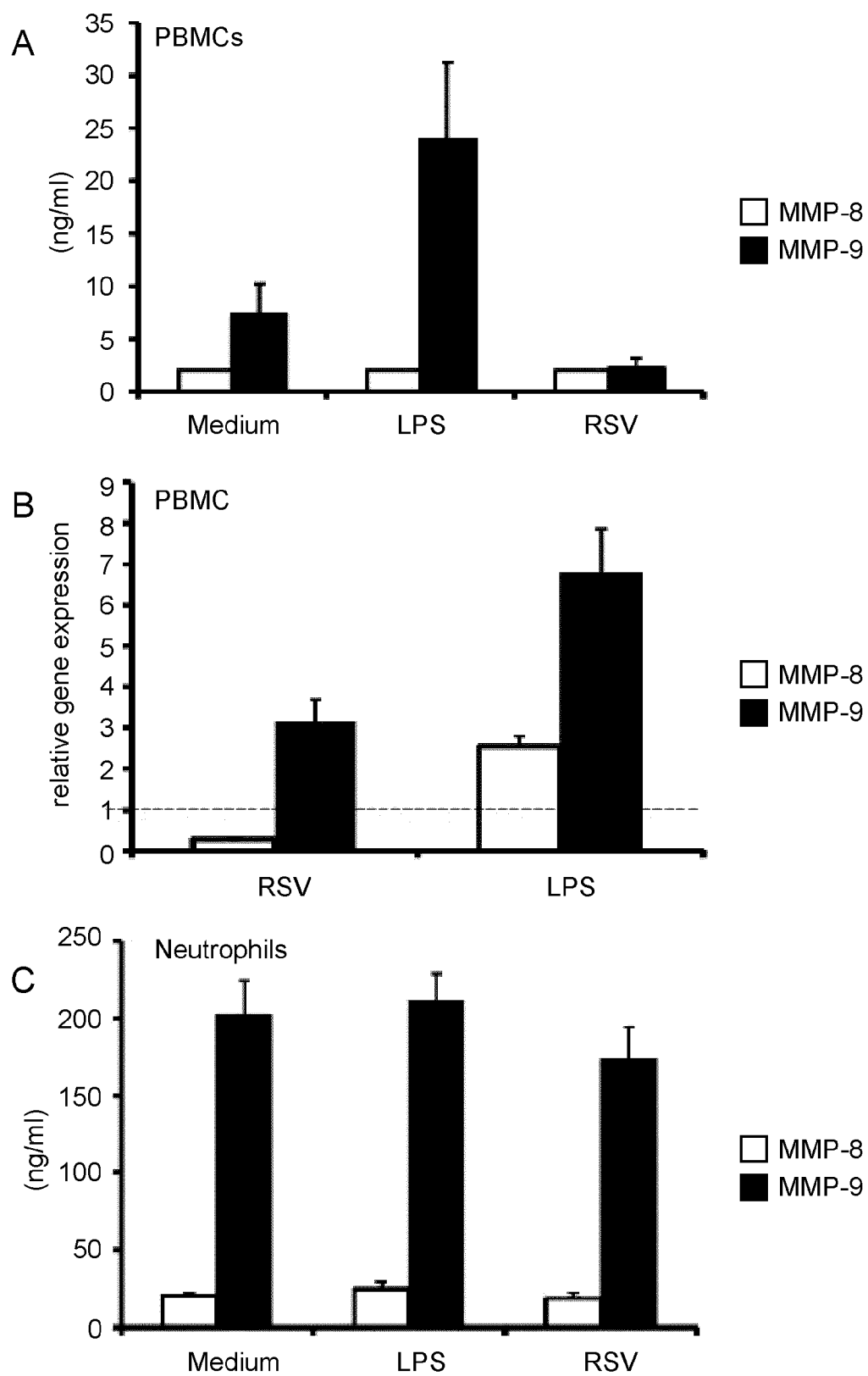


FIG. 9

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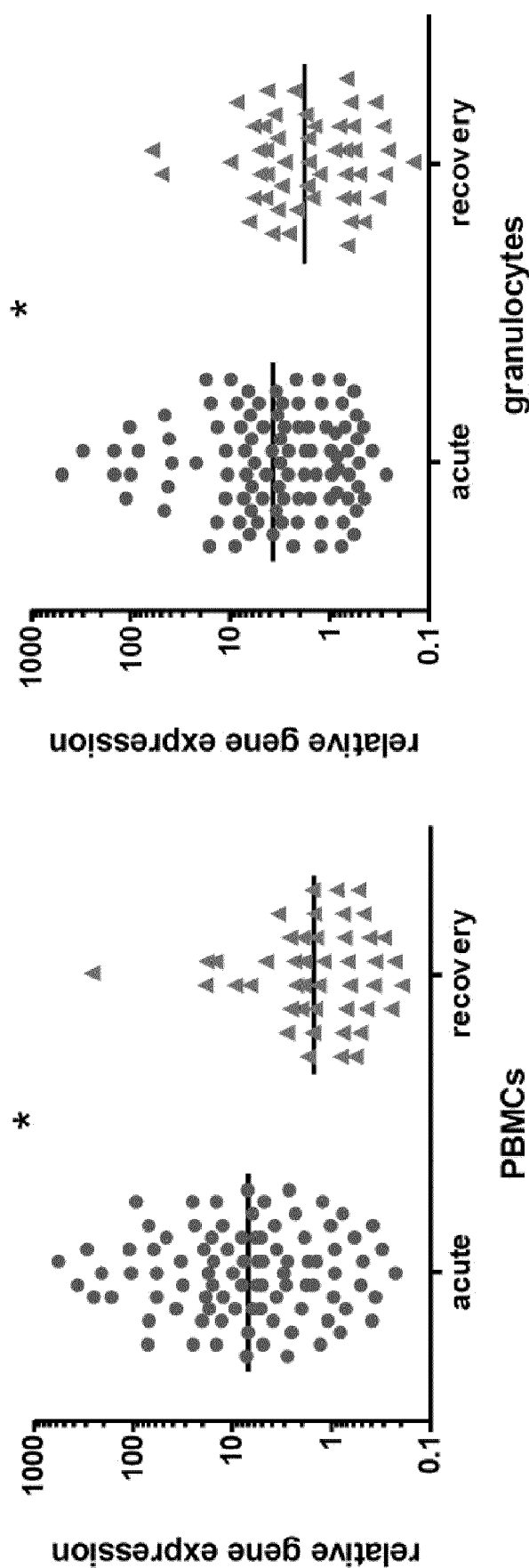


FIG. 10

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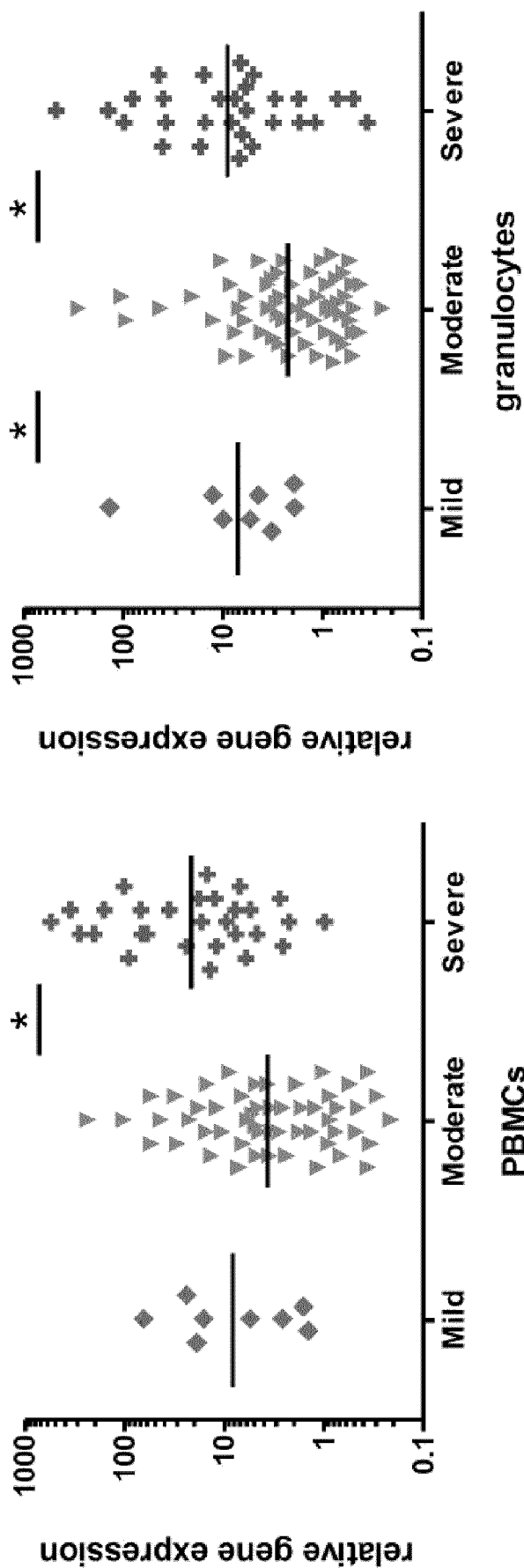


FIG. 11

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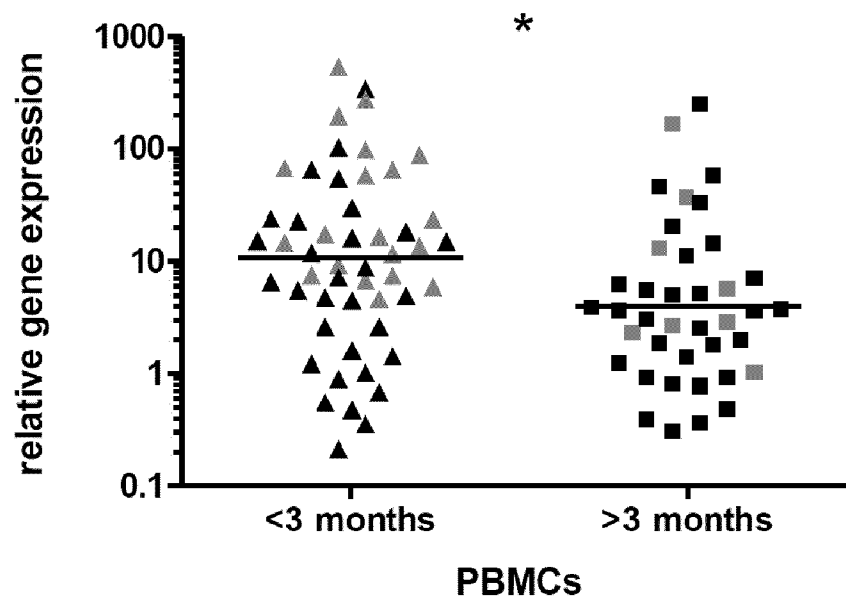


FIG. 12

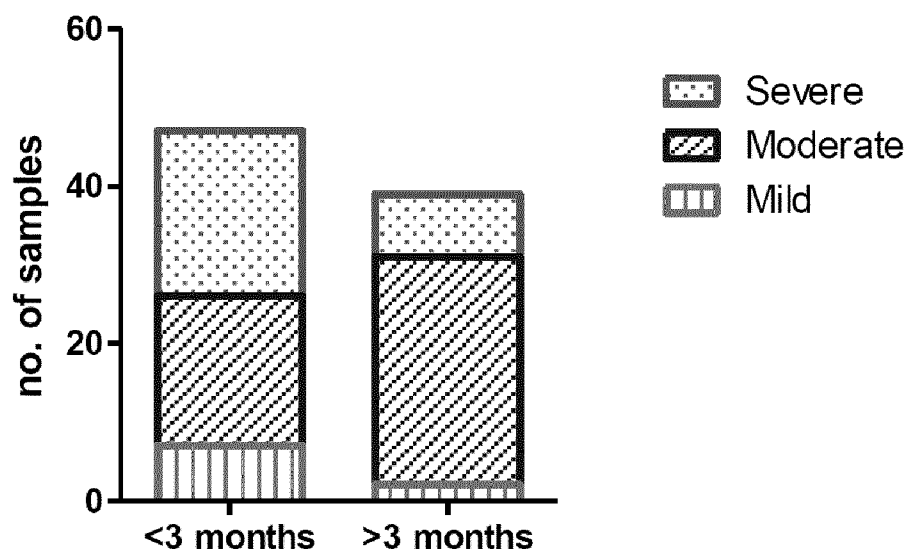


FIG. 13