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Soluble mannose receptor levels in blood correlate to disease severity in patients with community-acquired pneumonia

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ABSTRACT

Purpose: Community-acquired pneumonia (CAP) is the most common form of pneumonia and is a leading infectious cause worldwide. Identification of patients that are at risk to develop severe disease has proven to be a major challenge. Soluble mannose receptor (sMR; sCD206) is a new serum marker for macrophage activation. Recent studies showed that sMR levels are increased in patients suffering from severe infections making it a potential biomarker for improved discrimination of disease severity. For measuring sMR, no standardized assay is available. Aim of this study is to develop an assay for standardized measurement of sMR. Next, this assay was used to assess sMR plasma levels for its ability to predict severe disease development in a patient cohort for community-acquired pneumonia.
Methods: We developed a well-validated sandwich ELISA that enables standardized measurement of sMR in plasma and serum samples. Repeatability was tested by calculating the percentage coefficient of variation (%CV) within and between runs and within and between operators. sMR levels were assessed in a cohort of 100 patients with community-acquired pneumonia.
Results: All %CV values were < 10%, indicating low variation. Higher sMR levels were observed in patients with severe disease when compared to patients without severe disease development ($p = 0.004$). Patients with sMR levels between 100–430 ng/ml had 22.7% chance to develop severe disease whereas patients with levels between 430–1000 ng/ml had 33.3% chance to develop severe disease.
Conclusions: We suggest that sMR has potential as a new biomarker for the prediction of disease severity in patients with community-acquired pneumonia.

1. Introduction

Community-acquired pneumonia (CAP) is the most common form of pneumonia and a leading infectious cause of morbidity and mortality among adults worldwide. A wide spectrum of microorganisms, including bacteria, viruses, fungi and parasites, can cause CAP, of which *Streptococcus pneumoniae* is responsible for most cases. Identifying patients that are at risk to develop severe disease (i.e. intensive care unit (ICU) admission and/or death) is a major challenge. Several studies have shown that certain infection markers are (at least partly) able to predict disease development in CAP [1–5]. Ito and coworkers investigated C-reactive protein (CRP) and procalcitonin (PCT) as biomarkers for disease prognosis in a cohort of 365 CAP patients. They concluded that assessing serial PCT measurements next to CRP analysis, has added value for predicting prognosis and initial treatment failure [3]. Also plasma soluble urokinase plasminogen activator receptor (suPAR) has been studied during the last few years and results showed that this biomarker is suitable for the prediction of disease severity,

Abbreviations: AUC, area under curve; CAP, community-acquired pneumonia; CI, confidence interval; CRP, C-reactive protein; %CV, coefficient of variability; ED, emergency department; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit; IPD, invasive pneumococcal disease; LIS, laboratory information system; PCT, procalcitonin; PnAT, pneumococcal antigen test; ROC, receiver operating characteristic; sMR, soluble mannose receptor; suPAR, soluble urokinase plasminogen activator receptor; TMB, tetramethylbenzidine; WBC, white blood cell

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admission time and risk of mortality [4,5]. Although promising, we are still in need for new biomarkers in order to predict disease prognosis and severity more precisely.

The soluble mannose receptor (sMR; sCD206) is a new serum marker for macrophage activation and a recent study from Rødgaard-Hansen and coworkers showed that often high concentrations (cutoff > 430 ng/ml) are found in hospitalized patients, suffering from infections when compared to healthy controls (≤430 ng/ml) [6]. The mannose receptor is primarily expressed by macrophages and dendritic cells and its extracellular domains are able to bind several ligands including mannose, collagen and sulfated carbohydrates. Its functions include endocytosis, antigen presentation and induction of immune responses. MR is able to bind a wide range of microorganisms, including *S. pneumoniae*. The soluble form of the receptor is still able to bind its ligands. In another study of Rødgaard-Hansen and coworkers, it is reported that sMR levels were increased in patients with invasive pneumococcal disease (IPD). In addition, they showed that sMR levels are associated with mortality and suggested that sMR may be a biomarker for prediction of fatal outcome in IPD patients [7]. Although encouraging, replication of these initial results in independent patient cohorts is hampered by the fact that no standardized and validated assay for measuring sMR is available. As a consequence, it is difficult to compare results from different studies is a reliable way.

The goal of this study was to develop a well-validated sandwich enzyme-linked immunosorbent assay (ELISA) that enables standardized measurement of sMR in plasma and serum samples. This newly developed assay was used to measure sMR levels in patients with community-acquired pneumonia. Soluble MR was assessed for its ability to predict severe disease development in comparison to the infection markers CRP, PCT and suPAR [8].

2. Methods

2.1. Ethics statement

This study is part of a larger study that was performed at the Jeroen Bosch Hospital in' s-Hertogenbosch, The Netherlands [8]. The local Medical Ethical Committee stated that this study did not need specific review. Anonymous use of remnant whole blood and plasma samples was approved by the Internal Review Board of the Jeroen Bosch Hospital. In addition, they approved that data were retrieved from the laboratory information system (LIS) and waived the need for informed consent (study number 2014.11.03.01). All data in this study were retrospectively obtained and analyzed blinded.

2.2. Patients

Patients visiting the emergency department (ED) presenting symptoms of lower respiratory tract infection and for whom a urinary pneumococcal antigen test (PnAT

BinaxNOW, Alere Health BV, Tilburg, The Netherlands) was requested, were included in the study. Further inclusion criteria were: 1) age ≥ 18 years and 2) clinical suspicion of community-acquired pneumonia (CAP). Inclusion period ranged from December 2014 to May 2016. Chest X-rays were used to diagnose CAP. Patients were divided in two groups: with or without severe disease development in which severe disease was defined as admittance to the ICU and/or death. Patients with comorbidities (multiple myeloma, morbus Kahler, rheumatoid arthritis or morbus Wegener), missing data or inferior plasma quality were excluded. Gender, date of birth, blood culture results, white blood cell (WBC) counts and CRP levels were retrieved from the laboratory information system and were reported before [8].

2.3. sMR measurements

sMR levels were defined with a new *in vitro* assay developed for the quantitative determination of sMR in plasma and serum (cat# HK381, Hycult Biotech, Uden, The Netherlands). The assay was performed according to manufacturer instructions. Briefly, samples and standards were incubated in microtiter wells coated with antibodies recognizing sMR. After washing, plates were incubated with biotinylated tracer antibodies that bind to the captured sMR. Streptavidin-peroxidase conjugate was added and allowed to bind to the biotinylated tracer antibody. Addition of tetramethylbenzidine (TMB) substrate started an enzymatic reaction thereby producing a colored product which can be measured. The reaction was stopped after 30 min. by adding oxalic acid and the absorbance at 450 nm was measured using a spectrophotometer. sMR quantification was achieved by plotting the absorbance of samples relative to a set of recombinant sMR standards ranging from 0 to 200 ng/ml.

2.4. Biomarkers determination

Results regarding biomarkers other than sMR (WBC count, CRP, PCT and suPAR) were determined according to the manufacturer's instructions and reported before [8].

2.5. Statistical analyses

SPSS (Version 22, IBM) was used to perform the statistical analyses. The Chi-Square test was used to test for differences in age categories and gender distribution between groups. For analysis of sMR levels, stem-and-leaf plots and quantile-quantile plots were used to judge whether data fit the normal distribution. As the data did not follow a normal distribution, Mann-Whitney U tests were performed for analysis of sMR levels in different groups. A p value of ≤ 0.05 was considered statistically significant. Analyses of Receiver operating characteristics (ROC) curves were performed to establish how well sMR levels were able to distinguish between mild and severe disease development. ROC curves showed sensitivity versus 1-specificity such that area under the curves (AUC) varied from 0.5 to 1.0, with higher values indicating increased discriminatory capability.

3. Results

3.1. Patient characteristics

A total of 121 patients were initially included in this cohort. Twenty-one patients were excluded from the study due to comorbidities (multiple myeloma, morbus Kahler, rheumatoid arthritis or morbus Wegener), missing data or inferior plasma quality. The remaining 100 were included in the analyses. Average age of the patient population was 69 years (range 19–93). Sixty-one percent of the patients was male *versus* 39% female. Twenty-seven of the 100 patients developed severe disease as defined by ICU admission and/or death. The variables age, WBC count, and CRP did not differ between patients who developed mild or severe disease. Distribution of these variables were reported before [8].

3.2. Development and characterization of the sMR sandwich ELISA

A newly developed sandwich ELISA was used to measure sMR. The optimum combination of the capture and detection antibodies (both murine monoclonal antibodies) was determined based upon the most optimal signal/noise ratio. All obtained signals were specific for sMR protein as no signals were obtained in absence of capture or detection antibody (data not shown). The assay was linear between 0–200 ng/ml. Over this concentration range, the relationship between absorbance and sMR concentration yielded a coefficient of determination of 0.99 (Fig. 1). When concentrations appear to be higher than 200 ng/ml, they should be re-analyzed using a higher dilution.

Matrix effects occur when the target analyte interacts with matrix

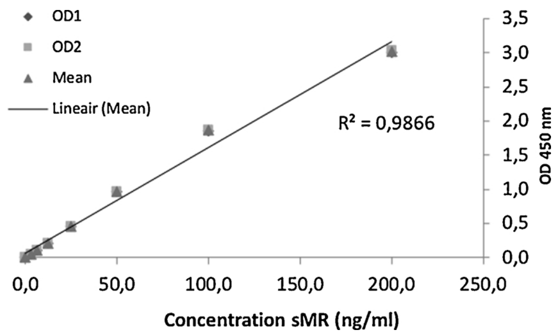

Fig. 1. sMR standard curve. Representative results of a sMR ELISA standard curve in dublicate showing linear response between absorbance and sMR concentration ranging from 0 to 200 ng/ml. Abbreviations: sMR = soluble Mannose Receptor, OD = optical density, R^2 = coefficient of determination, nm = nanometer.

components in plasma or serum samples. This may result in erroneous sample readings. Increasing the dilution factor will reduce matrix component binding and reduce the matrix effect. In order to investigate whether these matrix effects occur in the sMR sandwich ELISA and to determine the optimal dilution factor to minimize these effects, dilution linearity experiments were performed. Pooled serum samples and pooled plasma samples anticoagulated with either sodium citrate, heparin or ethylenediaminetetraacetic acid (EDTA), were 2-fold serial diluted to establish an endogenous measurable concentration over at least 3 dilutions. For each sample dilution, the coefficient of variability (%CV) was calculated. A %CV of ≤ 20 was considered as minimal matrix effects.

Results showed that the %CV over 5 dilutions for respectively sodium citrate, heparin, EDTA and serum were 8.9, 9.5, 12.1 and 8.9, indicating minimal matrix effects. Serum samples yielded at least 3 fold lower levels of sMR when compared to sodium citrate, heparin and EDTA anticoagulated samples (Table 1). Optimal sample dilution factor ranges between 4 and 64 fold. To further assess assay accuracy, spike recovery experiments were performed. A known amount of recombinant sMR was added to serum samples and samples anticoagulated either with sodium citrate, heparin and EDTA. Percentage recovery ((observed [sMR] / expected [sMR])*100%) was calculated and results showed a recovery of > 95% for all spiked samples (data not shown). Subsequently, intra-assay variation (multiple determinations of a single sample in a single test run) and inter-assay variation (multiple determinations of a single sample in several assay runs performed by different operators) was assessed. Also here, %CVs were calculated showing that both inter- and intra-assay variation were < 10%, indicating low variation between both runs and operators (Table 2). The assay was used to quantify sMR concentrations in a small set of samples (n = 7) derived from healthy volunteers. Mean sMR concentration for this small set was 250 ± 37.7 ng/ml.

Table 1
Analysis of matrix effects.

	Sodium citrate	Heparin	EDTA	Serum
Mean (ng/ml)	181	233	226	60
SD	16.2	22.2	27.4	5.4
%CV	8.9	9.5	12.1	8.9

For each sample, linearity of dilutions was determined to assess matrix effects. Samples were 2-fold serial diluted to establish a measurable concentration over at least 3 dilutions. Variability for each serial dilution was assessed by calculating %CV. Presented means and %CV were calculated over a total of 5 dilutions. A %CV of ≤ 20 was considers as minimal matrix effects. Abbreviations: SD = standard deviation, %CV = coefficient of variability, EDTA = ethylenediaminetetraacetic acid.

Table 2
Inter- and intra-assay variation.

		Sample 1	Sample 2	Sample 3	Sample 4
Intra-assay variation					
Operator 1	Mean aliquot 1-3 (ng/ml)	189	228	186	160
	%CV	4.6	3.5	5.5	2.4
Operator 2	Mean aliquot 1-3 (ng/ml)	199	243	198	170
	%CV	2.7	4.3	3.4	2.5
Inter-assay variation					
	Mean Operator 1 & 2 (ng/ml)	194	236	192	165
	%CV	4.5	4.9	5.2	4.2

Intra-assay variation (multiple determinations of a single sample in a single test run) and inter-assay variation (multiple determinations of a single sample in several assay runs performed by different operators) was assessed. To determine intra-assay variation, 4 samples were tested by 1 operator in 1 test run. For each sample, 3 independent aliquots were made and after testing the means and %CV were calculated between aliquots. To assess inter-assay variation, the means and %CV were calculated between the test runs from operator 1 and 2. A %CV < 10% indicates low variation. Abbreviations: %CV = coefficient of variability.

3.3. *sMR levels and prediction of severe disease development in patients with community-acquired pneumonia*

The newly developed assay was used to measure sMR levels in a cohort of 100 patients with community-acquired pneumonia. Next, sMR levels in this cohort were assessed for their ability to predict severe disease development.

Mean value for sMR levels in this cohort of 100 patients was 404 ± 422.8 ng/ml. Comparison of sMR levels between patient groups showed that levels were significantly higher in patients with severe disease development (n = 27) when compared to patients without severe disease development (n = 73) (611 ± 701.8 ng/ml *versus* 311.8 ± 231.2 ng/ml

$p = 0.004$). This study is part of a larger study in which, next to sMR, also the markers WBC count, CRP, PCT and suPAR were assessed for their ability to predict severe disease development [8]. The levels for these infection markers were measured in exactly the same patients as those in which sMR was measured. Also suPAR and PCT showed a significant association with severe disease development ($p = 0.001$ and $p = 0.046$ respectively). In contrast, no correlation between disease severity and the established inflammatory biomarkers WBC count and CRP was observed [8].

In order to calculate the average chance to develop severe disease based upon sMR concentrations, we used a sMR concentration of 430 ng/ml as cut-off for low/high and a concentration > 1000 ng/ml as very high as reported Rødgaard-Hansen and coworkers [6]. Patients with higher sMR levels showed to have a higher average chance to develop severe disease (Fig. 2a). When sMR levels were between 100 and 430 ng/ml, the change to develop severe disease was 22.7%. However, patients with sMR levels between 430 and 1000 ng/ml had an average risk of 33.3% to develop severe disease. In 4 patients with levels above 1000 ng/ml, the chance for severe disease development was 75.0%.

ROC curves analysis was performed for sMR next to the markers suPAR, PCT, CRP and WBC for their ability to predict severe disease progression (Fig. 2b). AUC for sMR is 0.690 in comparison to 0.717 (suPAR), 0.636 (PCT), 0.568 (CRP) and 0.528 (WBC).

Results for suPAR, PCT, CRP and WBC were previously reported [8].

4. Discussion

The main aim of this study was to develop a well-validated

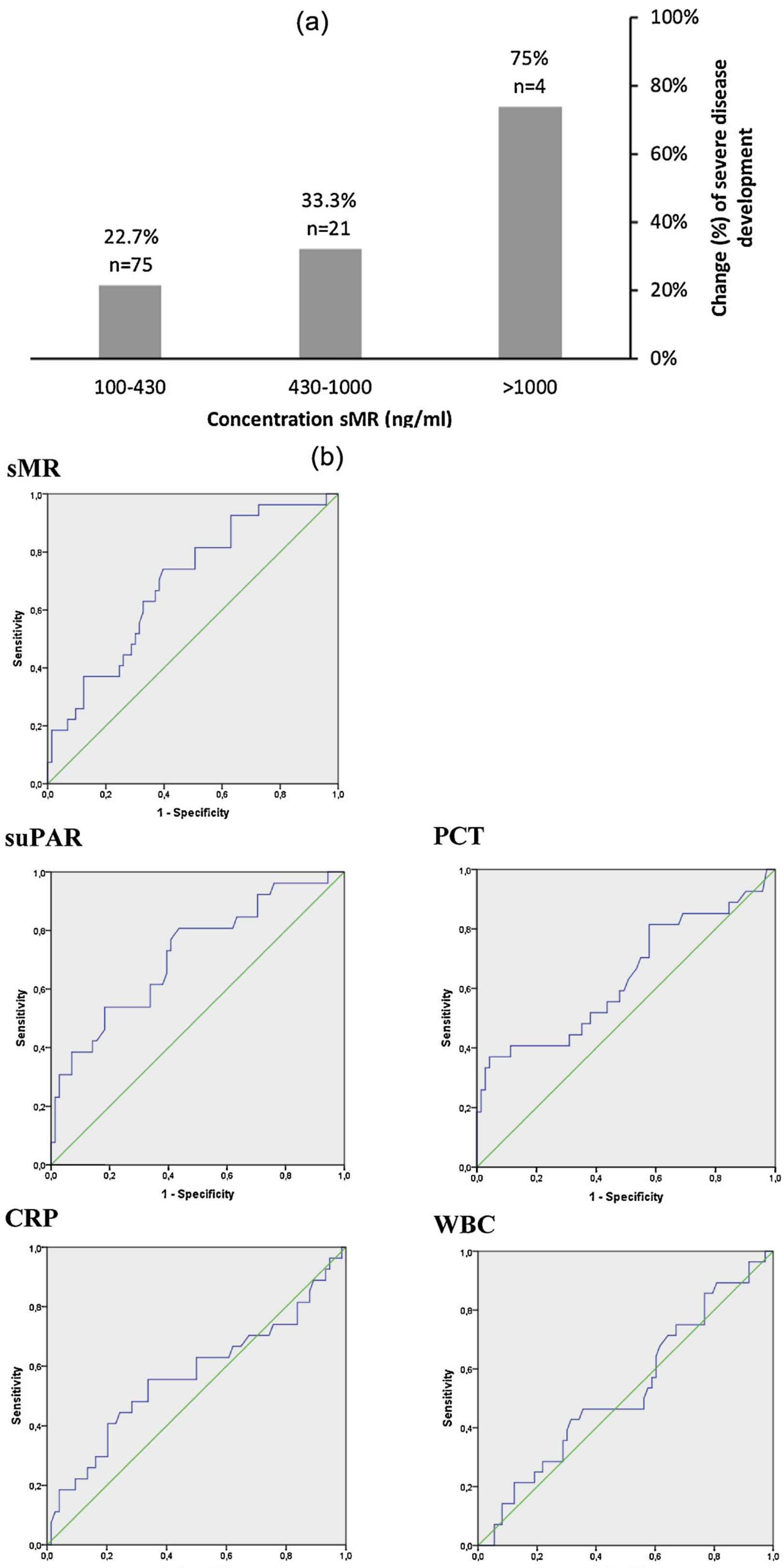

Fig. 2. (a) Different concentrations of sMR and the chance (%) of severe disease development. The number *n* above the bars represent the number of patients in that group. A sMR concentration of 430 ng/ml was used as cut-off for low/high and a concentration > 1000 ng/ml as very high as reported Røgaard-Hansen and coworkers [6]. Abbreviations: sMR = soluble Mannose Receptor. (b) ROC curves. ROC curves analyses were performed for sMR next to the markers suPAR, PCT, CRP and WBC for their ability to predict severe disease progression. AUC for sMR is 0.690 in comparison to 0.717 (suPAR), 0.636 (PCT), 0.568 (CRP) and 0.528 (WBC). AUCs for suPAR, PCT, CRP and WBC were previously reported [8].

sandwich ELISA for the measurement of sMR. Next, we used this assay to measure sMR levels in a cohort of patients with community-acquired pneumonia and investigated whether the obtained levels correlated with disease severity. We developed a new assay that is able to quantify sMR in plasma and serum samples with high accuracy and high reproducibility. Our results indicate that sMR has, next to the established markers WBC count, CRP and PCT, added value for predicting severe disease development in our cohort of patients with community-acquired pneumonia.

Our results are in line with the results reported by Rødgaard-Hansen and coworkers [7]. They explored whether sMR serum concentrations were related to disease outcome in IPD. They reported a significant difference in the median sMR concentrations between survivors (0.71, CI = 0.65-0.82) and non-survivors (1.38, CI = 0.85–1.70), $p < 0.001$. For patients in this cohort that were under the age of 75, sMR serum levels were even higher in non-survivors (median = 1.67, CI = 1.33-2.04) when compared to survivors (median = 0.73, CI = 0.66-0.87), $p < 0.001$. They concluded that sMR is a potential valuable biomarker for severe infections. Another study investigated whether sMR has potential as a biomarker that could be used to discriminate between septic and non-septic patients [9]. They showed that sMR levels were significantly higher in septic patients compared with non-septic patients and healthy controls ($p < 0.001$ for both comparisons) and concluded that sMR is an promising biomarker for sepsis.

Our overall conclusion that PCT, suPAR and sMR are valuable markers to predict disease severity in patients with community-acquired pneumonia, is in line with previously reported results [3,5,7,10]. However, the well-established infection and inflammation markers WBC count and CRP were not found to be associated with severe disease development in our study cohort. These results suggest that these well-established infection markers are less suitable for predicting disease severity when compared to the markers sMR, PCT and suPAR.

Most studies investigating sMR in relation to disease reported corresponding results being that levels are positively correlated with severity status. However, comparing sMR levels between these studies is difficult due to the fact that no well-validated assay for the detection of sMR was available. Standardized interpretation of results is hampered by variation in the used methods for sMR detection. Standardized testing is of utmost importance since it represents true assessment whereby individual performances can be compared to other performances. During this study, a well-validated sandwich ELISA was developed that enables standardized measurement of sMR in human plasma and serum samples. This assay will enable future studies to investigate the role of sMR as a biomarker in other infectious and inflammatory diseases.

Although our initial results regarding sMR as a biomarker for severe disease development in community-acquired pneumonia are promising, this study is limited by its small sample size. In total, 100 patients were evaluated of which 27 developed severe disease. Our initial results should be validated in future studies that include larger patient cohorts.

To conclude, we developed a new sandwich ELISA that is able to quantify sMR in plasma and serum samples with high accuracy and high reproducibility. Our results showed that sMR levels correlated with disease severity in a cohort of 100 patients with community-acquired pneumonia. In contrast, the well-established infection markers WBC count and CRP were not found to be associated with severe disease. We suggest that sMR, next to the markers suPAR and PCT, could have added value for predicting disease severity in community-acquired pneumonia.

Declaration of interest

Sandra Leijtens and Erik J.M. Toonen are employees of Hycult Biotechnology b.v.

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Ethical approval

This study is part of a larger study that was performed at the Jeroen Bosch Hospital in 's-Hertogenbosch, The Netherlands [8]. The local Medical Ethical Committee stated that this study does not needed specific review. Anonymous use of remnant whole blood and plasma samples was approved by the Internal Review Board of the Jeroen Bosch Hospital.

Informed consent

The Internal Review Board of the Jeroen Bosch Hospital approved that data was retrieved from the laboratory information system (LIS) and waived the need for informed consent (study number 2014.11.03.01).

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