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Research paper

# Blood biomarker algorithms for the diagnosis of mycoplasma pneumoniae respiratory infections

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A B S T R A C T

The correct diagnosis of acute infections as to bacteria, mycoplasma or virus is a clinical challenge and has a great impact on the therapeutic decisions. Current diagnostic tests of mycoplasma pneumoniae infections of the respiratory tract such as PCR and serology are either somewhat unreliable or slow and do not entirely meet the clinical needs of accurate and fast diagnosis. The aim of this report was to examine a panel of candidate biomarkers and their capacity to distinguish mycoplasma pneumoniae respiratory infections from respiratory infections caused by either bacterial or virus.

*Method:* Patients with confirmed etiology of their acute respiratory infections ($n$ = 156) were included of which 28 patients were diagnosed with mycoplasma pneumoniae. Blood was taken before any antibiotics treatment and analysed for Azurocidin (HBP), Calprotectin, CRP, Human Neutrophil Lipocalin (HNL), Interferon γ-induced Protein 10 kDa (IP-10), Procalcitonin (PCT), Thymidine Kinase 1 (TK1), TNF-Related Apoptosis-Inducing Ligand (TRAIL).

*Results:* Individually the concentrations of IP-10, TK1 and P-HNL distinguished mycoplasma pneumoniae from bacterial infections with AUCs of 0.79–0.85. However, in combination, TK1 with either IP-10 or P-HNL showed an AUC of 0.97–0.95. In the distinction between mycoplasma pneumoniae and viral respiratory infections CRP, Calprotectin and TRAIL showed individual AUCs of 0.94–0.84. Together with either P-HNL dimer or PCT, CRP showed AUCs of 0.97.

*Conclusion:* Our results indicate that it may be possible to design useful diagnostic algorithms of biomarkers that could help distinguish mycoplasma pneumoniae from respiratory infections caused by bacteria or virus. The development of rapid point-of-care assays based on such algorithms could be clinically useful tools in the therapeutic decision-making.

## 1. Introduction

The correct diagnosis of acute infections as to bacteria, mycoplasma or virus is a clinical challenge and has a great impact on the therapeutic decision. In our previous reports we showed that the measurement of HNL in serum or after in vitro activation is a powerful and accurate biomarker in the distinction between bacterial and viral infections (Venge et al., 2015a; Venge et al., 2019; Venge and Xu, 2019). However, the diagnostic distinction could be improved further by adding biomarkers such as IP-10 or TRAIL to the diagnostic algorithm reaching sensitivities and specificities of between 90 and 95%.(Venge et al., 2019) The diagnosis of mycoplasma pneumoniae infections is fairly complicated and takes several hours to days and involves PCR and serology (Meyer Sauteur et al., 2019). In a recent report from the Bio-X cohort we showed that plasma levels of Calprotectin were elevated in patients with mycoplasma pneumoniae pneumonia and superior to Procalcitonin and Azurocidin as diagnostic biomarkers of the mycoplasma pneumoniae infection and the distinction to viral respiratory infections (Havelka et al., 2020). The aim of this report was to examine the diagnostic distinction between mycoplasma pneumoniae and bacterial or virus infections even further. We applied the same approach as mentioned above i.e. measuring a number of candidate biomarkers for these distinctions and combined the results in the best and hopefully useful diagnostic algorithms. Azurocidin, Calprotectin and HNL originate from circulating neutrophils (Dale et al., 1985; Spitznagel, 1990; Xu et al., 1994), whereas TRAIL (Beyer et al., 2019), IP-10 (Luster and Ravetch, 1987), PCT (Muller and Becker, 2001) and TK1 (Gronowitz et al., 1984) (Jagarlamudi et al., 2020) have several origins. HNL was measured in EDTA plasma by an HNL Dimer specific assay but also after fMLP activation of neutrophils in whole blood.

## 2. Methods

The Bio-X cohort of patients has been described in detail previously (Venge et al., 2015a). In addition to the infected patients 144 healthy controls were recruited with an average age of 43.6 ± 12.8 years consisting of 57 males (age 41.3 years±12.7) and 87 females (age 45.0 years ±12.8).

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Briefly, the inclusion criteria were fever >38 °C and signs and symptoms of acute respiratory infections. The clinical diagnosis of the causes of the infection was complemented with objective microbiological/serological testing. Blood was drawn at their first visit and before start of antibiotics treatment. The study was approved by the ethics committee of Uppsala.

Patients with confirmed etiology of their acute respiratory infection were 156. Of these patients 86 had a bacterial infection, men $n = 41$ (age 54 years IQ range 41–71) women $n = 45$ (age 43 years IQ range 29–66), 42 a viral infection men $n = 23$ (Age 49 IQ range 39–73) women $n = 19$ (Age 40 IQ range 25–57) and 28 mycoplasma, men $n = 14$ (age 42.5 years IQ range 34–61) women n = 14 (age 36.5 years IQ range 22–44). The estimated duration of the mycoplasma infection was 168 h, 130–196 h (median and IQ range) as compared to 96 h (IQ range 64–172 h) for bacterial infections and 89 h (IQ range 53–142 h) for viral infections, respectively.

## 3. Biomarker assays

HNL was measured in whole blood after activation with fMLP as described previously (Venge et al., 2015b) or in EDTA-plasma (Diagnostics Development, Uppsala, Sweden) by an HNL assay configured to specifically catch the dimer of the molecule and subsequently called HNL Dimer. Azurocidin (HBP) (HK352, Hycult Biotech, Uden, Te Netherlands) and Calprotectin ((Gentian AS, Norway) were measured in EDTA-plasma. IP-10 (Invitrogen, Thermo Fisher Scientific), PCT (Termo Fisher Scientifc Frederick, MD, USA), TK1 (Arocell AB, Uppsala, Sweden) and TRAIL (Affymetrix,Thermo Fisher Scientific) were measured in serum. All biomarkers were run according to the manufacturer's instructions for use. Imprecision of duplicate samples were between 4 and 10% CV for all assays. The assays were run blinded to the diagnosis of the patients.

### 3.1. Statistics

Data is expressed as medians and interquartile ranges or full ranges as indicated. Comparisons of groups were performed by the non-parametric Mann-Whitney's test for independent groups or the Kruskal-Wallis ANOVA. The clinical performances of the biomarker assays were estimated by receiver operating characteristics (ROC) analyses. The diagnostic performance of combinations of biomarkers was expressed as Area Under the ROC-curve (AuROC) and calculated by logistic regression analysis.

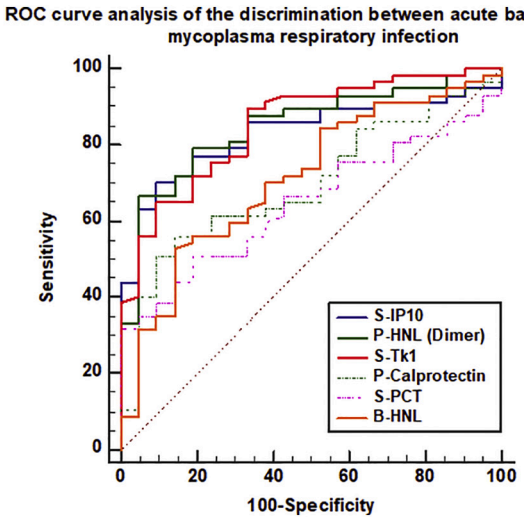
For the calculations of the statistics, MedCalc Statistical Software version 19.1.5 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2020) was used.

## 4. Results

In Table 1 we show the distributions in plasma/serum of the nine biomarkers in patients with acute respiratory infections caused by mycoplasma pneumoniae, bacteria or virus. The actual distributions of the individual results are shown in Fig. 1 a-i in the supplement. As compared to the findings in healthy subjects all biomarkers except S-TRAIL, were significantly elevated in patients with mycoplasma pneumoniae infections. As compared to bacterial infections the concentrations of P-Calprotectin ($p < 0.001$), P-CRP ($p < 0.01$), S-IP-10 ($p < 0.001$), S-TK1 ($p < 0.001$) were elevated in mycoplasma pneumoniae infections, whereas the concentrations of P-HNL Dimer ($p < 0.001$) and B-HNL ($p < 0.001$) were significantly lower. For S-PCT, S-TRAIL and P-Azurocidin no differences in concentrations between mycoplasma pneumoniae and bacterial infections were seen. As compared to viral infections P-Calprotectin ($p < 0.001$), P-CRP ($p < 0.001$), B-HNL ($p < 0.001$), S-TK1 ($p < 0.001$) were elevated in mycoplasma pneumoniae infections whereas S-TRAIL was lower in mycoplasma pneumoniae infections ($p < 0.001$). The concentrations of P-Azurocidin, S-PCT, P-HNL Dimer and S-IP-10 were similar in serum/plasma from patients with viral or mycoplasma pneumoniae infections.

**Table 1**
Biomarker distinction between mycoplasma pneumoniae and bacterial or viral infections and healthy non-infected controls. The non-parametric test Mann Whitney was used for the calculation of statistical differences between groups.

| Biomarker | Healthy<br>Median (IQ range) | Bacterial<br>Median (IQ range) | Viral<br>Median (IQ range) | Mycoplasma<br>Median (IQ range) | Mycoplasma vs<br>Healthy<br>Bacterial<br>Viral |
|---|---|---|---|---|---|
| P-Azurocidin (HBP) | 2.82 μg/L<br>(1.39–4.31) $n = 144$ | 10.1 μg/L<br>(4.7–25) $n = 95$ | 8.5 μg/L<br>(3.5–17) $n = 38$ | 6.1 μg/L<br>(4.3–10) $n = 22$ | P < 0.0001<br>$P = 0.007$<br>P=Ns |
| P-Calprotectin | 0.60 mg/L<br>(0.43–0.91) $n = 143$ | 2.37 mg/L<br>(1.58–3.62) n = 95 | 1.59 mg/L<br>(0.99–2.33) $n = 38$ | 3.42 mg/L<br>(2.79–5.33) $n = 23$ | P < 0.0001<br>$P = 0.0004$<br>$P < 0.0001$ |
| P-CRP | 0.94 mg/L<br>(0.54–1.80) $n = 134$ | 109 mg/L<br>(45–203) $n = 93$ | 35 mg/L<br>(19–54) $n = 38$ | 144 mg/L<br>(108–197) $n = 23$ | P < 0.0001<br>P = 0.01<br>$P < 0.0001$ |
| B-HNL | 96 μg/L<br>(72–127) $n = 98$ | 320 μg/L<br>(211–523) $n = 58$ | 134 μg/L<br>(108–164) $n = 27$ | 218 μg/L<br>(141–281) $n = 22$ | P < 0.0001<br>$P = 0.0015$<br>$P = 0.0003$ |
| P-HNL Dimer | 36 μg/L<br>(31–42) $n = 143$ | 68 μg/L<br>(53–93) $n = 97$ | 42 μg/L<br>(31–58) $n = 38$ | 43 μg/L<br>(37–54) n = 23 | $P = 0.002$<br>$P < 0.0001$<br>P=Ns |
| S-IP-10 | 39 μg/L<br>(16–78) $n = 144$ | 319 μg/L<br>(149–644) $n = 95$ | 1138 μg/L<br>(526–2283) n = 38 | 1039 μg/L<br>(704–1333) n = 23 | P < 0.0001<br>P < 0.0001<br>P=Ns |
| S-Procalcitonin | 0.04 μg/L<br>(0.03–0.06) n = 143 | 0.20 μg/L<br>(0.08–0.67) n = 97 | 0.11 μg/L<br>(0.07–0.21) $n = 37$ | 0.10 μg/L<br>(0.07–0.17) n = 23 | P < 0.0001<br>$P = 0.015$<br>P=Ns |
| S-TK1 | 0.25 μg/L<br>(0.19–0.29) n = 143 | 0.35 μg/L<br>(0.26–0.56) n = 95 | 0.40 μg/L<br>(0.30–0.53) n = 38 | 0.81 μg/L<br>(0.51–1.19) n = 22 | P < 0.0001<br>P < 0.0001<br>$P = 0.0012$ |
| S-TRAIL | 49 ng/L<br>(38–65) n = 144 | 32 ng/L<br>(25–62) n = 95 | 83 ng/L<br>(59–128) n = 38 | 36 ng/L<br>(25–59) n = 22 | $P = 0.014$<br>P=Ns<br>P < 0.0001 |




| Variable | AUC | SE | 95% CI |
|---|---|---|---|
| S-IP10 | 0.85 | 0.038 | 0.77 to 0.91 |
| P-HNL (dimer) | 0.79 | 0.048 | 0.71 to 0.87 |
| S-TK1 | 0.82 | 0.045 | 0.74 to 0.89 |
| P-Calprotectin | 0.70 | 0.057 | 0.60 to 0.78 |
| P-CRP | 0.62 | 0.056 | 0.53 to 0.71 |
| P-Azurocidin | 0.64 | 0.054 | 0.54 to 0.73 |
| S-TRAIL | 0.61 | 0.066 | 0.49 to 0.71 |
| S-Procalcitonin | 0.66 | 0.054 | 0.56 to 0.74 |
| B-HNL | 0.71 | 0.066 | 0.59 to 0.81 |

**Fig. 1.** ROC-curve analysis of the distinction between mycoplasma pneumoniae and bacterial infections.

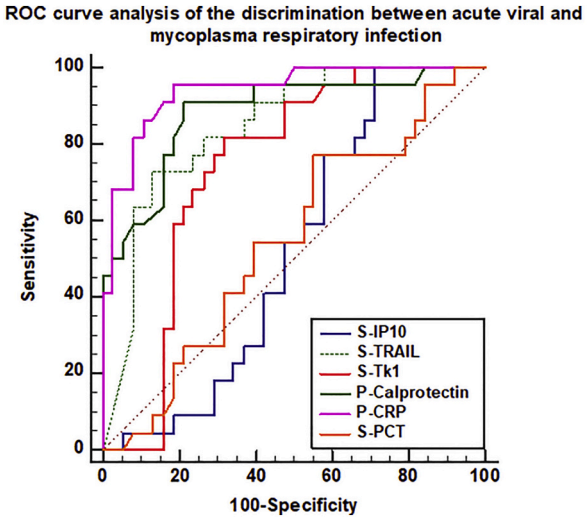
The distinction between mycoplasma pneumoniae respiratory infections from respiratory infections caused by bacteria was estimated by ROC curve analysis. As shown in Fig. 1 all biomarkers but S-TRAIL showed a significant distinction. Three biomarkers, S-IP-10, P-HNL Dimer and S-TK1 stood out with AUCs of 0.79–0.85. In a logistic regression analysis (Fig. 3) these three biomarkers each added significantly to the model and resulted in an AUC of 0.97 with 88% correctly classified cases. In Fig. 4 we eliminated S-IP-10 from the analysis and show a minor reduction in AUC but a percent correctly classified proportion of cases of 90%.

The distinction between mycoplasma pneumoniae respiratory infections and respiratory infections caused by virus is shown in Fig. 2. P-Calprotectin, P-CRP and S-TRAIL showed AUCs of 0.84–0.94. B-HNL and S-TK1 also showed significant distinctions, although at lower levels, AUCs 0.76 and 0.74 respectively, whereas S-IP-10, P-HNL Dimer, P-Azurocidin and S-PCT showed no significant distinction. However, in the logistic regression analysis S-PCT together with P-CRP added significantly to the discrimination (Fig. 5) with an AUC of 0.97 and 93% correctly classified cases. By omission of S-PCT, P-HNL dimer added significantly to the distinction with unaltered AUC of 0.97 and marginally lowered percentage of correctly classified cases, 91.5% (not shown).

Based on the results from the above we constructed ratios between selected biomarkers. For the discrimination between mycoplasma pneumoniae and bacterial respiratory infections the ratio between the results of TK1 and HNL dimer in plasma increased the AUC over either of these biomarkers to 0.90. A ratio between IP-10 and P-HNL dimer did not increase the AUC over IP-10 alone. Previous reports advocated a ratio between CRP and PCT (Neeser et al., 2019) and an AUC of 0.79 was achieved in our cohort for the discrimination between mycoplasma pneumoniae and bacterial infections. However, in the discrimination between mycoplasma pneumoniae and viral respiratory infections the AUCs of the ratios between CRP and PCT or between CRP and P-HNL were similar i.e. 0.93.

## 5. Discussion

The clinical diagnosis of mycoplasma pneumoniae pneumonia from pneumonia caused by bacteria or virus is a challenge and rely currently on clinical signs and symptoms, PCR-based diagnosis and serology (Meyer Sauteur et al., 2019; Meyer Sauteur et al., 2018). Mild mycoplasma pneumoniae pneumonia may progress into severe disease why early detection and treatment may be important. In this report we show the results of nine different blood-based biomarkers and their capacity to distinguish mycoplasma pneumoniae pneumonia from lower respiratory tract infections caused by bacteria or virus. Primarily these nine




| Variable | AUC | SE | 95% CI |
|---|---|---|---|
| S-IP10 | 0.51 | 0.074 | 0.38 to 0.64 |
| P-HNL (dimer) | 0.52 | 0.075 | 0.39 to 0.65 |
| S-TRAIL | 0.84 | 0.051 | 0.72 to 0.92 |
| S-TK1 | 0.74 | 0.065 | 0.61 to 0.84 |
| P-Calprotectin | 0.87 | 0.049 | 0.76 to 0.94 |
| P-CRP | 0.94 | 0.030 | 0.85 to 0.98 |
| S-Azurocidin | 0.53 | 0.074 | 0.40 to 0.66 |
| P-Procalcitonin | 0.55 | 0.077 | 0.41 to 0.68 |
| B-HNL | 0.76 | 0.071 | 0.62 to 0.87 |

**Fig. 2.** ROC-curve analysis of the distinction between mycoplasma pneumoniae and viral infections.

**Blood biomarker discrimination between acute bacterial and mycoplasma respiratory infections**
**Logistic regression analysis model 1** (Enter p<0.01 and remove p>0.05)

| Variable | Coefficient | Std. Error | Wald | P |
|---|---|---|---|---|
| P-HNL (dimer) | 0.1044 | 0.0376 | 7.70 | 0.0055 |
| S-IP-10 | -0.0016 | 0.0006 | 6.36 | 0.0116 |
| S-Tk1 | -9.183 | 3.191 | 8.28 | 0.0040 |
| Constant | 1.386 | 1.885 | 0.54 | 0.462 |

| Variables not included in the model | | |
|---|---|---|
| Calprotectin | Area under the ROC curve (AUC) | 0.97 |
| P-Azurocidin | 95% Confidence interval | 0.90 to 1.00 |
| B-HNL | Per cent correctly classified | 88% |
| S-TRAIL | | |
| P-CRP | Sensitivity: 91% (81-97%) | |
| PCT | Specificity: 80% (56-94%) | |

**Fig. 3.** The logistic regression analysis of the distinction between mycoplasma pneumoniae and bacterial infections. The biomarkers included in the model as independent contributors to the distinction are shown as well as the ROC-curve analysis and sensitivity and specificity of the algorithm.

**Blood biomarker discrimination between acute bacterial and mycoplasma respiratory infections**
**Logistic regression analysis model 2, no IP-10** (Enter p<0.01 and remove p>0.05)

| | Coefficient | Std. Error | Wald | P |
|---|---|---|---|---|
| P-HNL (dimer) | 0.101 | 0.031 | 10.72 | 0.0011 |
| S-Tk1 | -7.971 | 2.44 | 10.61 | 0.0011 |
| Constant | -0.256 | 1.455 | 0.031 | 0.860 |

| Variables not included in the model | | |
|---|---|---|
| S-Calprotectin | Area under the ROC curve (AUC) | 0.95 |
| P-Azurocidin | 95% Confidence interval | 0.87 to 0.99 |
| B-HNL | Percent correctly classified | 90% |
| S-TRAIL | | |
| P-CRP | Sensitivity: 96% (88-100%) | |
| S-PCT | Specificity: 71% (48-89%) | |

**Fig. 4.** The logistic regression analysis of the distinction between mycoplasma pneumoniae and bacterial infections. The biomarkers included in the model as independent contributors to the distinction are shown as well as the ROC-curve analysis and sensitivity and specificity of the algorithm. In this analysis the results of IP-10 were omitted.

biomarkers were chosen for their potential diagnostic value in the distinction between bacterial and viral infections, but during the study it became obvious that they might also be used in the diagnosis of mycoplasma pneumoniae infections. Thus, in the distinction to bacterial infections three of these biomarkers seemed of particular interest. Those were IP-10, TK1 and the Dimeric form of HNL. The concentrations of IP-10 and TK1 were raised in mycoplasma pneumoniae infections which added to their diagnostic performance. This was contrasted by HNL Dimer that was lower in mycoplasma pneumoniae as compared to bacterial infections. However, in the logistic regression analysis all three biomarkers added significantly to the diagnostic performance resulting in a very high AUC and a percent correctly classified number of patients of 88%. IP-10 was the biomarker that contributed the least, which made us test the performance in the absence of this biomarker. The omission of IP-10 did not change the diagnostic performance, which means that the combination of only two biomarkers was able to correctly classify 90% of the patients. The sensitivities of the algorithms were > 90% for both, but the specificities were lower. Similar to a recent report we constructed ratios between selected biomarker (Neeser et al., 2019). Thus, the ratio between TK1 and P-HNL dimer seemed to be the superior diagnostic ratio for the discrimination between mycoplasma pneumoniae and bacterial infections and in line with the results of the logistic regression. This was contrasted by the high diagnostic performance of the CRP/PCT ratio in the discrimination between mycoplasma pneumoniae and viral infections. These latter results support previous findings in the discrimination between mycoplasma pneumoniae and viral infections (Neeser et al., 2019). However, the diagnostic performance as judged by AUC was no better for the ratio as compared to CRP alone. The

**Blood biomarker discrimination between acute viral and mycoplasma respiratory infections**
**Logistic regression analysis. Model 1** (Enter p<0.01 and remove p>0.05)

| Variable | Coefficient | Std. Error | Wald | P |
|---|---|---|---|---|
| S-PCT | -14.63 | 6.52 | 5.03 | 0.02 |
| P-CRP | 0.055 | 0.0136 | 16.4 | 0.0001 |
| Constant | -3.44 | 1.048 | 10.7 | 0.0010 |

**Variables not included in the model**

P-Calprotectin
P-Azurocidin
S-Tk1
S-TRAIL
S-IP10
P-HNL (dimer)
B-HNL

| | |
|---|---|
| **Area under the ROC curve (AUC)** | **0.97** |
| 95% Confidence interval | 0.91 to 1.00 |
| **Percent of cases correctly classified** | **93%** |

Sensitivity: 86% (65-97%)
Specificity: 96% (85-99%)

**Fig. 5.** The logistic regression analysis of the distinction between mycoplasma pneumoniae and viral infections. The biomarkers included in the model as independent contributors to the distinction are shown as well as the ROC-curve analysis and sensitivity and specificity of the algorithm.

obvious interpretation of these findings is that CRP is increased also in bacterial infections, but PCT only in bacterial infections and not in mycoplasma pneumoniae infections. The highly raised CRP does not by itself indicate mycoplasma pneumoniae, but has to be complemented by a biomarker that is unaltered by mycoplasma pneumoniae such as PCT. In this regard the ratio between CRP and P-HNL dimer served the same purpose with an AUC of 0.93 i.e. similar to the AUC of CRP/PCT. These diagnostic performances are quite interesting, since they lend themselves to the development of assays with short response times of <15 min. The caveat though is the low number of patients included in this study. Thus, the diagnostic performances of the algorithms have to be confirmed in additional studies. Another limitation is the absence of children in our study cohort. However, one strength of this study was the fact that all patients were antibiotics naïve i.e. all blood samples were obtained at first visit and before any antibiotics was given.

In a previous report we showed that Calprotectin distinguished mycoplasma pneumoniae pneumonia from viral causes of lower tract infections (Havelka et al., 2020). In the present report we show that TRAIL has a similar performance, but that CRP showed the best performance. However, it needs to be emphasized that the results of CRP may be biased since this biomarker was known to the adjudicator. In the logistic regression analysis, the performance of CRP was supported by PCT resulting in very high accuracy. Thus, the complete absence of any difference in the PCT levels between mycoplasma pneumoniae and viral infection in combination with very high concentrations of CRP gave rise to an impressive specificity of 96% in combination with a decent sensitivity of 86%. Thus, comparing the best diagnostic algorithms for diagnosing mycoplasma pneumoniae infection and distinguish this infection from either bacterial or viral infection, the biomarker combinations are different. In the case of distinction mycoplasma pneumoniae from bacterial infections we see very high sensitivities, which means that very few patients with mycoplasma pneumoniae infections are missed but at the expense of specificity. For the distinction between mycoplasma pneumoniae and viral infections specificity is very high which means that a patient with symptoms from the respiratory tract and very high CRP in combination with normal or only slightly raised PCT or P-HNL dimer most likely suffers from a mycoplasma pneumoniae infection. In cases with bacterial infections high CRP concentrations are also common findings, but in such cases other biomarkers such as HNL are needed to confirm the diagnosis (Venge et al., 2015a).

This limited study has clearly indicated a way forward in the development of rapid biomarker assays for the diagnosis of acute respiratory infections as to their bacterial, mycoplasma pneumoniae or viral causes. The distinction between bacterial and viral respiratory infections has been well documented and involves HNL with an accuracy by its own of about 90% (Venge and Xu, 2019). In this report we show that the combination of plasma concentrations of the dimeric form of HNL in combination with TK1 has an accuracy of 84% in the distinction of bacterial from mycoplasma pneumoniae infections. For the distinction of mycoplasma from viral infections, the combination of CRP and PCT resulted in an accuracy of 91%.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2020.112908.

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