Journal of Immunological Methods 474 (2019) 112627

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

HNL (Human Neutrophil Lipocalin) and a multimarker approach to the distinction between bacterial and viral infections

Per Venge[a,c,*], Ann-Katrin Eriksson[c], Sofia Holmgren[c], Lena Douhan-Håkansson[a], Christer Peterson[a,c], Shengyuan Xu[a,c], Staffan Eriksson[d], Daniel Garwicz[a], Anders Larsson[a], Karlis Pauksen[b]

[a] Department of Medical Sciences, Clinical Chemistry, Uppsala University, Uppsala, Sweden
[b] Department of Medical Sciences, Infectious Disease, Uppsala University, Uppsala, Sweden
[c] Diagnostics Development a P&M Venge AB company, Uppsala, Sweden
[d] Arocell AB, Uppsala, Sweden

A B S T R A C T

Objectives: The distinction between bacterial and viral causes of acute infections is a major clinical challenge. In this report we investigate the diagnostic performance in this regard of nine candidate biomarkers together with HNL (Human Neutrophil Lipocalin).

Methods: Blood was obtained from patients with symptoms of infectious (n = 581). HNL was measured in whole blood (B-HNL) after pre-activation with the neutrophil activator fMLP or in plasma (P-HNL). Azurocidin also known as heparin-binding protein (HBP), Calprotectin, PMN-CD64, CRP (C-reactive protein), IP-10 (Interferon γ-induced Protein 10 kDa), PCT (Procalcitonin), TK1 (Thymidine kinase 1), TRAIL (TNF-related apoptosis-inducing ligand) were measured in plasma/serum. Area under the ROC (receiver operating characteristics) curve (AuROC) was used for the evaluation of the clinical performance of the biomarkers.

Results: Side-by-side comparisons of the ten biomarkers showed large difference in the AuROC with B-HNL being the superior biomarker (0.91, 95% CI 0.86–0.95) and with the other nine biomarkers varying from AuROC of 0.63–0.79. The combination of B-HNL with IP-10 and/or TRAIL increased the diagnostic performance further to AuROCs of 0.94–0.97. The AuROCs of the combination of CRP with IP-10 and/or TRAIL were significantly lower than combinations with B-HNL 0.87 (95% CI 0.83–0.91).

Conclusion: The diagnostic performance of whole blood activated HNL was superior in the distinction between bacterial or viral infections. The addition of IP-10 and/or TRAIL to the diagnostic algorithm increased the performance of B-HNL further. The rapid analysis of HNL, reflecting bacterial infections, together with biomarkers reflecting viral infections may be the ideal combination of diagnostic biomarkers of acute infections.

1. Introduction

The rapid and accurate distinction between the causes of respiratory infections is a major clinical need in order to reduce the misuse of antibiotics (Hopstaken et al., 2005; Dupuy et al., 2013; Quenot et al., 2013; Laxminarayan et al., 2013). Currently the measurements of white blood cell numbers and/or biomarkers such as CRP (C-reactive protein) are widely used to assist the doctor in this distinction. It is, however, well documented that neither of these measures, although rapid, are sufficiently sensitive and specific to satisfy these needs. HNL (Human Neutrophil Lipocalin) is a protein stored in and released from the secondary granules of human neutrophils. In our previous studies we showed that the measurements of HNL in serum or after activation of the neutrophils in whole blood might fulfill the unmet clinical needs since they showed superior sensitivities and specificities as compared to the contemporary biomarkers, but also to some of the more recent biomarkers such as Procalcitonin and the expression of CD64 in neutrophils (Yu et al., 2016; Venge et al., 2015a; Venge et al., 2015b; Xu et al., 1995). However, many other biomarkers have recently been proposed to be used for the distinction between acute infections caused by either bacteria or virus. Some of these biomarkers originate, as HNL, from circulating neutrophils i.e. Azurocidin (Heparin Binding Protein, HBP) (Linder et al., 2009a) and Calprotectin (Sander et al., 1984). Others, such as TRAIL (TNF-Related Apoptosis-Inducing Ligand) (Oved et al., 2015a), IP-10 (Interferon γ-induced Protein 10 kDa) (Hayney et al., 2017) and TK1 (Thymidine kinase 1) (Kumar et al., 2016a) have several cellular origins, but been proposed as useful biomarkers in this regard.

The aim of this report was to compare the measurements of HNL side-by-side with several potentially useful biomarkers for their potency in the distinction between bacterial and viral causes of acute infections, but also to combine these biomarkers in order to find the most powerful

* Corresponding author at: Department of Clinical Chemistry, University Hospital, SE-75185 Uppsala, Sweden.
E-mail address: per.venge@medsci.uu.se (P. Venge).

https://doi.org/10.1016/j.jim.2019.06.018
Received 31 January 2019; Received in revised form 19 June 2019
Available online 23 June 2019

algorithm for this distinction.

2. Methods

The total study cohort included 725 participants. Patients with signs and symptoms of acute infections were 253 males (age 52.7 ± 20.0 years ±SD) and 328 females (age 46.4 ± 19.3 years). The 144 healthy controls i.e. subjects without apparent infection and other disease, had an average age of 43.6 ± 12.8 years and consisted of 57 males (age 41.3 years ± 12.7) and 87 females (age 45.0 years ± 12.8).

In total 581 patients with signs and symptoms of acute infections i.e. infectious symptoms of a duration of < one week, were recruited as described previously (Venge et al., 2015b; Venge et al., 2015a). The type and location of the infections was also described previously. Inclusion criteria were fever > 38 °C and signs and symptoms of acute infection. Known to the adjudicator were in addition to clinical findings, CRP, white blood cell counts, X-ray and all microbiological test results. Based on this information patients were judged to have either bacteria or virus as their causing infectious agent. Thus, in 288 of the patients a microbiological confirmation of the clinical diagnosis was achieved and in these patients the diagnosis as to bacterial or viral infections as well as to etiology were regarded "certain" and confirmed. Exclusion criteria were known chronic viral infection, such as human immunodeficiency virus infection and patients on antibiotics treatment. In addition, children under the age of 18 years and patients that could not give informed consent were excluded from this study. The patients were admitted to the infectious disease department at the University Hospital in Uppsala or to a primary care unit in Uppsala during a two-year period and included consecutively. All patients were evaluated blinded to the biomarker results by the same adjudicator (KP). In cases of uncertainty of the clinical diagnosis a second opinion from the infectious specialist in charge of the patient was obtained. Radiological findings were based on the analysis of a radiologist. The adjudication was performed without the knowledge of the biomarker results. Blood was drawn before start of antibiotics treatment. The study was approved by the ethics committee of Uppsala.

Patients with confirmed etiology of their acute infection were 288 (49.6% of all patients) (Venge et al., 2015a). Of these patients 185 had a bacterial infection (101 females, age median 44 years, range 18–87 years, 84 males, age median 59 years, range 18–90 years), 54 a viral infection (27 females, age median 41, range 19–88 years, 27 males age median 50, range 24–92 years), 26 mycoplasma infection and 23 bacterial infection as a secondary infection to Influenza. Patients with mycoplasma infection or bacterial infection secondary to a viral infections were not included in the calculations of this report. Patients with symptoms of infections, but in whom a confirmed etiology could not be established, were excluded from all calculations.

3. Biomarker assays

HNL was measured in whole blood after activation with fMLP (N-formyl-met-leu-phe) as described previously (Venge et al., 2015b). Briefly, whole blood was incubated for 20 min at 37 °C with fMLP 5×10^{-8} M. The activation process was terminated by adding ice-cold EDTA and the plasma harvested by centrifugation. HNL was measured in plasma by ELISA. HNL was also measured in EDTA-plasma without prior activation of whole blood (Diagnostics Development, Uppsala, Sweden).The expression of CD64 on polymorphonuclear leukocytes was evaluated by flow cytometry as described (Fjaertoft et al., 2005b). CRP was measured in plasma by the routine department of clinical chemistry at the University Hospital. Azurocidin (HBP i.e. Heparin Binding Protein) (Hycult Biotech, Uden, The Netherlands) and Calprotectin (Gentian Diagnostics, Moss, Norway) were measured in EDTA-plasma. IP-10 (Invitrogen, Thermo Fisher Scientific), Procalcitonin (Thermo Fisher Scientific), TK1 (Arocell AB, Uppsala, Sweden) and TRAIL (Affymetrix, Thermo Fisher Scientific) were measured in serum. Whole blood, EDTA-plasma and serum were obtained simultaneously. After 30 min at room temperature plasma and serum were obtained by centrifugation and stored frozen (-70 °C) until analysis. All biomarkers were run according to the manufacturer's instructions for use. Imprecision of duplicate samples were between 4 and 10% CV (Coefficient of Variation) for all assays.

4. Statistics

Data was expressed as medians and interquartile ranges or full ranges as indicated. Comparisons of groups i.e. healthy controls, patients with confirmed bacterial infection and patients with confirmed viral infection, were performed by the non-parametric Mann-Whitney's test for independent groups. In order to estimate the clinical performances of the biomarker assays receiver operating characteristics (ROC) analyses were performed and comparisons of areas under the curves analyzed by c-statistics. Youden index J was calculated by the formula: J = max [Sensitivity + Specificity – 1] and used to estimate the optimal discriminatory concentration of the biomarker. The diagnostic performance of combinations of biomarkers was expressed as Area Under the ROC-curve (AuROC) and calculated by logistic regression analysis. In the logistic regression analysis patients with confirmed bacterial infection were included as positive cases and patients with confirmed viral infections included as negative cases. Logistic regression analysis was performed in either of two ways. One was entering all variables (biomarkers) into the model and the other using the procedure of backward exclusion of variables. The criteria of entering variables was if $p < .05$ and remove if $p > .1$.

For the calculations of the statistics, MedCalc Statistical Software version 18.2.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018) was used.

5. Results

In Figs. 1-10 we show the distributions of the biomarker concentrations in blood, serum or plasma in patients with confirmed etiology of their acute infections in comparison to the results of healthy references. It is seen from these results that the biomarkers were raised in bacterial infections in comparison to the results of healthy references. However, one exception was the findings with TRAIL, since the results of TRAIL in bacterial infections were significantly lower than the healthy references. Most biomarkers were raised in bacterial infections as compared to viral infections. However, the results of IP-10, TK1 and TRAIL were significantly higher in viral infections as compared to bacterial infections (Supplemental Table 1).

In the next step the diagnostic power of the individual biomarkers was evaluated by ROC-curves in the distinction between bacterial and viral causes of the acute infection (Fig. 11). All biomarkers had a significantly raised AuROC. However, the AuROCs varied between 0.63 and 0.91. TK1, HBP and PCT were the three biomarkers with lowest AuROC. The three neutrophil biomarkers Calprotectin, CD64 and P-HNL had intermediate AuROCs between 0.70 and 0.72, whereas TRAIL and IP-10 had higher AuROCs of 0.79. CRP with an AuROC of 0.81 is shown for comparison keeping in mind that the results of this biomarker was known to the adjudicators. The highest AuROC was seen for whole blood HNL after activation (0.91, 95% CI 0.86–0.95). The AuROC of B-HNL was significantly larger than for any of the other biomarkers ($p = .003$-$p < .0001$). The optimal cut-offs for the individual biomarkers were calculated by the Youden index and shown in supplemental Table 2.

In order to investigate the diagnostic performance of combinations of biomarkers, a logistics regression analysis was performed including all biomarkers. As entered in the model B-HNL ($p < .0001$), IP-10 ($p = .0059$) and TRAIL ($p = .0130$) added significantly and independently to the discrimination between bacterial and viral infections with an AuROC of 0.95 (95%CI 0.91–0.98) (Table 1 and

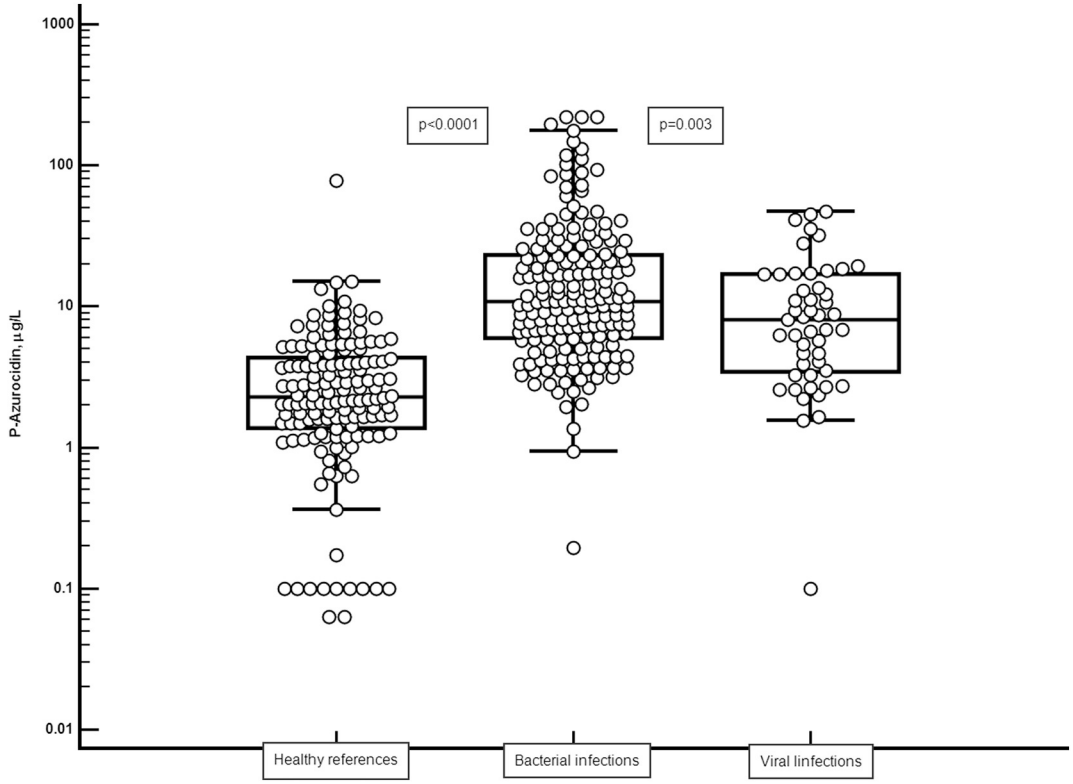

Fig. 1. In Fig. 1 the concentrations of Azurocidin (Heparin binding protein) in plasma are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

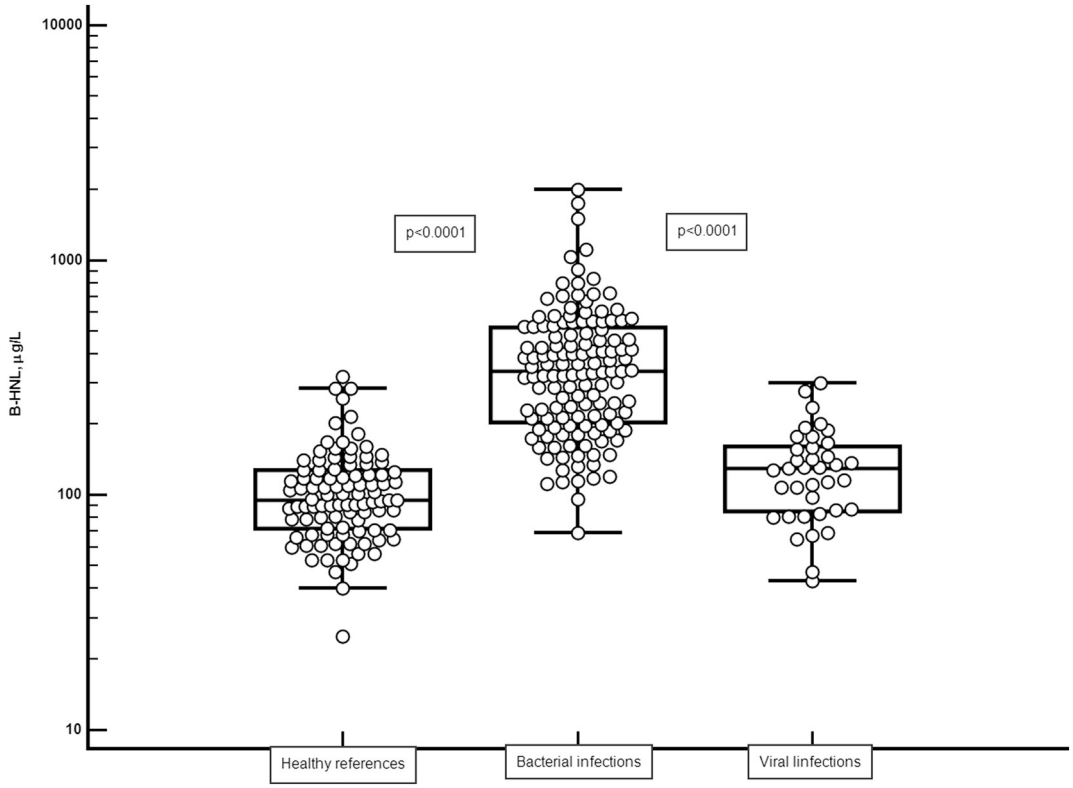

Fig. 2. In Fig. 2 the concentrations of HNL in whole blood after activation are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

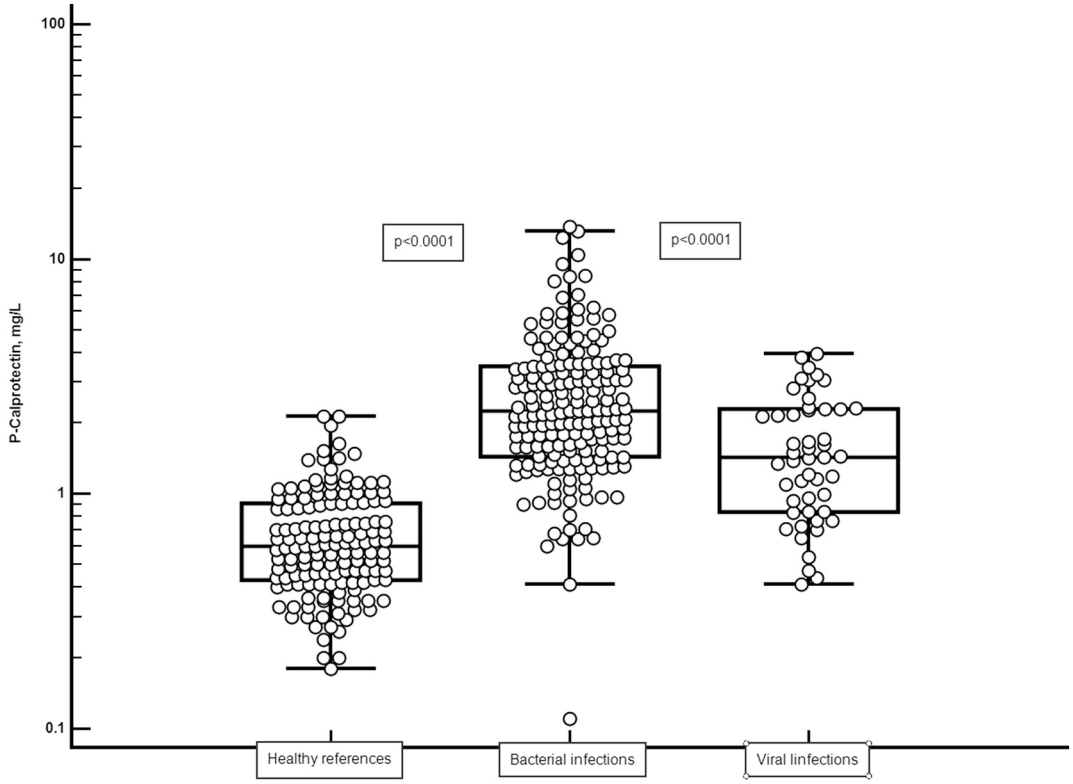

Fig. 3. In Fig. 3 the concentrations of Calprotectin in plasma are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

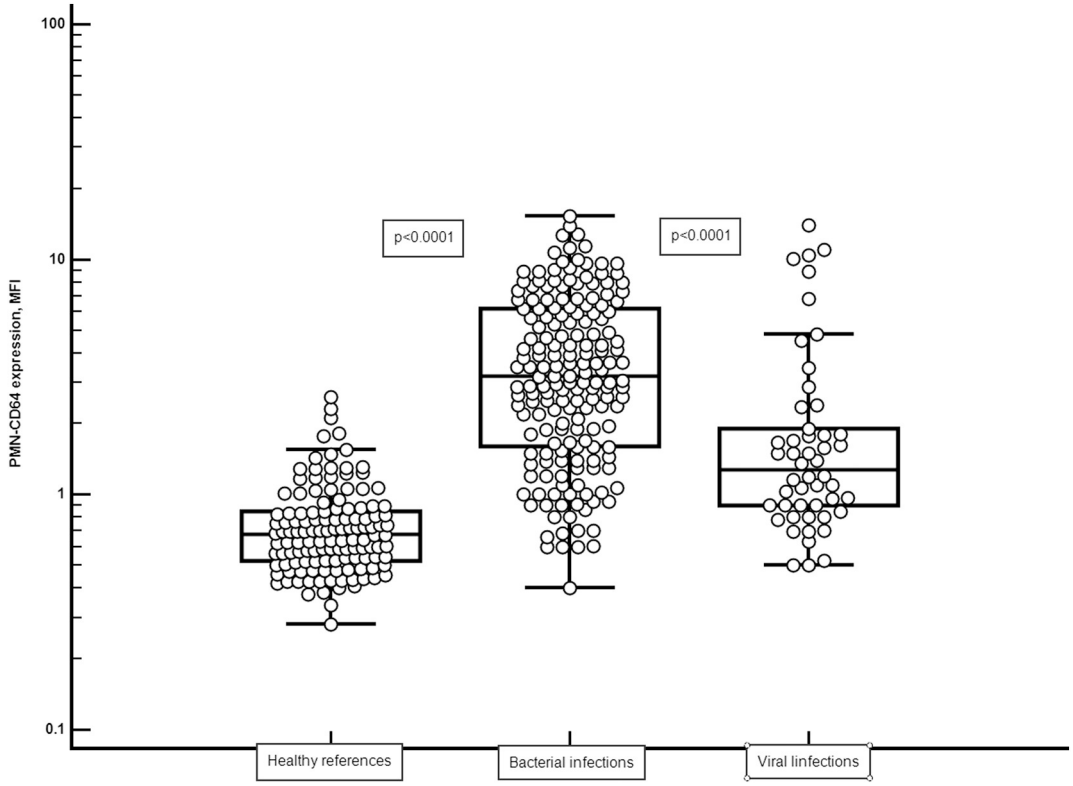

Fig. 4. In Fig. 4 expressions of CD64 on polymorphonuclear leukocytes (PMN) are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

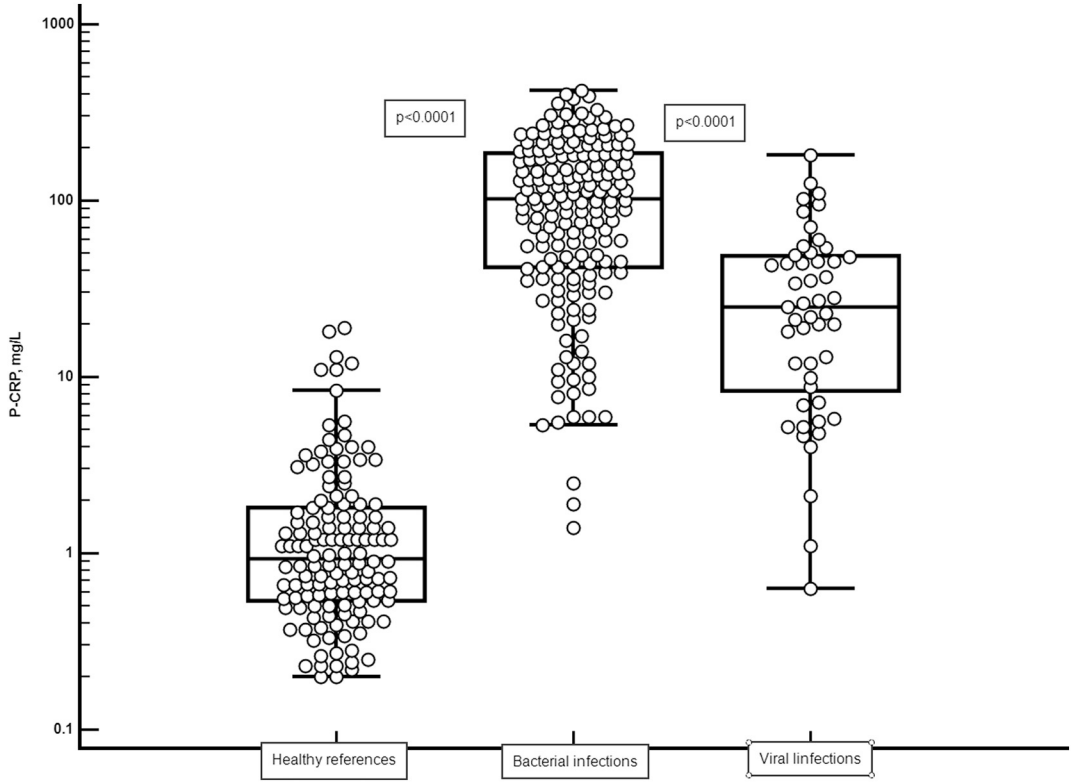

Fig. 5. In Fig. 5 the concentrations of CRP in plasma are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

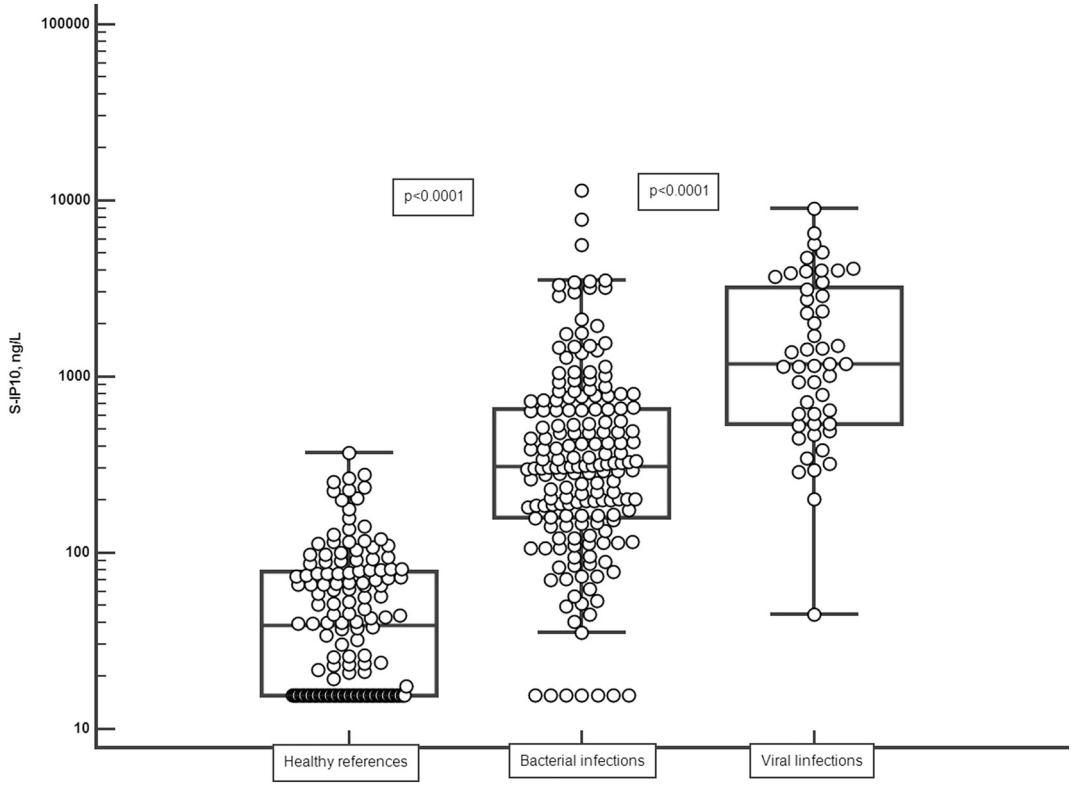

Fig. 6. In Fig. 6 the concentrations of IP-10 in serum are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

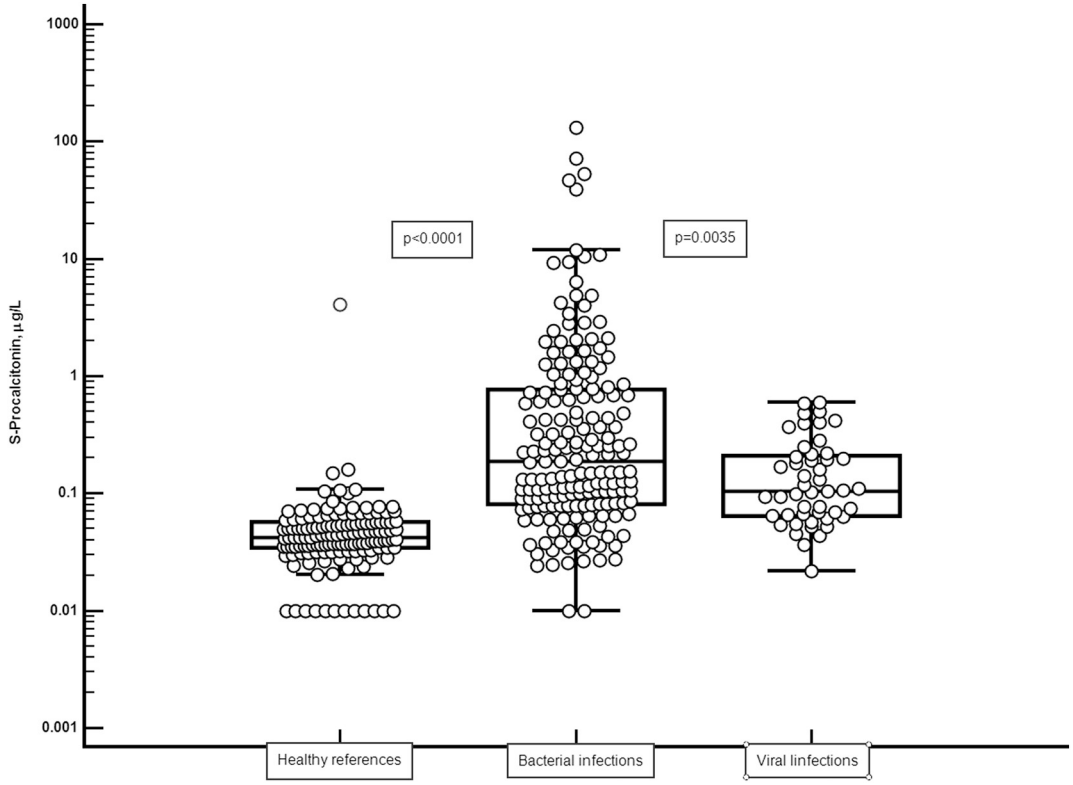

Fig. 7. In Fig. 7 the concentrations of Procalcitonin in serum are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

Supplemental Table 3). When patients with respiratory infections were evaluated separately only B-HNL (p = .0038) and TRAIL (p = .0012) (Table 2) added independently to the discrimination between bacterial and viral causes of their infections.

By the exclusion of the whole blood assays i.e. B-HNL, CD64 expression on neutrophils and blood neutrophil counts (B-PMN) and only calculating on plasma/serum biomarkers except CRP an AuROC of 0.86 (95% CI 0.81–0.90) was achieved. In this model Calprotectin (p = .004), PCT (p = .02), TK1 (p = .008) and IP-10 (p = .0001) contributed independently (supplemental Table 4a). When CRP was added the AuROC increased, however non-significantly, to 0.90 (95% CI 0.86–0.94) and with CRP as the most powerful biomarker (p < .0001) together with TK1 (p = .01), PCT (p = .03) and IP-10 (p = .0001) as significant contributors to the discrimination between bacterial and viral causes of the infections (supplemental Table 4b).

Fig. 12 shows the comparison of AuROCs of some combinations of biomarkers with the greatest potential. As can be seen the addition of IP-10 and/or TRAIL to CRP increased the AuROC from 0.81 (0.76–0.86, 95%CI) to 0.87 (95%CI 0.83–0.91), p = ns. However, the AuROCs were similar whether either IP-10 or TRAIL was added. The addition of CRP to HNL affected marginally the AuROC of HNL alone (0.91, 0.86–0.95 vs 0.92, 0.88–0.96 95%CI), whereas the addition of IP-10 and TRAIL to HNL increased the AuROC significantly (0.96, 0.92–0.99 95% CI), p = .03. The AuROC of this latter combination was the same as the AuROC with all 10 biomarkers included in the model. However, when B-HNL was omitted from the model i.e. only including plasma/serum biomarkers, the AuROC was significantly reduced (0.98, 0.94–0.99 vs 0.90, 0.86–0.94, 95%CI) p = .002. The AuROCs of B-HNL and TRAIL or IP-10 were the same i.e. 0.94 (0.89–0.07, 95% CI).

Several of the biomarkers were correlated to each other. A correlation matrix is shown in the supplemental Table 5. The neutrophil associated biomarkers were significantly and positively correlated to each other and to CRP, whereas the correlations of these biomarkers with IP-10 and TRAIL were significant but negative. IP -10 was significantly and positively correlated to TK1 and TRAIL.

6. Discussion

In our previous reports we showed that HNL in serum (Yu et al., 2016; Xu et al., 1995; Bjorkqvist et al., 2004) or as measured after activation of whole blood (Venge et al., 2015b; Venge et al., 2015a) was superior to any other known biomarker, such as procalcitonin in plasma or CD64 expression on neutrophils, in the distinction between bacterial or viral causes of acute infections. Such data makes HNL a very interesting candidate for the future management of acute infections in order to avoid the abuse of antibiotics (Laxminarayan et al., 2013; Dupuy et al., 2013). However, the most widely used biomarker in the management of acute infections is currently CRP and CRP has indeed been a valuable tool in the management of patients with acute infections (Hopstaken et al., 2003). However, it is also well known that CRP will react to almost any process in the body that involves inflammation, which makes CRP quite unspecific. In our Bio-X study CRP was known to the adjudicator and used to classify patients into those likely having a bacterial or viral cause of their infection. In this study we evaluated an even larger panel of potential candidate biomarkers for the distinction between bacterial or viral causes of acute infections. The goal was to achieve the best possible discriminator of the two causes of infections. It was therefore of great interest that the combination of our whole blood assay of the activation of blood neutrophils to release their specific protein HNL and a couple of other biomarkers in serum made this distinction quite accurate, since we achieved areas under the ROC curves of close to 100%. This was true whether we evaluated our whole cohort of verified infections or restricted the evaluation to those patients who presented with respiratory disease (data not shown). A distinction to this magnitude has only been achieved in our previous studies on serum measurement of HNL (Xu et al., 1995; Yu et al., 2016).

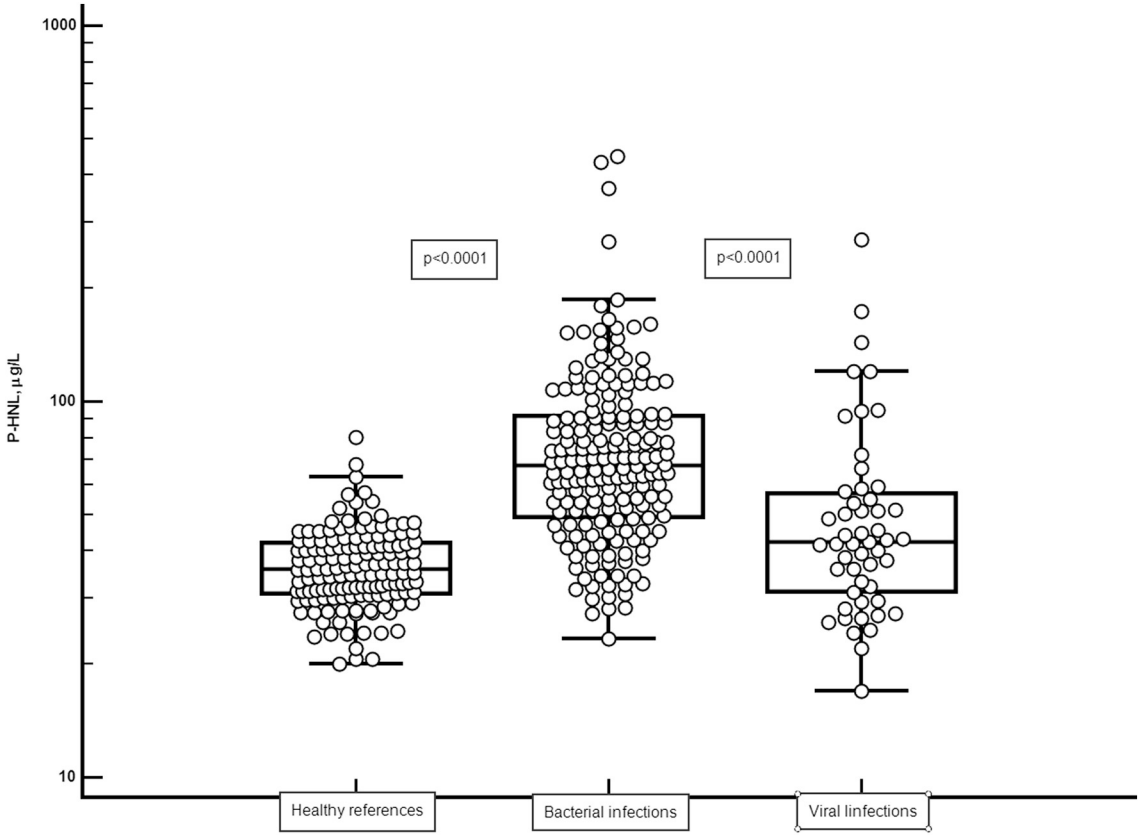

Fig. 8. In Fig. 8 the concentrations of HNL in plasma are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

However, as discussed before serum measurements put demanding requirements on the end-user, since the results are dependent on length of coagulation and ambient temperature. The biomarkers that seemed to complement B-HNL most strongly were IP-10 (Interferon γ-induced Protein 10 kDa) and TRAIL (TNF-Related Apoptosis-Inducing Ligand) and indeed these biomarkers have recently shown to be interesting discriminators in these diagnoses and in particular in combination with CRP (Oved et al., 2015b). As shown in this study both IP-10 and TRAIL have diagnostic potentials as individual biomarkers and are as such somewhat superior to any of the other biomarkers except B-HNL. We therefore evaluated the combination of these biomarkers with several other candidate biomarkers. Disappointedly we could not repeat what has been shown recently, since the combination of the three biomarkers CRP, IP-10 and TRAIL did not perform significantly better than either of these alone. It was not until we combined the results of these two latter biomarkers with the results of B-HNL, that we revealed their real potential. B-HNL reflects the response of the body to a bacterial challenge and IP-10 and TRAIL also reacts to viral infections. Indeed, the concentrations of TRAIL were even lower during bacterial infections than those seen in healthy non-infected controls. Thus, the addition to the specific bacterial biomarker of two biomarkers with a viral profile seemed very successful.

The other biomarkers tested did not as individual biomarkers show any promise as powerful diagnostic means to distinguish bacterial from viral infections. As discussed before PCT did not add much to the diagnostic distinction between bacterial and viral causes of acute infections (Ip et al., 2007; Toikka et al., 2000; Le et al., 2015) although numerous publications have advocated PCT as a tool to manage antibiotics treatment of such patients (Muller and Becker, 2001; Meili et al., 2015; Drozdov et al., 2013). Possibly the severity of infection in our patients was less than in many of those studies, although our previous results on sepsis also showed the diagnostic superiority of HNL over PCT (Martensson et al., 2013). Likewise, HBP (Azurocidin), a protein secreted from the primary granules and secretory vesicles of neutrophils and advocated for the diagnosis and monitoring of sepsis (Linder et al., 2009b) did not have the capacity to distinguish between bacterial and viral infections. Nor did HBP show up in any of our logistic regression analysis as a significant contributor to this distinction. Calprotectin is a well know biomarker of inflammatory bowel disease and the measurements of this protein in feces is included in the management guidelines (Fagerhol, 2000). However, originating from sequestered neutrophils Calprotectin has also been analyzed in serum/plasma in a number of diseases, including infections, with variable success (Jonsson et al., 2017). Our results showed a moderate, but significant capacity of Calprotectin to distinguish between bacterial and viral acute infections. This capacity was most obvious when we excluded CRP from our calculations in the logistic regression analysis. As shown Calprotectin and CRP are highly correlated, which may explain these results.

Thymidine kinase (TK1) originates from all dividing cells and is used to monitor cancer cell growth (Kumar et al., 2016b) but was also shown in previous reports to be increased in viral infections (Gronowitz et al., 1984). As an individual biomarker TK1 did not have the capacity to distinguish between bacterial and viral infections. However, in the logistic regression analysis in which only plasma/serum biomarkers were analyzed TK1 contributed significantly to this distinction. Plasma HNL was shown in this report for the comparison with B-HNL. As such plasma HNL is clearly inferior to B-HNL. However, in the logistic regression analyses in the absence of B-HNL, plasma HNL contributed significantly. Indeed, we showed in previous studies on sepsis that plasma HNL was a better diagnostic biomarker than CRP and PCT in the discrimination between SIRS (Systemic Inflammatory Reaction Syndrome) and sepsis (Martensson et al., 2013).

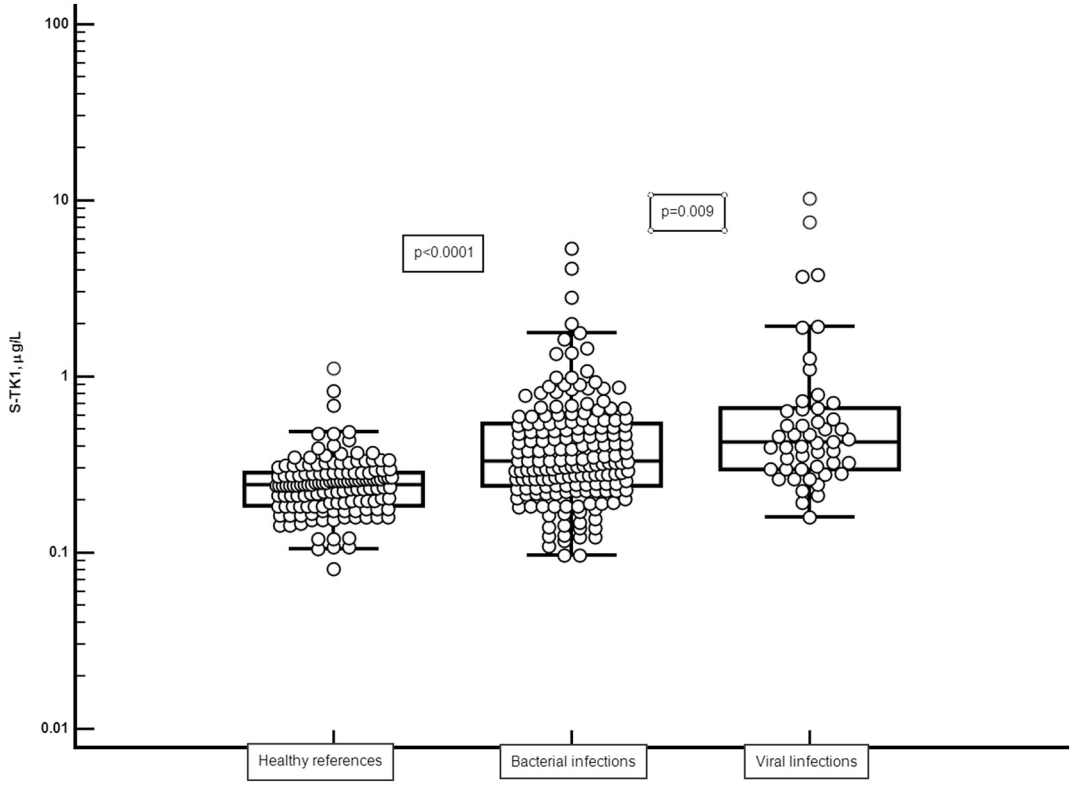

Fig. 9. In Fig. 9 the concentrations of TK1 in serum are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

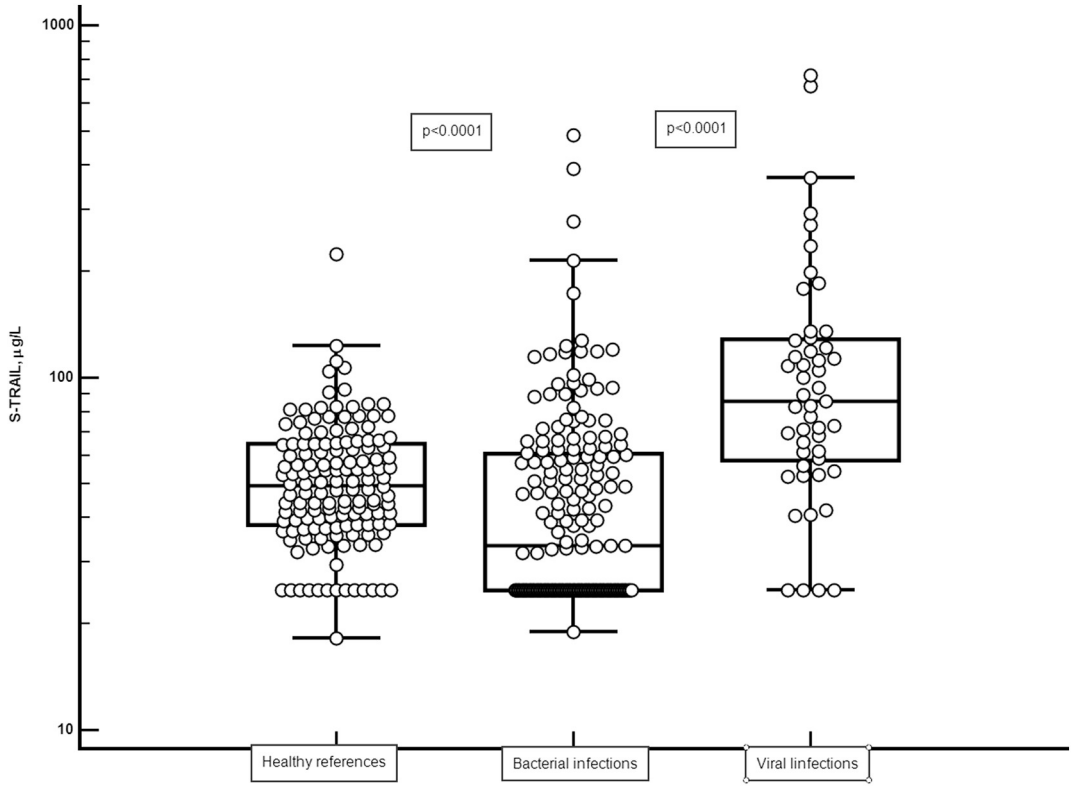

Fig. 10. In Fig. 10 the concentrations of TRAIL in serum are shown in healthy persons as compared to patients with either bacterial or viral infections. The statistical difference between results of those with bacterial infections and healthy or virally infected patients was evaluated by Mann-Whitney non-parametric test and shown on the figure. The box plot indicates median and quartile ranges.

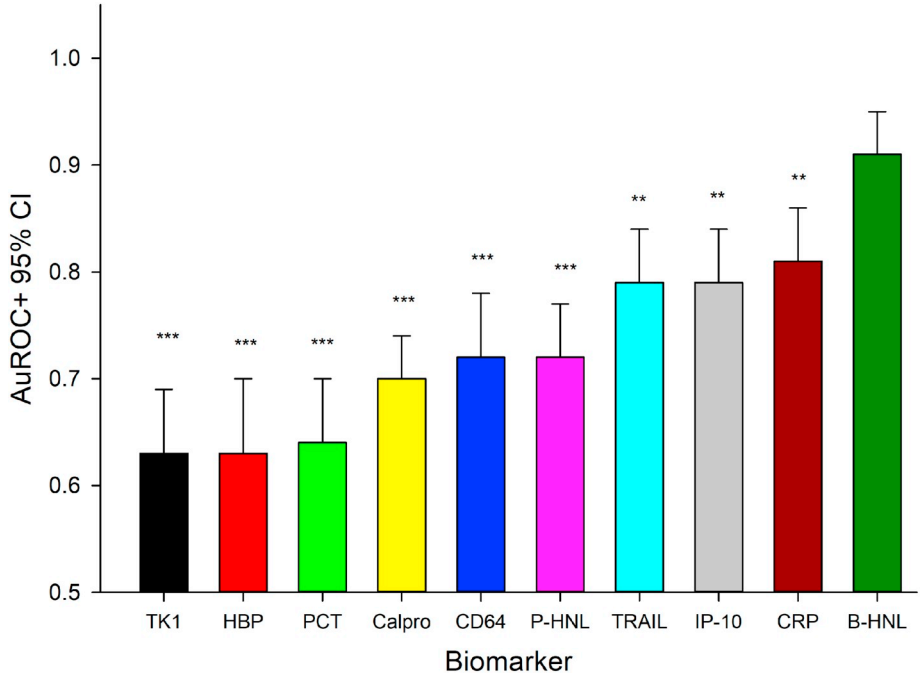

Fig. 11. A comparison between individual biomarkers in their distinction between bacterial and viral infections. The results are given as AuROC and 95% CI.

Table 1
Logistic regression analysis. Dependent variable: Infectious agent bacteria or virus. Method: Backward exclusion of variables. Enter if $p < .05$ and remove if $p > .1$.

Variable	Coefficient	Std. error	Wald	p
B-HNL	0.027182	0.0059658	20.7602	<0.0001
S-TRAIL	−0.018305	0.0052353	12.2256	0.0005
S-IP-10	−0.00074243	0.00023859	9.6829	0.0019
Area under the ROC curve (AUC)			0.961	
Standard Error			0.0143	
95% Confidence interval			0.920 to 0.985	

Not included in the model as independent biomarkers were Calprotectin, TK1, PCT, Azurocidin, P-HNL, CRP, CD64 and blood neutrophil counts.

Table 2
Logistic regression analysis. Dependent variable: Infectious agent bacteria or virus. Selection respiratory infections. Method: Backward exclusion of variables. Enter if $p < .05$ and remove if $p > .1$.

Variable	Coefficient	Std. Error	Wald	p
TRAIL	−0.040789	0.012580	10.5122	0.0012
B-HNL	0.028850	0.010131	8.3811	0.0038
Area under the ROC curve (AUC)			0.964	
Standard Error			0.0197	
95% Confidence interval			0.89 to 0.99	

Not included in the model as independent biomarkers were Calprotectin, TK1, IP-10, PCT, Azurocidin, P-HNL, CRP, CD64 and blood neutrophil counts.

HNL is a fairly complicated molecule with several origins and several names i.e. NGAL (Neutrophil Gelatinase Associated Lipocalin) or Lipocalin 2 (Xu and Venge, 2000). In blood the major origin is blood neutrophils in which cells HNL exists in a preformed state (Cai et al., 2010). The production of HNL may, however, be induced in epithelial cells such as in the kidney tubular cells in patients with acute kidney injury (AKI) (Cai et al., 2009). If measured in serum or plasma, such production of HNL could potentially affect the diagnostic performance of HNL. HNL originating from neutrophils is to a substantial extent released in the dimeric form, whereas HNL released from epithelial cells is in the monomeric form. This means that assays should be able to

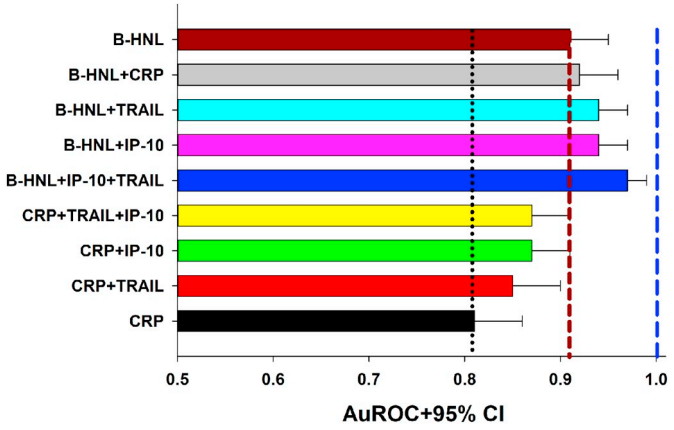

Fig. 12. A comparison between individual and combinations of biomarkers in their distinction between bacterial and viral infections. The results are given as AuROC and 95% CI. The three vertical lines are given for comparison of AuROC of CRP only, B-HNL only or AuROC of 1.0.

distinguish these forms for optimal diagnostic performance. As shown recently the configuration of the assay by certain antibody pairs enables such distinction when measured in serum or plasma (Yu et al., 2016). In the present report we have circumvented these potential confounders by measuring directly what is released from the circulating neutrophils.

The limitation of our study was the accuracy in the diagnosis i.e. in the distinction between bacterial and viral causes of the respiratory infections. Such distinction is notoriously difficult (Hopstaken et al., 2003), but very important if one wishes to investigate any biomarker for these purposes. In this report we made an effort to make an accurate diagnosis by microbiologic testing of various body fluids, but with even more extensive testing we might have reached higher diagnostic accuracies of HNL of > > 0.9. Among all patients included in the study, a certain and confirmed microbiological diagnosis was only achieved in 50% (228/581) which may also be seen as a limitation. The exclusion of approximately 50% of the patients is explained by the difficulties to obtain a documented exact microbiological diagnosis. Of those excluded, several patients had both a documented viral and bacterial

infection concomitantly and many hade viral upper respiratory tract infections where no microbiological tests were performed or negative for influenza A or B but not tested for other virus. Also in a proportion of patients with bacterial infections a documented bacterial culture was missing because of early initiation of antibiotic treatment before cultures were obtained. However, what seems very important in our study is that the diagnostic performance of HNL in the side-by-side comparison with other potential candidate biomarkers still was the superior biomarker, but also that the diagnostic performance of HNL may be increased further by adding biomarkers such as IP-10 and/or TRAIL. Another limitation of the study is the lack of children among our patients nor did we include patients admitted to the intensive care unit. This means that we cannot generalize our results into these groups, although HNL was measured in serum in children in earlier studies and shown to have an interesting diagnostic performance (Fjaertoft et al., 2005a). The strength of our study was the fact that the biomarkers were judged against each other and with the diagnostic limitations affecting the biomarkers equally. It should, however, be emphasized that our studies on whole blood activation and the measurement of the release of HNL are explorative, since the technology used for this purpose is not practical and has to be replaced by more appropriate assay formats e.g. in the form of rapid point-of-care assays. In a recent study the three-biomarker algorithm was found superior to HNL either measured in serum or plasma (Ashkenazi-Hoffnung et al., 2018). The plasma HNL results reported were in line with our own findings in that report. Unfortunately, however, the study failed to follow the instructions for serum preparations, which seriously invalidated the comparison with serum HNL as the discriminator. Thus, the increased concentrations of HNL in serum as compared to plasma are the results of active release from the blood neutrophils. Such active release is prevented by keeping blood at +4 °C, as was the case in the study by Ashkenazi-Hoffnung et al.(Ashkenazi-Hoffnung et al., 2018). In a very recent study on PCT, IP-10 and TRAIL similar results were obtained to our findings for the indivdual protein biomarkers and also showed that the combination of biomarkers increased the discrimination between bacterial and viral infections (van der Does et al., 2018).

We conclude that the diagnostic performance of whole blood activated HNL is superior to any other known biomarker in the distinction between acute infections caused by bacteria or virus. The rapid and accurate analysis of HNL, which reflects the response of the body to a bacterial challenge of the body, together with one or two biomarkers reflecting viral infections may be the ideal diagnostic biomarker combination in our fight against misuse of antibiotics. The development of a point-of-care assay that will allow the diagnosis to be made within 10 min from a finger stick blood is ongoing and this method should aid the doctor in the decision of whether to treat the infection or not and hopefully reduce the misuse of antibiotics.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.06.018.

References

Ashkenazi-Hoffnung, L., Oved, K., Navon, R., Friedman, T., Boico, O., Paz, M., Kronenfeld, G., Etshtein, L., Cohen, A., Gottlieb, T.M., Eden, E., Chistyakov, I., Srugo, I., Klein, A., Ashkenazi, S., Scheuerman, O., 2018. A host-protein signature is superior to other biomarkers for differentiating between bacterial and viral disease in patients with respiratory infection and fever without source: a prospective observational study. Eur. J. Clin. Microbiol. Infect. Dis. 37, 1361.

Bjorkqvist, M., Kallman, J., Fjaertoft, G., Xu, S., Venge, P., Schollin, J., 2004. Human neutrophil lipocalin: normal levels and use as a marker for invasive infection in the newborn. Acta Paediatr. 93, 534.

Cai, L., Borowiec, J., Xu, S., Han, W., Venge, P., 2009. Assays of urine levels of HNL/NGAL in patients undergoing cardiac surgery and the impact of antibody configuration on their clinical performances. Clin. Chim. Acta 403, 121.

Cai, L., Rubin, J., Han, W., Venge, P., Xu, S., 2010. The origin of multiple molecular forms in urine of HNL/NGAL. Clin. J. Am. Soc. Nephrol. 5, 2229.

Drozdov, D., Dusemund, F., Muller, B., Albrich, W.C., 2013. Efficacy and safety of procalcitonin-guided antibiotic therapy in lower respiratory tract infections. Antibiotics. (Basel) 2, 1.

Dupuy, A.M., Philippart, F., Pean, Y., Lasocki, S., Charles, P.E., Chalumeau, M., Claessens, Y.E., Quenot, J.P., Guen, C.G., Ruiz, S., Luyt, C.E., Roche, N., Stahl, J.P., Bedos, J.P., Pugin, J., Gauzit, R., Misset, B., Brun-Buisson, C., 2013. Role of biomarkers in the management of antibiotic therapy: an expert panel review: I - currently available biomarkers for clinical use in acute infections. Ann. Intensive Care 3, 22.

Fagerhol, M.K., 2000. Calprotectin, a faecal marker of organic gastrointestinal abnormality. Lancet 356, 1783.

Fjaertoft, G., Foucard, T., Xu, S., Venge, P., 2005a. Human neutrophil lipocalin (HNL) as a diagnostic tool in children with acute infections: a study of the kinetics. Acta Paediatr. 94, 661.

Fjaertoft, G., Pauksen, K., Hakansson, L., Xu, S., Venge, P., 2005b. Cell surface expression of FcgammaRI (CD64) on neutrophils and monocytes in patients with influenza A, with and without complications. Scand. J. Infect. Dis. 37, 882.

Gronowitz, J.S., Källander, C.F.R., Diderholm, H., Hagberg, H., Petterson, U., 1984. Application of an in vitro assay for serum thymidine kinase: results on viral disease and malignancies in humans. Int. J. Cancer 33, 5.

Hayney, M.S., Henriquez, K.M., Barnet, J.H., Ewers, T., Champion, H.M., Flannery, S., Barrett, B., 2017. Serum IFN-gamma-induced protein 10 (IP-10) as a biomarker for severity of acute respiratory infection in healthy adults. J. Clin. Virol. 90, 32.

Hopstaken, R.M., Muris, J.W., Knottnerus, J.A., Kester, A.D., Rinkens, P.E., Dinant, G.J., 2003. Contributions of symptoms, signs, erythrocyte sedimentation rate, and C-reactive protein to a diagnosis of pneumonia in acute lower respiratory tract infection. Br. J. Gen. Pract. 53, 358.

Hopstaken, R.M., Stobberingh, E.E., Knottnerus, J.A., Muris, J.W., Nelemans, P., Rinkens, P.E., Dinant, G.J., 2005. Clinical items not helpful in differentiating viral from bacterial lower respiratory tract infections in general practice. J. Clin. Epidemiol. 58, 175.

Ip, M., Rainer, T.H., Lee, N., Chan, C., Chau, S.S., Leung, W., Leung, M.F., Tam, T.K., Antonio, G.E., Lui, G., Lau, T.K., Hui, D.S., Fuchs, D., Renneberg, R., Chan, P.K., 2007. Value of serum procalcitonin, neopterin, and C-reactive protein in differentiating bacterial from viral etiologies in patients presenting with lower respiratory tract infections. Diagn. Microbiol. Infect. Dis. 59, 131.

Jonsson, N., Nilsen, T., Gille-Johnson, P., Bell, M., Martling, C.R., Larsson, A., Martensson, J., 2017. Calprotectin as an early biomarker of bacterial infections in critically ill patients: an exploratory cohort assessment. Crit. Care Resusc. 19, 205.

Kumar, J.K., Aronsson, A.C., Pilko, G., Zupan, M., Kumer, K., Fabjan, T., Osredkar, J., Eriksson, S., 2016a. A clinical evaluation of the TK 210 ELISA in sera from breast cancer patients demonstrates high sensitivity and specificity in all stages of disease. Tumour Biol. 37, 11937.

Kumar, J.K., Aronsson, A.C., Pilko, G., Zupan, M., Kumer, K., Fabjan, T., Osredkar, J., Eriksson, S., 2016b. A clinical evaluation of the TK 210 ELISA in sera from breast cancer patients demonstrates high sensitivity and specificity in all stages of disease. Tumour Biol. 37, 11937.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K., Wertheim, H.F., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D., Cars, O., 2013. Antibiotic resistance-the need for global solutions. Lancet Infect. Dis. 13, 1057.

Le, B.J., Hausfater, P., Chenevier-Gobeaux, C., Blanc, F.X., Benjoar, M., Ficko, C., Ray, P., Choquet, C., Duval, X., Claessens, Y.E., 2015. Diagnostic accuracy of C-reactive protein and procalcitonin in suspected community-acquired pneumonia adults visiting emergency department and having a systematic thoracic CT scan. Crit. Care 19, 366.

Linder, A., Christensson, B., Herwald, H., Bjorck, L., Akesson, P., 2009a. Heparin-binding protein: an early marker of circulatory failure in sepsis. Clin. Infect. Dis. 49, 1044.

Linder, A., Christensson, B., Herwald, H., Bjorck, L., Akesson, P., 2009b. Heparin-binding protein: an early marker of circulatory failure in sepsis. Clin. Infect. Dis. 49, 1044.

Martensson, J., Bell, M., Xu, S., Bottai, M., Ravn, B., Venge, P., Martling, C.R., 2013. Association of plasma neutrophil gelatinase-associated lipocalin (NGAL) with sepsis and acute kidney dysfunction. Biomarkers 18, 349.

Meili, M., Muller, B., Kulkarni, P., Schutz, P., 2015. Management of patients with respiratory infections in primary care: procalcitonin, C-reactive protein or both? Expert. Rev. Respir. Med. 9, 587.

Muller, B., Becker, K.L., 2001. Procalcitonin: how a hormone became a marker and mediator of sepsis. Swiss Med. Wkly. 131, 595.

Oved, K., Cohen, A., Boico, O., Navon, R., Friedman, T., Etshtein, L., Kriger, O., Bamberger, E., Fonar, Y., Yacobov, R., Wolchinsky, R., Denkberg, G., Dotan, Y., Hochberg, A., Reiter, Y., Grupper, M., Srugo, I., Feigin, P., Gorfine, M., Chistyakov, I., Dagan, R., Klein, A., Potasman, I., Eden, E., 2015a. A novel host-proteome signature for distinguishing between acute bacterial and viral infections. PLoS One 10, e0120012.

Oved, K., Cohen, A., Boico, O., Navon, R., Friedman, T., Etshtein, L., Kriger, O., Bamberger, E., Fonar, Y., Yacobov, R., Wolchinsky, R., Denkberg, G., Dotan, Y., Hochberg, A., Reiter, Y., Grupper, M., Srugo, I., Feigin, P., Gorfine, M., Chistyakov, I., Dagan, R., Klein, A., Potasman, I., Eden, E., 2015b. A novel host-proteome signature for distinguishing between acute bacterial and viral infections. PLoS One 10, e0120012.

Quenot, J.P., Luyt, C.E., Roche, N., Chalumeau, M., Charles, P.E., Claessens, Y.E., Lasocki, S., Bedos, J.P., Pean, Y., Philippart, F., Ruiz, S., Gras-Leguen, C., Dupuy, A.M., Pugin, J., Stahl, J.P., Misset, B., Gauzit, R., Brun-Buisson, C., 2013. Role of biomarkers in the management of antibiotic therapy: an expert panel review II: clinical use of biomarkers for initiation or discontinuation of antibiotic therapy. Ann. Intensive Care 3, 21.

Sander, J., Fagerhol, M.K., Bakken, J.S., Dale, I., 1984. Plasma levels of the leucocyte L1 protein in febrile conditions: relation to aetiology, number of leucocytes in blood, blood sedimentation reaction and C-reactive protein. Scand. J. Clin. Lab. Invest. 44, 357.

Toikka, P., Irjala, K., Juven, T., Virkki, R., Mertsola, J., Leinonen, M., Ruuskanen, O., 2000. Serum procalcitonin, C-reactive protein and interleukin-6 for distinguishing bacterial and viral pneumonia in children. Pediatr. Infect. Dis. J. 19, 598.

van der Does, Y., Rood, P.P.M., Ramakers, C., Schuit, S.C.E., Patka, P., van Gorp, E.C.M., Limper, M., 2018. Identifying patients with bacterial infections using a combination of C-reactive protein, procalcitonin, TRAIL, and IP-10 in the emergency department: a prospective observational cohort study. Clin. Microbiol. Infect. 24, 1297.

Venge, P., Douhan-Hakansson, L., Garwicz, D., Peterson, C., Xu, S., Pauksen, K., 2015a. Human neutrophil lipocalin as a superior diagnostic means to distinguish between acute bacterial and viral infections. Clin. Vaccine Immunol. 22, 1025.

Venge, P., Hakansson, L.D., Garwicz, D., Peterson, C., Xu, S., Pauksen, K., 2015b. Human neutrophil lipocalin in fMLP-activated whole blood as a diagnostic means to distinguish between acute bacterial and viral infections. J. Immunol. Methods 424, 85.

Xu, S., Venge, P., 2000. Lipocalins as biochemical markers of disease. Biochim. Biophys. Acta 1482, 298.

Xu, S.Y., Pauksen, K., Venge, P., 1995. Serum measurements of human neutrophil lipocalin (HNL) discriminate between acute bacterial and viral infections. Scand. J. Clin. Lab. Invest. 55, 125.

Yu, Z., Jing, H., Hongtao, P., Furong, J., Yuting, J., Xu, S., Venge, P., 2016. Distinction between bacterial and viral infections by serum measurement of human neutrophil lipocalin (HNL) and the impact of antibody selection. J. Immunol. Methods 432, 82.