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Human neutrophil lipocalin in fMLP-activated whole blood as a diagnostic means to distinguish between acute bacterial and viral infections

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ABSTRACT

The distinction between causes of acute infections is a major clinical challenge. Current biomarkers, however, are not sufficiently accurate. Human neutrophil lipocalin (HNL) in serum distinguishes acute infections with high accuracy, but in the emergency setting the assay time should be <15–20 min, which excludes the use of serum samples. The aim was therefore to develop a novel rapid assay principle and test its clinical performance.
Methods: Serum and neutrophils obtained from 84 infected and 20 healthy subjects were used in the experimental study. 725 subjects (144 healthy controls and 581 patients with signs and symptoms of acute infections) were included in the clinical study. HNL was measured in EDTA-plasma by ELISA or in heparinized whole blood after fMLP activation by a prototype point-of-care assay.
Results: Increased release of HNL from neutrophils after activation with fMLP was seen already after 5 min incubation. The release of HNL from purified neutrophils after 15 min incubation with fMLP was significantly correlated to the HNL concentrations in serum obtained from the same patient ($r = 0.74$, $p < 0.001$). In the distinction between healthy controls and patients with bacterial infections, the areas under the ROC-curves were 0.95 (95% CI 0.91–0.97) and 0.88 (95% CI 0.84–0.91) for HNL in fMLP-activated whole blood and EDTA-plasma, respectively, ($p < 0.001$) and in the distinction between bacterial and viral infections 0.91 (95% CI 0.86–0.95) and 0.76 (95% CI 0.70–0.81), respectively ($p < 0.001$).
Conclusion: The clinical performance of HNL in fMLP-activated whole blood was superior to HNL in EDTA-plasma and similar to HNL in serum. The procedure can be adopted for point-of-care testing with response times of <15 min.

1. Introduction

Human neutrophil lipocalin (HNL) is a protein of many names such as lipocalin 2 and neutrophil gelatinase associated lipocalin (NGAL) (Xu and Venge, 2000). The main source of HNL is the neutrophil granulocyte, whereas the production of the protein may be induced in epithelial cells under certain conditions (Cai et al., 2010; Nielsen et al., 1996). Immunoassays were developed to measure HNL/NGAL in various bodily fluids and the concentrations in blood were shown to be raised in patients having bacterial infections and in urine in patients with acute kidney injury (Martensson et al., 2012; Xu et al., 1994, 1995; Mishra et al., 2005). Thus, serum concentrations of HNL were shown to discriminate between acute bacterial and viral infections with high likelihood and showed positive (PPV) and negative predictive values (NPV) in this distinction of >90% (Xu et al., 1995). The discriminatory power of HNL in serum in this regard was further confirmed in additional studies on children and adults indicating that HNL measurements in serum could be a clinically useful biomarker for the distinction between bacterial or viral causes of acute infections (Bjorkqvist et al., 2004; Fjaertoft et al., 2005). However, the high discriminatory power of HNL was seen only with measurements in serum and not with EDTA-plasma. This suggested that the neutrophils in the test tube ex vivo continued to release their HNL during the hours following the collection of the blood specimen and that this release activity reflected the state of activation of the neutrophils that was induced by the bacterial challenge but not by virus.

To be useful in the emergency department or in the doctor's office the total assay time from collecting the blood to result of any biomarker should be short i.e. <15–20 min, which is the philosophy behind the development of point-of-care (POC) assays (Pfafflin and Schleicher, 2009; Bingisser et al., 2012). Such requirements are not possible to meet with serum measurements of HNL, since, as indicated above, this requires the pre-activation of the neutrophils for hours. In this report we have tested the possibility that activation of the neutrophils in the blood by the well-established neutrophil activator tri-peptide fMLP

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(Fletcher and Gallin, 1983), might circumvent the problem and in fact be used to mimic the neutrophil activation occurring during whole blood coagulation and replace the need to measure HNL in serum after a prolonged incubation. To test this hypothesis we have collected blood from a large cohort of patients with signs of acute infection and compared the diagnostic performance of HNL concentrations in whole blood after activation with fMLP with HNL in non-activated plasma.

2. Patients

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 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).

Patients with confirmed etiology of their acute infection were 288 (49.6% of all patients). Of these patients 185 had a bacterial infection, 54 a viral infection, 26 mycoplasma infection and 23 bacterial infection as a secondary infection to Influenza. Remaining patients with uncertain diagnosis were 293 (50.4%).

The study was approved by the regional ethics committee of Uppsala.

3. Methods

Inclusion criteria of patients in the study was fever >38 °C and signs and symptoms of an acute infection. Exclusion criteria were known chronic viral infection, such as human immunodeficiency virus infection or hepatitis. 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 (n = 449) or to a primary care unit in Uppsala (n = 132). Blood was drawn before start of antibiotics treatment.

The patients were classified into three groups (1: Verified etiology 2: Possible etiology and Unknown etiology) and in this report only results from patients with a verified etiology of their acute infection, bacteria or virus, were included. Verification included in addition to clinical findings, white blood cell counts, CRP, positive chest-X-ray supported by positive culture or PCR-testing of samples from the respiratory tract and IgG/IgM serology. Bacterial infections were also supported by cultures from blood, urine, stool, wounds abscesses, and respiratory tract when appropriate. Tonsillitis was diagnosed by rapid test for group A streptococcus and supported by positive culture. Endocarditis had positive blood cultures and findings on echocardiography. In bacterial infections the results of plasma CRP were median 115 mg/L, range 1.4–419 mg/L and in viral infections median 17 mg/L, range 0.2–181 mg/L. Corresponding results for WBC were median 10.6×10^9/L, range 3.0–29.8 $\times 10^9$/L for bacterial infections and median 6.5×10^9/L, range 1.6–19.0 $\times 10^9$/L for viral infections.

4. Activation of neutrophils

Blood was obtained from healthy controls or from patients with acute infections. Neutrophils were purified from heparinized blood by density gradient centrifugation over isotonic 67% of Percoll (Pharmacia, Uppsala, Sweden). After centrifugation the mononuclear cells in the interphase were removed. The erythrocytes in the bottom of the tube were lysed by hypotonic shock in ice-cold water for 1 min. The solution was normalized by adding an equal volume of 2 times higher concentration of phosphate-buffered saline (PBS). The neutrophils were then washed twice with PBS. The neutrophils obtained by this procedure had a purity of >95%.

Cells were resuspended at a cell density of 3×10^6 cells/mL in HBSS. For stimulation, separated aliquots (150 μL) of cells were exposed to a volume of 300 μL of fMLP (Sigma) at various concentrations (10^{-8}–10^{-7} mol/L) at 37 °C and at various time periods as indicated under results. The release of HNL was terminated by the addition of 2 vol. of ice-cold buffer and then centrifuged at 600 *g* for 5 min, at 4 °C. A volume of 675 μL of supernatant was removed and mixed with 100 μL of 0.5% CTAB in 0.15 M NaCl. The released HNL in the supernatant was measured by a radioimmunoassay as described previously (Xu et al., 1994). The released HNL concentrations in the supernatant were calculated as relative concentrations on the basis of the total granule HNL content determined after extraction with 0.5% CTAB in 0.15 NaCl.

5. Assay of HNL

HNL was measured either in EDTA-plasma or in heparinized blood after pre-activation with the tri-peptide fMLP (formyl-Methionine-Leucine-Phenylalanine (BioXtra Sigma-Aldrich, St. Louis, MO, USA)). Measurements in plasma were performed by means of a double monoclonal ELISA kit provided by Diagnostics Development (Uppsala, Sweden) and run according to the instructions of the provider. The imprecision of the ELISA assay was <6% CV. HNL in whole blood was assayed by a prototype POC assay on the Meritas®-platform (Fiomi Diagnostics, Uppsala, Sweden). Pre-activation of whole blood was performed by the incubation of whole blood with 10^{-7} mol/L fMLP for 20 min at 37 °C after which the blood was applied to the Meritas cartridge.

6. Statistics

Data is expressed as means ± SD or geometric means with 95% confidence interval (CI) wherever appropriate. Comparisons of two groups, either dependent or independent, were performed by Student's paired or unpaired t-test and comparisons of >2 groups by one-way analysis of variance (ANOVA). Pearson's linear correlation was applied. P-values < 0.05 were considered significant. 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. For the calculations the statistics programs MedCalc Statistical Software v. 14.8.1 (MedCalc Software, Ostend, Belgium) and Statistica 64 v. 12 (Statsoft, Tulsa, OK, USA) were used.

7. Results

7.1. Activation of neutrophils by fMLP

Neutrophils purified from blood of patients and healthy controls were exposed to various concentrations of fMLP and incubated for 15 min at 37 °C. After this, the samples were centrifuged and the supernatants assayed for the presence of HNL. The optimal concentration of fMLP for release of HNL was found to be 5×10^{-8} mol/L. In order to study the kinetics of release of HNL, purified cells were incubated for different lengths of time. Significant release was seen after 5 min of incubation and increased further by prolonged incubation (Fig. 1).

In order to test the possibility that the release propensity of neutrophils after incubation with fMLP reflected the release of HNL in whole blood after coagulation, the release of HNL from neutrophils purified from 84 patients with acute infections and 20 healthy non-infected subjects was compared to the serum concentrations of the respective subject. As can be seen in Fig. 2 a significant and linear correlation ($r = 0.74$, $p < 0.001$) was obtained between the supernatant and serum concentrations of HNL, which suggests that fMLP activation of neutrophils in whole blood or by coagulation reflects similar mechanisms.

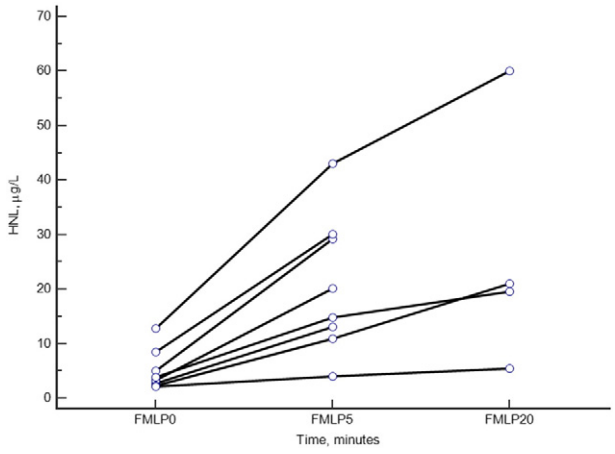

Fig. 1. The kinetics of the release of HNL from purified blood neutrophils after incubation with fMLP, 5×10^{-8} mol/L for 0, 5 and 20 min.

7.2. Clinical results

Heparinized whole blood and EDTA-plasma were collected from 581 patients with fever >38 °C and symptoms of infection and from 144 apparently non-infected healthy subjects. Without knowledge of the investigated biomarker results the infected patients were classified as having a bacterial or viral cause of their disease. In 288 the etiology of their acute infection was confirmed by objective tests. The study group included patients with verified etiology of their infections, but with the exclusion of mycoplasma infections and patients with mixed infections. This group consisted of 383 subjects (144 healthy non-infected controls, 185 with bacterial infections and 54 with viral infections).

7.2.1. The analytical performance of the HNL assay

The monoclonal antibody pair used in the prototype POC assay on the Meritas®-platform was the same as used in the ELISA of HNL. The measuring range was 2 to 640 µg/L. The CV profile showed an imprecision of the POC assay of <10% above 75 µg/L. The POC assay correlated well to the ELISA ($r = 0.959$, Fit POC assay $= 1.04 \times$ ELISA $+ 2.4$).

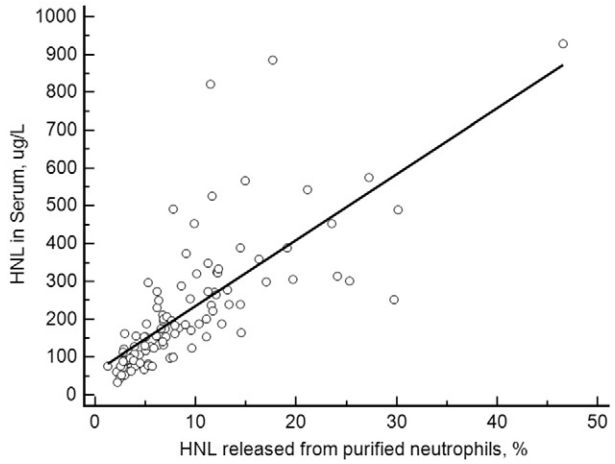

Fig. 2. The correlation between the release of HNL from purified blood neutrophils and the concentration of HNL in serum in 104 samples obtained from 20 healthy non-infected subjects and 84 patients with various kinds of acute infections. The correlation coefficient was $r = 0.74$, $p < 0.0001$.

7.2.2. The diagnostic performance of HNL

In Figs. 3a and b the HNL concentrations in whole blood after fMLP activation for 20 min at 37 °C and in EDTA-plasma are shown. As compared to HNL concentrations in fMLP-activated whole blood of healthy subjects (geometric mean 98 µg/L, 95% CI 90–107 µg/L), the concentrations in patients with bacterial (geometric mean 337 µg/L, 95% CI 300–379 µg/L) ($p < 0.001$) and viral infections (geometric mean 117 µg/L, 95% CI 101–136 µg/L) ($p < 0.05$) were significantly raised. The concentrations of HNL in plasma of healthy subjects were 35 µg/L (geometric mean, 95% CI 34–36 µg/L) with significantly higher concentrations in patients with bacterial infections, 64 µg/L (geometric mean, 95% CI 60–69) ($p < 0.001$) and in patients with viral infections, 43 µg/L (geometric mean 95% CI 38–49) ($p < 0.001$). It is apparent that the overlap between healthy individuals and patients with bacterial infection is greater with EDTA-plasma. On average, the additional amounts of HNL released from the neutrophils in whole blood activated by fMLP were 2.8-fold for healthy subjects, 5.3-fold for patients with bacterial infections and 2.7-fold for those with viral infections. Thus, no additional release of HNL over healthy subjects was seen with fMLP-activated whole blood in those with viral infections.

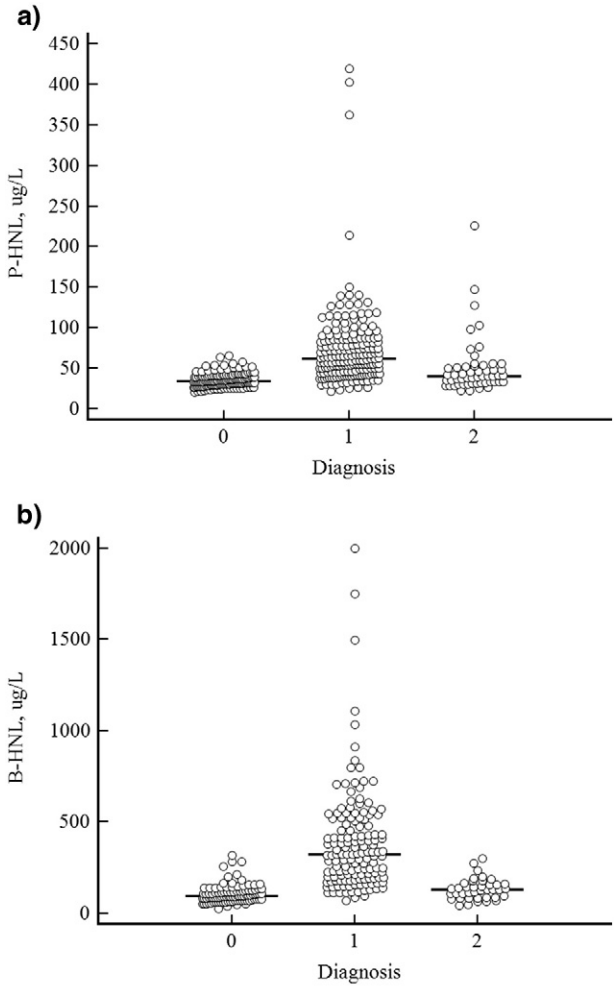

Fig. 3. a. Plasma concentrations of HNL in healthy non-infected subjects (0), patients with bacterial infections (1) and patients with viral infections (2). The concentrations in bacterial infections were significantly higher than in the two other groups ($p < 0.001$). b. HNL concentrations in whole blood activated by fMLP in healthy non-infected subjects (0), patients with bacterial infections (1) and patients with viral infections (2). The concentrations in bacterial infections were significantly higher than in the two other groups ($p < 0.001$).

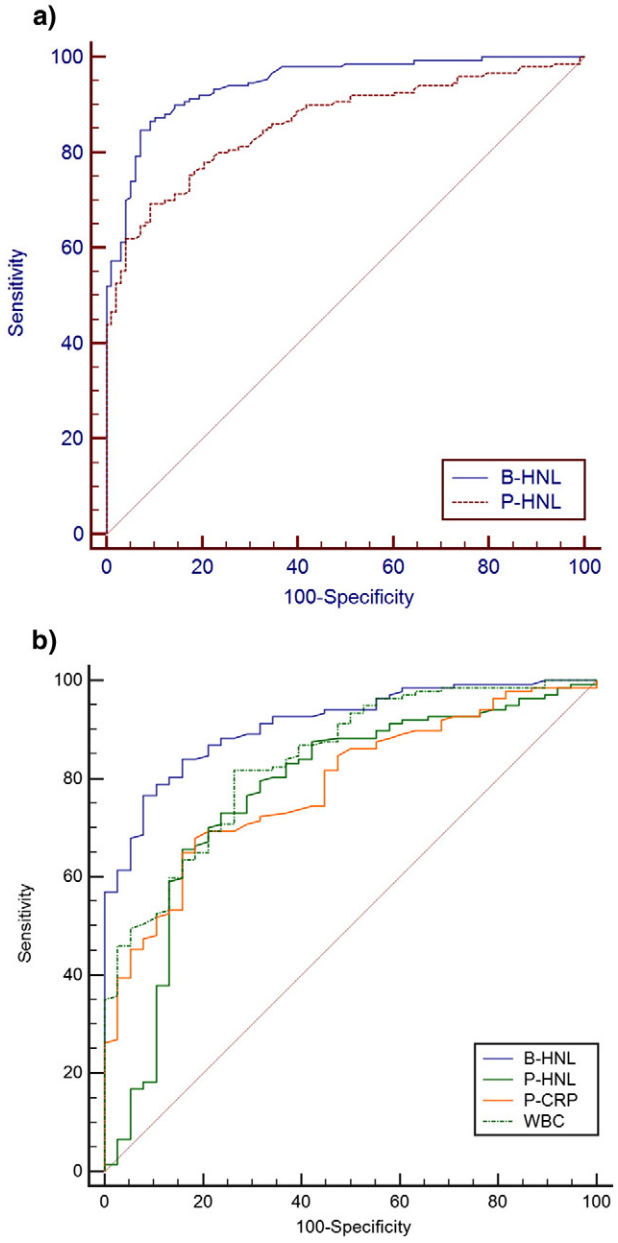

Fig. 4. a. Receiver operating characteristic curves of HNL in fMLP-activated whole blood (B-HNL) in the distinction between healthy non-infected and bacterially infected subjects. b. Receiver operating characteristic curves of HNL in fMLP-activated whole blood (B-HNL) in the distinction between bacterially and virally infected subjects. The clinical performance characteristics are given in Table 1. For comparison the ROC curves of plasma HNL and CRP as well as of white blood cell counts (WBC) are shown.

Figs. 4a and b show the diagnostic performances of the two assays. In Fig. 4a the distinction between healthy non-infected subjects and those with confirmed bacterial infection is shown by means of receiver operating characteristics (ROC) curves. The area under the curve (AUC) for the fMLP-activated whole blood was 0.95 (95% 0.91–0.97) as compared to 0.88 (95% CI 0.84–0.91), $p < 0.001$, for HNL in EDTA plasma (Table 1). At the optimal discrimination of 168 μg/L for fMLP-activated whole blood NPV was 80% (95% CI 71–87%) and PPV 95% (95% CI 90–98%) with Lr+ of 11.9 (95% CI 6–24) and Lr− of 0.16 (95% CI 0.1–0.2). For EDTA-plasma at the optimal HNL concentration of 43.6 μg/L the NPV was 73% (95% CI 65–79%) and the PPV 88% (95% CI 82–92%) with Lr+ of 5.3 (95% CI 3.5–7.9) and Lr− of 0.27 (95% CI 0.2–0.4).

In the distinction between bacterial and viral infections the AUC for fMLP-activated whole blood was 0.91 (95% CI 0.86–0.95) and for plasma 0.76 (95% CI 0.70–0.81), $p < 0.001$ (Fig. 4b). At the optimal concentration of 142 μg/L for fMLP-activated whole blood, based on the ROC curves, the NPV 71% (95% CI 54–85%) and PPV 91% (95% CI 85–95%) with Lr+ 2.7(95% CI 1.7–4.2) and Lr− 0.11 (95% CI 0.06–0.2). Corresponding figures for HNL at the concentration of 33.5 μg/L in EDTA-plasma were NPV 60% (95% CI 41–77%) and PPV 83% (95% CI 77–88%) and Lr+ of 1.40 (95% CI 1.2–1.7) and Lr− 0.2 (95% CI 0.1–0.4). Thus, the clinical performance of HNL in fMLP-activated whole blood was superior to HNL in EDTA-plasma both in the distinction between healthy subjects and bacterial infections and in the distinction between bacterial and viral infections. For comparison also the results of plasma levels of CRP and white blood cell counts are shown although this data may be biased by the fact that the results were known to the adjudicator.

8. Discussion

Symptoms of acute infections are probably the most common reason for seeking health care worldwide. In a preliminary survey of a large primary health care center in Sweden, 49% of all patients who visited the center did so because of symptoms of acute infections. The judgment of the doctor of whether to treat the infection with antibiotics is commonly based on clinical symptoms and in some cases supported by laboratory tests such as white blood cell counts, CRP, rapid tests for bacterial or viral agents (Hopstaken et al., 2003). The sensitivities and specificities of such tests, however, often preclude the accurate distinction between various causes of infections with the consequent prescription of antibiotics just in case (Graffelman et al., 2004; Hopstaken et al., 2005). The unnecessary prescription of antibiotics or sales of antibiotics without any prescription over the counter adds to the rapid development of antibiotics resistance, which is seen as a serious threat to mankind (Laxminarayan et al., 2013; Nathan and Cars, 2014). Thus, the development of diagnostic tools that are accurate and easily available are highly desirable. In previous publications we showed that the neutrophil secretory protein HNL may be a prominent step in this direction, since serum concentrations of HNL showed accurate distinction between bacterial or viral cause of acute infections with negative and positive predictive values of >90% and superior to white blood cell counts and CRP (Bjorkqvist et al., 2004; Fjaertoft et al., 2005; Xu et al., 1995). However, in order to make HNL easily available and attractive to the primary care physician or to the emergency doctor the results of HNL concentrations in blood should be reported within 15–20 min

Table 1
Clinical performances of HNL in fMLP activated whole blood and in EDTA-plasma.

ROC cut-off	AUC	Sensitivity	Specificity	NPV	PPV	LR+	LR−
Bacterial infection vs healthy controls							
Blood (168 μg/L)	0.95 *** (0.91–0.97)	85% (78–90%)	93% (86–97%)	80% (71–87%)	95% (90–98%)	11.9 (6–24)	0.16 (0.1–0.2)
Plasma (43.6 μg/L)	0.88 (0.84–0.91)	77% (70–83%)	85% (79–91%)	73% (65–79%)	88% (82–92%)	5.3 (3.5–7.9)	0.27 (0.2–0.4)
Bacterial infection vs viral infection							
Blood (142 μg/L)	0.91*** (0.86–0.95)	93% (87–97%)	66% (49–80%)	71% (54–85%)	91% (85–95%)	2.7 (1.7–4.2)	0.11 (0.06–0.2)
Plasma (33.5 μg/L)	0.76 (0.70–0.81)	93% (89–97%)	33% (21–48%)	60% (41–77%)	83% (77–88%)	1.40 (1.2–1.7)	0.2 (0.1–0.4)

*** $p < 0.001$ blood vs plasma.

with similar performance as serum measurement of HNL in the laboratory. In this study we have investigated the possible use of plasma or whole blood for this purpose and show that whole blood measurements after activation with the neutrophil activator fMLP is the superior assay for the identification of patients with bacterial infections. More importantly was the fact that normal concentrations of HNL with high likelihood ruled-out bacteria as the causative agent of the acute infections.

Our findings in this study that plasma measurements of HNL were less diagnostic in the distinction between bacterial and viral infections than the previously reported findings with serum indicate a unique quality of serum. This quality is due to the additional release to the extracellular environment of HNL during coagulation. This release is an active process which is time and temperature dependent and most likely reflects the inherent state of activity of the neutrophils in the blood. In blood from healthy subjects we previously showed that the addition of released HNL to plasma after coagulation was on average 55% as compared to 79% in viral and 326% in bacterial infections (Xu et al., 1995). Our attempts in this study to mimic coagulation activation of HNL release by the activation of the blood neutrophils with fMLP resulted in similar figures i.e. with similar additional contributions in blood from healthy and virally infected patients but with substantially higher addition of released HNL in bacterially infected patients. Thus, again emphasizing that the propensity of blood neutrophils obtained from patients with bacterial infections to release HNL is much higher than seen in patients with viral infections or healthy subjects. It was therefore reassuring that the clinical performance of HNL levels in fMLP-activated whole blood was similar to previous results obtained with serum and indicates that fMLP-activation of whole blood may be suitable for point-of-care applications.

In the comparisons of clinical performance we estimated the diagnostic performance of the distinction between bacterial and healthy in addition to the distinction between bacterial and viral infections. In these comparisons we included only those patients in whom the infectious etiology was objectively supported. This approach obviously reduced the numbers of patients in the two cohorts by 60% but was taken to minimize the numbers of false classifications, since it is well known that the true distinction between different causes of infections, bacterial or viral, based on clinical judgment only is difficult (Hopstaken et al., 2003).

One limitation in the comparisons of HNL with other biomarkers was the fact that both CRP and WBC were used in the clinical judgment of the diagnosis. A bias towards these two biomarkers precluded the accurate evaluation of their diagnostic performance. In viral infections the considerable overlap with bacterial infections, however, indicated that CRP is less useful in this distinction, which corroborates with current knowledge of the diagnostic power of CRP (Andreeva and Melbye, 2014; Calvino et al., 2014; Dupuy et al., 2013; Hausfater, 2014; Ip et al., 2007; Toikka et al., 2000). In our earlier studies serum measurements of HNL were clearly superior to CRP and also showed differences between CRP and serum HNL in kinetics after start of antibiotics treatment, since serum concentrations of HNL normalized within 2–3 days in contrast to CRP which stayed elevated several days after the infection had subsided. Thus, HNL seems to more closely reflect the ongoing infection than CRP. Another limitation of CRP as a diagnostic means in acute infections is the low specificity of CRP since this biomarker is found raised in most other inflammatory diseases. HNL, on the other hand, was found non-elevated in patients with active rheumatoid arthritis (Torsteinsdottir et al., 1999).

We conclude from this study that neutrophil release of HNL in whole blood induced by the neutrophil activator fMLP is increased in blood obtained from patients with bacterial infections as opposed to blood from healthy controls and blood form patients with viral infections. The increased propensity of neutrophils in this regard mimics the propensity of neutrophils to release HNL at coagulation. Similar to recent studies on serum HNL, the clinical performance of HNL in fMLP activated whole blood showed high positive and negative predictive values in the distinction between acute bacterial and viral infections. Building point-of-care applications based on this principle with a response time of less than 15 min should be the next goal in the development of diagnostic tools as aids in the reduction of antibiotics abuse.

Disclosures and potential conflicts of interest

Per Venge and Shengyuan Xu own worldwide patent rights to measure HNL in bodily fluids. Per Venge is a shareholder of Diagnostics Development

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