The dynamics of circulating heparin-binding protein (HBP) - implications for its use as a biomarker

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Abstract

Background Heparin Binding Protein (HBP) is a promising new biomarker for the development and severity of sepsis. To guide the use of HBP as a biomarker it is important to understand the factors that may lead to false positive or negative results. The mechanisms that could lead to falsely elevated HBP levels include inappropriate release and inadequate clearance of HBP by the responsible cells and organs. HBP is presumably released only by neutrophils and the organs responsible for its elimination are unknown. Therefore, in this study we aimed to determine whether non-neutrophil cells can be a source of HBP in the circulation and which organs are responsible for its removal.

Results We measured HBP in two cohorts of neutropenic patients and found that 12% and 19% of patients in each cohort respectively had detectable HBP levels. *In vitro*, we found that three leukemia-derived monocytic cell lines and healthy CD14 + monocytes constitutively released detectable levels of HBP. Next we injected HBP intravenously in rats found that plasma levels of HBP decreased rapidly, with a distribution half-life below 10 minutes and an elimination half-life of 1–2 hours. We measured HBP levels in the liver, spleen, kidneys, lungs, and urine using both ELISA and immunofluorescence quantitation and found that the majority of HBP was present in the liver and a small amount was present in the spleen. Immunofluorescence imaging indicated that HBP is associated mainly with hepatocytes in the liver and monocytes/macrophages in the spleen.

Conclusions HBP can be found in some neutropenic patients. Other than neutrophils, malignant myeloid cells and monocytic cells may be an additional source of HBP. HBP disappears rapidly from the circulation and distributes primarily to liver hepatocytes and spleen monocytes/macrophages. The impact of hematologic malignancies and liver diseases on plasma HBP levels should be explored further in clinical studies.

Introduction

Mortality in sepsis is greatly reduced by early diagnosis and treatment [1], and therefore biomarkers for early detection of sepsis are a key to reducing its mortality. Heparin binding protein (HBP, also known as azurocidin or cationic antimicrobial protein of 37 kDa/CAP37) is a promising new prognostic biomarker that is elevated in the plasma of sepsis patients up to 12 hours before a drop in blood pressure is detected [2]. Plasma HBP levels above 15-30ng/mL are associated with mortality and organ failure in sepsis [3–5]. Rapid HBP testing is becoming available at clinical chemistry departments of certain hospitals in anticipation of its routine use in the future. If HBP is to be used clinically as a biomarker it is important to understand its origin, its kinetics in the blood, and its eventual removal from the circulation in order to identify conditions that could lead to false positive and negative results.

HBP is presumably expressed only in neutrophils and stored in their secretory vesicles and azurophilic granules [6]. There is so far no evidence that HBP is released by any other cells in the body. There are reports of HBP expression in endothelial cells of rats [7, 8] and in the kidneys of mice [9], but since mice

and rats lack the gene for HBP [10, 11], these findings are likely due to artefacts. Therefore, it is assumed that people with low levels of circulating neutrophils will have low or no plasma HBP, leading to possible false negative results in such patients. However, this assumption has never been confirmed in an appropriate cohort.

Once HBP is released into the blood, it is important to know how long it remains in the circulation to determine how frequently it should be measured in patients. Several reports suggest that circulating HBP is short lived. Serial measurements of HBP every four hours in septic shock patients indicated that HBP levels fluctuate widely between measurements [12]. Repeated measurements of HBP in patients undergoing cardiac surgery with cardiopulmonary bypass showed that HBP levels were reduced 3-fold in only five minutes after protamine reversal at the end of the surgery [13]. In mice, HBP plasma levels were measured at the end of an experiment following intravenous HBP infusion and only 1% of the administered HBP remained after 1 hour [14]. The pharmacokinetics of a single bolus dose of HBP have never been determined.

If HBP is targeted to specific organs then certain organ failures may lead to false positive or negative results. HBP can bind to and be taken up by endothelial cells [15] and monocytes [16, 17], but it is unclear what proportion of circulating HBP is associated with these cells *in vivo*. It is unknown to which compartments circulating HBP distributes and which cells in these compartments take up HBP.

The aims of this study were 1) determine whether circulating HBP can be detected in patients with neutropenia, 2) to determine the half-life of HBP in the blood, and 3) to determine the organs and cell types to which HBP distributes.

Methods

Ethics

Blood sample collection from patients and healthy donors was approved by the Swedish Ethical Review Authority. Cohort 1 had registration number 2015/628; cohort 2 had registration number 2015/828 with change/addition in decision number 2017/27. Pooled blood from healthy donors for extraction of CD14+ monocytes was collected under registration number 2020:24. Blood from healthy donors for extraction of neutrophils and unsorted monocytes was collected under registration number 2013/728. The local Ethical Committee for Animal Research approved the experimental protocol for animal experiments (registration number M143-16).

Patient enrollment

HBP levels are elevated in people with infection and sepsis [3–5] but are generally very low in healthy people (median ranges from 6.3-12 ng/mL in published studies [18–20]). Therefore, we included two different cohorts of neutropenic patients – one with clinically stable patients with few infections (Cohort 1) and one with febrile neutropenia (and therefore more likely to have infections) (Cohort 2).

Cohort 1 consisted of patients from a previously published study [21], which included patients 18 years or older that were eligible for platelet transfusions were recruited from the Haematology Department, Skåne University Hospital in Lund, Sweden, between February and September 2016. All patients gave written informed consent prior to inclusion. Blood samples were drawn before the platelet transfusion was given and were centrifuged at the accredited central laboratory of the hospital and the plasma was stored frozen at –80 °C within 2 h from sample time.

For cohort 2, patients were enrolled from three hospitals in Sweden between 2016 and 2019: Skåne university hospitals in Lund and Malmö and Helsingborg hospital. The inclusion criteria were age of at least 18 years and febrile neutropenia. Blood samples were collected at admission for patients presenting at emergency care, and at onset of fever for patients already admitted to the hospital at the time of inclusion. Informed consent was by an opt-out procedure after sampling and inclusion. Blood samples were centrifuged within 2 hours to obtain plasma, which was then stored frozen at –80 °C.

In both cohorts, neutropenia was defined as a neutrophil count of less than $0.5x10^9$/L or less than $1.0x10^9$/L and decreasing. Only patients with neutropenia were included in this study. In both cohorts some patients had been included up to three times, on separate occasions. Only values from the first inclusion instance were included in this study. Data on clinical parameters, including neutrophil counts and clinical diagnoses, were collected from the patients' medical charts. Values below the detection limit for neutrophil counts (0.1 x 10^9 cells/L) were not reported as a numeric value, and so we replaced them with half of the value of the detection limit (0.05 x 10^9 cells/L) to be able to use them in statistical analyses.

Isolation of CD14-positive monocytes

Primary CD14-positive monocytes were isolated from pooled blood from healthy donors by density gradient centrifugation and magnetic bead separation. Briefly, 10 ml of leukocyte concentrate were diluted 1:1 with 0.9% NaCl. Twenty milliliters of that mixture was layered on to 20 mL of Lymphoprep (AxisShield) and centrifuged for 20 min at 700 x g without brake. Erythrocytes were lysed with pure water for 15 s. Purified mononuclear cells were re-suspended in MACS buffer and CD14 microbeads were added (both Miltenyi Biotec). Cells were then sorted with a LS column using a MACS separator (both Miltenyi Biotec) and re-suspended in serum-free Roswell Park Memorial Institute (RPMI)-1640 medium (ThermoFisher) and then used for further experiments.

Neutrophil and mononuclear cell isolation

Blood was collected from healthy human donors using EDTA anticoagulant. Neutrophils and mononuclear cells were separated from human blood using polymorphprep (Axis-Shield) according to the manufacturer's directions with some modifications as described previously [22]. Briefly, blood was layered on the polymorphprep and centrifuged at 370 x *g* for 30 minutes. The mononuclear cell layer and neutrophil layer were separated and washed with phosphate buffered saline (PBS). Contaminating

erythrocytes were lysed with water for 20 seconds and then washed with PBS. The cell pellet was re-suspended in Hank's buffered saline solution (HBSS) and used for further experiments.

Cell lines

Immortalized human endothelial cells (EA.hy926; American Type Culture Collection/ATCC), primary human lung microvascular endothelial cells (HMVEC-L; Lonza), erythroleukemia cells from a chronic myelogenous leukemia (K562; ATCC), acute myeloid leukemia cells (HNT-34 and MOLM-13; DSMZ-German Collection of Microorganisms and Cell Cultures), lymphoblastic cells from biphenotypic B myelomonocytic leukemia (MV-4-11; ATCC), T-acute lymphoblastic leukemia cells (DND-41; DSMZ-German Collection of Microorganisms and Cell Cultures), and acute monocytic leukemia cells (THP-1; ATCC) were cultured according to the manufacturers' specifications. Endothelial cells were grown to confluence in culture plates and then washed and fresh serum-free media was added. Cells were incubated at 37°C for 24 hours and cell supernatants were collected. All leukemia cell lines, isolated mononuclear cells and isolated CD14-positive monocytes were diluted to a concentration of $1x10^6$ cells/mL in serum-free RPMI-1640 medium (ThermoFisher) and added to culture plates. Cells were incubated at 37°C for 3 hours and then centrifuged to collect supernatants.

Preparation of neutrophil secretory vesicle secretion

The only neutrophil-derived protein in secretory vesicles is HBP [23]. Neutrophils were isolated from three different donors as above. A total of $25x10^6$ cells were re-suspended in Hanks buffered saline solution (HBSS, from Life Technologies) containing calcium and magnesium. Secretory vesicle release was stimulated by crosslinking of CD18 as described [23]. Briefly, samples were incubated with anti-CD18 antibody clone IB4 (Adipogen; 3 µg per 10^6 cells) for 30 minutes at room temperature. Cells were washed twice and then incubated with $F(ab')_2$ fragment of goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) at a 1:20 dilution for 10 minutes at 37°C. The samples were then centrifuged to remove the cells and HBP level in each sample was measured by ELISA prior to use. This solution was diluted and immediately used for injection in rats.

Preparation of total neutrophil lysate

Neutrophils were isolated from six different donors as above. A total of $25x10^6$ isolated human neutrophils were washed and re-suspended in sterile water and incubated for 10 minutes at room temperature. Cells were disrupted by sonication with 4 pulses of 30 seconds each. The samples were then centrifuged to remove cell debris and HBP level in each sample was measured by ELISA prior to use. This solution was diluted and immediately used for injection in rats.

Rat model

Rats and other rodents do not carry a direct homologue of HBP [10, 11], but larger animals such as pigs and dogs, which have an HBP homologue, are ethically complicated and would require a prohibitively

large amount of HBP. A literature search indicated that human HBP can affect rat cells and organs and interact with the plasma proteins from rats suggesting that they carry receptors that interact with HBP (**Table 1**). Therefore we chose to use rats to explore the kinetics and distribution of circulating HBP. Adult male rats (Taconic, 250-270g, 40 rats in total) were used. Animals were treated in accordance with the National Institutes of Health for the Care and Use for Laboratory animals.

Table 1. Effects of human HBP in rats described in the literature

Effect	Reference
HBP enhanced phagocytic activity of macrophages from neutropenic rats	Soehnlein *et al. (2008)* [24]
HBP induced blood-retinal barrier breakdown in rats, blocked by aprotinin	Skondra *et al. (2008)* [25]
HBP expression supposedly detected by immunohistochemistry and northern blot in rat aortic and rat brain endothelial cells	Lee, T. D. *et al.* (2002) [7], Pereira *et al.* (1996) [8]
HBP increased PKC activity in rat brain endothelial cells	Pereira *et al.* (1996) [26]
HBP formed strong complexes with ceruloplasmin from rat, rabbit, dolphin, dog, horse, and mouse plasma	Sokolov *et al.* (2010) [27]

Anesthesia was induced with 4-5% isoflurane (Baxter), then maintained at 3.5% via a mask while the trachea was intubated. Following intubation, the animals were mechanically ventilated and anesthesia was maintained using isoflurane at 1.5% for the duration of the experiment. Body temperature, measured rectally, was maintained at 37°C throughout the experiment. The left femoral vein and the left femoral artery were cannulated. A blood sample was collected from the artery prior to the start of the experiment. Recombinant human HBP (kindly provided by Axis-Shield) at doses of 3 μg/kg (n=4 rats), 6 μg/kg 4 (n=4 rats), 160 μg/kg (n=12 rats) or 320 μg/kg (n=7rats), or neutrophil lysate (containing HBP 3 μg/kg; n=4 rats) or CD18 crosslinking-induced neutrophil secretion (containing HBP 3 μg/kg; n=4 rats) or an equal volume of saline solution (300 μL; n=5 rats) was then injected in a single bolus dose into the left femoral vein. As this was an exploratory study, randomization, blinding, and *a priori* power calculations were not used. Blood samples (150μL each) were collected via the left femoral artery to tubes with citrate anticoagulant and then centrifuged to obtain plasma at the time points shown in each figure. A solution of 10mM EDTA filled the arterial catheter in between blood collection time points longer than 1 minute to prevent coagulation in the catheter. At the end of the experiment urine was collected by squeezing the bladder. Rats were sacrificed at various time points following injections (indicated in the figure legends) and the liver, spleen, kidney, and lung were removed and processed as described below.

HBP ELISA

Human plasma samples were measured in duplicate using an HBP ELISA (Axis-Shield) at a 1:40 dilution according to the manufacturer's directions. According to the manufacturer, the lower limit of detection (LOD) is 5.9 ng/mL in plasma samples when diluted 1:40, which corresponds to an LOD of 0.148 ng/mL for the assay. Rat plasma, organ lysate and urine were first validated in the assay as described in the

supplementary information and in **supplementary tables S1** and **S2**, and used at the dilutions described therein. Any samples that were above the upper limit of quantitation were diluted further until they were within the quantitation range. Because values obtained from the standard curve, any values below the detection limit were used as-is without further processing (i.e. imputation).

Pharmacokinetic analysis of plasma HBP

To calculate the pharmacokinetics of HBP, first all HBP values below the detection limit were removed due to the uncertainty of the measurement below this limit. HBP-values were log-transformed and plotted. Visual inspection of the graphs indicated that the values follow an alpha and beta phase with a distinct change in slope around 10 minutes. Thus, for each rat the beta phase was modeled using a linear regression including time points equal to or greater than 10 minutes, and the coefficients were extracted. Based on this analysis, a prediction of the elimination between 1 and 10 minutes was subtracted from the actual values between 1 and 10 minutes and a linear regression was fitted for the alpha phase. The means of the coefficients for the alpha and beta phase were calculated and transformed into half-lives

For rats injected with 160µg/kg HBP, calculation of the slope was not possible for the beta phase for six of the rats because four of them were sacrificed at 10 minutes after injection for measurement of HBP levels in the organs, and two of them were measured only at late time points where the values had dropped below the detection limit, resulting in a lack of available values. In the primary analysis no calculation was made for the alpha phase for these rats and only the 7 rats with detectable HBP after 10 minutes were used for calculation of the half-lives for the alpha and beta phases. For rats injected with 320µg/kg HBP, all rats were used for calculation of both alpha and beta phases. For all rats we additionally calculated the alpha phase without subtraction of the predicted beta phase.

Preparation of organ lysates

A section of the left lobe of the liver (approximately 1.1 grams), half of the spleen, one kidney and one lung were collected and placed in 600µL of tissue-protein extraction reagent (Thermo Fisher) immediately after removal from the rat and kept on ice. Organs were homogenized using ceramic beads (MoBio) in a TissueLyser (Qiagen). Tissue homogenates were incubated on ice for 30 minutes and then centrifuged to remove tissue debris. Total protein in the lysate was determined by Bicinchoninic acid (BCA) assay kit (Thermo Fisher) according to the manufacturer's directions using bovine serum albumin as the standard. HBP concentrations in rat organ lysates were normalized to total protein levels.

Immunocytochemistry of organs

A section of the left lobe of the liver, half of the spleen, one kidney and one lung were cut into 4 x 4 mm pieces and fixed in 4% paraformaldehyde (Sigma Aldrich) for 48 hours and then transferred to 70% ethanol. Following dehydration, samples were embedded in paraffin (Histolab Products AB). After rehydration and antigen retrieval, tissue sections were incubated overnight with a rabbit antiserum against HBP at a 1:3000 dilution and one of the following antibodies: monoclonal mouse-anti-rat

endothelial cell antigen 1 (RECA-1; Abcam; clone RECA-1; a pan-endothelial cell marker), monoclonal mouse-anti-rat CD68 (Abcam; clone ED1; a pan-monocyte/macrophage marker), monoclonal mouse-anti-rat asialoglycoprotein receptor (ASGR1; Invitrogen; clone 8D7; a hepatocyte marker) at a 1:100 dilution. HBP antiserum from rabbit was kindly provided by Heiko Herwald, originally obtained as described previously [28]. Samples were then stained with secondary Alexa Fluor (AF)-647 conjugated goat-anti-rabbit and AF-568 conjugated goat-anti-mouse Fab2' antibody fragment. Coverslips (Menzel-glazer; #1.5 thickness) were mounted on the samples using mounting media with ProLong Gold anti-fade reagent with 4,6 diamidino-2-phenylindole (DAPI; Life Technologies).

Fluorescence imaging and quantification

Images were obtained using Nikon A1RHD confocal system confocal microscope with a 20x objective. For fluorescence quantification, images of 4-6 different areas of each section were obtained and the total fluorescence (integrated density) in each image was quantified using Image J.

Statistical analysis

We first determined whether data were normally distributed by using a Shapiro-Wilk test and by visual examination of histograms and quintile-quintile (QQ) plots. Data from the patient cohorts were not normally distributed so non-parametric tests were used. Data from rats were normally distributed so parametric tests were used. When comparing medians in the clinical cohorts, Mann Whitney U-test was used, and when comparing relative frequencies then Fisher's exact test was applied. In the rat experiments, one-way ANOVA with *post hoc* Holm-Šídák's multiple comparisons test was used to compare the means. P-values below 0.05 were considered significant.

Results

HBP in neutropenic patients

We enrolled two cohorts of neutropenic patients with low numbers of circulating neutrophils (**Figure 1**), summarized in **Table 2**. In cohort 1, consisting of patients in need of platelet transfusions with chemotherapy-induced bone marrow aplasia and neutropenia, 93% of patients had hematological malignancies and were more often treated with antibiotics and steroids prior to enrollment. In cohort 2, consisting of patients with neutropenic fever, only 60% had hematological malignancies and fewer patients were treated with antibiotics and steroids prior to enrollment. Patients in cohort 2 had higher temperature (38.5 IQR 38.2-38.8 vs 37.2 IQR 36.8-37.4 °C; P<0.0001) and more patients with infection (33 (63%) vs 1 (3%); P<0.0001), indicating that patients in cohort 1 were included in a clinically stable phase while patients in cohort 2 had more acute illness. However median c-reactive protein (CRP) was not significantly different between the two cohorts (63 IQR 29-129 vs 43 IQR 12-101 mg/dL; P=0.251). In cohort 2 there was no significant difference in HBP levels in patients with infection vs with no infection (0.0 IQR 0.0-6.5 vs 0.5 IQR 0.0-4.7 ng/mL; p=0.846)) or in patients with vs without sepsis (0.7 IQR 0.0-13.0 vs 0.16 IQR 0.0-4.6 ng/mL; p=0.528).

Table 2. Characteristics of two cohorts of neutropenic patients

	Cohort 1 (n=41)			Cohort 2 (n=52)		
	HBP < LOD (n=36)	HBP > LOD (n=5)	All patients (n=41)	HBP < LOD (n=42)	HBP > LOD (n=10)	All patients (n=52)
HBP (ng/mL); median (IQR)	0.0 (0.0-1.7)	30 (17-33)	0.0 (0.0-1.8)	0.0 (0.0-1.1)	11 (9.1-17)	0.2 (0-4.9)
Demographics						
Age (years) ; median (IQR)	72 (49-75)	57 (44-70)	56 (44-69)	66 (54-72)	68 (64-70)	66 (55-71)
Females; n (%)	11 (31%)	1 (20%)	12 (29%)	19 (45%)	5 (50%)	24 (46%)
Measurements at time of sampling; median (IQR)						
Temperature (°C)	37.2 (36.7-37.4)	37.0 (36.8-37.1)	37.1 (36.7-37.4)	38.5 (38.2-38.8)	38.5 (38.0-38.9)	38.5 (38.2-38.8)
CRP (mg/L)	40 (12-88)	100 (89-139)	43 (12-101)	69 (32-110)	108 (7-261)	69 (30-129)
Leukocytes (x10^9/L);	0.1 (0.1-0.3)	0.6 (0.2-0.9)	0.1 (0.1-0.4)	0.3 (0.1-0.8)	0.6 (0.2-1.0)	0.4 (0.1-0.9)
Neutrophils (x10^9/L)	0.05 (0.05-0.1)	0.1 (0.1-0.2)	0.05 (0.05-0.1)	0.05 (0.05-0.1)	0.2 (0.1-0.2)	0.05 (0.05-0.1)
Platelets (x10^9/L)	7 (5-10)	25 (8-26)	8 (5-10)	41 (13-115)	33 (23-91)	39 (15-113)
Comorbidities; n (%)						
Cardiovascular disease	2 (6%)	0 (0%)	2 (5%)	8 (19%)	2 (20%)	10 (19%)
COPD	1 (2%)	0 (0%)	1 (2%)	2 (5%)	0 (0%)	2 (4%)
Liver disease	1 (3%)	0 (0%)	1 (3%)	0 (0%)	1 (10%)	1 (2%)
Kidney disease	0 (0%)	0 (0%)	0 (0%)	4 (10%)	0 (0%)	4 (8%)
Diabetes	2 (6%)	0 (0%)	2 (5%)	3 (7%)	2 (20%)	5 (10%)
Infection	0 (0%)	1 (20%)	1 (2%)	24 (57%)	9 (90%)	33 (63%)
Sepsis[1]				8 (19%)	4 (40%)	12 (23%)
Concomitant treatments (prior to inclusion); n (%)						
Steroids	34 (94%)	3 (60%)	37 (90%)	23 (52%)	5 (50%)	27 (52%)
Cytostatic drugs	33 (92%)	2 (40%)	35 (85%)	38 (90%)	10 (100%)	48 (92%)
Antibiotics	34 (94%)	4 (80%)	38 (93%)	26 (62%)	4 (40%)	30 (58%)
Heparin or LMWH	0 (0%)	0 (0%)	0 (0%)	3 (7%)	2 (20%)	5 (10%)
Warfarin	0 (0%)	0 (0%)	0 (0%)	3 (7%)	0 (0%)	3 (6%)
G-CSF	1 (3%)	1 (20%)	2 (5%)	14 (33%)	4 (40%)	18 (35%)
Malignancy; n (%)						
Leukemia	22 (61%)	5 (100%)	27 (66%)	11 (26%)	3 (30%)	14 (27%)
MDS	1 (3%)	2 (40%)	3 (7%)	3 (7%)	0 (0%)	3 (6%)
AML	14 (39%)	2 (40%)	16 (39%)	4 (10%)	3 (30%)	7 (13%)
Other	9 (19%)	1 (20%)[2]	8 (20%)	4 (10%)	0 (0%)	4 (8%)
Myeloma	6 (17%)	0 (0%)	6 (15%)	7 (17%)	0 (0%)	7 (13%)
Lymphoma	3 (8%)	0 (0%)	3 (7%)	8 (19%)	2 (20%)	10 (19%)
Other - solid tumor	0 (0%)	0 (0%)	0 (0%)	14 (33%)	4 (40%)	18 (35%)
Other- no malignancy	3 (8%)	0 (0%)	3 (7%)	2 (5%)	1 (10%)	3 (6%)

LOD = limit of detection (5.9ng/mL); HBP = heparin binding protein; IQR = interquartile range; CRP = C-reactive protein; COPD = chronic obstructive pulmonary disease; NSAID =

non-steroidal anti-inflammatory drugs; LMWH = low molecular weight heparin; G-CSF = granulocyte colony stimulating factor; MDS = myelodysplastic syndrome; AML = acute myeloid leukemia.

[1]Sepsis was defined using Sepsis-3 criteria [29].

[2]This patient had a mixed phenotype acute leukemia.

Even though all patients were neutropenic, in cohort 1 five patients (12 %) had HBP above the lower limit of detection (median HBP 30 ng/mL IQR 17-33 ng/mL) and in cohort 2 ten patients (19 %) had HBP above the limit of detection (11 ng/mL IQR 9.1-17 ng/mL). The numbers of patients with HBP above the lower limit of detection was not significantly different between the two cohorts, p=0.408. In both cohorts, patients with detectable HBP had significantly higher neutrophil counts than did patients with HBP below the limit of detection (Cohort 1: 0.1 IQR 0.1-0.2 vs 0.05 IQR 0.05-0.1 x10^9 cells/L; p=0.032. Cohort 2: 0.2 IQR 0.1-0.2 vs 0.05 IQR 0.05-0.1 ng/mL; p=0.015). However, not all patients with HBP above the detection limit had detectable neutrophils. In cohort 1 and 2 there were 3 and 5 patients respectively with neutrophil counts at or below the detection limit (≤0.1 x 10^9 cells/L). Of these 8 patients, 6 (75%) had acute myeloid leukemia, one (13%) had follicular lymphoma and one (13%) had a non-hematological malignancy (pancreatic cancer). This led us to explore the possibility whether malignant white blood cells, particularly those of myeloid origin, can release HBP.

HBP release by non-neutrophil cell types

To examine the possibility that HBP might be released by non-neutrophil cell types, we measured HBP in the supernatant of various cells. CD14-positive mononuclear cells and cells derived from acute monocytic leukemia (MOLM-13 and THP-1) and biphenotypic myelomonocytic leukemia (MV-4-11) constitutively released detectable levels of HBP into the supernatant. Unsorted mononuclear cells, immortalized endothelial cells (EA.hy 926), primary lung endothelial cells (HMVEC-L) and cell lines derived from acute myelomonocytic leukemia (HNT-34), chronic myelogenous leukemia (K562), and T-cell acute lymphoblastic leukemia (DND-41) did not produce detectable HBP in the supernatant (**Figure 2**).

Kinetics of circulating HBP

To determine how long HBP remains in the circulation, we injected rats with various concentrations of recombinant human HBP. We first injected rats with 64 µg/kg of HBP (**Figure 3A**) and the plasma concentration one minute after injection was 1478 ± 301 ng/mL, which is, in our experience, at the high end of the range of physiological concentrations measured during sepsis and septic shock. The plasma HBP concentration was below the detection limit 15 minutes after the injection in most rats and therefore pharmacokinetic analysis was not possible in these rats. When rats were injected with 160 µg/kg (**Figure 3B** and **Supplementary figure S1**) or 320 µg/kg (**Figure 3C** and **Supplementary figure S2**) of HBP, the concentration decreased rapidly for the first 10 minutes and then more slowly indicating distinct distribution and elimination phases. In rats injected with 160 µg/kg of HBP, the distribution half-life was 8 min and the elimination half-life was 72 min (**supplementary table S3**). Since 6 rats lacked values for

time points past 10 minutes the alpha phase was also calculated without subtraction of the predicted beta phase values thus using all 12 rats. The distribution half-life was then calculated to 4 minutes (**supplementary table S3**). The results for rats injected with 320 µg/kg of HBP were in the same range with a distribution half-life of 14 min and an elimination half-life of 61 min (**supplementary table S4**).

Organ distribution of HBP

To identify the organs that take up HBP, we measured HBP levels in the liver, spleen, kidneys, and lungs in rats injected with 160 µg/kg HBP after 10 min and 60 min. HBP concentrations were measured by ELISA and normalized for the total protein concentration in the lysate (**Figure 4A**). Liver samples had the highest HBP level both at 10 min (55.3 ± 11.9 ng HBP/mg total protein) and at 60 min (42.0 ± 16.1 ng/mg), which was significantly higher than all other organs ($p<0.0001$). The spleen had the second-highest amount of HBP both at 10 min (17.6 ± 5.4 ng/mg) and 60 min (6.9 ± 5.2 ng/mg). The lung and kidney had the lowest HBP levels (kidney at 10 min: 2.3 ± 2.1 ng/mg; kidney at 60 min: 0.8 ± 0.1 ng/mg; lung at 10 min: 0.3 ± 0.1 ng/mg; lung at 60 min: 0.2 ± 0.1 ng/mg). HBP levels at the two time points did not differ significantly in any of the organs. We additionally measured HBP levels in two rats each at 6 hours and 24 hours after the injection and found that HBP levels in the liver and spleen decreased over time were no longer detectable at 24 hours (**Supplementary figure S3A**). We saw a similar organ distribution pattern in rats injected with 320 µg/kg HBP (**Supplementary figure S3B**). Quantification of fluorescence in sections of organs stained for HBP confirmed that the liver had a high level of HBP (**Figure 4B and C**). The HBP level in the other organs was not quantifiable because it was too similar to the background fluorescence level of the tissue (**Figure 4B, controls**).

In urine, HBP was below the limit of detection for rats injected with 160 µg/kg (10 minutes: 0.6 ± 1.2 ng/mL; 60 minutes: 3.2 ± 4.0 ng/mL) and for rats injected with 320 µg/kg (60 minutes: 1.6 ± 1.6 ng/mL).

Low dose and native HBP

Because HBP can affect permeability [14], which could conceivably affect its distribution, we also injected rats with a very low dose of HBP (3µg/kg) that resulted in plasma levels below the detection limit within 5 minutes after injection and was therefore not likely to significantly affect permeability. We found that this low dose of HBP had a similar distribution pattern at 40 minutes after injection (**Figure 4D**). The majority of HBP was found in the liver (1.4 ± 0.5 ng/mg), some was found in the spleen (0.2 ± 0.04 ng/mg), and very little in the kidneys (0.03 ± 0.02 ng/mg) and the lungs (0.09 ± 0.04 ng/mg). Urine from these rats had 0 ng/mL HBP.

To account for the possibility that recombinant proteins may differ from their native counterparts, which may affect their distribution, we also injected rats with native HBP obtained from isolated human neutrophils. We tested HBP released from the secretory vesicle compartment (secreted HBP) and HBP released by lysis of the cells (lysate HBP) (both at 3 µg/kg) (**Figure 4D**). Forty minutes after injection, native HBP was found mainly in the liver (secreted HBP: 1.5 ± 0.3 ng/mg; lysate HBP: 1.0 ± 0.2 ng/mg) with some in the spleen (secreted HBP: 0.4 ± 1.6 ng/mg; lysate HBP: 0.3 ± 0.2 ng/mg), and very little in

the kidneys (secreted HBP: 0.09 ± 0.06 ng/mg; lysate HBP: 0.3 ± 0.2 ng/mg). However, rats injected with native secreted and lysate HBP tended to have more HBP in the lungs (0.43 ± 0.30 and 0.40 ± 0.19 ng/mg respectively) compared to rats injected with recombinant HBP (0.09 ± 0.04 ng/mg), although this difference was not statistically significant (p=0.149 and 0.129 respectively). Urine from these rats had 0 ng/mL HBP.

Cell types associated with HBP

To determine with which cell types take up or bind HBP in the targeted organs, sections of liver (**Figure 5A**) and spleen (**Figure 5B**) taken 10 minutes after intravenous injection with 160µg/kg HBP were stained for human HBP and for rat markers of hepatocytes (ASGPR), endothelial cells (RECA) and monocytes/macrophages (CD68). In the liver, HBP was associated with hepatocytes (ASGPR-positive cells). Endothelial cells (RECA-positive cells) and monocytes/macrophages (CD68-positive cells) were devoid of HBP. In the spleen, strong HBP staining was very occasionally found associated with monocytes/macrophages (CD68-positive cells).

Discussion

In this study we found that, although HBP levels are low in most patients with neutropenia, some patients had detectable levels of plasma HBP. Some monocytic leukemia cell lines and healthy CD14+ monocytes were found to constitutively express HBP. Circulating HBP has a rapid distribution phase where plasma levels drop rapidly for the first 10 minutes, followed by a slower elimination phase. We found that HBP distributes mainly to hepatocytes in the liver and a smaller proportion to monocytes/macrophages in the spleen.

In studies of HBP as a biomarker, neutropenic patients are typically excluded because they are expected to have falsely negative results, however we found that HBP was detectable in a surprisingly large proportion of neutropenic patients, although there was no difference in HBP levels between patients with and without sepsis. In both cohorts patients with detectable HBP had significantly higher neutrophil counts, implicating neutrophils as the main source of circulating HBP. It is likely that even though these patients had low numbers of neutrophils, the present neutrophils were activated and therefore able to produce detectable HBP levels. However, some patients with detectable HBP had neutrophil counts below detectable levels. These patients were mostly afflicted with acute myeloid leukemia, indicating the possibility of malignant myeloid cells as a non-neutrophil source of HBP. Indeed, we found that cell lines derived from malignant monocytic cells constitutively expressed high HBP levels. We also found that healthy CD14+ monocytes constitutively expressed low, but detectable, HBP levels, thus pointing to monocytic cells as a possible source of circulating HBP.

We found that HBP circulates in the blood for a short time, with a distribution half-life below 10 minutes and an elimination half-life between 1-2 hours. This finding is in line with previous observations in humans [12, 13] and in mice [14] which indicated that plasma HBP levels change rapidly between measurements. HBP has pro-inflammatory and vascular permeability-inducing effects that can be

dangerous in situations such as sepsis [6], and therefore rapid removal of HBP from the circulation is likely a protective mechanism against these detrimental effects. Patients with sepsis typically have HBP levels above 30ng/mL and often much higher [3–5], indicating that sepsis patients either have impaired clearance of HBP or that their neutrophils are continuously releasing HBP to keep plasma levels elevated, or both.

We found that HBP distributes mainly to the liver where it is associated with hepatocytes. This is in line with previous findings that plasma HBP is correlated with plasma bilirubin [12]. HBP is an inactive serine protease [30] with several glycan chains, 80% of which are non-sialylated [31]. Many serine protease-serpin complexes are taken up rapidly by hepatocytes via the low-density lipoprotein (LDL) receptor family [32], especially via LDL receptor-related protein 1 (LRP1) which takes up neutrophil elastase [33], a close relative of HBP [30]. On the other hand, non-sialylated glycoproteins are taken up rapidly by hepatocytes via the Ashwell-Morell (asialoglycoprotein) receptor [34]. Thus it is conceivable that HBP could associate with one or both of these receptors. Future studies of specific knockdown of these candidate receptors in hepatocyte cell lines and injection of competing ligands in animal models could shed light on the receptors and intracellular uptake pathways of HBP, but such experiments were beyond the scope of the present study.

We also found that some HBP was present in the spleen where it was associated with monocytes/macrophages, which is in line with previous findings that monocytes can internalize HBP [16]. It is likely that some of the circulating monocytes had internalized plasma HBP and migrated to the spleen [35] which explains why, although we found detectable HBP levels in the spleen, HBP staining was only associated with a small number of monocyte/macrophage cells.

It is somewhat surprising that we found no HBP in the kidney or the urine. Glomerular filtration is size- and charge-selective, preferentially allowing cationic proteins [36] and proteins below 45 kDa [37] to cross the filtration barrier. HBP is highly cationic [30] and has a molecular weight of 35.5 kDa [28] so it would be expected to pass freely through the glomerular filtration system. However, our findings are in line with previous studies showing that, even though elevated plasma HBP is associated with acute kidney injury [38, 39], renal clearance of HBP was found to be very low in healthy individuals and in burn patients, only increasing slightly when renal function was impaired [18]. Together these findings indicate that HBP is not primarily cleared by healthy kidneys.

Implications for HBP as a biomarker

Differentiation of neutropenic fever and neutropenic sepsis is a large clinical challenge. Attempts have been made to stratify patients with neutropenic fever with high risk of complications from those with low risk of complications. The most validated tool for stratification is Multinational Association for Supportive Care in Cancer (MASCC) [40]. Among hematological patients the sensitivity of MASCC has been as low as 58%, the specificity 87%, positive predictive value 84% and negative predictive value 64% [41]. Because of the low numbers of patients with elevated HBP, the two cohorts in this study were likely too small to detect differences in HBP levels in patients with and without sepsis. However, given

that HBP is a promising biomarker in patients with normal neutrophil levels, it could be interesting to explore whether HBP might have an additive effect to the performance of stratification scores for sepsis in larger cohorts of neutropenic patients. This would likely require a far larger cohort to establish relevant cutoff values for HBP in these patients.

The short half-life of HBP has several implications for its use as a biomarker. In the setting of surgery it is advantageous to have a biomarker that rapidly normalizes following the temporary inflammatory stimulus of surgery, making post-surgical measurements less likely to be confounded by falsely elevated results. This was found to be particularly important in cardiac surgery with cardiopulmonary bypass which has particularly high levels of neutrophil activation and inflammation [13]. On the other hand the combination of rapid clearance and rapid release from neutrophils could cause values to fluctuate greatly between measurements, as was observed in a previous study [12] and could therefore affect their interpretability. The kinetics of plasma HBP should therefore be considered when deciding how frequently to measure HBP.

Our finding that HBP distributes mainly to the liver opens the possibility that liver dysfunctions, especially those involving impairment of hepatocyte function, could lead to falsely elevated plasma levels of HBP. Additionally our finding that malignant monocytic leukemia cells can constitutively release HBP indicates an additional potential source of false-positive results. The effect of specific liver diseases and hematological malignancies, and the extent to which they affect plasma HBP levels, should be explored further in appropriate cohorts of patients.

Limitations

This study had several obvious limitations. First, because of the low numbers of patients with elevated HBP, the two cohorts in this study were likely too small to detect differences in HBP levels in patients with and without sepsis. The two cohorts were also collected at different times and we can't exclude differences in sample handling that could have led to different HBP levels. Cohort 1 samples had been freeze-thawed once before while Cohort 2 samples had not, however we have found that HBP levels remain stable over several freeze-thaw cycles so we do not expect this to have a great effect on the results (unpublished in-house data).

Additionally, rats don't possess endogenous HBP so we can't be sure that our findings are applicable to humans. The lack of endogenous HBP may have had some advantage however, as we can be sure that there was no endogenous HBP release interfering with the results. Animals that possess HBP include sheep, pigs, cows, primates, elephants, whales, bats, and their relatives [10, 11] – all of which are problematic (or impossible) to include in laboratory experiments. Studies in sheep or pigs are possible, but these are expensive and more ethically complicated. Therefore, this study is only a first step toward elucidating the clearance pathways of HBP.

We mainly used recombinant HBP which may be processed differently than native HBP in neutrophils. Native HBP undergoes several proteolytic and glycosylation steps [28], and is stored in both azurophilic

granules and secretory vesicles [42], which contain different glycosidases [43]. Additionally sialic acids can undergo spontaneous modifications [44] which could affect the results. Therefore recombinant HBP could have different glycosylation patterns than native HBP. We partially addressed this limitation by examining the organ distribution of native HBP derived from both secretory vesicles and whole cell lysate, and by applying minimal handling of the samples (i.e. we did not purify HBP) to reduce the possibility of spontaneous modifications. However we were unable to extract sufficient amounts of HBP to be able calculate the pharmacokinetics of native HBP in this study.

Finally, the pharmacokinetic studies were intended only to determine the approximate lifespan of circulating HBP, so they were done in only a few animals. Samples were taken at varying time points because we used the same rats for various exploratory analyses in order to reduce the total number of rats needed for the study. This variation in sampling times also may have affected the analysis. Thus, the calculations should be carefully interpreted, and as a result we do not focus on the exact values but rather on relative time scale. In spite of these limitations our findings indicate a very quick distribution phase and an elimination phase with a half-life of 1-2 hours, which is consistent with prior findings in the literature.

Conclusions

HBP can be found in some neutropenic patients. Malignant myeloid cells and monocytic cells may be a source of HBP, in addition to neutrophils. HBP disappears rapidly from the circulation and distributes primarily to liver hepatocytes, with some uptake into monocytes/macrophages in the spleen. The impact of hematologic malignancies and liver diseases on plasma HBP levels should be explored future in clinical studies.

Declarations

Ethics approval and consent to participate

Blood sample collection from patients and healthy donors was approved by the Swedish Ethical Review Authority. Cohort 1 had registration number 2015/628; cohort 2 had registration number 2015/828 with change/addition in decision number 2017/27. In cohort 1 all patients gave written informed consent prior to inclusion while in cohort 2 informed consent was obtained by an opt-out procedure after sampling and inclusion. Pooled blood from healthy donors for extraction of CD14+ monocytes was collected under registration number 2020:24. Blood from healthy donors for extraction of neutrophils and unsorted monocytes was collected under registration number 2013/728. The local Ethical Committee for Animal Research approved the experimental protocol for animal experiments (registration number M143-16).

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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PB: The Anna and Edwin Berger Foundation, ALF grant # 86626

Authors' contributions

JF conceptualized the study, carried out experiments, analyzed and interpreted the data, and wrote the first draft of the manuscript. FK carried out pharmacokinetic analyses. EW carried out immunofluorescence analyses of rat organs. PG and LM provided plasma samples and clinical data for cohort 2 and aided in analysis of the results. TK provided the plasma samples and clinical data for cohort 1 and aided in analysis of the results. PB aided in conceptualization and analysis of the animal experiments. AL aided in conceptualization and analysis of all parts of the study. All authors contributed to interpretation of the data and editing of the manuscript. All authors read and approved the final manuscript.

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Figures

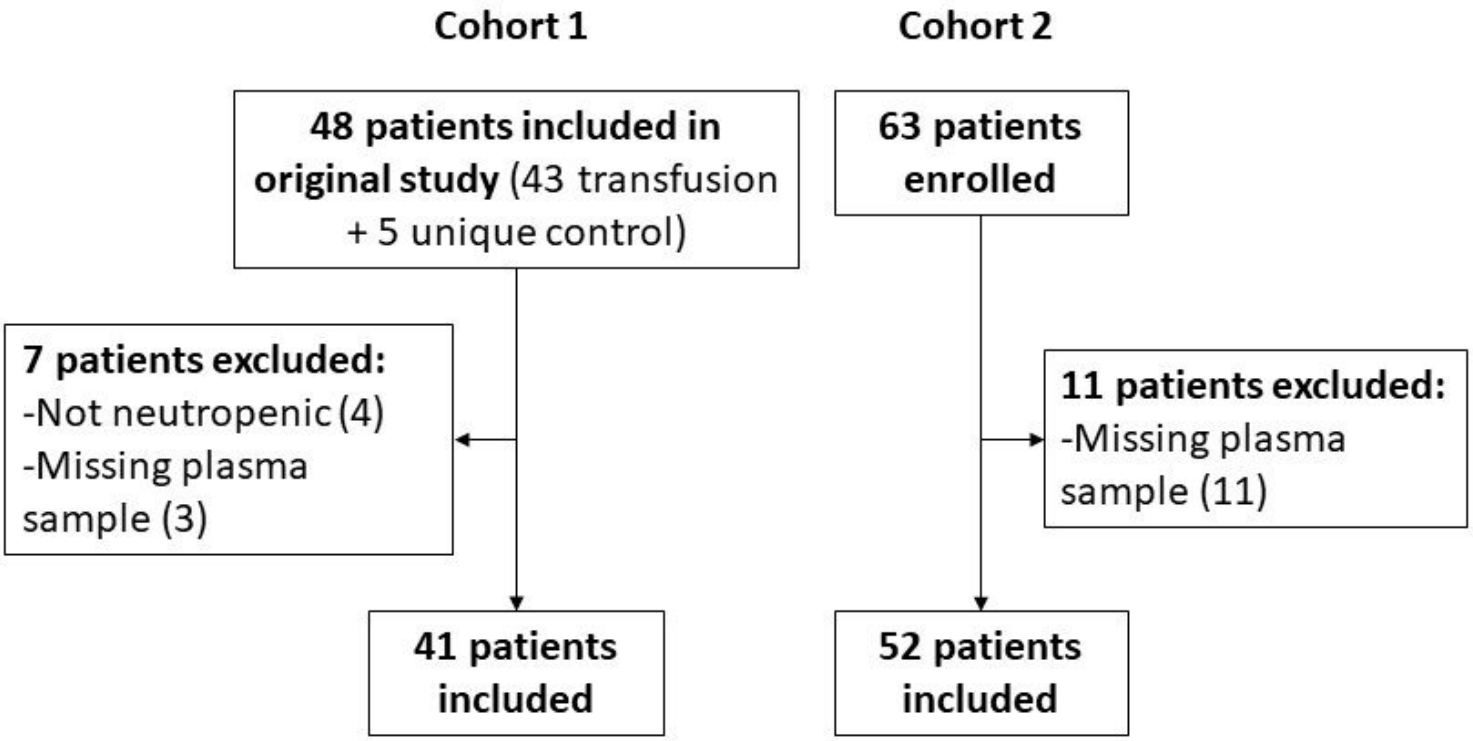

Figure 1

Flow chart showing patient enrolment, exclusion, and inclusion of patients in the present study.

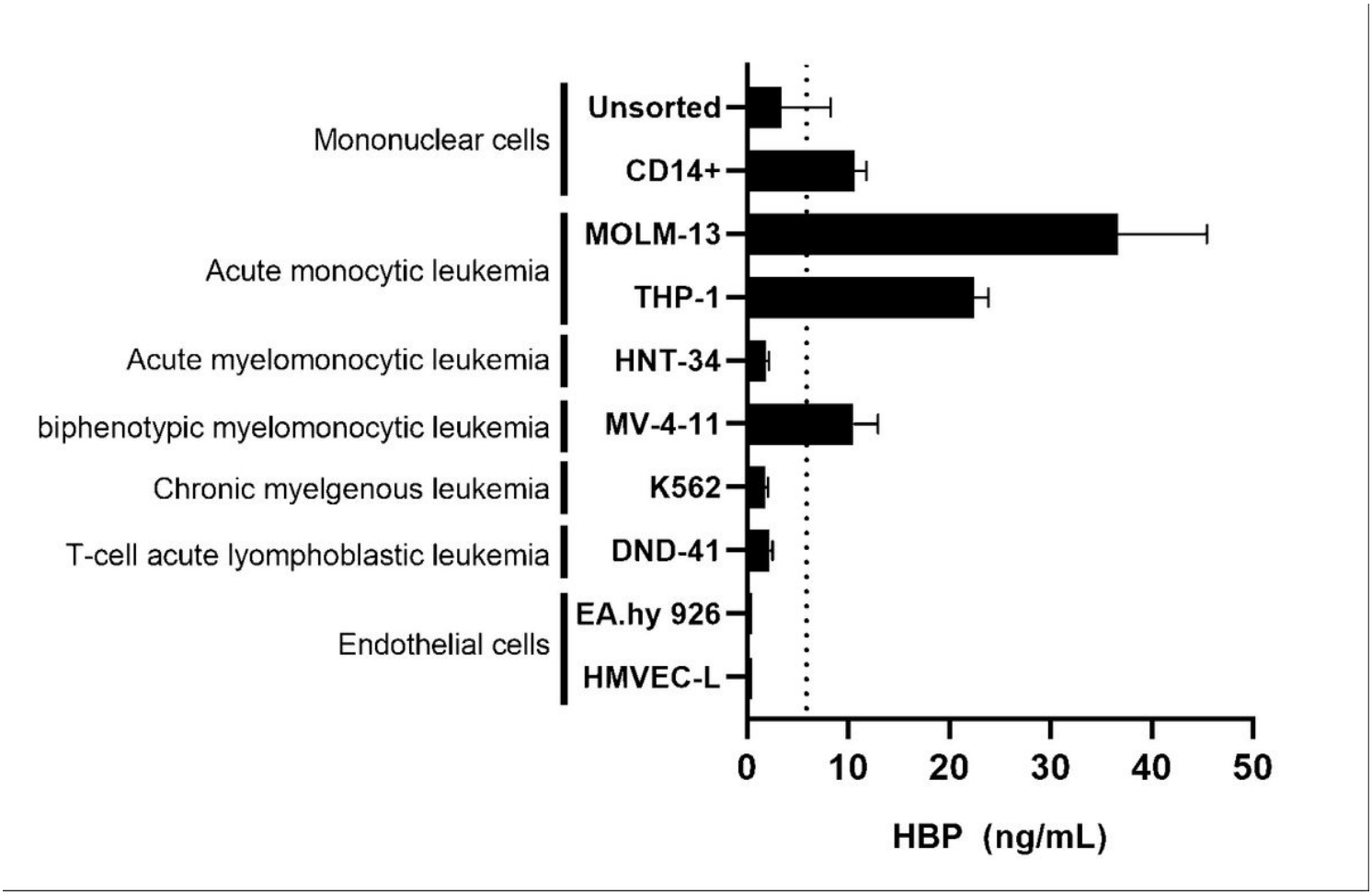

Figure 2

Release of HBP by non-neutrophil cell types. Various cell lines or primary cells were incubated for three hours at 37 °C and supernatants were collected and HBP concentration was measured. Data are the mean and whiskers are standard deviation (n=4 replicates). Dashed line is the detection limit of the ELISA (5.9 ng/mL at sample dilution of 1:40). Bars are the mean and whiskers are standard deviation, n=4 experiments in each group, except for CD14+ monocytes which were n=2 pooled donor blood bags.

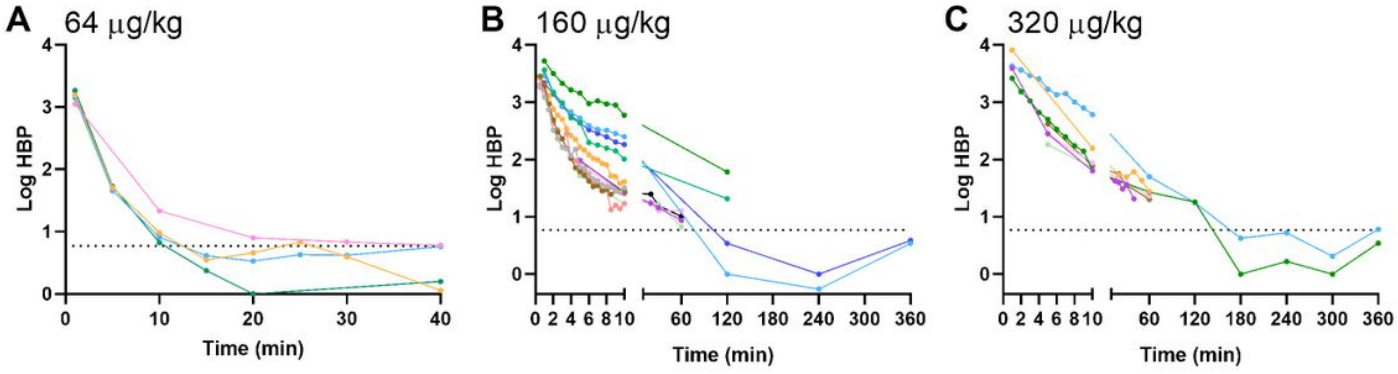

Figure 3

Plasma HBP over time. Plasma HBP levels over time in rats injected intravenously with recombinant human HBP at a dose of A) 64 µg/kg, B) 160 µg/kg and C) 320 µg/kg. Data points from each individual rat are shown connected with a colored line. The dashed line indicates the lower limit of detection (5.9 ng/mL).

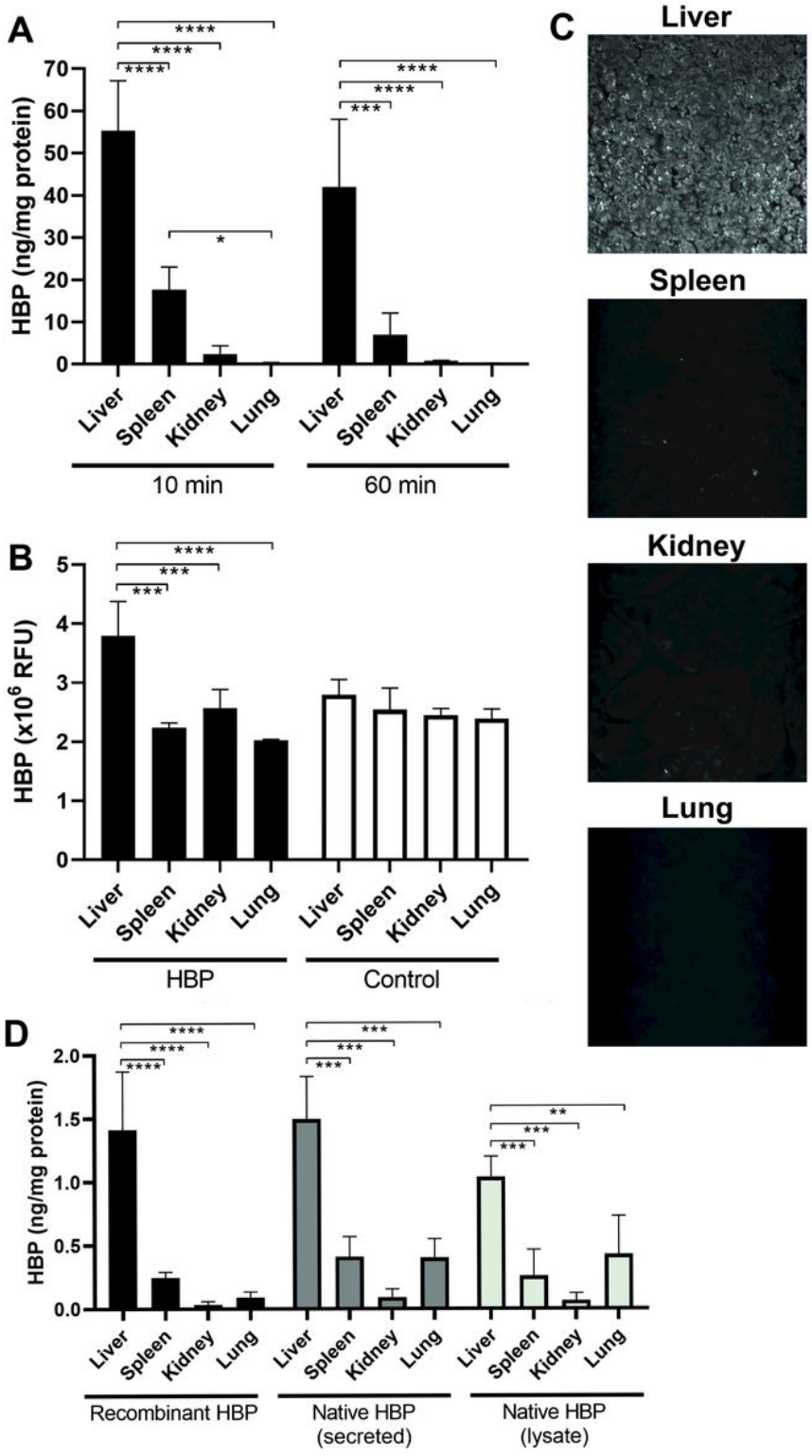

Figure 4

Circulating HBP primarily distributes to the liver and spleen. A-C) Rats were injected with recombinant human HBP at a dose of 160 µg/kg. A) HBP levels normalized for total protein concentration in lysates of liver, spleen, kidney, and lung 10 and 60 minutes after injection. B) Immunofluorescence quantification of HBP levels in tissue sections, in relative fluorescence units (RFU). C) Representative fluorescence images of organ sections stained for HBP. D) HBP levels at 40 minutes after injection of HBP at a dose of 3 µg/kg normalized for total protein concentration in lysates of liver, spleen, kidney, and lung from rats injected with either recombinant HBP, native HBP secreted from neutrophil secretory vesicle (secreted HBP), or native HBP from total neutrophil lysate (lysate HBP). Bars are the mean and whiskers are standard deviation, n=4 in each group. Means were compared using one-way ANOVA with post hoc Holm-Šídák's multiple comparisons test.

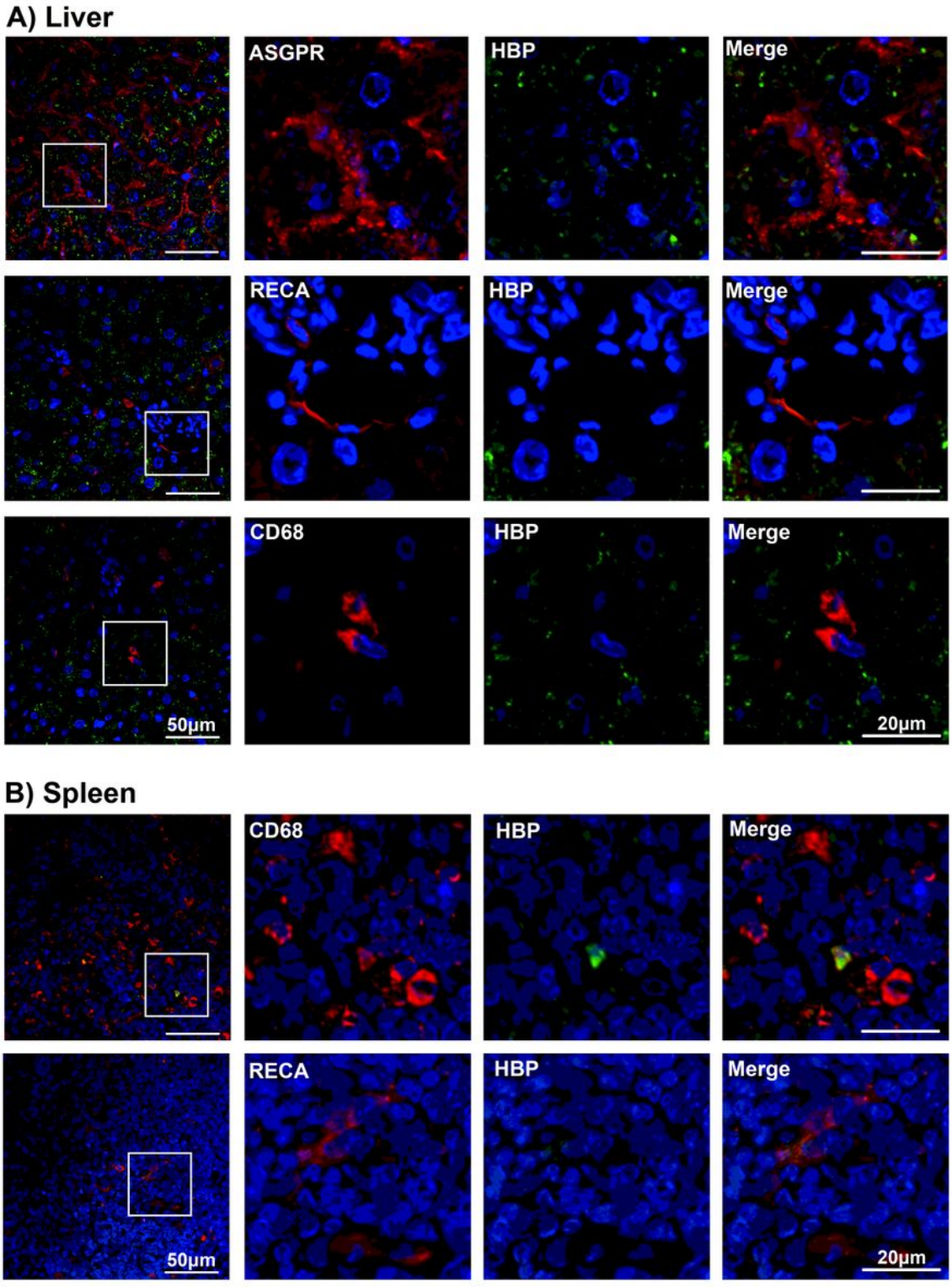

Figure 5

HBP in the liver is associated with hepatocytes in the liver and some macrophages in the spleen. Ten minutes after intravenous injection of 160µg/kg HBP, A) liver and B) spleen sections were stained for HBP (green) and general markers for hepatocytes (ASGPR), endothelial cells (RECA) and monocytes/macrophages (CD68) in red. DNA was visualized by DAPI in blue.

Supplementary Files

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