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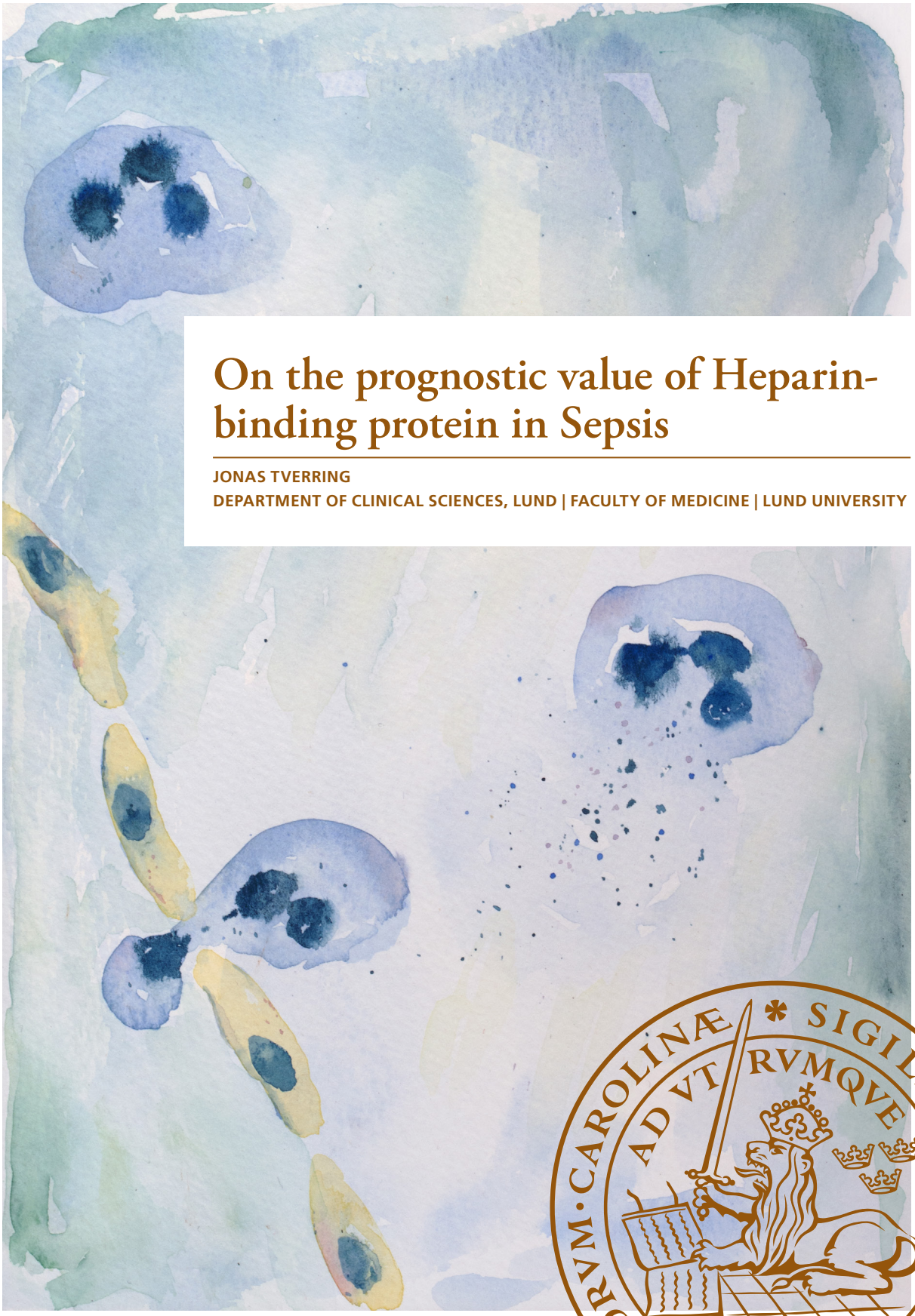

On the prognostic value of Heparin-binding protein in Sepsis

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On the prognostic value of Heparin-binding protein in Sepsis

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Abstract

Sepsis causes major morbidity and mortality worldwide. There is wide consensus that biomarkers have an important role in sepsis research and patient care. Heparin-binding protein (HBP) is a neutrophil-derived, proinflammatory and vascular permeability-inducing protein and a promising novel sepsis biomarker. In this thesis, we investigate some aspects of the prognostic accuracy and added value of single and repeated HBP measurements in plasma at the emergency department (ED) and the intensive care unit (ICU) regarding infection-related organ dysfunction (OD) and sepsis survival.

In Paper I, we conducted a prospective, observational, convenience sample study (NCT02366650) and recruited patients with affected vital signs regardless of infection suspicion at the ED of four centres in three countries in 2015 to 2016. Among 524 included patients, we found that plasma HBP on ED admission had an area under the receiver operating characteristics curve (AUC) of 0.73 (95% CI: 0.68-0.78) to discriminate the pre-specified primary endpoint of infection-induced OD within 72 hours compared to an AUC of 0.82 (0.78-0.86) for C-reactive protein and 0.69 (0.64-0.74) for procalcitonin.

In Paper II, we did a *post hoc* study including patients with severe sepsis or septic shock and available plasma samples on ICU admission from the prospective, observational FINNAKI study conducted in 17 Finnish ICUs in 2011 to 2012. In a total of 511 patients, addition of plasma HBP to a prediction model including age, simplified acute physiology score II, and creatinine 48 hours pre-ICU increased the AUC from 0.78 (0.73–0.84) to 0.82 (95% CI: 0.77–0.87) regarding the primary endpoint of acute kidney injury (AKI) stage 2-3 from 12 hours up to 5 days.

In Paper III, we conducted an observational, convenience sample study recruiting patients with suspected septic shock at two general mixed ICUs in Skåne, Sweden, and sampled patients for plasma from ICU admission and every 4 hours for 3 days. Among 24 included patients, we found that plasma HBP is highly variable in concentration between 4-hour measurements and that every 100 ng/mL increase in HBP (range 0 to 932 ng/mL) corresponded to 1.4 mmHg decrease in mean arterial pressure in a linear mixed-effects model adjusted for time, noradrenaline dose and vasopressin use (95% CI: -1 to -2.3 mmHg, p=0.04).

In Paper IV, we performed another *post hoc* investigation of the FINNAKI study, this time including longitudinal sampling for plasma HBP up to seven times during the first five days (hour 0, 12, 24, 36, 48 and day 3 and 5). We pre-published a statistical analysis plan (ISRCTN15560762). In a total of 652 patients, we found that longitudinal HBP adds a small but statistically significant prognostic value to a prediction model (including age, sex, functional performance pre-ICU, sequential organ failure assessment score, lactate and pre-existing chronic health conditions) regarding the primary endpoint of 90-day survival in a complete case analysis (HR 1.06, 95% CI: 1.01 to 1.12, n=576, p=0.019) and in a *post-hoc* analysis using multiple imputation and nonlinear HBP over time (HR 1.26, 95% CI: 1.11 to 1.43, p<0.001) but not in the pre-specified analysis using multiple imputation and linear HBP over time (HR 1.11, 95% CI: 0.93 to 1.31, p=0.245).

In summary, the findings from this thesis indicate that plasma HBP does not outperform current biomarkers to prognosticate infection-induced OD within 72 hours in patients in the ED with and without suspected infection, that plasma HBP can add prognostic value to known risk factors regarding development of sepsis-related AKI in the ICU, that repeatedly measured plasma HBP concentrations can be highly variable over time during sepsis and that longitundinal HBP adds little prognostic value to known risk factors regarding 90-day survival in ICU patients with sepsis.

Key words: Sepsis; Biomarker; Heparin-binding protein; Diagnostic accuracy; Added value

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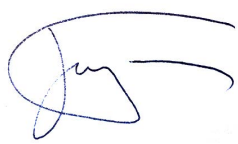
Signature  Date 15th Dec 2021

On the prognostic value of Heparin-binding protein in Sepsis

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“Study hard what interests you the most in the most undisciplined, irreverent and original manner possible”

— Richard Feynmann, 30 November 1965

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Abbreviations

α	Significance level
ß	Statistical power
AIC	Akaike information criterion
AKI	Acute kidney injury
AUC	Area under the receiver operating characteristic curve
APACHE	Acute physiologic assessment and chronic health evaluation
ARDS	Acute respiratory distress syndrome
CAP37	Cationic antimicrobial protein of 37 kD
cfNRI	Category-free net reclassification index
CI	Cardiac index
CRP	C-reactive protein
CSF	Cerebrospinal fluid
ELISA	Enzyme linked immunosorbent assay
FP	False positives
FPF	False positive fraction
HBP	Heparin-binding protein
HDU	High dependency unit
HR	Hazard ratio
IDI	Integrated discrimination improvement
ISRCTN	International standard randomised controlled trial number
LMWH	Low-molecular weight heparin
LR+	Positive diagnostic likelihood ratio
LR-	Negative diagnostic likelihood ratio
LR $\chi 2$	Chi square likelihood ratio
MAP	Mean arterial pressure
NEJM	New England journal of medicine
NA	Noradrenaline
NPV	Negative predictive value

NRI	Net reclassification index
OD	Organ dysfunction
OR	Odds ratio
P	Prevalence
PCT	Procalcitonin
POC	Point-of-care test
PPV	Positive predictive value
R_{var}	Variance of predicted risk
RCT	Randomised controlled trial
SAPS	Simplified acute physiology score
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
SVRI	Systemic vascular resistance index
TP	True positives
TPF	True positive fraction
qSOFA	Quick SOFA score

Glossary

Sepsis	refers to the sepsis-3 definition (1)
Severe sepsis	refers to the sepsis-2 definition (2)
Septic shock	refers to the sepsis-3 definition if mentioned alone or the sepsis-2 definition if mentioned together with severe sepsis

List of papers in thesis

I. Kahn F, **Tverring J**, Mellhammar L, Wetterberg N, Bläckberg A, Studahl E, Hadorn N, Kahn R, Nueesch S, Jent P, Ricklin ME, Boyd J, Christensson B, Sendi P, Åkesson P, Linder A. Heparin-Binding Protein as a Prognostic Biomarker of Sepsis and Disease Severity at the Emergency Department. Shock. **2019** Dec;52(6):e135-e145.

II. **Tverring J**, Vaara ST, Fisher J, Poukkanen M, Pettilä V, Linder A; FINNAKI Study Group. Heparin binding protein (HBP) improves prediction of sepsis-related acute kidney injury. Ann Intensive Care. **2017** Oct 18;7(1):105.

III. **Tverring J**, Nielsen N, Dankiewicz J, Linder A, Kahn F, Åkesson P. Repeated measures of Heparin-binding protein (HBP) and procalcitonin during septic shock: biomarker kinetics and association with cardiovascular organ dysfunction. Intensive Care Med Exp. **2020** Sep 10;8(1):51.

IV. **Tverring J**, Vaara ST, Torisson G, Fisher J, Poukkanen M, Nielsen N, Pettilä V, Linder A; FINNAKI Study Group. Added value of Heparin-binding protein in prognosticating 90-day survival in intensive care unit patients with sepsis. [Unpublished manuscript]. **2021**.

List of papers not in thesis

Tverring J, Åkesson A, Nielsen N. Helmet continuous positive airway pressure versus high-flow nasal cannula in COVID-19: a pragmatic randomised clinical trial (COVID HELMET). Trials. **2020** Dec 3;21(1):994.

Mellhammar L, Linder A, **Tverring J**, Christensson B, Boyd JH, Sendi P, Åkesson P, Kahn F. NEWS2 is Superior to qSOFA in Detecting Sepsis with Organ Dysfunction in the Emergency Department. J Clin Med. **2019** Jul 29;8(8):1128.

Mellhammar L, Linder A, **Tverring J**, Christensson B, Boyd JH, Åkesson P, Kahn F. Scores for sepsis detection and risk stratification - construction of a novel score using a statistical approach and validation of RETTS. PLoS One. **2020** Feb 20;15(2):e0229210.

Forsvall A, Fisher J, Cardoso JFP, Wagenius M, **Tverring J**, Nilson B, Dahlin A, Bratt O, Linder A, Mohanty T. Evaluation of the Forsvall biopsy needle in an ex vivo model of transrectal prostate biopsy - a novel needle design with the objective to reduce the risk of post-biopsy infection. Scand J Urol. **2021** Jun;55(3):227-234

Ingefors E, **Tverring J**, Nafaa F, Jönsson N, Karlsson Söbirk S, Kjölvmark C et al. Low 30-day mortality and low carbapenem-resistance in a decade of Acinetobacter bacteraemia in South Sweden. Infection Ecology and Epidemiology. **2022**;12(1):1-9.

1 Thesis introduction

"Worldwide, sepsis is one of the most common deadly diseases, and it is one of the few conditions to strike with equal ferocity in resource-poor areas and in the developed world. When sepsis is quickly recognized and treated, lives are saved but health care providers need better training because they are the critical link to preventing, recognizing and treating sepsis."

- K Reinhart, NEJM, 2017 (3)

Infectious diseases have posed a threat to the well-being of man since the very beginning. Major progress towards controlling infections appeared late in human history and was dominated by improved living standards, hygiene, pasteurisation and vaccine development. With the introduction of antibiotics around the 1950s it was the perception of some western leaders that the fight against infections had been won. Government and pharmaceutical investments declined accordingly, and funding was concentrated to research on cardiovascular disease and cancer. There has been a partial swing of the pendulum in the last few years as the epidemiology and burden of infectious diseases have been clarified (4). The World Health Organization declared sepsis a Global Health Priority in 2017 (3).

One persisting challenge in sepsis care is to discriminate which individuals will go on to develop severe disease. This was the focus of paper I in the thesis. We measured the concentration of Heparin-binding protein (HBP) in a blood test in a study recruiting participants at the emergency department and estimated the accuracy to prognosticate sepsis development. It is common practice to use infectious biomarker in this way, to add to the prognostic information from symptom history and vital signs. Estimating this, the size of HBP's added prognostic value, was done in paper II and paper IV. It is also common practice to measure biomarkers repeatedly over a few days to visualise trends over time as an added source of prognostic information. This was the focus of paper III and IV for HBP. Paper II focused on the special case of sepsis-related acute kidney injury (AKI) prognostication in the intensive care unit. Another persisting challenge in sepsis research is that of novel immunomodulatory therapies. This is not the focus of any

of the included papers. But this subject is so intimately connected to the current concept of sepsis and to the potential future use of biomarkers that it will appear both in the thesis introduction and in the discussion.

There is wide consensus that biomarkers are important in the care of patients with sepsis and in sepsis research. More than 250 biomarkers have been investigated for sepsis prognostication but only two are in widespread clinical use in Europe and North America (procalcitonin and C-reactive protein) (5). The key explanation is the inherent complexity of sepsis pathophysiology, but it also suggests an inadequacy in the design of hitherto performed biomarker studies. Only time will tell if HBP will be the third biomarker in sepsis to advance from bench to bedside.

The thesis is dispositioned to begin with a three-part introduction. It starts out with a perspective of what the sepsis entity is today and how it came to be. Next is a detailed account of what we knew about HBP prior to this thesis. Thirdly there is an overview of classical and contemporary statistical concepts for prognostic biomarker evaluation. Next is a summarized account of the aims, rationale, methods and results of the four papers in the thesis. Lastly there is a discussion. This begins in the narrow, focusing on future perspectives for HBP research based on the findings from this thesis and end in a wider discussion on the nuances and consequences of the sepsis definition and current guidelines and the role of biomarkers in sepsis research.

2 Introduction to sepsis

It is possible that we do not understand the epidemiology and pathogenesis of sepsis well enough to do a good study.

- RC Bone, Crit Care Med, 1995 (6)

2.1 Modern definitions

2.1.1 Roger C Bone (1941-1997)

The modern sepsis definition is essentially based on the thinking of a critical care physician named Roger C Bone (1941-1997). Bone published a randomised controlled trial (RCT) on high dose methylprednisolone in sepsis in the New England Journal of Medicine (NEJM) in 1987 (7). The inclusion criteria for this trial became in essence the modern definition of sepsis when the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SSCM) Consensus Conference Committee, led by Bone, published their conclusions in 1992 (8). Bone and others had recognized that previous sepsis trials like that of Schumer *et al.* in 1976 (9) had included very heterogenous study populations. This hampered the interpretation of study results and were due to inconsistent or lacking sepsis definitions they argued (10). Bone proposed that bacteraemia be defined by a positive blood culture, that septicaemia no longer be used and that sepsis be defined as a suspected infection plus the systemic response to an infection, which in turn was defined by tachycardia, tachypnoea and hypothermia or hyperthermia (11). Leucocytosis was added in the consensus paper to form the systemic inflammatory response syndrome (SIRS). SIRS was said to appear under many conditions but be termed sepsis when caused by an infection (8). The consensus paper further added the term severe sepsis to be used when sepsis was associated with an organ dysfunction (defined by lactic acidosis, oliguria or acute alteration of mental status)

and a subgroup thereof, septic shock, as defined by the presence of sepsis-induced hypotension (8). The authors made clear that the definitions should be updated as new knowledge on sepsis was gained.

The 1992 definitions were naturally influenced by the conceptual ideas of the pathogenesis of severe infections at the time, where endotoxin-induced hyperinflammation was the prevailing explanatory model (12). Bone recognized already in 1996 that this represented a one-sided and flawed model of the pathogenesis of sepsis and that anti-inflammatory signalling was just as prevalent during severe infections as pro-inflammatory drivers, often occurring as a "mixed antagonist response syndrome" (13). He further recognized that, even though the definition had been readily adopted by the scientific community and led to more homogenous inclusion criteria for clinical trials, the resulting trial populations were still highly heterogenous. He concluded that this had surely contributed to the repeatedly non-beneficial outcomes of several recent interventional trials in sepsis (14-17). He further stated that "it is possible that we do not understand the epidemiology and pathogenesis of sepsis well enough to do a good study" (6). Bone regrettably passed away two years later.

2.1.2 Sepsis-2 and sepsis-3

Despite a growing discontent with SIRS (18), a renewed conference meeting in 2001 did not find support to revise the original 1992 definition beyond a slight update of some physiological cut-offs ("sepsis-2"). They also did not add the use of sepsis biomarkers as had been proposed (2). In 2016 however, there was a major update to the definitions, aptly named sepsis-3 (1). The arguments for a revision of the definition were that SIRS often occurred outside of severe infections, were too often not present during severe infections, were not necessarily correlated to severe illness and that recent advances into the understanding of the pathogenesis of sepsis were more complex than indicated by SIRS. SIRS had also been constructed wholly without the support of data, where sepsis-3 would be data-based. Singer *et al.* stated that "Sepsis should be defined as life-threatening organ dysfunction caused by a dysregulated host response to infection" (1). The authors removed the phrasing *severe* sepsis, stating that all sepsis should be considered severe, and simplified the physiological definitions of organ dysfunction (OD) by adopting Vincent *et al.*'s sequential organ failure assessment (SOFA) score (19, 20), which was also data-based. The new definition implies a shift from easily recognisable bedside vital signs into more blood-sampling-heavy markers of organ dysfunction. It is appreciable from the early writings of Bone that the sepsis definition had a double rationale to him, by both enabling RCT uniformity and improving sepsis recognition This is now lost in the new definition, in exchange for a closer correlation to the outcome of sepsis. The sepsis-3 authors tried to ameliorate this through the construction of a quick SOFA score (qSOFA) based on altered mentation,

tachypnoea and hypotension. qSOFA's performance has since been discredited (21) which may have been based on a flawed statistical approach. They used data-driven identification of the few variables that were most strongly correlated to the outcome, which inevitably favours specificity, when early sepsis recognition should prioritize sensitivity and hence include non-dichotomized predictors for all organ systems. Because other early warning scores (e.g., National Early Warning Score 2) outperform both qSOFA and SIRS in this regard (22), and for its data-based transparency and relative simplicity, the shift from SIRS to SOFA appear reasonable. The SOFA score also offers a staging for sepsis severity, beyond the dichotomy of earlier definitions, which was asked for in the 1992 consensus paper (8).

2.2 Areas of progress and frustration

We have seen an increased interest in sepsis in the last couple of decades. This has been true across health care systems (23), among decision-makers (24), in international organisations (3, 25) and in the general public (26). This increased attention to sepsis is both timely and appropriate when considering the relatively low government spending into sepsis research (27) compared to the high costs (28) and extensive disease burden (4). This intensified effort in sepsis research has been successful in some aspects more than others.

2.2.1 Improved supportive care

Knowledge on the optimal supportive care for critically ill patients with sepsis have improved (29). This includes randomised controlled trials supporting balanced crystalloids as the best initial resuscitation fluid (30, 31), a target mean arterial pressure no higher than 65 mmHg (32, 33), the lack of benefit from early goal directed therapy (34), a recommended lower tidal volume ventilation strategy (35, 36) and benefit from prone ventilation (37), a recommended restrictive haemoglobin concentration transfusion trigger of 70 g/L (38) and a moderately high glucose control target of ~ 8-10 mmol/L (39) to name a few. The survival rates of patients with sepsis in the intensive care unit (ICU) appear to be steadily improving in conjunction with this increasing knowledge based on data from Australia and New Zealand in 2000 to 2012 (40). This is however contrasted by more recent data from Wales which indicates that no such improvement could be seen in the general wards (41). The reason for this inconsistency is surely multifaceted, but it could arguably be connected to the heavy focus on critical care in sepsis research and sepsis guidelines (42). This is in spite the fact that the majority of hospitalized patients with sepsis are treated outside of the ICU and often suffer from higher mortality rates (43).

2.2.2 Immunomodulation and sepsis subtypes

We have unfortunately witnessed a continued series of non-beneficial trials testing novel immunomodulatory therapies in sepsis in the last couple of decades. This is in spite of a rise in interest from the pharmaceutical industry and a wide array of tested hypothesis, both trying to enhance and to antagonize the host response, with more than 100 tested substances (44). The predominant explanation for this has not changed since the writings of Bone from more than 20 years ago; the sepsis population is too heterogenous (44, 45). The current movement in sepsis research to differentiate which subpopulation of sepsis patients may benefit from which intervention is the construction of sepsis sub-pheno/endo-types/classes (46). These subtypes are often based on very large sets of data, including omics-technology and use advanced statistical approaches like machine learning to derive two or more subclasses (46). Ironically, sepsis subtypes are suffering from heterogeneity issues themselves (47). There are single *post-hoc* examples where endotypes have succeeded in stratifying treatment response, such as the study by Wong *et al.* focusing on paediatric sepsis and corticosteroid therapy (48). The subtype-approach is relatively new and may prove successful but still has everything left to prove. A major challenge is that patients can often change which endotype they belong to during the course of illness (49).

2.3 The role of biomarkers

As previous investigations have made clear, there will be no holy grail biomarker to perfectly prognosticate sepsis and predict the outcome of different treatments (50). Based on the complexity of sepsis, it is reasonable to consider multiple biomarkers for different purposes, sometimes in combinations as biomarker panels (5). The different roles of biomarkers can be divided into at least 8 different categories according to the FDA-NIH Biomarker Working Group (51) and are listed in Table 1 together with an explanation and an example from sepsis or infectious diseases.

Table 1. Potential roles of biomarkers in sepsis.

PURPOSE	EXPLANATION	EXAMPLE	APPLICABLE IN SEPSIS	RELEVANCE FOR HBP
Susceptibility	Potential for developing a disease	INR levels for sepsis susceptibility (52)	Yes	No
Prognostic	Likelihood of clinical event	Lactate for death from sepsis (53)	Yes	Yes
Diagnostic	Confirm prescence of disease	Glactomannan for Aspergillosis diagnosis (54)	No	No
Predictive	Identify likelihood for positive or negative drug response	CRP levels for corticosteroids in COVID-19 (55)	Yes	Maybe
Response	Biologial drug response	Blood culture negativity for s. aureus bacteremia (56)	Yes	Probably not
Monitoring	Repeated disease status assessment	PCT for antimicrobial response (57)	Yes	Maybe
Safety	Likelihood of risk for drug toxicity or adverse event	Serum creatinine for risk of renal failure from vancomycin (58)	Yes	Probably not
Surrogate endpoint	Strong mechanistic or epidemiological rationale for endpoint correlation	Sputum culture negativity for resolution of Tuberculosis (59)	Maybe	No

CRP; C-reactive protein, s. aureus; Staphylococcus aureus, INR; International Normalized Ratio, PCT; Procalcitonin

As is clear from the examples in Table 1, we are already using multiple biomarkers for different purposes in sepsis and the statement that only CRP and PCT are in widespread clinical use is a relative truth depending on your definition of an infectious biomarker. The foremost demand in the clinic is for better *prognostic* biomarkers to enable early discrimination of which individuals risk developing severe disease. Prognostic markers have also been used for trial participant selection (60). A major desire in interventional sepsis research is to find *predictive* biomarkers that can differentiate which patients benefit more from which [immunomodulatory] treatment (61). The current biomarkers CRP, PCT and lactate are already used in the clinic as *response* or *monitoring* biomarkers, or even as *surrogate endpoints*, and appear relatively capable, even though they have not been proved to contribute to survival (62, 63). *Diagnostic* biomarkers as described by the FDA-NIH group is not relevant for sepsis or at least redundant, because diagnosing sepsis has no gold standard other than the readily available clinical and laboratory characteristics that defines it. Differentiating bacterial from viral infection and infection from inflammation is however highly desirable and have been shown for PCT (64, 65).

2.4 Acute kidney injury

2.4.1 Background

Acute kidney injury (AKI) is common in sepsis. It occurs in approximately one fifth of hospitalised patients with severe sepsis (based on ICD codes) (66) and in about half of patients with severe sepsis in the ICU (67). Sepsis is also the most common cause of AKI among the critically ill (68). AKI is associated with increased hospital mortality (66), long-term mortality (69) and progression in chronic kidney disease (70). The current definition for acute kidney injury (AKI) comes from the Kidney Disease Improving Global Outcomes (KDIGO) initiative (71). AKI is staged from 1 to 3 based on an increase from baseline serum creatinine and a low urine output. Both creatinine and urine output are recognised to be late markers of kidney distress. The former have to be accumulated after a decrease in filtration before an increase is identifiable in serum and the latter have to be tightly observed for 6-12 hours to enable diagnosis according to the definition (71). There is a wide consensus that earlier markers for kidney distress are needed, "the troponin of the kidney", to enable earlier recognition and timely intervention (72).

2.4.2 Current biomarkers

The most widely studied biomarker for this purpose is Neutrophil gelatinase-associated lipocalin (NGAL) which have been studied in both urine and plasma (73). NGAL is interestingly a granule protein just like HBP, although it is found in secondary granule instead of the secretory vesicles and azurophilic granule. NGAL is believed to be involved in iron transport and can thereby have a bacteriostatic effect (74). It was first suggested as an AKI biomarker because it was highly upregulated in an animal model of ischemic AKI (75). NGAL showed promising prognostic ability in early investigations but this was not validated in later studies (73). Instead, the most promising current biomarker for AKI is the combined urine test for tissue inhibitor of metalloproteinases-2 (TIMP-2) and insulin-like growth factor binding protein-7 (IGFBP-7) (76, 77). TIMP-2 and IGFBP-7 are markers for cell cycle arrest and the fact they appear to be the highest performing markers for sepsis-related AKI say something important about pathophysiology.

2.4.3 Pathophysiology

The classical explanation for AKI, like many organ dysfunctions in sepsis, is that of macro-hemodynamic compromise and decreased oxygen delivery (i.e., ischemia) (78). This paradigm is increasingly being questioned (79, 80). Both animal studies (81) and studies in humans (82) show that renal blood flow is generally maintained

or increased in sepsis. Histopathologic examinations are also not consistent with extensive ischemic tubular necrosis or apoptosis (83, 84). In recent animal studies, ischemic renal failure models have failed to uniformly induce AKI. AKI was also commonly occurring in a large study of patients with non-severe pneumonia without hemodynamic instability (85). The current theory of sepsis-related AKI is instead dominated by a model of inflammation-induced (86) and functional, adaptive cellular change, at least in the first 24-48 hours (79, 80). In essence, circulating endogenous cytokines (e.g., TNF-α) and pathogen-derived inflammatory signals (e.g., LPS) reach the kidney as an alarm signal for danger, leading to microvascular change and triggers tubular epithelial cells into protective cell cycle arrest at the transient expense of kidney function (79).

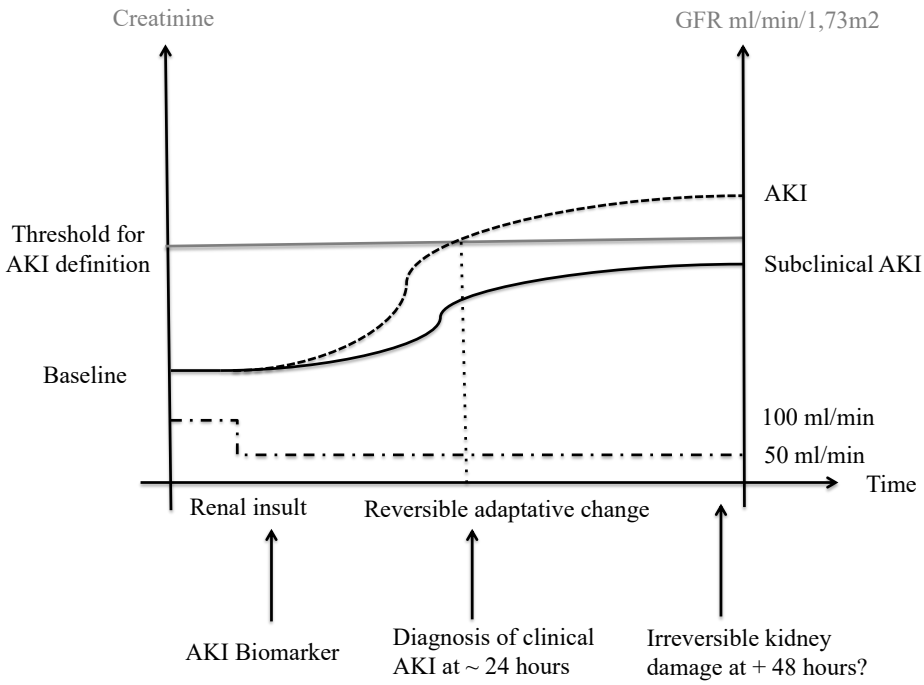

Figure 1. The potential role for novel AKI biomarkers. After a renal insult, such as septic shock, there is a decrease in glomerual filtration and urine production and creatinine begins accumulating. No clinical AKI diagnosis can be made at this point and potential interventions may be delayed compared to when they could have been initiated if there was an early AKI biomarker signalling danger.

2.4.4 Implications for therapy

This updated model also says something about the adequate clinical response to sepsis-related AKI, which have classically been seen as a trigger for a higher blood

pressure target and increased fluid administration. Not only have an increased focus on reaching haemodynamic targets not succeeded in ameliorating AKI in the early goal directed therapy (EDGT) trials (87), there is also data to support that a restrictive approach to fluids may reduce the incidence of AKI (88) and possibly the need for renal replacement therapy (89). There is furthermore an imminent risk that oliguria combined with increased fluid administration leads to fluid accumulation which is widely recognised to negatively impact survival in sepsis (90, 91). The functional and at least initially non-destructive nature of AKI should instead lead to an avoidance of further kidney damage, e.g., by reducing nephrotoxic drugs and avoiding radiocontrast (71) and by an intensified focus on treating the root infectious process by timely source control and adequate antimicrobial therapy (92). It is the role of novel AKI biomarkers to enable the early recognition of reversible danger in the kidney, preferably before a recognisable fall in function, and necessarily before extensive cellular damage.

3 An overview of Heparin-binding protein

HBP is a neutrophil-derived protein that acts as an amplifier of inflammatory responses and induces capillary leakage. During sepsis there is a significant increase in HBP in plasma, and the levels correlate with the development of hypotension and circulatory failure.

- Linder *et al.*, J Innate Immun, 2010 (93)

3.1 Discovery and nomenclature

The original identification of Heparin-binding protein (HBP) occurred as a consequence of the global quest for new antibiotics (94). Shafer *et al.* isolated the protein in 1984 while they were investigating granule extracts of neutrophils for *in vitro* antimicrobial activity against *Salmonella typhimurium* (95). They named the protein cationic antimicrobial protein of 37000 dalton (CAP37) for its positive charge, molecular weight and wide bactericidal activity. A similar protein of 29 kD was isolated by an independent research group, Gabay *et al.*, in 1989 (96). They also found the protein in neutrophil granules and that it was bactericidal against *Escherichia coli, Enterococcus faecalis* and *Candida albicans*, and they suggested the name azurocidin based on its location within the azurophilic granulae (97). The name (human) Heparin-binding protein (hHBP) was first described by a third research group, Flodgaard *et al.*, in 1991 (98). They described a protein with strong binding to heparin due to a high number of positively charged basic amino acids. Flodgaard *et al.* subsequently recognized that their hHBP, Shafer and Pereira *et al.*'s CAP37 (99) and Gabay *et al.*'s azurocidin (96) had an identical N-terminal sequence and must be the same protein. The three names are still used somewhat interchangeably in the literature, although HBP is more common in clinical studies on prognostic accuracy and will therefore be favoured in this thesis.

3.1.1 History in Lund

One day in the mid 1990's, Heiko Herwald, now a professor of Infection Medicine in Lund, found himself in the same elevator as Maria A Olofsson. They discussed a new protein that her associate, Hans Flodgaard and colleagues had found, called hHBP. Flodgaard, working in Copenhagen at Novo Nordisk at the time, was enthused by this new protein and wanted to tease out its biological role and rang Heiko the very next day. Together with Lennart Lindbom at Karolinska university, Heiko, Olofsson and Flodgaard published a key paper on HBP's role in neutrophil-evoked vascular permeability in 2001 (100). Heiko and Flodgaard identified HBP's location in secretory vesicles and azurophilic granules the next year (101) and they published on HBP's connection to *streptococcus pyogenes*-derived M protein together with Lars Björck, professor emeritus at Infection Medicine in Lund, in 2004 (102). Björck came up with the idea of investigating if HBP could be used as a sepsis biomarker in the clinic and handed the project to Per Åkesson, senior consultant at the Infectious Diseases clinic in Lund and Björck's former PhD student. Åkesson in turn gave the project to his PhD student at the time, Adam Linder, who is now my PhD supervisor.

3.2 Biological role

3.2.1 Origin

Structurally, HBP belongs to the serprocidin family of serine proteases, but it has lost its cleaving ability during evolution making it a "sterile enzyme" with a different biological role (103). HBP is expressed with two other granule proteins (elastase and proteinase 3) (104) under very tight transcriptional control. It is expressed only in the early development stage of neutrophils (promyelocytes) in the bone marrow and not in mature neutrophils (105). Some authors have suggested that HBP is expressed in other cell types from experiments in rats (106) and rabbits (107) but they are plausibly due to artefacts because neither of these animals have the HBP-responsible gene (AZU1) in their genome (108). The majority of HBP is found in the tertiary (azurophilic) granule that have a low propensity to degranulate (~74% of HBP) and less in the secretory vesicles that have a high propensity to degranulate (~18% of HBP) and even less in the plasma membrane (~8% of HBP) (101, 109).

3.2.2 Pro-inflammation

Apart from its wide antimicrobial properties, HBP acts as an early proinflammatory and immunomodulatory alarm signal (110). HBP's most important biological

function is probably its ability to enable immune cell extravasation through interacting with the endothelium to regulate vascular permeability (111).

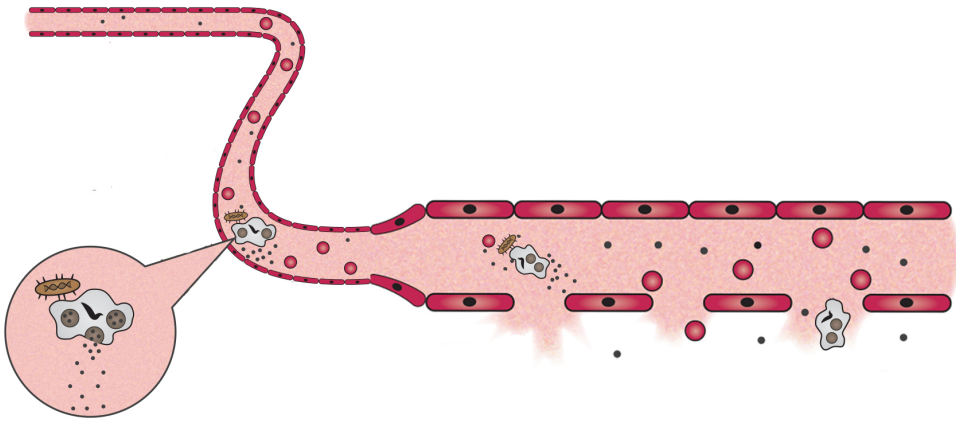

Figure 2. Activated neutrophils release HBP which enables extravasation.

Circulating neutrophils are typically the first immune cells to be recruited to a site of infection (112). Upon activation, for instance by interaction with a bacterial antigen (102, 113), neutrophils release up to 89% of total HBP within 30 minutes (99) from the secretory vesicles and azurophilic granules (101). After release, HBP can neutralize LPS (99) and acts as a chemotactic agent for neutrophils (114), monocytes (99) and T-lymphocytes (115). HBP also prolongs survival times of monocytes and induces LPS-associated TNF- α release (116), monocyte arrest (117) and differentiation into macrophages (98).

3.2.3 Vascular permeability

HBP release can also occur from neutrophil activation after contact with the inflamed endothelium (112). When the gel-like glycocalyx which covers the inside of the endothelium is shed during sepsis it exposes cellular adhesion molecules (118). HBP contributes to neutrophil adhesion (107, 119) and induces cell contraction (120) and Ca^{2+}-dependent cytoskeletal rearrangement in the endothelium, probably through the PKC – Rho kinase pathway (119, 121, 122). This leads to intercellular gap formation (100) and allows neutrophil (112) and monocyte (123) extravasation into the infected tissue. The side effect of this essential biological function is increased macromolecular efflux (100), i.e., vascular leak (119). HBP has also been shown to affect endothelial mitochondrial function (124, 125).

3.2.4 Effects on other cell lines

After extravasation, neutrophils release further HBP from azurophilic granules into the tissue (101) enhancing bacterial killing and also affecting other cells. HBP is chemotactic for fibroblasts and may have a function in wound healing (120) and induces migration in smooth muscle cells in the endothelium and may have a role in atherosclerosis (126). HBP has also been shown to produce LPS-like acute kidney injury (AKI) in a mouse model and HBP induces IL-6 production as an alarm signal in renal epithelial cells (127), possibly through macrophage activation (128).

3.3 Physiological relationship to current biomarkers

C-reactive protein (CRP) and procalcitonin (PCT) are often used as comparators to novel infectious biomarkers because they are well studied, display good prognostic accuracy and are in widespread clinical use. Even though CRP and PCT are evolutionary preserved, their precise biological roles have been challenging to define.

3.3.1 Procalcitonin

Procalcitonin's goal end hormone calcitonin is involved in calcium and bone homeostasis and found in the thyroid gland (129). Its precursor gene (CALC1) however, is expressed in multiple tissues during sepsis (130) and PCT levels increase rapidly within 4-6 hours after endotoxin injection in human volunteers (131). Why PCT is increased and whether PCT is harmful or beneficial in sepsis is not entirely clear. Exogenous PCT have been associated with increased mortality in experimental animal models (132) and immunoneutralization seem to reverse this effect (133). PCT have also been shown to augment nitric oxide production in inflamed smooth muscle cells (134). On the contrary, some evidence indicate that PCT can neutralize bacterial endotoxin (135) and decrease LPS-associated TNF-α release (136).

3.3.2 C-reactive protein

CRP is an acute-phase protein produced primarily in the liver (137) as part of an organised immune response and is found deposited in sites of acute inflammation (138). CRP can activate the complement cascade (139) and work as an opsonin for bacteria (140) but have also been associated with important anti-inflammatory effects. This seems to occur in both acute infection and in autoimmune conditions, possibly through affecting IL-10 production and T regulator cells (141).

3.3.3 Relationship to HBP

Some indirect evidence indicates that HBP may be located upstream of CRP and PCT in the inflammatory cascade and may therefore possibly be detectable earlier in plasma. In a study in hamsters, injection of TNF-α (400 μg/kg) increased PCT levels 25-fold but injection of PCT (30 μg/kg) induced no change in TNF-α in healthy or septic animals (142). CRP is well-known to be detectable at earliest 12-24 hours after an inflammatory insult (143) and one of the major drivers of CRP-production is IL-6 (144). HBP is prefabricated, rapidly released and has been shown to induce both TNF-α release in monocytes (116) and IL-6 release in renal epithelial cells (127).

3.4 Plasma levels and kinetic profile

While single plasma HBP measurements early in the disease course are relatively well-studied, plasma HBP dynamics over time are only recently being clarified.

3.4.1 Plasma levels

Healthy individuals are expected to have plasma HBP at or below the lower limit of detection for the standard HBP assay (5.9 ng/mL) (145, 146). Most prognostic studies have used HBP cut-offs around 15 to 30 ng/mL to discriminate patients at risk of infection-related non-beneficial outcomes but the upper range for HBP in patients with severe sepsis have often been well over 200-1000 ng/mL (147-149). The relationship between an increased risk for a non-beneficial outcome and continuous HBP have appeared relatively linear. This is true even for very high HBP concentrations, well beyond the typical cut-offs, although with wide confidence intervals.

3.4.2 Kinetics

Patients with “single-insult” inflammatory syndromes seem to have a relatively predictable HBP dynamic over time. The plasma levels generally return to normal within 24-48 hours, as seen in a study of 10 patients with burns (145), a study of 30 patients undergoing cardiothoracic surgery (150) and another study of 83 patients post cardiac arrest (151). During sepsis however, HBP levels seem to stay elevated for longer and have more inter-individual variation (152). This difference can plausibly be attributed to ongoing neutrophil activation during sepsis, since circulating neutrophils are the only credible source of HBP in blood. HBP was also elevated for up to 5 days in patients with severe trauma, where a high proportion of patients had shock and organ dysfunction (153).

3.4.3 HBP clearance

A prolonged elevation in HBP could also be an effect of disturbed HBP clearance, the mechanism of which remains to be described in detail. Despite HBP being cationic and small, HBP does not seem to be cleared by the kidney to any significant degree (154) and HBP levels are not visibly elevated in patients with chronic kidney disease (147). A recently submitted manuscript indicates that liver hepatocytes may be responsible for HBP clearance from the circulation and that the half-life of circulating HBP is very short, probably below 10 minutes for distribution and below 1-2 hours for elimination, after a single injection of recombinant HBP in rats (155). Note also that the rats were injected with a very high dose of HBP (25-125 μg) to produce detectable levels after 15 minutes and had initial plasma HBP concentrations well above 1000 ng/mL. Furthermore, in a study of HBP-induced acute kidney injury (AKI) concentrations of above 5 μg/mL were used to produce significant IL-6 production in renal cells (127).

3.4.4 Biological plausibility

If neutrophil extravasation is considered to be the key biological role of HBP and vascular leakage its principal side effect, then a high local concentration and short half-life makes physiological sense.

3.5 Assays and shareholders

3.5.1 The sandwich ELISA

The classical method for detecting and quantifying HBP in plasma is the sandwich enzyme linked immunosorbent assay (ELISA) which takes about 3 hours in your standard research laboratory. Briefly, a diluted plasma sample is added to a well which is firmly coated with an antibody specific to the antigen of interest (here HBP). Unbound antigens are then washed away before another antibody is added to well, also specific to HBP, but with a different binding site, i.e., sandwiching the antigen. The second antibody is linked with an enzyme and after another washing of the well, a chromogenic substrate is added which is cleaved by the enzyme and leads to a colour development. The relative intensity of the colour corresponds to the amount of antigen in the sample and can be estimated using a spectrophotometer.

Most of the early clinical studies on HBP published from 2009 to 2014 (93, 148, 151, 152, 156) used a version of an “in-house”-ELISA method originally developed by Tapper *et al.* in 2002 (101). Studies that are published from around 2015 and forward had generally begun using commercial HBP ELISA kits (149). All HBP

analyses in this thesis were done using Axis-Shield Diagnostics' HBP ELISA assay (cat. # FMHBP100IUO). Today, several similar ELISA assays for HBP/azurocidin/CAP37 are commercially available from Abcam (cat. # ab213755), Thermo-Fisher (cat. # EH39RB), RayBio (cat. # ELH-AZU1), Acro Biosystems (cat. # AZ1-H5225), Eagle biosciences (cat. # AZD36) and Booster Bio (cat. # EK1161) to name a few.

3.5.2 Point of care tests

There is also a point of care (POC) test with a detection time of 18 minutes offered by Chinese Hangzhou Joinstar Biomedical, who have signed a sub-license with Axis-Shield, based on a fluorescence quantitative immunochromatography assay (FIGA). The Jet-iStar 800 point of care device (cat. # FGCOV100) was used in this study (157) and was recently validated against Axis Shield's HBP ELISA assay (158). Joinstar's POC test is in common clinical use in China (159), but one study raises concerns over reproducibility and present a novel time-resolved fluorescence immunoassay (TRIFA) with improved sensitivity from 0.11 ng/mL to 530 ng/mL compared with 5.9 to 200 for FIGA (160). In the context of an active but paused recruiting trial, there has also been a HBP test that takes 20-30 minutes in the clinical chemistry department in Lund, Sweden, using a Cobas c 502 module as part of the cobas® 8000 modular analyzer series from Roche Diagnostics.

3.5.3 Patents

In 2009, a patent (WO2008151808A1) was filed by Hansa Biomedical AB in Lund with Lars Björck, Bertil Christensson, Heiko Herwald, Adam Linder and Per Åkesson as listed inventors (161). The HBP Assay have been licensed from Hansa Biopharma to Axis-Shield Diagnostics, an affiliate of Abbott, and Hansa have the right to royalties from Axis-Shield. Axis-Shield have provided the majority of the HBP ELISA kits for the studies in this thesis free of charge but have otherwise not been involved in the design of the studies, interpretation of results, writing of the manuscript or the decision submit the reports for publication.

3.6 Overview of clinical studies

3.6.1 Timeline

The pivotal study on HBP's prognostic ability in sepsis was published in 2009 by Linder *et al.* (148). The study included 233 adult febrile patients with suspected infection at Lund university hospital's infectious disease clinic's emergency

department (ED). The study found that plasma HBP measured at hospital admission had an area under the receiver operating curve (AUC) of 0.95 to discriminate patients who developed severe sepsis or septic shock during hospitalisation from those who did not. The AUC was 0.85 for 0.PCT, 0.69 for CRP and 0.79 for lactate. The following ten years saw a series of around 15-20 publications on the prognostic accuracy of plasma HBP in the English language, covering several inflammatory clinical syndromes such as acute respiratory distress syndrome (ARDS), influenza, undifferentiated shock, trauma, burn, acute kidney injury and cardiac arrest and were typically published with Adam Linder or Heiko Herwald as co-authors. The last two years have seen about as many English language publications as the preceding ten, from several independent authors, predominantly Chinese, further covering adult-onset Still's disease, pancreatitis, COVID-19 and community-acquired pneumonia. The increased interest for HBP in China may be linked to HBP having been recommended alongside PCT as a prognostic marker for sepsis in 2014 by "The powerful committee of The Consensus of Chinese Emergency Medicine Experts on Early Prevention of Sepsis" (162). Between 2013 and 2021 there have furthermore been at least 15 Chinese language publications in Chinese journals according to a recent systematic review and meta-analysis by Wu *et al.* (159). HBP have been the focus of at least two additional reviews in the last five years (163, 164). Table 3 lists a summary of English language publications on the prognostic accuracy of plasma HBP from 2009 to 2021 based a non-systematic literature search.

3.6.2 Non-plasma HBP studies

HBP has also been assessed as a biomarker in other body fluids than plasma. In 2018 Tydén *et al*. (165) analysed HBP in bronchoalveolar lavage fluid (BALF) from 12 anaesthetized pigs and found elevated HBP levels in the 6 pigs with ventilator-induced lung injury compared to pigs with normal tidal volumes (median 1144 vs 89 ng/mL). No difference was seen in HBP in plasma. Paulsson *et al.* published a clinical study in 2021 on HBP in BALF and bronchial wash (BW) and found HBP to be 100% sensitive and 100% specific at a cut-off of 206 ng/mL in discriminating 28 patients with ventilator-associated pneumonia from 20 healthy controls (166). Ren *et al.* analysed HBP in cerebrospinal fluid (CSF) in 2021 and found an AUC of 0.86 for HBP to discriminate 118 children with purulent meningitis from 110 children with viral meningitis and 80 controls (167). The AUC for leucocyte count in CSF was not provided for comparison. HBP have also been analysed in the sputum of 19 children (6-18 years) with cystic fibrosis in 2018 and found to have an AUC of 0.80 for > 10% decrease in change in first forced expiratory breath (ΔFEV1) (168).

3.6.3 Pre-registered studies

There is wide consensus that study protocols and pre-registration should be done more often in observational studies to increase the validity of findings (169-171). Table 2 lists the currently unpublished pre-registered studies on HBP's prognostic accuracy.

Table 2. Summary of registrered unpublished studies on HBP's prognostic ability in WHO's International Clinical Trials Search Portal.

Recruitment status (updated date)	Author	n_{pat}	Sites (country)	Sampling at	Population	Primary objective	NCT no.
Completed (25sept2017)	Arnold *et al.*	1055	1 (USA)	24-48-72 hours from ED adm.	ED patients sepsis alert or PRIO => 15	Prognostic accuracy for severe sepsis or septic shock within 72 hours	NCT02533011
Recruiting (31oct2019)	Halldorsdottir & Herwald *et al.*	60	1 (Swe)	1-5 hours post Heparin-infusion	ICU patients planned for surgery	Can heparin in clinical doses lower the level of plasma HBP?	NCT04146493
Active, not recruiting (9jan2019)	Corbin & Axis-Shield *et al.*	571	5 (USA)	probably ED adm.	ED patients with suspected infection	Prognostic accuracy for sepsis within 72 hours	NCT03113721
Completed (24may2021)	Tverring *et al.*	652	17 (Finland)	Day 0-5 from ICU adm.	ICU patients with severe sepsis or septic shock	Added value regarding 90-day survival	ISRCTN15560762
Recruiting (2mar2020)	Xinping *et al.*	300	1 (China)	Unknown	Children with and without sepsis	Unknown	ChiCTR2000030364
Pending (9nov2020)	Heng *et al.*	230	1 (China)	Unknown	Patients with aseptic SIRS or sepsis	Unknown	ChiCTR2000037900

NCT; National clinical trial (ClinicalTrials.gov); ChiCTR; Chinese Clinical Trial Registry, ISRCTN; International Standard Randomised Controlled Trial Number

Table 3. Non-systematic overview of English language studies on plasma HBP's prognostic ability. Population and n_{pat} refer to primary endpoint analysis.

Year	Authors	n_{pat}	Sites	Population (adults)	Sampling at	Endpoint and comparison	Outcome measure
2009	Linder *et al.* (148)	233	1 (Swe)	Fever + suspected infection at hospital adm.	ED adm.	Severe sepsis / shock during admission vs. local infection	AUC HBP 0.95 PCT 0.85 CRP 0.69 Lactate 0.79
2010	Berkestedt & Herwald *et al.* (172)	56	1 (Swe)	ICU patients with sepsis or neurosurgery controls	ICU adm.	90-day mortality vs. alive	$p > 0.05$
2012	Linder *et al.* (152)	151	1 (Swe)	Severe sepsis in ICU	Day 0-6 from ICU adm.	28-day mortality vs. alive	HR 4.1 (95% CI: 1.3 to 12.6) at 15 ng/mL
2012	Chew, Herwald & Linder *et al.* (146)	53	1 (Swe)	Undifferentiated shock in ICU	Diagnosis of shock	Infectious cause vs. other	24 vs 27 ng/ml $p = 0.29$
2013	Kaukonen & Herwald *et al.* (173)	29	4 (Finland)	Influensa H1N1 / ARDS in ICU	ICU adm.	Severe sepsis / shock vs. not	83 vs 83 ng/mL
2013	Lin *et al.* (174)	106	1 (China)	ARDS or pulm. aedema in ICU	ICU adm.	ARDS vs. CPE	AUC HBP 0.82
2013	Johansson & Herwald *et al.* (175)	47	1 (Swe)	Trauma in ICU	ICU adm.	ARDS vs not	AUC HBP 0.75
2013	Dankiewicz & Linder *et al.* (151)	84	1 (Swe)	Cardiac arrest	6 h / 12h post CA	Poor vs. good cerebral performance	AUC HBP 0.68/0.70 APACHE 0.76 SOFA 0.64
2013	Llewelyn *et al.* (176)	219	1 (England)	Undifferentiated general ICU and HDU	ICU adm.	Sepsis during hospital stay vs. not	AUC HBP 0.61 PCT 0.84 IL-6 0.81
2015	Linder *et al.* (149)	487	7 (Swe, USA, CA)	1 of 3 SIRS + no OD + final infection diagnosis	ED adm.	Severe sepsis / shock within 72 hours vs. local infection	AUC HBP 0.80 PCT 0.70 CRP 0.70 Lactate 0.64
2016	Linder & Bentzer *et al. (119)*	341	27 (Canada, AUS, USA)	Septic shock in ICU	ICU adm.	Fluid overload correlation PF-ratio 100 mmHg below vs above (adj.)	r=0.13, p=0.01 47 vs 22 ng/ml (p=0.03)
2016	Tydén & Herwald *et al.* (177)	278	1 (Swe)	Mixed ICU patients with arth. catheter	ICU adm.	unadj. 30-d mortality (SAPS 3 adj.)	AUC HBP 0.64 (p>0.05)
2016	Zanfaly *et al.* (178)	90	1 (Egypt)	ICU patients with sepsis	ICU adm.	Severe sepsis / shock vs. sepsis	AUC HBP 0.95 PCT 0.58
2017	Fisher & Linder *et al.* (127)	138	27 (CA, AUS, USA)	ICU patients with septic shock	18h post shock	Acute kidney injury (AKI) stage 0 vs. 2	AUC HBP 0.85 (aOR p<0.05)
2017	Tydén & Herwald *et al.* (179)	245	1 (Swe)	Mixed general ICU patients	ICU adm.	AKI stage 3 in 7 days vs. stage 0-1	AUC HBP 0.70

2018	Halldorsdottir & Herwald *et al.* (153)	97	1 (Swe)	Post-injury trauma patients in ICU	Day 1, 3 and 5 from ICU adm.	Sepsis-3 within 48 hours of sampling vs. not	AUC HBP 0.48-0.60-0.64 (*p*>0.05)
2018	Liu *et al.* (180)	118	1 (China)	Patients with severly affected vital signs	ED adm.	Uncomplicated vs. severe sepsis	Median HBP *p*=0.04
2018	Ipek *et al.* (181)	106	1 (Turkey)	ST-elevation myocardial infarction	ED adm.	STEMI vs. healthy controls (n=30)	AUC HBP 0.71
2019	Zhou *et al.* (182)	125	1 (China)	ICU + ward patients with final infection diagnosis	Any time during adm.	Sepsis vs. local infection (sepsis-3)	AUC HBP 0.89 PCT 0.86 CRP 0.70
2020	Olinder & Herwald *et al.* (183)	15	1 (Swe)	General ICU patients with septic shock	1-6 days post ICU adm.	Longitudinal SOFA score correlation	*p*=0.14
2020	Saridaki *et al.* (157)	178	10 (Greece)	COVID-positive	ED adm.	PF-ratio <150mmHg & mechanical ventilation vs not	HBP aOR 3.1 (1.0-9.1) IL-6 aOR 9.2 (3.2-26.4)
2020	Pajenda *et al.* (184)	60	1 (Austria)	Undifferentiated sepsis-patients	Daily from ED adm.	Acute kidney injury vs. not	AUC mean/peak HBP 0.61/0.67
2020	Mellhammar & Linder *et al.* (185)	941	8 (Swe, USA, CA, Switz.)	Acutely ill undifferentiated ED patients	ED adm.	Sepsis, ICU or death within 72 hours vs. not	AUC HBP+vitals 0.73 NEWS2 0.69
2021	Sjöbeck & Herwald *et al.* (186)	202	1 (Swe)	Suspected acute pancreatitis	ED adm.	Mild vs severe Fluid balance correlation	AUC HBP 0.46 *p* > 0.3
2021	Cai *et al.* (187)	141	1 (China)	Community-aquired pneumonia	ED adm.	Final bacterial vs viral cause (fungi excluded)	AUC HBP 0.93 PCT 0.89 Leucocyte 0.83
2021	Mellhammar & Linder *et al.* (158)	29	1 (Swe)	COVID-positive	Max in 72h from ED adm.	OD during adm. vs. not	AUC HBP 0.88
2021	Zhong *et al.* (188)	343	1 (China)	Acute pancreatitis	ED adm.	Severe vs non-serve	AUC HBP 0.79 PCT 0.85
2021	Tian *et al.* (189)	59	1 (China)	Sepsis-3 or Adult-onset Still's disease	Not defined	Sepsis-3 vs. Adult-onset Still's disease	AUC HBP 0.65 (95 CI: 0.51-0.80)
2021	Elsayed *et al.* (190)	66	1 (Egpyt)	Undifferentiated ICU patients	ICU adm.	Septic shock vs. not (time interval not defined)	AUC HBP 0.82 PCT 0.55 Lactate 0.77 Leucocyte 0.77
2021	Shu *et al.* (191)	66	1 (China)	Suspected first acute pancreatitis within 48h	24h from ED adm.	Persistent organ failure vs. not	AUC HBP 0.82 APACHE 0.76

AUC; area under the receiver operating curve, ED; emergency department, CPE; cardiopulmonary eadema, SIRS; systemtic inflammatory response syndrome, HDU; high dependency unit, ICU; intensive care unit, AUS; australia, STEMI; ST-elevation myocardial infarction, NEWS2; new early warning score 2, CA; Canada, Swe; Sweden, APACHE; acute physiology and chronic health evaluation, Adm; admission, PF-ratio; arterial oxygen pressure to fraction of inspired oxygen ratio, SOFA; sequential organ failure assessment score, SAPS; simplified acute physiology score

4 The statistical evaluation of a prognostic biomarker

Ignore statistical significance for this situation. Go with a pre-specified statistical analysis plan and stick to it.

- F Harrell, personal communication, 19 Oct 2021

4.1 Classic concepts

4.1.1 Sensitivity and specificity

The fundamental accuracy of a binary biomarker (positive versus negative) to detect binary disease status (disease versus no disease) is commonly described through sensitivity and specificity in medical research (192). We consider a good biomarker to be highly sensitive, i.e., that a positive test identifies the majority of individuals that actually carry the disease, and highly specific, i.e., that a negative test can be trusted to identify individuals where the disease is not present. Sensitivity is also known as true positive fraction (TPF) and specificity as true negative fraction or 1 – false positive fraction (FPF) (193). These can easily be calculated from a two-by-two table. We use example data from MS Pepe's 2004 text book in Table 4 (193).

Table 4. Two-by-two table with examples for binary test accuracy of binary disease status.

Disease Prevalence = 69.8% (P)	**No disease (D^0)**	**Disease present (D^1)**	**n =**
Biomarker negative (B^0)	327 (B^0D^0) true negative	208 (B^0D^1) false negative	535 (n_{B0})
Biomarker positive (B^1)	115 (B^1D^0) false positive	815 (B^1D^1) true positive	930 (n_{B1})
n =	442 (n_{D0})	1023 (n_{D1})	1465 (n_{tot})

$$\text{Sensitivity} = B^1D^1/n_{D1} = 815/1023 = 80\% \text{ (TPF)}$$

$$\text{Specificity} = B^0D^0/n_{D0} = 327/442 = 74\% \text{ (1 – FPF)}$$

4.1.2 Predictive values

Sensitivity and specificity represent a biomarker's frequency of correct classification for each disease state. These measures are thought to represent inherent biomarker performance. A clinically relevant alternative is to look at how well the test predicts the true disease status in a population (194). This can be calculated by including the prevalence of disease in the study population, i.e., the number of people with the disease divided by the people at risk, and then be reported as the positive predictive value (PPV) and negative predictive value (NPV) (193).

$$\text{PPV} = B^1D^1 / n_{B1} = 815 / 930 = 88\ \%$$

$$\text{NPV} = B^0D^0 / n_{B0} = 327 / 535 = 61\%$$

It is important to appreciate how heavily the predictive values rely on the population prevalence. In our example, even though specificity was relatively high at 74%, NPV was only 61%. If we studied a different population or disease with a lower prevalence of say 5%, then the NPV would be 99% at the same level of sensitivity and specificity, while the PPV would only be 14%.

4.1.3 Power, Prediction, P values

A relevant parallel to measures of biomarker accuracy in medical research are power and sample size calculations. Here, your desired power (ß) is your sensitivity, and your significance level (α) is 1 – specificity (FPF) and the prevalence (P) are the number of true hypotheses divided by the number of tested hypotheses in the research field (193). Because biology is complicated and many initial hypotheses in

medicine are false, a lot of studies reporting statistically significant results are in fact also false in analogy with the PPV calculation. This occurs even when widely accepted levels of power calculation are used. If the power is 80% and the significance level is 5% and 5% of tested hypotheses are actually true, then only 45% of significant findings represent the truth. Increasing the power to 90% only increases the positive predictive value to 48%. If instead 25% of hypotheses are true, then 84% of significant findings are true when using ß=0.8 and α=0.05. Setting α at 0.005 as suggested by some (195) would results in PPV 89% for P=5% and ß=0.8. A relevant analogy is multiple significance testing correction which is used within but not between studies (196). The problem with false positive statistically significant findings is clearly much greater if no power calculation has been performed or if research is being done without a thorough hypothesis. These circumstances alone can explain much of the replication crisis in biomedical research (197). Others argue that raising the bar for statistical significance will aggravate other more severe issues in biomedical research like "P hacking", selective reporting and publication bias (198).

4.1.4 Diagnostic likelihood ratios

Another way of describing the prognostic value of a binary biomarker is through positive and negative diagnostic likelihood ratios (LR^+ and LR^-) (199). They are literally the ratios of the likelihood of the observed test results in those with versus without the disease. The major benefit of diagnostic likelihood ratios is that they quantify the increase in knowledge about the presence of disease that is gained through the test (193), unfortunately on the odds scale. The post-test odds of disease are equal to the pre-test odds of disease multiplicated by the likelihood ratio. For this relationship, diagnostic LR is also known as Bayes [multiplication] factors relating prior and posterior distributions (193). Diagnostic LR does not depend on the prevalence of disease and is therefore seen as a compromise between classification probabilities and predictive values. Diagnostic LR scale (0, ∞) is such that a perfect test has $LR^+ = \infty$ and $LR^- = 0$. They can be straightforwardly calculated from sensitivity and specificity.

LR^+ = Sensitivity / (1 – specificity) = (815/1023)/(1-327/442) = 3.06

LR^- = (1 – sensitivity) / (specificity) = (1-815/1023)/(327/442) = 0.27

Pre-test odds (O) of disease = P/(1-P) = 0.698/(1-0.698) = 2.31

According to the diagnostic LR the pre-test odds of disease are hence increased to 7.21 (2.31*3.06) by a positive test and decreased to 0.62 (2.31*0.27) by a negative test. Odds ratios (OR) are sometimes approximated into risk ratios in cohort studies when the event is rare, but this cannot be done in this situation because a positive

test is not a rare event. If we know the prevalence however, we can calculate the predicted probabilities from the diagnostic LR. On that note, OR has a simple relation to diagnostic LR so that $OR = LR^{+} / LR^{-}$ (200).

4.1.5 The receiver operating characteristic curve

Most biomarkers are not simply positive or negative but can take on a range of values, sometimes ordinal, but most commonly continuous, like the plasma concentration of HBP. The receiver operating characteristic (ROC) curve is a tool for describing the performance of such markers (201). It is a measure of discrimination based on ranks. What the ROC does in practice is plot the sensitivity (TFP) on the y-axis and 1 – specificity (FPF) on the x-axis for all biomarker cut-offs. The potential application of ROC curves in medicine were described already in the 1960s (202). It is typically reported alongside sensitivity and specificity for a chosen or pre-determined cut-off, with or without predictive values and diagnostic LR. The most suitable cut-off for a biomarker is best decided based on the clinical situation it will be used in, i.e., whether sensitivity or specificity is favoured. In sepsis recognition in the emergency department for instance, it is reasonable to favour a sensitive biomarker so that people at risk are not sent home without treatment or surveillance, while it is reasonable favour specificity in markers for localised prostate cancer in the elderly, where a subsequent prostate biopsy risk causing considerable harm. The ROC curve describes a specific biomarker's range of such trade-offs. The ROC curve also provides means for meaningful comparison of continuous biomarkers' performance (193). This can be done visually as in Figure 3 for the overall biomarker performance across cut-offs but it can also provide information on different biomarkers' tendency to overperform in sensitivity or specificity or vice-versa, across different cut-offs.

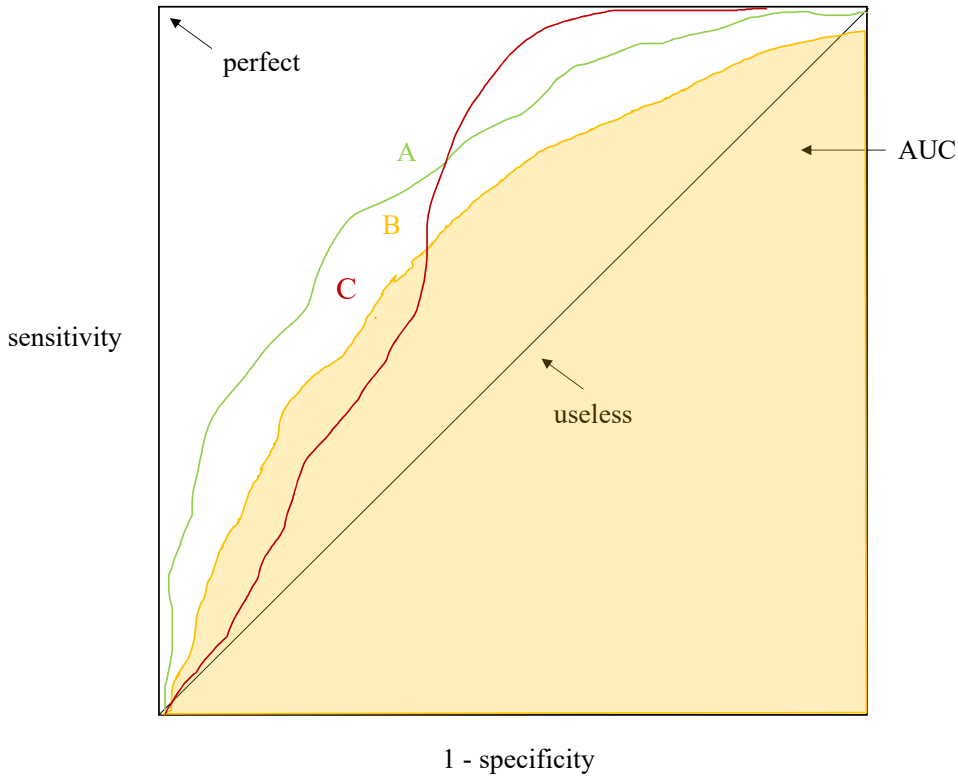

Figure 3. The receiver operating characteristic (ROC) curve. Biomarker A is clearly a better performer than B across all visible cut-offs. Biomarker C however looks to be the most sensitive marker at certain cut-offs, at the expense of specficity, while it is the worst performer at a different cut-off. All biomarkers clearly have an area under the curve above the "coin flip" threshold of 0.5.

4.1.6 Area under the ROC

Meaningful comparisons can also be done by the use of numerical summary indices. The area under the ROC curve (AUC) is the most widely used. The AUC spans from 0.5 (chance or coin flip) to 1.0 (perfect discrimination). The AUC has an interesting mathematical interpretation. It represents the probability that biomarker results from a randomly chosen subject with the disease is correctly ranked as higher risk than a randomly chosen subject without the disease (203). The clinical utility of AUC is less clear because patients do not present themselves as random diseased and non-diseased pairs (193). Pepe prefers to interpret AUC as a an average sensitivity over the whole range of specificity (193).

Sometimes there are relevant co-variate effects on binary or continuous biomarker results. In the case of HBP this might be neutrophil count (155) since HBP is neutrophil-derived and leukopenia is not uncommon in severe sepsis. Another factor

may be sample haemolysis or lag time to lab handling which could affect the biomarker values and be regarded as co-variate effects (if these samples are not directly excluded). Methods have been developed to adjust for such basic co-variate effects into an adjusted ROC curve (204). For more advanced investigations into biomarker performance and its relation to other markers or co-variates it is reasonable to move on to regression modelling and maximum likelihood.

4.2 Added value

4.2.1 The why

The above-mentioned measures for classification and prediction relate to the accuracy of a single biomarker on its own. These are valid and important measures for a basic investigation of novel biomarkers to ensure an adequate association and discriminatory ability towards the outcome. But even when these measures indicate good accuracy, the biomarker may still contain little clinically applicable added value because the biomarker-conveyed information may be same as is already available to the treating physician (205). Clinicians very rarely, if ever, make decisions in a complete void of other relevant prognostic factors. This is particularly relevant in sepsis research, an area where comprehensive analyses of added value for investigated biomarkers appears to be rare (5, 50, 206). When evaluating a patient with suspected sepsis in the emergency department (ED) the clinician will have information on age and vitals and often also symptom history, medications, comorbidities and lab results, all of which hold potential prognostic value regarding the development of sepsis or similar outcomes of interest. Even in the prehospital setting, health-care workers will have some prognostic information about the patient. In the intensive care unit (ICU) the available information is of course extensive. A clinically relevant analysis of the value of a biomarker in an observational study should convey this relationship of what is already known to the clinician at the time of biomarker sampling and to what degree the addition of the biomarker carries additional knowledge towards the outcome (207, 208).

4.2.2 The what

A fundamental construct for much of statistical modelling is the likelihood function. This reflects the probability, or “likelihood,” of obtaining the actual observed data using the variables fitted in a model (209). The goal then for a biomarker seeking to prove added value is to increase the likelihood of a model that includes the factors that the clinician already knows at the bedside. The addition of a biomarker can be formally tested for statistical significance using a likelihood ratio test between the

model with the biomarker versus the nested model without the biomarker (210). What is more important (and more complicated) than statistical significance in this situation however, is to estimate the incremental size of the added value from the biomarker to determine if it constitutes a clinically significant addition (211).

4.2.3 The how

If there is already an established prediction model in clinical use that is relevant for your outcome of interest, then it is only natural to use this model for investigation of biomarker added value. A classic example is the addition of cholesterol to the Framingham score (212). This is most often not the case when assessing a novel biomarker. The investigator will then need to build a new model using clinically relevant variables (205). The construction of a valid clinical prediction model is a formidable endeavour in its own right and the subject of statistical reviews (213) and whole text books (200). The process is briefly summarized below. The statistical jargon "prediction model" and "predictors" are used even when referring to the evaluation of a prognostic biomarker.

4.2.4 Prediction model development

Selection of predictors

Two examples of established methods for combining clinically relevant variables into prediction models are logistic regression for binary outcomes and proportional hazards regression (e.g., Cox) for time-to-event outcomes (200). If we first assume that we have a well-grounded hypothesis and a relevant outcome and that appropriate data have been collected from a sufficient amount of individuals (commonly > 10 events per variable in the model (214)) and that there is not a large degree of missing data (i.e., below 5% (215)) then the first step for the investigator is to choose which predictors to include in the model (205). This can be done using data-driven automated "stepwise" methods testing all candidate predictors but including only those that are most strongly associated with the outcome, but this may lead to overfitting or biased and unstable models (216, 217). A better approach is to choose predictors that represent different and comprehensive aspects of the clinical situation and have a former known association with the outcome based on earlier studies and the clinical investigators' expertise (200). An example would be age, comorbidities, lactate and measures of organ dysfunction for sepsis survival.

Variable transformation and model specification

The next statistically demanding step is to look at the included variables more closely for non-linear relationship with the outcome, influential observations, apparent outliers, small categories, collinearity, the need for interaction terms and

then transform variables and model specification accordingly and finally check model assumptions (200). Next is checking model performance and validity.

Model calibration

The baseline for model credibility is determining whether the constructed model predicts the outcome in agreement with the observed data, in analogy with the likelihood function (213). Meaning if the model predicts 23% of patients develop sepsis 72 hours from ED admission, then there should be approximately 23 out of 100 patients that do so in the dataset. This can be checked by plotting the predicted outcome on the x-axis versus observed outcome on the y-axis in a calibration plot, while reporting the intercept (also called calibration-in-the-large, ideal 0) and slope (ideal 1). Pseudo R^2 can be used as a measure of model fit (200).

Model discrimination and validation

Next is to assess the discriminatory ability of the prediction model using the AUC for binary endpoints and the corresponding C (for concordance) statistic for time-to-event endpoints (218) using the continuous predicted values from the model. The last step is to assess model overfitting, i.e., the over-adaptation of the model to your data compared to the performance in real-world data. A recommended approach is bootstrap resampling (209). Bootstrapping creates hundreds of new fictive datasets from the original dataset against which the model is validated to mirror external validity and may for instance lead to a decrease in AUC from 0.82 to 0.78 for a small dataset (213).

4.2.5 Quantifying added value

The classic way of evaluating the incremental value for a biomarker added to a prediction model is through increase in area under the ROC (ΔAUC) (208). The AUC together with the C statistic are valid measures for improved classification depending on overall rank, although significance testing for ΔAUC between nested models using standard software is not advisable (219). ΔAUC and change in C statistic have however proved to have low power to prove added value despite the addition of a valuable marker. This is plausibly because clinically important changes in risk for part of the population may be hidden in overall ranks (220, 221). The difficulty in proving an increase in AUC have led to a development of new measures to quantify added value. The net reclassification index (NRI) focuses on how many patients are moved into a new risk strata when adding a biomarker (222). While this measure appears clinically intuitive and have been adopted in many studies it is sensitive to the size, number and cut-offs of strata which are seldomly pre-specified (223). NRI is also sensitive to model miscalibration, meaning it can indicate that an invalid model is better performing than a valid model (224, 225). A category free "continuous" NRI have been proposed but this may instead overestimate the value

of a new marker when there is very little change in risk so that weak or uninformative markers appear clinically relevant (226, 227). The integrated discrimination improvement (IDI) is instead a measure of absolute change in risk (222) which is related to the pseudo R^2 but can also be affected by infinitesimally small changes in risk and can be difficult to interpret (220). It has also been noted that both the "new" two-category NRI and IDI are actually new names for old measures (IDI = mean risk difference (MRD) (205) and NRI = $-\Delta$FPR). Furthermore NRI does not actually compare the performance between the old and the new model, but is rather a comparison of the old and new risk values for each person (224), which does not necessarily translate into improvement on a population level (228). Needless to say, risk reclassification have not proved to be a methodologically valid replacement for ΔAUC (229). The Brier score (230) have been proposed as it is a proper scoring rule (i.e., not sensitive to model miscalibration) but it can also be difficult to interpret because it mirrors both calibration and discrimination in one measure and its maximum value depends on the incidence of the outcome (220). One could also look at change in pseudo R^2 (220, 221) or change in Akaike or Bayesian information criterion (220) but these may be equally difficult to interpret clinically. Harrell suggests that the fraction of new information can be efficiently calculated using the chi square likelihood ratio (LR χ^2) or variance of predicted risk (R_{var}) from the model with versus without the biomarker added (221).

$$\text{Fraction of new information} = 1 - \text{pre-test LR } \chi^2 / \text{post-test LR } \chi^2$$

$$\text{Fraction of new information} = 1 - \text{pre-test } R_{var} / \text{post-test } R_{var}$$

In Harrell's example, the addition of cholesterol to age and sex added a fraction of new information of 0.18 based on LR χ^2 or 0.17 based on R_{var} (221). It is fair to say that the research community, despite agreeing on the importance of quantifying the added value of a biomarker, have yet to agree on how it should optimally be done (220).

4.3 Net benefit

4.3.1 Including the clinical setting

As mentioned previously, there is an inherent difficulty in translating statistical measures of calibration and discrimination into impact on clinical decision-making. Net benefit (NB) is a decision analytic technique that incorporates the clinical setting into the evaluation of a marker or a prediction model (220). This is achieved through specifying an "exchange rate" or "weight" based on clinical judgement

between the relative benefit of correctly identifying disease (such as detecting a cancer) versus the "cost" of a false-positive result (such as the harms of performing an unnecessary biopsy) (231). Vickers *et al.* define NB as follows:

$$\text{Net Benefit} = \text{benefit} - (\text{harm} \times \text{exchange rate})$$

Benefit could also be referred to as true positives (TP) and harm as cost or false positives (FP) and the exchange rate as weight. Using sepsis as an example, say we are facing patients in triage, and we have the choice of admitting patients or sending them home. While hospital admission will most often (but certainly not always) be beneficial for patients being evaluated for sepsis, there is a cost related to admitting everyone due to a scarcity of hospital beds. If we can accept admitting a maximum of 10 people per one person developing sepsis, then the exchange rate is 1:9. Assume next that your triage would normally admit 25 patients with sepsis out of 100 evaluated individuals. Let's say you introduce a biomarker called HBP to be added to the existing vital sign-based triage system that results in 122 persons being admitted out of which 28 develop sepsis. Does HBP confer net benefit in this example? Without HBP the NB was 16.7% (0.25-0.75*1/9) and with HBP NB is now 17.6% (0.28-0.94*1/9). Q.E.D.

4.3.2 Decision curve analysis

The unit of NB is true positive fraction (or sensitivity) indicating that a triage strategy with HBP would find an additional 9 patients with sepsis out of 1000 people at risk. A criticism to NB is that it is only a measure of rank order so that it does not consider the size of increase in risk, which could be very small and still appear as benefit. Another criticism is that the exchange rate can be viewed as arbitrary and is variable between practitioners. The authors suggestion is to plot the NB in a decision curve for a wide range of exchange rates based on the predicted risk from the model with versus without the biomarker added (231). An example is presented in Figure 4 using the fictional case above. The take home message is that HBP is only helpful for a subset of exchange rate preferences.

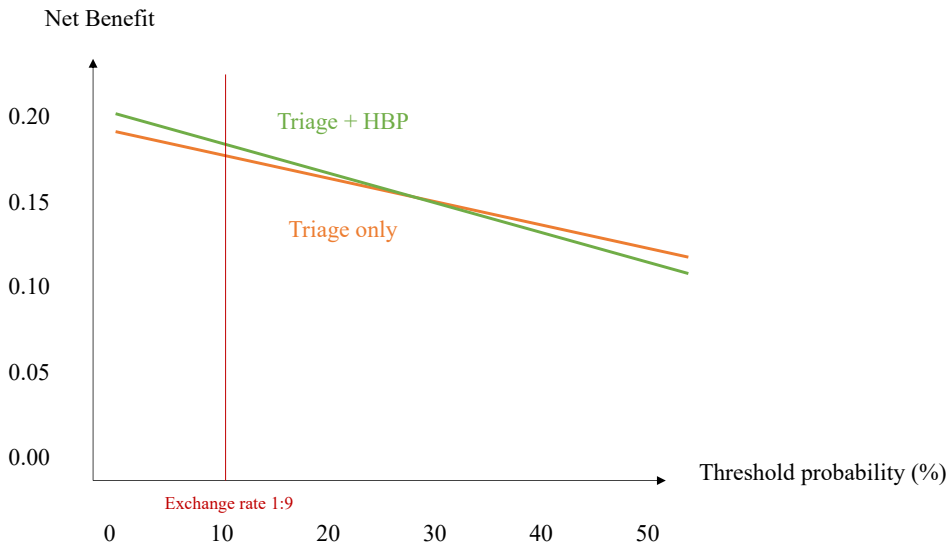

Figure 4. Example of net benefit decision curve analysis using fictional data. Notice how "Triage only" performs better if we choose to accept sending home half of patients who develop sepsis while "HBP added" performs better as long as we accept to "over-admit" more 3.2 persons for every one person who develop sepsis.

4.4 Diagnostic randomised controlled trials

4.4.1 Gold standard

Observational studies on biomarker accuracy cannot provide direct evidence on the effect of biomarker introduction to clinical practice (232). For this purpose, well-designed "diagnostic" randomised controlled trials (RCT) constitute gold standard (233). With an RCT, you do not need to worry about model calibration or quantifying the incremental value or translating statistical jargon to clinical usefulness. It should already be built into the trial design by using a control group, an appropriate biomarker cut-off and a clinically relevant endpoint. The downside is that it is much more challenging and costly to perform compared to an observational study.

4.4.2 A design challenge

Designing and reporting a diagnostic RCT for novel biomarker evaluation is not trivial. It will often require the design and detailed reporting of biomarker-associated interventions apart from choosing a relevant study population, endpoint and biomarker cut-off. Such studies are often referred to as "test-treatment-trials" (234).

A recent review pointed to an extensive problem with standardisation and reporting in such trials (235) suggesting that they are wasted if not describing interventions in detail, by adhering to the TIDieR checklist for instance (236). There is arguably an opposite trade-off as well. An overly detailed stepwise interventional plan based on biomarker results may deviate too much from standard-of-care to be generalizable and will lead to difficulty in separating what was the effect of the biomarker versus the effect of the intricate intervention.

4.4.3 Pragmatic trials

The most straightforward design for biomarker evaluation that would allow trial results to be attributable to the biomarker itself, would be a simple randomisation between revealing the biomarker result to the treating clinician for half the population and hiding it from the other half and then watching what happens (Design I in Fig. 5). The issue is that clinicians will by definition not know what to make of a novel biomarker result and hence we cannot expect group separation. This would however be feasible for a marker in wide clinical use such as CRP, if found motivated and ethical. But, for a novel biomarker like HBP, you would have to at least provide a basic interpretation for a positive or negative result such as “low”, “moderate” or “high” risk of sepsis within 72 hours (Design II in Fig. 5). An example of such a study is the TRIAGE III trial where ED physicians were cluster randomised to see or not see suPAR levels along with corresponding unadjusted mortality rates (237). The effect of such an intervention will of course be difficult to predict on beforehand and it may be difficult to generalize to other populations based on individual physician’s interpretation of risk. But this simple design holds a great advantage in the purity of its evaluation of the introduction of a prognostic biomarker simply because it is pragmatic (238).

4.4.4 Test-treatment trials

Connecting a test result to an intervention can make it easier to interpret the results of biomarker introduction and hence the results of a trial. But it doesn’t make it easy, and it results in an assessment of the entire treatment-test-strategy so that a weak intervention may discard a perfectly accurate biomarker (239). The most elegant way of doing this methodologically is by first splitting the population based on a positive or negative test result and then randomising either group to intervention or control so that you end up with a four groups (Design III in Fig. 5) (240). If two tests are being compared, you can assess both tests in all participants and then randomly disclose either test for half the population (239). The benefit of this design is that you can effectively deduce the details for the difference in effect between intervention and control based on biomarker allocation. If you have intervention-vs-control effect in A) only the biomarker positive group, you are dealing with a

prognostic biomarker or if you have B) effect in both positive and negative groups, but a larger effect among positive patients, you have found a *predictive* biomarker or if you observe C) the intervention has equal effect among positive and negative patients you are looking at a useless or coin flip biomarker.

A major difficulty in designing such a trial is finding the appropriate intervention for the condition. Choosing an intervention without proven effect for the whole population can be considered overly risky for a costly RCT but can be feasible if preceded by well-conducted observational studies. Choosing a clearly superior treatment overall makes control groups not ethically feasible. Instead, test-treatment trials often include an intervention that is presumably reasonably effective, but cumbersome, costly or have side effects so that it is not feasible to perform in all patients. An example is radiological work-up or antibiotics de-/escalation (241) or speciality physician consultation (242) or escalation to a higher level of care. In these instances, the investigators may be tempted to only randomise biomarker positive patients and keeping biomarker negative patients as control (Design IV-VI in Fig. 5) because of the difficulties in defending a costly or harmful intervention in the biomarker negative (null hypothesis) group. The problem with these designs is that it is impossible to determine if the accuracy of the biomarker contributed to the outcome of the trial or if a coin flip would have been equally effective since you have no intervention-control comparison in the biomarker negative group (243). Because many test-treatment trials are complex with stepwise treatment algorithms, use ≥ 2 biomarker cut-offs, evaluate ~ 4 subgroups and because sample size calculation are often based on weak foundation, some authors suggest that it would be appropriate to employ adaptive trial design in test-treatment trials, using pre-determined interim analyses that can adjust allocation in parallel with trial recruitment (239).

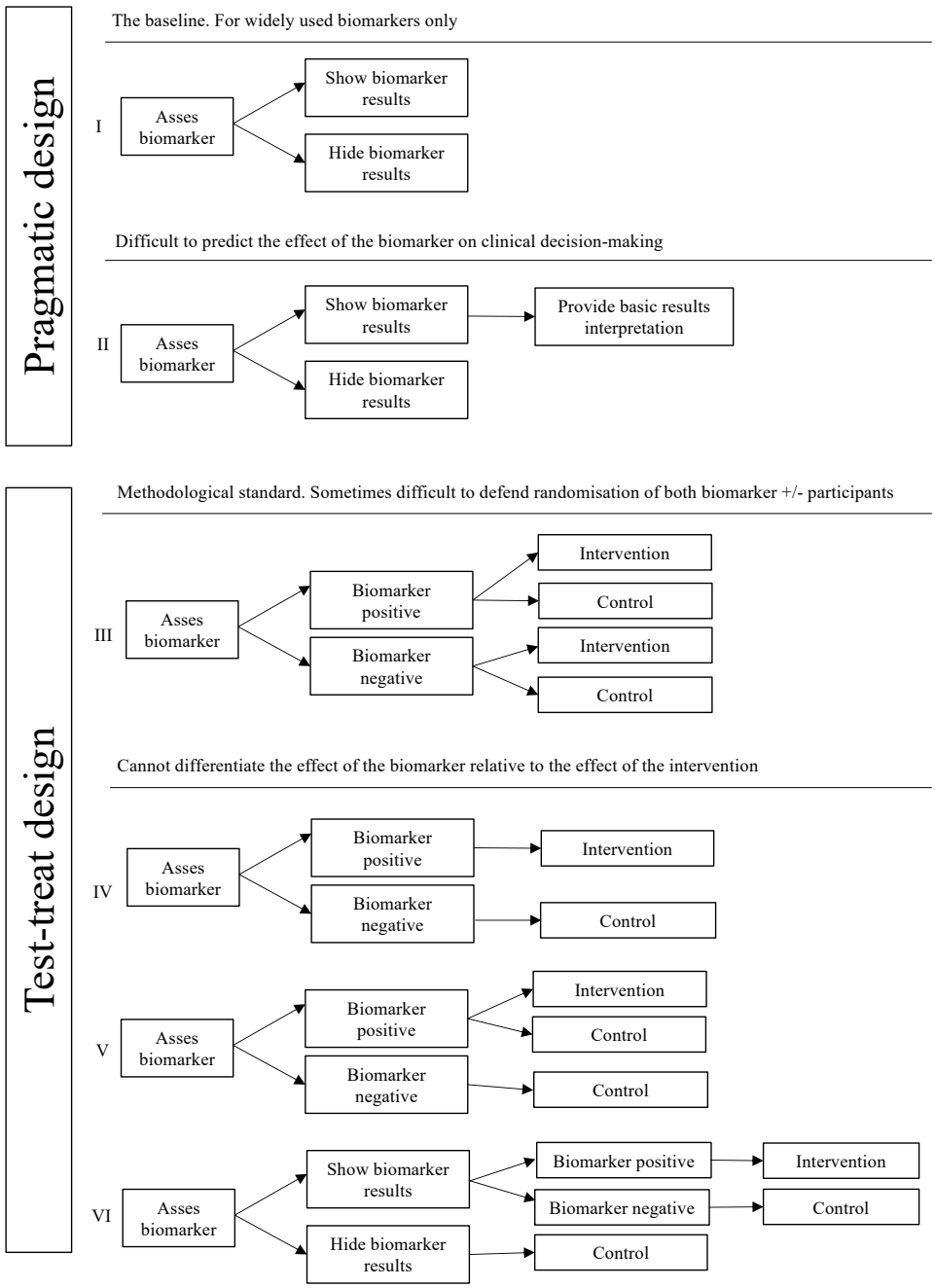

Figure 5. Diagnostic randomised controlled trial design examples.

5 Thesis aims and rationale

5.1 Overall aim

The overall aim of the thesis has been to investigate the prognostic accuracy and added value of single and repeated measurements of Heparin-binding protein as a clinical biomarker in the emergency department and the intensive care unit regarding infection-related organ dysfunction and sepsis survival.

5.2 Specific aims

5.2.1 Paper I

To evaluate HBP as a prognostic biomarker for infection-induced organ dysfunction among patients seeking medical attention at the emergency department regardless of whether there was a suspicion of infection or not; and to compare the prognostic properties of HBP to other biomarkers currently in use.

5.2.2 Paper II

To investigate whether plasma HBP would add predictive value to known clinical risk factors regarding development of sepsis-related acute kidney injury (AKI).

5.2.3 Paper III

To describe the kinetics of plasma HBP during septic shock using frequent sampling, and to investigate an association between plasma HBP concentration and measures of cardiovascular organ dysfunction severity over time.

5.2.4 Paper IV

To investigate if a single HBP measurement or if repeated HBP measurements will provide added prognostic value to clinically available risk factors regarding 90-day survival in ICU patients with sepsis.

5.3 Overall rationale

There is wide consensus that biomarkers have an important role in sepsis research and in the care of patients with sepsis (5) and previous studies have supported the accuracy for plasma HBP in sepsis prognostication (148, 149). There are several questions regarding plasma HBP that remains to be answered to enable optimal use in the clinic. These questions include plasma concentration kinetics over time, prognostic ability in patients without infection and an evaluation for the added prognostic value of HBP to clinically available risk factors.

5.4 Specific rationale

5.4.1 Paper I

The "Help in the Emergency Room to detect Organ dysfunction (HERO)" study (NCT02366650) was conceived around 2015 because there was an interest from the research group and a request from reviewers and editors to investigate HBP's prognostic accuracy (with focus on specificity) on sepsis development among patients both with and without a suspected infection at the ED. The research groups' previous studies on HBP's prognostic accuracy, a single-centre study from 2009 (148) and a multi-centre study from 2013 (244), had only recruited patients with suspected infection.

5.4.2 Paper II

The "FINNAKI-HBP" study was conceived in 2015 when there was a wide consensus that better biomarkers for acute kidney injury (AKI) were needed to improve diagnosis and enable earlier intervention (72). The study took form as collaborative effort between the principal investigator for the observational FINNAKI study who had plasma samples and patient data, and our research group which was already involved in a mechanistic project on HBP and AKI at the time (127).

5.4.3 Paper III

The "HBP Kinetics in Septic Shock (KISS)" study was conceived around 2014 because there was very little published data on how plasma HBP levels change over time, particularly in tighter than daily sampling (152). Knowledge on basic biomarker kinetics was considered essential for the efficient use of HBP in the clinic by our research group, in analogy with how we use current infectious biomarkers like CRP and PCT. Support from clinical data regarding HBP's role in sepsis pathophysiology was also of interest.

5.4.4 Paper IV

The "Finnish AKI repeated measurements (FA-repeat)" study (ISRCTN15560762) was conceived in 2017 to further investigate plasma HBP concentration dynamics over time in a larger cohort and to translate repeatedly measured HBP into prognostic value. The use of repeated measurement to improve prognostication is common for current infectious biomarkers (245, 246) but had not been studied for plasma HBP. Previous studies on HBP and sepsis survival also lacked a comprehensive adjusted survival analysis and did not report on added prognostic value (152, 174, 177).

6 Thesis methods

6.1 Overall methods

Setting, population and data

All investigations were conducted as observational studies using hospitalised patient data and clinical plasma samples on individuals above 18 years of age. All patient data were collected from patient-specific CRF:s or electronic medical journals. All data collection was blinded to the biomarker test results. There were no sample size calculations performed for either study.

Ethics

All study participants were asked to participate in the study and to give their written informed consent and if unable, their next-of-kin was asked for permission. All studies were approved by local ethical boards in Lund and Helsingborg (Dnr 2014/741 and 2016/271), in Helsinki, Finland (Ref # 18/13/03/02/2010), in Bern, Switzerland (KEK 315/14), and Vancouver, Canada (H11-00505).

Biomarker analyses

All plasma samples were centrifuged and stored at -80°C prior to analyses. HBP concentrations were analysed in duplicates using Axis-Shields commercial HBP ELISA (Axis-Shield Diagnostics, Dundee, UK) according to the manufacturer's directions. Intra-test variability was controlled through repeated analyses when the coefficient of variation (%CV) was above 10% and analyses were performed with positive and negative controls. Procalcitonin was analysed using ADVIA Centaur BRAHMS PCT assay. Other blood tests and biomarkers were analysed according to routine at the local clinical chemistry departments.

Definitions

AKI was defined and staged using the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines using both daily serum creatinine and hourly urine output measurements (71). Sepsis-2 was defined according to the 2001 consensus conference (2) and sepsis-3 according to the 2016 consensus publication (1).

6.2 Specific methods

6.2.1 Paper I

Setting, eligibility and sampling

The "HERO study" (NCT02366650) was conducted as a prospective, multicentre, observational, convenience sample study recruiting patients at the emergency department with affected vital signs (respiratory rate >25 breaths per minute, heart rate >120 beats per minute, altered mental status, systolic blood pressure <100mmHg, oxygen saturation <90% without oxygen, oxygen saturation <93% with oxygen and/or reported oxygen saturation <90%) at two Swedish (Skåne University Hospital, Lund and Helsingborg Hospital, Helsingborg), one Swiss (Inselspital University Hospital, Bern) and one Canadian (St Paul's Hospital, Vancouver) academic centres in 2015 to 2016. Plasma samples were collected within 2 hours from ED admission.

Endpoints, definitions and statistics

The primary endpoint was infection-induced organ dysfunction within 72 hours from ED admission as pre-specified in Clinicaltrials.gov (NCT02366650). The secondary analysis used a composite endpoint called *critical infection* and was defined as death or ICU admission within 24 hours or ≥ 5 SOFA-score within 12-24 hours from ED admission. The primary endpoint was evaluated using AUC and the secondary endpoint analyses included AUC, multivariable logistic regression, fictive stochastic cohort generation with varying proportion of infected patients from 10-100% and local weighted scatterplot smoothing. Analyses were performed on complete cases.

6.2.2 Paper II

Setting, eligibility and sampling

The "FINNAKI-HBP study" was a *post hoc* study including patients with severe sepsis or septic shock diagnosed on day one and who had plasma samples available from admission from the prospective, observational, multicentre FINNAKI study (67). The FINNAKI study had consecutively included all emergency ICU admissions and the elective admissions with an ICU stay of above 24 hours from seventeen Finnish ICUs during a 5-month period (1 September 2011–1 February 2012) and reported the incidence, risk factors and 90-day mortality of patients with AKI. Plasma samples were collected within 2 hours from ICU admission.

Endpoints and statistics

The primary endpoint was development of AKI stage 2-3 from 12 hours after ICU admission up to 5 days. A clinical prediction model was constructed using all baseline characteristics as candidate predictors and selection of variables to be included in the final model was done using stepwise univariable logistic regression (cut-off $p > 0.3$) and multivariable logistic regression (cut-off $p > 0.1$). The added value for HBP to the prediction model was evaluated using change in AUC, category-free net reclassification index (cfNRI) and integrated discrimination improvement (IDI). Secondary endpoints were evaluated using Kaplan–Meier survival curve and log rank (Mantel–Cox) test, independent-sample t tests and Mann–Whitney U test. Analyses were performed on complete cases.

6.2.3 Paper III

Setting, eligibility and sampling

The "HBP KISS study" was a dual-centre, observational, convenience sample study conducted at two general mixed ICUs in Sweden, Skåne University Hospital in Lund in 2014 and Helsingborg Hospital in September 2016 to February 2018. Patients with suspected septic shock (sepsis-3) were screened for inclusion. Plasma sample collection was started within 2 hours from ICU admission and repeated every 4 hours for 3 days, or until death or ICU discharge.

Endpoints, definitions and statistics

This exploratory study did not investigate primary or secondary endpoints in the traditional sense but focused on biomarker kinetics and association to surrogate measures of cardiovascular organ dysfunction. Longitudinal HBP and PCT in plasma were comparatively associated with mean arterial pressure (MAP) and noradrenaline (NA) dose as primary outcome measures and systemic vascular resistance index (SVRI, from non-invasive monitoring) as an exploratory outcome measure. Generalized linear mixed-effects regression were the primary statistical method used without adjustments in a crude model, with adjustments for time since ICU admission, NA dose, vasopressin (yes/no) and/or cardiac index (CI) in the simpler models and additionally age, gender, chronic heart failure (yes/no), Simplified acute physiology (SAPS) 3 score, SOFA score and lactate in the fully adjusted models. Analyses were performed on complete cases (including at least one HBP concentration).

6.2.4 Paper IV

Setting and eligibility and sampling

The "FA-repeat study" (ISRCTN15560762) was an ancillary *post-hoc* study of the FINNAKI study (67) including patients with severe sepsis or septic shock diagnosed on the day of ICU admission and who had at least one plasma sample available from the first five days of ICU stay. Plasma was sampled on ICU admission and repeatedly up to seven times during the first five days (hour 0, 12, 24, 36, 48 and day 3 and 5), referred to as "longitudinal HBP".

Endpoints, definitions and statistics

The primary endpoint was 90-day survival. The primary objective was to investigate the added prognostic value of longitudinal HBP regarding the primary endpoint. The secondary objectives included an evaluation of the added value of a single HBP concentration on ICU admission and on single- and longitudinal HBP's unadjusted association with 90-day survival. The pre-published statistical analysis plan involved the construction of a clinical prognostic survival model (including age, sex, functional performance pre-ICU, SOFA score, lactate and pre-existing chronic health conditions) and an evaluation for the incremental value of biomarker addition to that model, both as single measurement in an adjusted Cox model and in the form a longitudinal linear mixed-effects joint survival model. Analyses were performed using both multiple imputation for missing data and complete cases as sensitivity analyses.

7 Thesis results

7.1 Overall results

Plasma HBP did not outperform current biomarkers in prognosticating infection-induced OD within 72 hours in ED patients with and without suspected infection. HBP showed potential as a novel candidate for prognosticating sepsis-related AKI when measured at ICU admission. HBP sometimes displayed considerable variability in plasma concentration over time in ICU patients with sepsis. Repeated measurements of plasma HBP in patients in the ICU with sepsis seem to add limited prognostic value to known risk factors regarding 90-day survival.

7.2 Specific results

7.2.1 Paper I

Population

A total of 718 patients were recruited out of which 30 were excluded due to missing or extreme leucocyte count, 146 due to sample haemolysis (visual inspection) and 18 for other reasons. This left 524 included patients to be divided into 5 groups based on observed infection probability from expert medical chart review by two consultants in infectious disease: I. proven bacterial infection (n=96), II. probable infection (n=84), III. probably no infection (n=69) IV. proven non-infection (n=236) and V. viral infection (n=39) (Fig. 1, paper I).

Primary endpoint analysis

The primary endpoint analysis revealed an AUC of 0.73 (0.68-0.78) for HBP, 0.82 (0.78-0.86) for CRP and 0.69 (0.64-0.74) for PCT to discriminate infection-induced organ dysfunction within 72 hours from ED admission when including all patients regardless of observed infection probability (n=524) (Table S7, paper I). When only looking at patients confidently classified as infected or not infected (group I or IV, n=332), the AUC for HBP was 0.82 (95% CI: 0.76-0.87) versus 0.87 (0.83-0.92) for CRP and 0.79 (0.70-0.82) for PCT (Table 2, paper I). Also including haemolytic

samples did not affect HBP's AUC much (0.72, 95% CI: 0.67-0.76, n=670) (Table S8, paper I). An exploratory analysis using multiple fictive datasets with a varying proportion of infected patients based on data from group I and IV indicated that CRP had superior AUC compared to HBP when the proportion of infected patients were ≤ 66% and inferior AUC when ≥ 67% (Fig. 7 in thesis and Fig. 5 in paper I).

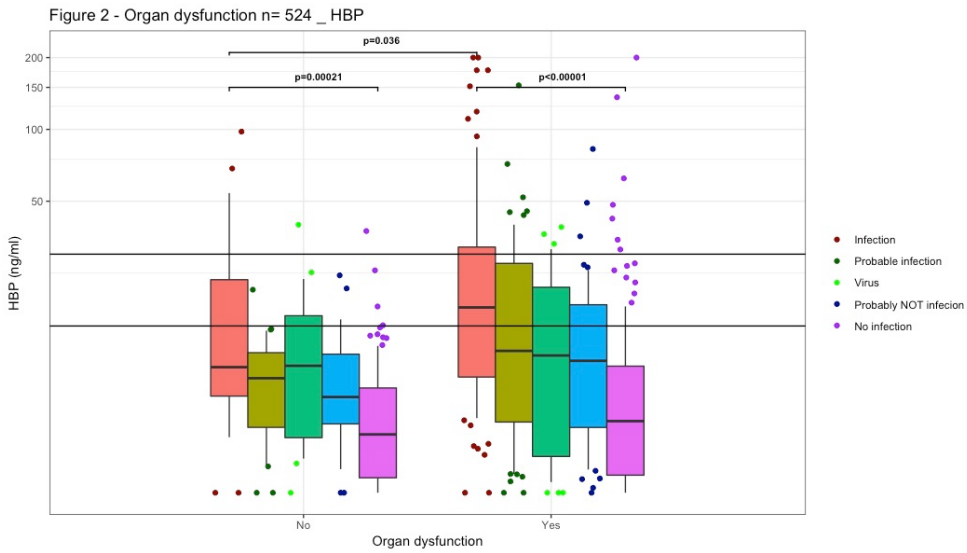

Figure 6. Levels of HBP are shown for patients with and without OD and divided in 5 classes depending on the likelihood of infection. Boxes represent the first, second and third quartile with whiskers extending to the 10th and 90th percentile. HBP-values >200 ng/mL was plotted at 200 ng/mL. Horizontal lines represent 15 and 30 ng/mL.

Secondary endpoints analyses

HBP AUC for *critical infection* was 0.88 (95% CI: 0.77-0.99) versus 0.86 (0.73-0.99) for CRP and 0.76 (0.60-0.93) for PCT in group I and IV (n=332) (Table 3, paper I). When all groups were included (n=524) the AUC was 0.87 (0.79-0.95) for HBP, 0.81 (0.72-0.91) for CRP and 0.76 (0.65-0.87) for PCT (Table S7, paper I). For *critical infection*, HBP had superior discriminatory ability compared to all other biomarkers across the entire fictively modelled infection proportion range (10-100%) (Fig. 7 in the thesis and 5 in paper I). In an exploratory logistic regression analysis using the biomarker as the outcome and OD and final infection diagnosis as co-variates, CRP was strongly associated with infection but not significantly associated with OD while HBP were significantly associated with both (Table 4, paper I). Median HBP was also uniformly numerically higher in a stepwise fashion for each group of increasing observed infection probability (Fig. 6 in thesis and Fig. 2 in paper I) and for every additional failing organ in group I versus IV (Fig. 3, paper I), but with considerable range overlap. The risk for *critical infection* appeared to increase in a linear fashion with increasing HBP concentrations, although a steeper slope can be seen at 0-50 ng/mL versus >50 ng/mL (Fig. S3, paper I).

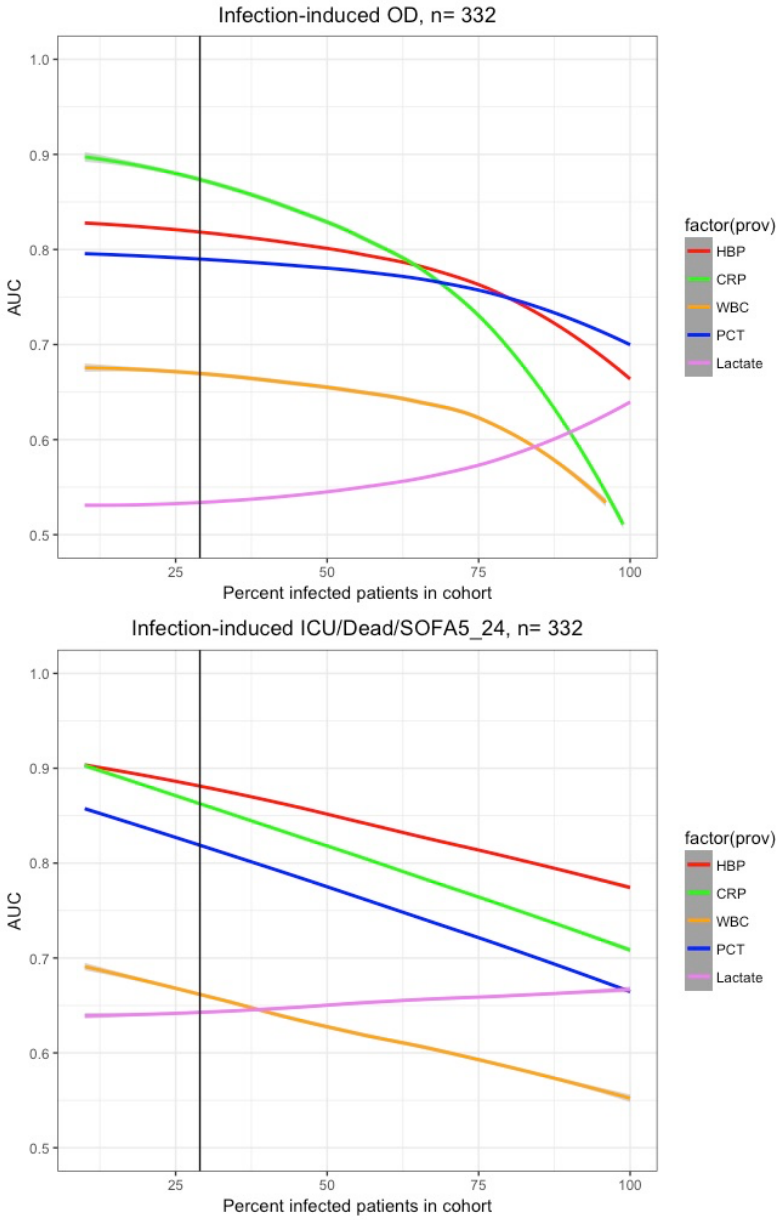

Figure 7. The discriminatory properties of biomarkers change with the frequency of infection in the cohort. Infected and non-infected patients (group I and IV) were included in this plot (n=332, group I and IV). Samples from the two populations (infected, group I and non-infected, group IV) were drawn to mimic cohorts with the proportion of infected patients ranging from 10% to 100% in steps of 1%. For each proportion of infected patients 100 stochastic cohorts were drawn with replacement and the AUC-values for the different biomarkers were calculated and the median was calculated and plotted. The vertical line represents the proportion of infected patients in the original whole cohort (group I-V, n=524).

7.2.2 Paper II

Population and prediction model

A total of 601 patients were included from the original FINNAKI cohort (n=2901) excluding 2194 patients who did not have severe sepsis on admission and 106 patients without a plasma sample from admission or missing sample ID (Fig. 1, paper II). Ninety patients developed AKI stage 2-3 within 12 hours from ICU admission leaving 511 eligible patients for the primary endpoint analysis (Fig. 1, paper II). The final prediction model included the variables age, SAPS II without points for renal failure or age, and serum creatinine up to 48 hours pre-ICU (Table 1, paper II).

Primary endpoint analysis

Adding categorized plasma HBP on ICU admission to the prediction model increased the AUC to 0.82 (95% CI: 0.77–0.87) from 0.78 (0.73–0.84) for the risk model alone (n=489) (Table 2, paper II). cfNRI for events was 37.4% (95% CI: 18.6–55.1), 24.6% (15.2–34.0) for non-events and IDI event was 0.042 (0.02–0.63) and IDI non-event was 0.011 (0.003–0.019) (Table 2, paper II). Continuous plasma HBP alone had AUC 0.70 (95% CI 0.64–0.76, n=511) to prognosticate the primary endpoint, which ranged from 0.66 to 0.72 in five sensitivity analyses (n range 114-601) (Table S5, paper II).

Secondary endpoints analyses

Patients with plasma HBP above 20 ng/mL had higher unadjusted 28-day mortality compared to patients with a lower plasma HBP (28 vs. 21%, p=0.03, n=601) (Fig. 2, paper II). Mean fluid balance within 24 hours from ICU admission was higher in patients with a higher plasma HBP (≥ 20 ng/ml) compared to patients with a lower plasma HBP on ICU admission (+ 2452 ml vs. + 1031 ml, p<0.001) (Fig. 2, paper II). Maximum SOFA score within 5 days from ICU admission was higher in patients with higher plasma HBP compared to patients with lower HBP on ICU admission (mean points 9.1 vs. 7.4, $p < 0.001$, n=601).

7.2.3 Paper III

Population and samples

A total of 24 patients (1 non-infectious shock), 2 in Lund and 22 in Helsingborg (out of 88 eligible patients) were included. Included patients provided 341 reliable plasma samples (average 15 per patients) out of 370 collected samples because 21 were excluded for sample haemolysis (> 1 g/L haemoglobin) and 8 due to prolonged lag time to lab handling (> 1 hour) (Fig. 1, paper III).

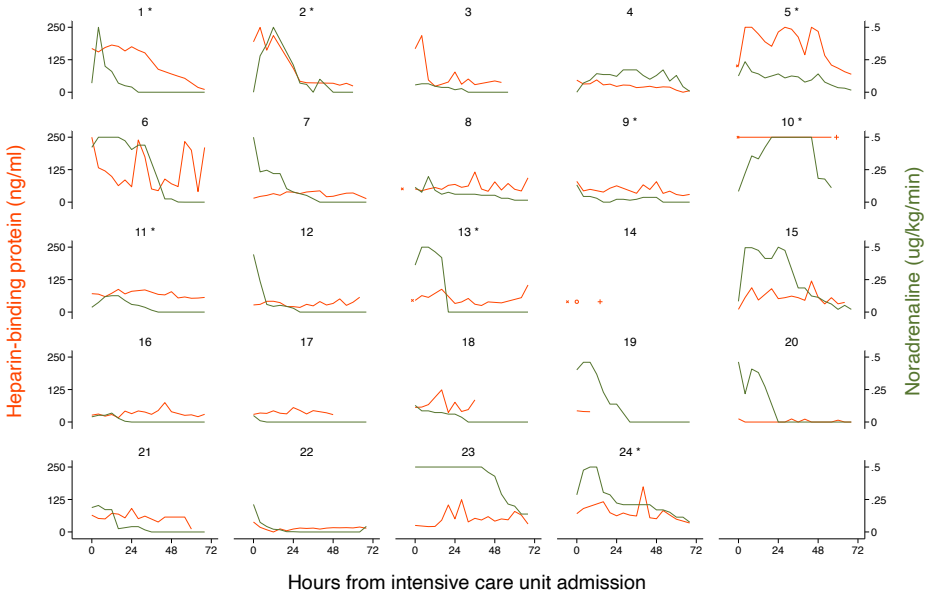

Figure 8. Plasma HBP and NA doses for all 24 patients during the first 72 h of ICU stay. HBP and NA values in this graph are truncated at 250 ng/ml and 0.5 µg/kg/min, respectively. Eight patients are marked with an "*" indicating that they had a significant correlation between plasma HBP and NA dose according toSpearman's R. "x" marks patients' HBP at the ED, if available. Deaths within 72 h are marked with a "+" (n =2). Patient id 12 only had one HBP measurement in ICU prior to death, marked with an "o". Patient id 10 is also viewed separately in Fig. S2, paper III, because of repeated HBP levels above 250 ng/mL

Primary, secondary and exploratory analyses

The kinetics of plasma HBP was highly variable over time, with occasional > 2-fold increases and decreases in between 4-hour measurements, sometimes resembling a peak-and-baseline pattern (Fig. 8 in thesis and Fig. 2, paper III). PCT decreased steadily over time in practically all patients (Fig. S1, paper III). Every 100 ng/mL increase in HBP (range 0 to 932 ng/mL) corresponded to 1.4 mmHg decrease in MAP in a model adjusted for time, NA dose and vasopressin (95% CI: -1 to -2.3 mmHg, p=0.04). The association was mostly driven by patients with very high HBP

and severe cardiovascular OD (Fig 3, paper III). HBP was not associated with MAP in the fully adjusted model. Every 100 ng/mL increase in HBP was further associated with 30% increase in NA dose in an unadjusted model (95% CI 3 to 60%, p=0.03, n_{obs}=340) and 99 dyne s cm^{-5} m^{-2} decrease in SVRI in a model adjusted for time, CI and NA dose (95% CI −36 to −162, p = 0.002, n_{pat} = 13) (Fig. S3, paper III). PCT had a stronger association to NA dose than HBP in an unadjusted model (Akaike information criterion -1092 versus -1030) but was not significantly associated to NA dose, MAP or SVRI in any model including an adjustment for time. The area under the curve for HBP was slightly more closely related to the area under the curve for NA dose than it was to the area under the curve for PCT (R=0.49 vs. 0.35 and p=0.04 vs. 0.10).

7.2.4 Paper IV

Population and samples

A total of 652 patients were included, providing 647 plasma samples on ICU admission and 2565 longitudinal samples (Fig. 1, paper IV). One-hundred-ninety patients died within 90 days (29%) (Fig. 1, paper IV). The individual plasma HBP concentrations over time can be viewed in Figure 9 (Fig. 4 in paper IV).

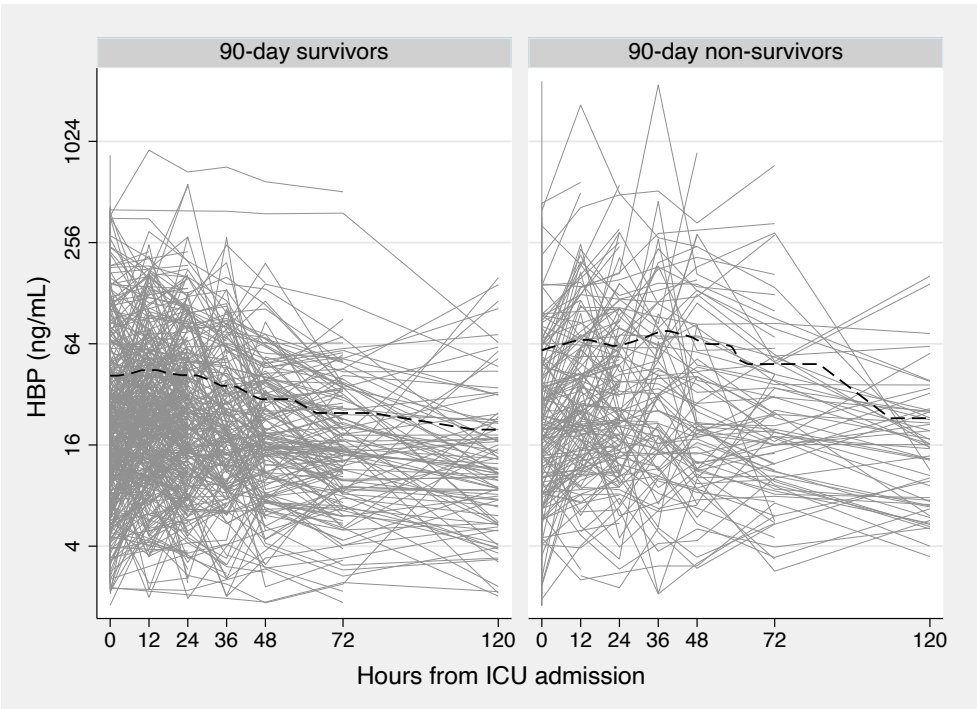

Figure 9. Spaghetti plot on individual plasma HBP concentrations over the first five days in the ICU. The y-axis is on the log scale and HBP values below 1.8 (n_{obs}=67) have been excluded for ease of viewing purposes. The dotted lines represent Kernel-weighted local polynomial smoothing of HBP values over time.

Primary analysis

The added value of longitudinal HBP was statistically significant in a complete case analysis (HR 1.06, 95% CI: 1.01 to 1.12, p=0.019, n=576) and in a *post-hoc* analysis using multiple imputation and nonlinear HBP over time (HR 1.26, 95% CI: 1.11 to 1.43, p<0.001) but not in the pre-specified analysis using multiple imputation and linear HBP over time (HR 1.11, 95% CI: 0.93 to 1.31, p=0.245). The size of the added value is best appreciated graphically (Fig. 10 in thesis and Fig. 4 in paper IV). Longitudinal HBP decreased slightly over time in both survivors and non-survivors, sometimes with considerable variability between measurements (Fig. 3, paper IV).

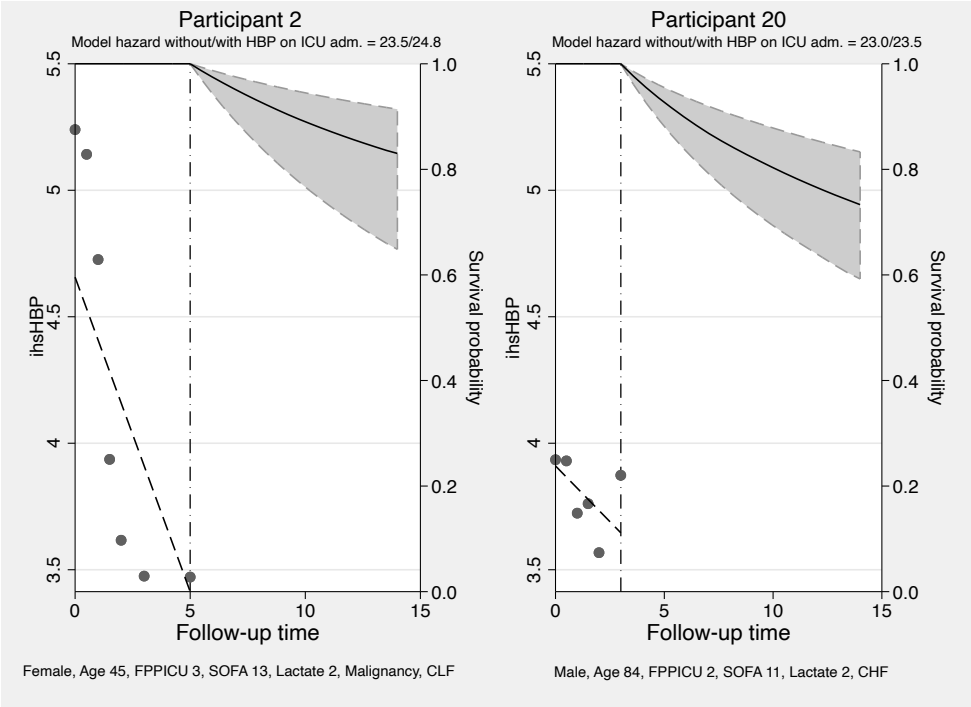

Figure 10. Predicted survival probability up to day 14 for participant 2 and 20. This is based on estimates from the complete case joint prognostic model analysis and individually available linear longitudinal ihsHBP over time (dots and dotted line) Notice how participant 2 had a lower survival probability at baseline (higher HR) compared to participant 20, both with and without HBP on ICU admission, but after taking the change of longitudinal ihsHBP into account, the survival probability was higher for participant 2 at day 14. Actual survival time was 47 days for participant 2 and 10 days for participant 20. Population median ihsHBP is 3.6 and range is 0 to 8.4.

HR; Hazard ratio, CLF; Chronic liver disease, SOFA; Sequential organ failure assessment score on day 1, CHF: Chronic heart failure, HBP; Heparin-binding protein, ihsHBP; Inverse hyberbolic sine transformed HBP, FPPICU; Functional performance before intensive care unit admission (0-3), Lactate; maximum lactate 24h pre/post ICU admission

Secondary analyses

A single HBP concentration on ICU admission did not add statistically significant value to the clinical prognostic model (p=0.39) and the estimated size of added value was minimal (+0.01) (Fig. 2, paper IV). Both HBP on ICU admission (LR test p=0.03) and longitudinal HBP (HR 2.08, 95% CI: 1.75 to 2.47, p<0.001) were significantly associated with 90-day survival in unadjusted models.

8 Thesis discussion

8.1 Future perspectives on HBP

8.1.1 HBP in repeated measurements

Findings from both paper III and IV indicate that plasma HBP concentrations can be highly variable over time in ICU patients with sepsis. A few other studies have reported repeated sampling for HBP (152, 153, 183, 184) but without individual-level graphs or analyses. This finding implies a difficulty in using HBP in once or twice daily sampling, assuming relative linearity, like we do with current infectious biomarkers such as CRP and PCT. It could also have implications for future plasma HBP studies which may or may not consider tighter repeated sampling.

Future potential research questions include:

1. Does plasma HBP vary over time in tighter sampling than every 4 hours in ICU patients with sepsis?
2. Does HBP vary to the same degree in less severely ill patients with suspected infection at the ED?

Limitations

A relevant enquiry is whether the observed high plasma HBP variability over time is merely a biproduct of a flawed biomarker assay. This can be argued to be improbable, based on consistent findings from two different cohorts, where paper III was designed with this specific objective in mind. We also used a commercial assay and included positive and negative controls and duplicate testing with re-testing when the coefficient of variation was above 10% between tests. We further considered what our research group believes to be the prominent error sources to the HBP analyses, namely neutrophil count, sample haemolysis and lag time to lab handling. However, this issue cannot be 100% ruled out until externally validated, since the biomarker results did originate from the same manufacturer's ELISA assay and were analysed in the same laboratory by mostly the same individuals.

8.1.2 HBP and AKI

The results from paper II support the added value of HBP for sepsis-related AKI prognostication through increasing the prediction model's AUC from 0.78 to 0.82. There is additional support from one mechanistic study (127) and from three clinical cohorts (127, 179, 184) reporting good prognostic accuracy for HBP and sepsis-related AKI. Earlier AKI recognition could potentially lead to modified management and reduce damage to renal cells.

Future potential research questions include:

1. What is the prognostic performance of plasma HBP compared to urine [TIMP-2*IGFBP-7] in a head-to-head comparison in the same cohort?
2. What is the clinical benefit of early and accurate AKI prognostication [when assessed in a diagnostic randomised controlled (test-treatment) trial]?

Limitations

The investigation in paper II has limitations. It was a *post-hoc* design using available samples and data, stepwise predictor selection, complete case analyses and did not include a head-to-head comparison to competing AKI biomarkers. Still, the major approach and findings should be valid. They did catch the interest of the primary [TIMP-2*IGFBP-7]-investigators (247). However, when it comes to the evaluation of sepsis-related AKI biomarkers, a direct evaluation of clinical usefulness constitutes a particularly pressing issue. The reason is that the gold standard definition of AKI, using urine output and serum creatinine increase (71), represent a late and coarse representation of what is going on in the kidney and does not easily translate into appropriate interventions (72) besides optimizing supportive sepsis management and avoiding aggravating renal injury (248). A test-treatment trial would be the most appropriate way to motivate the introduction of plasma HBP, or of any novel AKI biomarker, into clinical use.

8.1.3 HBP in the ED

A few important aspects on plasma HBP's role in ED prognostication was clarified in paper I. HBP does not perform as well as CRP in discriminating infection versus no infection in undifferentiated ED patients with affected vitals. This is because HBP is also elevated in patients without infection, showing increasing levels with an increasing number of dysfunctional organs. A single plasma HBP on ED admission does however perform well in prognosticating severe disease from infection within 24 hours in this cohort, and HBP's prognostic performance increases with an increasing proportion of infected individuals in the cohort.

The ED still holds the highest potential for HBP's possible clinical usefulness. Approximately a fifth of patients with infection and intact organ function develop

sepsis within 24 hours (249). It is difficult to clinically discriminate who needs hospital admission and intensified management. The available prognostic information is relatively scarce and hospital beds are definitely scarce. Nonetheless, a direct evaluation of the clinical usefulness of HBP introduction remains to be done.

Future potential research questions include:

1. Does the introduction of HBP to the ED result in a clinically beneficial and relevant outcome compared to control [in a pragmatic diagnostic RCT]?
2. Does HBP add prognostic value to clinically available risk factors regarding development of sepsis among patients in the ED?
3. What is the prognostic value of longitudinal HBP starting from ED admission?

Limitations

Paper I had strengths in that its population and primary endpoint was pre-specified and that it was performed prospectively at four centres in three countries. Its limitations are primarily that many analyses were performed on a limited proportion of convenience included patients depending on the final observed infection probability based on medical charts review by three co-authors of the paper (blinded to the HBP result of course). This impairs external validity. But, since the pre-specified primary outcome analysis was performed across all five subgroups and because all other analyses are performed and visibly presented across different parts of the population as sensitivity analyses in the paper and in supplements, it is possible for the reader to determine which finding is generalisable. The categorisation of patients into five subgroups based on observed infection probability is also most certainly truer to the clinically challenging reality of deciding which patient if suffering from infection and who is not, compared to simple dichotomization, with the downside of adding complexity to the findings' interpretation.

8.1.4 HBP in the ICU

The findings from paper III point to an association between plasma HBP and cardiovascular organ dysfunction severity. Paper IV indicates that the added value for plasma HBP to prognosticate 90-day survival is small. Several previous studies have reported on plasma HBP's association to sepsis severity, both in unadjusted (178, 180) and adjusted analyses (119, 157), and some have shown plasma HBP to be accurate in prognosticating sepsis survival (62, 76, 79) while others have not (146, 172, 182). The relentless question is whether the information conveyed by HBP can be translated into something clinically useful. This is particularly challenging in the ICU since the clinician will have extensive information at the

bedside and the patient is already at the highest level of care. For discussions like the goals of care or the withdrawal of life-sustaining support the added information size from a biomarker would have to be very large to have a clinical impact.

Future potential research questions include:

1. Does repeatedly measured HBP confer prognostic information regarding infection complications in the non-septic ICU patient?
2. Is HBP an accurate prognostic biomarker for fluid accumulation in the ICU?

Limitations

Paper IV was a *post-hoc* study, using the same cohort as paper II. Neither data collection, nor plasma sampling was originally performed with HBP evaluation in mind which may have introduced errors to the HBP analyses and bias in the data analyses. The primary results were strengthened by originating from a thorough pre-published statistical analysis plan, although relying on relatively novel approach of joint modelling and added value.

8.1.5 HBP and therapeutics

In paper III we described an association between plasma HBP, liver failure and circulatory failure which resembled sepsis subtype δ as described by Seymour *et al.* (46). HBP has been investigated *in vitro* to cause increased vascular permeability in endothelial cells (26) and inflammation in renal cells (127, 250). HBP has been studied *in vivo* in a murine model to cause increased renal vascular leak and interstitial haemorrhage (127) and acute lung injury (26). The permeability-increasing effects of HBP have been shown to be inhibited *in vitro* by HBP-specific antibodies (100), Dextran sulphate (100), unfractionated heparin (26), low-molecular weight heparin (LMWH) (127) and albumin (250). These findings suggest that there may be a role for plasma HBP as a predictive biomarker on interventional efficacy and as a therapeutic target.

Future potential research questions include:

1. Does a sudden increase in longitudinal HBP indicate benefit from timely albumin infusion [in a test-treatment RCT]?
2. Do individuals with an elevated plasma HBP benefit from unfractionated heparin infusion or higher doses of LMWH [in a test-treatment RCT]?
3. Does HBP elevation enable simple differentiating of participants that respond beneficially to corticosteroids [in a test-treatment RCT]?

Limitations

Wise from past disappointments (44), the above hypotheses based on hitherto published associations from observational data and cellular or small sample animal models must reasonably be regarded to have low prior probability to prove benefit in an RCT (or a low prevalence and a low positive predictive value). A reasonable next step could be to co-operate with investigators of previous randomised controlled trials testing heparin- (251) albumin- (252) or corticosteroid therapy (253), to evaluate if HBP levels can discriminate relative therapeutic benefit retrospectively. In the event of clearly positive findings with indisputable group separation it could be reasonable to design a full size RCT directly thereafter. If findings were encouraging but ambiguous, a reasonable second step could be to perform a pilot trial using a small sample size and surrogate endpoints to prove feasibility and provide priors for sample size calculations and biomarker cut-offs.

8.2 Heterogeneity and focus on infectious disease

8.2.1 The evolving purpose of the sepsis entity

The primary rationale for the 1992 sepsis definition was to homogenize study populations to allow identification of novel immunomodulatory therapies. Bone identified the SIRS-based sepsis model as a flawed already in 1996. The current SOFA-based sepsis-3-defintion arrived in 2016. The updated definition states in theory that sepsis is the result of a "dysregulated host response". The problem is that sepsis-3 does not include any criteria to support that this is happening in the patient. It only considers organ dysfunction. As we learned in the introductory section on AKI, the host response can be well-regulated and adaptive even when organs are not performing their functions normally. Sepsis has become an encompassing term to include all infections without further differentiating than a hospital mortality of 10% (1). The sepsis-3 authors are most certainly aware that the sepsis definition is not apt to serve as inclusion criteria for novel immunomodulatory therapies as was Bone's original intent. Instead, the sepsis entity has come to serve a different purpose – recognition. While previous iterations of WHO's global burden of disease considered maternal sepsis, HIV/AIDS, pneumonia in children under five, malaria and tuberculosis as separate conditions (254) they can now be estimated under one umbrella term, sepsis (255). This may sound trivial. But being able to level sepsis with other umbrella terms such as "cardiovascular disease" and "cancer" represent a major and ongoing achievement that the sepsis research community will not want to readily abandon by narrowing the sepsis definition in future iterations. Settling on the term severe infectious disease could have had similar practical meaning but not the same political impact.

8.2.2 Infectious origin as a sepsis sub-type

The current COVID-19 pandemic has enabled exceptions from the repeated failures of immunomodulatory interventions in sepsis. First came the reports on the benefit of corticosteroids (256) and next the benefit from IL-6-inhibitors (55, 257) and more recently the benefit from JAK-inhibitors (258). These achievements have naturally been facilitated by the unique opportunity to recruit many study participants during a short period of time. But the RECOVERY trial with its 6425 participants with COVID-19 (256) was not much larger than the ADRENAL trial of corticosteroids recruiting 3800 participants with sepsis (253). Instead, it says something about the potential benefit of singling out one infecting pathogen in consecutive trials in sepsis. It almost goes without saying that patients with the same site of infection and the same infecting pathogen are more likely to benefit from the same intervention. But many sepsis trials do not report the site of infection and the responsible pathogen adequately and even fever pre-define sub group analysis on such basis (259). There are previous evidence on benefit from immunomodulatory therapy based on infectious origin subgroup, but they should be interpreted with great caution as always regarding *post-hoc* subgroup analyses. Patients with pneumococcal pneumonia had larger benefit from activated protein C in the original trial (260), patients with gram-positive infections experienced harm from TLR-4-receptor antagonist targeting gram-negative-derived endotoxin (261) and patients with bacterial meningitis had survival benefit from corticosteroids in bacterial meningitis of pneumococcal origin but not otherwise (262, 263). It poses a challenge to recruit a population that is homogenous in infectious origin, but it is conceivable using contemporary rapid art identification techniques (264, 265). It is a simple concept that deserves more attention in future sepsis trials. The infecting pathogen can be classified as a sepsis subtype (47).

8.2.3 Infectious disease in definitions and guidelines

Besides lacking criteria for the assumed dysregulated immune response, the sepsis definition also lacks criteria for infection (1). This absence risks implying that determining who is suffering from an infection is trivial, which it is certainly not. That is why we divided the study cohort in paper I into five categories depending on infection probability, which can be a challenge even for two infectious disease consultants. A study from 2016 showed that diagnosing sepsis is subjective and highly variable among experienced intensivists (266). Rhee *et al.* write in their discussion that "although the terminology and criteria for organ dysfunction are being updated, this new definition still relies on clinicians' judgement of whether infection is present, as well as whether organ dysfunction is attributable to infection". This lack of stringency will inevitably contribute to sepsis trial population heterogeneity and worse yet, may impede the optimal treatment of our patients. What the sepsis definition giveth in recognition it taketh away in precision.

The SSC guidelines

The Surviving Sepsis Campaign (SSC) guidelines originate from the realm of immunomodulatory therapy like much else in sepsis. It was originally a result of Eli Lilly's marketing strategy for Xigris (recombinant human activated protein C) (267). The fifth iteration of SSC guidelines were just updated in 2021 and are in widespread clinical use (29). The focus on the infectious process is unfortunately largely lacking from the SSC guidelines as well. The authors touch on antimicrobial resistance, antifungal therapy and a general recommendation to perform source control and search for alternative diagnoses but fail to mention how the site of infection or the infecting pathogen may affect the recommended approach. The treating physician thereby risks missing that patients with *Staphylococcus aureus* bacteraemia of unknown origin require a completely different work-up and treatment approach than do patients with *Escherichia coli* urinary tract infection (56, 242). The SSC authors may feel that such details are outside of their scope but their guideline document with 93 recommendations and 653 references do give the impression of being comprehensive in most other regards. This focus on the infectious process can be argued to be particularly important in the Anglo-Saxon system where infectious disease physician are seldomly involved in the acute or critical care of sepsis patients. It is a chicken and egg situation. The characteristics of the sepsis definitions and the guidelines are the natural result of the individuals who wrote them. They were almost exclusively critical care physicians. Marshall writes that what SIRS had was “face validity”, meaning that the patients who fulfilled the criteria looked like the kind of patients a critical care physician would clinically recognise as sepsis (44). It is about time that infectious disease physicians get into the game.

8.2.4 Adaptive or maladaptive host response

What the sepsis definition authors were plausibly trying to get at with the concept or SIRS and a dysregulated host response, is the vague moment when the immune systems’ accumulated actions transcend from being adaptive and beneficial to being maladaptive and harmful to the individual. This would constitute a theoretically reasonable place to try immunomodulatory therapies. But pinning it down in practice is complicated. COVID-19 provides the most straightforward example. Because the SARS-CoV-2 virus replicates and spreads mostly in first five days of infection (268), and most patients seek care around day eight or nine and the disease severity peaks and inflammatory biomarkers peak somewhere around day ten or eleven, it is fairly easy to understand that the RECOVERY trial found support for corticosteroid therapy in patients with symptom duration of more than one week (256). Things get more complicated when we take the example of *streptococcus pyogenes*. This bacteria colonizes the upper respiratory tract in up to 20% of children asymptomatically (269) but the same bacteria in the upper respiratory tract of other

individuals can lead to treatment-resistant and fatal septic shock (270). The shift from adaptive to maladaptive host response in this case is not a simple mechanism of time. It poses a challenge to identify such cases before the disease becomes treatment resistant. Individual genetics may be a good place to start (271).

8.2.5 A model for immunomodulation

Figure 11 displays a conceptual hypothetical two-dimensional model for identifying individuals with a theoretically increased probability of benefit from immunomodulatory therapy in sepsis.

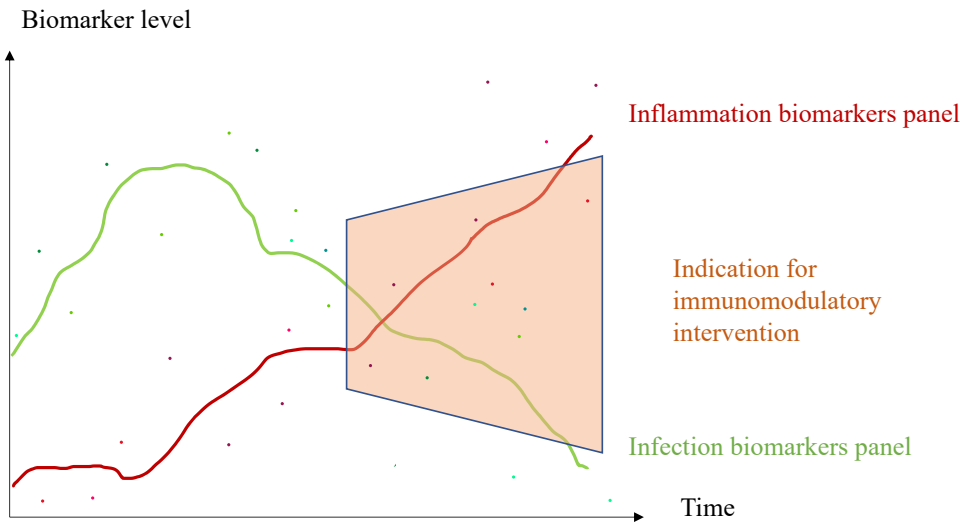

Figure 11. Two-dimensional longitudinal biomarker model concept for indication of immunomodulatory therapy in sepsis. Dots are hypothetical actual biomarker values, with shades of red representing the inflammatory biomarkers and shades of green the infection biomarkers. The lines are hypothetical polynomial smoothing of biomarker panels. The orange box defines the hypothetical temporal opportunity for therpaeutic immunomodulation.

The model consists of two longitudinal biomarker panels, because patients may change subclasses over time (272). The infection biomarker panel contains biomarkers of microbial load (e.g., PCR cycle threshold, virulence factors, microbial degradation products or antigen-antibody-compounds). The inflammatory biomarker panel includes markers of host response (e.g., IL-6, HBP or CRP). The panel could include categorized biomarker cut-offs if biologically feasible. An example could be that the model suggests benefit from immunomodulation when 4 out of 9 biomarkers are above the cut-off in the inflammatory panel it and no more than 2 out of 5 biomarkers in the infection panel are above their cut-off. Cut-offs and choice of biomarkers in each panel could change depending on the type of therapy. You could call the overall concept

“predictive enhancement” in contemporary sepsis research jargon (48). Any resemblance to the viral load of SARS-CoV-2 versus CRP over time in relation to the time of indication for corticosteroids is unintentional (256).

8.3 Concluding remarks

Severe infectious diseases will continue to challenge modern medicine for all foreseeable future. This thesis has contributed to clarify some aspects of the prognostic value of the novel biomarker Heparin-binding protein. The collected results from the four papers may appear predominantly negative. They should be viewed in the larger context of all peer-reviewed investigations on HBP. Elucidating both positive and negative aspects of novel strategies are an essential part of medical research. It is becoming clearer that HBP’s highest potential for clinical usefulness is among individuals with suspected infection at the emergency department in accordance with the original clinical study from 2009 (148). Whether HBP actually confers clinical benefit to patients would best be answered through a well-designed randomised controlled trial.

9 Popular science summary (Swedish)

Vad är sepsis?

Enligt ett konsensusmöte från 2016 bör sepsis teoretiskt definieras som en livshotande organdysfunktion orsakad av ett dysreglerat immunsvar mot en infektion. I praktiken fungerar sepsis-definitionen som ett samlingsbegrepp för all allvarlig infektionssjukdom. Sepsis drabbar ungefär 40 000 personer årligen i Sverige. Ungefär en fjärdedel av alla patienter med sepsis på sjukhus dör inom 90 dagar. Överlevare lider inte sällan av långtidskonsekvenser i form av kognitiva besvär och nedsatt funktionsgrad. Behandlingen av sepsis grundar sig på tidig och korrekt antibiotikabehandling och operation av eventuell infektionshärd samt organ-understödjande behandling såsom syrgas eller kärlsammandragande läkemedel. Mängden pengar som satsats på forskningen om sepsis har varit liten de sista 50 åren jämfört med de ekonomiska satsningarna inom hjärt-kärl-sjukdomar och cancer. Intresset för sepsis har dock växt bland beslutsfattare de sista åren i takt med att man klarlagt hur många som drabbas. År 2017 beslutade världshälsoorganisationen sig för att betrakta sepsis som en global hälsoprioritet. Kännedomen bland Sveriges befolkning om vad sepsis är har ökat från 21% år 2015 till 59% år 2021.

Varför denna avhandling?

Det finns flera kvarstående utmaningar för att optimera omhändertagande av patienter med sepsis. En avgörande utmaning är att tidigt prognostisera vilka individer som löper störst risk att drabbas av sepsis. På en akutmottagning bland personer med tecken på infektion är det ungefär en på fyra. Här finns en teoretisk potential för ett blodprov att underlätta identifieringen av den person som behöver stanna på sjukhus medan de andra kan gå hem. Koncentrationen av Heparin-bindande protein (HBP) kan på labbet mätas i ett sådant blodprov och har i flera tidigare studier visat sig ha mycket god träffsäkerhet för att avgöra vilka individer som löper störst risk att drabbas allvarligt av en infektion. Det är emellertid okänt hur pass träffsäker HBP är för att prognostisera ett allvarligt utfall bland individer utan misstanke om infektion. Det är vanligt att infektionsmarkörer mäts upprepat över flera dagar för att utvärdera om insatt behandlingen fungerar. Detta har tidigare inte studerats i tillräcklig utsträckning för HBP. Det övergripande syftet med de fyra

delarbetena i denna avhandling är sammanfattningsvis att utvärdera HBP's träffsäkerhet för att prognostisera allvarliga konsekvenser av en infektion bland patienter med, eller med risk för, sepsis, utifrån enstaka eller upprepade blodprov. Det förlängda syftet är att förbättra omhändertagandet av patienter med sepsis.

Vad är visar avhandlingens fyra delarbeten?

I delarbete I visade sig inte HBP vara lika bra som en av dagens använda infektionsmarkör CRP för att prognostisera utveckling av sepsis. Detta undersöktes bland 524 personer med och utan infektion som rekryterats vid fyra akutmottagningar i tre länder åren 2015 till 2016. Däremot presterade HBP bäst i att identifiera de individer som löpte risk för ett väldigt allvarligt utfall från sin infektion såsom död eller intensivvård.

Av delarbete II framgick att koncentrationen av HBP i blod vid ankomst till intensivvårdsavdelningen kan tillföra prognostiskt värde avseende utveckling av sepsis-relaterad akut njurskada utöver de riskfaktorer som redan är kända för den behandlade läkaren. Analysen gjordes på 511 deltagare som tidigare inkluderats i en finsk studie år 2011 till 2012. Resultaten antydde vidare att en förhöjd nivå av HBP var associerat med högre sjukdomsgrad och en positiv vätskebalans.

I delarbete III upptäcktes att koncentrationen av HBP ibland kan fördubblas eller halveras på bara fyra timmar under ett sjukdomsförlopp med septisk chock på intensivvårdsavdelningen. Undersökningen gjordes bland 24 personer som rekryterades under åren 2014 och 2016 till 2018 vid två sjukhus i Skåne. Blodprov togs var fjärde timme under tre dygn. Det visade sig också att nivån av HBP var relaterad till graden av funktionsnedsättning i hjärt- och kärlsystemet.

I delarbete IV studerades också upprepade mätningar av HBP, och koncentrationen visade sig även i denna undersökning ofta variera över tid. HBP mätt i 7 upprepade blodprov över 5 dagar på intensivvårdsavdelningen verkade också kunna tillföra ett litet prognostiskt värde, men var sannolikt av så pass liten storlek att det inte skulle anses kliniskt användbart. Analysen gjordes på 652 personer som ingick i samma finska studie som användes i delarbete II. Endast ett blodprov vid ankomst till intensivvårdsavdelningen tillförde inget motsvarande prognostiskt värde till redan kända riskfaktorer.

Vilken forskningsfrågeställning är viktigast framöver?

För att få ett tillförlitligt stöd för huruvida introduktionen av HBP i klinisk vardag medför en relevant förbättring för patienter med sepsis, eller med risk för sepsis, bör en randomiserad kontrollerad klinisk prövning genomföras.

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