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Full Length Article

Serum amyloid A – A prime candidate for identification of neonatal sepsis

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ABSTRACT

Neonatal sepsis is common, lethal, and hard to diagnose. In combination with clinical findings and blood culture, biomarkers are crucial to make the correct diagnose. A Swedish national inquiry indicated that neonatologists were not quite satisfied with the available biomarkers. We assessed the kinetics of 15 biomarkers simultaneously: ferritin, fibrinogen, granulocyte colony-stimulating factor (G-CSF), interferon (IFN)-γ, interleukin (IL)-1β, −6, −8, −10, macrophage inflammatory protein (MIP)-1β, procalcitonin, resistin, serum amyloid A (SAA), tumor necrosis factor (TNF)-α, tissue plasminogen activator-3 and visfatin. The goal was to observe how quickly they rise in response to infection, and for how long they remain elevated. From a neonatal intensive care unit, newborns ≥28 weeks gestational age were recruited. Sixty-eight newborns were recruited to the study group (SG), and fifty-one to the control group (CG). The study group subjects were divided into three subgroups depending on clinical findings: confirmed sepsis (CSG), suspected sepsis (SSG) and no sepsis. CSG and SSG were also merged into an entire sepsis group (ESG) for sub-analysis. Blood samples were collected at three time-points; 0 h, 12–24 h and 48–72 h, in order to mimic a “clinical setting”. At 0 h, visfatin was elevated in SSG compared to CG; G-CSF, IFN-γ, IL-1β, −8 and − 10 were elevated in SSG and ESG compared to CG, whereas IL-6 and SAA were elevated in all groups compared to CG. At 12–24 h, IL-8 was elevated in ESG compared to CG, visfatin was elevated in ESG and SSG compared to CG, and SAA was elevated in all three groups compared to CG. At 48–72 h, fibrinogen was elevated in ESG compared to CG, IFN-γ and IL-1β were elevated in SSG and ESG compared to CG, whereas IL-8 and SAA were elevated in all three groups compared to CG. A function of time-formula is introduced as a tool for theoretical prediction of biomarker levels at any time-point. We conclude that SAA has the most favorable kinetics regarding diagnosing neonatal sepsis, of the biomarkers studied. It is also readily available methodologically, making it a prime candidate for clinical use.

1. Introduction

Neonatal sepsis is a common and serious condition, which can develop rapidly and cause morbidity or death if not treated properly. Thus, it remains a major health problem throughout the world. Every year an estimated 30 million newborns acquire an infection, and 1–2 million of them die [1]. About 10% of the newborn in Sweden are admitted to a neonatal unit [2], and some 16% of the admitted suffer at least one episode of infection [2]. The Swedish National Board of Health and Welfare showed in 2014 that sepsis causes about 14% of neonatal death in Sweden [3].

Neonatal sepsis is a condition that continues to puzzle us with its variety of symptoms, its unpredictability, and the difficulty of correctly diagnosing it. There is to date no solitary biomarker nor combination of biomarkers available for correctly discriminating neonatal sepsis from trauma, tissue damage or even the normal birth process, especially in

Abbreviations: CRP, C-reactive protein; CG, control group; CSG, confirmed sepsis group; ESG, entire sepsis group; G-CSF, granulocyte colony-stimulating factor; EOS, early-onset neonatal sepsis; GA, gestational age; IFN, interferon; IL, interleukin; PCT, procalcitonin; MIP, macrophage inflammatory protein; PROM, premature rupture of membranes; SAA, serum amyloid A; SSG, suspected sepsis group; TNF, tumor necrosis factor; tPA, tissue plasminogen activator.

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preterm infants [4–7]. Levels of the readily available sepsis biomarkers either rise too slowly or drop too fast for clinicians to be certain about catching a sepsis in development – or for clinicians to be able to eliminate sepsis as a reason for deterioration.

Interleukin (IL)-6, IL-8 and procalcitonin (PCT) alongside C-reactive protein (CRP) seem to be the most widely used sepsis biomarkers. The cytokines IL-6 and IL-8 rise very quickly in response to sepsis, but they also return to normal levels quickly, sometimes before CRP has even begun to rise [8,9]. Procalcitonin rises fairly slowly, after about 6–8 h, and has a half-life of 20–24 h, placing it kinetically in-between IL-6/IL-8 and CRP [8]. Tumor necrosis factor alfa (TNF)-α is another quick-acting biomarker, release starting at approximately 30 min, having a half-life of about 70 min [10]. This cytokine regulates the release of IL-1β, which is released within the hour and peaks within 5–10 h [10,11]. One might argue that a combination of a fast acting and a slow acting biomarker solves this issue, but even though the fast-acting biomarkers rise quickly enough, they also generally drop below the cut-off limit before the slow acting biomarkers have even begun to rise. Sampling in this interval might render a false-negative outcome, hence the need for serial sampling. More recently, kinetically less well-known markers like resistin [12,13], visfatin [13], macrophage inflammatory protein (MIP)-1β [11,14,15] and serum amyloid A (SAA) [14,16,17] have surfaced, showing potentially useful results, alongside traditional acute phase reactants like ferritin [18] and fibrinogen [19], and also tissue plasminogen activator (tPA)-3, a known inflammatory regulator, though with unknown role in regulating sepsis response in the newborn [19].

In these times, one also needs to address the issue of antibiotic stewardship. A biomarker that securely excludes sepsis would be of enormous benefit. Blood cultures are considered "gold standard" when it comes to concluding the sepsis diagnose. However, the sensitivity of blood cultures is low in the neonatal population, because of limited bacteremia, the isolated use of aerobic cultures and the small volume of blood used for culture [20–22]. Despite all effort, we are still no closer to the "optimal sepsis biomarker" proposed by Ng in 2004 [1]. An ideal marker would – in our view – be specific for sepsis, rise quickly, and stay elevated for a prolonged time, to secure detection, and minimize the need of repeated blood sampling, and in turn, excessive use of antibiotics.

An electronic inquiry was sent to physicians in charge of infection at all neonatal wards in Sweden. The purpose of this was to evaluate which biomarkers that are in use in order to diagnose and follow the course of neonatal sepsis within Sweden. Participants were also asked how satisfied they were with the available diagnostics. The results of this questionnaire made us realize that there is no consensus on infection diagnosis in newborns in Sweden, that many tests are used per patient, and that there is indeed room for improvement (J. Bengnér, P—O. Gäddlin, M. Faresjö, Biomarkers of Choice in Diagnosing and Following the Course of Neonatal Sepsis, Swedish Data, J. Clin. Imm., in press).

The objective of this study was therefore to improve identification of sepsis in neonates, by means of using sepsis biomarkers. In order to mimic a real-life clinical situation, we chose to do the blood sampling at the NICU's regular intervals. The main outcome was to characterize biomarker kinetics during the first days of sepsis, aiming to find biomarkers that rose quickly in response to sepsis, and then maintained their elevated levels during 72 h, differing from the levels in the control group. A secondary objective was to try to mathematically describe the kinetics of sepsis biomarkers during the first 72 h of infection.

2. Materials and methods

2.1. Ethical considerations

Study blood samples were obtained at the same time as regular blood sampling; no extra arterial or venous punctures were made. All the subjects' parents were given information orally and in writing, and written consent was obtained. The study was approved by the Regional Research Ethics Board, Linköping, Sweden.

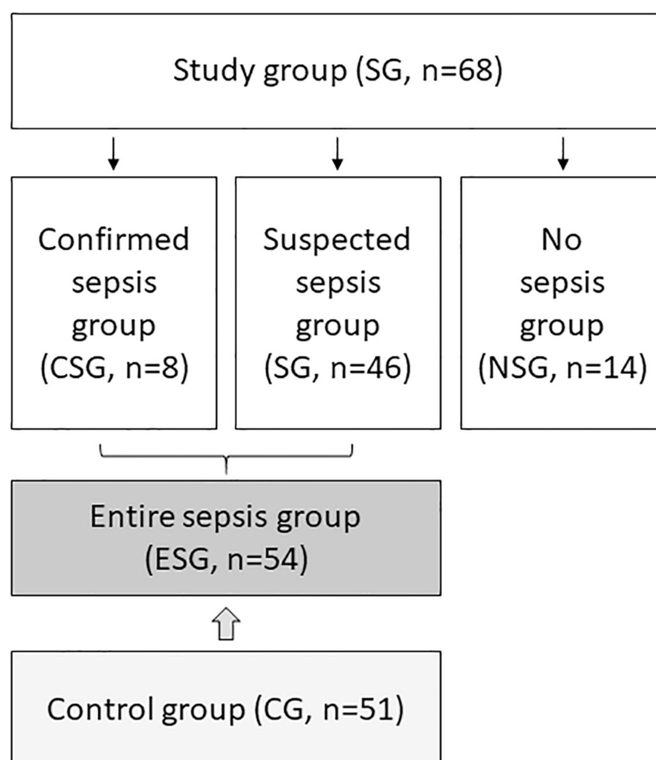

Fig. 1. The study group (SG) consists of neonates born after completed gestation week 27. Following discharge, the 68 participants in SG were subsequently divided into three sub-groups: confirmed sepsis group (CSG) judged by clinician as a confirmed sepsis patient, suspected sepsis group (SSG) judged by clinician as a probable sepsis and no sepsis group (NSG) initial clinical suspicion of sepsis withdrawn due to inadequate rise in CRP and/or IL-6 levels, improvement in clinical course, no relevant growth in blood or superficial cultures. CSG and SSG were merged into the entire sepsis group (ESG). The control group (CG) consists of neonates admitted to the NICU for several different reasons besides sepsis.

2.2. Study cohort

This prospective study is a cooperation between The Paediatric Clinic at Ryhov County Hospital, the School of Health and Welfare at Jönköping University, the Department of Laboratory Medicine at Region Jönköping County and the School of Engineering Sciences at the University of Skövde.

All neonates were recruited from the same neonatal intensive care unit (NICU), at the Paediatric Clinic at Ryhov County Hospital, in Jönköping, Sweden, which is a level III neonatal unit at a secondary care hospital, managing approximately 4.000 births annually.

2.3. Background characteristics

From the mothers of the participating neonates, premature rupture of membranes (PROM; ≥18 h), CRP rise (≥20 mg/L) and/or fever (≥38.0 °C) during the last 24 h before birth, known colonization with Group B-streptococci (GBS), occurrence of antibiotic administration antenatally due to above reasons, and mode of delivery were recorded. Regarding the neonates, gestational age at delivery, birth weight and Apgar score were recorded in both the control group and the sepsis group. Additionally, in the sepsis group, clinical signs that made the clinician suspect sepsis were recorded (tachypnoea, tachycardia/unstable heart rate, apnoea, fever, paleness, other), alongside culture results and the clinician's opinion on the diagnosis.

Table 1
All participants in the entire study group (ESG), displaying age of first blood sampling, all growth in cultures, and levels of IL-6 and CRP as detected by routine analysis.

Group	Patient #	Age at first blood sampling (h)	Blood culture, growth	Nasopharyngeal culture, growth	Outer ear canal culture, growth	IL-6 (ng/L), 0 h	CRP (mg/L), 0 h	CRP (mg/L), 12-24 h	CRP (mg/L), 48-72 h
CSG	1	362.0	none	Pneumococci, Moraxella	none	798	<5	60	16
	2	0.2	none	Pneumococci	Pneumococci	100,000	<5	31	12
	3	24.8	GBS + CoNS	GBS	GBS	2149	104	148	74
	4	23.1	*E. coli*	none	E. coli	n.d.	52	n.d.	n.d.
	5	128.5	CoNS+S. aureus	none	none	1035	42	111	63
	6	17.7	none	GBS	GBS	221	34	54	14
	7	27.4	none	GBS	GBS	n.d.	n.d.	47	22
	8	6.5	none	GBS	GBS	8697	<5	66	n.d.
SSG	9	8.8	none	none	none	n.d.	33	79	35
	10	3.0	none	none	none	1332	<5	50	19
	11	98.3	none	none	none	n.d.	30	22	38
	12	5.1	none	none	none	972	47	82	n.d.
	13	9.8	none	none	none	800	14	n.d.	15
	14	37.2	none	none	none	n.d.	n.d.	45	11
	15	10.4	none	none	none	1842	7	21	10
	16	4.2	none	none	none	2246	<5	21	12
	17	7.2	none	none	none	1586	<5	36	21
	18	4.8	none	none	none	417	<5	26	12
	19	55.4	none	none	none	17	41	25	20
	20	4.4	none	none	none	2629	90	221	56
	21	13.8	none	none	none	427	6	22	<5
	22	18.7	none	none	none	346	27	32	6
	23	6.3	none	none	none	1150	<5	58	20
	24	5.1	none	none	none	5000	<5	26	7
	25	22.6	none	none	none[a]	48	51	43	13
	26	3.5	CoNS[b]	none	none	2181	6	58	21
	27	5.8	none	none	none	544	<5	20	6
	28	18.4	none	none	none	355	33	54	n.d.
	29	38.8	none	none	none	8	37	43	10
	30	17.5	none	none	none	1201	13	n.d.	9
	31	39.4	none	none	none	15	63	33	<5
	32	6.4	none	none	none	3776	<5	78	53
	33	17.7	none	none	none	52	133	54	19
	34	77.6	none	none	none	144	8	14	43
	35	32.2	none	none	none	n.d.	n.d.	32	14
	36	13.8	none	GBS[b,c]	GBS[b]	10,189	28	47	24
	37	27.2	none	none	none	n.d.	58	27	8
	38	18.8	none	none	none	3056	121	56	12
	39	44.9	none	none	none	n.d.	49	28	16
	40	5.7	none	none	CoNS[b]	933	<5	89	49
	41	17.3	none	none	none	164	18	44	15
	42	11.6	none	GBS[b]	GBS[b]	2868	6	19	7
	43	29.3	none	none	none	61	<5	53	40
	44	0.8	none	none	none	2820	<5	n.d.	n.d.
	45	3.5	none	none	E. coli[b]	153	60	56	18
	46	n.d.	none	GBS[b]	GBS[b]	576	14	50	6
	47	19.4	none	none	none	657	47	62	34
	48	1.6	none	none	none	n.d.	n.d.	6	23
	49	21.4	none	none	none	46	32	36	15
	50	2.8	none	none	none	968	<5	16	11

[a] Mixed skin flora.
[b] regarded irrelevant.
[c] Hemophilus parainfluenzae.

2.4. *The study group*

The study group (SG; $n = 68$) consists of neonates born after completed gestation week 27, younger than 28 days at admission, admitted to the NICU on suspicion of sepsis, or neonates admitted to the NICU for other reasons, which developed suspected sepsis within the first 28 days of life. Exclusion criteria were known immunologic disease in the mother, mother treated with chemotherapy or immune modulating therapy, treatment with high doses of corticosteroids, baby with terminal illness, suspicion of oncologic or immunologic disease, or refusal to participate in the study.

Following discharge, the 68 participants in SG were subsequently divided into three sub-groups following laboratory and culture findings, combined with clinical signs (as illustrated in Fig. 1):

Confirmed sepsis group (CSG; $n = 8$): judged by clinician as a confirmed sepsis patient. Showed clinical signs associated with sepsis (tachypnoea; apnoea; tachycardia; bradycardia, compromised peripheral circulation; cyanosis; fever ≥38.0 °C; low muscle tone; feeding intolerance), a rise in CRP at any time point >40 mg/L and/or a rise in IL-6 > 1000 ng/L at the first time point, relevant bacterial growth in blood and/or two superficial cultures (naso-pharyngeal, ear). Three infants in this group had relevant growth in blood cultures, the remaining five had growth of group B streptococci or *E. coli* in two superficial cultures (Table 1).

Suspected sepsis group (SSG; $n = 46$): judged by clinician as a probable sepsis, following the typical clinical pattern. Clinical course with recorded symptoms (as above), a rise in CRP at any time point >40 mg/L and/or a rise in IL-6 > 1000 ng/L at the first time point, but no relevant

Table 2
Timing of blood sampling for the entire sepsis group (ESG) and control group (CG) at 0 h, 12-24 h and 48-72 h presented as median (range). Results in hours of age.

Study group	0 h	12–24 h	48–72 h
ESG ($n = 54$)	17.7 (0.2–362.0)	37.1 (12.2–381.5)	72.2 (52.7–419.0)
CG (n = 51)	3.4 (0.3–105.8)	22.7 (13.1–87.2)	52.8 (25.6–121.6)
p-value	$p < 0.05$	$p < 0.05$	$p < 0.01$

growth in blood or superficial cultures (as above) as presented in Table 1.

No sepsis group (NSG; $n = 14$): initial clinical suspicion of sepsis withdrawn due to inadequate rise in CRP and/or IL-6 levels, improvement in clinical course, no relevant growth in blood or superficial cultures. Participants in NSG are excluded from presentation of data with exception for analysis of function of time for SAA.

The confirmed sepsis group and SSG showed very similar results at all parameters in preliminary data assessment. Due to the small number of participants in CSG, we also merged CSG and SSG into a separate group of 54 individuals, referred to below as the *entire sepsis group* (ESG).

2.5. The control group

We included a control group ($n = 51$) for reference (Fig. 1). The control group (CG) consists of neonates admitted to the NICU for several different reasons besides sepsis, *e.g.* prematurity, low birth weight, hypoglycemia – reflecting the average neonatal patient. Inclusion criteria: all admitted neonates born after completed gestation week 27, younger than 28 days at admission. Exclusion criteria as in the sepsis group, including suspicion of sepsis. The two groups were not matched regarding gestational age, birth weight or gender.

2.6. Blood sampling

Study blood samples of approximately 100 μL were in both groups drawn at 0 h (sample 1), 12–24 h (sample 2) and 48–72 h (sample 3) later. "0 h" was in the SG defined as "as soon as possible following suspicion of sepsis"; usually within the hour. Blood sampling at 0 h, besides the study blood sample, consisted of blood for routine analysis of CRP and IL-6 alongside other tests ordered by the clinician in charge. At 12–24 h and at 48–72 h, additional blood samples for analysis of CRP were drawn in the SG. In the CG, "0 h" coincided with the time of the first blood sampling for other purposes according to clinical routine. All study blood samples were immediately transferred to the laboratory for centrifugation. Serum was stored frozen at −80 °C, until all blood sample collection for the study were finished. A total of 306 study blood samples was obtained: 164 in SG and 142 in the CG. The time-points for blood sampling at 0, 12–24 and 48–72 h did not differ between ESG and CG. Median and time range values are presented in Table 2, expressed as hours since birth. Early onset neonatal sepsis (EOS) defined as onset before 72 h after birth are seen in majority of children included in ESG (median: 17.7 h at 0 h). Only four subjects were sampled after 72 h of absolute age.

2.7. Detection of immune markers by multiplex fluorochrome technique

Acute phase and pro-inflammatory markers were analysed in sera with multiplex flourochrome technique (Luminex, Bio-Rad, Austin, Texas, USA). Procalcitonin, ferritin, fibrinogen, SAA and tPA-3 were analysed from hundredfold (1:100) diluted serum samples, using the Bio-Plex Pro human Acute Phase 5-plex panel (Bio-Rad Laboratories, Hercules, California, USA). Granulocyte colony-stimulating factor, IFN-γ, IL-1β, IL-6, IL-8, IL-10, MIP-1β and TNF-α were analysed from fourfold (1:4) diluted serum samples, using the Bio-Plex Pro 8-plex complete kit (Bio-Rad Laboratories). Additionally, resistin and visfatin were analysed using the Bio-Plex Pro Resistin set and the Bio-Plex Pro Visfatin set (Bio-Rad Laboratories), respectively, both from fourfold (1:4) diluted serum. A Bio-Plex 200™ system (Luminex xMAP™ Technology, Austin, TX, USA) was used for identification and quantification of each cytokine/chemokine, and the threshold was set to a minimum of 50 individual microspheres per region. Raw data (median fluorescence intensity [MFI]) for each reaction were analysed using Bio-Plex Manager™ Software 5.0. All serum samples were run on the same instrument and the same batch of all included reagents were used throughout all analysis. One positive control, analysed for all markers, was included in all analysis. To obtain sample concentration values, a five-parameter logistic equation was used to calculate each standard curve. The cut-off values enforced for minimum detectable concentrations for each immunological marker were as follows: ferritin (38.64 pg/mL), fibrinogen (12.73 pg/mL), G-CSF (50.7 pg/mL), IFN-γ (34.37 pg/mL), IL-1β (7.55 pg/mL), IL-6 (26.11 pg/mL), IL-8 (38.4 pg/mL), IL-10 (34.31 pg/mL), MIP-1β (6.3 pg/mL), PCT (5.30 pg/mL), resistin (38.3 pg/mL), SAA (1.51 pg/mL), TNF-α (60.79 pg/mL), tPA (4.77 pg/mL) and visfatin (1071 pg/mL). Coefficient of variation (CV) for inter-assay ranged from 4.24–17.45 for the different markers (the lowest inter-assay was detected for IL-6 and the highest inter-assay for tPA).

2.8. Statistics

Background characteristics were statistically analysed using two-tailed Chi-square test for analysis of categorical outcomes, and unpaired, two-tailed Mann-Whitney non-parametric *U* test for analysis of non-normally distributed data. Significance level was set to $p < 0.05$.

The suspected sepsis group (SSG) and confirmed sepsis group (CSG) as well as the entire sepsis group (ESG) were compared to control group (CG), again using the Mann-Whitney non-parametric U test: unpaired, two-tailed. To correct for multiple statistical comparisons, the probability level was set to <0.001 by using the Šídák multiple comparison test, adjusted for 45 tests (15 biomarkers at three time points).

Statistical analyses were performed and graphs produced using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.9. Regression analysis

In order to estimate the variation of time for the biomarker levels as functions of time, the median concentration values at the center of the time intervals for each biomarker were used. This yielded three median values, *i.e.* at 0 h, 18 h and 60 h, respectively. In the present study, second order polynomials according to $f(x) = a + bx + cx^2$ were used to fit the data points, thus enabling the capture of both linear and quadratic regression trend outcomes. The function value $f(x)$ corresponds to the biomarker concentration level at any given point in time, x, and the unit of time was in hours in the present study. For each biomarker, three median concentration values were collected in a vector $F = [f_{0h}, f_{18h}, f_{60h}]^T$ and the corresponding time points were collected in a matrix $X_i = [1\ (0h)_i\ (0h)_i^2; 1\ (18h)_i\ (18h)_i^2; 1\ (60h)_i\ (60h)_i^2]$, where i indicates the biomarker and 0 h, 18 h and 60 h are the mid points of the time intervals in the present study. The coefficients a, b and c, respectively, were determined by the vector $C = [a, b, c]^T = (X^T X)^{-1} X^T F$. Note that the coefficients will be different for each biomarker as well as the choice of time unit (*i.e.* hours in the present study). Nevertheless, with these coefficients, functions of time for the concentration levels for each biomarker can be established.

3. Results

3.1. Demographics

There were more mothers with fever in ESG compared to CG ($p = 0.0475$), and more cesarean deliveries in CG than in ESG ($p = 0.0398$).

Table 3
Demographic data on mothers and infants, the entire study group (ESG) compared to the control group (CG).

		PROM[a]	GBS[b] colonization	Fever	CRP[c] rise	Antenatal antibiotics	Vacuum extraction	Cesarean delivery
Mothers' background data								
ESG (n=54)	*n*	14	3	4	7	17	6	14
CG (n=51)	*n*	9	4	0	6	17	5	23
	p-value [e]	0.3053	0.6386	0.0475	0.8522	0.8394	0.8539	0.0398

		Apgar score 1 min	Apgar score 5 min	Apgar score 10 min	GA[d] at delivery (wks)	Birth weight (g)		Sex
Infants' background data								
ESG (n=54)	*Median*	9	10	10	40.4	3777	*Males*	23
	Range	1-10	2-10	4-10	31.9-42.0	2045-4870	*Females*	31
CG (n=51)	*Median*	9	9	10	34.9	2510	*Males*	20
	Range	3-10	2-10	5-10	29.3-41.1	670-5095	*Females*	31
	p-value [f]	0.1307	0.2032	0.0226	<0.0001	<0.0001	*p-value* [e]	0.7251

[a]Premature rupture of membranes, [b]Group B-streptococci, [c]C-reactive protein (CRP), [d]gestational age of the infant at delivery, in weeks, [e]Chi-square test, two-tailed, [f]Mann-Whitney non-parametric *U*-test: two-tailed, unpaired.

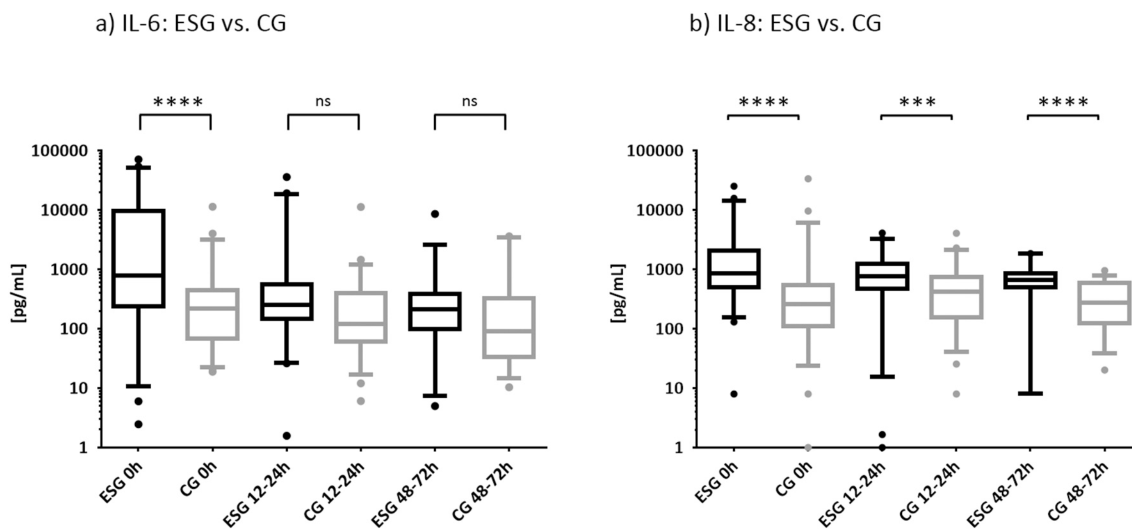

Fig. 2. Levels of IL-6 (a) and IL-8 (b) detected in pg/mL, the entire sepsis group (ESG) *versus* the control group (CG). Presented grouped at time-intervals 0 h, 12–24 h and 48–72 h. Box-and-whisker plot, whiskers at 5th and 95th percentiles. The y-axes are consequently logarithmic to the 10th order, due to high dynamic range.

Apgar score at 10 min was lower in CG than in SG ($p = 0.0226$). Gestational age ($p < 0.0001$) and birth weight ($p < 0.0001$) also differed in-between groups. Other than that, no other differences were recorded regarding demographics. Background data on mothers and infants are presented in Table 3.

3.2. Immune markers

3.2.1. Interleukin-6 and -8

Interleukin-6 was elevated at 0 h in ESG ($p < 0.0001$, Fig. 2a), CSG ($p < 0.001$) and SSG ($p < 0.0001$) (Table 4) compared to CG. Interleukin-8 was elevated in ESG ($p < 0.0001$, Fig. 2b) and SSG ($p < 0.0001$), but not in CSG at 0 h (Table 3). At 12–24 and 48–72 h, there were no differences in secretion of IL-6 between the groups, whereas IL-8 was elevated at 12–24 h in ESG ($p < 0.001$), and at 48–72 h in ESG ($p < 0.0001$), CSG ($p < 0.001$) as well as in SSG ($p < 0.0001$) compared to CG (Fig. 2b, Table 4).

3.2.2. G-CSF, interferon-γ and interleukin-1β and − 10

Secretion of G-CSF was increased at 0 h in both ESG ($p < 0.0001$) and SSG ($p < 0.0001$), compared to CG, but not at 12–24 h or at 48–72 h (Fig. 3a, Table 4). Interferon-γ was increased at 0 h in ESG ($p < 0.001$) and SSG ($p < 0.001$) compared to CG, and again at 48–72 h in ESG ($p < 0.0001$) and SSG ($p < 0.001$) compared to CG (Fig. 3b, Table 4).

Interleukin-1β was elevated at 0 h in ESG ($p < 0.0001$) and in SSG ($p < 0.001$) compared to CG, and again at 48–72 h; $p < 0.0001$ in ESG and $p < 0.0001$ in SSG compared to CG (Fig. 4a).

Interleukin-10 was increased only at 0 h in ESG ($p < 0.0001$) and in SSG ($p < 0.001$) compared to CG (Fig. 4b, Table 4).

Table 4
Secretion of biomarkers presented for the entire sepsis group (ESG), including both the confirmed sepsis group (CSG) as well as the suspected sepsis group (SSG) compared to the control group (CG) at different time intervals; 0 h, 12–24 h and 48–72 h.

		Ferritin	Fibrinogen	G-CSF	IFN-γ	IL-1β	IL-6	IL-8	IL-10
0 hours									
ESG (n=50)	*Median [pg/mL]*	128197	4394	1309	1511	69	783	849	182
	Range	9-641548	3-9909	3-296392	2-6925	2-1457	2-70836	8-25118	4-8167
CSG (n=8)	*Median [pg/mL]*	148717	4370	30138	2556	156	10219	3385	456
	Range	9-353139	2246-9909	3-296392	5-6925	2-742	230-53694	8-25118	27-4528
SSG (n=42)	*Median [pg/mL]*	121865	4394	1270	1435	68	637	730	175
	Range	9-641548	3-9544	17-206236	25-6068	5-1457	2-70836	130-13312	4-8167
CG (n=53)	*Median [pg/mL]*	75504	2939	299	550	37	216	260	81
	Range	9-635398	432-27709	3-2323	5-3572	0-3891	19-11322	1-33488	14-766
	p-value ESG vs CG	0.1324	0.0374	<0.0001	<0.001	<0.0001	<0.0001	<0.0001	<0.0001
	p-value CSG vs CG	0.4080	0.1910	0.0140	0.0358	0.0172	<0.001	0.0049	0.0052
	p-value SSG vs CG	0.1579	0.0580	<0.0001	<0.001	<0.001	<0.0001	<0.0001	<0.001
12-24 hours									
ESG (n=40)	*Median [pg/mL]*	163549	3646	719	1163	57	249	758	152
	Range	9-392060	3-10568	12-135279	5-6807	2-515	2-35865	1-4085	23-3768
CSG (n=7)	*Median [pg/mL]*	267838	3593	2935	1547	62	157	1121	319
	Range	112603-349714	2391-6584	213-135279	273-3805	29-339	2-19065	333-2039	90-517
SSG (n=33)	*Median [pg/mL]*	143199	3700	453	1107	56	255	725	144
	Range	9-392060	3-10568	12-9212	5-6807	2-515	26-35865	1-4085	23-3768
CG (n=48)	*Median [pg/mL]*	69938	1824	345	409	33	120	418	73
	Range	9-653552	287-6789	36-21209	5-3030	0-266	6-11205	8-4046	11-792
	p-value ESG vs CG	0.0078	0.0020	0.0014	0.0150	0.0136	0.0121	<0.001	0.0243
	p-value CSG vs CG	0.0062	0.0634	0.0012	0.0371	0.0458	0.3645	0.0039	0.0157
	p-value SSG vs CG	0.0428	0.0047	0.0138	0.0465	0.0395	0.0116	0.0063	0.0950
48-72 hours									
ESG (n=38)	*Median [pg/mL]*	141877	2831	393	1170	59	212	661	116
	Range	42077-517876	1482-15776	13-3241	41-6371	0-225	5-8570	8-1846	17-580
CSG (n=7)	*Median [pg/mL]*	255752	2820	568	1482	62	414	873	113
	Range	122145-502520	2343-3788	264-1702	575-6105	42-188	39-2284	397-1341	65-580
SSG (n=31)	*Median [pg/mL]*	139317	2842	376	963	58	211	638	117
	Range	42077-517876	1482-15776	13-3241	41-6371	0-225	5-8570	8-1846	17-453
CG (n=39)	*Median [pg/mL]*	68395	936	222	309	16	90	274	42
	Range	1467-482250	165-12191	47-845	2-2427	1-101	10-3600	20-957	12-864
	p-value ESG vs CG	0.0051	<0.001	0.0086	<0.0001	<0.0001	0.1106	<0.0001	0.0011
	p-value CSG vs CG	0.0176	0.1077	0.0098	0.0028	0.0028	0.1122	<0.001	0.0144
	p-value SSG vs CG	0.0198	0.0012	0.0400	<0.001	<0.0001	0.2192	<0.0001	0.0047

		MIP-1β	PCT	Resistin	SAA	TNF-α	tPA-3	Visfatin
0 hours								
ESG (n=50)	*Median [pg/mL]*	2685	1637	51094	8532	386	2119	6280
	Range	6-168700	2-12295	489-169851	0-96320	12-3152	2-28889	178-41503
CSG (n=8)	*Median [pg/mL]*	3163	2706	84923	11818	986	3678	3412
	Range	6-168700	2-9692	626-165250	8024-20660	12-2553	1537-19191	178-27235
SSG (n=42)	*Median [pg/mL]*	2685	707	41340	6436	343	1931	6688
	Range	270-14772	2-13785	489-169851	0-96320	12-3152	2-28889	659-41503
CG (n=53)	*Median [pg/mL]*	2512	707	24518	59	198	2528	2345
	Range	6-25240	2-13785	1-282045	0-64009	1-3077	228-34017	58-46435
	p-value ESG vs CG	0.7788	0.2223	0.6490	<0.0001	0.0020	0.6922	0.0020
	p-value CSG vs CG	0.7629	0.1866	0.2581	<0.0001	0.0250	0.4403	0.5952
	p-value SSG vs CG	0.8331	0.3591	0.9094	<0.0001	0.0065	0.4679	<0.001
12-24 hours								
ESG (n=40)	*Median [pg/mL]*	2853	1567	48996	11819	352	2364	7433
	Range	3-26095	104-19225	348-253485	0-29261	8-3881	2-17741	165-51996
CSG (n=7)	*Median [pg/mL]*	2864	3520	36305	13605	381	3704	4813
	Range	1476-26095	1649-13059	25884-165123	9854-22846	160-1366	1721-15059	1212-51996
SSG (n=33)	*Median [pg/mL]*	2841	1484	50383	11699	321	1877	7844
	Range	3-6735	104-19225	348-253485	0-29261	8-3881	2-17741	165-43458
CG (n=48)	*Median [pg/mL]*	2257	516	31624	921	152	1428	2994
	Range	14-6580	2-15143	670-167851	0-25248	2-1199	48-25937	41-42575
	p-value ESG vs CG	0.0759	0.0029	0.1580	<0.0001	0.0316	0.5578	<0.001
	p-value CSG vs CG	0.4057	0.0078	0.1628	<0.0001	0.0266	0.3104	0.2164
	p-value SSG vs CG	0.0876	0.0159	0.2801	<0.0001	0.1051	0.7798	<0.001
48-72 hours								
ESG (n=38)	*Median [pg/mL]*	2648	1210	28141	8435	331	1255	5605
	Range	4-8771	2-13059	256-109885	126-31500	12-1629	292-67758	178-41936
CSG (n=7)	*Median [pg/mL]*	2484	1278	23949	12810	337	1537	5882
	Range	1087-3578	632-13059	1999-86440	4318-22452	202-1629	1190-10614	736-31801
SSG (n=31)	*Median [pg/mL]*	2721	1068	30274	8113	324	1255	5328
	Range	4-8771	2-4577	356-109855	126-31500	12-1239	292-67758	178-41936
CG (n=39)	*Median [pg/mL]*	2224	334	24943	499	100	406	1490
	Range	618-6961	2-15782	11981-208375	0-40950	7-999	117-17235	38-46557
	p-value ESG vs CG	0.0980	0.1783	0.8493	<0.0001	0.0011	0.0560	0.0017
	p-value CSG vs CG	0.7420	0.0316	0.7390	<0.0001	0.0071	0.2062	0.0526
	p-value SSG vs CG	0.0741	0.4600	0.7223	<0.0001	0.0062	0.0859	0.0039

Mann-Whitney non-parametric *U*-test, two-tailed, unpaired. Significance level set to $p < 0.001$ according to Šídák multiple comparison test, adjusted for 45 tests. Significant results are highlighted with white text on grey background.

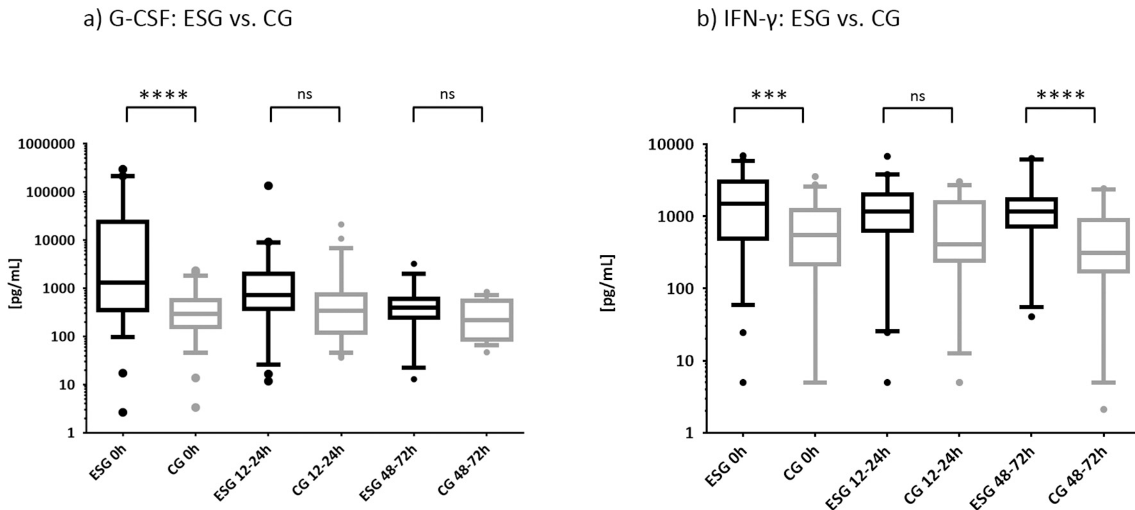

Fig. 3. Levels of G-CSF (a) and IFN-γ (b) detected in pg/mL, the entire sepsis group (ESG) *versus* the control group (CG). Presented grouped at time-intervals 0 h, 12–24 h and 48–72 h. Box-and-whisker plot, whiskers at 5th and 95th percentiles. The y-axes are consequently logarithmic to the 10th order, due to high dynamic range.

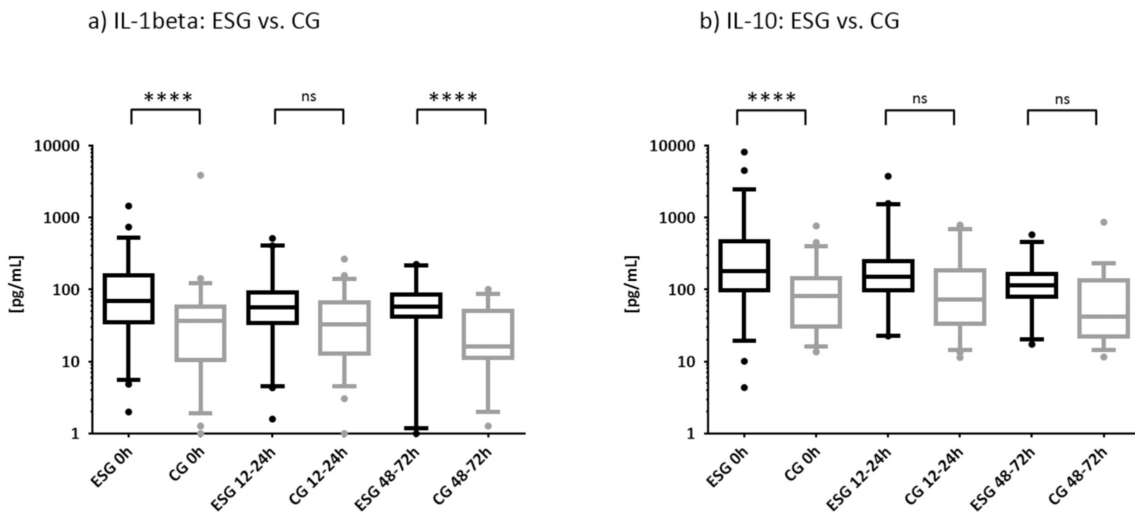

Fig. 4. Levels of IL-1β (a) and IL-10 (b) detected in pg/mL, the entire sepsis group (ESG) *versus* the control group (CG). Presented grouped at time-intervals 0 h, 12–24 h and 48–72 h. Box-and-whisker plot, whiskers at 5th and 95th percentiles. The y-axes are consequently logarithmic to the 10th order, due to high dynamic range.

3.2.3. Serum amyloid A

Serum amyloid A was the only biomarker that turned out elevated at all three time-intervals (0 h, 12–24 h and 48–72 h) in all sepsis-groups (SSG, CSG och ESG) compared to CG (consequently $p < 0.0001$, Fig. 5).

3.2.4. Visfatin, fibrinogen, ferritin, MIP-1β, resistin, tPA-3, procalcitonin and TNF-α

Visfatin was elevated at 0 h in SSG compared to CG ($p < 0.001$), and at 12–24 h in ESG and SSG compared to CG (both $p < 0.001$, Table 4). Fibrinogen was increased only at 48–72 h in ESG compared to CG ($p < 0.001$) (Table 4).

The immune markers ferritin, MIP-1β, procalcitonin, resistin, TNF-α and tPA-3 were all secreted to the same extent in all groups compared to the control group at all time intervals (Table 4).

3.3. Variation of biomarker levels as function of time

Variation of concentration during the time-period: 0–72 h were estimated for the biomarkers which had the most favorable kinetics: IL-1β, IL-6, IL-8, IFN-γ, PCT and SAA. Median concentration values at the center of the time intervals for each biomarker was used. This yielded three median values, *i.e.* at 0 h, 18 h and 60 h, respectively, presented for the entire sepsis group (ESG) in Table 5. Graphs to illustrate the function of time for SAA are shown in all study groups (*i.e.* CSG, SSG, NSG, ESG and CG; Fig. 6a-e) and the difference between ESG and CG is presented in Fig. 6f.

4. Discussion

Sweden is usually considered a modern country from a healthcare

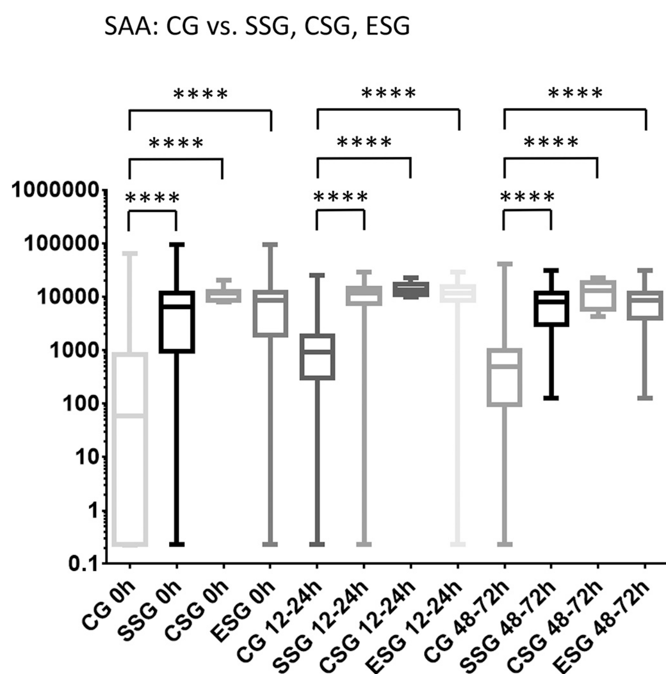

Fig. 5. Levels of SAA detected in pg/mL, the suspected sepsis group (SSG), confirmed sepsis group (CSG) and entire sepsis group (ESG) *versus* the control group (CG). Presented grouped at time-intervals 0 h, 12–24 h and 48–72 h. Box-and-whisker plot, whiskers at 5th and 95th percentiles. The y-axes are consequently logarithmic to the 10th order, due to high dynamic range.

Table 5
Variation of biomarker levels as a function of time, the entire sepsis group (ESG), select biomarkers.

Immune marker	Function of time
IL-1β	$f = 0.0335 \times {}^{2}–2.5\times + 76$
IL-6	$f = 0.482 \times {}^{2}–39.5\times + 763$
IL-8	$f = 0.0609 \times {}^{2}–6.9\times + 771$
IFN-γ	$f = 0.567 \times {}^{2}–47.3\times + 1625$
PCT	$f = 0.183 \times {}^{2}–24.4\times + 1658$
SAA	$f = -4.055 \times {}^{2} + 214.1\times + 7566$

f = concentration of the biomarker [pg/mL]; x = time (h) from 0 h.

perspective, with great access to data and outcome in neonatology. It is a relatively small country, managing some 110.000 births annually, distributed over 37 neonatology wards. However, there are no national guidelines when it comes to diagnosing sepsis. Our inquiry indicated that there is room for improvement in the field of diagnostics of neonatal sepsis. The goal of this study was to survey a broad range of possible sepsis markers simultaneously, in a clinical setting. Of top priority was to find markers with useful kinetics: markers that rise early in response to sepsis, preferably a solitary biomarker whose levels was maintained significantly elevated throughout the first 72 h of sepsis. Results on the well-known markers IL-6 and IL-8 turned out as expected, whereas PCT showed to be of less importance in this material. Major findings include the significantly elevated SAA at all time intervals.

Gestational age at birth and birthweight did vary significantly inbetween SG and CG, due to the groups reflecting a typical neonatal ward, rather than being age- and/or weight-matched. This, and the increased number of deliveries by cesarean section in the CG, is most probably due to the CG comprising a larger number of prematurely born infants. Also, four mothers presented with fever before delivery in the SG, making for a significant difference. These are expected differences, bearing in mind that the SG should contain infected babies, and early-onset sepsis would commence with the baby being infected by its mother's bacteria while still in the womb [23]. The generally low numbers of mothers with a known GBS colonization might be explained by Sweden not screening for this. Prenatal antibiotics were given to mothers with PROM, to mothers with impending premature delivery, and on suspicion of infection.

Two individuals in SSG presented with growth of GBS in two superficial cultures and a CRP >40 mg/L were judged "probable sepsis" by a clinician, because of lack of convincing clinical signs associated with sepsis, and were therefore grouped in SSG rather than in CSG.

In vivo, we do not know the exact time when the infection starts. Because of this, and to reflect a real-life clinical situation, we chose to draw blood samples within time intervals (*i.e.* 12–24 h; 48–72 h) rather than on a predetermined theoretical time point. The timing of blood sampling in the two groups was relatively consistent and revealed that the ESG consisted of mainly EOS.

One of the most frequently used immune marker, IL-6, behaved like suspected; it rises quickly in response to infection, and therefore shows a highly significant rise in all groups compared to the control group at initial infection (i. e. 0 h), as described by Ng et al. [24], among others. With IL-6 having a peak at about 6 h, it is more useful for initiating sepsis treatment than CRP [9]. However, it vanishes quickly from serum during the next 48 h, demonstrated by the less than significant rise in all groups already at 12–24 h, making it less useful for tracking the patient's response to infection. We expected IL-8 to follow the pattern of IL-6. However, IL-8 did turn out significantly elevated in SSG as well as ESG compared to CG at 0 h, but also at 48–72 h in all the three groups studied compared to the controls.

Granulocyte colony-stimulating factor (G-CSF) has also been proposed as an early and sensitive marker of bacterial infection [25–27]. In our cohort, G-CSF does behave much like IL-6, suggesting it belongs to the fast-acting marker kinetic group. Interleukin-1 β also shows good potential, being elevated in the entire sepsis group both at 0 h and again at 48–72 h. This is in line with several previous studies, indicating that IL-1β is a good marker for prognostics and for monitoring therapeutic efficiency in neonatal sepsis [15,28–30].

Interleukin-10 is claimed to have a role in the anti-inflammatory process [10], and a majority of studies report elevated IL- 10 in neonatal sepsis [11,15,31,32]. This is also in agreement with our findings, indicating this interleukin to be an early rising immune marker for sepsis.

Even though procalcitonin did not reach significantly elevated levels in any of the study groups compared to the control group at any of the time-intervals, it shows a small increase in levels in all study groups compared to the controls at 12–24 h. This result contradicts several other investigators' findings [4,8,26,33–35], who appoint PCT a good medium fast-acting biomarker for sepsis. Results do conflict on sensitivity and specificity [36], making it a difficult marker to interpret. Perhaps this is due to a physiological rise in all groups, shown to occur in premature infants after normal birth by Turner et al. [36]. Furthermore, despite TNF-α being widely studied, showing results similar to CRP [37,38], it did not show any significant differences at any time interval in our cohort.

Interferon-γ may act as an indicator of viral infections [38] but this interferon can also be induced by other agents. This interferon shows significant differences at 0 h and again at 48–72 h, in SSG and ESG compared to CG, but not in CSG at any time interval, suggesting that some subjects in SSG were in fact affected by viral infections. Resistin and visfatin are both recently discovered adipokines, which to our knowledge as yet are unused in clinical practice, nevertheless showing interesting results in a few smaller clinical trials of newborn infants [12,13]. Theoretically, both should rise even faster than IL-6, since they act as precursors [13]. While visfatin proved somewhat promising results, being significantly elevated in SSG and ESG at several time-points (at 0 h and 12–24 h), in accordance with findings by other investigators [13], resistin however did not. Nor did ferritin, MIP-1β or tPA-3 show any significant differences, while fibrinogen was just barely significantly

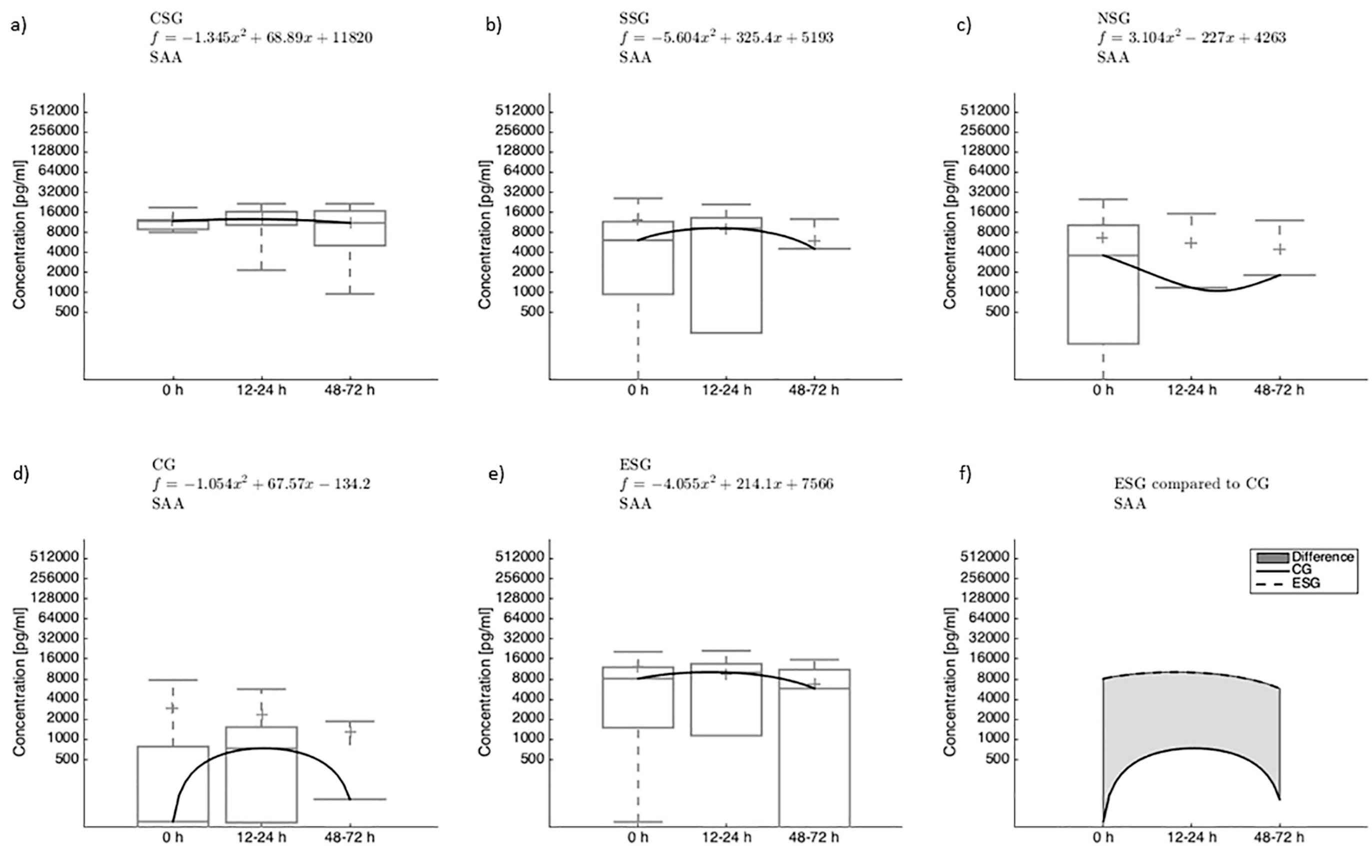

Fig. 6. Function of time for SAA detected in pg/mL, the confirmed sepsis group (CSG; a), suspected sepsis group (SSG; b), no sepsis group (NSG; c), control group (CG; d), entire sepsis group (ESG; e) and the difference between ESG and CG (f).

elevated at 48–72 h in the entire sepsis group compared to the controls.

Serum amyloid A is a precursor protein in inflammation-associated reactive amyloidosis [17]. Induced by IL-1, IL-6, TNF-α and gram-negative bacteria lipopolysaccharides, it is also proven to be an accurate marker for the diagnosis of neonatal sepsis [16,17,26,34,39,40]. Serum amyloid A might theoretically rise in response to other causes than infection, such as intraventricular haemorrhage, birth trauma and asphyxia, but it does not seem to pass the placental barrier [16]. Rising earlier and more sharply than CRP, SAA is of prognostic value as early as eight hours after sepsis onset and keeps this usefulness at 96 h after the first suspicion of sepsis [17,41]. Our results second this, in that SAA displays the greatest difference between the septic group and the control group, at all studied time intervals. Furthermore, SAA seems to be a useful biomarker for discriminating between positive and negative cultures of neonatal sepsis [40]. In EOS, SAA also seems valuable for monitoring efficacy of antibiotic treatment at 48–96 h and later, even passing 96–144 h [42]. We conclude that serum amyloid A has the most favorable kinetics regarding diagnosing and following the course of neonatal sepsis, in this assay of biomarkers.

The function of time-formula provides a potential tool for prediction of relevant biomarker levels at any time-point, where only the measured concentration is inserted. This can be a useful complement to laboratory analysis performed at a few time-points. Thus, concentrations of sepsis biomarkers can theoretically be calculated at any given time-point up to 72 h. In clinical use, one might use this to try to predict the course of the infection and efficacy of treatment. Since this is a theoretical tool, it is important to keep in mind that biological variation may impact the real outcome.

5. Conclusions

Sepsis in the neonate population is a difficult diagnosis to make, and perhaps even harder to rule out. Reliable biomarkers for sepsis play a vital role in this, and there is to date no perfect solitary biomarker in clinical use. Several studies have concluded that a combination of biomarkers is the way forward, usually by combining a fast-acting marker like IL-6 with PCT and/or CRP. This study assesses the expression of SAA as a neonatal sepsis marker over several days, showing favorable kinetic results for use as a solitary sepsis biomarker. Kinetically, SAA looks promising enough to be used as a solitary marker. Before this can be implemented, further studies need to be performed. We would need to evaluate how GA and birth weight affect the infants' ability to secrete SAA in response to sepsis. Optimal cut-off values for SAA as a neonatal sepsis marker would need to be established. With further development, we will hopefully one day get closer to Ng's "one sepsis marker to rule them all", which will confirm or exclude sepsis, minimizing the need for serial sampling and greatly reducing the use of unnecessary antibiotics.

Declaration of Competing Interest

None.

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