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Relationships among the concentrations of 25 inflammation-associated proteins during the first postnatal weeks in the blood of infants born before the 28th week of gestation

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Abstract

Background—Inflammation appears to be involved in processes leading to organ damage in preterm newborns, yet little is known about the relationships among elevated concentrations of inflammation-associated proteins in the blood of preterm newborns.

Methods—In this exploratory study, we used an electrochemiluminescence method to measure 25 proteins in blood obtained on postnatal day 1 (range 1–3), day 7 (range 5–8), and day 14 (range 12–15) from 939 children born before the 28th week of gestation and evaluated to what extent those whose concentration of each protein was elevated (defined as in the highest quartile for gestational age and day the specimen was obtained) also had an elevated concentration of every other protein the same day or on a day 1 or 2 weeks later ($p < .0001$).

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Author contributions

Alan Leviton played a role in every aspect of the ELGAN Study and played major roles in data analysis and manuscript preparation. Elizabeth Allred played a major role in designing the data collection forms and the database management system. She is also the person most responsible for maintaining data quality and for data analysis. In addition, she has read and edited multiple drafts of the manuscript and offered comments.

Hidemi Yamamoto played a major role in maintaining the high quality of blood protein measurements. She participated in data analyses, and has read and edited multiple drafts of the manuscript and offered comments.

Raina N Fichorova was most responsible for the high quality of the blood protein measurements. She participated in data analyses, and has read and edited multiple drafts of the manuscript and offered comments.

Conflict of interest statement

The authors do not see how they might benefit financially from publication of this manuscript, nor do they have any financial stake in any commercial organization that might benefit.

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Results—On each of the 3 days assessed, elevated concentrations of 17 proteins were associated with elevated concentrations of 15 or more of the other 24 proteins. VEGF, VEGF-R1, VEGF-R2 were among these proteins, while IGFBP-1 was associated with 13 other proteins on day 7. An elevated concentration of 8 proteins on day 1 predicted an elevated concentration of 10 or more proteins on day 7, while an elevated concentration of only two proteins on day 7 were associated with elevated concentrations of 10 or more proteins on day-14. Few associations were seen between day 1 and day 14.

Conclusions/inferences—Inflammation is a diffuse process in ELGANs, with elevated concentrations of cytokines, chemokines, adhesion molecules, matrix metalloproteinases, a growth factor and its receptors, as well as a growth factor binding protein associated with each other the same day, as well as on subsequent days.

Keywords

Preterm newborn; Inflammation; Biomarkers

1. Introduction

Systemic inflammation in the preterm newborn has been associated with lung [1], bowel, and brain damage [2–6]. Sometimes the inflammation is extensive.

One view of the inflammation is that the elevated concentration of a single protein (or a process associated with this single protein) is sufficient to damage the organ [7]. This view is supported by those able to diminish damage or symptoms by limiting the function of one protein in newborn rodents [8, 9].

Another view is that inflammation is considerably more complex and involves so many proteins that damage is likely to reflect multiple components of diffuse processes [10, 11]. The view that inflammation is complex and involves many proteins is supported by the finding that a single inflammatory stimulus can influence the expression of thousands of genes [12], and by evidence that drugs once considered to influence only one protein probably have diffuse anti-inflammatory effects [13, 14].

If, as we expected, inflammation involves many proteins, then concentrations of inflammation-associated proteins in the blood of extremely low gestational age newborns (ELGANs) should vary with one another, either at the same time or from one time interval to another. Here we examine the relationships among 25 inflammation-related proteins we measured in blood obtained during the first postnatal weeks from a very vulnerable group, infants born before the 28th week. Our findings document that inflammation in the preterm newborn is broad, highly interconnected, and sustained for more than just a few days.

2. Methods

2.1 The ELGAN Study

The ELGAN study was designed to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in ELGANs (the acronym for Extremely Low Gestational Age Newborns) [15]. During the years 2002–2004, women who did or might deliver before 28 weeks gestation at one of 14 institutions in 11 cities in 5 states were asked to enroll in the study. The enrollment and consent processes were approved by the individual institutional review boards.

Mothers were approached for consent either upon antenatal admission or shortly after delivery, depending on clinical circumstance and institutional preference. 1249 mothers of

1506 infants consented. The 939 children who comprise the sample for this report had blood specimens collected during the first two postnatal weeks, and a neurodevelopmental assessment at approximately 24 months post-term equivalent.

2.2 Newborn variables

The gestational age estimates were based on a hierarchy of the quality of available information. Most desirable were estimates based on the dates of embryo retrieval or intrauterine insemination or fetal ultrasound before the 14th week (62%). When these were not available, reliance was placed sequentially on a fetal ultrasound at 14 or more weeks (29%), LMP without fetal ultrasound (7%), and gestational age recorded in the log of the neonatal intensive care unit (1%).

2.3 Blood spot collection

Drops of blood were collected on filter paper (Schleicher & Schuell 903) on the first postnatal day (range: 1–3 days), the 7th postnatal day (range: 5–8 days), and the 14th postnatal day (range: 12–15 days). All blood was from the remainder after specimens were obtained for clinical indications. Dried blood spots were stored at -70°C in sealed bags with desiccant until processed.

2.4 Elution of proteins from blood spots

For protein elution, 12mm punched biopsies of the frozen blood spots were submerged in 300 μL phosphate buffered saline containing 0.1% Triton X100 (Sigma-Aldrich, St. Louis, MO) and 0.03% Tween-20 (Fisher, Hampton, NH), vortexed for 30 seconds, and incubated on a shaker for 1 hour at 4°C . The buffer and biopsy were then transferred over the filter of a SpinX tube (Corning Fisher), centrifuged at $2000 \times g$ and the filtered eluted blood collected. An additional wash of the punch was performed in 100 μL for a final elution volume of 400 μL .

2.5 Protein measurements

In this exploratory, descriptive evaluation, proteins were measured in duplicate in the Laboratory of Genital Tract Biology of the Department of Obstetrics, Gynecology and Reproductive Biology at Brigham and Women's Hospital, Boston using the Meso Scale Discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD), an electrochemiluminescence system that has been validated by comparisons with traditional ELISA [16, 17] and produces measurements that have high content validity [18–21]. The multiplex assays measuring up to 10 proteins simultaneously were optimized to allow detection of each biomarker within the linearity range of the eluted samples. The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations (pg/mL) using interpolation from several log calibrator curves. Split quality control blood pools tested on each plate showed inter-assay variation of less than 20% for each protein. The total protein concentration in each eluted sample was determined by BCA assay (Thermo Scientific, Rockford, IL) using a multi-label Victor 2 counter (Perkin Elmer, Boston, MA). The measurements of each analyte were then normalized as pg specific protein per mg total protein. The central tendency and dispersion tendencies of each protein are presented elsewhere [21] We defined the top quartile in each of nine distributions of concentrations of each protein defined by the three postnatal days the blood was collected, and by each of the three gestational age groups (23–24, 25–26, and 27 weeks).

The following are the 25 proteins whose concentrations were measured: two acute phase reactants, C-Reactive Protein (CRP) and Serum Amyloid A (SAA), a lysosomal protein made by neutrophils, Myeloperoxidase (MPO), six cytokines and their receptors,

Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6), Interleukin-6 Receptor (IL-6R), Tumor Necrosis Factor- α (TNF- α), (TNF-R1), Tumor Necrosis Factor Receptor-2 (TNF-R2), six chemokines, Interleukin-8 (IL-8; CXCL8), Monocyte Chemotactic Protein-1 (MCP-1; CCL2), Monocyte Chemotactic Protein -4 (MCP-4; CCL13), Macrophage Inflammatory Protein-1 β (MIP-1 β ; CCL4), Regulated upon Activation, Normal T-cell Expressed, and [presumably] Secreted (RANTES; CCL5), Interferon-inducible T cell Alpha-Chemoattractant (I-TAC; CXCL11), four adhesion molecules, Intercellular Adhesion Molecule -1 (ICAM-1; CD54), Intercellular Adhesion Molecule -3 (ICAM-3; CD50), E-selectin (CD62E), Vascular Cell Adhesion Molecule-1 (VCAM-1; CD106), two metalloproteinases, Matrix Metalloproteinase-1 (MMP-1), Matrix Metalloproteinase-9 (MMP-9), and finally a growth factor, two of its receptors, and a binding protein of another growth factor, Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial Growth Factor Receptor-1 (VEGF-R1; Flt-1), Vascular Endothelial Growth Factor Receptor-2 (VEGF-R2; KDR), and Insulin Growth Factor Binding Protein-1 (IGFBP-1).

2.6 Data analysis

We evaluated two broad hypotheses. The first hypothesis is that the concentrations of inflammation-associated proteins in the blood of ELGANs do not vary with one another. We use the term “inflammation-associated” broadly to encompass proteins with either pro-inflammation or anti-inflammation capabilities. The second hypothesis we evaluated is that an elevated concentration of an inflammation-associated protein in the blood of ELGANs on one day does not predict an elevated concentration of the same or another inflammation-associated protein in the blood one or two weeks later.

Because we have emphasized the proportion of the sample whose concentration of each protein is in the top quartile, we continued to use the top quartile as our focus. Consequently, we evaluated the hypothesis that children whose concentration of a protein is in the top quartile do not differ from their peers with a lower concentration in their tendency to have a concentration of another protein in the top quartile. Pair-wise associations for both the first and second hypotheses were evaluated with Fisher’s exact test (two-tailed). Because we constructed 2775 two-way tables, we balanced our desire to avoid type 1 and type 2 errors by selecting a p-value < 0.0001 as statistically significant. At this p-value we would not expect statistical significance to occur randomly in even one table.

3. RESULTS

3.1 Same day co-occurrence: overview (Table 1) (Summary of associations in Supplement Tables 1–3)

When one protein in a sample had a concentration in the top quartile, other proteins also tended to have elevated concentrations on the same day. On day 1, for example, the concentrations of four proteins varied with the concentrations of 20 or more other proteins (TNF-R1, TNF-R2, I-TAC, E-SEL), the concentrations of 17 proteins varied with the concentrations of 10–19 or more other proteins, and the concentrations of only four proteins varied with the concentrations of less than 10 other proteins (MCP-1, MCP-4, VEGF-R1, IGFBP-1). On the other hand, receptors for TNF-alpha tended to be associated with a number of proteins that was similar to that of TNF-alpha.

With few exceptions, the number of associations between proteins did not seem to vary appreciably in a linear trend with the day of the specimen. Of the three exceptions, only MMP-1 showed a linear decline in the number of associations over the two weeks of assessments, while IGFBP-1 increased prominently after the first week, and MCP-1 and

MCP-4 displayed modest increases between days 1 and 14. The number of associations with RANTES declined prominently in specimens after day 7.

3.2 Same day co-occurrence: Day 1 associations (Table 2) (Qualitative display of Supplement Table 1)

We have included Tables 2–4 and 6–8 because they provide a sense of the relationships between individual proteins. The main impression provided by Table 2 is that of a plethora of associations.

Children who had elevated concentrations of both CRP and SAA also tended to have elevated concentrations of MPO, IL-1 β , TNF- α , TNF-R1, TNF-R2, IL-8, I-TAC, ICAM-1, ICAM-3, VCAM-1, E-SEL, and VEGF. IL-6 and IL-6R are not included in this group.

The expected associations among IL-1beta, IL-6, TNF-alpha, and IL-8 are prominent, as are associations of these proteins with adhesion molecules (ICAM-1, ICAM-3, VCAM-1, and E-SEL) and several other proteins including TNF-R1, TNF-R2, I-TAC, MMP-9, VEGF, and VEGF-R2.

The tendency to have elevated concentrations of receptors for TNF-alpha varied with the tendency to have an elevated concentration of TNF-alpha. This receptor-ligand relationship applied to one of the receptors for VEGF (VEGF-R2), but not the other (VEGF-R1), nor did it apply to IL-6R and IL-6.

3.3 Same day co-occurrence: Day 7 associations (Table 3) (Qualitative display of Supplement Table 2)

Children who had elevated concentrations of both CRP and SAA on day 7 also tended to have elevated concentrations of MPO, IL-6, TNF- α , TNF-R1, TNF-R2, IL-8, MIP-1 β , I-TAC, ICAM-1, E-SEL, VEGF-R2, and IGFBP-1. On day 7 IL-1beta is not among these proteins.

The associations among IL-1beta, IL-6, TNF-alpha, and IL-8 continued, as did their associations with TNF-R1, TNF-R2, MIP-1 β , ICAM-3, E-SEL, MMP-9, VEGF, and VEGF-R1. On the other hand, elevated concentrations of IL-1beta, IL-6, TNF-alpha, and IL-8 were no longer all associated with elevated concentrations of ICAM-1, VCAM-1, I-TAC.

While a concentration in the top quartile of IGFBP-1 on day 1 was associated with elevated concentrations of only four proteins, on day 7 with an elevated concentration of IGFBP-1 was associated with elevated concentrations of CRP, SAA, IL-6, TNF-R1, TNF-R2, MCP-1, MCP-4, I-TAC, ICAM-3, VCAM-1, E-SEL, VEGF-R1, VEGF-R2, and IGFBP-1. The tendency to have elevated concentrations of receptors for TNF-alpha varied with the tendency to have an elevated concentration of TNF-alpha, as did VEGF receptors with its ligand. In contrast, however, this receptor-ligand relationship was not seen with IL-6R and IL-6.

3.4 Same day co-occurrence: Day 14 associations (Table 4) (Qualitative display of Supplement Table 3)

Children who had elevated concentrations of both CRP and SAA on day 14 also tended to have elevated concentrations of MPO, IL-1 β , IL-6, TNF- α , TNF-R1, TNF-R2, IL-8, MIP-1 β , I-TAC, ICAM-1, E-SEL, VEGF-R2, and IGFBP-1.

Concentrations in the top quartile of IL-1beta, IL-6, TNF-alpha, and IL-8 continued their tendency to occur together. Children who had elevated concentrations of all of these proteins

also tended to have elevated concentrations of MIP-1 β , ICAM-1, ICAM-3, VCAM-1, E-SEL, MMP-9, VEGF, VEGF-R1, and VEGF-R2.

On day 14, a tendency to have an elevated concentration of IL-6R finally varied with the tendency to have an elevated concentration of IL-6. The ligand-receptor relationships for TNF-alpha and for VEGF seen on day 7 continued to be evident on day 14.

3.5 Different day relationships: overview (Table 5) (Summary of associations in Tables 6–8 and Supplement Tables 4–6)

The Latin phrase, “*vaticinium ex eventu*” or “foretelling after the event” has sometimes been called postdiction and sometimes retrodiction. Here we use the term postdiction to indicate the information provided by an elevated concentration of a protein on one day about elevated concentrations of that or other proteins on a previous day a week or two earlier. We consider three patterns of relationships between elevated concentrations of proteins. One pattern is of equal numbers of prediction and postdictions. This is seen for CRP, IL-1beta, TNF-R1, RANTES, and VEGF-R2. This pattern is also seen for five additional proteins that had very low numbers of both predictions and postdictions (IL-6, MCP-1, MCP-4, MMP-1, IGFBP-1).

Another pattern is of a larger number of predictions than postdictions. This pattern of preferential predictability was seen between days 1 and 7 for SAA, TNF-alpha, TNF-R2, I-TAC, ICAM-1, and VCAM-1, and between days 7 and 14 for CRP, SAA, IL-6, and IGFBP-1.

The last pattern, of a larger number of postdictions than predictions, is characterized by MPO, IL-6R, IL-8, MIP-1 β , MMP-9, VEGF, and VEGF-R1 between days 1 and 7, and for ICAM-3, E-SEL, and VEGF between days 7 and 14.

The relationships between concentrations on days 1 and 14 were very weak indeed. For example, no elevated day-1 concentration predicted the elevated day-14 concentration of more than 2 proteins, nor was any day-14 protein postdicted by more than 2 day-1 protein elevations.

3.6 Different day relationships: Day 1 and day 7 (Table 6) (Qualitative display of associations in Supplement Table 4)

Unlike the qualitative tables for co-occurrences on each day (Tables 2–4), the qualitative tables for different day relationships have data above and below the diagonal running from the upper left to the lower right. This diagonal identifies the 21 proteins whose elevated concentration on day 1 predicted an elevated concentration on day 7, and whose day-7 elevated concentration postdicted an elevated day-1 concentration. The four proteins not on the diagonal are SAA, IL-6, MCP-1, and IGFBP-1.

Going down each column identifies which proteins are predicted by an elevated concentration of the protein listed (by number) at the head of each column. For example, an elevated concentration of MPO on day 1 (column 3) predicts elevated day-7 concentrations of only a small irregular group of proteins. In contrast, MPO on day 7 (row 3) is predicted by elevated day-1 concentrations of every protein with seven exceptions, IL-6, MCP-1, MCP-4, MMP-1, VEGF-R1, VEGF-R2, and IGFBP-1.

An elevated concentration of IL-6 on day 1 was not associated with an elevated concentration of any protein on day 7, just as an elevated concentration of IL-6 on day 7 was not associated with an elevated concentration of any protein on day 1. The same was seen for IGFBP-1.

3.7 Different day relationships: Day 7 and day 14 (Table 7) (Qualitative display of associations in Supplement Table 5)

Only four proteins (MPO, IL-1beta, MMP-9, and IGFBP-1) are not on the diagonal running from the upper left to the lower right for days 7 and 14.

An elevated concentration of MMP-1 on day 7 did not predict an elevated concentration of any protein on day 14. Similarly, an elevated concentration of VEGF on day 7 was not associated with an elevated concentration of any protein on day 14 other than itself. An elevated concentration of MMP-1 on day 7 was associated with an elevated concentration of only MMP-1 and RANTES on day 14, while an elevated concentration of IGFBP-1 on day 7 was associated with elevated concentrations of CRP, TNF-R1, IL-8, MCP-4, E-SEL, and VEGF-R2.

3.8 Different day relationships: Day 1 and day 14 (Table 8) (Qualitative display of associations in Supplement Table 6)

What is most impressive about this table is the relative paucity of associations. Only seven proteins were on the diagonal for days 1 and 14 (IL-6R, MCP-4, MIP-1 β , RANTES, I-TAC, MMP-1, and VEGF-R2). The only three off-diagonal associations are day-1 TNF-R1 with day-14 VCAM-1 and VEGF-R, and day-1 RANTES with day-14 ICAM-3.

4. Discussion

4.1 wide-spread inflammation

Our most impressive finding is that inflammation is so widespread. Of the 25 proteins we measured, the concentrations of 15–20 tended to be high when the concentration of any other protein was in the top quartile.

Each of the cytokines, chemokines, and adhesion molecules whose concentration we measured can contribute to the traffic of cells out of blood vessels, and into surrounding parenchyma [22, 23]. Each of these categories, plus the other proteins we measured, has the potential to influence how the traffic of cells influences danger elimination, debris removal, and repair, or contributes to organ damage [24, 25].

Others have found that the concentrations of many inflammation-associated proteins tend to vary together [10, 14]. In addition, an inflammatory stimulus given to rodents influences the expression of thousands of genes [12].

Thus our finding such close relationships does not come at a surprise. We do not know, however, of any other study of the day-by-day relationships among inflammation-associated proteins in ELGANs.

4.2 changes with postnatal age

The concentrations of some proteins in ELGANs increase with increasing postnatal age [21]. With few exceptions, though, (RANTES, MMP-1), the number of significant relationships did not change with postnatal age, suggesting that the relative increases in concentration were not much different among the various proteins.

The relationships between protein concentrations one week with protein concentrations the next week, however, sometimes changed dramatically. The pre-partum and intra-partum stimuli for an inflammatory response in the days near delivery might differ from those that result in inflammation that begins a week or two later [26]. Perhaps that is why we see so few relationships between protein concentrations on days 1 and 14.

4.3 Duration of inflammation/half life of proteins

The large number of proteins on the diagonal (from upper left to lower right) of tables with data from two separate days a week apart indicates that an elevated concentration of a protein at one time is associated with an elevated concentration of the same protein a week later. In adults, the half-life of most of the cytokines is short (almost invariably only a few hours) [27–29], while the acute phase reactant CRP has a half-life that is almost a full day [30]. The half life of most inflammation-associated proteins in preterm newborns, however, is not known. Thus, we do not know if the associations in our data set between measurements of the same protein one week apart reflect long half lives, persistent or recurrent inflammatory stimuli, or feedback loops [31, 32].

4.4 proteins traditionally considered not inflammatory vary with well-accepted inflammation-associated proteins

We found that elevated concentrations of VEGF and its receptors, as well as of IGFBP-1 are associated with elevated concentrations of cytokines, chemokines, and adhesion molecules. In the recent past, VEGF, traditionally viewed as an angiogenic growth factor, its receptors, and IGFBP-1 were not viewed as inflammatory proteins. More recently, however, VEGF is increasingly recognized for its inflammation relationships [33–38] as are VEGF receptors [19, 39, 40].

IGFBP-1 also appears to have inflammation relationships not previously recognized. For example, IGFBP-1 is one of a small group of proteins whose amniotic fluid concentrations are strongly associated with histological chorioamnionitis and funisitis in humans [41]. In addition, plasma concentrations of IGFBP-1 in animals, also increase during infection [42].

These findings by others, plus what we report here, indicate that some growth factors, their receptors and binding proteins are related to elevated concentrations of inflammation-associated proteins and other inflammatory phenomena.

4.5 Measures of relatedness

Correlation coefficients describe relationships between the central tendencies of the two measures. We, however, are less interested in the central tendency than in tendencies to have elevated concentrations. This interest reflects our perception that high concentrations convey information about organ-damage propensities in preterm humans [1, 4, 5, 43]. Consequently, rather than correlation coefficients, we calculated exact p values for the tendency of concentration in the top quartile of a protein to be associated with a concentration in the top quartile of a different protein on the same day.

Transitional matrices quantify the probability of an event or characterization given the presence/absence or levels of the event or characteristic during the previous time interval. In light of our preference to focus on elevated concentrations, we could have presented transitional matrices limited to concentrations in the top quartile. To keep changes from one week to another as symmetrical with what we presented for same day relationships, we present exact p values for the tendency of a protein concentration in the top quartile on one day to be associated with a concentration in the top quartile of the same or a different protein on another day one or two weeks later.

4.6 Strengths

Our study has several strengths. First, we included a large number of infants, making it unlikely that we have missed important associations due to lack of statistical power. Second, we selected infants based on gestational age, not birth weight, in order to minimize confounding due to factors related to fetal growth restriction [44]. Third, our protein data are

of high quality, and have high content validity [18–21]. Fourth, we concentrated on elevated concentrations, which are most likely to convey biologically-important information [1, 4, 5, 43]. Fifth, we chose a p value criterion of relatedness ($p < .0001$) that took into account the plethora of comparisons while balancing a desire to minimize both type 1 and type 2 inferential errors [45–48].

This paper has several limitations. First, this was an exploratory analysis, which should be viewed as generating hypotheses, rather than a test of any of them. Second, since this is an observational study, causation should not be inferred from any of the relationships found.

4.7 Conclusions/inferences

The inflammatory response in ELGANs is diffuse. Therapies and prophylactics are likely to be most effective if they can deal with complex inflammatory processes rather than merely one or a small group of proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Blood concentrations of inflammation-related proteins tend to vary with one another.
- High concentrations on day 1 predict high concentrations on day 7.
- Concentrations of VEGF, VEGF-R1, VEGF-R2, and IGFBP-1 vary with these proteins

Table 1

Summary of Tables 2–4. The number of proteins whose concentration in the top quartile (for gestational age on that day) was associated with a concentration in the top quartile of other proteins on the same day. An upward-directed outline arrow (Δ) indicates that a positive relationship is significant at $p < 0.0001$. A downward-directed solid arrow (▼) indicates a statistically significant inverse relationship.

		Day 1 (Table 2)		Day 7 (Table 3)		Day 14 (Table 4)	
	Protein	Δ	▼	Δ	▼	Δ	▼
1	CRP	14		15		14	
2	SAA	16		14		15	
3	MPO	18	1	19		18	
4	IL-1β	19		13		18	
5	IL-6	12		18		20	
6	IL-GR	16		15		18	
7	TNF-α	18		19		19	
8	TNF-R1	23		23		21	
9	TNF-R2	21		23		21	
10	IL-8	17		21		17	
11	MCP-1	6		11	1	10	
12	MCP-4	9		13		17	
13	MIP-1β	18		19		17	
14	RANTES	14		13		7	
15	I-TAC	20		21		14	
16	ICAM-1	19	1	19		14	
17	ICAM-3	19	1	19		20	
18	VCAM-1	16		20		15	
19	E-SEL	21		23		21	
20	MMP-1	13	1	6		7	
21	MMP-9	16	1	15	1	15	
22	VEGF	18		18		16	
23	VEGF-R1	5		19		16	
24	VEGF-R2	19		21		21	

		Day 1 (Table 2)		Day 7 (Table 3)		Day 14 (Table 4)	
	Protein	Δ	▼	Δ	▼	Δ	▼
25	IGFBP-1	4	3	13		10	

Table 2
Co-occurrence of two proteins in the highest quartile on days 1

The numbers at the head of each column represent the proteins identified in the left 2 columns. The arrows identify proteins whose concentrations in the top quartile vary significantly with each other (pair-wise Fisher's exact test and two-sided $p < 0.0001$). The upward-pointing outlined arrows (Δ) indicate significant positive relationships while the downward-pointing solid arrows ($\blacktriangledown$) indicate significant inverse relationships. Cells are empty when relationships are not statically significant.

	Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	CRP																								
2	SAA																								
3	MPO																								
4	IL-1β																								
5	IL-6																								
6	IL-6R																								
7	TNF-α																								
8	TNF-R1																								
9	TNF-R2																								
10	IL-8																								
11	MCP-1																								
12	MCP-4																								
13	MIP-1β																								
14	RANTES																								
15	I-TAC																								
16	ICAM-1																								
17	ICAM-3																								
18	VCAM-1																								
19	E-SEL																								
20	MMP-1																								
21	MMP-9																								
22	VEGF																								
23	VEGF-R1																								
24	VEGF-R2																								

	Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
25	IGFBP-1			▼		Δ						Δ	Δ					▼						Δ	

Table 3
Co-occurrence of two proteins in the highest quartile on day 7

The numbers at the head of each column represent the proteins identified in the left 2 columns. The arrows identify proteins whose concentrations in the top quartile vary significantly with each other (pair-wise Fisher's exact test and two-sided $p < 0.0001$). The upward-pointing outlined arrows (Δ) indicate significant positive relationships while the downward-pointing solid arrows ($\blacktriangledown$) indicate significant inverse relationships. Cells are empty when relationships are not statically significant.

	Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	CRP																								
2	SAA	Δ																							
3	MPO	Δ	Δ																						
4	IL-1β		Δ	Δ																					
5	IL-6	Δ	Δ	Δ	Δ																				
6	IL-6R			Δ																					
7	TNF-α	Δ	Δ	Δ	Δ	Δ	Δ																		
8	TNF-R1	Δ	Δ	Δ	Δ	Δ	Δ	Δ																	
9	TNF-R2	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ																
10	IL-8	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ															
11	MCP-1	Δ				Δ			Δ	Δ	Δ														
12	MCP-4						Δ		Δ	Δ	Δ	Δ													
13	MIP-1β	Δ	Δ	Δ	Δ	Δ		Δ	Δ	Δ	Δ														
14	RANTES			Δ			Δ			Δ	Δ			Δ											
15	I-TAC	Δ	Δ	Δ		Δ	Δ	Δ	Δ	Δ	Δ		Δ	Δ	Δ										
16	ICAM-1	Δ	Δ	Δ		Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ									
17	ICAM-3			Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ	Δ	Δ	Δ									
18	VCAM-1	Δ				Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ							
19	E-SEL	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ						
20	MMP-1								Δ					Δ	Δ	Δ		Δ	Δ						
21	MMP-9			Δ	Δ		Δ	Δ	Δ	Δ	Δ	▼		Δ	Δ	Δ		Δ		Δ					
22	VEGF			Δ	Δ		Δ	Δ	Δ	Δ	Δ			Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		
23	VEGF-R1			Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	
24	VEGF-R2	Δ	Δ	Δ		Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ

	Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
25	IGFBP-1	Δ	Δ			Δ			Δ	Δ		Δ	Δ			Δ	Δ		Δ	Δ				Δ	Δ

Table 4

Co-occurrence of two proteins in the highest quartile on days 14

The numbers at the head of each column represent the proteins identified in the left 2 columns. The arrows identify proteins whose concentrations in the top quartile vary significantly with each other (pair-wise Fisher's exact test and two-sided $p < 0.0001$). The upward-pointing outlined arrows (Δ) indicate significant positive relationships while the downward-pointing solid arrows ($\blacktriangledown$) indicate significant inverse relationships. Cells are empty when relationships are not statically significant.

	Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	1	CRP																							
	2	SAA	Δ																						
	3	MPO	Δ	Δ																					
	4	IL-1β	Δ	Δ	Δ																				
	5	IL-6	Δ	Δ	Δ	Δ																			
	6	IL-6R			Δ	Δ	Δ																		
	7	TNF-α	Δ	Δ	Δ	Δ	Δ	Δ																	
	8	TNF-R1	Δ	Δ	Δ	Δ	Δ	Δ	Δ																
	9	TNF-R2	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ															
	10	IL-8	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ														
	11	MCP-1				Δ		Δ	Δ	Δ	Δ	Δ													
	12	MCP-4			Δ	Δ	Δ		Δ	Δ		Δ	Δ												
	13	MIP-1β	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ	Δ											
	14	RANTES					Δ						Δ		Δ										
	15	I-TAC	Δ	Δ				Δ		Δ			Δ	Δ	Δ	Δ									
	16	ICAM-1	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ				Δ	Δ								
	17	ICAM-3		Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ	Δ	Δ	Δ		Δ	Δ						
	18	VCAM-1			Δ		Δ		Δ	Δ		Δ	Δ	Δ		Δ	Δ	Δ	Δ	Δ					
	19	E-SEL	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ	Δ	Δ	Δ	Δ					
	20	MMP-1					Δ						Δ		Δ	Δ		Δ	Δ		Δ				
	21	MMP-9			Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ				Δ	Δ		Δ					
	22	VEGF			Δ	Δ	Δ	Δ	Δ		Δ		Δ	Δ	Δ			Δ			Δ	Δ			
	23	VEGF-R1			Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ					Δ	Δ	Δ	Δ	Δ	Δ	Δ	
	24	VEGF-R2	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ

	Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
25	IGFBP-1	Δ	Δ		Δ	Δ			Δ	Δ						Δ			Δ	Δ					Δ

Table 5

Summary of Tables 6–8. The total number of proteins whose concentration in the top quartile on one day was associated with a concentration in the top quartile of the same and other proteins one or two weeks later. These numbers summarize the number of upward-directed arrows (Δ) (indicators of relationships significant at $p < 0.0001$) in Tables 6, 7, and 8. No inverse relationships were statistically significant.

	Protein	Day 1 $\rightarrow$ Day 7 (Table 6)		Day 7 $\rightarrow$ Day 14 (Table 7)		Day 1 $\rightarrow$ Day 14 (Table 8)	
		Day 1 predicts day 7	Day 7 predicted by day 1	Day 7 predicts day 14	Day 14 predicted by day 7	Day 1 predicts day 14	Day 14 predicted by day 1
1	CRP	9	7	9	5	0	0
2	SAA	6	0	6	2	0	0
3	MPO	6	18	3	3	0	0
4	IL-1 β	9	7	1	1	0	0
5	IL-6	0	0	14	1	0	0
6	IL-GR	5	10	2	4	1	1
7	TNF- α	13	5	2	0	0	0
8	TNF-R1	11	9	8	8	2	0
9	TNF-R2	12	4	5	8	0	0
10	IL-8	8	12	5	4	0	0
11	MCP-1	1	0	5	2	0	0
12	MCP-4	1	2	6	5	1	1
13	MIP-1 β	7	12	2	2	1	1
14	RANTES	5	6	3	4	2	1
15	I-TAC	12	3	5	3	1	1
16	ICAM-1	15	3	4	5	0	0
17	ICAM-3	10	19	5	14	0	1
18	VCAM-1	13	5	8	11	1	2
19	E-SEL	18	13	5	11	0	0
20	MMP-1	3	1	2	3	1	1
21	MMP-9	6	16	0	5	0	0
22	VEGF	6	19	1	6	0	0
23	VEGF-R1	0	6	5	4	0	1
24	VEGF-R2	8	8	10	10	1	1

	Protein	Day 1 → Day 7 (Table 6)		Day 7 → Day 14 (Table 7)		Day 1 → Day 14 (Table 8)	
		Day 1 predicts day 7	Day 7 predicted by day 1	Day 7 predicts day 14	Day 14 predicted by day 7	Day 1 predicts day 14	Day 14 predicted by day 1
25	IGFBP-1	0	0	6	0	0	0

Table 6
Protein elevations on day 1 associated with protein elevations on day 7

The upward facing arrows (Δ) identify proteins listed at the top of each column whose concentration on day 1 was positively and significantly associated with a concentration in the top quartile on day 7 of the protein listed on the left (pair-wise Fisher's exact test and two-sided $p < 0.0001$).

Day 7 level in top quartile		Day 1 measure in the top quartile (column heads are those of proteins listed on left)																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	CRP	Δ	Δ				Δ		Δ						Δ					Δ					Δ	
2	SAA																									
3	MPO	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			
4	IL-1β	Δ		Δ			Δ	Δ							Δ					Δ						
5	IL-6																									
6	IL-6R	Δ	Δ			Δ	Δ	Δ					Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			Δ		
7	TNF-α			Δ	Δ		Δ		Δ							Δ				Δ						
8	TNF-R1			Δ	Δ		Δ	Δ	Δ					Δ	Δ	Δ	Δ	Δ	Δ	Δ						
9	TNF-R2							Δ								Δ			Δ	Δ						
10	IL-8			Δ			Δ	Δ	Δ	Δ		Δ		Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ				
11	MCP-1																									
12	MCP-4										Δ								Δ							
13	MIP-1β			Δ	Δ		Δ		Δ				Δ		Δ	Δ	Δ	Δ		Δ	Δ	Δ	Δ			
14	RANTES							Δ					Δ		Δ	Δ	Δ	Δ		Δ				Δ		
15	I-TAC	Δ														Δ			Δ							
16	ICAM-1	Δ														Δ				Δ						
17	ICAM-3	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			Δ		Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			
18	VCAM-1														Δ			Δ	Δ	Δ					Δ	
19	E-SEL			Δ			Δ	Δ	Δ	Δ			Δ		Δ	Δ	Δ	Δ	Δ	Δ		Δ	Δ			
20	MMP-1																				Δ					
21	MMP-9	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ		Δ	Δ	Δ	Δ	Δ		Δ		Δ	Δ			
22	VEGF	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ		Δ	
23	VEGF-R1							Δ	Δ		Δ								Δ	Δ				Δ		
24	VEGF-R2						Δ	Δ					Δ		Δ	Δ	Δ	Δ	Δ	Δ				Δ		Δ

Day 1 measure in the top quartile (column heads are those of proteins listed on left)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Day 7 level in top quartile																										
	25	IGFBP-1																								

Table 7
Protein elevations on day 7 associated with protein elevations on day 14

T The upward facing arrows (Δ) identify proteins listed at the top of each column whose concentration on day 7 was in the highest quartile (for gestational age on that day) was significantly associated with a concentration in the top quartile on day 14 of the protein listed on the left (pair-wise Fisher's exact test and two-sided $p < 0.0001$).

Day 14 level in top quartile		Day 7 measure in the top quartile (column heads are those of proteins listed on left)																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	CRP	Δ	Δ		Δ																				Δ	Δ
2	SAA	Δ	Δ																							
3	MPO				Δ				Δ																Δ	
4	IL-1β				Δ																					
5	IL-6				Δ																					
6	IL-6R				Δ	Δ												Δ						Δ		
7	TNF-α					Δ																				
8	TNF-R1				Δ		Δ	Δ		Δ	Δ								Δ					Δ		Δ
9	TNF-R2	Δ	Δ		Δ			Δ	Δ							Δ				Δ					Δ	
10	IL-8	Δ			Δ				Δ																Δ	
11	MCP-1									Δ	Δ															
12	MCP-4				Δ					Δ	Δ								Δ							Δ
13	MIP-1β				Δ							Δ														
14	RANTES											Δ		Δ					Δ		Δ				Δ	
15	I-TAC											Δ		Δ		Δ										
16	ICAM-1	Δ						Δ	Δ							Δ									Δ	
17	ICAM-3	Δ	Δ	Δ	Δ			Δ		Δ	Δ				Δ			Δ	Δ	Δ				Δ	Δ	Δ
18	VCAM-1					Δ		Δ	Δ		Δ					Δ	Δ	Δ	Δ	Δ				Δ	Δ	Δ
19	E-SEL	Δ			Δ	Δ	Δ			Δ		Δ						Δ		Δ				Δ	Δ	Δ
20	MMP-1											Δ				Δ					Δ					
21	MMP-9	Δ	Δ		Δ				Δ							Δ										
22	VEGF			Δ				Δ								Δ			Δ	Δ				Δ		
23	VEGF-R1			Δ				Δ										Δ						Δ		Δ

		Day 7 measure in the top quartile (column heads are those of proteins listed on left)																								
Day 14 level in top quartile		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
24	VEGF-R2	Δ	Δ			Δ			Δ	Δ							Δ		Δ	Δ					Δ	Δ
25	IGFBP-1																									

Table 8
Protein elevations on day 1 associated with protein elevations on day 14

The upward facing arrows (Δ) identify proteins listed at the top of each column whose concentration on day 1 was in the highest quartile (for gestational age on that day) was significantly associated with a concentration in the top quartile on day 14 of the protein listed on the left (pair-wise Fisher's exact test and two-sided $p < 0.0001$)

Day 14 level in top quartile		Day 1 measure in the top quartile (column heads are those of proteins listed on left)																						
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
CRP																								
SAA																								
MPO																								
IL-1β																								
IL-6																								
IL-6R																								
TNF-α																								
TNF-R1																								
TNF-R2																								
IL-8																								
MCP-1																								
MCP-4																								
MIP-1β																								
RANTES																								
I-TAC																								
ICAM-1																								
ICAM-3																								
VCAM-1																								
E-SEL																								
MMP-1																								
MMP-9																								
VEGF																								
VEGF-R1																								

		Day 1 measure in the top quartile (column heads are those of proteins listed on left)																								
Day 14 level in top quartile		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
24	VEGF-R2																								Δ	
25	IGFBP-1																									