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Systemic immune activation profiles in streptococcal necrotizing soft tissue infections: A prospective multicenter study

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ABSTRACT

Objective: Early stages with streptococcal necrotizing soft tissue infections (NSTIs) are often difficult to discern from cellulitis. Increased insight into inflammatory responses in streptococcal disease may guide correct interventions and discovery of novel diagnostic targets.
Methods: Plasma levels of 37 mediators, leucocytes and CRP from 102 patients with β-hemolytic streptococcal NSTI derived from a prospective Scandinavian multicentre study were compared to those of 23 cases of streptococcal cellulitis. Hierarchical cluster analyses were also performed.
Results: Differences in mediator levels between NSTI and cellulitis cases were revealed, in particular for IL-1β, TNFα and CXCL8 (AUC >0.90). Across streptococcal NSTI etiologies, eight biomarkers separated cases with septic shock from those without, and four mediators predicted a severe outcome.
Conclusion: Several inflammatory mediators and wider profiles were identified as potential biomarkers of NSTI. Associations of biomarker levels to type of infection and outcomes may be utilized to improve patient care and outcomes.

1. Introduction

Necrotizing soft tissue infections (NSTIs) have high rates of morbidity and mortality [1]. *Streptococcus pyogenes* (group A streptococcus; GAS) is the major cause of monomicrobial (type 2) NSTIs [2], while *Streptococcus dysgalactiae* (SD) is an emerging etiologic cause [3,4]. Streptococcal NSTIs are frequently associated with bacteremia, septic shock, organ failure and death [5,6]. Although GAS and SD are the major microbial etiologies also of cellulitis, systemic and local severity signs are less prominent, surgery is rarely needed and complication rates are low [7,8]. Of concern, early NSTIs may be mistaken for cellulitis [1,6,9,10]. This poses a major threat to many NSTI patients, as it may delay surgery and antibiotic therapy, the most important measures to reduce organ failure, sequelae and death [11–13]. The diagnosis of NSTI is still based on surgical exploration and confirmation [9].

Cytokine profiles in sepsis are well characterized [14,15], but studies

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have failed to identify plasma mediators that reliably diagnose or prognosticate septic shock and mortality [14,15]. Therefore, combinations of mediators are advocated [14,15]. Cytokines have also been explored as diagnostic and prognostic biomarkers in specific infectious diseases [16,17]. Apart from streptococcal toxic shock syndrome (STSS) [18,19], inflammatory profiles of streptococcal NSTIs and cellulitis are insufficiently described [20–22].

Improved biomarker-based tools are needed to support the clinician in establishing the diagnosis of NSTIs at an early stage of the disease. For streptococcal skin and soft tissue infections (SSTIs) in particular, there is a wide and overlapping spectrum of disease that requires investigation of differential inflammatory patterns. This may advance the understanding of the pathogenesis, lead to development of new diagnostic and prognostic tools and possibly identify potential targets of treatment. In a large Scandinavian multicenter patient cohort of NSTIs, we recently demonstrated that inflammatory profiles differ between polymicrobial and monomicrobial infections and by the presence of shock [23]. In the present study, we explore the systemic immune activation pattern of the streptococcal NSTI cases, comparing and contrasting them to streptococcal cellulitis.

2. Materials and methods

2.1. Setting & patients

This work was conducted as part of the INFECT-study, a Scandinavian multicenter study, with a prospectively included cohort of 409 NSTI cases in total (ClinicalTrials.gov (NCT01790698)) [2]. Cases caused by GAS ($n = 126$) and SD ($n = 27$) were selected, and of the 153 streptococcal cases identified [2,5], 102 were strictly monomicrobial (GAS, $n = 88$; SD, $n = 14$) and included for further analysis in this study.

Two control groups with a total of 43 cases were included. The cellulitis control group consisted of four operated (surgery disproved NSTI), and 19 non-operated cellulitis cases with confirmed or probable GAS or SD etiology defined by positive culture or serology as described elsewhere [8]. The healthy control group consisted of patients admitted for elective orthopaedic surgery ($n = 20$), with blood samples taken prior to surgery (Copenhagen University Hospital, Denmark, Regional ethics committee permit: H-2-2014-071). Ethical approval and patients' consent are as described previously [2,20].

2.2. Clinical characteristics

Clinical and laboratory data were acquired according to the INFECT-study protocol [2]. Outcomes registered in the NSTI cohort included septic shock at admission, along with death and/or amputation. Septic shock was defined as use of vasopressor and lactate $\geq$2 mmol/L [24]. Severe outcome was defined as amputation and/or death within 90 days from admission. Analyzing this outcome, we restricted inclusion to cases with an extremity as a primary site of infection and excluded cases amputated prior to arrival at study hospital.

2.3. Plasma biomarker analysis

Blood was collected into 10 mL EDTA vacuum tubes and kept on ice until processed (15–90 min max). Plasma was collected after centrifugation (2500 G, for 10 min at 20 °C), aliquoted into cryotubes and stored at −80 °C until further analysis.

A 32 and a 5-plex customized multiplex assay (Magnetic Luminex Assay, R&D Systems, Inc.; Abingdon, UK) were used to analyze interleukins: IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-22, IL-36β; chemokines: CCL2, CCL4, CCL5, CXCL8, CXCL10; soluble adhesion molecules: *E*-selectin, ICAM-1, VCAM-1; matrix metalloproteases: MMP-1, MMP-8, MMP-9; growth factors: G-CSF; and molecules designated 'others': C5/C5a, Collagen IVα1, Collagen Iα1, Fas-Ligand, Galectin-3, MPO, Pentraxin-3, Resistin, S100A8, S100A9, thrombomodulin and TNFα. In addition, IL-23 and IL-33 were analyzed using ELISA technique (R&D Systems; Abingdon, UK), whereas C-reactive protein (CRP) and leucocytes were analyzed according to laboratory diagnostic routines. Analyte concentration measurements were performed according to manufacturer's instructions. An overview of the analyzed mediators and mode of analysis is detailed in Supplementary Table 1. For concentrations outside their respective detection ranges (out of range, OOR), an imputation strategy was applied, as described previously [23]. Due to high rates of OOR measurements, the analytes IL-1ra and IL-33 were omitted for further analysis. For more details, see Supplementary methods. In total, 37 systemic plasma mediators together with leucocytes and CRP were included in the analyses.

2.4. Experimental model: In vitro stimulation

The level of selected mediators was measured in an in vitro stimulation experiment using human peripheral blood mononuclear cells (PBMC) isolated from healthy blood donors exposed to supernatants and heat-inactivated GAS and SD isolates from NSTI cases, and in human umbilical vein endothelial cells (HUVEC) exposed to supernatants from the bacterial-stimulated PBMCs. For details, see Supplementary methods.

2.5. Statistical analysis

Analyses were done using R programming language (R Core Team, 2019) and IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY). For categorical variables Fisher's exact test or χ^2 test were used as appropriate. Due to the non-normality of the data, Mann-Whitney *U* test was applied for continuous variables. Due to the unequal group size of the clinical categories compared, a resampling procedure (iteration) of the data results was performed. The resampling included 10^4 iterations based on 90% of the smallest group size used for each comparison. An average *p*-value of all comparisons was used to evaluate the final results.

All tests were two-sided and differences were considered significant at a p-value <0.05. The Benjamini-Hochberg adjustment for multiple testing was applied [25].

The discriminant ability of the most significant biomarker differences was evaluated through receiver operation characteristic (ROC) curves and the corresponding area under the ROC curve (AUC) was calculated. For correlation analysis Spearman's correlation (rho, ρ) was calculated.

Random forest (RF) models were built in R (rfPermute), including key clinical variables, as described elsewhere [23]. All RF classification models were constructed using 10^5 decision trees, selecting six random cytokines at every split of the tree. The standard Mean Decrease in Accuracy and Mean Decrease Gini index were used as measures of importance of every cytokine in the final classification model. The Gini index and respective *p*-values are presented herein. For more details, see Supplementary methods.

Additionally, we performed unsupervised hierarchical cluster analysis, where the cases and biomarkers are categorized based on relatedness and combined with a heat map to visualize correlations. The mediators were $\log_{10}$ transformed and converted to *z*-score [26], before analysis using J-Express [27]. Euclidean distance and complete linkage were used. Details concerning analysis of the in vitro stimulation tests are provided in Supplementary methods.

Network of associations between mediators were built, using the Probabilistic Context Likelihood of Relatedness on Correlation (PCLRC), as described elsewhere [28]. PCLRC gives a measure of association and the probability of likelihood in occurrence of the relationship between the mediators. Associations with a probability >95% were kept in the analysis. Different networks were built separately for GAS and SD cases, with and without septic shock.

Differential connectivity analysis was applied to compare the mediators in the association networks. Connectivity can be interpreted as how much one mediator is significant or affects the entire system (of all mediators) as it takes into account both the number and strength of connections. Differential connectivity can be interpreted as the difference in the significance or effect of one mediator in the two situations. The higher the difference, the higher change in the role that mediator plays in the two comparing situations. The analysis was performed as described elsewhere [23,29].

3. Results

3.1. Clinical characteristics

Among the 102 monomicrobial streptococcal NSTI cases, 88 were caused by GAS and 14 by SD. Demographics and characteristics of these, and the 23 streptococcal cellulitis controls, are summarized in Table 1. Comorbid conditions were prevalent among both NSTI and cellulitis patients, and most infections were located in the extremities. Bacteremia, septic shock, treatment in ICU, and death were more frequent among NSTI cases. Details on risk factors, pre- and peroperative findings, treatment including time from admission to primary surgery and total number of operations per patient in the NSTI cohort have been described previously [5].

3.2. Mediator levels in streptococcal necrotizing soft tissue infections and cellulitis

In total, 29 of 37 plasma mediators, in addition to CRP, were significantly elevated in the NSTI compared to the cellulitis cases, of which 28 showed an AUC above 0.80 (Table 2). In the multivariate RF model adjusting for age, gender and septic shock, a set of nine mediators differentiating NSTI and cellulitis were identified, based on discriminatory power according to Gini index values. Predictors identified in the model were IL-1β, TNF-α, CXCL8, MMP-8, IL-6, Pentraxin-3, IL-22, CCL4 and S100A8, that all displayed AUC values >0.86. Additionally, a comparison between NSTI and cellulitis cases without septic shock was performed, in which all the same mediators were predictors in the RF model, with exception of IL-6 (Supplementary Table 2). In this latter comparison, CRP also showed a significant result, in both the univariate- and multivariate analyses. Several of the plasma mediators with significant findings in the RF model even displayed higher AUC in this latter comparison.

Comparing cellulitis cases and healthy controls, 18 mediators were significantly higher in cellulitis, whereas three mediators were more elevated among the controls (Supplementary Table 3).

3.3. Mediator levels associated to severity and outcome in necrotizing soft tissue infections

In streptococcal NSTIs with septic shock at admission, 25 mediators were significantly elevated compared to cases without shock. In

Table 1
Demographics, clinical characteristics and outcomes in patients with streptococcal NSTIs and cellulitis.

	GAS NSTIs	SD NSTIs	All NSTIs	Cellulitis	
	n = 88	n = 14	*n* = 102	n = 23	*p*-value [a]
Demographics					
Age (years)	59.5 (48–69)	67.5 (60–73)	60.5 (48–70)	43.0 (38–62)	**0.009**
Sex, male gender	44 (50)	8 (57)	52 (51)	16 (70)	0.106
BMI [b]	25.9 (23.4–30.2)	25.6 (23.1–28.1)	25.9 (23.3–29.4)	27.7 (23.43–33.8)	0.233
Underlying condition					
Significant comorbidity [c]	52 (59)	12 (86)	64 (63)	12 (52)	0.348
Active smoker	15/77 (19.5)	3/12 (25)	18/89 (20)	3/19 (16)	1.000
High alcohol consumption [d]	6/63 (9.5)	4/11 (36)	10/74 (13.5)	0/19 (0)	0.205
Outcome variables					
Blood culture positive BHS	46 (52)	8 (57)	54 (52.9)	3 (13)	**0.001**
Septic shock (at baseline)	55 (62.5)	9 (64.3)	64 (63)	3 (13)	**<0.001**
IVIG treatment	64 (73)	8 (57)	72 (70.6)	0 (0)	**<0.001**
Amputation [e]	11 (12.5)	3 (21.4)	14 (13.7)	0 (0)	0.070
Mortality (day 90)	8 (9)	5 (36)	13 (12.7)	0 (0)	0.124
Mortality and/or amputation	16 (18.2)	7 (50)	23 (22.5)	0 (0)	**0.007**
SOFA score, day 1	9 (6–12) [f]	11 (8–13) [g]	9 (6–12)	8 (5–13) [h]	- [i]
SAPS II, day 1	40 (33–55) [f]	56 (47–63.5) [g]	42 (34–58)	32 (23.5–48.5) [h]	- [i]
Hospitalization at					
ICU/HDU	88 (100)	14 (100)	102 (100)	4 (17.4)	**<0.001**
Primary site of infection					
Head/neck	11 (12.5)	1 (7.1)	12 (11.8)	9 (39.1)	–
Upper extremities	38 (43.2)	1 (7.1)	39 (38.2)	5 (21.8)	–
Lower extremities	34 (38.6)	12 (85.8)	46 (45.1)	9 (39.1)	–
Abdomen/anogenital	5 (5.7)	0 (0)	5 (4.9)	0 (0)	–

Abbreviations: NSTIs: Necrotizing soft tissue infections, GAS: *Streptococcus pyogenes* (Group A streptococcus), SD: *S. dysgalactiae*, BMI: Body mass index, BHS: β-hemolytic streptococci, IVIG: Intravenous immunoglobulin, ICU: Intensive care unit, HDU: High dependency unit, SOFA: Sequential Organ Failure Assessment score, SAPS II: Simplified Acute Physiology Score.
The data are given as median values with interquartile range (IQR) and numbers, percentage in parentheses. Statistical significance highlighted in bold.
[a] Represents comparison of all NSTI cases vs cellulitis cases (either χ^2test or Mann-Whitney *U* test, as appropriate).
[b] Missing two in the GAS cohort.
[c] Active malignancy, chronic obstructive pulmonary disease or asthma, current or previous cardiovascular disease, diabetes mellitus, chronic kidney failure, chronic liver disease, rheumatoid disease, immunodeficiency/immunosuppression.
[d] Intake as defined by Madsen et al. 2018 [24].
[e] Including also those amputated before admission to study hospital. *Amputation* defined as the surgical removal of all or part of a limb or extremity.
[f] Missing data for two cases.
[g] Missing data for one case.
[h] Data only available for four of the cellulitis cases (i.e. the four cases with suspected NSTI, in whom surgery later showed cellulitis).
[i] Statistical comparison not performed due to low number of cases with available data in the cellulitis cohort.

Table 2
Biomarker levels in streptococcal NSTIs vs cellulitis.

	NSTIs	Cellulitis	Mann-Whitney U[a]	AUC [c]	RF[d]	
	n = 102	*n* = 23	*p*-value[b]		Gini index	*p*-value
Interleukins						
IL-1α	54 (46–64)	30 (21–40)	***	0.893	0.37	1.00
IL-1β	17 (12–30)	0.2 (0.2–1.9)	***	**0.910**	3.37	**<0.01**
IL-2	1011 (561–1367)	402 (236–626)	NS	–	0.25	1.00
IL-4	227 (184–263)	120 (98–151)	***	0.892	0.34	1.00
IL-6	775 (185–7523)	20 (15–78)	***	**0.894**	1.85	**<0.01**
IL-10	70 (40–116)	13 (6–36)	*	0.813	0.29	1.00
IL-12p70	197 (127–263)	22 (0.6–88)	***	0.835	0.46	1.00
IL-13	1464 (1154–1688)	738 (603–950)	***	0.905	0.41	1.00
IL-17A	30 (16–70)	7 (3–11)	***	0.851	036	1.00
IL-18	566 (372–1024)	341 (270–490)	*	0.732	0.35	1.00
IL-22	120 (100–139)	62 (42–77)	***	**0.906**	1.49	**0.03**
IL-23	1857 (307–7146)	622 (37–10,026)	***	0.582	1.75	0.06
IL-36β	18 (15–20)	8 (8–12)	***	0.892	0.91	0.27
Chemokines						
CCL2	868 (380–2050)	153 (119–271)	***	0.882	1.01	0.22
CCL4	880 (763–989)	525 (488–626)	***	**0.919**	1.45	**0.03**
CCL5	6575 (2332–12,480)	5104 (1630–9727)	NS	–	0.57	0.97
CXCL8	43 (18–226)	6 (4–13)	***	**0.920**	2,20	**<0.01**
CXCL10	5001 (617–383,052)	282 (177–916)	NS	–	0.36	1.00
Adhesion molecules						
E-selectin	145,171 (97,983–208,659)	42,453 (33,220–67,367)	***	0.861	0.44	1.00
ICAM-1	667,966 (544,604–876,826)	355,418 (224,411–518,623)	**	0.821	1.16	0.21
VCAM-1	43×10^5 (33×10^5–63.1×10^5)	29.4×10^5 (12.7×10^5–34.3×10^5)	**	0.803	0.26	1.00
Matrix metalloproteases						
MMP-1	1566 (973–3112)	620 (453–877)	***	0.850	0.59	0.96
MMP-8	31,555 (11,820–65,403)	1846 (758–6632)	***	**0.930**	2.10	**<0.01**
MMP-9	5527 (3226–16,509)	8145 (6358–13,664)	NS	–	1.00	0.48
Growth factors						
G-CSF	2465 (437–24,977)	155 (128–314)	***	0.848	0.65	0.86
Other						
C5/C5a	21,703 (14,176–32,769)	30,665 (13,715–60,214)	NS	–	0.64	0.95
Pentraxin-3	15,993 (6549–26,957)	880 (478–5373)	***	**0.884**	1.92	**0.03**
TNFα	35 (22–58)	10 (7–12)	***	**0.938**	2,56	**<0.01**
S100A8	1014 (690–1799)	328 (213–528)	***	**0.864**	1.26	**0.04**
S100A9	2628 (1508–4569)	568 (450–1349)	***	0.816	1.18	0.29
MPO	41,121 (31,004–55,724)	20,150 (15,294–26,418)	**	0.838	0.26	1.00
Fas-ligand	36 (26–53)	36 (23–47)	NS	–	0.40	1.00
Thrombomodulin	12,731 (9028–16,146)	5764 (4919–7030)	***	0.908	1.87	0.06
Galectin-3	3318 (2771–3956)	2524 (2208–3169)	NS	–	0.53	0.99
Collagen IV α1	2549 (1632–3745)	940 (610–1247)	***	0.902	078	0.73
Collagen I α1	9552 (7104–19,088)	6457 (4605–10,049)	NS	–	0.55	0.98
Resistin	55,062 (40,589–64,903)	19,083 (14,410–39,174)	***	0.818	0.27	1.00
Leucocytes[e]	14.3 (8.1–20.6)	9.6 (7.1–12.5)	NS	–	0.52	1.00
CRP[f]	272 (191–361)	54 (36–151)	***	0.814	1.52	0.21

Abbreviation: NSTIs: Necrotizing soft tissue infections. Unit of measurement: pg/mL. Data are presented as median values with interquartile ranges (IQR). Bold face indicates significant findings in both Mann-Whitney *U* test, RF and AUC.

[a] Calculated after resampling as described in the methods section.

[b] Mann-Whitney U test *p*-values after Benjamini-Hochberg adjustment: * ≤0.05; ** ≤0.01; *** ≤0.005. NS: non-significant.

[c] AUC: Area under receiver operating curve. AUC values are presented exclusively for biomarkers with statistically significant differences obtained after Benjamini-Hochberg adjustment.

[d] RF: Random forest. *No. of trees: 100.000. Split: 6. Repetitions: 100. Accuracy: 92.7%. Age, gender and septic shock are clinical parameters included in the RF modelling.*

[e] Unit of measurement: $\times 10^9$/L. Missing six in the GAS cohort.

[f] Unit of measurement: mg/L. Missing five in the GAS cohort.

contrast, the concentration of MMP-9 was higher in the group without shock. The RF model, with adjustment for age and gender, identified eight independent relevant predictors of shock (high Gini index), with an associated AUC >0.80; IL-4, IL-6, IL-36β, CCL2, CXCL8, G-CSF, Pentraxin-3 and S100A8 (Table 3).

A significant positive correlation was seen between Sequential Organ Failure Assessment (SOFA) score at the day of admission and 30 out of 37 plasma mediators, but not CRP or leucocytes. Two mediators had a significant negative correlation (Supplementary Table 4).

No associations of severe outcome and mediators were detected by univariate analysis. In the RF model, four mediators (IL-6, IL-10, G-CSF and Collagen IV α1) were associated with severe outcome, all with an AUC value above 0.70 (Table 4).

3.4. Mediator levels by streptococcal etiology

There were no significant differences in mediator levels in NSTI caused by GAS compared to SD in univariate analysis. According to the RF model, however, three markers higher in GAS cases (CXCL10, *E*-selectin, and S100A9) differentiated the two etiologies, all with AUC >0.70 (Table 5). Restricting analysis to cases with septic shock generated similar results (Supplementary Table 5).

3.5. Identification of biomarker profiles related to clinical categories using unsupervised hierarchical clustering

To explore further the differential inflammatory profiles of the NSTI and cellulitis cohorts, we performed an unsupervised hierarchical

Table 3
Biomarker levels in streptococcal NSTIs with and without septic shock.

	Septic shock	Non-shock	Mann-Whitney U[a]	AUC [c]	RF[d]	
	$n = 64$	$n = 38$	p-value[b]		Gini index	p-value
Interleukins						
IL-1α	60 (53–70)	46 (39–50)	***	0.826	1.47	0.20
IL-1β	20 (14–34)	12 (7–17)	***	0.736	0.94	1.00
IL-2	1209 (822–1538)	583 (411–1017)	***	0.784	1.13	0.88
IL-4	250 (222–286)	182 (152–213)	***	**0.832**	2.40	**<0.01**
IL-6	2676 (586–37,017)	216 (86–558)	***	**0.829**	2.72	**<0.01**
IL-10	91 (58–166)	44 (18–67)	***	0.786	1.24	0.14
IL-12p70	218 (181–292)	131 (75–196)	***	0.776	1.21	0.72
IL-13	1562 (1303–1793)	1218 (955–1474)	***	0.739	0.80	1.00
IL-17A	42 (20–90)	19 (12–30)	***	0.731	1.25	0.91
IL-18	566 (397–1106)	546 (291–856)	NS	–	1.27	0.91
IL-22	127 (116–151)	101 (87–121)	***	0.777	1.03	0.98
IL-23	2726 (486–8356)	1460 (196–4961)	NS	–	0.85	1.00
IL-36β	19 (17–23)	15 (12–16)	***	**0.847**	2.28	**<0.01**
Chemokines						
CCL2	1325 (754–2914)	387 (211–594)	***	**0.847**	2.79	**0.02**
CCL4	951 (937–1040)	767 (699–835)	***	0.813	1.82	0.09
CCL5	6056 (2155–14,518)	7490 (3684–12,088)	NS	–	1.17	0.98
CXCL8	74 (38–493)	19 (12–31)	***	**0.806**	2.42	**0.04**
CXCL10	19,426 (1298–385,626)	981 (371–11,054)	***	0.726	1.24	0.88
Adhesion molecules						
E-selectin	156,013 (118576–214,019)	121,227 (71708–185,216)	NS	–	1.00	1.00
ICAM-1	737,901 (568,783–916,275)	616,286 (527,608–771,348)	NS	–	0.69	1.00
VCAM-1	49.9×10^5 (37.2×10^5–70.5×10^5)	39.4×10^5 (21.3×10^5–52.9×10^5)	NS	–	0.93	1.00
Matrix metalloproteases						
MMP-1	1961 (1366–4374)	1243 (856–1875)	*	0.682	0.91	1.00
MMP-8	45,030 (25367–73,789)	14,578 (8403–35,893)	***	0.730	1.20	0.92
MMP-9	4210 (2628–8410)	15,353 (5114–30,679)	*** [e]	0.748 [e]	1.64	0.38
Growth factors						
G-CSF	7544 (1989–60,986)	441 (269–1099)	***	**0.858**	3.82	**0.02**
Other						
C5/C5a	21,255 (13315–32,528)	22,401 (15613–34,755)	NS	–	1.46	0.77
Pentraxin-3	20,872 (10,810–30,902)	5735 (3163–12,991)	***	**0.837**	3.24	**<0.01**
TNFα	47 (29–72)	22 (17–30)	***	0.812	1.92	0.09
S100A8	1329 (939–2143)	707 (490–865)	***	**0.820**	3.85	**<0.01**
S100A9	3124 (1549–5714)	2151 (1477–3395)	NS	–	0.98	1.00
MPO	47,213 (36,078–61,287)	31,046 (24,733–40,880)	***	0.732	1.61	0.40
Fas-ligand	41 (31–70)	30 (20–38)	**	0.719	1.70	0.31
Thrombomodulin	13,813 (10,269–16,996)	11,424 (7955–13,081)	*	0.678	1.60	0.50
Galectin-3	3564 (2973–4189)	3058 (2606–3503)	NS	–	0.76	1.00
Collagen IV α1	3101 (2105–4597)	1846 (1365–2905)	***	0.736	1.09	0.99
Collagen I α1	10,861 (7826–23,185)	8030 (5302–13,384)	NS	–	1.00	1.00
Resistin	57,395 (45,002–67,308)	46,847 (31,006–60,136)	NS	–	0.63	1.00
Leucocytes [f]	11.9 (7.2–19.4)	16.5 (11.9–20.8)	NS	–	0.97	1.00
CRP [g]	284 (149–377)	270 (200–337)	NS	–	1.13	0.99

Unit of measurement: pg/mL. Data are presented as median values with interquartile ranges (IQR). Bold face indicates significant findings in both Mann-Whitney *U* test, RF and AUC.

[a] Calculated after resampling as described in methods.

[b] Mann-Whitney *U* test *p*-values after Benjamini-Hochberg adjustment: * ≤0.05; ** ≤0.01; *** ≤0.005. NS: non-significant.

[c] AUC: Area under receiver operating curve. AUC values are presented exclusively for biomarkers with statistically significant differences obtained after Benjamini-Hochberg adjustment.

[d] RF: Random forest. *No. of trees: 100.000. Split: 6. Repetitions: 100. Accuracy: 72.6%. Age and gender are clinical parameters included in the RF modelling.*

[e] Non-shock>Septic shock.

[f] Unit of measurement: $\times 10^9$/L. Missing six in the GAS cohort.

[g] Unit of measurement: mg/L. Missing five in the GAS cohort.

cluster analysis, including septic shock, severe outcome, and streptococcal etiology of NSTIs. Four main clusters were detected, as shown in Fig. 1. The clusters with high or intermediate mediator levels (denoted cluster 1a and 1b, respectively) included 87% (20/23) of the cases with severe outcome ($P = 0.002$), and 85% (57/67) of the cases with septic shock ($P < 0.001$). Cluster 1a comprised GAS cases only. Sixteen of the 19 non-operated cellulitis cases were grouped in cluster 2, showing, in general, low levels of mediators.

3.6. Network and connectivity analysis

Network connectivity analysis was applied to assess interactions and to identify key response nodes among the mediators. Differences in connectivity were evident within the GAS cohort, whereas few significant connections were detected in the SD cohort (Supplementary Table 6). In the mediator-mediator association networks, a similar pattern was seen. Several associations among mediators were retrieved for the GAS cohort, with distinctive connections and strong power of the connections, of which the connection between IL-17A and S100A9 in septic shock cases was most evident. In contrast, associations were generally weak in the SD cohort (Supplementary Fig. 1).

3.7. Biomarker responses after in vitro stimulation

In vitro stimulation of PBMC and human umbilical vein endothelial cells (HUVEC) with GAS and SD was performed to corroborate the host

Table 4
Biomarker levels in streptococcal NSTIs by outcome.

	Severe outcome [a]	Non-severe outcome	Mann-Whitney U[b]	AUC [d]	RF[e]	
	$n = 16$	$n = 65$	p-value[c]		Gini index	p-value
Interleukins						
IL-1α	65 (54–70)	53 (46–60)	NS	–	0.55	0.10
IL-1β	26 (15–40)	17 (11–24)	NS	–	0.28	0.96
IL-2	1099 (741–1441)	1038 (561–1253)	NS	–	0.33	0.85
IL-4	231 (217–286)	220 (179–261)	NS	–	0.29	0.94
IL-6	9885 (2599–66,661)	506 (168–1947)	NS	**0.795**	0.97	**0.04**
IL-10	127 (62–312)	63 (40–90)	NS	**0.702**	0.94	**0.03**
IL-12p70	250 (206–329)	182 (127–231)	NS	–	0.52	0.31
IL-13	1575 (1282–1837)	1409 (1154–1626)	NS	–	0.30	0.91
IL-17A	33 (17–167)	27 (17–61)	NS	–	0.51	0.37
IL-18	561 (387–1473)	593 (386–800)	NS	–	0.29	0.94
IL-22	134 (112–196)	120 (101–134)	NS	–	0.52	0.28
IL-23	2972 (101–6935)	1886 (519–8376)	NS	–	0.30	0.96
IL-36β	19 (16–23)	17 (15–20)	NS	–	0.26	0.96
Chemokines						
CCL2	988 (612–2556)	775 (343–1791)	NS	–	0.40	0.66
CCL4	913 (837–1033)	835 (760–955)	NS	–	0.34	0.76
CCL5	2906 (1430–10,603)	6861 (2892–12,481)	NS	–	0.84	0.10
CXCL8	230 (64–643)	33 (16–81)	NS	–	0.61	0.26
CXCL10	4355 (893–222,004)	6878 (824–383,300)	NS	–	0.35	0.74
Adhesion molecules						
E-selectin	120,734 (80,256–193,336)	143,440 (103,933–187,719)	NS	–	0.44	0.60
ICAM-1	748,585 (507,738–1,002,047)	654,278 (544,169–863,507)	NS	–	0.56	0.31
VCAM-1	5.2×10^6 (4.6×10^6–7.7×10^6)	4.1×10^6 (3.2×10^6–6.3×10^6)	NS	–	0.47	0.58
Matrix metalloproteases						
MMP-1	2518 (1772–7568)	1405 (937–2736)	NS	–	0.38	0.79
MMP-8	51,096 (23,720–75,962)	24,620 (11,130–52,946)	NS	–	0.66	0.26
MMP-9	3217 (2320–10,830)	5701 (3844–21,384)	NS	–	0.43	0.68
Growth factors						
G-CSF	26,117 (3279–421,222)	1465 (430–8386)	NS	**0.747**	0.97	**0.03**
Other						
C5/C5a	17,937 (12,831–2247)	22,579 (14,177–37,416)	NS	–	0.52	0.51
Pentraxin-3	20,679 (11,514–25,231)	12,991 (5330–27,959)	NS	–	0.30	0.98
TNFα	36 (28–84)	29 (21–48)	NS	–	0.30	0.92
S100A8	1849 (1020–2720)	915 (703–1341)	NS	–	0.57	0.19
S100A9	1795 (1084–4787)	2616 (1477–4213)	NS	–	0.65	0.23
MPO	55,629 (38,269–80,820)	37,231 (29,011–53,639)	NS	–	0.60	0.32
Fas-ligand	34 (25–87)	38 (27–52)	NS	–	0.68	0.25
Thrombomodulin	13,813 (10,301–18,267)	12,407 (9102–15,802)	NS	–	0.28	0.94
Galectin-3	3580 (3259–4123)	2370 (2628–3664)	NS	–	0.32	0.94
Collagen IV α1	4131 (2243–6203)	2347 (1554–3300)	NS	**0.734**	1.37	**<0.01**
Collagen I α1	9341 (6926–19,013)	9130 (7160–17,516)	NS	–	0.83	0.17
Resistin	57,395 (49,644–67,740)	54,208 (31,893–63,171)	NS	–	0.29	0.97
Leucocytes [f]	9.3 (6.6–23.7)	13.9 (7.7–19.3)	NS	–	0.55	0.40
CRP [g]	168 (110–309)	271 (192–364)	NS	–	0.46	0.66

Unit of measurement: pg/mL. Data are presented as median values with interquartile ranges (IQR). Bold face indicates significant finding.

[a] Severe outcome: Death or amputation within 90 days. Only cases with NSTIs affecting extremities were included. Those amputated before arrival at study hospital were excluded. *Amputation* defined as the surgical removal of all or part of a limb or extremity.

[b] Calculated after resampling as described in methods section.

[c] Mann-Whitney U test p-values after Benjamini-Hochberg adjustment: * ≤0.05; ** ≤0.01; *** ≤0.005. NS: non-significant.

[d] AUC: Area under receiver operating curve. AUC values are presented exclusively for biomarkers with statistically significant differences obtained after Benjamini-Hochberg adjustment or RF modelling.

[e] RF: Random forest. *No. of trees: 100.000. Split: 6. Repetitions: 100. Accuracy: 79.7%. Age, gender and septic shock, hyperbaric oxygen treatment (HBOT) and intravenous immunoglobulin (IVIG) treatment are clinical parameters included in the RF modelling.*

[f] Unit of measurement: $\times 10^9$/L. Missing three.

[g] Unit of measurement: mg/L. Missing two.

responses measured in plasma (Fig. 2). Overall GAS triggered a higher IFNγ, TNFα, and IL-1β response in PBMC as compared to SD. Similarly, when exposing HUVECs to the stimulated PBMC supernatants, the highest release of CXCL10 and *E*-selectin responses were generally observed following stimulation with supernatants of PBMCs exposed to GAS. In contrast, stimulation with heat-killed SD bacteria induced a higher IL-1β and E-selectin response as compared to heat-killed GAS, but not for S100A9, which was higher in GAS.

4. Discussion

This study demonstrates a profound immune activation in NSTI caused by β-hemolytic streptococci and the way it differs from non-necrotizing infections with the same pathogens. We have identified several single mediators and broader host response profiles associated to type of infections, streptococcal species, disease severity and outcome. The findings illustrate that different immunological pathways and pathogenic processes are involved or predominate along the spectrum of moderate and severe streptococcal SSTIs. The different patterns observed in our study can be exploited to advance the development of much needed new diagnostic tools to aid clinical decisions and management.

In NSTI, early recognition, surgical debridement and appropriate antimicrobial therapy contribute to reduce mortality and improve

Table 5
Biomarker levels in monomicrobial NSTIs caused by either GAS or SD.

	GAS	SD	Mann-Whitney U[a]	AUC [c]	RF[d]	
	n = 88	n = 14	*p*-value[b]		Gini index	*p*-value
Interleukins						
IL-1α	55 (46–66)	49 (46–60)	NS	–	0.98	0.80
IL-1β	17 (12–29)	18 (9–29)	NS	–	1.14	0.69
IL-2	1018 (557–1395)	758 (585–1211)	NS	–	1.22	0.63
IL-4	227 (183–270)	223 (184–231)	NS	–	0.94	0.89
IL-6	702 (184–3570)	4603 (413–20,540)	NS	–	1.43	0.29
IL-10	74 (39–135)	60 (44–69)	NS	–	1.20	0.66
IL-12p70	199 (129–273)	183 (121–246)	NS	–	0.91	0.96
IL-13	1489 (1172–1729)	1282 (1016–1498)	NS	–	0.85	1.00
IL-17A	31 (17–74)	18 (11−30)	NS	–	1.17	0.81
IL-18	594 (384–1031)	423 (344–585)	NS	–	1.13	0.89
IL-22	123 (100–144)	112 (102–117)	NS	–	1.04	0.85
IL-23	2316 (525–8612)	508 (15–2521)	NS	–	1.68	0.40
IL-36β	18 (15–21)	16 (15–19)	NS	–	1.00	0.76
Chemokines						
CCL2	917 (405–1962)	671 (181–2070)	NS	–	1.29	0.47
CCL4	868 (761–1007)	890 (765–914)	NS	–	1.76	0.06
CCL5	6766 (2918–15,495)	2516 (1162–9006)	NS	–	1.18	0.81
CXCL8	38 (18–202)	101 (40–683)	NS	–	1.56	0.21
CXCL10	9616 (784–383,497)	893 (149–1508)	NS	**0.726**	4.19	**0.01**
Adhesion molecules						
E-selectin	150,022 (116,004–212,739)	93,763 (61,876–108,329)	NS	**0.742**	2.18	**0.04**
ICAM-1	692,105 (569,574–883,187)	507,738 (395,507–833,182)	NS	–	2.13	0.06
VCAM-1	4.2×10^6 (3.3×10^6–6.3×10^6)	4.6×10^6 (3.5×10^6–6.3×10^6)	NS	–	0.74	1.00
Matrix metalloproteases						
MMP-1	1566 (1067–3167)	1637 (665–2437)	NS	–	1.08	0.85
MMP-8	33,640 (12481–65,414)	22,312 (9384–38,223)	NS	–	1.00	0.93
MMP-9	5608 (3349–15,353)	4420 (1446–23,091)	NS	–	2.28	0.11
Growth factors						
G-CSF	1830 (431–16,675)	11,025 (892–39,393)	NS	–	0.93	0.96
Other						
C5/C5a	23,522 (15,547–34,128)	14,136 (11,511–21,060)	NS	–	2.76	0.08
Pentraxin-3	17,157 (6336–28,517)	11,873 (6549–19,059)	NS	–	0.95	0.99
TNFα	35 (22–61)	29 (26–37)	NS	–	0.96	0.96
S100A8	1014 (647–1803)	1037 (711–1758)	NS	–	1.02	0.83
S100A9	3059 (1685–5450)	1075 (671–1776)	NS	**0.834**	2.71	**0.02**
MPO	42,537 (32,105–57,588)	34,100 (27,343–50,148)	NS	–	1.38	0.47
Fas-ligand	36 (27–55)	32 (20–41)	NS	–	1.18	0.75
Thrombomodulin	12,972 (8845–16,336)	12,014 (9217–13,659)	NS	–	1.11	0.91
Galectin-3	3318 (2770–4011)	3259 (2888–3647)	NS	–	0.93	0.98
Collagen IV α1	2604 (1823–4188)	2051 (1599–3353)	NS	–	0.67	1.00
Collagen I α1	10,052 (7496–23,772)	6863 (3586–14,472)	NS	–	1.43	0.61
Resistin	55,283 (42,589–65,419)	48,427 (31,788–67,177)	NS	–	1.19	0.81
Leucocytes [e]	14.2 (8.2–20.4)	15.7 (6.6–23.7)	NS	–	1.53	0.31
CRP [f]	296 (200–368)	148 (66–261)	NS	–	2.44	0.08

Abbreviations: GAS: *Streptococcus pyogenes* (Group A streptococcus), SD: *S. dysgalactiae*. Unit of measurement: pg/mL. Data are presented as median values with interquartile ranges (IQR). Bold face indicates significant finding.

[a] Calculated after resampling as described in methods.

[b] Mann-Whitney U test p-values after Benjamini-Hochberg adjustment: * ≤0.05; ** ≤0.01; *** ≤0.005. NS: non-significant.

[c] AUC: Area under receiver operating curve. AUC values are presented exclusively for biomarkers with statistically significant differences obtained after Benjamini-Hochberg adjustment or RF modelling.

[d] RF: Random forest. No. of trees: 100.000. Split: 6. Repetitions: 100. Accuracy: 81.5%. *Age, gender and septic shock are clinical parameters included in the RF modelling.*

[e] Unit of measurement: $\times 10^9$/L. Missing six in the GAS cohort.

[f] Unit of measurement: mg/L. Missing five in the GAS cohort.

patient outcomes [9,13]. It is therefore a great concern that misdiagnosing at admission is frequent [9]. Use of scorings systems like the LRINEC score [30], based on routine laboratory values, has turned out to be of limited value as adjuncts to clinical judgement [31,32]. In our study, however, several inflammatory mediators and mediator profiles showed promising diagnostic and prognostic accuracy, outperforming both CRP and leucocytes.

Data on use of biomarkers in the diagnosis of NSTIs are scarce [21,23,33]. Hansen et al. [20] studied cytokine responses in NSTIs, observing higher levels of IL-6 and TNFα in streptococcal compared to other NSTIs. A contemporary retrospective study, revealed IL-6 as the most accurate cytokine to distinguish NSTIs from severe SSTIs [33]. We have previously explored the pathogenesis in NSTIs in the INFECT cohort identifying differential host-pathogen-interactions by etiology [34,35]. Recently, in the same cohort we identified that thrombomodulin was a promising general marker for NSTI, whereas G-CSF, S100A8 and IL-6 were associated to septic shock [23]. The present study is restricted to GAS and SD etiology, and biomarker candidates that may be used for separating streptococcal NSTIs from streptococcal cellulitis are unraveled. Based on data herein, biomarker profiles (including e.g. IL-1β, CXCL8, TNFα and possibly thrombomodulin) could become valuable adjunctive tools to rapidly decipher severe (i.e. NSTI) from less severe (i.e. cellulitis) infections. Although thrombomodulin was not one of the mediators reaching significance in the RF model, as opposed to our former study, it displayed a very high AUC (0.91) and a low *p*-value ($P = 0.059$).

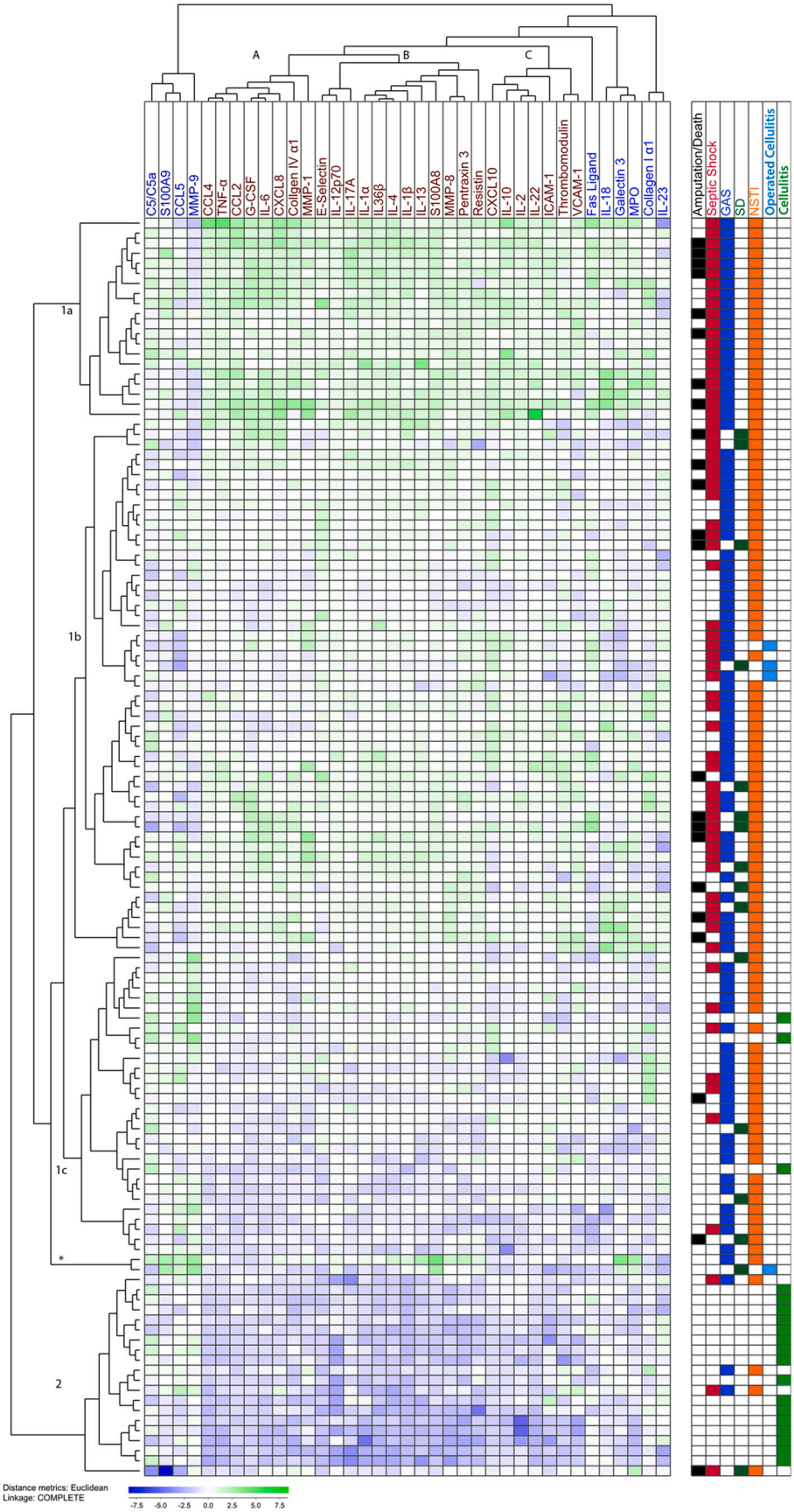

Fig. 1. Unsupervised hierarchical clustering analysis of plasma levels of 37 mediators in 102 NSTI patients and 23 cellulitis patients with streptococcal etiology. Euclidean distance and complete linkage were applied in the clustering analysis. The dendrogram on the top (biomarkers) and on the left side (cases) of the heat map form clusters. The threshold is set to midpoint of the longest branch.

Cluster 1a is made by cases with generally high levels of biomarkers, while cluster 1b represent cases with intermediately high values of biomarkers, cluster 1c represent neutral/low values and cluster 2 represents low levels of biomarkers. * constitutes a group of two cases, situated between cluster 1a and 1b/1c with respect to biomarker levels.

The biomarkers tested generates a pattern where most cytokines, chemokines and adhesion molecules form one main, middle cluster (marked red). This main cluster can be subdivided in the sub-clusters, A, B and C. Cluster A contains main pro-inflammatory cytokines (IL-6, TNF-α), main chemokines (CCL2, CCL4, CXCL8) and some others (G-CSF, Collagen IV α1, MMP-1). Cluster B contains pro-inflammatory cytokines (IL-1α, IL-1β, IL-12p70, IL-17A, IL-36 β), cytokines involved in adaptive immune responses (IL-4, IL-13) and E-selectin, MMP-8, Pentraxin-3, S100A8 and Resistin. Cluster C contains anti-inflammatory cytokines (IL-10, IL-22), IL-2 cytokine involved in the adaptive immune response, one chemokine (CXCL10), as well as adhesion molecules and endothelial markers (ICAM-1, VCAM-1, Thrombomodulin). Most mediators outside the main cluster (marked blue) are not cytokines, chemokines or adhesion molecules (C5/C5a, S100A9, MMP-9, Fas-ligand, Galectin 3, MPO, Collagen 1α1) except the cytokines IL-18, IL-23 and the chemokine CCL5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

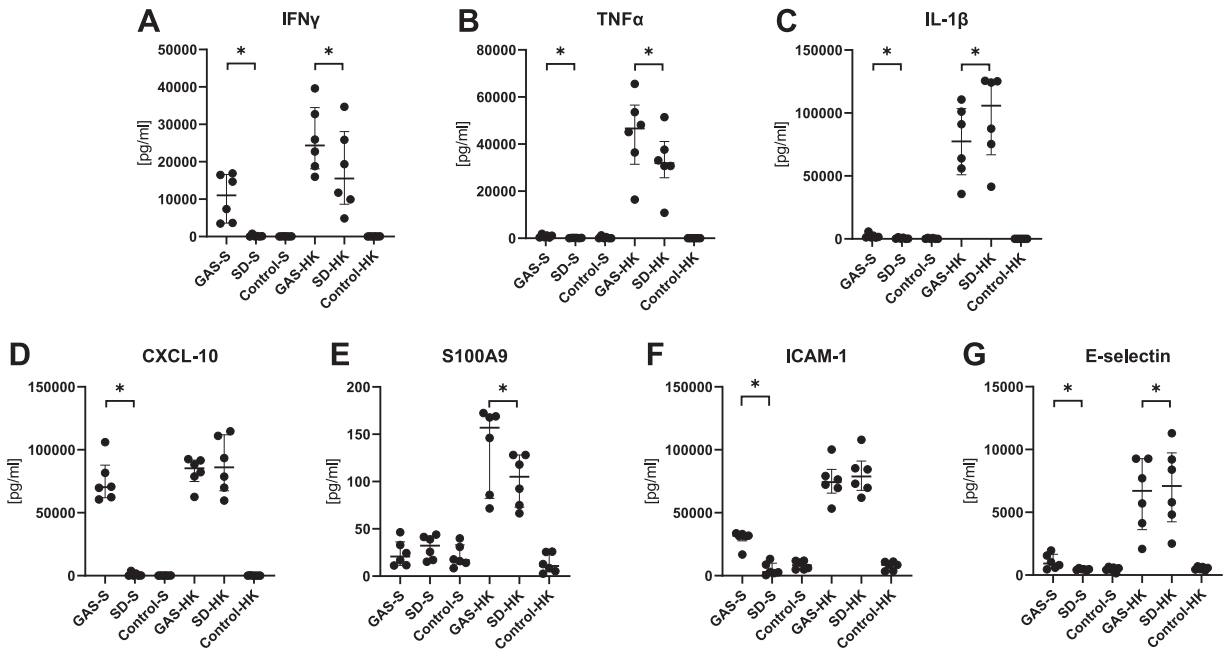

Fig. 2. Cell responses during in vitro stimulation with GAS and SD. Concentrations of selected analytes in cell culture media after stimulation of PBMC (A-C) or HUVEC (D-E). Isolated PBMCs were stimulated with bacterial supernatant (S) or heat-killed bacteria (HK) for 24 h. The supernatants of the PBMC stimulations were then used for stimulation of HUVEC cells for another 24 h. Bacterial culture media and PBS were used as negative controls for supernatant and heat-killed bacteria stimulations, respectively. Stars indicate significance (p-value<0.05).

Distinguishing GAS from SD NSTIs based on clinical findings, is difficult, and of limited clinical importance. Of the three mediators displaying discriminant ability in our study, CXCL10 appear as a central chemokine in GAS NSTIs, as previously published [23,34], and merits further investigation.

Broad biomarker profiles may also be useful additional tools for risk stratification and prognostic evaluation. In the hierarchical cluster analysis, we found that 87% of the patients with a severe outcome, and 85% of those with septic shock clustered in two groups with similar profiles. However, the method is descriptive, complex and not feasible to apply in every-day-clinical practice. Nevertheless, hierarchical cluster analysis may contribute to creation of novel hypotheses. Multiplex profiling or use of combinations of biomarkers offers advantages compared to single biomarkers, as it can portray the concomitant pro- and anti-inflammatory pattern expressed by the patient [17,36]. The powerful pro-inflammatory response in streptococcal NSTI, especially when eliciting STSS, is probably a main reason for mortality [37,38]. In two studies of NSTIs of all etiologies, both pro-inflammatory (IL-1β, IL-6, G-CSF and TNFα) and anti-inflammatory (IL-10) cytokines were associated with severity, mortality or amputation [20,39]. Bulger and co-workers showed that higher levels of plasma chemokines and cytokines (TNFα, IL-6, CXCL8, and CCL2) were correlated to poorer clinical outcome [40]. In our study, we found that IL-6, along with IL-10, G-CSF and Collagen IV α1 could predict severe outcome in streptococcal NSTI patients, all four displaying biomarker potential (AUC >0.70). It is crucial that such profiles are used in conjunction with risk assessment involving clinical factors that are highly associated to outcome, such as underlying comorbidities, the extent of the infection, and the severity of organ dysfunction.

In streptococcal disease, bacterial toxins, but also immune cells and other host factors contribute to tissue damage and systemic inflammatory reactions [38,41,42]. In GAS NSTI, STSS is frequent and $>$ 60% in our GAS NSTIs cohort had septic shock [5,18]. Key mediators of STSS are the superantigens [19,43], which activate T cells in an unconventional manner resulting in a massive cytokine response, including release of IL-6, CXCL8, and CCL2, which all were independent markers of shock in our study [38,44]. At-present, 13 superantigens have been identified in GAS [38,43]. In contrast, *SpeG* is currently the only superantigen gene identified in a substantial number of SD isolates [43], and its activity and involvement in toxic shock is unclear. Although the profound immune activation observed in the present study may reflect a major role for superantigens in the systemic response to streptococcal NSTIs, it is unclear to what extent the differences observed between GAS and SD infections are due to superantigen activity. Previous findings have demonstrated that the local cytokine response in severe SSTIs caused by GAS resembles that of a systemic superantigen induced response [19], but the role for superantigens at the tissue level in less severe infections, like cellulitis, is not clear.

Notably, IL-1β levels were elevated in NSTIs compared to cellulitis. This pro-inflammatory cytokine has been inferred as a key mediator facilitating GAS NSTIs [38,45].

Network analyses revealed strong associations between several mediators in NSTIs caused by GAS. The connections may reflect the predominant role of certain virulence factors, including superantigens, in activating pathways. In contrast, the absence of strong connectivity in SD NSTI networks may reflect lack of superantigen activity. Also, it could reflect the diversity of this population, where comorbidities and age may have a greater impact on the host responses observed. Of note, this result may be influenced by the low number of SD cases. Nevertheless, the in vitro stimulation experiments suggest that also SD has the ability to induce a broad and strong activation of the immune system.

Together with S100A8, S100A9 constitutes the heterodimer calprotectin, a well-known damage-associated molecular patterns (DAMPs) molecule, highly concentrated in e.g. phagocytes [46]. It has been shown that IL-17A can induce S100A8 and S100A9 expression in keratinocytes [47]. Furthermore, the complex S100A8/100A9 is a known endogenous activator of Toll-like receptor 4, subsequently promoting endotoxin-induced septic shock [48], and has been associated with increased risk of mortality in septic shock patients [49]. In our study, the powerful association of S100A9 and IL-17A in GAS septic shock cases suggests that the same pathway may also be involved in gram-positive sepsis.

A main limitation of this study is the low number of severe cellulitis cases in the control group, but resampling was applied to overcome the

uneven numbers of patients in the different groups. Moreover, the profound differences seen between NSTI and cellulitis cases would mitigate the under-powered situation of the comparison. Collection of plasma was done in a standardized fashion, but only at the study hospitals, and not at admittance to the primary hospital (for the NSTI cohort). However, for the referred patients, median time from admission at primary hospital to admission at study hospital was not more than14 and 18 h, (for SD and GAS cases, respectively) [5].

Major strengths of this study include a predefined study protocol, and the multicenter prospective patient enrollment, contributing to inclusion of a homogenous patient cohort. This cohort of cases caused by GAS and SD is the largest to date. In addition, this study included comparable control cohorts. The statistical methods applied decreased the likelihood of committing type 1 errors. These strengths made it possible to identify robust associations pointing at specific immunological pathways and biomarkers in SSTIs of streptococcal etiology. The study therefore also adds to the identification of candidate targets for personalized therapy in streptococcal NSTI.

In summary, this prospective study of streptococcal NSTIs compared with cellulitis cases, identified systemic inflammatory mediators significantly associated to type of infection as well as severity and prognosis, both single mediators and wider profiles. The study also highlights interactions and patterns of immune activation that may direct search for future targets for therapy in NSTIs.

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Author contribution

A.N.T. is project coordinator of the INFECT study. E.R., T.B. and S.S. conceived the biomarker study. O.H., S.S. and M.N. are national investigators and have contributed to study design and coordinated study conduct. M.B.M. is responsible for the database and contributed to patient inclusion and data collection, as did E.R., T.B., S.S., O.O., T.N., N. H., M.N. and O.H. E.R., L.M.P.M., S.J., T.B., S.S., K.A.M., J.K.D., E.S., V. A.P.M.d.S., M.S., and A.N.T. contributed to analysis or interpretation of data or both. E.R. drafted the publication. All authors contributed to the writing and approved the final version.

Declaration of Competing Interest

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and no potential conflicts are reported.

Some of the data were presented at a lecture at the **21st Lancefield International Symposium for Streptococci and Streptococcal Diseases 2022**, 7th - 10th June.

Data availability

The entire raw data with and without imputed values are available at DOI: 10.5061/dryad.f1vhhmgw4.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2023.109276.

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