Center for Infectious Medicine,
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STREPTOCOCCUS PYOGENES INFECTIONS AND TOXIC SHOCK SYNDROME

– MOLECULAR EPIDEMIOLOGY AND IMMUNOTHERAPY

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Cover photograph: gram-staining of group A streptococci.

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ABSTRACT

Streptococcus pyogenes, also known as group A streptococcus (GAS), is an important human pathogen causing a wide variety of diseases. One of the most severe diseases is streptococcal toxic shock syndrome (STSS), which is associated with high mortality rates. Toxic shock syndrome (TSS) may also be caused by *Staphylococcus aureus.* Superantigens have been identified as key mediators of STSS and staphylococcal TSS. Intravenous polyspecific immunglobulin (IVIG) has been suggested as adjunctive therapy in TSS, since it contains neutralising antibodies against streptococcal and staphylococcal superantigens, as well as bacterial opsonising antibodies.

To assess the safety and efficacy of IVIG as adjunctive therapy in STSS, we conducted a multicenter placebo-controlled trial (paper I). The trial was prematurely terminated due to a low incidence of disease in the participating countries. Results were obtained from 21 enrolled patients; 10 of whom received IVIG and 11 placebo. The primary objective was mortality over 28 days, and a trend to decreased mortality was observed in the IVIG group, 10% versus 36% in the placebo group. A significant decrease in sepsis-related organ failure assessment (SOFA) score was noted in the IVIG-group, whereas no change was seen in the placebo group. The IVIG cases obtained a significantly increased plasma superantigen-neutralising activity against their own isolate following IVIG administration, whereas no change could be noted among patients in the placebo group.

In paper II we tested whether superantigen-containing culture supernatants from streptococcal and staphylococcal severe sepsis isolates were inhibited to an equal extent by IVIG. Three different IVIG preparations were tested and found to be highly efficient in neutralising the superantigens. Most supernatants were completely inhibited at concentrations between 0.5 - 2.5 mg IVIG/ml. Importantly, culture supernatants from *S. pyogenes* isolates were consistently inhibited to a higher extent as compared to those of *S. aureus* isolates.

In paper III and IV, results from active surveillance of invasive GAS infections in Denmark and Sweden during 2001-02 and 2002-04, respectively, are described. The yearly incidences were similar, 2.0-3.4 /100 0000 inhabitants, as was the prevalence of the severe manifestations STSS and necrotising fasciitis (NF), which were seen in approximately 10% of the cases. However, differences were observed in outcome with a mortality rate of 25% and 14.5% in Denmark and Sweden, respectively. Also the GAS type distribution varied between the two studies. *emm*1 was the most prevalent type (32%) in the Danish study in comparison to the new types *emm*89 (16%) and 81 (14%) that dominated in the Swedish study. Non-invasive GAS isolates were collected and analysed in parallel with the invasive in both studies, and the type distribution differed significantly from the invasive isolates. Differences in presence of superantigen genes were seen between isolates of different *emm*-types and also between invasive and non-invasive isolates. A combination of PFGE-analysis and superantigen profiling revealed subclones within the *emm*-types with higher invasiveness than others (paper IV). Further, IVIG treatment in patients with STSS was significantly associated with improved outcome. 20 out of 72 patients with STSS were given IVIG, with a mortality of 15% as compared to 48% among patients not receiving IVIG (paper IV)

This thesis provides further support of IVIG therapy in STSS, and the *in vitro* analyses revealed that a higher dose of IVIG may be needed in staphylococcal TSS in order to achieve protective antibody levels. The thesis also provides new insights in the molecular epidemiology of invasive GAS disease.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Darenberg J**, Ihendyane N, Sjölin J, Aufwerber E, Haidl S, Follin P, Andersson J, Norrby-Teglund A and the StreptIg Study Group. Intravenous immunoglobulin G therapy in streptococcal toxic shock syndrome: a European randomized, double-blind, placebo-controlled trial. Clinical Infectious Diseases 2003, 37:333-40.

II. **Darenberg J**, Söderquist B, Henriques Normark B, and Norrby-Teglund A. Differences in potency of intravenous polyspecific immunoglobulin G against streptococcal and staphylococcal superantigens: Implications for therapy of toxic shock syndrome. Clinical Infectious Diseases 2004; 38:836-42

III. Ekelund K, **Darenberg J**, Norrby-Teglund A, Hoffman S, Bang D, Skinhøj P, Konradsen HB. Variations in *emm* type among group A streptococcal isolates causing invasive or noninvasive infections in a nationwide study. Journal of Clinical Microbiology 2005; 43:3101-9.

IV. **Darenberg J**, Luca B, Jasir A, Sandgren B, Schalén C, Norgren M, Romanus V, Norrby-Teglund A, Henriques Normark B. Molecular and clinical characteristics of invasive Group A streptococcal infections in Sweden. Manuscript

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
APSGN	Acute post-streptococcal glomerulonephritis
CA-MRSA	Community acquired methicillin resistant *S. aureus*
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
CpG	Cytidine-phosphate-guanosine
Efb	Extracellular fibrinogen binding protein
GAS	Group A Streptococci
GRAB	G-related α2-macroglobulin binding protein
HMGB-1	High mobility group 1 box protein
IL	Interleukin
IVIG	Intravenous polyspecific immunglobulin G
LPS	Lipopolysaccaride
LTA	Lipoteichoic acid
MHC	Major histocompability complex
MLST	Multi locus sequence typing
MODS	Multiorgan dysfunction syndrome
MRSA	Methicillin resistant *S. aureus*
NF	Necrotising fasciitis
NT	Non-typable
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PBP	penicillin binding protein
PepG	Peptidoglycan
PFGE	Pulse field gel electrophoresis
PV	Panton-Valentine
RHD	Rheumatic heart disease
SAg	Superantigen
SAPS	Simplified acute physiology score
SCIN	Staphylococcal compliment inhibitor
SE	Staphylococcal enterotoxin
SOF	Serum opacity factor
SOFA	Sepsis-related organ failure assessment
Spe	Streptococcal pyrogenic exotoxin
SSA	Streptococcal superantigen
STSS	Streptococcal toxic shock syndrome
TAFI	Thrombin-activable fibrinolysis inhibitor
TcR	T-cell receptor
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1

1 INTRODUCTION

1.1 GENERAL ASPECTS ON SEPSIS

Sepsis and its complications are of major clinical importance since they are associated with significant morbidity and mortality. Sepsis ranges in severity from mild systemic inflammation without significant clinical consequences to multisystem failure in septic shock associated with high mortality rates (table 1). The overall mortality in sepsis has been calculated to be around 30% (1) and increases with severity, with mortality rates of 40-80% in patients with septic shock (2, 3).

Table 1. Sepsis and related syndromes

Diagnosis	Definition
Bacteraemia	Infection of the blood stream or other normally sterile site
Sepsis	Bacteraemia + clinical symptoms of systemic infection
Severe sepsis	Sepsis + multiorgan dysfunction syndrome (MODS)
Septic shock	Severe sepsis + hypotension

According to definitions in (4).

Despite improvements in medical care, the incidence of sepsis is progressively increasing in developing countries (2, 5, 6, 7, 8). An epidemiological study of over 10 million patients with sepsis in the US between 1979 and 2000 showed an 8.7% increased incidence of sepsis (from 82.7 to 240.2 per 100 000 population), and both fungal and gram-positive bacteria as causative pathogens increased over time (2). Several studies have noted a shift in epidemiology of sepsis in developed countries, going from a dominance of gram-negative to gram-positive bacterial pathogens (2, 9). This change may be attributed to improved medical technology, expanded use of invasive devices, and increased numbers of patients at risk to develop sepsis, such as immunocompromised patients and elderly. Also improved therapies against gram-negative pathogens and the fact that antibiotic resistance in gram-positive pathogens has increased may also in part explain this shift in epidemiology (2, 10, 11).

One of the most severe manifestations of sepsis is streptococcal toxic shock syndrome (STSS) caused by the gram-positive bacterium, group A streptococcus (GAS). STSS is characterised by hypotension and multiorgan failure early in the course of infection, and may affect young previously healthy individuals. The fatality rate of STSS commonly exceeds 50% (12-14). Toxic shock syndrome (TSS) may also be caused by gram-positive *Staphylococcus aureus*, and can then present as either menstrual or non-menstrual TSS. Staphylococcal TSS is usually associated with lower mortality, ranging from 3-5% (15). Diagnostic criteria for staphylococcal TSS and STSS are summarised in table 2.

Table 2. Diagnostic criteria for staphylococcal and streptococcal toxic shock syndrome

Staphylococcal toxic shock syndrome[a]	Streptococcal toxic shock syndrome[b]
1. Fever 2. Hypotension 3. Diffuse macular rash with subsequent desquamation 4. ≥ 3 of the following organ systems involved: i. Liver ii. Blood iii. Renal iv. Mucous membranes v. Gastrointestinal vi. Muscular vii. Central nervous system 5. Negative serologies for measles, leptospirosis, and Rocky Mountain spotted fever, as well as negative blood or cerebral spinal fluid cultures for organisms other than *S. aureus*	1. Isolation of group A streptococci from a i. Normally sterile site → definite case ii. Non-sterile site → probable case 2. Hypotension 3. ≥ 2 of the following signs: i. Renal impairment ii. Liver involvement iii. Generalised erythamatous macular rash that may desquamate iv. Coagulopathy v. Soft tissue necrosis vi. Adult respiratory distress syndrome

[a] Diagnostic criteria as defined in (16). Additionally, proposed revision of diagnostic criteria for TSS includes (*a*) isolation of *S. aureus* from a mucosal or normally sterile site, (*b*) production of TSS-associated superantigen by the infecting isolate, (*c*) lack of antibodies to the implicated toxin at the time of acute illness, and (*d*) development of antibodies to the toxin during convalescence (17).

[b] Diagnostic criteria as defined in (18)

Table partly adapted from (15).

1.2 PATHOPHYSIOLOGY OF SEPSIS

Inflammation is the body's normal response to infection. The initial response to an infection involves a pro-inflammatory state with release of mediators such as tumor necrosis factor alpha (TNFα), interleukin 1 and 6 (IL-1, IL-6) and platelet activating factor (PAF). These mediators have multiple overlapping effects designed to induce appropriate responses that can control the infection and repair existing, and limit new, damage. To ensure that the effects of the pro-inflammatory mediators do not become destructive, the body launches compensatory mediators, such as IL-4 and IL-10, which normally down-regulate the initial pro-inflammatory state.

In microbial sepsis and septic shock, the regulation of the early response to infection is lost, due to microbial products that induce a generalised activation of the host defence systems including the inflammatory response, complement, coagulation and fibrinolytic systems. This activation leads to loss of homeostasis of several important host pathways, and a hyper-inflammatory and coagulatory state (19, 20) (figure 1).

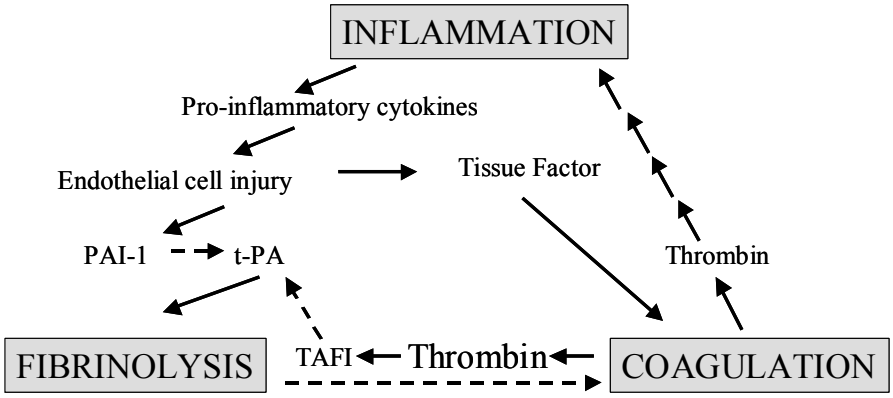


Figure 1. *Inflammation, coagulation and fibrinolysis are intimately linked and directly influenced by the septic process. Pro-inflammatory mediators activate the coagulation through induction of tissue factor, which in turn results in production of thrombin and subsequent formation of fibrin clot formation. Tissue-plasminogen activator (t-PA) triggers the conversion of plasminogen to plasmin, the main effector molecule of fibrinolysis. Pro-inflammatory cytokines and thrombin stimulates release of plasminogen activator inhibitor (PAI-1) from platelets and the endothelium. Thrombin also activates thrombin-activatable fibrinolysis inhibitor (TAFI). Fibrinolysis, which is the body's normal response to remove formed clots, is inhibited by TAFI and PAI-1 and fibrinolysis is therefore effectively suppressed during sepsis. Plain and dashed arrows indicate activation and inhibition, respectively.*

Several microbial factors can activate these hyper-inflammatory responses. In gram-negative sepsis, the main causative factor is the lipopolysacharide (LPS), a major cell wall constituent of gram-negative bacteria, which through interaction with the Toll-like receptor (TLR) 4 induce potent pro-inflammatory responses. In gram-positive sepsis, the implicated microbial products include the cell wall components, peptidoglycan and lipoteichoic acid which also interact with TLRs, as well as secreted factors, including the potent superantigens, discussed in more detail below (21) (figure 2). A consequence of this microbial induced activation of the pro-inflammatory and pro-coagulatory cascades is excessive release of pro-inflammatory and hypotensive molecules, such as cytokines, nitric oxide, endothelins, tissue damaging proteinases, lipid mediators, kinins and nitric oxide, which are all central mediators of the systemic effects seen in severe sepsis and septic shock, i.e. circulatory collapse and organ failure (22, 23). The classical sepsis-associated cytokines include IL-1, IL6, IL-8, IL-12, TNFα and beta, and interferon gamma (IFNγ). Late mediators of sepsis include high mobility group 1 box protein (HMGB-1) (24, 25) and macrophage migration inhibitor factor (MIF) (26) (figure 2).

This improved knowledge of the pathophysiology of sepsis generated a lot of interest in the use of immunotherapy in sepsis. Following years of disappointment in clinical trials evaluating mostly anti-cytokine therapies in sepsis, recent trials have reported improved outcome in severe sepsis or septic shock with activated protein C, (27), tight glycemic control (28), early goal-directed therapy, including enhanced fluid resuscitation, transfusion and vasopressor therapy (29), as well as low dose corticosteroids (30). The absolute reduction in mortality ranged from 4.6 to 16% in these studies, with the most significant improvement reported for the early-goal treatment.

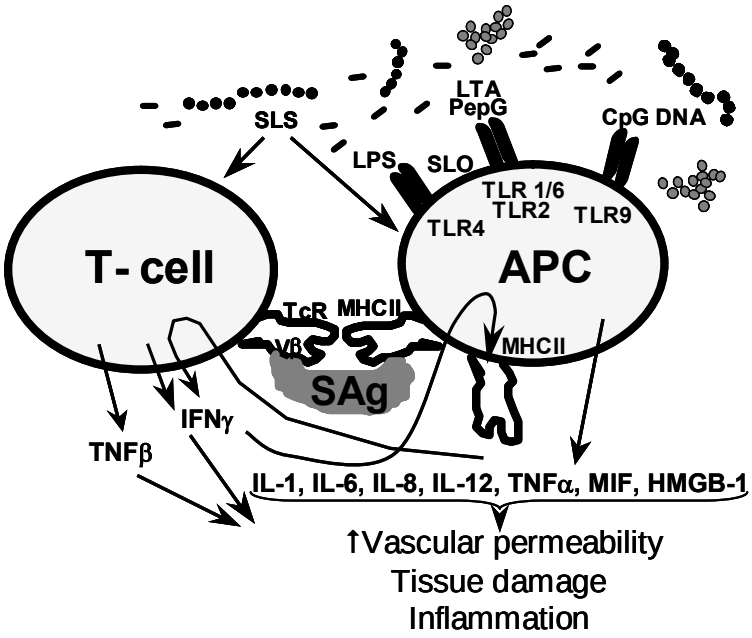


Figure 2. *Induction of pro-inflammatory responses by microbial virulence factors. Lipopolysaccaride (LPS), produced by gram-negative bacteria, and lipoteichoic acid (LTA) peptidoglycan (PepG), and unmethylated cytidine-phosphate-guanosine (CpG) DNA, from gram-positive bacteria, activate antigen-presenting cells (APC) through Toll-like receptors (TLRs). Superantigens (SAg), binds to the major histocompability complex (MHC) class II molecules on APC and the Vβ chain on the T-cell receptor (TcR) on T-cells, and activates both cell-types. The activated cells releases pro-inflammatory cytokines, which results in increased vascular permeability, causes tissue damage and inflammation and also affects other host defence systems such as the coagulation- and fibrinolytic systems.*

1.3 *STREPTOCOCCUS PYOGENES* INFECTIONS

Streptococcus pyogenes, also called group A streptococcus (GAS), is a strictly human pathogen that can cause a wide spectrum of diseases, ranging from mild upper respiratory tract and skin infections, to invasive life threatening conditions, as well as post-streptococcal sequelae. A recent review of population-based data demonstrated that GAS is an important cause of morbidity and mortality (table 3), as GAS was estimated to be the eight most common source of global mortality due to a single pathogen (31).

1.3.1 Infections and sequelae

The bacteria are spread through direct or indirect contact between individuals and the most common ways for the bacteria to get access to the human body is through the throat or pre-existing wounds or cuts of the skin. It has been estimated that about 2-6% of the population are asymptomatic carriers of GAS, most commonly in their respiratory tract (32, 33). These healthy carriers likely function as reservoirs of bacterial isolates capable to cause disease in other persons that are more susceptible.

GAS is the most common cause of tonsillopharyngitis, often seen in children and adolescents. Although this is an infection most commonly spread through person-to-person contact, there have been pharyngitis outbreaks of GAS of food-borne origins,

as reviewed in (34). Poor preparation and handling of the food, often by someone with skin lesions on their hands, seems to be a common trend for these outbreaks, which further also have shorter incubation periods and higher attack rates, as compared to droplet transmission.

The most common GAS infections of skin and soft tissue are impetigo (pyoderma), erysipelas and cellulitis. Impetigo is limited to the epidermis and characterised by clusters of small blisters that expands and ruptures within 24 hours. In erysipelas and cellulitis the bacteria have penetrated the epidermis, and these conditions are manifested by local signs of inflammation (pain, erythema and heat) and, in most cases, also by fever and leukocytosis. Erysipelas involves the superficial layers of the skin and cutaneous lymphatics. The the erythema in erysipelas is well demarcated from surrounding skin. Cellulitis, on the other hand, extends more deeply into the subcutaneous tissue and the demarcation between involved and non-involved is less distinct (35). Necrotising fasciitis (NF) is the most severe tissue infection caused by GAS and affects deep subcutaneous tissue and fascia. It is characterised by extensive progressive destruction of fascia and fat, and is often associated with marked systemic symptoms (36).

STSS is a severe form of GAS bacteremia and was first introduced in the mid 1980s (37) in response to a significant resurgence of severe invasive streptococcal infections (38). Beginning in the early 1980s, reports began to appear describing not only an increased mortality (35 - 48%) due to GAS bacteremia, but also a syndrome of toxic shock and multiorgan failure and in many cases rapidly progressive soft tissue infection. This clinical entity was described as STSS due to similarities in clinical features with staphylococcal TSS (table 2). STSS is commonly seen in association with skin- or soft-tissue infections, including NF. In a population-based surveillance of NF in Canada, 47% percent of the cases were associated with the presence of STSS, and those who met the criteria for STSS had a mortality of 67% as compared to 4.9% in those who did not (39).

GAS may also cause puerperal sepsis in pregnant or post-partum women. Historically, this used to be a severe and life threatening condition, accounting for up to two thirds of the deaths in post-partum mothers of 18th and late 19th centuries (40). Puerperal sepsis usually occurs between one or two days after delivery, but may also occur during pregnancy, where the infection is associated with a significant risk for both mother and child. The incidence of puerperal sepsis has decreased during recent years, mainly due to improved hygiene and awareness at the delivery wards. However, outbreaks of puerperal sepsis occasionally still occur, and asymptomatic carriers among the staff are often identified as plausible sources of these outbreaks (41, 42).

Streptococcal infections may also cause post-infectious complications, such as the non-suppurative diseases acute glomerulonephritis and acute rheumatic fever. These post-streptococcal sequalae are relatively uncommon in developed countries, although case reports or even outbreaks may occasionally occur, but they constitute a major health problem in developing countries (31) (table 3). Acute glomerulonephritis occurs primarily in children and young adults, usually seen one to

three weeks after GAS-associated skin or throat infection. The disease is characterised by acute inflammation of renal glomerulus with edema, hypertension, hematuria and urinary sediment abnormalities. Crowding, poor hygiene and poverty are factors associated with outbreaks of acute glomerulonephritis. Acute rheumatic fever is a multisystem disease, which manifest as an inflammation of the joints, heart, central nervous system, skin and subcutaneous nodules. It typically develops after a GAS throat infection. In its most severe form, acute rheumatic fever develops into rheumatic heart disease, a major cause of acquired heart disease. The reported minimum prevalence of rheumatic heart disease is estimated to 15.6 million cases with 282 000 new cases and 163 000 deaths each year (31) (table 3). The disease resembles autoimmune diseases, and most likely results in part from the production of autoreactive antibodies and T-cells shown to cross-react with components of the GAS and host tissues.

Table 3 Estimates of the frequency of GAS infections globally and in Sweden

Disease	Predominant region	Annual No. of cases	Case fatality rates (%)
Globally[a] *Superficial:*			
Pharyngitis	Global	616 million	--
Severe:			
Invasive	Global	663 000	25
RHD	Developing countries	282 000	1.5
APSGN	Developing countries	472 000	1
Sweden[b]			
Superficial:	Not applicable	300 000*	
Severe:			
Invasive	Not applicable	250-300	15.5
RHD	Not applicable	None reported	--
ASPGN	Not applicable	None reported	--

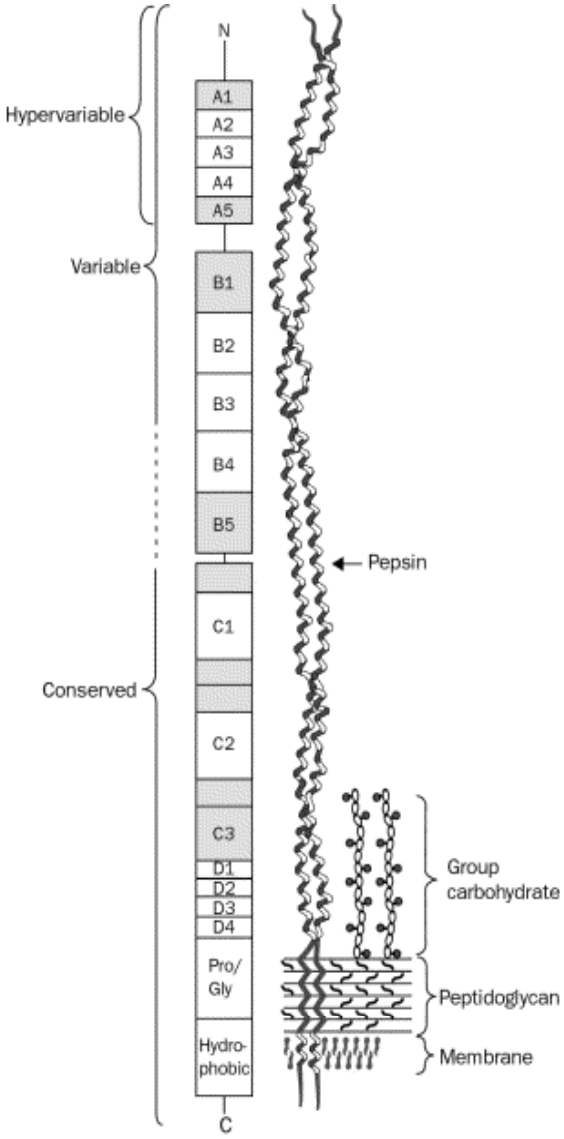
RHD, rheumatic heart disease; APSGN, acute post-streptococcal glomerulonephritis
[a] Data from Carapetis *et al.*, Lancet Infect Dis 2005 (31)
[b] Data from Eriksson *et al.*, CID 2003 (43) and paper IV
* Data not available, estimated number

1.3.2 Characterisation and classification

The classification of streptococci is based on their haemolysis pattern on blood agar plates. There are three groups of haemolysis patterns; α, β and γ, which correspond to partial, complete or no lysis of the red blood cells, respectively. The β-haemolytic streptococci, to which GAS belongs, was further classified into different serological groups (A-V) by Rebecca Lancefield in 1933, based on their carbohydrate composition. Among these, the main human pathogens are group A and B streptococci. Traditionally, serotyping targeting the T-antigen on the surface of GAS (44), the serum opacity factor or the M-protein (45) was used to further characterise GAS isolates. The M-and T-types are closely linked, and only certain combinations are possible. Most often, one M-type is associated with one specific T-type (or a T-type complex) (46), (table 4).

There are about 25 distinct T-types described, but complex agglutination patterns are quite common, which results in a more differentiated type distribution (46). Commonly, about 10% of isolates are non-typable for the T-antigen. The function of the T-protein was unknown until recently, when shown to represent the backbone of a the pilus-like structure of GAS (47). This suggests that the T-proteins may be important virulence factors contributing to adhesion and invasion of target cells, as is the case for pili in gram-negative bacteria.

GAS can also be classified into about 80 different serotypes, based on serological reactivity with the hypervariable N-terminus of the M-protein (figure 3). Over the last 10 years, the serological method has largely been replaced by sequence typing of the 5' portion of the *emm*-gene, encoding the hypervariable N-terminus of the M-protein. The main reason for this methodological shift was problems with a large number of non-typable isolates by serology (48). Since the sequence typing was introduced, more than 150 different sequence types have been described (49). With the sequence based methods, it was also shown that some of the previously designated M-types shared the same *emm*-sequence type (46, 50) (table 4). The M-proteins are important virulence factors of GAS, as described further in sections below.



The serum opacity factor (SOF) is a type-specific enzyme, associated with certain M-serotypes. T-typing and SOF inhibition test has been used in combination to identify certain M-types, such as T1 (SOF-) M1, or T1 (SOF+) M68 (table 4). The SOF typing can also be assessed by *sof* gene detection (46).

Pulse field gel electrophoresis (PFGE) analysis is a tool to investigate genetic relationships between isolates, which is especially useful in studies of isolates from outbreaks (42, 52). Another method to investigate how isolates are related to each other is by multi locus sequence typing (MLST), which is based on comparative sequence analysis of internal fragments of seven housekeeping genes (53, 54).

Figure 3. *Characteristics of the dimeric alpha-helical coiled-cold structure of the M-protein on the cell surface of GAS. The N-terminal hypervariable part differs between different M-types, which provide the molecular basis for M- or emm-typing. Also the group A specific carbohydrates and the peptidoglycan layer of the cell wall are indicated above the cell membrane. Adapted from Bisno et al., Lancet 2003 (51).*

Table 4. Prevalent M-/*emm*-types and their SOF/*sof* and T-pattern correlations [a]

M /*emm*-type	SOF/ *sof*	No. of isolates	T-agglutination patterns [b] (No. of isolates per pattern or complex, respectively)
1	-	4107	**1** (3979); NT (110); 1/3/13/B3264 (18)
2	+	742	**2** (528); 2/28 (146); 8/25/imp19 (56); NT (12)
3	-	2171	**3/13/B3264** (1959); NT (202); 1 (10)
4	+	1543	**4** (1478); NT (30); 8/25/imp19 (16); 4/28 (14); 3/13 (5)
5	-	616	**5/27/44** (442); NT (166); 11/12 (8)
9	+	142	**9** (97); 14 (20); 5/9 (17); 9/3/B3264 (3); NT (4) ; 11/12
11	+	339	**11**, 11/12 (311); NT (26); 28 (2)
12	-	2585	**12**, 11/12 (2396); NT (189)
18	-	456	**18** (127); NT (192); 8/imp19/27 (125); 14 (7); 8/25, 5/27/44, 23 (5); **9/18**
22	+	681	12 (391); 12/3/13/B3264, 13/B3264 (219); 11/12 (50); NT (17); **22** (4)
28	+	1412	28, 4/28 (1329); NT (35); 11/28, 8/28 (37); 3/13/B3264 (9); 4
33	-	72	**3/13/B3264** (60); NT (8); 8/25 (4)
41	-	95	**3/13/B3264** (78); **NT** (17); Misc (4)
43	-	88	**3/13/B3264** (68); NT (18); Misc (2)
49	-	185	**14** (98); NT (56); 8/14/25/imp19 (18); 3/13/B3264 (9); 12; Misc (3)
58	+	158	**8/25/imp19** (81); NT (45); 2/28 (18); 2/8/25 (11); 12 (2); 14/25
61/44[c]	+	198	**5/27/44** (146); 11/12 (24); NT (21); 8/25/imp19 (7) **11**, **9/11**
68	+	34	3/13/B3264 (16); 12, 11/12 (5); 1 (6); 6 (3); 4 (2); NT (2)
73	+	165	**3/13/B3264** (156); NT (6); Misc (3)
75	+	912	**8/25/imp19** (885); NT (18); 14 (4); 13 (3); 6; 2/8/14
76	+	78	8/25/imp19 (40); **12**, 11/12, 12/B3264 (30); 22; NT
77/27L[c]	+	505	**3/13/B3264** (209); 28, 13/28 (194); 9, 9/13/28 (45); NT (19); 8/25, 8/28 (10); 5/27/44
78	+	186	**11**, 11/12 (158); NT (16); 3/13, 3/13/B3264/5/27/44 (10); 5/11/27; 14/25
81	+	71	**3/13/B3264** (37); NT (11); 12, 12/B3264 (5); 8 (5); 4 (4); 6 (3); 14 (2); 23; Misc (3)
82	+	202	**5/27/44** (154); NT (32); 3/13/B3264 (11); 11/12 (3); 4 (1); 8/25 (1)
83	-	77	**3/13/B3264** (67); NT (10)
87	+	94	**28** (80); NT (8); 11/12 (4); imp19 (1); 6 (1)
89	+	518	**11**, 11/12 (314); 3/13/B3264 (115); NT (86); 4; 28; 27
92	+	98	8/25/imp19, **imp19** (86); 3/13/B3264 (5); NT (5); 28 (2)
94[d]	+	81	**3/13/B3264**, **B3264** (77); NT (2); 6; 11
114[d]	+	129	NT (66); **11/12** (47); 14 (10); **12/B3264**; 5/11; 9; imp19; 5; 1

[a] Table partly adapted from Johnson *et al*. JCM 2006 (46). Selected types are the most common M-/*emm*-types (n>60, except type 68) from the original table, which included more than 40 000 clinical isolates collected between 1953 and 2004.

[b] T-patterns of the same M-/*emm*-types considered distinctly different are separated by semicolons whereas closely related patterns are separated by commas, followed by the cumulative number of isolates with this pattern. Patterns not followed by a number indicate either a single observation or a very small number of observations. NT indicates non-typable; Misc (miscellaneous), which indicate patterns that are incompletely identified or confirmed. Patterns obtained with reference strains for each M-/*emm*-type are indicated by bold font.

[c] Sharing the same *emm*-sequence type. In addition also 38/40, 50/62 and 65/69 are *emm*-type identical.

[d] These types have not been designated any M-types, but only *emm* sequence types (which is the case for any types ≥ 94).

1.3.3 Epidemiology

In the late 1980s, reports began to appear that described a drastic increase in frequency of highly aggressive invasive GAS infections, including STSS and NF cases, with high mortality rates. Such outbreaks was reported from several parts of the world including Europe, the US, Canada, New Zeeland, and Japan (38). Epidemiological studies showed that the majority of these outbreaks, although reported from different countries and on different continents, were caused predominantly by GAS strains of serotypes M1 and M3 (55). However, also other serotypes, including non-typables, are known to cause these diseases (55, 56).

National surveillance studies of invasive GAS infections conducted after this world-wide outbreak, demonstrated that M1T1 and/or M3T3 remained common in the community (14, 57-62, paper III). However, in a national surveillance study on invasive GAS disease conducted during 1996-97, M28T28 isolates dominated both among invasive and non-invasive isolates (43). This study also reported a high incidence of puerperal sepsis, 22.4 / 100 000 of those at risk, as compared to an overall incidence of invasive GAS infections at 2.9 / 100 000 population. This is in agreement with reports of a strong association between M28 isolates and puerperal sepsis (42, 63, 64, paper IV). Functional analyses revealed that this association is likely attributed to the R28 protein expressed on the surface of this M-type (65). Stålhammar-Carlemalm *et al.* demonstrated by use of isogenic mutants with and without the R28 protein that R28 promotes binding of the bacteria to cervix epithelial cells and that the binding could be abolished by antibodies directed towards the protein (65).

Other disease manifestations caused by GAS has also been correlated to certain M-types. For example, skin infections with M-type 81 has been shown to be an important source of GAS skin infections (54, 57, paper IV). M-types 49 and 12 on the other hand have been demonstrated in pyoderma-associated respectively pharyngitis-associated acute post streptococcal glomerulonephritis (51, 56). M-type 5 has been commonly associated with outbreaks of acute rheumatic fever, but also M-types 3, 6 and 18 are correlated to this disease manifestation (56, 66).

Differences have also been seen when comparing invasive with non-invasive isolates. As mentioned, M1 and or M3 commonly dominates among invasive isolates, whereas M-types 12, 4 and 28 has been shown to be more prevalent among non-invasive isolates, M12 in particular among pharyngitis isolates (43, 67, 68, papers III and IV). Although epidemiologic studies have proven that there is a link between certain types and disease manifestations, it is worth emphasising that there is a significant overlap in disease potential, and the reported associations are far from exclusive.

The prevalence of different M-types in the society over time has also been shown to vary, both over time and with the geographic area studied (69-71). One theory to explain this phenomenon is lack of herd immunity to specific GAS isolates, due to low prevalence of these types in the population during prior years, which would allow emergence of these clones within the susceptible population.

1.3.4 Virulence factors

GAS expresses several virulence factors, both cell associated and secreted, which in various ways interact with the host to promote growth, dissemination, and survival of the organism, as summarised in table 5. The majority of the membrane-bound molecules, including capsule, M and M-like proteins, C5a peptidase, and fibronectin-binding proteins, are important for evasion of phagocytosis and bacterial adherence to host cells and tissue, whereas several of the secreted proteins such as peptidases, streptolysins, superantigens, and proteases contribute to spread and growth of the bacteria and result in induction of inflammatory responses (reviewed in 56, 72, 73). The biological and clinical consequences of the interplay between GAS virulence factors and host cells include inflammation, and in the severe cases, tissue injury, multiorgan failure and shock.

The M-proteins are classical virulence determinants of GAS by virtue of their ability to mediate resistance to phagocytosis. Studies by Rebecka Lancefield demonstrated GAS strains expressing M-proteins could resist phagocytosis by human blood cells, but became sensitive when opsonic type-specific anti-M antisera were present (45). Three main mechanism have been suggested for M-protein mediated anti-phagocytosis, including (i) binding of the proteins that controls the complement, i.e. plasma protein factor H and a factor H-like protein thereby, obstructing the binding of the opsonic fragment C3b to the bacterial surface (74, 75); (ii) interaction with the C4b-binding protein (76, 77); and (iii) by binding of fibrinogen to the M-protein (78, 79). Recently, an additional property as a major neutrophil activator was ascribed the M1-protein. M-protein was found to form complexes with fibrinogen, which activated neutrophils by binding to β2-integrins with subsequent massive release of heparin binding protein, a prominent inducer of vascular leakage and consequently shock (80).

GAS also expresses M-like proteins that are structurally related to the M-proteins (81-83). Many M-like proteins have immunoglobulin binding properties and can interact with the Fc-portion of IgG and or IgA (56). Several of these proteins also exhibit specificity for other plasma proteins, such as albumin, kininogen and plasminogen (84). In 1999 a novel GAS surface molecule was identified and named G-related α_2-macroglobulin binding protein (GRAB), based on its capacity to bind α_2-macroglobulin (85). It was proposed that GRAB could protect the bacteria from degradation by proteases produced by phagocytic cells or by the streptococci itself, since α_2-macroglobulin is the most abundant protein inhibitor present in serum (85). In a subsequent study, it was shown that α_2-macroglobulin bound to protein GRAB trapped the streptococcal cysteine protease, also called the streptococcal pyrogenic exotoxin B (SpeB), which in this complex lost its ability to degrade proteins but remained proteolytically active against small peptides such as the antimicrobial peptide LL-37 (86).

The pro-inflammatory molecules of GAS include the cell-wall components peptidoglycan and lipoteichoic acid, unmethylated CpG DNA, and superantigens. The

superantigens have been implicated as central players in the pathogenesis of STSS and necrotising fasciitis, by virtue of their potent immunomodulatory effects. As superantigens they interact (without prior cellular processing) with sites located outside the antigen-binding cleft of the major histocompatiblity complex (MHC) class II molecules on antigen presenting cells, and the Vβ-region of the T-cell receptor (figure 2) (87). Superantigens bind specifically to a set of T-cells expressing certain Vβ-chains and although there are overlaps in Vβ specificities among different superantigens, each superantigen has specificity for a unique Vβ repertoire. The crosslinking of antigen presenting cells and T-cells results in activation of both cell types. A conventional antigen activates 0.0001-0.01% of the resting T-cell population, as compared to up to 20% by superantigen activation, which subsequently results in expression of excessive amounts of cytokines. There are several distinct superantigens produced by GAS, including streptococcal pyrogenic exotoxins (Spe) A, B, C, F, G, H, I, J, L, M (88-90), streptococcal superantigen (SSA) (91), and the streptococcal mitogenic exotoxin Z (SmeZ) (92). Most GAS strains express several different superantigens, although the repertoire of superantigen genes varies between GAS strains. Some of the genes are universally present in all strains regardless of serotype, whereas others are only seen in distinct lineages. It seems likely that several different superantigens can trigger invasive GAS diseases and that more than one superantigen are produced by the bacteria during the infection.

Table 5 Major GAS virulence factors

Action/mechanism	Virulence factor
Adherence/ colonisation	Capsule M protein Fibronectin-binding protein Collagen-binding proteins Lipoteichoic acid
Anti-phagocytic	Capsule M-protein M-like proteins C5a peptidase Streptococcal inhibitor of complement (SIC) Streptococcal cystein protease (SpeB) Cell envelope proteinase (SpyCep)
Spreading of infection	DNAses Hyaluronidase Plasminogen-binding proteins Streptokinase
Systemic toxicity and pro-inflammatory activity	Streptolysins O and S Superantigens: SpeA-M, SmeZ, SSA Peptidoglycan Lipoteicoic acid CpG DNA
Inhibition of proteolysis	GRAB α_2-macroglobulin-binding proteins
Induction of vascular leakage	M-protein

1.3.5 Interactions with the host

In an attempt to identify factors determining severity of GAS infections, studies were conducted to assess whether M1T1 isolates causing severe or non-severe GAS during the outbreaks in the late 1980s, were similar or whether distinct subtypes were responsible for the different disease manifestations observed (93, 94). These studies revealed that clonally related GAS strains could cause disease of starkly varying severity: hence, emphasising the influence of host factors for the outcome of infection.

A crucial role for humoral immunity against GAS virulence factors was demonstrated already in the 1960s when Rebecka Lancefield showed that opsonic antibodies against the M-protein conferred resistance against infection (45). This study also revealed that the protection is serotype specific and the individual remains susceptible to infection by other serotypes.

Analyses of the outbreaks in the late 1980s, showed that invasive cases had significantly lower levels of opsonic anti-M1 and neutralising anti-superantigen antibodies, than did the healthy controls or patients with tonsillitis (95-100). Importantly, equally low levels of protective antibodies were found in cases with severe (i.e. STSS and/or NF) or non-severe invasive M1T1 infection (94, 97). Taken together, these data suggested that although protective immunity contributed to susceptibility to invasive disease, it did not determine the severity of infection. It was later shown that the severity of infection was strongly associated with the propensity of the individual to respond with a high or a low inflammatory response upon challenge with streptococcal superantigens (101). This was demonstrated to be a consequence of the HLA class II type of the patients as certain HLA class II haplotypes conferred strong protection against severe systemic GAS disease, whereas others were associated with risk of severe disease (102). Furthermore, a relation between HLA class II type and levels of superantigen induced inflammatory response was found, with the risk haplotypes promoting significantly higher cytokine responses as compared to the protective haplotypes.

Another important host-pathogen interaction that likely contributes to the pathogenesis of GAS infections is the internalisation of host cells. GAS is typically considered an extracellular pathogen, but studies have revealed that it may reside intracellularly in host cells (103-107). A recent analysis of a human biopsy material demonstrated a prolonged intracellular persistence of viable GAS in host cells, mainly macrophages, at the local site of infection during acute severe soft tissue infections (108). The data suggested that this represents an important immune escape mechanism, which promotes bacterial persistence and potentially also dissemination.

1.3.6 Treatment and prevention

Conventional therapy of invasive GAS infections has consisted of antimicrobials, and when necessary in severe invasive disease, support of vital functions for patients with STSS and surgery for those patients with NF. GAS are uniformly susceptible to benzylpenicillin and other β-lactam antibiotics, and penicillin remains the cornerstone

in antibiotic treatment of group A streptococcal infections. In severe invasive GAS infections, such as STSS, NF and myositis, it is recommended that the β-lactam antibiotic should be combined with clindamycin. The main reason for using clindamycin is that it offers freedom of the inoculum effect. The targets for the β-lactams are the penicillin binding proteins (PBP), enzymes responsible for the formation of the peptidoglycan in the bacterial cell wall. The PBPs are expressed during the log-phase of growth, but in large inocula, which often are the case in severe infections, the bacteria may reach stationary phase of growth, where the PBPs are down-regulated (the Eagle effect) (109-111). Such effect has been shown in a mouse model of GAS myositis, where penicillin was ineffective if the therapy was delayed with two hours or more (112). Mice receiving clindamycin had survival rates of 100%, 100%, 80%, and 70%, when treatment was delayed with 0, 2, 6, and 16.5 hours, respectively. Another advantage of clindamycin is related to its mechanism of action, i.e. inhibition of protein synthesis, including the important M-protein and superantigens (113-117). The use of clindamycin were also evaluated in epidemiological studies on severe invasive GAS infections, where it was shown to be significantly associated with improved outcome (118, 119).

Unlike many other bacteria, GAS have not developed resistance towards penicillin, which makes uncomplicated infections easy to treat. However, resistance to macrolides and clindamycin have been seen worldwide, and the approach for severe disease is therefore to combine penicillin and clindamycin treatment, since at least penicillin provides coverage against 100% of GAS isolates.

Research towards development of a safe and efficacious vaccine against GAS infections is ongoing, and would be of major importance, especially in low-income countries where the prevalence of post-streptococcal glomerulonephritis and rheumatic fever are high, causing a significant impact on public health. Different vaccine approaches in the field include mucosal vaccines containing the conserved portion of the M protein (120, 121) or other known conserved surface proteins like the C5a peptidase (122, 123). Another approach is development of multivalent vaccines consisting of type-specific M protein epitopes of prevalent M-types, designed to evoke serum bactericidal antibodies (124, 125). However, this approach has been questioned mainly due to the large numbers of different M-types circulating, and by the dynamics in the epidemiology at a given geographic area (69-71). It is also of major importance that none of the epitopes the vaccine is directed toward, cross-react with human tissue. As mentioned previously, the M-protein contains epitopes with homology to human tissue, resulting in antibody responses that cross-react with, among others, human myosin. Hence, it is crucial that M-protein vaccines are free from such deleterious eptiopes. Furthermore, in a recent study by Sandin *et al*. (126), it was shown that antibodies directed towards the three different surface exposed regions the M5 protein (the hypervariable B and C repeat regions respectively (figure 3) had different opsonising capacities *in vitro* as compared to *in vivo*. The finding could be explained by the ability of the B and C repeat regions to bind to plasma proteins, which in turn could inhibit antibody binding to these regions. The study further emphasises the importance of understanding the molecular properties of epitopes at physiological settings, in order to be able to choose appropriate epitopes for functional vaccine development.

1.4 *STAPHYLOCOCCUS AUREUS* INFECTIONS

Staphylococcus aureus, a gram-positive coccus, is often a part of the normal skin flora. It is one of the most common pathogens causing minor lesions such as skin abscesses, wound infections as well as more severe infections such as septicaemia, endocarditis, septic arthritis and TSS (127). The primary site of infection is often a local skin infection from which the bacteria may spread and infect deeper tissue or enter the blood stream. Patients with breaches in the skin due to surgery, central venous lines or burns are particularly at risk to develop *S. aureus* infections. Nosocomial infections caused by *S. aureus* have increased over the last decades, and are becoming increasingly important with the emergence of methicillin resistant *S. aureus* (MRSA), that are resistant not only to methicillin but also towards several of the most commonly used antibiotics. Commonly MRSA encompass between 10 and 60% of all *S. aureus* isolates isolated in hospitals. As a result, vancomycin is often the only remaining antibiotic to use, and clinical studies have already reported the occurence of MRSA isolates with reduced susceptibility, or even resistance towards vancomycin, which pose a real threat if spreading (128, 129). During recent years, MRSA infections have been reported to be acquired also in the community, and further been shown to cause outbreaks in many different settings and populations worldwide, especially in children (130). Community acquired (CA)-MRSA have primarily been associated with skin and soft tissue infections, but can also cause severe invasive disease including NF and necrotising pneumonia (131-133).

1.4.1 Virulence factors

S. aureus express several virulence factors, of which a large number are secreted proteins such as enzymes, cell surface components, toxins and superantigens, summarised in table 6. Many of the *S. aureus* cell-surface bound proteins are believed to be important for the virulence, especially for the adherence of the bacterium to specific cells or tissue of the host and thereby contribute to successful colonisation and persistence. Proteins identified as adhesins include the fibronectin binding proteins (134, 135), a collagen binding protein (136), fibrinogen binding proteins (137, 138), a vitronectin binding protein (139), an elastin binding protein (140) and the extracellular adherence protein (141). Another cell-surface bound protein is Protein A, that attenuates opsonisation and phagocytosis by binding to the Fc-portion of the IgG molecule and thereby masks this complement binding site on the IgG molecule (142). Protein A was also recently shown to induce inflammation through binding to TNFR1, a TNF α-receptor (143). This Protein A-TNFR1 binding was shown to have a central role in the pathogenesis of *S. aureus* pulmonary diseases, such as pneumonia (143). One of the fibrinogen binding proteins, the extracellular adherence protein (Eap) has in addition also been shown to interfere with transendothelial migration of neutrophils, and also with T-cell proliferation (144-147).

Examples of virulence factors conferring antiphagocytic effects are the extracellular fibrinogen binding protein (Efb), the chemotaxis inhibitory protein of *S. aureus* (CHIPS) and staphylococcal compliment inhibitor (SCIN) which are all shown to interfere with the complement system (148-152).

Of the factors contributing the systemic toxicity and pro inflammatory effects seen in severe *S. aureus* infections, the staphylococcal superantigen are most prominent. At least 24 staphylococcal superantigens are known today, including allelic variants, (153), and the ones implicated in TSS are described in more detail below. Another important virulence factor, especially prevalent among CA-MRSA cases, is the Panton-Valentine (PV) leukocidin. It is a two component toxin that kills leukocytes by creating pores in the cell, and detected in fewer than 5% of *S. aureus* isolates in a general hospital, but identified as a stable marker for CA-MRSA worldwide (154, 155). The inflammatory responses of PV leukocidin include the ability to induce granule secretion in polymorphonuclear leukocutes and release of inflammatory mediators (156).

Also *S. aureus* was until recently mainly regarded as a non-invasive pathogen, but it has now been shown that the bacteria can invade many types of cells, by the formation of a fibronectin bridge between the bacterial fibronectin-binding proteins and α5β1 integrin molecules that triggers internalisation (157-162). The internalisation has been associated with (i) persistent and relapsing infections, due to their intracellular localisation which protects the bacteria from host defence and antibiotics, (ii) apoptosis of the invaded cell, leading to engulfment of the apoptotic bodies of macrophages, and (iii) necrosis of the invaded cells leading to further spread of the bacteria (163, 164).

Table 6. Major *S .aureus* virulence factors

Action/mechanism	Virulence Factor
Adherence/colonisation:	Fibronectin binding protein A and B Fibrinogen binding proteins; Efb, EAP and Clf A and B Collagen binding protein Elastin binding protein Extracellular adherence protein (EAP)
Anti-phagocytic	Capsule Protein A Clumping factor A (Cfl A) Extracellular fibrinogen binding protein (Efb) Staphylococcal complement inhibitor (SCIN) Staphylokinase Chemotaxis inhibitory protein (CHIPS) Extracellular adherence protein (EAP)
Inhibition of neutrophil chemotaxis	Chemotaxis inhibitory protein (CHIPS) Extracellular adherence protein (EAP)
Systemic toxicity and pro-inflammatory activity	α-haemolysin γ-haemolysin Panton-Valentine leukocidin Leukocidin E/D and M/F Superantigens: staphylococcal enterotoxins A-E, G-Q and TSST-1 Exfoliative toxin A and B (SEA, SEB) Peptidoglycan Lipoteichoic acid CpG DNA Protein A

1.4.2 Epidemiology of staphylococcal toxic shock syndrome

Todd *et al.* (165) initially brought the illness to attention in 1978 with the report of TSS as a major systemic illness associated with non-invasive *S. aureus* infections in children. In the early 1980's there were increasing numbers of staphylococcal TSS cases among young women using high absorbency tampons, and the presence of *S. aureus* localised to cervical or vaginal colonisation was found (166-169).This caused a major interest in the pathogenesis of this disease and already in 1981 a secreted protein, that was highly associated with menstrual TSS, was identified and named toxic shock syndrome toxin-1 (TSST-1) (36, 170). TSST-1 was later shown to be a superantigen, and to be responsible for almost all cases of menstrual associated TSS. This association is likely due to TSST-1's unique ability to cross mucosal barriers, unlike the other staphylococcal superantigens, the enterotoxins (SE) (171). Underlying mechanisms that contribute to an increased risk of TSS under menstruation include an environment that promotes bacterial growth, in addition to a break in the mucosa that enhances absorption of the toxin. The incidence of TSS has decreased significantly since the 1980s, mainly due to the increased awareness of the role of tampons in menstrual TSS.

In non-menstrual TSS, several superantigens have been implicated, including most commonly TSST-1, staphylococcal enterotoxin (SE) B and SEC, although SEG and SEI also have been reported in a few cases. Non-menstrual TSS can occur in association with several different staphylococcal infections, the most common manifestations include post-surgical TSS, influenza associated TSS, recalcitrant erythematous desquamating syndrome, and TSS associated with use of contraceptive diaphragm (172-174). The mortality of both subtypes of staphylococcal TSS ranges between 3-5%, which is indeed lower than for streptococcal TSS, but is still a significant cause of death world-wide (15). Staphylococcal TSS patients do not normally have detectable bacteraemia, yet clinical features of the disease are systemic, suggesting that staphylococcal TSS results from an intoxication with bacterial products (15).

1.4.3 Humoral immunity

A crucial role of humoral immunity against superantigens has been demonstrated also for the protection against invasive staphylococcal disease. In healthy adults, antibodies against TSST-1 can regularly be detected (175, 176), and an antibody response against TSST-1 can usually be demonstrated following septicaemia with a TSST-1 producing *S. aureus* strain (177, 178). Patients with low antibody levels or with a deficient antibody response against the staphylococcal superantigens seem to be at risk for contracting TSS or recurrence of TSS (175, 179-182). The failure of some individuals to make antibodies to TSST-1, may be an parallel to the finding that TSST-1 is an poor immunogen in rabbits. As high as 50% of rabbits hyperimmunised with TSST-1 failed to develop antibodies against the toxin, even though humoral immune responses towards other antigens remained intact in these animals (183).

1.5 INTRAVENOUS IMMUNOGLOBULIN THERAPY IN SEPSIS

During the last two decades, significant advances have been made in the sepsis field revealing a complex interplay between several microbial factors and host defence systems. This has allowed for novel therapeutic strategies, ranging from interventions with defined microbial factors to host-derived mediators. Adjunctive therapy with intravenous immunoglobulin represents such an immunotherapeutic approach. Initially, the studies focused on the use of monoclonal immunoglobulins directed against either endotoxin or the pro-inflammatory cytokine TNF-α. Although, these monoclonal antibodies resulted in drastically improved survival in experimental models, none succeeded in lowering the mortality of sepsis in large phase III clinical trials, (reviewed in 184, 185).

Polyspecific intravenous immunoglobulin G (IVIG) has also been evaluated as adjunctive immunotherapy in sepsis, and a number of controlled, relatively small, clinical trials have been conducted with varying outcome (table 7).

Table 7. Randomised clinical trials of polyclonal immunoglobulin therapy in sepsis

Patient group	Study design	IVIG preparation	Mortality[a] (%) Control[b]	Mortality[a] (%) IVIG	Sign.	Ref.
Neonatal sepsis	SC	IgG	1/28 (3.6)	2/28 (7.1)	NS	(186)
Neonatal sepsis	SC	IgAMG	9/24 (37.5)	6/20 (30)	NS	(187)
Neonatal sepsis	DB, SC	IgM-enriched	6/30 (20)	1/30 (3.3)	0.04	(188)
Neonatal sepsis	DB, MC	IgG	5/17 (20)	0/14 (0)	0.05[c]	(189)
Neonatal sepsis	MC	IgG	7/25 (28)	7/25 (28)	NS	(190)
Neonatal sepsis	DB, SC	IgM-enriched	6/30 (20)	1/30 (3.3)	NS	(191)
Neonatal sepsis	SC	IgG	4/15 (26.7)	2/20 (10)	NS	(192)
Post-op sepsis and endotoxemia	DB, SC	IgG	19/22 (86.4)	15/24 (62.5)	NS	(193)
Sepsis/shock	SC	IgG	9/12 (75)	7/12 (58)	NS	(194)
Severe abdominal sepsis and surgery	DB, SC	IgM-enriched	13/27 (48.1)	8/29 (27.6)	NS	(195)
Surgical sepsis (sepsis score>20)	DB, MC	IgG	22/33 (67)	11/29 (37.9)	0.04	(196)
Post-op sepsis	SC	IgAMG	13/17 (76.5)	8/18 (44.4)	NS	(197)
Gram-negative septic shock	SC	IgAMG	9/28 (32)	1/27 (4)	0.006	(198)
Sepsis score 12-27, APACHE II 20-35	DB, MC	IgG	M; total of 653 patients		NS	(199)
STSS	DB, MC	IgG	4/11 (36.4)	1/10 (10)	NS	paper I

IVIG, intravenous immunoglobulin; Sign., significance; Ref., reference; DB, double-blinded; MC, multicenter; SC, single-center; G-, gram-negative; M, missing; STSS, streptococcal toxic shock syndrome; NS, not signinficant

[a] Mortality day 28

[b] Controls received placebo or no intervention

[c] Mortality day 7. No difference between the groups day 56.

The Cochrane group conducted a meta-analysis of IVIG therapy in sepsis, which included randomised sepsis trials that compared IVIG with either placebo or no intervention (200). The analysis showed a significant reduction in mortality by IVIG, but the authors acknowledged that the included studies were all small and the totality of evidence was insufficient to support a strong conclusion of benefit. Importantly, this meta-analysis did not include the large score-based immunoglobulin treatment in sepsis study that included 653 patients and failed to demonstrate a reduction in 28-day mortality (199). Hence, the data available to date are not sufficient to recommend the use of polyclonal IVIG for the entire group of sepsis, but studies indicate that certain septic subgroups, such as STSS, are likely to benefit from IVIG (as discussed in more detail below).

1.5.1 Intravenous immunoglobulin therapy in toxic shock syndrome

The finding that low levels of protective antibodies against important virulence factors, among others superantigens, correlated with invasive streptococcal and staphylococcal disease highlighted the importance of antibodies in protection against these infections, and suggested that immunoglobulins might be a potential adjunctive therapy. An advantage of IVIG is that it is pooled from several thousands of donors and hence, exhibits a high polyspecificity, which would ensure coverage of the many different superantigens and bacterial serotypes (96, 201, 202).

IVIG has been commonly used in Kawasaki disease, which is a disease in which superantigens have been implicated in the pathogenesis (203). Clinical improvement after IVIG-therapy of patients with severe invasive *S. pyogenes* infections, including TSS, necrotising fasciitis, and necrotising myositis, has been reported in several case reports (12, 13, 204-212). Further support for the use of IVIG as adjunctive therapy in STSS was obtained through an observational cohort study (213), a randomised controlled clinical trial (paper I) and in the Swedish enhanced surveillance of invasive GAS infections (paper IV), table 8.

The current medical literature as well as standard surgical and medical reference textbooks advocate an early and aggressive surgical approach for patients with suspected or proven necrotising fasciitis (217-223).Drainage plus complete removal of all infected tissue is felt to be lifesaving (222, 223). However, in a recent study high-dose IVIG was given to seven patients with severe soft tissue infections caused by GAS, in combination with antimicrobial treatment and a conservative surgical approach (216). Six of the seven patients also had STSS. All seven patients survived, which suggests that the use of IVIG for GAS soft tissue infections may allow an initial non-operative or minimal operative approach which can limit the need to perform immediate wide debridements and amputations in unstable patients. The results of this small observational study clearly warrant additional investigations of the use of IVIG in this clinical setting.

Table 8. Clinical studies of the use of intravenous immunoglobulin as adjunctive therapy in severe *S. pyogenes* infections.

Clinical Diagnosis	No. of Patients	Case Fatality Rate (%)	GAS Serotype/ sequence type	Reference
STSS	1	0	M1	(209)
STSS	1	0	M49	(205)
STSS	1	0	M1	(208)
STSS	1	0	M74	(206)
STSS	1	0	NA	(12)
STSS	5	20	M1	(211)
STSS	IVIG: 21	33	48% M1, 19% M3	(213)
	No IVIG: 32	66	6% M1, 28% M3	
STSS + NM	1	0	M1	(207)
STSS + NF	1	0	NA	(210)
STSS + NF	1	0	NA	(204)
STSS + NF	1	0	NA	(212)
STSS ± NF	10	10	20% T1, 20%T3, +NA	(13)
STSS	IVIG:10	10	Mix	paper I
	No IVIG: 11	36		
STSS ± NF	IVIG: 20	15	40% M1, 15% 89	paper IV
	No IVIG: 52	48	25% M1, 51% 89	
NF ± NM	IVIG: 16	19	Mix	(214)
	No IVIG: 4	25		
NF	IVIG: 10	10	35% M1, 25% M3	(39)
	No IVIG: 67	37		
NF	IVIG: 6	0	Mix	(215)
NF ± STSS	7	0	43% M1 + Mix	(216)

NF, necrotising fasciitis; STSS, streptococcal toxic shock syndrome; NM, necrotising myositis; NA, not available.

Clinical efficacy of IVIG in staphylococcal TSS is not as well documented, and is limited to three case reports (224-226). The recent case report describes a 14 years old boy with sepsis caused by a community acquired MRSA isolate, producing the exotoxin Panton-Valentine (PV) leukocidin (226). PV leukocidin-positive MRSA isolates have recently been associated with severe, rapidly progressive, systemic disease including soft tissue infections and necrotising pneumonia with significant mortality (227, 228). The current patient had septicaemia and disseminated foci of infection, including necrotising pneumonia, septic arthritis of the left knee, and deep vein thrombosis of the left leg (Hampson 2006). Although the antibiotic therapy was changed to antibiotics to which the isolate was sensitive for *in vitro*, the patient did not clinically improve over seven days. At this point, IVIG was administered and was followed by clinical improvement and a sustained fall in inflammatory markers. The patient was discharged to the ward five days later and has since been discharged home.

A multicenter clinical trial have investigated prophylactic treatment of low birth weight infants with donor selected IVIG to prevent nosocomial staphylococcal sepsis infections (228). The donors were selected based on high titers of anti-staphylococcal antibodies. Although not statistically significant, differences were found between treatment groups, with fewer episodes *S. aureus* sepsis, candedemia and reduced mortality. This is an important finding, since neonatal children are in need of

prolonged hospitalisation, and nosocomial staphylococcal infections represent a major problem.

IVIG is generally considered a safe drug with no severe side effects. However recently, concerns have been raised about thrombotic complications after IVIG administration, most commonly reported in idiopathic thromobocytopenia and neuroautoimmune disorders (229-232), but also includes a case report of this complication in a STSS patient (12). Several causes of IVIG mediated thromboses have been postulated, including increased plasma and blood viscosity, platelet activation, and cytokine-mediated vasospasm (229, 232). It has also been shown that IVIG preparations may be contaminated with coagulation factor IX (233). However, thrombotic complications are not uncommon in STSS patients, regardless of receiving IVIG or not, since the coagulation system is also involved in the disease. The patients should thus be closely monitored, and the recommended IVIG infusion rates not be exceeded. Adjunctive therapy targeting the coagulation cascade, as activated protein C, should also be considered.

1.5.2 Mechanistic actions of IVIG in toxic shock syndrome

Mechanisms of actions that may contribute to the beneficial effect of IVIG in both autoimmune and systemic inflammatory diseases include blockade of Fc-receptors on reticuloendothelial cell system and phagocytic cells, modulation of Fc-receptor expression, interference with activated complement, modulation of cytokine responses, modulation of immune cell functions, interaction with idiotype-antiidiotypic network, antigen-neutralisation, and selection of immune repertoires (reviewed in 234, 235).

Mechanistic actions that directly relate to invasive staphylococcal and streptococcal infections include superantigen-neutralisation, opsonising antibodies that promote phagocytosis and bacterial clearance, as well as cytokine modulation (figure 4). The defined role of opsonising antibodies in IVIG has been debated, mainly based on a study assessing the role of IVIG in a murine model of streptococcal NF in which no effect of IVIG on bacterial clearance was observed (236). However, this lack of effect may well be due to the fact that human immunoglobulins are not efficient opsonins for mouse phagocytes. In patients with severe invasive GAS infections, increased levels of opsonising anti-M1 antibodies have been found in plasma post IVIG-infusion as compared to pre-treatment samples (96), and it therefore seems likely that increased bacterial clearance through opsonising antibodies against GAS is a mechanistic action of IVIG contributing to clinical efficacy in these infections.

IVIG contain high levels of superantigen neutralising antibodies that have been shown to potently inhibit the proliferative and cytokine-inducing capacity of both staphylococcal and streptococcal superantigens *in vitro* at physiological concentrations of IVIG (96, 237-244, paper II). Specific antibodies against other important virulence factors, including DNaseB, streptolysin O and PV leukocidin, have also been demonstrated in IVIG preparations (211, 245, 246).

Another important property of IVIG, that is likely to contribute to clinical efficacy in TSS, is its ability to modulate cytokine responses through direct superantigen-neutralisation, as well as through immunomodulatory activities believed to include Fc-interactions, soluble immune components, and induction of regulatory cytokines (reviewed in 234, 235). IVIG has demonstrated powerful inhibition of superantigen-induced lymphokine production *in vitro*, with the strongest suppression seen for the Th1 cytokines IFNγ and TNFβ, as their production was almost completely abolished (241, 244, 247, 248). This inhibitory effect was seen, although to a lesser extent, even when addition of IVIG was delayed 24 hours post stimulation with superantigen (247, 248), hence, indicating that additional mechanisms of IVIG aside from antigen-neutralisation contribute to the potent inhibitory effect. Since the Th1 type of cytokines is the hallmark of a superantigen response, the powerful inhibition of these cytokines by IVIG most likely represents a major mechanistic action of IVIG contributing to clinical efficacy in TSS.

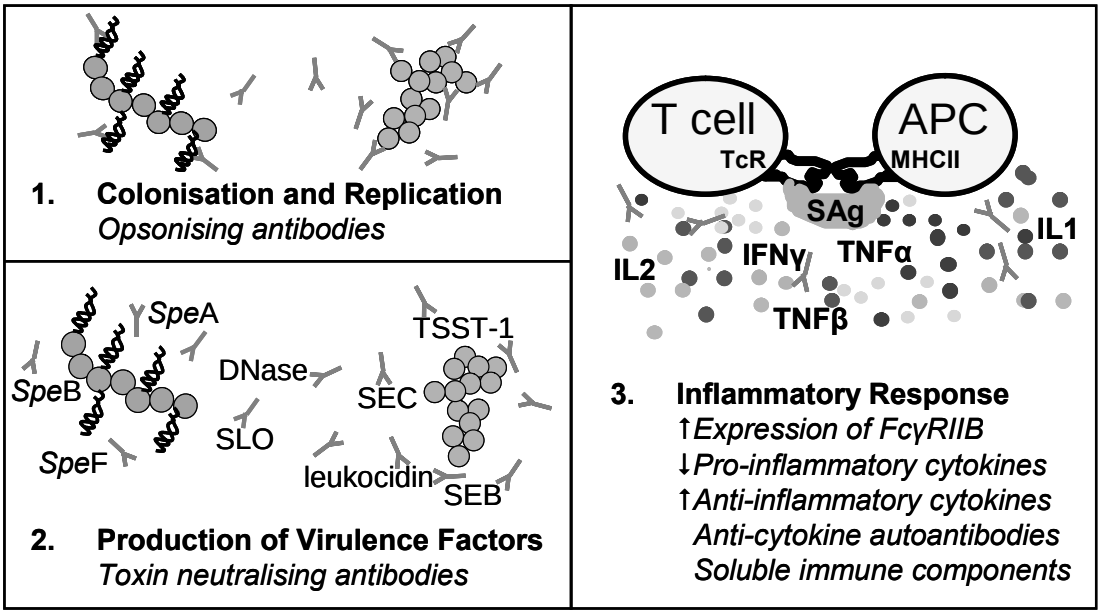


Figure 4. *Proposed mechanistic actions for IVIG in STSS and staphylococcal TSS. 1-3 represent different stages in the pathogenesis. The proposed actions of IVIG are shown in italic text.*

2 AIMS OF PRESENT INVESTIGATION

- To document the efficacy and safety of IVIG in streptococcal toxic shock syndrome

- To compare the potency of IVIG against streptococcal and staphylococcal superantigens

- To investigate the epidemiology of invasive GAS disease in Denmark and Sweden

3 MATERIALS AND METHODS

3.1 CLINICAL TRIAL

A multi-center placebo-controlled double-blinded clinical trial of IVIG in STSS involving 17 hospitals in Sweden, Norway, Finland and the Netherlands were conducted (paper I). Patients were included according to inclusion and exclusion criteria as specified in table 9.

Table 9. Inclusion and exclusion criteria for the StreptIg-001 study

Inclusion criteria:	• ≥18 years of age
	• Probable or suspected STSS, according to the definitions (18) (table 2)
Exclusion criteria:	• Known hypersensitivity to IVIG
	• Underlying disease expected to cause death within 3 months

The patients were randomly assigned to receive IVIG or equal volume placebo (1% albumin). The study drug was given intravenously for three consecutive days at 1g/kg of body weight day 1 and 0.5 g/kg days 2 and 3. The dose was chosen based on a previous case-control study (213) in which doses ranging from 0.4 to 2 g/kg bodyweight were used, as well as results from *in vitro* assays demonstrating 100% superantigen-inhibition at these concentrations (242, 243). The primary objective of the trial was to determine whether IVIG compared to placebo resulted in a statistically significant decrease in mortality over the first 28 days. The secondary objective was to evaluate whether there was statistical differences between the two groups in time of resolution of shock, time of no further progression of the tissue infection, mortality day 180, and differences in mental-, renal-, respiratory- or cardiovascular problems at day 180. Safety of the study drugs was assessed by reviewing non-serious adverse events, serious adverse events, disease related events, indices of organ function (hepatic, renal, pulmonary, cerebral, cardiac, metabolic, and haematological) and deaths of all causes. All patients, or their next of kin, were informed and gave their consent to participate in the study. The study was approved by the ethical committee at the Karolinska Institutet (and also regional committees) and by the drug agency authorities in respective countries.

Blood samples were obtained from the patients before study drug treatment at days 1-3, after treatment day 3 and at follow-ups at days 28 and 180. The patients were closely monitored and clinical information was registered in a case report form for each patient. Clinical isolates were obtained from 17/21 enrolled patients.

3.2 SURVEILLANCE OF INVASIVE GROUP A STREPTOCOCCAL INFECTIONS

Active surveillance of invasive group A streptococcal infections was conducted in Denmark between January 2001 to August 2002 (paper III), and in Sweden between April 2002 to December 2004 (paper IV). The isolates were collected at all microbiological laboratories in each country, and clinical information was obtained from questionnaires that were filled out by the responsible clinician for each patient. The questionnaires included questions concerning severity of disease, treatment, outcome, underlying diseases/conditions and origin of infection. Clinical information was obtained for 98% and 88% of Danish and Swedish patients respectively. Invasive isolates (n=201 and n=746 in Denmark and Sweden, respectively) were collected and characterised as described below.

The studies also included collection of GAS strains causing non-invasive throat and skin infections in respective country (n=335 and n=773 in Denmark and Sweden, respectively), as well as 17 isolates from the throats of healthy carriers in Denmark. The non-invasive isolates were sampled at GPs at different locations of Denmark, and divided into two collection rounds, since the major part of swabs sent in initially originated from patients with tonsillitis (part A, January-October 01) and a request had to be sent out in order to receive more samples from patients with impetigo (part B, November 01-August 02). Samples sent in during these two periods also originated from different parts of Denmark. The Swedish non-invasive isolates were collected at six of the microbiological laboratories (at the University hospitals in Lund, Göteborg, Linköping, Umeå, Karolinska Huddinge and Karolinska Solna). Ten consecutive isolates were requested each month, of which five were isolated from throat- and five from skin samples, during the period between February 03 and June 04.

In cases where blood samples were collected for humoral immunity analysis (n=148 from patients and additionally n=717 from GAS negative individuals/ controls), informed consent had been obtained from the patients prior to sample collection (paper III). These studies were approved by the scientific ethical committees for Copenhagen and Fredriksberg, the Danish data protection agency and the ethical committee at the Karolinska Institutet, respectively.

3.3 ISOLATE CHARACTERISATION

3.3.1 T-typing

T-typing was performed on all GAS isolates included in paper I, II and IV. The isolates were typed by a standard slide agglutination method using commercial antisera against the different T-antigens.

3.3.2 emm-typing

The *emm*-types of GAS isolates in paper II-IV were determined by direct sequencing of the N-hypervariable portion of the *emm*-gene. In short: DNA preparations from each isolates were used to amplify the gene by polymerase chain reaction (PCR), and the product used as the template for the PCR-sequencing reaction, using a primer starting from the beginning of the hypervariable part of the gene. The products were sequenced and the sequences obtained compared to the CDC library of known *emm*-sequences, available on internet (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm).

3.3.3 Superantigen genotyping

PCR amplification using primer pairs specific for each superantigen gene was performed to detect the presence of genes encoding seven (paper I) or nine (papers II and IV) or ten (paper III) of the streptococcal superantigenes. In papers II and IV, a multiplex PCR system was used (70), that enabled reactions with up to 8 of the primer pairs present in the same reaction. The genes investigated were *spe*A, *spe*B, *spe*F, *spe*G, *spe*H, *ssa* and *smeZ* in paper I, and in addition also *spe*C and *spe*J in the other papers as well as *spe*I in paper III. See respective paper for details.

3.3.4 Superantigen production by S. aureus isolates

In paper II, twenty *S. aureus* strains obtained from patients with severe sepsis or TSS were used. Expression of the superantigens SEA-D and TSST-1 by these isolates was determined by reversed passive latex agglutination (RPLA) analyses of bacterial culture supernatants using the commercial kits SET-RPLA and TST-RPLA. In a RPLA assay, the antibodies towards specific antigens (in our case SEA-D and TSST-1 respectively) are attached to latex particles, and when the antigen is present, the latex particles are cross-linked which results in a visible (positive) latex agglutination reaction. The isolates were also tested for expression of exfoliative toxin A and B by real-time (RT) PCR assays. The concept of RT quantitative PCR is, in short, that the target mRNA is reversely transcribed, amplified, detected, and quantified.

3.3.5 Pulse field gel electrophoresis (PFGE) analysis

The PFGE technique is a method developed by Schwartz and Cantor in 1984 to assess relationships between isolates. It uses rare-cutting restriction enzymes to digest the whole genomic bacterial DNA, followed by separation of the fragments on an agarose gel. To overcome the limitations of a conventional agarose gel electrophosesis, that can only separate short DNA fragments, the electrical field used for the PFGE is periodically altered resulting in a multidirectional electric field. Larger fragments have larger difficulties to reorient than smaller, resulting in size dependent gel separations of fragments. Different restriction enzymes can be used for DNA cleavage. We used *Sma* I, which is commonly used for GAS. Bionumerics' software was used to analyse and compare the banding patterns. Invasive and non-

invasive isolates of selected *emm*-types in paper IV (*emm*89, 81, 77, 28 and 12, total n= 846) were subjected to PFGE analysis.

3.4 CYTOKINE ASSESSMENT AT THE SINGLE CELL LEVEL

In order to assess if there were differences in cytokine levels and cell types in the patient blood of the different treatment groups in the IVIG-study (paper I), peripheral blood mononuclear cells (PBMC) were isolated from the blood by Ficoll-Hypaque gradient centrifugation, transferred to adhesion glass slides, and fixed with 2% formaldehyde. The cells were immunohistochemically stained for the cytokines TNFα and IFNγ, as well as CD3 and CD68 as markers for T-cells and monocytes/macrophages, respectively, as described in detail (249). Frequencies of cytokine producing cells and specific cell types were assessed by direct microscopy, where 500-700 cells were counted per field.

3.5 ASSESSMENT OF PLASMA CYTOKINE LEVELS

The concentrations of the pro-inflammatory cytokines IL6 and IL8 in patients' plasma were determined by multiplex cytokine analyses using Fluorokine MAP kits and the Luminex instrument (paper I).

3.6 ASSESSMENT OF HUMORAL IMMUNITY

Presence of anti-streptolysin O (ASOT) and anti-DNase B (ASDB) titers in patients' plasma as well as in plasma from GAS negative controls, was determined by use of standard techniques (250) (paper III). Superantigen-neutralising titers were determined in patients' plasma or IVIG using a functional cell culture assay previously described in detail (241). In this assay, bacterial culture supernatants, which contain a crude mix of extracellular proteins produced by the bacteria such as superantigens, were prepared from isolates obtained from respective patients. These supernatants were used to stimulate PBMC isolated from blood from healthy donors. The cells were cultured in the presence of either patient plasma (paper I and III), IVIG (paper I and II) or the positive control 5% fetal calf serum which does not contain antibodies against streptococcal or staphylococcal superantigens. Each patient's plasma was tested for inhibitory activity against its own isolate (i.e. the bacterial culture supernatant). After 72 h, the cells were pulsed and the proliferative response assessed by 3H-uptake. The neutralising activity of the plasma or IVIG was obtained by relating the proliferative response obtained in the presence of plasma/IVIG with that obtained in cultures with only fetal calf serum. See papers for further details.

4 RESULTS AND DISCUSSION

4.1 INTRAVENOUS IMMUNOGLOBULIN THERAPY IN STREPTOCOCCAL TOXIC SHOCK SYNDROME (PAPER I)

In this study we have assessed the efficacy and safety of IVIG in STSS, and we provide support for the use of IVIG as adjunctive therapy for this disease. Despite adequate and prompt antibiotic therapy and intensive care support, the mortality due to STSS remains high, commonly exceeding 50%, which clearly emphasises the need for adjunctive therapy in this disease. A beneficial effect of IVIG has been reported in several case reports (204-212), as well as in an observational cohort study of IVIG-therapy conducted in Canada, which reported a significantly reduced mortality rate in IVIG-treated cases as compared to controls (213). However, this latter study includes confounding factors that could affect mortality including historical controls and an increased usage of clindamycin among IVIG-treated cases.

To further document the clinical efficacy of IVIG in STSS, we conducted a multicenter placebo-controlled trial designed to evaluate the efficacy and safety of IVIG in this disease. This trial was an investigator-driven study and involved 17 hospitals in Sweden, Norway, Finland, and the Netherlands. The study was pre-objected to include 120 patients evenly randomised to the IVIG or placebo group. However, due to a low incidence of STSS in the participating countries during the study period, patient recruitment was slow, and the trial was prematurely terminated after enrolment of 21 patients, including 10 who received IVIG and 11 who received placebo. GAS strains were isolated from 17 of the patients. In one of the patients, the strain could not be cultured due to prior antibiotic therapy, but GAS was implicated as the causative agent based on a positive rapid antigen test. In three patients enrolled as suspected STSS cases, non-GAS strains were cultured, including *Pseudomonas aeroginosa*, group B streptococcus in combination with *S. aureus*, and a mixed anaerobic infection where gram-positive cocci were identified by gram-staining and direct microscopy.

The IVIG and placebo groups were well matched with respect to clinical and demographic characteristics, but differed in isolate characteristics. Serotypes T1 and T3 were over-represented among isolates in the placebo group, and consequently the *spe*A gene, which is commonly found in these serotypes, was more prevalent in isolates from the placebo group. Importantly, all GAS isolates harboured genes encoding for several different superantigens, and there was no difference between the two treatment groups with respect to the total number of superantigen genes detected. Although, the M1 and M3 serotypes have been commonly associated with outbreaks of severe invasive infections, also other serotypes are known to cause severe and even fatal infections (67, 95, 251-258). This point is further empathised in the current study, where the isolates of the fatal cases included serotypes of T1, T3, T4 and a non-typable isolate, and also by differences seen of their superantigen gene profiles as two of the isolates lacked the *spe*A gene. Hence, we find it unlikely that the over-representation of the M1 and M3 serotypes in the placebo-group would significantly affect the endpoints of the study.

Analysis of the primary endpoint, i.e. mortality over 28 days, revealed a trend to reduced mortality rates in IVIG-treated cases as compared to those receiving placebo (10% versus 36%). Importantly, this trend to a reduced mortality rate was supported by a significant reduction in sepsis-related organ failure (SOFA) score demonstrated in the IVIG group on days 2 and 3 (figure 5). No change in SOFA score could be noted in the placebo group.

The safety of the study drugs was also an endpoint. In total, 6 severe adverse events, i.e. deaths, and 12 adverse events/disease related events were reported, but none of these were reported as related to the study drug.

We also performed *in vitro* studies to see whether we could detect differences between the IVIG and placebo groups with respect to plasma superantigen-neutralising activity and cytokine levels. Cytokine analyses revealed highly varying levels of IL8 and IL6 in the patients' acute phase plasma, and higher levels of IL8 could be noted in the placebo group. However, both groups showed an equal decrease in the levels of serum IL6 and IL8 on day 2. The ability of plasma to neutralise superantigens produced by the patient's own isolate was tested by *in vitro* cell culture experiments, and in agreement with previous studies on patients with invasive GAS infection, all patients had low neutralising plasma activity pre-treatment. A significant increase in superantigen-neutralising activity following administration of IVIG could be seen, whereas the plasma neutralising activity remained low in the placebo group. This is in agreement with previous reports demonstrating that IVIG confers neutralising anti-superantigen antibodies to the patients (213, 241, 242).

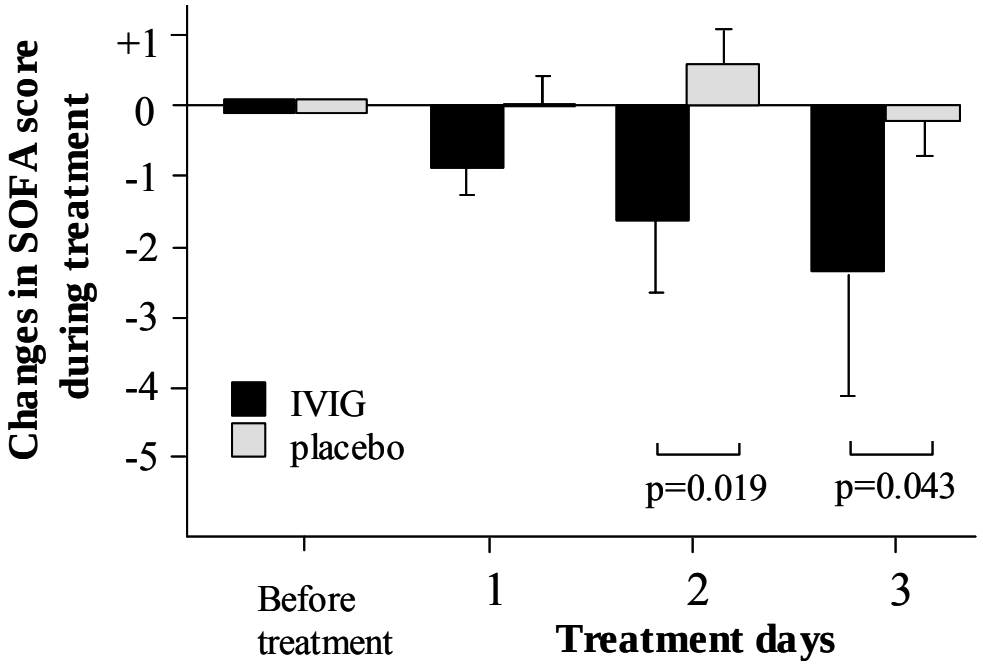


Figure 5. *Assessment of sepsis-related organ failure assessment (SOFA) scores during treatment in trial of Intravenous immunoglobulin (IVIG) therapy in streptococcal toxic shock syndrome (STSS). The figure shows the change in SOFA score (mean ± SE) during treatment in IVIG (black bars) or placebo (grey bars). Baseline SOFA scores, i.e. pre treatment, were 10.8 and 11.5 in the IVIG group and placebo groups respectively. Differences were analysed by the Mann-Whitney U-test and p-values are indicated in the figure. Figure adapted from (259)*

Previous reports have indicated that there is a rapid consumption of the relevant, i.e. M-specific and anti-superantigen, antibodies in streptococcal TSS cases and that multiple doses of IVIG may be required to maintain an efficient level of neutralisation of superantigens (213, 241, 242). In this study, the patients received three infusions of IVIG with the highest dose (1g/kg body weight) given at day 1, followed by 0.5 g/kg body weight on days 2 and 3. Comparison of plasma samples collected after the first dose of IVIG day 2 and before the second dose day 3, also indicated a rapid consumption of antibodies, since 83% of the samples showed a reduction in neutralising activity from day 2 to day 3.

Taken together, the results of this trial with a trend to reduced mortality rate and a significant improvement in organ function, provide support for the use of IVIG as adjunctive therapy in STSS patients. Although it would be desirable to have a controlled trial to provide definite proof of clinical efficacy in this disease setting, considering the high mortality rates it seems reasonable that the clinical and functional data that are available today be sufficient to recommend that IVIG be used in conjunction with conventional therapy for STSS patients.

4.2 DIFFERENCES IN POTENCY OF INTRAVENOUS IMMUNOGLOBULIN AGAINST STREPTOCOCCAL AND STAPHYLOCOCCAL SUPERANTIGENS (PAPER II)

In paper I, one of the patients in the IVIG group did not obtain neutralising activity in her plasma following administration of IVIG. This patient had a mixed infection with group B *streptococcus* and *S. aureus*. Since the group B streptococcal isolate did not induce a proliferative response when tested *in vitro*, the plasma-neutralisation assay was performed using the *S. aureus* isolate. Hence the data reflected a complete lack of plasma neutralizing activity against staphylococcal superantigens even after the patients had received 3 doses of IVIG. This finding was in striking contrast to the STSS patients who all obtained neutralising activity against the streptococcal superantigens following administration of IVIG, in agreement with previous studies (213, 241, 242). To further investigate this, we conducted this study in which the neutralising activity of IVIG against streptococcal and staphylococcal superantigens was compared. The study confirmed our previous observation, inasmuch as IVIG was significantly less potent in neutralising staphylococcal, as compared to streptococcal, superantigens.

This study is of importance since IVIG has been reported as adjunctive immunotherapy in superantigen-mediated TSS. Although, there is good clinical data to support the use of IVIG in STSS, the clinical data of IVIG in staphylococcal TSS is limited to only three case reports (224-226). *In vitro* studies have demonstrated the presence of antibodies against defined streptococcal and staphylococcal superantigens in IVIG (237-240). Here we show, for the first time, that culture supernatants prepared from clinical *S. aureus* isolates, which contain a mixture of superantigens and other exotoxins, are functionally inhibited by IVIG. We chose to use culture supernatants from clinical isolates, since this more closely mimics the clinical situation with potential synergistic or additive activities between superantigens and other secreted factors. The isolates used were all obtained from TSS or severe sepsis

cases, and represented different serotypes and/ or different superantigen expression profiles. The *S. pyogenes* isolates used were predominantly of serotype M1T1 (65%), but also other isolates of other serotypes were included. SAg genotyping revealed that all streptococcal isolates harboured genes encoding for 5-8 different superantigens. As expected, all strains harboured the chromosomally encoded *spe*B, *spe*F, *spe*G (all except one) and *sme*Z genes, whereas the other superantigen genes varied among the serotypes. The *S. aureus* isolates produced at least one superantigen each, SEA-D and/or TSST-1, but none of the isolatesstrains were positive for either exfoliative toxin A or B. All culture supernatants prepared from the isolates were potent inducers of proliferative responses. Similar to the previous reports of IVIG-mediated broad-spectrum inhibition of streptococcal superantigens (reviewed in 259), IVIG seems to exhibit a broad polyspecificity against staphylococcal superantigens since all staphylococcal isolates were equally inhibited regardless of their superantigen expression profile.

The study showed a significant difference in the potency of IVIG to inhibit streptococcal or staphylococcal isolates. The reason for this difference in inhibitory activity against streptococcal and staphylococcal superantigens is unknown, but is likely due to differences in quality and/or quantity of antibodies directed to these toxins. In a study by Holtfreters *et al*., it was shown that *egc*-encoded superantigens i.e. SEG, I, and M-O, were significantly less neutralised by plasma from health donors or by IVIG, than were other staphylococcal superantigens (260). The *S. aureus* isolates included in paper II were not tested for the presence of *egc*-encoded superantigens. However, the genes have been reported in 26–51% of *S. aureus* sepsis isolates (261), and hence, the results of a reduced inhibition of staphylococcal superantigens by IVIG may be, at least in part, contributed to the *egc*-superantigens. A differential neutralising activity of IVIG has previously been reported for defined streptococcal superantigens, with the superantigen SpeA being less efficiently neutralised in comparison to other streptococcal superantigens. In these studies, no correlation between ELISA activity against purified streptococcal superantigens and neutralising activity of IVIG could be demonstrated (243, 244). This indicated that the functional quality of the antibody is the most relevant factor, a finding that has also been reported in other studies (97, 100).

Previous studies have demonstrated that different IVIG preparations and different batches of the same preparation may contain varying levels of neutralising activity against *S. pyogenes* superantigens (243, 244). In this study we tested five preparations each of Gamunex® and Gamimune®, and they all showed superior neutralising activity against streptococcal and staphylococcal superantigens as compared to the one lot of Endobulin® S/D tested. Inter-batch variation could also be demonstrated, since two lots of Gamunex® differed in their neutralisation of *S. aureus* exotoxins. This was the only significant difference found within and between the different Gamunex® and Gamimune® lots. We have previously found an almost complete (98%) inhibition of streptococcal superantigens by Endobulin S/D (paper I and data not shown), and hence, the observed difference in neutralising activity of Endobulin S/D in comparison to Gamunex® or Gamimune® is likely the result of batch variation.

Difference in the degree of IVIG-induced neutralising activity between staphylococcal and streptococcal culture supernatants was relatively small, ranging between 6–11% at physiological concentrations of IVIG. However, we believe that even this small difference in neutralising activity may have an impact on clinical efficacy, inasmuch as the staphylococcal TSS patient in the IVIG trial (paper I) had only 41% neutralising activity in the plasma post-therapy, as compared to 98% for the streptococcal TSS patients. This was in concordance with *in vitro* data that showed reduced IVIG neutralising activity against the staphylococcal isolate, 65% as compared to 99% for the streptococcal isolates (paper I). Furthermore, a correlation between the *in vitro* neutralising activity of the IVIG preparation and the plasma neutralising activity following therapy has previously been reported for streptococcal TSS patients (243). Hence, together the data suggest that even slight variations in superantigen-neutralising activity may be clinically important, and that a higher dose may be needed to obtain protective antibody levels in the treatment of staphylococcal TSS. Importantly, our *in vitro* data demonstrate that 100% inhibition of *S. aureus* superantigens can be obtained at the higher doses of IVIG, and it is thus likely to obtain neutralising titers also in the patients.

4.3 EPIDEMIOLOGICAL STUDIES OF INVASIVE GROUP A STREPTOCOCCAL INFECTIONS (PAPERS III AND IV)

In these two papers we describe the epidemiology of invasive GAS disease in Denmark during 2001-2002 and in Sweden during 2002-2004, respectively. In Sweden, 746 isolates from patients with invasive disease were collected over the study period, which corresponds to annual incidences between 2.0 and 3.0 per 100 000 inhabitants. Somewhat lower incidences were reported in Denmark where 201 invasive cases were identified, corresponding to incidence rates ranging from 1.8 to 2.8 / 100 0000 (262). However, it seems likely that this difference merely reflects fluctuations over time, as the highest incidences in Denmark were noted in 2002, i.e. partly over-lapping with the Swedish study. These incidence rates demonstrate that the prevalence of invasive GAS infections has remained at a relatively high level in Sweden, as similar rates were reported from national surveillance studies conducted during 1996-97 and 1994-95. However, this incidence is higher than that seen before and during the M1T1 outbreak in 1987-1989 when the reported incidence ranged between 1.8 and 2.6 per 100 000 inhabitants (258). This general increase in number of invasive GAS over time was also seen in the prevalence of Danish isolates (262), and also among many other European countries (263).

A typical season variation was seen with increased numbers of invasive cases during the winter months. Similar trends in age distribution were seen in the Danish and Swedish material, with an increase of invasive disease with higher age and also among younger children (figure 5). The relatively high number of invasive isolates from children of 0-9 years of age may reflect the frequent occurrence of tonsillitis and impetigo within this age group, as well as the fact that young children are more sensitive towards infections since their immune system is not fully developed. This is also underscored by the mean age of 2.5 years among the 26 Swedish children with invasive disease in this age group. However, selection bias may have contributed to the higher number of non-invasive isolates from the younger age groups, since

children are more frequently visiting general practioners and being sampled. Non-invasive isolates sampled from skin were more common than throat isolates in age groups from 40 and upwards among Swedish patients. This might be due to the higher prevalence of skin infections among the elderly. Diabetes are more prevalent in elderly people, consistent with the finding that diabetes was the most prevalent underlying disease reported for the invasive cases where skin- and tissue infections were common complications.

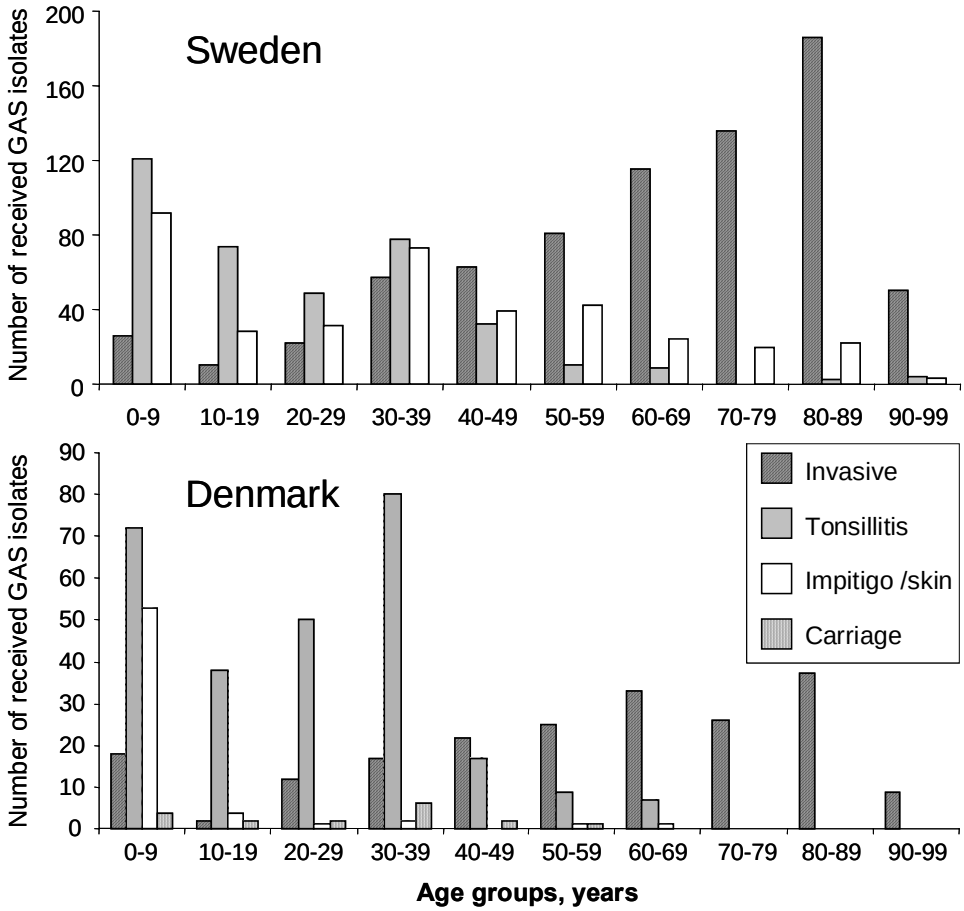


Figure 5. *Age distribution of invasive GAS infections collected during the Danish and Swedish surveillance studies. The non-invasive isolates from patients with either tonsillitis, impetigo/ skin infections or from healthy carriers were sampled during parts of the same periods. See materials and methods and papers III and IV for details.*

The severe manifestations, STSS or NF, occurred in approximately 10% of the invasive cases, which is similar to rates previously reported (14, 43). However, the overall case fatality rate was significantly lower in the Swedish material as compared to the Danish (14.5% and 25%, respectively; p= 0.008, Fisher's exact test) (table 10). Improved outcome was also seen among STSS patients with a case fatality rate of 39% and 55% in the Swedish and Danish material, respectively (table 10). The underlying rationale for the difference in mortality between the countries remains to be elucidated.

Table 10. Selected disease manifestations of patients with invasive GAS disease in the present studies and one previous Swedish study.

Clinical manifestation	Denmark [a] 2001-02 No. Cases (%) fatal %	Sweden 2002-04 [b] No. Cases (%) fatal %	Sweden 1996-97 [c] No. Cases (%) fatal %
Tot. invasive cases	201 (100) 25	654 (100) 14.5	144 (100) 16
Puerperal sepsis	8 (4.0) 13	17 (2.6) 0	16 (11) 6.3
NF	13 (6.5) 31	62 (9.5) 19.0	3 (2.1) NA
STSS	20 (10.0) 55	74 (11.3) 38.9	22 (15.2) 45

NF, Necrotising fasciitis, STSS, streptococcal toxic shock syndrome; NA, non available
[a] Fatal cases, death ≤ 30 days after positive culture, data obtained from the Central Office of Civil Registration in Denmark.
[b] Based on data from 654/ 746 (88%) returned questionnaires.
[c] Based on 144/255 (56%) returned questionnaires, see (43).
The groups of clinical presentations may be overlapping.

Interestingly, there was a marked difference in the type distribution among invasive isolates between the two studies, and also in comparison to the most recent Swedish surveillance study by Eriksson *et al.* performed in 1996-97 (43). In Denmark, *emm*-type 1, which previously has been associated with severe disease and fatal outcome (51, 55, 56, 264) was the most prevalent type, isolated from 32% of patients with invasive disease, followed by *emm*-types 28 (20%), 89 (10%) and 12 (5.5%) (table 11). In contrast, the four most prevalent *emm*/T-types among invasive cases in the present Swedish study were *emm*89 T3/13/B3264 (16%), *emm*81 T3/13/B3264 (14.1%), *emm*28 T28 (13.9%) and *emm*1 T1 (12%) (table 11). If the difference in mortality rates discussed above would be contributed to the higher prevalence of *emm*1 isolates in Denmark, this should have been reflected in higher percentages of STSS and NF cases, which in fact were lower as compared to Sweden (table 10). Hence, it seems unlikely that the presence of *emm*1 would have affected the mortality rates considerably. Among the Swedish isolates, *emm*1 T1 was the most common type among patients with STSS, but *emm*89 T3/13/B3264 was second most prevalent among STSS cases and the most prevalent type among fatal cases, which demonstrate that also this type is a cause of severe disease. The results from the most recent Swedish surveillance study performed between 1996 and 1997 showed yet another type distribution, since the most prevalent type then were *emm*28 T28 accounting for 31% of invasive isolates followed by T3/13/B3296 (~14%), T12 (12%), T8/25/imp19 (5%) and *emm*1 T1 (4.7%) (43). Hence, paper IV demonstrates a significant change in type distribution among invasive cases in Sweden during the 1990s and beginning of 2000.

To our knowledge, in no previous surveillance studies have *emm*-types 89 and 81 isolates been the dominant types, which make the findings from the Swedish study most interesting. Further, in the material presented by Johnson *et al. emm*89 isolates were most commonly associated with T-type 11 (46) (table 4), but in the present study, the vast majority of *emm*89 isolates belongs to T-types of the cluster 3/13/B3264.

When looking at the outcome and type distribution among the patients with different disease manifestations among Swedish patients, *emm*28T28 was the most common type among women with puerperal sepsis (table 11), which is in line with previous

observations that this type is commonly associated with puerperal sepsis (42, 63, 64). This association has been suggested to be attributed to the R28 protein expressed in particular by M28 isolates, which was shown to represent a novel type of GAS adhesin promoting binding to cervico-vaginal cells (65).

Table 11. *emm*-type distribution among invasive isolates, and disease manifestations, shown as percentage of respective *emm*-type and in descending order.

Denmark		Sweden [a]											
All invasive		All invasive		Puerperal sepsis		NF wo STSS		NF		STSS		Fatal	
n= 201		n= 746		n= 17		n= 30		n= 62		n= 74		n= 94	
1	32.3	**89**	16.0	**28**	35.3	**81**	23.3	**1**	22.6	**1**	28.4	**89**	18.8
28	19.9	**81**	14.1	**89**	15.4	**28**	20.0	**81**	14.5	**89**	13.5	**1**	14.6
89	10.0	**28**	13.9	**1**	11.8	**4**	13.3	**28**	12.9	**81**	12.2	**81**	11.5
12	5.5	**1**	11.9	**12**	11.8	**1**	10.0	**4**	9.7	**28**	10.8	**28**	10.4
4	5.0	**12**	6.3	**2**	5.9	**12**	10.0	**89**	8.1	**12**	6.8	**12**	8.3
6	3.5	**77**	5.9	**22**	5.9	**89**	6.7	**12**	6.5	**77**	5.4	**77**	8.3
77	2.5	**4**	5.9	**73**	5.9	**77**	6.7	**77**	4.8	**4**	4.1	**22& 3**	4.3
Other:	21.0	**Other:**	26.0	**Other:**	0.0	**Other:**	10.0	**Other:**	21.0	**Other:**	18.9	**Other:**	18.1

NF, Necrotising fasciitis; STSS, streptococcal toxic shock syndrome; wo, without.
[a] Sub-division of Swedish patients with invasive disease into manifestation groups is based on 654/ 746 returned questionnaires. The groups may be overlapping.

Further, results of paper IV showed that *emm*81 T3/13/B3264 isolated were more common among patients with skin and tissue involvement, as seen both among NF cases and among patients with any symptoms from skin or tissue or the skin stated as the probably port of entry of infection, as well as among non-invasive isolates originating from skin (tables 11, 12 and table 2 paper IV). When comparing NF patients without STSS to all NF patients, *emm*-type 1 and fatal outcome were associated with the addition of STSS, whereas *emm*-type 81 were more common in NF patients without STSS (table 11 and table 2 paper IV).

Although, the overall gender distribution among patients in the Danish and Swedish studies were similar, 47% and 49% were of male gender respectively, interesting differences were seen in the type distribution among male and female. *emm*28 isolates were significantly more common among female patients, and *emm*81 significantly more common among men (paper IV). Fiftynine percent of invasive and non-invasive isolates of *emm*-type 28, and 64% of invasive isolates alone, were isolated from women. Sixty percent of invasive and non-invasive *emm*81, 63% of invasive isolates alone, came from men. This is also in part reflected in the disease manifestations since *emm*28 is associated with puerperal sepsis and females, patients with skin tissue involvement/entry included 52% males and *emm*81 the most prevalent type (16.7%), NF without STSS, 57% males and *emm*81 most prevalent (23%) (table 11 and tables 1 and 2 paper IV).

In order to relate characteristics of GAS isolates causing invasive disease to isolates circulating in the community during the same period, non-invasive isolates were collected and characterised in parallel with the invasive in both the Danish and Swedish study. The distribution of the most prevalent types among non-invasive

isolates is shown in table 12. Isolates of *emm*-type 6 were more common in the Danish study, which also was seen among the invasive isolates. *emm*-type 89 and 81, on the other hand, were more prevalent in the Swedish study, also in parallel with the invasive isolates. Other similarities and discrepancies could also be seen. In concordance with previously reports, isolates of *emm*12, 4 and 28 were common among the non-invasive isolates (43, 67), and *emm*-type 12 was associated with tonsillitis as it was the most prevalent type in this group of patients, also in line with previous reports (68). An interesting difference were the *emm*4 isolates among skin isolates, as it was the most prevalent type (26%) among the non-invasive Danish isolates, whereas only 8% of the non-invasive Swedish isolates were *emm*4 (table 12). As the number of healthy carriers with GAS isolates sampled from their throats, were relatively few, it is hard to draw any conclusions from these. However *emm*28 were the most prevalent type in this group, and the types presented are similar to the ones of the other Danish non-invasive groups (table 12). The types of the Swedish isolates were more diverse as compared to the Danish, and comprised 54 different *emm*-types among non-invasive isolates as compared to 25 among the Danish. This can also bee seen in the percentage of "other types", presented in table 12. The same were also seen among invasive isolates, where the Swedish isolates presented 56 different *emm*-types and the Danish 26. However, this is likely due in part to the increased numbers of isolates included in the Swedish study.

Table 12. *emm*-type distribution among the non-invasive isolates, and disease manifestations, shown as percentage of respective *emm*-type and in descending order.

Denmark [a]								Sweden [b]					
All Non-invasive		Tonsillitis		Impetigo		Carriers		All Non-invasive		Tonsillitis		Skin origin	
n= 352		n= 273		n= 62		n= 17		n=773		n=389		n=384	
12	16.5	**12**	17.9	**4**	25.8	**28**	35.2	**28**	16.2	**12**	20.1	**81**	18.5
4	15.9	**6**	17.6	**28**	22.6	**4**	17.6	**12**	14.2	**28**	16.7	**28**	15.6
6	14.2	**4**	13.2	**1**	14.5	**12**	11.7	**81**	10.7	**4**	10.0	**89**	8.6
28	12.2	**1**	9.2	**12**	11.3	**6**	5.9	**4**	9.1	**1**	9.3	**12**	8.3
1	9.9	**28**	8.4	**6**	1.6	**1**	5.9	**89**	8.0	**89**	7.5	**4**	8.1
Other:	31.3	**Other:**	33.7	**Other:**	24.1	**Other:**	23.5	**Other:**	41.8	**Other:**	42.3	**Other:**	40.1

[a] The distributions of the five most prevalent types among the total of Danish non-invasive isolates are shown

[b] The distribution of the five most common types of each group are show, which were the same type as of the total, except for *emm*1 among Swedish tonsillitis cases.

In order to investigate the humoral response to the infection, the superantigen-neutralising capacity and levels of antibodies against streptolysin O and DNase B were determined in sera from 33 patients with invasive disease and compared to levels of 91 patients with non-invasive disease in the Danish study. The levels of anti-streptolysin O and anti-DNase B antibodies were significantly higher among patients with invasive or non-invasive disease, as compared to GAS negative controls. The frequency of superantigen-neutralising activity in sera was relatively low, as only 30% of sera from patients with non-invasive disease and 58% of sera from patients with invasive disease showed neutralising activity towards the patients' own infecting strains. These findings of a higher response in invasive cases is in sharp contrast to

previous studies in which lack of protective humoral immunity was evident in patients with invasive GAS disease who had significantly lower levels of both opsonising antibodies towards their infecting isolates and neutralising anti-superantigen antibodies, than patients with tonsillitis or healthy controls did (95-100). However, these studies were performed with material collected during the M1T1 outbreak, whereas the present study is conducted during a period without pronounced outbreaks and with a wide distribution of types circulating in the community. Another explanation is that these responses do not reflect the immunity at the time of infection but rather the response to the current infection, which is consistent with the fact that most sera were obtained at least three days after onset of infection. This would also explain the lack of a positive correlation between protective antibody titers and age of the patients. One interesting finding in the neutralising activity was the fact that the vast majority of patients could be divided into a responder (75-100% inhibition) or a non-responder with essentially no inhibitory capacity. Genetic predisposition attributed to the patient's HLA class II type has previously been shown to control the superantigen-mediated inflammatory response and consequently severity of infection (102). Whether this could also contribute to the antibody responses remains to be investigated.

Superantigens are known to be central mediators of the systemic effects seen in invasive GAS infections. In order to investigate possible differences in superantigen gene profiles, all invasive and non-invasive isolates were genotyped for 10 and 9 of the streptococcal superantigens in the Danish and Swedish study, respectively. The isolates harbored in average 5.2 of superantigen genes (paper IV) with no signficicant difference among invasive and non-invasive isolates. However, the results revealed significant differences between invasive and non-invasive isolates in the prevalence of certain superantigens. In the Danish material, *emm*1 isolates from invasive cases had a higher prevalence of the *spe*C and *ssa* genes, but lower prevalence of the *spe*A genes, than did non-invasive isolates. The opposite were seen among Swedish *emm*1 isolates where the *spe*C gene were significantly less prevalent among invasive than non-invasive isolates. Among *emm*28 isolates, *spe*A and *ssa* were significantly more prevalent among invasive as compared to non-invasive isolates in the Danish study, whereas the opposite were found for *spe*A and *emm*28 in the Swedish. These results indicate that there are differences in the regional distribution of isolates, even within the same types. In the Danish material, there was also a shift over time in the prevalence of the *spe*A and *ssa* genes among the invasive *emm*1 isolates, with significantly higher prevalence of both genes during the first half of the study. These data indicate that the superantigen gene profile may be a useful tool in discriminating clones within the same type, discussed further below. The data is in agreement with the hypothesis that there is not one superantigen responsible for the systemic effects seen in severe GAS infections but rather that all superantigens have the capacity to trigger these effects in susceptible individuals and there are likely several superantigens involved in each patient.

To further investigate the genetic relationships between the Swedish isolates, PFGE analysis were performed on isolates belonging to the major prevalent *emm*-types with the exception of the *emm*1 isolates, which have previously been reported to be highly related (94, 265). All invasive and non-invasive isolates of *emm*-types 89, 81, 77, 28

and 12 (n=844) were analysed and the vast majority of isolates (97%) clustered according to their *emm*-type with no discrimination between invasive or non-invasive isolates. Nor could the analysis discriminate between any specific disease manifestation or outcome of infection. However, different banding patterns within each *emm*-types, revealed potential sub-groups within each type, except for *emm*81 isolates that were all more closely related (table 3 in paper IV). At this stage, we decided to combine the PFGE-patterns with the superantigen profiles, to further investigate clonal relationships of the isolates. These revealed superantigen profiles among *emm*81 isolates and sub-groups of the *emm*89, and 12 isolates which differed significantly in prevalence among invasive and non-invasive cases. Among one sub-group of *emm*89 isolates and among *emm*81 isolates, specific superantigen profiles were found that were significantly more common among invasive isolates, and among one sub-group of *emm*12 isolates the opposite were found for a specific superantigen profile significantly more common among non-invasive isolates. A specific superantigen profile among *emm*1 isolates were found to be more prevalent among invasive, and the opposite found for one of the profiles among *emm*4 isolates (table 3 in paper IV). These findings are of interest since they indicate that there may exist sub-groups of isolates with higher invasiveness within an *emm*-type. However, this is currently being debated since the prevalence of specific virulence-associated clones appears to reflect their prevalence in the normal population (264, 266).

The questionnaires also included information on the use IVIG therapy in patients with invasive GAS infections. The results showed that IVIG had been used in some cases, all with STSS. Of the 20 Danish STSS cases, treatment was known in 19, and 5 (26%) of these received IVIG. The mortality in the IVIG-treated group was 20% as compared to 64% among non treated (K. Ekelund, Hvidovre Hospital, personal communications). Among the 74 Swedish STSS cases, the outcome was known for 72. Of these, 20 (28%) had received IVIG and in this IVIG-treated group the mortality was 15% as compared to 48% among STSS patients who had not received IVIG ($p<0.0142$) (table 6 in paper IV). Comparisons between the two treatment groups showed that the IVIG-treated patients were younger, but importantly, these patients had a higher number of organ failures and presence of NF indicative of a more severe course of infection, than in the untreated group. This makes the difference in outcome between the treatment groups even more impressive. In April of 2004, the Swedish drug agency board published treatment recommendations for sepsis in which IVIG treatment was recommended for patients with STSS. Following this publication, a positive trend to increased IVIG therapy was seen; from 21% to 47% of STSS patients received IVIG.

Here we have highlighted the main results from national surveillance in Denmark and in Sweden, and we describe interesting similarities as well as differences. These studies are both part of a larger European network, called StrepEuro. StrepEuro was designed to provide uniform and comparable epidemiological data on invasive GAS infections in Europe, and 11 different countries. The surveillance has recently been completed and the database is currently being analysed. This project is anticipated to greatly advance the knowledge of incidence of these infections, clinical manifestations, and antibiotic resistance in relation to spread of specific GAS clones.

5 CONCLUDING REMARKS

- The clinical trial demonstrated a reduced mortality rate and a significant improved organ function among STSS patients receiving IVIG as compared to placebo.

- Further support for the use of IVIG as adjunctive therapy was provided by the national surveillance study of invasive GAS infections, which revealed a significant improved survival among STSS patients receiving IVIG.

- *In vitro* studies revealed that a higher dose of IVIG was required to achieve 100% neutralisation of the staphylococcal superantigens as compared to the streptococcal superantigens. Hence, if IVIG is used as an adjunctive therapy in staphylococcal TSS, a higher dose (i.e. 2 g/kg body weight) is likely required to achieve protective effect.

- National surveillance of invasive GAS infections was conducted in Sweden and in Denmark during a time period without large outbreaks. Differences were seen in the outcome of disease, and in GAS type distribution. In Sweden, two novel *emm*-types, *emm*89 and 81, were found to be the most prevalent types in the community. Specific subpopulations of isolates particularly prone to cause invasive disease were identified through a combination of *emm*-typing, superantigen profiling and PFGE analysis. Furthermore, the superantigen-neutralising activity in sera obtained from patients with invasive or non-invasive patients were relatively low, which is in contrast to previous findings obtained during outbreak periods.

In conclusion, this thesis provides further support for the use of IVIG in STSS, both through the clinical trial as well as through the surveillance data. The thesis also demonstrates important changes in the epidemiology of invasive GAS infections in Sweden, among others the introduction of new types in the community. This further emphasises the importance of surveillance studies conducted during non-outbreak episodes.

6 SAMMANFATTNING PÅ SVENSKA

Streptococcus pyogenes, även kallad grupp A streptokocker (GAS), är en både vanlig och viktig orsak till sjukdom hos människan. Denna bakterie är den vanligaste orsaken till halsfluss (tonsillit) och är även orsak till andra sjukdomar som tex hudsjukdommen rosfeber. Ibland kan GAS orsaka mer allvarliga sjukdomstillstånd; ca 250-300 personer i Sverige blir årligen sjuka i blodförgiftning (sepsis) orsakad av GAS. Hos en liten andel av dessa patienter kompliceras sjukdomstilståndet ytterligare då patienten utvecklar necrotiserande fascit (NF), en allvarlig hud-/mjukdelsinfektion där vävnaden bryts ner, och/eller så kallat ”streptococcal toxic shock syndrome” (STSS) då patienten är både i klinisk chock och har multiorgansvikt. Dödligheten i dessa två allvarligaste sjukdomstillstånd är rapporterad ofta så hög som 30-80% av fallen. Intravenöst imunoglobulin (IVIG) har föreslagits som tilläggsbehandling vid STSS eftersom det bla innehåller antikroppar som neutraliserar de speciella toxin, sk. superantigen. Dessa toxin är viktiga bakteriella faktorer delaktiga i det svåra sjukdomsförloppet i STSS och NF.

I det första arbetet i avhandlingen presenteras resultaten från en randomiserad multicenterstudie av IVIG-behandling hos patienter med STSS. Studien genomfördes vid sammanlagt sjutton olika sjukhus i Sverige, Norge, Finland och Nederländerna. Som en följd av oväntat långsam patientrekrytering, då incidensen av STSS-patienter bland medverkande sjukhusen var låg, tvingades studien desvärre avslutas i förtid. Under de nästan 2½ år som studien pågick inkluderades 21 patienter, varav 10 erhöll IVIG och 11 placebo, utöver ordinarie behandling. Det främsta målet med studien var att studera eventuella skillnader i överlevnad efter 28 dagar. Trots att materialet var litet, noterades en tendens till ökad överlevnad bland de patienter som fick IVIG, 10% jämfört med 36% i placebogruppen. Vi noterade även en signifikant sänkning av ”sepsis-related organ failure assessment” (SOFA) poäng hos IVIG-behandlade patienter jämfört med de övriga. Denna bedömning ger ett poängbaserat mått på hur allvarligt sjuk patienten är. Blodprov togs före och efter behandling, och analyser visade att de patienter som fått IVIG påvisade en signifikant bättre förmåga att neutralisera superantigen som producerats av varje patients eget bakterieisolat. Någon sådan förändring kunde inte ses bland de prov som tagit från placebo-patienterna.

En av patienterna som inkluderats som en misstänkt STSS-patient i den kliniska prövningen visade sig ha ”toxic shock syndrome” (TSS) orsakad av *Staphlococcus aureus*, vilken i likhet med STSS är en superantigenmedierad sjukdom. Patienten erhöll IVIG och i analyser av blod kunde vi se att superantigen inte neutraliserades i lika hög grad som hos patienter med STSS. Detta fynd ledde oss till delarbete II, där vi jämför IVIGs förmåga att neutralisera olika superantigener, antingen från GAS eller *S. aureus* isolat, *in vitro*. Isolaten som användes i försöken var samtliga från patienter med allvarlig sepsis. Tre olika IVIG-preparats förmåga att neutralisera superantigenerna från de olika bakterieisolaten undersöktes. Vi såg att samtliga preparat hämmade superantigen från både GAS och *S. aureus* isolat, däremot krävdes

högre koncentrationer av IVIG för full hämning av *S. aureus*-superantigen än för dem från GAS. Dessa fynd är även av klinisk relevans om man vill behandla *S. aureus* TSS med IVIG, eftersom det då kan behövas en större dos IVIG för att uppnå skyddande nivåer av antikroppar hos dessa patienter.

I delarbete III och IV beskrivs resultat från två nationella övervakningsstudier av invasiv GAS-sjukdom, genomförda i Danmark och Sverige under 2001-02 respektive 2002-04. De bakteriologiska laboratorierna i de båda länderna deltog genom att skicka in all invasiva isolat under respektive studieperiod. Klinisk data för varje patient erhölls genom enkätsvar från behandlande läkare. Insidensen i de båda länderna var likartad och varierade mellan 2,0 och 3,4 per 100 000 invånare och år. Likaså var andelen patienter med de allvarligaste sjukdomsbilderna NF och STSS likartad och drabbade omkring 10% av patienterna årligen. Däremot noterades en skillnad i dödlighet mellan de båda studierna, då 25% av patienter med invasiv GAS infektion avled i den danska studien, jämfört med 14,5% i den svenska. Även typfördelningen bland isolaten skiljde de båda studierna åt. *emm*-typ 1 var den vanligast förekommande typen i den danska studien (i 32% av fallen), medan de båda nya typerna *emm*89 (16%) och *emm81* (14%) var vanligast i den svenska. Parallellt med de invasiva isolaten insamlades även icke-invasiva isolat från patienter med hals- eller hudsjukdom, samt några isolat från friska bärare. Materialet jämfördes med det invasiva, och vi fann även här skillnader i typfördelningarna, både mellan ivasiva och icke-invasiva isolat samt mellan länderna. Vi utförde också ytterligare en rad olika karaktärsbestämningar av alla isolat vi samlat in, bland annat undersöktes vilken genuppsättning varje isolat hade för olika superantigen. I den svenska studien utfördes dessutom släktskapsanalyser mellan isolat av de vanligast förekommande *emm*-typerna. Vidare visade enkätsvaren att IVIG behandling signifikant förbättrade utgången för patienter med STSS. 20 av 72 patienter behandlades med IVIG och endast 15% av dessa avled. Bland övriga 52 patienter var mortaliteten 48%. I april 2004 publicerade läkemedelsverket nya rekommendationer för behandling av sepsispatienter. Dessa inkluderar IVIG vid behandling av STSS, och efter detta iakttogs en ökad användning av IVIG bland STSS-patienterna i studiematerialet, från 21% till 47%.

Denna avhandling styrker ytterligare IVIG som tilläggsbehandling vid STSS. *In vitro*-analyser visade att en högre dos av IVIG kan behövas för att nå skyddande nivåer av antikroppar vid TSS orsakad av *S. aureus*. Avhandlingen ger oss också nya insikter om molekylärepidemiologin för invasiva GAS-sjukdomar.

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9 APPENDIX (PAPERS I-IV)