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Nygren, David

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DAVID NYGREN
DEPARTMENT OF CLINICAL SCIENCES, LUND | FACULTY OF MEDICINE | LUND UNIVERSITY

Fusobacterium necrophorum

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- from tonsillar carriage to Lemierre's syndrome

David Nygren

LUND
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Abstract:

Fusobacterium necrophorum is the main causative agent of Lemierre's syndrome, where the bacteria, following a benign initial oropharyngeal infection, cause septic thrombophlebitis of the internal jugular vein with subsequent pulmonary embolisation. Despite that data are lacking to suggest so, *F. necrophorum* has been described as normal tonsillar flora for decades. We investigated tonsillar carriage in Sweden and Zambia among mainly adolescents and young adults (Paper I). These are the age groups most affected by its infections. *F. necrophorum* was found to be present in one in five asymptomatic participants in Sweden, yet was rarely identified in Zambia. Given the finding of a relatively high tonsillar carriage rate in Sweden, the impact of a finding of *F. necrophorum* in pharyngotonsillitis was evaluated (Paper II). Interestingly, *F. necrophorum* was found to be a very prevalent finding and equally associated with the development of complications as *Streptococcus pyogenes*.

Antibiotic therapy in non-streptococcal pharyngotonsillitis has decreased in recent decades, yet simultaneously concerns of an increase in complications have been suggested. To investigate the incidence rate of invasive infections with *F. necrophorum*, an eight-year nationwide study in Sweden was performed (Paper III). All invasive presentations of *F. necrophorum* increased, including Lemierre's syndrome. Finally, the hallmark of Lemierre's syndrome was studied, i.e., the septic thrombophlebitis. We investigated the effects of anticoagulant therapy in Lemierre's syndrome and found that while harm due to treatment was rare, so were the benefits (Paper IV). Finally, plasma from all patients with Lemierre's syndrome in Skåne in 2017-2021 was compared with controls with other severe infections to investigate differentially expressed proteins by mass spectrometry and generate hypotheses on thrombogenesis (Paper V). Several thrombogenic pathways were identified, including endothelial injury, platelet activation, and linkage of innate immunity with coagulation. These thrombogenic pathways are hypothesised to be important in the development of septic thrombophlebitis and specific proteins highlighted.

In this thesis, we trail *F. necrophorum* from tonsillar carriage (Paper I) to pharyngotonsillitis (Paper II) and onwards to Lemierre's syndrome and other invasive infections (Papers III-V). We highlight the specifics of each presentation, evaluate the treatment and hypothesise on pathogenesis through epidemiological and translational studies. Our most important findings include reproting a nationwide increase of incidence across all invasive presentations, a geographical difference in tonsillar carriage, the establishment of *F. necrophorum* as a pathogen in pharyngotonsillitis, and generation of hypothesised thrombogenic pathways in Lemierre's syndrome.

Key words: *Fusobacterium necrophorum*, tonsillar carriage, pharyngotonsillitis, Lemierre's syndrome, septic thrombophlebitis, jugular vein thrombosis

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Fusobacterium necrophorum
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David Nygren

LUND
UNIVERSITY

Supervisor: Karin Holm

Co-supervisor: Gustav Torisson

Faculty of Medicine
Department of Clinical Sciences, Lund

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“One could retire with such scientific luggage as his;

yet that is not the character of André Lemierre”

– Marc Burnt on André Lemierre in 1921

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Preface

Before Karin presented the ideas of the projects that became this thesis, I knew little about *Fusobacterium necrophorum.* Based on its name, I realised that this might be serious. The genus name *Fusobacterium* describes the shape (spindle-shaped bacteria) whereas the species name *necrophorum* is a bit more morbid. It could freely be translated as 'the bringer of death', and perhaps rightly so. When Professor André Lemierre described the syndromic presentation of a throat infection followed by the development of septic jugular thrombophlebitis and systemic embolisation in young adults and adolescents in Paris in 1936, 90% of cases described died from the disease. As you will be aware if you finish this read, Lemierre's syndrome is luckily not as incurable today, yet the bacterium and its syndrome still deserve attention.

In this thesis, I will try and guide you from the asymptomatic tonsillar carriage of the bacterium in adolescents and young adults, to when a subset of these individuals develops pharyngotonsillitis. Some will suffer local complications, mainly in the form of peritonsillar abscess or recurrence of pharyngotonsillitis. In a rare few, Lemierre's syndrome will occur, and its incidence, clinical presentation, pathogenesis, and treatment will be highlighted.

List of Papers

Papers included in this thesis, which will be referred to in the text as Paper I-V.

Paper I

Nygren D, Brorson E, Musonda M, Wasserstrom L, Johansson Å, Holm K. Geographical differences in tonsillar carriage rates of *Fusobacterium necrophorum* – A cross-sectional study in Sweden and Zambia, *Anaerobe*, **2021**;69:102360

Paper II

Nygren D, Wasserstrom L, Holm K, Torisson G. Associations Between Findings of *Fusobacterium necrophorum* or β-Hemolytic Streptococci and Complications in Pharyngotonsillitis – A Registry-Based Study in Southern Sweden. *Clinical Infectious Diseases*, **2023**;76(3);e1428-e1435

Paper III

Nygren D, Holm K. Invasive infections with *Fusobacterium necrophorum* including Lemierre's syndrome – An eight-year Swedish nationwide retrospective study. *Clinical Microbiology and Infection*, **2020**;26(8):1089.e7–1089.e12.

Paper IV

Nygren D, Elf J, Torisson G, Holm K. Jugular Vein Thrombosis and Anticoagulation Therapy in Lemierre's Syndrome – A Post Hoc Observational and Population-Based Study of 82 Patients, *Open Forum Infectious Diseases*, **2021**;8:ofaa585

Paper V

Nygren D, Torisson G, Happonen L, Mellhammar L, Linder A, Elf J, Yan H, Welinder L, Holm K. Proteomic characterization of plasma in Lemierre´s syndrome. **2023**; Manuscript submitted.

List of Papers not in thesis

Alsved M, **Nygren D**, Thuresson S, Fraenkel C-J, Medstrand P, Löndahl J. Size distribution of exhaled aerosol particles containing SARS-CoV-2 RNA. *Infectious Diseases*, **2023**;55(2):158-63

Nygren D, Mölstad U, Thulesius H, Hillman M, Broman LM, Tanash H, Landin-Olsson M, Rasmussen M, Thunander M. Low prevalence of mild Alpha-1-antitrypsin deficiency in hospitalized COVID-19-patients. *International Journal of General Medicine*. **2022**;15:5843-8

Alsved M, **Nygren D**, Thuresson S, Medstrand P, Fraenkel C-J, Löndahl J. SARS-CoV-2 in exhaled aerosol particles from covid-19 cases and its association to household transmission, *Clinical Infectious Diseases*, **2022**;75(1):e50-e56

Nygren D, Oldberg K, Holm K. Short blood culture time-to-positivity in *Fusobacterium necrophorum* bacteremia is associated with Lemierre's syndrome, *Anaerobe*, **2022**;73:102474

Nygren D, Norén J, De Marinis Y, Holmberg A, Fraenkel C-J, Rasmussen M. Association between SARS-CoV-2 and exposure risks in health care workers and university employees – a cross-sectional study, *Infectious Diseases*, **2021**;53:460-8

Nygren D, Älverbrandt M, Sunnerhagen T, Fagman E, Ostenfeld E, Rasmussen M. Aortitis caused by *Abiotrophia defectiva*: Description of two cases, *Infectious Disease Reports*, **2018**;10(3):7746

Nygren D, Nilson B, Rasmussen M. "A Case of Recurrent Erysipelas Caused by Streptococcus mitis Group," *Case Reports in Infectious Diseases*, **2018**:5156085

Nygren D, Hård af Segerstad C, Ellehuus Hilmersson C, Elf J, Ekelund U, Lundager Forberg J. "Goda resultat när akutläkare diagnostiserade djup ventrombos" *Läkartidningen.* **2018**;115

Nygren D, Stoyanov C, Lewold C, Månsson F, Miller J, Kamanga A, Shiff CJ. Remotely Sensed, Nocturnal, Dew Point Correlates with Malaria Transmission in Southern Province, Zambia: A Time-Series Study. *Malaria Journal*. **2014**;13:231

Nygren D, Isaksson, AL. Battling malaria in rural Zambia with modern technology: a qualitative study on the value of cell phones, geographical information systems, asymptomatic carriers and rapid diagnostic tests to identify, treat and control malaria. *Journal of Public Health in Africa.* **2014**;5(1):171

Abbreviations

16S-rRNA	16S ribosomal Ribonucleic Acid
95% CI	95% Confidence Interval
Ct	Cycle threshold
DIA	Data Independent Acquisition
EBV	Epstein-Barr Virus
GAS	Group A Streptococci (*Streptococcus pyogenes*)
GCS	Group C Streptococci (commonly SDSE)
GGS	Group G Streptococci (commonly SDSE)
gyrB	Gyrase B
ICD-10	International Classification of Diseases, 10th revision
ICU	Intensive Care Unit
IQR	Interquartile Range
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RADT	Rapid Antigen Detection Test (for GAS)
rpoB	RNA polymerase B
SDSE	*Streptococcus dysgalactiae* subspecies *equisimilis*
SOFA	Sequential Organ Failure Assessment

Thesis at a glance

Paper	Aim	Method	Results	Conclusion
I	To investigate the tonsillar carriage of *Fusobacterium necrophorum* in Sweden and Zambia.	A cross-sectional study of tonsillar carriage of *F. necrophorum* in 15-25-year-olds in Sweden (n=100) and participants aged 15 or above in Zambia (n=282). PCR was used to identify tonsillar carriage.	In Swedish 15-25-year-olds the tonsillar carriage rate was 21%. In the same age group in Zambia, the tonsillar carriage rate was 3%. Above 25 years of age in Zambia, the tonsillar carriage rate was 1%.	In Swedish adolescents and young adults, tonsillar carriage was relatively common, whereas geographical differences were apparent with low carriage rates seen in all ages in Zambia.
II	To investigate associations between microbiological findings in pharyngotonsillitis and the development of complications.	A retrospective analysis of pharyngotonsillitis cases tested for *F. necrophorum* and beta-hemolytic streptococci (n=3700). The association of microbiological findings with the development of complications (30 days) was investigated with logistic regression.	28% had *F. necrophorum*, 13% GCS/GGS, 10% GAS and 54% had negative results. Complications were common (20%). *F. necrophorum* (OR 1.8; 95%CI 1.5-2.1) and GAS (1.9; 1.5-2.5) were associated with complications, whereas GCS/GGS were negatively associated (0.7; 0.4-0.98).	*F. necrophorum* was similarly associated with complications as GAS, whereas GCS/GGS had fewer complications than cases testing negative. Testing for beta-haemolytic streptococci beyond GAS with RADT is questioned.
III	To describe the incidence and presentation of invasive infections with *F. necrophorum* in Sweden.	A nationwide, population-based descriptive retrospective study of all invasive infections with *F. necrophorum* in Sweden 2010-17 (n=300). Patients were grouped as Lemierre's syndrome, head and neck infections without Lemierre's syndrome, and non-head and neck infections and compared.	The incidence rate of invasive infections with *F. necrophorum* increased from 2.9 to 5.0 cases/million/year from 2010-13 to 2014-17. Lemierre's syndrome increased from 1.0 to 1.7 cases/million/year. Patients with Lemierre's syndrome had low mortality (2%) yet commonly required ICU-admission (43%).	Invasive infections with *F. necrophorum*, including Lemierre's syndrome, increased during the study period. An updated clinical description of Lemierre's syndrome and other invasive presentations is presented.
IV	To investigate the impact of the presence of jugular vein thrombosis in Lemierre's syndrome, and assess the impact of anticoagulation therapy on outcomes.	A nationwide, population-based retrospective cohort study of all cases with Lemierre's syndrome radiologically investigated for jugular vein thrombosis (n=82). The impact of therapeutic, prophylactic, or no anticoagulation therapy was investigated in patients with jugular vein thrombosis (n=51).	Patients with jugular vein thrombosis had lower platelets (median 76 vs 112*109/L, p=0.04), took longer to defervesce (12 vs 7 days, p=0.03) yet had similar 30-day mortality and major sequelae when compared to patients without jugular thrombosis. No differences in study outcomes among patients with jugular vein thrombosis in relation to anticoagulation were seen.	Patients with Lemierre's syndrome with jugular vein thrombosis were more severely affected, yet with a similar prognosis. Regardless of anticoagulation, study outcomes were similar.
V	To generate hypotheses on why patients with Lemierre's syndrome develop septic thrombophlebitis.	A case-control study comparing Lemierre's syndrome (n=8) with other severe infections (n=15) without thrombosis, using DIA liquid chromatography tandem mass spectrometry of plasma.	23 differentially expressed proteins were seen of which 16 have previously been associated with thrombosis. 14 had suggested prothrombotic effects (e.g., CD44-antigen, neutrophil defensin-1). 2 had suggested antithrombotic effects (e.g., Plastin-2).	Platelet activation, endothelial injury, and linkage of innate immunity with thrombogenesis are hypothesised to be important pathogenic pathways through which septic thrombophlebitis develops.

Thesis introduction

> "To anyone instructed as to the nature of these septicaemias it becomes relatively easy to make a diagnosis on the simple clinical findings. The appearance and repetition several days after the onset of a sore throat (and particularly of a tonsillar abscess) of severe pyrexial attacks with an initial rigor, or still more certainly the occurrence of pulmonary infarcts and arthritic manifestations, constitute a syndrome so characteristic that mistake is almost impossible"
>
> – André Lemierre 1936

This quote originates from the paper "On certain septicaemias due to anaerobic organisms" published by Professor André Lemierre in 1936 in The Lancet (1). After reviewing hundreds of medical records of patients with invasive *F. necrophorum* infection in Sweden, I know that despite its peculiar presentation, identification of the syndrome is often missed, and it is mainly due to its rarity that we do not think of it. Thankfully, the availability of microbiological diagnostics in our part of the world has improved markedly since the earliest descriptions of the syndrome (1, 2, 3, 4, 5), aiding clinicians of today.

Even though it carries his name, André Lemierre was not the first to describe the syndrome or the bacterium. It was rather his continuous publications of case reports and series describing the syndrome that led to it being referred to as Lemierre´s syndrome first in the 1980s (6). Despite several papers in the pre-antibiotic era mentioning the role of *F. necrophorum* as part of our normal oral or tonsillar flora, a cause of pharyngotonsillitis, pharyngeal abscesses as well as involved in invasive disease, the evidence of these statements was scarce. Following the introduction of antibiotics, *F. necrophorum* appeared to diminish in clinical importance. In fact, during the 1980s, when the syndrome was termed Lemierre's, it was cited as forgotten with numerous papers since describing it as the "forgotten disease" (7, 8, 9, 10). Since then, the interest in the pathogen has increased yet again and as will become apparent later in this thesis, often thanks to improved microbiological methods. During these last decades, it has increasingly been recognised as an important pathogen in common infections (e.g., pharyngotonsillitis (11, 12) and peritonsillar abscess (13)) in addition to its rare but severe presentations (e.g., Lemierre's syndrome) (14). Interestingly, with each decade, the incidence rates have been reported to increase when examining data from studies originating in high-

income countries such as the United Kingdom (15), Canada (16), Denmark (17, 18, 19), Finland (20), and Sweden (21).

Taken together, the goals of this thesis were several and meant to increase the understanding as we follow *F. necrophorum* from asymptomatic tonsillar carriage, to when it causes local pharyngeal infection, its complications, and finally its invasive disease. Despite increasing interest in *F. necrophorum* during the last decades, several unknowns remain. First, tonsillar carriage rates have varied, and data were missing globally. Second, whether a finding of *F. necrophorum* in a patient with pharyngotonsillitis was associated with more severe disease or the development of complications was not known. Third, increased incidence has been suggested in several papers including recent regional findings (22). Population-based data and an updated description of Lemierre's syndrome and the other invasive presentations were needed to follow up on whether the suggested increases have continued. Finally, septic thrombophlebitis as part of Lemierre's syndrome is increasingly reported as being treated with anticoagulation therapy (23), yet population-based studies of its effect were lacking, and recent meta-analyses of existing evidence have not provided consistent conclusions (23, 24). Similarly, the pathogenesis underlying septic thrombophlebitis in sepsis is poorly understood, and no prospective population-based studies of the pathogenesis in Lemierre's syndrome existed. With these gaps of knowledge in mind, the five papers of this thesis were designed.

Fusobacterium necrophorum

History

F. necrophorum infections in humans are caused by *F. necrophorum* subspecies *funduliforme*, whereas *F. necrophorum* subsp. *necrophorum* is an important pathogen in animals. When *F. necrophorum* was first described as a pathogen in 1884 (25), then called *Bacillus necrophorus* (26), it was identified as a cause of calf diphtheria and bovine liver abscess (27). The first description of a human infection with what is believed to have been *F. necrophorum*, dates back to 1891. When Schmorl and his assistant were conducting studies on rabbits with necrobacillosis, a term sometimes used synonymously to Lemierre's syndrome, the researchers developed abscesses on their fingers, with Gram negative filamentous bacteria found to be present (14, 28). This likely represented *F. necrophorum* subsp. *necrophorum* (the animal strain).

Instead, the first descriptions of what this thesis focuses on, infections in humans caused by *F. necrophorum* subsp. *funduliforme*, is commonly ascribed to Jean Hallé. During work on his thesis in 1898 he mainly investigated the bacteriology of the female genital tract (29). The bacterium he then identified was referred to as *Bacillus funduliformis.* As Riordan writes in an extensive and excellent review of literature on *F. necrophorum* (14), a review that has served as a library of historical knowledge throughout the work on this thesis, Jean Hallé described them as similar to *boudins* (French blood sausages) or slingshots and named them using the Latin term *fundula* (which directly translates as slingshot) to describe their characteristics. Luckily, he also made drawings of the pleomorphic morphology of the bacteria, for his analogies to be easier understood (Figure 1). Thus, the name *Fusobacterium necrophorum* subspecies *funduliforme,* translates to *'spindle-shaped bacteria which bring death* of the subspecies *that looks like slingshots'* (or French blood sausages). For the purposes of this thesis, I will stick to referring to it as *F. necrophorum*.

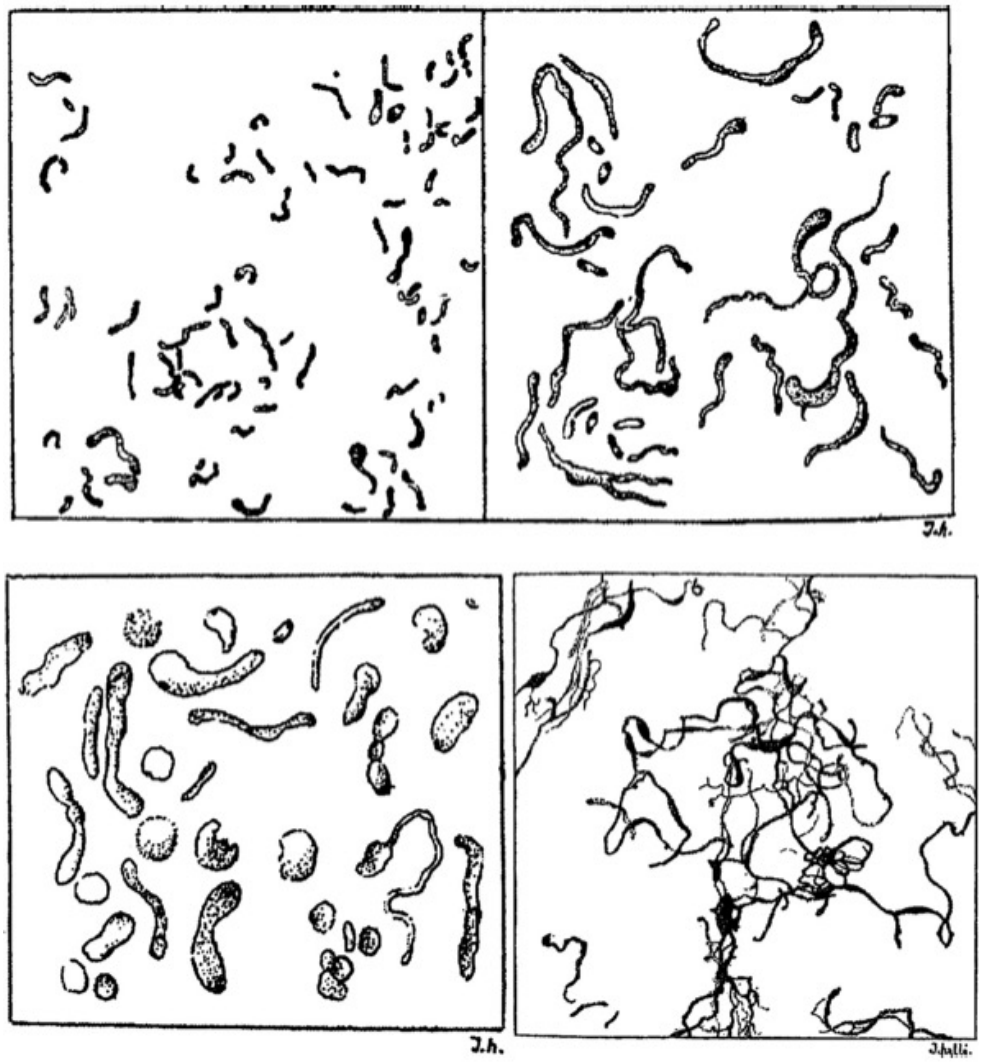

Figure 1: Drawings from the doctoral dissertation of Jean Hallé, 1898
These drawings by Jean Hallé highlight the pleomorphism seen in Gram stains of *F. necrophorum* subsp. *funduliforme* (29). The top left shows short, slightly curved rods from pus and tissues. The top right shows rare, giant forms. The bottom left shows vesicular forms sometimes found in cultures and the bottom right shows the most typical forms, seen in Gram stain from cultures with curling and tangling of short and long rods.

The first published case of what is now known as Lemierre's syndrome is commonly cited as a paper by Courmont and Cade from 1900 (2). Here, a case is presented of a patient with an initial complaint of a sore throat who then developed rigors and sepsis. On the subsequent autopsy, multiple lung abscesses (multifocal septic pulmonary embolisation) were seen. From pus and culture, pleomorphic anaerobic Gram negative bacilli were demonstrated. However, no detailed examination of the neck was described, why the presence of internal jugular vein thrombosis was not confirmed or rejected. Others (including André Lemierre) (14) have given credit to Veillon and Zaber (30) or Long (3) for the first descriptions of this syndromic presentation.

Some of the confusion is partly due to the misperception about the causative organism, in part due to deficient anaerobic culture methods. Misattribution to different streptococci, most commonly alpha-haemolytic, has often occurred. Nevertheless they remain relatively common as co-pathogens (14, 31). With improved anaerobic culture methods, the role of *F. necrophorum* (at that time still named *B. funduliformis*) was appreciated (32, 33, 34, 35). It is during this time (1930s) that Lemierre published frequently (1, 32). Thus, André Lemierre was not the first to discover *F. necrophorum* or describe the syndrome but instead earns credit for recognising it as a specific disease secondary to a specific pathogen with a specific presentation, again returning to the quote at the very beginning of this thesis (1). Many others also deserve credit for early and later contributions, including Alston, who described several cases in 1955 (36). His work as well as reviews by Brazier in 2006 (37) and Riordan in 2007 (14) are recommended for historical and fascinating reads.

Taxonomy

Now, let us focus on the confusion regarding the taxonomy of the bacterium. In Swedish, there is a saying that translates as "a dear child has many names", with the sentiment that we name those or that dear to us in many ways. In the book *"Anaerobic bacteria in human disease"* by Sydney Finegold (38) it is stated that *F. necrophorum* has been known by up to 52 names. Evidently, microbiologists are very fond of *F. necrophorum*.

F. necrophorum is part of the genus *Fusobacterium* (of the *Fusobacteriacae* family, *Fusobacteriales* order, and *Fusobacteria* phylum). Among species, *F. necrophorum* and *F. nucleatum* are the two most important in terms of human disease and are phylogenetically different (39, 40). As for many bacteria, the taxonomy has continuously been updated given sequencing data (40, 41, 42, 43, 44).

F. necrophorum is a strictly anaerobic, non-motile, non-spore-forming Gram negative bacillus. Two different subspecies are known and were already recognised

by André Lemierre's group (14), as one causing animal and one causing human infection. This has been confirmed (44, 45, 46, 47, 48), and while initially referred to as *F. necrophorum* biovar A (animal infections) and biovar B (human infections), they are now referred to as subsp. *necrophorum* (animal infections) and subsp. *funduliforme* (human infections) (46). The latter, while mainly causing infections in humans, is also found in the gastrointestinal tract of animals but is then described as commensal (46).

The routine identification of *F. necrophorum* subsp. *funduliforme* from cultures can be performed according to the description by Jensen et al. (49). This involves recognising the specific colony morphology of *F. necrophorum* subsp. *funduliforme*, to make use of its susceptibility to kanamycin and metronidazole, its smell of butyric acid, its chartreuse colour fluorescence, and recognising the beta-haemolysis on horse blood agar. This method is specific (49) and is used at some laboratories also in Sweden, mainly for identification in anaerobic throat swab cultures. We used a similar method when performing anaerobic cultures in Zambia in addition to the data presented in Paper I (data not published). However, for species identification of invasive infections, the method of choice is MALDI-TOF MS (50, 51). Confirmatory species identification with PCR as presented by Aliyu et al. (52) or Jensen et al. (53) based on the sequences of the *rpoB* and *gyrB*-genes is also used, particularly when MALDI-scores are not distinct. 16S-rRNA gene sequencing has also been shown to be useful as a diagnostic tool (54). In addition, 16S-23S rRNA has been used in phylogenetic studies (40), yet for these purposes targeted *rpoB*-based sequencing appears to have the highest resolution at the sub-species level (41, 44). Whole-genome sequencing has consistently identified *F. necrophorum* subsp. *funduliforme* from human throat cultures (and not subsp. *necrophorum*) (55), but rare reports of *F. necrophorum* subsp. *necrophorum* in human infections do exist (56). By whole-genome sequencing, isolates from patients with Lemierre's syndrome have been described to be homogenous and to exhibit high synteny (57).

Morphology

In addition to genetic differences between *F. necrophorum* subsp. *funduliforme* and subsp. *necrophorum*, differences in morphology are quite clear. *F. necrophorum* subsp. *funduliforme* are shorter, with a cocco-bacillary appearance in Gram stain (Figure 2) compared to the more rod-like appearance of subsp. *necrophorum.* On the agar plate, colonies of subsp. *funduliforme* are rounded, shiny, and creamy as compared to more umbonate, dull, and waxy in subsp. *necrophorum* (45, 47, 49, 58, 59) (Figure 3A-B).

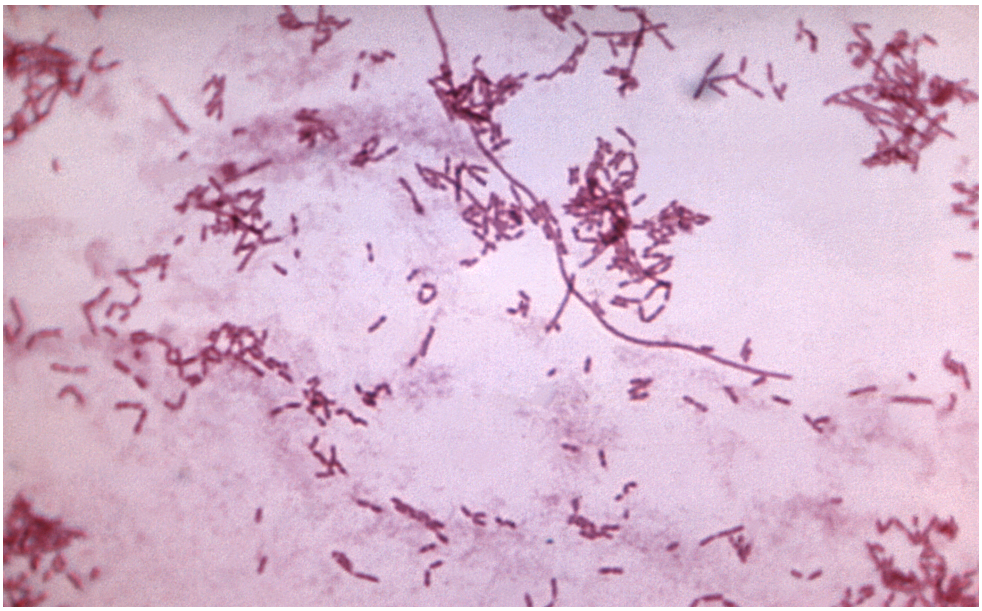

Figure 2: Gram stain of *Fusobacterium necrophorum* subspecies *funduliforme*.
Short, cocco-bacillary yet pleomorphic Gram negative rods are seen with tangling and curling. © PHIL, CDC, Atlanta, USA.

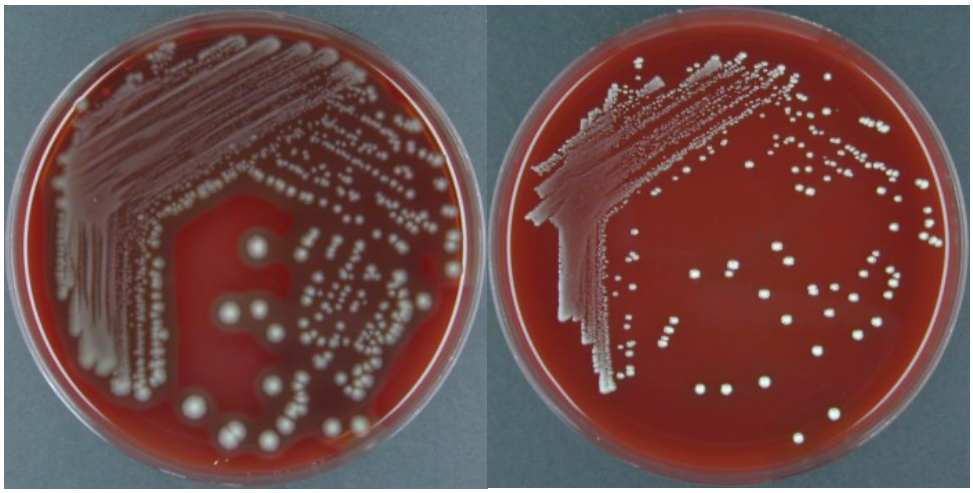

Figure 3A-B: Colony morphology of *F. necrophorum* subsp. *necrophorum* vs. *funduliforme*
Anaerobic cultures on Viande-Levure medium with 5% horse blood incubated for 48 hours. On the left (Figure 3A), are colonies of *Fusobacterium necrophorum* subsp. *necrophorum*, with a waxy and dull appearance and distinct haemolysin activity. On the right (Figure 3B) are colonies of *F. necrophorum* subsp. *funduliforme*, rounded, shiny, and creamy with less apparent haemolysis. © Online Veterinary Microbiology Atlas, Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark. https://atlas.sund.ku.dk/

Normal flora and tonsillar carriage

In early literature, *B. funduliformis,* as *F. necrophorum* was then called, was referred to as normal flora of the human gut, the female genital tract, and the oropharynx (1, 29, 32, 33, 60). This perception has stuck in the literature but there are very little data to support it. In fact, as Riordan points out (14), in the 1898 thesis from Hallé, it is described to be found in the healthy vagina, in exudates of patients with retained placentas and in the pus of Bartholin's abscesses, but on scrutiny, no data presented in the thesis seem to support these statements (29). Furthermore, when Dack (61) reviewed available data up until 1940 he did not describe evidence of its presence as normal flora, but still stated: "it is evident that the organism is probably a normal inhabitant of the mucous membranes of man". He also stated that the fact that the bacterium had not been identified in the colon, did not mean it is not there. I would claim it neither is proof it is.

Several of these conclusions have been reiterated without new data referenced and they appear to have become generally accepted (62, 63, 64). Interestingly though, by using culture methods, several attempts have been made to detect *F. necrophorum* as part of the normal human flora, yet these have all failed (14). In reports from gingival fluid (65), saliva (66), oral anaerobic flora (67, 68), periodontal pockets (69) and pharyngeal samples (70) presence of *F. necrophorum* indicating a role as a commensal has not been shown. However, other species of *F. necrophorum*, mainly *F. nucleatum*, are seen commonly. These studies represent Northern America, Europe, and Japan where most of the research on *F. necrophorum* has been performed. In the only previous studies of *F. necrophorum* from Sub-Saharan Africa, Falkler et al. investigated pathogens potentially causative of noma, in the Sokoto State of Nigeria, and found *F. necrophorum* to be almost overwhelmingly present (71, 72, 73). Following this finding, they investigated the role of *F. necrophorum* as normal oral flora in children at risk of noma, i.e. malnourished children living in the Sokoto state at that time. While *F. nucleatum* was commonly identified, *F. necrophorum* was rarely seen when the infection was not present (74).

Up until the turn of the millennium, no studies based on culture had found *F. necrophorum* to be present anywhere that could suggest it to be a commensal, why one would have been inclined to revise the conception of *F. necrophorum* as part of at least the oral or tonsillar flora as a fallacy. Then the PCR was introduced into the diagnostic arsenal by two important case-control studies from Aliyu et al. in 2004 (52) and Jensen et al. in 2007 (53). They tested cases with pharyngotonsillitis and asymptomatic controls by performing PCR on tonsillar swabs. Interestingly, among controls, Aliyu et al. did not find any positive sample from 100 healthy subjects (age 22-64 years, mean 40 years). On the contrary, Jensen et al. found a tonsillar carriage rate of 21% among controls. Controls were here younger and consisted of 42 healthy female student nurses and 50 healthy male soldiers (median age 22 years) with no

recent oropharyngeal infection or antibiotic therapy within two weeks. Jensen et al. concluded that *F. necrophorum* could be part of the normal human flora, in line with the previously unfounded assumptions, suggesting some evidence of carriage in one in five in specific groups (students and young soldiers) (53). While the findings from Aliyu et al. (52) and Jensen et al. (53) varied in terms of tonsillar carriage, with the main baseline difference being an age difference, both showed a higher presence of *F. necrophorum* among symptomatic cases than in controls. Subsequently, interest was ignited with repeated case-control studies focusing on pharyngotonsillitis as well as cross-sectional studies performed since, using PCR, culture, or both. Studies reporting tonsillar carriage rates among asymptomatic participants are summarised in Table 1.

Table 1:1-2: Summary of PCR and culture-based tonsillar carriage rates of *Fusobacterium necrophorum* from previous studies.

Table 1:1 PCR	Year and country	Age (mean or median, range)	Tonsillar carriage rate, n/tot (%)
Aliyu (52)	2004 UK	40 (22-64)	0/100 (0%)
Jensen (53)	2007 Denmark	22 (18-32)	19/92 (21%)
Ludlam (75)	2009 UK	20 (18-39)	29/362 (8%)[a]
Centor (76)	2015 US	24 (15-30)	17/180 (9%)
Hayakawa (77)	2018 Japan	33 (IQR 26-36)[b]	2/31 (7%)
Pallon (78)	2021 Sweden	8 (IQR 4-10)[b]	1/34 (3%)
Agerhäll (79)	2021 Sweden	19 (16-25)	20/217 (9%)
Total			**88/1016 (9%)**

Table 1:2 Culture	Year and country	Age (mean or median, range)	Tonsillar carriage rate, n/tot (%)
Jensen (80)	2015 Denmark	22 (10-40)	10/176 (6%)
Hedin (81)	2015 Sweden	31 (16-46)	4/128 (3%)
Kjaerulff (82)	2015 Denmark	29 (15-40)	9/100 (9%)
Hayakawa (77)	2018 Japan	33 (IQR 26-36)[b]	1/31 (3%)
Agerhälll (79)	2021 Sweden	19 (16-25)	8/217 (4%)
Total			**32/652 (5%)**

[a] Ludlam et al. (75) report higher rates of positivity within the control group but then later exclude participants with symptoms of sore throat. Data from only asymptomatic controls are presented here.
[b]Only interquartile range and not range reported in the references.

In short, tonsillar carriage rates among controls or asymptomatic study participants using PCR-methods have ranged from 0-21% (52, 53, 75, 76, 77, 79) with culture methods identifying fewer, 2-9% (77, 79, 80, 81, 82). Studies focusing on adolescents and young adults identify the highest tonsillar carriage rates and PCR appears to be a more sensitive method than anaerobic culture. Tonsillar carriage is rarely seen among children or older adults.

Following up on the Centor et al. study (76) presented above which reported tonsillar carriage rates of 9% by PCR, 16S rRNA gene sequencing was performed on cases and a subset of controls (n=30). Remarkably, 16S rRNA sequencing then identified *F. necrophorum* in 23/30 (77%) tested controls (83). Yet, in another study by Jensen et al. also based on 16S rRNA (84), *F. necrophorum* was not commonly seen among healthy controls. As shown elsewhere, since sequencing of the *rpoB*-gene appears to allow higher resolution and certainty of species and subspecies distinction than 16S rRNA-sequencing, one possibility is that closely related species could have been misidentified as *F. necrophorum* (41). Nevertheless, these findings are intriguing and need to be replicated in future tonsillar microbiome studies. In essence, most recent studies have been able to identify *F. necrophorum* in tonsillar samples from healthy asymptomatic individuals, particularly among adolescents and young adults, but at varying and possibly lower rates than expected if they were to be considered as a normal part of the tonsillar flora.

So far, we have focused on tonsillar and oral flora, but the historical description of *F. necrophorum* also refers to it as part of the microbiome of the female genital tract and the gastrointestinal system (29). Studies are scarce also here, yet recently the colorectal microbiota was investigated using *rpoB*-based sequencing of colorectal biopsies. Here, the overall presence of *F. necrophorum* subsp. *funduliforme* was seen in 13% (data seen in supplementary data 3 of the article). They also examined *faecal samples* in a subset of colorectal cancer patients and healthy controls but then found no trace of *F. necrophorum* (41). Regardless, given the finding from colorectal tissues as well as the association with invasive disease due to translocation from precipitating abdominal lesions (14, 21), it is likely that *F. necrophorum* resides in the gastrointestinal tract, yet far from as abundant as other *Fusobacterium* species (41, 85). Similarly, 16S rRNA-based studies of the normal flora of the female genital tract have not identified *Fusobacterium* species. While these are sometimes seen during bacterial vaginosis, or recently also in endometriosis (*F. nucleatum*) (86), other anaerobes are much more common (87, 88, 89). As for gastrointestinal infections, case reports on infections arising in the female genital tracts do exist but are even less common and when reported, have been seen mostly in the elderly with underlying disease or following instrumentation, *e.g.,* abortion or labor (14, 19, 36).

Transmission

If *F. necrophorum* is not commonly found in the normal flora, exogenous acquirement of the bacterium is necessary for infection to occur. But how is it transmitted? Ludlam et al. (75) analysed potential risk factors associated with a finding of *F. necrophorum* among university students. They suggested that transmission is related to a social lifestyle, with kissing identified as a risk factor for

testing positive for *F. necrophorum,* OR 3.2 (95% CI 1.1-9.5). Similar findings have previously been described for *Neisseria meningitidis* (90, 91) and EBV (92) where patterns of high incidence of symptomatic infection are also seen in adolescence. Except for the study by Ludlam et al. (75), no further studies have investigated transmission. While potential acquisition from animals has been proposed (36), this is rarely reported with the exception of cases such as when Schmorl and his assistant mistakenly inoculated themselves during laboratory work (28). The absence of publications on outbreaks of *F. necrophorum* pharyngotonsillitis within families suggests that there might be at least an extended latency from becoming a carrier to developing infection. However, it has been described that similar to what is seen in *Streptococcus pyogenes*, there is a second peak of isolation of *F. necrophorum* in the 30s, where an explanation suggested is that this could be due to transmission from children to their parents (11). Given the rarity of tonsillar findings of *F. necrophorum* in children (78, 93), this seems less plausible.

Virulence factors

The presence of two distinct subspecies of *F. necrophorum* affecting man vs. other animals means that studies on virulence and pathogenesis in *F. necrophorum* subsp. *necrophorum,* cannot be directly translated to human infections. *F. necrophorum* subsp. *necrophorum* causes life-threatening infections in cattle, sheep, dear, and wallabies (94, 95). Given its importance in animal infections, more data are available on virulence factors of *F. necrophorum* subsp. *necrophorum* than of *F. necrophorum* subsp. *funduliforme*. The term necrobacillosis is often used in both human and veterinary medicine. Among animals specific entities of necrobacillosis occur, such as calf diphtheria, bovine liver abscesses, and bovine foot rot. These are common and important within the food industry (94, 95). From a Nordic perspective, outbreaks of necrobacillosis in reindeer are also known to occur (96).

In terms of virulence, known virulence factors in, and pathogenic pathways caused by *F. necrophorum* subsp. *funduliforme* include activation of the contact system (97), complement evasion (98), binding and activation of plasminogen (99), LPS-production (100), haemolysin (101, 102), ability to adhere to and invade epithelial cells (103) and leukotoxin activity (104, 105). Yet, even higher LPS (100, 106), haemolysin (101, 102, 107), epithelial invasion (103) and leukotoxin activity (107, 108, 109, 110, 111) have been shown in *F. necrophorum* subsp. *necrophorum*, as well as the presence of haemagglutinin activity (101, 112, 113), platelet aggregation ability (114, 115, 116), and ability to adhere to endothelial cells (117). Newer approaches, including whole-genome sequencing, have further confirmed genotypic differences between subspecies. They have also highlighted that even though genotypic differences are seen between human isolates, these differences do not necessarily correlate with differences in how human infections present (55). Known

virulence factors are summarised in Table 2. In essence, while recent suggestions of contact system activation only have been described in *F. necrophorum* subsp. *funduliforme* (97), many virulence factors where thrombogenic effects are expected are more commonly seen in *F. necrophorum* subsp. *necrophorum* (106, 116, 117, 118).

Table 2 – Differences in virulence factors in *Fusobacterium necrophorum* subspecies *funduliforme* vs. subsp. *necrophorum*.

Virulence factors	*F. necrophorum* subsp. *funduliforme*	*F. necrophorum* subsp. *necrophorum*
Plasminogen activation	+	?
Contact system activation	+	?
Complement evasion	+	?
Epithelial adhesion and invasion	+	++
Platelet aggregation	-	+
Haemagluttinin	-	+
Leukotoxin activity	+	++
Virulence in rabbit and mouse model	-	++
LPS activity	+	++
Haemolysin activity	+	++
Adherence to endothelial cells	?	+

Adapted from Tan (102) and Holm (115).

Invasion

How *F. necrophorum* causes local or more invasive infection remains uncertain. Here, suggested factors associated with invasion are covered.

Local invasion

Bacteria commonly invade tissues by proteolysis, where either bacteria or the host generate or activate proteases (119). One of several possible mechanisms that this occurs through is the recruitment, binding, and activation of plasminogen. This has been described to occur on the bacterial surface of *F. necrophorum* subsp. *funduliforme* (99). From studies on mainly *F. necrophorum* subsp. *necrophorum* other factors suggested to be potentially important for invasion have also been described. These include protease secretion (119), haemolysin (101, 102, 120), and adhesins (117, 121).

Exotoxins

The most broadly studied virulence factor related to the invasiveness of *F. necrophorum* is leukotoxin, which is named after its toxicity aimed primarily at neutrophils. The leukotoxin gene of *F. necrophorum* was described in 2001 (122). But the first publication suggesting leukotoxic activity is from 1967 when rabbits and guinea pigs were injected with *F. necrophorum* subsp. *necrophorum* and were shown to develop strong inflammatory activation. Particularly, neutrophils adjacent to the infected area died (123). The leukotoxins have since been investigated in several studies (104, 105, 108, 109, 110, 111). Early studies focused on *F. necrophorum* subsp. *necrophorum* (the animal strain), including a genomic description of the leukotoxin gene in 2001 (122), whereas later studies have investigated the leukotoxin gene in *F. necrophorum* subsp. *funduliforme* from bovine isolates, and found it to be similar yet not identical (108, 111). Holm et al. confirmed the presence of leukotoxin genes in all of the human isolates of *F. necrophorum* subsp. *funduliforme* investigated (104).

Complement evasion

In a study by Friberg et al. (98), Factor H binding and subsequent complement evasion were described. They showed that higher binding capacity was associated with more severe disease. In brief, the complement system plays an important role in the innate immunity against common pathogens, and its activation leads to general proinflammatory responses, adaptive immune system activation, and direct proteolytic cascades that cause opsonisation and lysis of pathogens (124, 125). Factor H binding is a known mechanism through which the complement system is evaded. This is also a known virulence mechanism in *N. meningitidis* (126). The complement system can be activated either via the classical, lectin, or alternative pathway, where the classical pathway requires antigen: antibody complexes (adaptive immunity), the lectin pathway is triggered by recognition of pathogen-associated molecular patterns (i.e. direct activation by bacteria), and the alternative pathway by a mechanistically dissimilar process, where spontaneous hydrolysis of C3 occurs to C3b. This hydrolysis can be activated by contact with various protein, carbohydrate, or lipid structures on microorganisms. If it is triggered, an alternative pathway C3-convertase is formed, which starts to convert C3 (freely circulating in plasma) to C3b and C3a. This conversion also occurs downstream in the classical and lectin pathways. The C3b created then opsonise pathogens to guide phagocytosis, whereas C3a activates local inflammation. The C3-convertase is essential for the activation of these symptoms. Its function is stabilised by the binding of Factor B, yet Factor H has the same binding site as Factor B and when bound, it instead causes degradation of the C3 convertase, decreasing inflammatory response, opsonisation, and neutrophil attraction (125).

Immunity

As noted, Factor H binding and complement evasion are proposed mechanisms through which *F. necrophorum* evades the innate and adaptive immune system. While the importance of leukotoxins in human infections is not known, their presence indicates a role in evading primarily innate immunity (neutrophils). In terms of humoral immunity, there are several studies investigating antibody responses in animals (127, 128, 129), yet very few in humans (106). However, using whole-cell preparations of *F. necrophorum*, Klug et al. have demonstrated that *F. necrophorum* antibodies develop following peritonsillar abscess in most patients with cultures positive for *F. necrophorum* (130). Local and invasive pharyngeal infections mainly occur in previously healthy adolescents and younger adults, and there have been no signs of immune deficiencies being overrepresented among the affected (14).

On the contrary, invasive non-head and neck infections often occur among elderly or previously ill patients. It is common to find a locus minoris resistentiae in these patients and possibly, translocation of bacteria over damaged epithelial linings here causes invasion (18, 19, 131, 132).

Co-infection

There has been a debate as to whether *F. necrophorum* alone can cause pharyngeal infection or if it requires a concomitant infection to invade. Several publications report concomitant infection with *F. necrophorum* and EBV in Lemierre's syndrome, yet in part, reporting is likely impacted by false-positive results as well as publication bias (14, 43). Based on population-based data, EBV is a relatively rare co-infection in Lemierre's syndrome (11, 21). In studies on pharyngotonsillitis, rates of co-infection with EBV have been reported at 6% when prospectively investigated (81). However, co-pathogens are often seen also in other respiratory infections (133). To date, there are five prospective case-control studies investigating and reporting co-infections in *F. necrophorum* pharyngotonsillitis, with findings summarised in Table 3. In general, co-infections identified increase when broader microbiological analyses are performed and involve viral pathogens. An interesting finding is the co-infection rate with GAS reported by Hedin et al. (81), where 33% of patients positive for *F. necrophorum* also were positive for GAS. When investigated by Centor et al. (76), the co-infection rate of GAS and *F. necrophorum* however was lower (14%). Taken together, concomitant infection appears relatively common, yet there is also epidemiological evidence highlighting that monomicrobial infections with *F. necrophorum* occur suggesting that it can cause local (Table 3) and invasive infections on its own (13, 14, 130).

Table 3: Summary of prospective case-control studies investigating co-infection rates in *Fusobacterium necrophorum* pharyngotonsillitis

Study	Age inclusion criteria years	*F. necrophorum* of total n/tot (%)	*F. necrophorum* as sole finding n/tot (%)	Co-infection with GAS n/tot (%)	Co-infection with GCS/GGS n/tot (%)	Co-infection with EBV n/tot (%)	Co-infection with other respiratory virus n/tot (%)
Hedin (81)[a]	15-45	33/220 (15%)	14/33 (42%)	11/33 (33%)	2/33 (6%)	2/33 (6%)	5/33 (15%)
Centor (76)[b]	15-30	64/312 (21%)	47/64 (73%)	9/64 (14%)	7/64 (11%)	N/A	N/A
Kjaerulff (82)[b]	15-40	16/100 (16%)	12/16 (75%)	4/16 (25%)[c]		N/A	N/A
Hayakawa (77)[b]	>15	6/44 (14%)	4/6 (66%)	1/6 (17%)	1/6 (17%)	0/6 (0%)	N/A
Pallon (78)[a]	0-14	1/79 (1%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	1/1 (100%)
Total		**120/755 (16%)**	**77/120 (64%)**	**21/104 (20%)**	**10/104 (10%)**	**2/40 (5%)**	**6/34 (18%)**

[a] *F. necrophorum* results were based on culture, [b] *F. necrophorum* results were based on PCR, [c] Cultures were only reported as beta-hemolytic streptococci and not by Lancefield-antigen positivity or species.

Pharyngeal infections

Pharyngotonsillitis

While pharyngeal infection has been considered an essential part of Lemierre's syndrome, Lemierre himself then considered it as a secondary invader (60). The preceding pharyngeal infection in Lemierre's syndrome was for long assumed to involve *F. necrophorum* (14), but little data existed supporting *F. necrophorum* pharyngotonsillitis as a disease entity of its own. Therefore, interest was sparked when the PCR-based case-control study by Aliyu et al. identified *F. necrophorum* more commonly among cases (52). Jensen et al. designed another PCR in 2007, and then reported a staggering 48% *F. necrophorum*-positivity rate among cases with 21% positive among controls (53). Both these studies presented remarkable data at the time, yet enrolled cases at the laboratory and not in the clinic and had the risk of mainly selection bias that possibly impacted findings. In the study by Aliyu et al., age ranges differed between cases and controls and clinical data were lacking (52). Similarly, in the study by Jensen et al. (53), clinical data were lacking, patients were selected from non-streptococcal cases and the age range focused on ages prone to both tonsillar carriage and infection, partly explaining their findings. Batty et al. in 2005 (134). Amess et al. in 2007 (135) used culture instead of PCR on routine throat swabs sent to the laboratory but did not include controls in the study design. In 2008, Hagelskjaer et al described all diagnosed cases during three years in Denmark (136). In 2009, Ludlam et al. (75) investigated cases that presented to a general practitioner (any age) with university students as controls. However, controls could have symptoms, yet were then later excluded from comparisons, and all data were not presented making conclusions difficult. In summary, while all initial studies had some possibilities for improvement in study design, they consistently identified *F. necrophorum* in cases, and the initial case-control studies found *F. necrophorum* to be more common in cases than controls.

In recent years one narrative review (11) and two meta-analyses (12, 137) have summarised the role of *F. necrophorum* in pharyngotonsillitis. In total, there have been four prospective well-designed case-control studies performed in adolescents and young adults. These four studies were included in the meta-analysis by Malmberg et al. (137), who somewhat unusually used positive etiologic predictive value as the outcome, instead of odds ratio or risk difference. In the meta-analysis by Klug et al. (12), a systematic review and meta-analysis as per usual was

performed, why these two meta-analyses differ somewhat and add different perspectives. Another meta-analysis by Marchello et al. (138) investigated GCS and *F. necrophorum* in pharyngotonsillitis yet include a wider spectrum of studies including laboratory-based studies without controls and focus on the prevalence of a finding GCS or *F. necrophorum* in tonsillar swabs without investigating risk differences in cases compared to controls.

In 2015, three important case-control studies with a low risk of bias were published by Centor et al. (76), Hedin et al. (81), and Kjaerulff et al. (82). All three were well-designed prospective case-control studies enrolling patients with pharyngotonsillitis attending primary care and used either PCR or culture in cases and age-matched controls. These have been followed by a well-designed (yet with low power) case-control study by Hayakawa et al. in 2018 (77) who introduced data also from Japan and Pallon et al. in 2021 (78) who investigated pharyngotonsillitis in children in Sweden. The four studies investigating adolescents and young adults are summarised in Table 4.

Studies enrolling adolescents and younger adults, show higher rates of *F. necrophorum* in pharyngotonsillitis, ranging from 14-21% compared to 3-9% in controls. In the study of paediatric cases by Pallon et al. (all <15 years) (78), rates of *F. necrophorum* were low in both cases and controls. These findings were similar to previous findings from a paediatric cross-sectional study investigating the role of *F. necrophorum* in children by Van et al. (93), where it was rarely seen below 15 years of age (2%), yet more common among adolescents aged 14-20 years old (14%). No control group was enrolled in this study.

Table 4: Meta-analysis of prospective case-control studies with low risk of bias investigating the presence of *Fusobacterium necrophorum* in pharyngotonsillitis among adolescents and young adults.

Study	Age, cases, mean or median (range)	Age, control, mean or median (range)	Cases *F. necrophorum* n/tot (%)	Controls *F. necrophorum* n/tot (%)	Weight (%)	Risk difference (95%CI)
Hedin (81)[a]	33 (15-48)	31 (16-46)	33/220 (15%)	4/128 (3%)	31%	**0.11 (0.06-0.17)**
Centor (76)[b]	22 (15-30)	24 (15-30)	64/312 (21%)	17/180 (9%)	44%	**0.11 (0.06-0.17)**
Kjaerulff (82)[a]	28 (15-40)	29 (15-40)	16/100 (16%)	9/100 (9%)	18%	0.07 (-0.02-0.16)
Hayakawa (77)[b]	29 (24-37)	33 (26-36)	6/44 (14%)	2/31 (7%)	7%	0.07 (-0.06-0.24)
Total			119/676 (18%)	32/439 (7%)	100%	**0.10 (0.06-0.14)**

[a] Results were based on culture. [b] Results were based on PCR. Risk differences in bold indicate significant differences ($p<0.05$) between cases and controls. Adapted from Klug et al. (12).

Evidently, *F. necrophorum* is mainly identified among adolescents and young adults and pre-adolescent children appear to be at very low risk of *F. necrophorum* pharyngotonsillitis. When the association of bacterial finding (GAS or *F. necrophorum*) with pharyngotonsillitis was analysed using the etiological predictive value by Malmberg et al. (137), a finding of *F. necrophorum* had an etiological predictive value of 64% (95% CI 33-83%) based on the studies summarised above (Table 4). This could be compared to GAS which had a predictive etiological positive value of 93% (95% CI 83-99%). Unfortunately, the Hayakawa et al. study (77) was excluded from calculations for statistical reasons, yet this had a minor impact due to its low weight in comparison with the three others.

The etiological predictive value incorporates the carriage rate and the prevalence of the pathogen in those with the disease. It also includes a variable (theta) which assumes the ratio between the proportion of carriers of the pathogen among controls and those with a sore throat caused by another pathogen, typically a virus. When carriage is common, the etiological predictive value becomes lower. In referenced papers in Table 4, carriage of GAS was very low (2%) whereas carriage of *F. necrophorum* was higher (7%) (137). Interestingly, GAS tonsillar carriage here reported was considerably lower compared to what recently has been reported from a Swedish study investigating pharyngeal carriage of pathogens among adolescents and young adults. Here, GAS and *F. necrophorum* among asymptomatic adolescents and young adults in Sweden were found to be similarly common, both seen in 22/217 (10%) (79). Importantly, tonsillar carriage rates have been shown to vary by place, method of diagnostics, and time both for *F. necrophorum* (Table 1) as well as for other pathogens (91, 139, 140, 141).

In summary, the studies originating during the last 20 years implicate *F. necrophorum* as a potentially important pathogen in pharyngotonsillitis in adolescents and young adults, second only to GAS in how commonly it is found. To a certain extent, other beta-haemolytic streptococci have had a similar standing and are looked upon as potential pathogens in pharyngotonsillitis, mainly *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE*)*, previously mainly referred to as GCS and GGS (138, 142, 143) as they will be throughout this thesis.

The Cochrane reviews of antibiotic therapy compared to placebo in pharyngotonsillitis have shown that antibiotics reduce symptoms on days three and seven, reduce suppurative complications, e.g., otitis media within 14 days and peritonsillar abscess within two months as well as rheumatic fever (yet includes no reported rheumatic fever cases from studies performed after 1975). Most of these studies were performed when complications were more common and prevalence rates of GAS in pharyngotonsillitis were higher (144, 145). No randomised controlled trial has evaluated treatment in *F. necrophorum* or GCS/GGS pharyngotonsillitis specifically. However, symptom reduction on day three following antibiotic therapy was seen in a sub-group analysis of patients with

negative beta-haemolytic culture, yet with a weaker treatment effect than in those with positive GAS cultures (144, 146). Before our studies, voices had already been raised to expand testing to involve *F. necrophorum* in pharyngotonsillitis (147, 148).

Age

Both tonsillar carriage and pharyngeal infections with *F. necrophorum* are focused on a certain age range. While some data have indicated that younger children are at risk of complicated ear infections due to *F. necrophorum,* the majority of published case reports, case series and systematic microbiological studies focus on a median age at the turn of adolescence and adulthood (1, 13, 14, 15, 17, 18, 19, 21, 23, 24, 36, 37, 52, 53, 62, 79, 80, 81, 132, 134, 136, 149, 150, 151, 152), with an increase of local and invasive disease seen that begins in the early adolescence and starts to diminish after 30 years of age. While non-head and neck infection then begins to occur in an elderly population (mainly above 50 years) (14, 21), most cases and the focus of this thesis lies on adolescents and young adults where most infections are seen.

Why this pattern is seen remains elusive, however, as referred to previously, similar patterns are seen in meningococcal disease (90) and EBV (92, 153). Social factors are likely involved (75) and it is also possible that tonsillar growth up until the onset of puberty impacts the size and anaerobic conditions in tonsillar crypts that might increase with tonsillar growth (84). These effects are not completely consistently reported though, since the tonsillar size has also been described to decrease already from ten years of age which would not fit with the aforementioned explanation (154). Possibly, hormonal changes and their impact on immunity are also involved (155), yet plainly, knowledge is lacking.

Sex

Lemierre claimed both sexes to be equally affected (1) but several case series have since identified a male overrepresentation in pharyngotonsillitis (11), peritonsillar abscess (13, 155) and Lemierre's syndrome (15, 19, 31) due to *F. necrophorum.* In an impressive recent meta-analysis of published cases Valerio et al. collected individual data on 333 cases, defined as typical Lemierre's syndrome, and found 61% of cases to be male. When atypical cases (here defined as cases without evidence of *Fusobacterium* infection) were included, 59% were male of the total 712 cases (23). In a follow-up post hoc analysis of the same data with added focus on other important sex differences, none further were identified (156). Thus, there is conflicting evidence, with a male overrepresentation predominately seen in local pharyngeal infections including peritonsillar abscess, whereas the sex ratio in

Lemierre's syndrome is more uncertain with a possible slight male overrepresentation proposed.

Seasonality

Seasonality in *F. necrophorum* infections has been reported in a previous study by Brazier, based on data in England and Wales from 1990-2000 (15). Here, they described a peak in the late winter months (January-March). Hagelskjaer et al. reported a slight seasonal variation from their prospective study in Denmark 1998-2001 on all invasive cases of *F. necrophorum*, with an accumulation of cases in the late winter and early autumn months but exact numbers were not presented (18). In a prospective study on aetiology in pharyngotonsillitis, Hedin et al. reported seasonal variation for GAS and viral pathogens, but not for *F. necrophorum* (81). Pharyngotonsillitis cases regardless of cause are known to vary over the year, with increased cases seen during colder seasons (157), but these patterns have not been shown to be distinct for *F. necrophorum*.

Complicated pharyngeal infections

Peritonsillar abscess

Peritonsillar abscess is a known complication of *F. necrophorum*-infection (158, 159, 160) and during the pre-antibiotic era, it was commonly described in Lemierre's syndrome (1). The 2009 publication by Klug et al. (13) further highlighted its importance. In this Danish retrospective study of 847 cases with peritonsillar abscess, *F. necrophorum* was the most prevalent finding and identified by culture in 23%, followed by GAS in 17% and GCS/GGS in 5%. In most cases (81%), *F. necrophorum* grew in pure culture. In addition, seroconversion has been shown following *F. necrophorum* peritonsillar abscess (130).

The incidence data from the Klug et al. study (13) were also the highest presented thus far and the authors speculated that these numbers could be due to the restrictive antibiotic policy in place, where penicillin was used in pharyngotonsillitis only if a RADT was positive for GAS (13). Findings of *F. necrophorum* as the most prevalent pathogen in peritonsillar abscess have been repeated, e.g. in the US (161), where *F. necrophorum* was also found to be strongly associated with the recurrence of peritonsillar disease, and in Finland (162). Interestingly, while the peritonsillar abscess is generally considered a complication of pharyngotonsillitis and is reduced by antibiotic treatment of pharyngotonsillitis (144), a subset of patients develops peritonsillar abscess without prior pharyngotonsillitis. However, *F. necrophorum* has rarely been found among these cases. Since a potential reduction of

complications is one of the reasons to further investigate the treatment of pharyngotonsillitis due to *F. necrophorum*, these findings are important (163).

Recurrent tonsillitis

In the 2004 study that introduced PCR as a diagnostic in pharyngotonsillitis, Aliyu et al. reported that 40% of patients positive for *F. necrophorum* had recurrent or persistent pharyngotonsillitis (52). This led to several studies investigating this association (50, 75, 84, 134, 164) with most identifying *F. necrophorum* as a relatively common finding. In patients scheduled to undergo tonsillectomy due to chronic/recurrent tonsillitis or recurrent peritonsillar abscess, *F. necrophorum* was commonly found at inclusion (28%), surgery (30%), and at follow-up post-surgery (16%). While the rates of *F. necrophorum* decreased following surgery, this decrease was not significant (possibly partly due to missing data of repeated samples in a quarter of patients). However, a few participants also acquired *F. necrophorum* following tonsillectomy indicating that carriage varies over time and appears to be transient (50). In summary, when taking the available evidence into account, an association is seen between *F. necrophorum* and chronic or recurrent pharyngotonsillitis in addition to acute pharyngotonsillitis and peritonsillar abscess, but causality is not certain.

Retropharyngeal, parapharyngeal, and submandibular abscesses

Peritonsillar abscesses are the most common pharyngeal abscesses and are located between the tonsil and the superior pharyngeal constrictor muscle. Retropharyngeal or parapharyngeal abscesses are referred to according to the posterior or lateral location in the pharynx and occur within the retropharyngeal or parapharyngeal fascial space (which communicate laterally) (165). Parapharyngeal or retropharyngeal abscesses arise lateral to the superior pharyngeal constrictor muscle, whereas peritonsillar abscesses arise medially (166, 167). Posterior to the retropharyngeal space lies what is referred to as the ‘danger space’, since from here, direct communication to the mediastinum is possible. This route is associated with the dreaded complication of descending mediastinitis. Finally, the submandibular space is the last of the three most clinically important cervical spaces. It consists of several spaces in communication that are summarised as the submandibular, sublingual, and submental spaces. It is within these spaces that Ludwig’s angina occurs (168).

The parapharyngeal abscesses typically occur secondary to tonsillar infection but can also arise from dental or otogenic sources as well as indirectly from suppurative lymph node origin secondary to another airway infection (169). Patients who develop parapharyngeal abscesses are generally older than those who develop peritonsillar abscesses. A recent Danish study identified *F. necrophorum* in 17% of

parapharyngeal abscesses, with slightly higher rates in cases with concomitant peritonsillar abscess (170). Yet, findings were often polymicrobial with other non-beta-haemolytic streptococci (most commonly *S. anginosus*) and unspecified anaerobes as the most common findings. While less commonly seen in parapharyngeal abscesses compared to peritonsillar abscesses (13, 151, 171), findings suggest that *F. necrophorum* also play a role in the pathogenesis of parapharyngeal abscesses despite them affecting an older age range.

Retropharyngeal infections can occur following local trauma, such as upon accidentally swallowing a bone or after instrumentation of the oesophagus. They can also occur from the extension of parapharyngeal infections through lateral communication of the spaces or be of odontogenic or lymph node origin. As mentioned, the dreaded complication is gravitational drainage of the abscess causing descending mediastinitis (172). While *F. necrophorum* has been described as a pathogen in retropharyngeal infections, it is rarely reported and its importance here is less established (14, 21, 173, 174).

In the submandibular space, Ludwig's angina can occur, which is an aggressive polymicrobial infection that spread typically following a dental infection of the second or third mandibular tooth with airway compromise as the main risk (172). It has been described to be caused by *F. necrophorum*, yet this is rarely seen (175).

Invasive infections

Lemierre's syndrome and other invasive infections

History

Biography on André Lemierre (1875-1956)

A tribute published on his death described André Lemierre, Professor of Microbiology and Infectious Diseases at the Hospital Claude Bernard in Paris, as having limitless energy and enthusiasm and the ability to uncover the most obscure diagnoses based on careful history taking and clinical examination (176). For those interested in medical history (and with some French knowledge), this reference (177) covers Lemierre's and Sohier's mission to French prisoners of war in Germany during a louse-borne typhus (*Rickettsia typhi*) outbreak in 1942. In the archives of Uppsala University Library, I discovered a newspaper article from 1921 describing André Lemierre (Figure 4). Freely translated quotes (Figure 4) highlight his recognition in France long before his publications (1) on what half a century later became known as Lemierre's syndrome, and attached is also a caricature highlighting the diverse opportunities knowledge in culture provides (Figure 5):

> "Between the drinks, I had the possibility to get to know Lemierre better, to better appreciate him. He was no longer just a doctor, he was no longer so serious, I found human weaknesses in him, and they were charming. I discovered him to be an excellent musician, reader, expert, historian, artist. What else did I learn? He seemed to me more and more like perfection."
>
> "But to talk about Lemierre, let us proceed methodically. His career was that of a tremendously educated aspirant. Bright and clear; intern in 1900, hospital doctor in 1912, associate professor in 1913; not bad! But he could have been appointed even sooner if only the candidate's valour would have triumphed in these ardent struggles; his peers had since long already appointed him. Long before the judges!"
>
> "One can be a great scientist and a modest doctor, but Lemierre is the good doctor, in the best sense of the word."
>
> "It is likely that the next version of him will not appear until the December of the year 2000."

Biographie du Docteur André LEMIERRE

Le 30 juillet 1875 naissait, dans la Ville Lumière, un enfant du sexe masculin, qui fut prénommé André-Alfred. Je l'ai connu quelque 30 ans plus tard, assez changé et s'appelant LEMIERRE. Jeune interne, j'étais plein de respect pour ce brillant interne du service Widal ; il m'apparaissait très distant, très savant, aussi apprécié par son maître que par ses collègues et ses élèves; il était pour moi l'exemple inimitable du parfait interne, que j'aurais voulu devenir un jour; il était l'élève chéri du Grand Patron, sur lequel je n'osais lever les yeux qu'en tremblant.

J'avais bientôt l'occasion de retrouver LEMIERRE en salle de garde, où, petit étudiant imberbe, à peine sorti du lycée, j'assurais cependant (mais avec quels tremblements!) le service d'un autre lumineux interne en chirurgie qui venait d'avoir la bonne idée de se marier. *Inter pocula*, j'avais le moyen de mieux connaître, de mieux apprécier LEMIERRE; il n'était plus seulement médecin, il n'était plus aussi grave, je lui trouvais des faiblesses humaines, qui étaient charmantes; je le découvrais excellent musicien, lecteur averti, historien, artiste... que sais-je encore? Il m'apparaissait de plus en plus comme une perfection... Il y a 18 ans de cela (pauvres nous !) et, depuis, il n'a fait que s'améliorer encore; tous ses amis, et il n'a que des amis, vous le diront.

Mais, pour parler de LEMIERRE, procédons, comme lui, méthodiquement :

Sa carrière: Celle d'un candidat formidablement instruit, brillant et clair; interne en 1900, médecin des hôpitaux en 1912, professeur agrégé en 1913; ça n'est pas mal ! mais il aurait pu être nommé encore plus tôt dans les concours, si la seule valeur du candidat l'emportait dans ces luttes ardentes; il y a longtemps que ses collègues l'avaient nommé... avant les juges! Pendant la guerre il a été profondément apprécié partout où il a été envoyé ; médecin consultant d'armée, il a su remplir ce rôle délicat pour le plus grand bien des malades et des médecins, qu'il instruisait, sans en avoir l'air. La croix de guerre, le ruban rouge sont tombés tout naturellement sur son uniforme. Le voilà, cette année, secrétaire-général du Congrès de médecine; le voilà promu à l'immortalité, puisque sa tête est dans le « *Rictus* ». Est-ce là le summum de sa carrière ? Ceux qui le connaissent vous diront que ce n'est qu'un début. S'il est jamais professeur, les étudiants ne s'en plaindront pas, car son enseignement, net et précis, est extrêmement apprécié; signe particulier: sait faire salle comble en professant un cours... de thérapeutique ; autre signe particulier: ne peut promettre de place d'externe ou d'interne avant 1932 !

Ses travaux: Grand dépouilleur des journaux les plus indigestes, LEMIERRE a su glaner parmi le fatras des publications allemandes certains faits qu'il lui paraissait utile de vérifier ; il les reprenait, les simplifiait, y apportait la clarté latine et en tirait une œuvre originale de toute première valeur. Il commence par s'occuper de l'ensemencement du sang et, dans cette voie féconde, il persévère; depuis sa thèse de 1904, on peut dire que dans toute l'œuvre de LEMIERRE la *bacillemie erre* (ô! *Rictus!* que me fais-tu dire !), recherches sur la *septicémie éberthienne*, *coli-bacillaire*, sur la *pneumococcémie*, sur l'*hemio-culture dans les ictères*, sur l'*infection descendante des voies biliaires*, avec Abrami. — Faut-il rappeler que c'est avec LEMIERRE que le Pr Widal a poursuivi ses premières recherches sur la *rétention chlorurée*, recherches qui devaient bouleverser toute la pathologie rénale ? LEMIERRE attaque aussi le foie ; avec Abrami, puis avec Brulé, il donne de solides assauts à cette doctrine de l'*angiocholite ictérigène* qui régnait en maîtresse ; ses recherches sur les *hémoconies*, sur les *ictères dissociés* l'amènent à soutenir l'existence d'ictères dus à des lésions de la cellule hépatique; elles l'amènent aussi à soutenir le rôle prépondérant de la bile dans l'absorption intestinale des graisses. Ce n'est pas tout : il a étudié, chemin faisant, *le liquide céphalorachidien dans la syphilis*, les *états meningés;* les *réactions puriformes aseptiques*. On pourrait s'endormir avec un tel bagage scientifique; ce n'est pas le genre de LEMIERRE: il est tous les matins à 9 heures à l'hôpital Andral et n'en part qu'après avoir étudié à fond tous ses malades; ses publications se succèdent à la Société Médicale, où l'on apprécie les faits précis qu'il apporte, entourés d'une bibliographie très travaillée.

Le médecin. On peut être grand savant et médiocre médecin, mais LEMIERRE est le bon médecin, dans toute la beauté du terme. Il faut le voir interroger méthodiquement un malade, sans rien oublier ! Quand il vient me voir, pour une angine ou un rhume, je sens toujours qu'il fait un grand sacrifice à notre vieille amitié en ne recherchant ni mes réflexes achilléens, ni le signe d'Argyll Robertson ! La méthode et le flair classique le conduisent tout droit au diagnostic exact et tous les concurrents aux hôpitaux s'accordaient à déclarer LEMIERRE « incollable sur le malade ». On comprend ainsi ses succès de clientèle, mais il se défend contre l'envahissement... pour pouvoir « travailler ».

L'homme: Un grand travailleur, modeste et calme; minutieux et ponctuel. Un sage! La 3e République française en a ainsi au moins un... si le royaume grec n'en a plus sept. Toutes ses qualités ont fait à LEMIERRE une vie harmonieuse et heureuse; entouré de beaux enfants et d'une compagne charmante, sortie d'une des plus exquises familles chirurgicales, il peut se reposer dans les joies de la famille des fatigues d'une vie absorbante; je suis bien sûr que sa belle carrière médicale ne tient, au fond, qu'une place secondaire dans ses pensées.

Mais il faut me borner; si je devais dire tout le bien que chacun pense de l'ami LEMIERRE, *Rictus* serait ruiné par les frais et son prochain numéro risquerait fort de ne paraître qu'en décembre de l'an 2.000.

MARC BURNT.

Figure 4 – Biography in French on André Lemierre published in 1921 by Marc Burnt.
Photocopy of the original article included in "Biographie du Docteur Lemierre", a newspaper article by Marc Burnt, 1921. It is kept with letters signed by André Lemierre 1921-11-30 (Waller Ms fr-05578) at Uppsala University Library, Sweden.

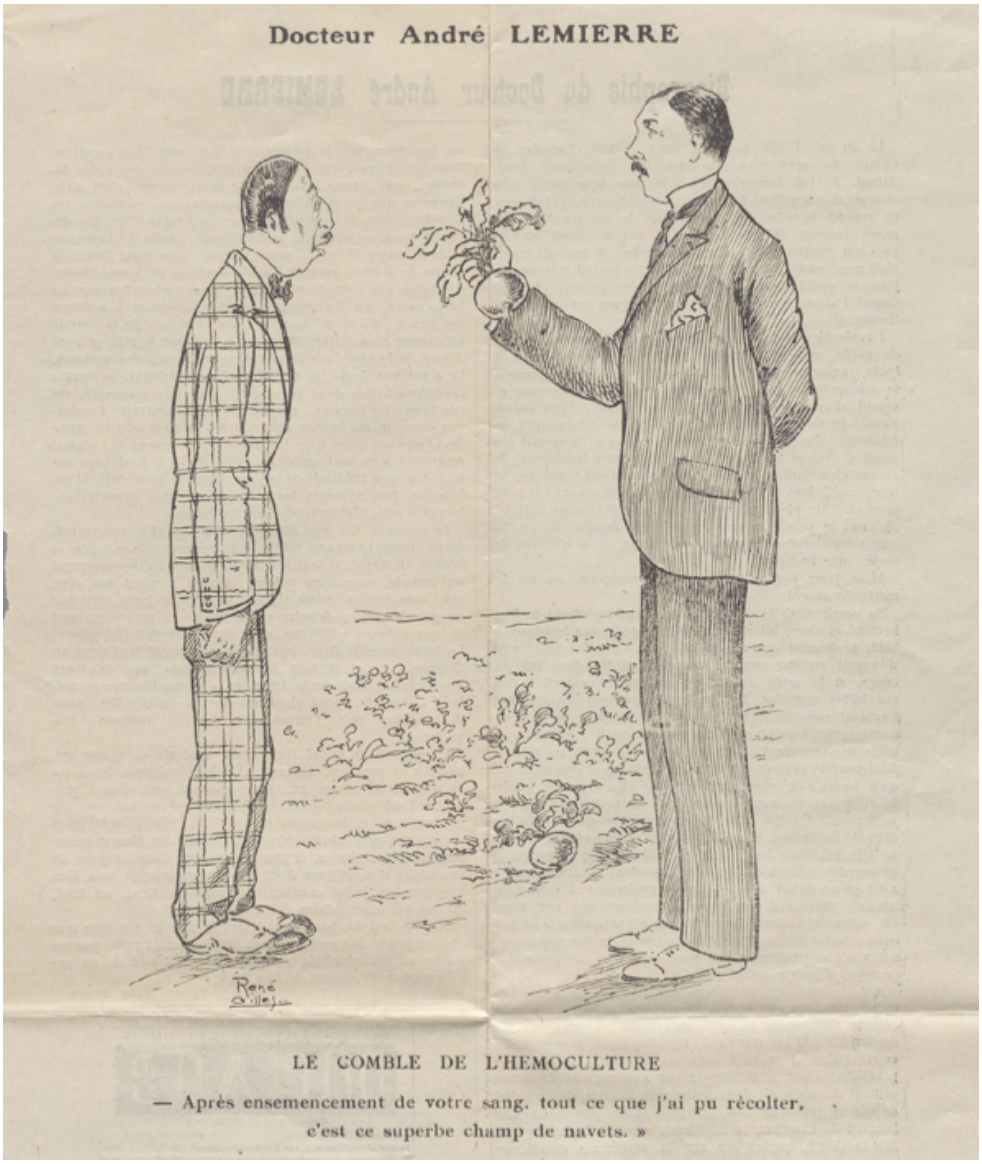

Figure 5 – Caricature of André Lemierre published in 1921. Le comble de l'hemoculture, caricature by René Oilles.

Freely translated the farmer tells André Lemierre, "After using your blood agar to sow my fields, this excellent field of Swedes (Swedish Turnips) became my harvest". Little did René Oilles know that Swedes would continue to culture an interest in André Lemierre's work a century later. The work is named, *Docteur André Lemierre: Le comble de l'hemoculture*, a caricature by René Oilles. It is included in "Biographie du Docteur Lemierre", a newspaper article by Marc Burnt, 1921. It is kept with letters signed by André Lemierre 1921-11-30 (Waller Ms fr-05578) at Uppsala University Library, Sweden.

Definitions

Now we change focus from historical curiosa to the syndrome itself (178). Its definitions have varied, complicating comparisons of incidence, why Riordan proposed a definition of microbiological and clinical criteria, similar to the original description of Lemierre's syndrome (1, 14). This definition consisted of:

1. History of an anginal infection during the preceding 4 weeks
2. Evidence of metastatic lesions in lungs or another remote site
3. Evidence of jugular vein thrombophlebitis or isolation of *F. necrophorum* or *Fusobacterium sp.* from blood cultures or a normally sterile site

This definition excludes what is often referred to as atypical cases in the literature, sometimes described to arise from dental or otogenic sources or even non-head and neck origin. *F. necrophorum* do cause septic thrombophlebitis also when not originated from pharyngeal infections, but not to the same extent and with much more varying presentations (14, 56, 152, 179, 180). In the recent meta-analysis by Valerio et al. (23) a definition of two criteria was used. They required a (i) primary head and neck infection to be followed by (ii) local thrombotic complication or a peripheral septic lesion. A similar definition was used by Gore in a separate yet concurrent meta-analysis (24). Valerio et al. subsequently also grouped the 712 cases they identified into typical and atypical Lemierre's syndrome, where typical Lemierre's syndrome required (i) an acute oropharyngeal infection, (ii) isolation of *Fusobacterium spp.* and (iii) either head/neck vein thrombosis or septic embolism (23). Holm et al. (21) defined Lemierre's syndrome as a preceding (4 weeks) anginal illness or other primary focus in the head and neck or compatible clinical findings, and *at least two* of the following: (i) isolation of *F. necrophorum* from blood cultures or a normally sterile site, (ii) evidence of internal jugular vein thrombophlebitis, or (iii) evidence of metastatic lesions in the lungs (multifocal pneumonia, or a typical rounded septic embolisation consolidation if only one pulmonary infiltrate was present). Hagelskjaer et al. (18) used a wider definition of Lemierre's syndrome in their prospective study in Denmark, where any infection arising in the head and neck region with signs of dissemination was included. Signs of dissemination included a positive blood culture alone; thus no signs of septic thrombophlebitis were required to fulfil their definition. When investigating incidence rates, these differences are important. For this thesis, incidence data from previous studies have been recalculated according to the following definition of Lemierre's syndrome, also used in Paper III-V (181, 182, 183):

1. Isolation of *F. necrophorum* from blood or another sterile site
2. An oropharyngeal infection precedes the invasive infection
3. Development of septic thrombophlebitis or signs of septic embolisation

Epidemiology

Most of the literature on Lemierre's syndrome is based on case reports and series, excellently reviewed among others by Riordan (14), Hagelskjaer et al. (62), Brazier (37), Gore (24), and Valerio et al. (23). Luckily, a few population-based studies also exist. Here, microbiological findings of invasive infection with *Fusobacterium* sp. or *F. necrophorum* are generally used as case definitions, and in studies on Lemierre's syndrome, microbiological criteria then typically require a finding of *F. necrophorum.* These studies are summarised in Table 5 with incidence and mortality rates provided. What becomes clear throughout, is a markedly elevated incidence within the age-range 15-25 years, and that incidence appears to increase with each decade.

Interestingly, following the introduction of antibiotics, Lemierre's syndrome seemed to disappear and was 'forgotten'. Since the 1980s and onwards, several publications have referred to it as the "forgotten syndrome" (7, 8, 9, 10). Indirect suggestions of a decrease can be seen in the surveys that Gunn performed during the 1950s, where he found that among cases with anaerobic Gram negative bacteraemia 31% had an oropharyngeal portal of entry, with thrombophlebitis of the internal jugular vein often described (184). Based on similar surveys 20 years later, only 1.5 % of the cases with anaerobic septicaemia then arose from the oropharynx (185). A common explanation proposed for this decrease has been the widespread use of antibiotics for pharyngeal infections (14). However, it is also likely that improved diagnostics and increased use of blood cultures play a role in the perceived increase that we are now seeing. Nevertheless, the use of antibiotics in pharyngotonsillitis has been increasingly restricted and continuously is (186). This is necessary for several reasons, yet, it is also feasible to be one of the causes of the reported increase in invasive *F. necrophorum* infections since the late 1980s (14) as well as the perceived increase of purulent complications to pharyngotonsillitis, such as peritonsillar abscess (13, 151, 187).

Table 5:1– Incidence data on Lemierre's syndrome

Lemierre's syndrome	Country and year	Incidence cases/million/year	Mortality rate n/tot (%)
Hagelskjaer (19)	Denmark 1990-95	0.4[a]	0/12 (0%)
Jones (149)	Soutwest England 1994-99	0.6[a]	N/A
Nohrström (20)	Helsinki, Finland 1994-2004	0.7[a]	0/5 (0%)
Hagelskjaer (18)	Denmark 1998-2001	1.3[a]	1/21 (5%)
Holm (21)	Skåne, Sweden 2000-15	1.7[a]	1/31 (3%)

[a]Incidence data have been estimated from the original publications to fit with a definition of Lemierre's syndrome as a preceding oropharyngeal infection with *F. necrophorum*-bacteremia (or cultured from another sterile site) with either septic thrombophlebitis or septic pulmonary embolisation.

Table 5:2 - Incidence data on *Fusobacterium necrophorum*-bacteremia

F. necrophorum bacteremia	Country and year	Incidence cases/million/year	Mortality rate n/tot (%)
Hagelskjaer (19)	Denmark 1990-95	1.5	12/49 (12%)
Jones (149)	Southwest England 1994-99	0.9	N/A
Brazier (15)	England and Wales 1990-2000	0.6	N/A
Nohrström (20)	Helsinki, Finland 1994-2004	2.3	1/23 (4%)
Hagelskjaer (18)	Denmark 1998-2001	3.8	N/A
Afra (16)	Calgary, Canada 2000-11	1.4	0/15 (0%)
Bank (17)	Denmark 2010-14	2.8	N/A
Holm (21)	Skåne, Sweden 2000-15	3.2	1/58 (2%)

As becomes apparent, most studies consist of data from the Nordic countries and the UK. While data from Northern America were represented by Afra et al. (16), recently Lee et al. (188) published data from Seoul, South Korea 2006-2021 where *F. necrophorum* infections also appear to be increasing. Incidence data are not reported in their study and the material does not solely consist of blood cultures. Potentially, a change of practice in performing more pharyngeal cultures can impact their finding yet none is reported. Nevertheless, increasing numbers of cases with *F. necrophorum* was reported (personal communication, Se Ju Lee). Furthermore, invasive infections with *F. necrophorum* have been reported from Oceania (189) as well as a few microbiologically verified reports from South America (190), yet no incidence data exist. In Africa, invasive infections with *F. necrophorum* have been described in noma (71, 72, 73) as well as in nosocomial infections based on data from Egypt (191). To my knowledge, no case of Lemierre's syndrome is yet to be identified and reported from Africa, likely due to the unavailability of anaerobic culture diagnostics in many places (192).

Clinical and laboratory presentation

Lemierre's syndrome mainly occurs among adolescents and young adults without prior medical history (18, 19, 21, 23, 62). Sometimes, the initial oropharyngeal infection has normalised on presentation to the hospital but generally is still present. Cervical lymphadenopathy or tenderness is commonly seen. The distension related to the typical internal jugular vein thrombophlebitis can be mistaken for lymphadenitis (14). Inflammatory markers such as C-reactive protein are typically high. The presentation is often mistaken for infectious mononucleosis, community-acquired pneumonia, or abdominal infection, since abdominal pain is a common presenting feature as is slight hyperbilirubinemia and elevated liver function tests (62). Some degree of renal impairment is common, as is thrombocytopenia, and in rare but severe cases clinically significant disseminated intravascular coagulation has been described (14). While the combination of a severely ill patient following a

sore throat, with signs of jugular vein thrombophlebitis and septic embolisation is very characteristic of Lemierre's syndrome (1), it is common that the diagnosis is first thought of following blood culture findings of *F. necrophorum*. Still, a third of bacteraemia cases from a study in Denmark were not recognised as Lemierre's syndrome despite culture findings, i.e., it appears the syndrome to some extent remains 'forgotten' (8, 18). Historically, the duration from when a sample was sent until a species was identified took several days. With the introduction of MALDI-TOF MS and automatised blood culture cabinet systems, this has become much more rapid (18). In recent work by our group not included in this thesis, blood cultures in Lemierre's syndrome patients commonly signalled within 24 hours, and strains were adequately confirmed as *F. necrophorum* using MALDI-TOF MS (or confirmed by targeted PCR when scores were below 2.0) (183). Thus, in our setting, while the syndrome is sometimes diagnosed before cultures return positive, quite commonly we as Infectious Disease physicians are very much aided by the Clinical Microbiology laboratory that quickly identifies cases admitted yesterday due to sepsis with a preceding sore throat as potential Lemierre's syndrome cases.

Septic embolism and systemic dissemination

Following the initial oropharyngeal infection and development of septic thrombophlebitis in Lemierre's syndrome, pulmonary symptoms occur, and at this stage patients commonly present to the hospital. The septic emboli to the lungs are initially typically seen as rounded pulmonary infiltrates, but hereafter often develop into multifocal pneumonia with the development of lung abscesses, pleurisy, and empyema (14, 19). Less commonly, they pass into the general circulation. In rare instances, such as with an open foramen ovale, general systemic arterial embolisation can occur (193, 194). Yet, secondary dissemination sometimes occurs without persistent foramen ovale, with common sites for lesions being joints, muscles, and the liver (23). The acknowledgement of septic embolisation is also involved in the definition of the syndrome, where commonly, either presence of jugular vein thrombosis or septic pulmonary embolisation is required. Intracranial complications are described but often occur after the continuous spread of infection (e.g., mastoiditis) or retrograde progression of septic thrombophlebitis with the development of sinus thrombosis (3, 14). Cases that develop sinus thrombosis are more associated with otogenic infections (18, 195, 196) but occur as part of typical Lemierre's syndrome cases as well (21). When described by Lemierre, joint manifestations were reported as common (1). Following the introduction of antibiotics they have become increasingly rare (14, 18, 21). In meta-analyses and case series, the frequency of dissemination beyond pulmonary embolisation is consistently higher (14, 23) than when compared to population-based data (18, 21), once again highlighting the impact of publication bias.

Antibiotic therapy

No randomised control trials have been performed to help us guide the treatment of Lemierre's syndrome. When André Lemierre's case series was published (1), antibiotics were not available, but a few papers document how the prognosis was transformed when penicillin therapy was given (14, 184, 197). Despite antibiotics, the response to treatment remains slow today, often requiring several days and even weeks for patients to defervesce with prolonged courses of antibiotics often given (21, 62). In the Holm et al. study (21), which described patients with Lemierre's syndrome in Region Skåne, Sweden 2000-15, antibiotic therapy was given for a median duration of 31 days, range 18-90 days. Commonly, antibiotic therapy for (2-) 4-6 weeks is suggested with emphasis put on individualisation (14, 21, 62, 150, 152, 178, 198, 199, 200). In the large meta-analysis of published cases from 2000-2017 by Valerio et al. (23), the antibiotic choice is reported. In cases with typical Lemierre's syndrome, which was defined to include *Fusobacterium sp.* in addition to direct or indirect signs of septic venous thromboembolism, the majority received metronidazole (61%), followed by penicillin (59%), cephalosporins (39%) and clindamycin (38%). Somewhat remarkably, 17% were also reported to have received quinolones and 15% macrolides (23). While not reported, it is reasonable to assume that these treatments were given empirically to cover atypical causes of pneumonia (e.g., legionellosis) or other co-infections.

Antimicrobial susceptibility

There is intrinsic resistance of *F. necrophorum* to aminoglycosides and quinolones, while tetracyclines have poor activity (14). In addition, macrolide resistance is common but varies (15, 201). Two older reports of findings of beta-lactamase production in *F. necrophorum* are unfortunately commonly cited as representative. The first study reports two cases without a denominator (202) and the second investigated 22 strains of *F. necrophorum* and found five to have beta-lactamase activity using nitrocefin disks (203). However, all other, more recent, and geographically varying investigations of antimicrobial susceptibility highlight that resistance to penicillin is rarely seen (at most a few percent). Similarly, resistance to clindamycin or metronidazole has hardly ever been described (14, 15, 16, 51, 59, 204, 205).

Surgical treatment

Surgery is a key component in many cases of Lemierre's syndrome, both before and after the introduction of antibiotics (14, 32). Historically, internal jugular vein ligation or even excision was performed in attempt to reduce continuous septic embolism (3). When pre-antibiotic mortality rates of 90% are considered (1), these

drastic measures are easier to understand. While jugular ligation is continuously discussed in the literature (206, 207, 208) and reported in case reports it has increasingly fallen out of favour (14), mainly due to favourable outcomes of septic thrombophlebitis with medical treatment alone. From population-based studies, it is evident that it is rarely used (18, 21). In a meta-analysis of published cases from 2000-17 by Valerio et al. jugular vein ligation or embolectomy was performed in 6% of cases with Lemierre's syndrome (23). In a similar meta-analysis performed by Gore, based on cases published from 1980-2017, jugular vein ligation or excision was performed in 4% (24). It is reasonable to assume that publication bias affects these numbers and overestimates its use.

More importantly, other surgical interventions for source control are recommended and commonly performed, e.g., drainage of pleural empyema or peritonsillar abscess. In the meta-analysis by Valerio et al., 42% of cases with Lemierre's syndrome underwent surgical abscess drainage (23). In the prospective and population-based data by Hagelskjaer et al. (18), when examining the cases with a throat infection with dissemination beyond only a positive blood culture, 31% developed pleural empyema, 19% subcutaneous abscesses, and 15% meningeal empyema or meningitis. 62% needed any kind of surgical intervention, typically drainage. Surgical ligation or excision of the internal jugular vein was not performed in any patient.

Anticoagulation therapy

The most characteristic feature of invasive infections with *F. necrophorum* is the association with septic venous thrombophlebitis. The prospective study by Hagelskjaer et al. (18) on its epidemiology did not include a systematic investigation of thrombophlebitis and one of their findings was that radiological evaluation of jugular vein thrombosis was not consistently performed. However, there is enough evidence based on retrospective analyses to conclude that invasive infection with *F. necrophorum* has a stronger association with the development of septic thrombophlebitis than other infections (14). Nevertheless, very little evidence exists on how to manage them, and recommendations are generally given based on opinion and very low to low quality evidence (14, 23, 147, 152). Similarly, knowledge is lacking on the causes of thrombogenesis, specifically for *F. necrophorum* subsp. *funduliforme*, as highlighted previously in this thesis.

In case series involving patients prior to the millennium approximately one in five of cases defined as Lemierre's syndrome received anticoagulation. The argument was then often raised that most patients responded well without treatment with anticoagulation (14, 209). Hagelskjaer et al. (18) similarly reported that "only a few patients with septic thrombophlebitis received anticoagulation therapy" in their prospective study and emphasise that anticoagulation therapy is problematic in patients requiring surgery. Holm et al. (21) later investigated treatment in patients

with confirmed jugular vein thrombosis (n=14). Six received anticoagulant treatment, two received prophylactic doses of anticoagulants and six did not receive any or only a few doses. Among patients not treated, no complications developed, whereas one patient developed progression of jugular thrombosis despite being treated with full dose anticoagulation. Due to the lack of strong signals suggesting benefits of treatment of septic thrombophlebitis, many have considered treatment not generally indicated and reserved it for patients with evidence of progression of thrombosis (152, 199, 208, 210, 211, 212).

On the contrary, data from septic pelvic thrombophlebitis is sometimes extrapolated to suggest that anticoagulation should be used in Lemierre's syndrome (213, 214, 215). While more commonly given in septic pelvic thrombophlebitis, the only randomised controlled trial performed did not show any benefit, and anticoagulation therapy has become increasingly controversial also here (214, 216). Finally, data from the treatment of non-septic venous thromboembolism is also generalised as an argument for treatment in Lemierre's syndrome, as is the safety of the *continuation* of anticoagulation in patients already treated for other indications when they become affected by infective endocarditis (23). While similarities could be drawn between septic and non-septic thrombophlebitis, similarities could also be drawn between infective endocarditis and septic thrombophlebitis, where infectious processes cause thrombi or vegetations to develop, with the main difference being arterial vs. venous vegetations. There is data to suggest also that those who develop infective endocarditis when already on anticoagulation therapy are less prone to present with septic embolisation before diagnosis and start of antibiotics, yet these effects disappear following antibiotic therapy (217, 218). However, an older non-randomised study has indicated a higher risk of bleeding among those treated with anticoagulants (219). But as an added treatment in itself in infective endocarditis, anticoagulation is not recommended. The only high-quality interventional study performed investigated anti-platelet therapy, and not anticoagulation, in infective endocarditis and then showed higher rates of bleeding and no improved outcomes (220).

In the latest meta-analyses of published cases with Lemierre's syndrome focusing on anticoagulation, performed by Gore (24) and Valerio et al. (23), their conclusions differ, where Valerio et al. suggest benefit and Gore does not. Still, despite no firm evidence for its benefit, anticoagulation therapy appears to be increasingly used in Lemierre's syndrome (23). In accordance with the findings of Valerio et al. (23), it is biologically plausible that anticoagulation therapy could be effective in clearing thrombosis or stopping the progression. Nonetheless, the main importance in the management of Lemierre's syndrome needs to be holistic. Given the very low quality of evidence, mainly based on case series and reports with conflicting findings, we cannot adequately answer the question of whether septic embolisation will increase or decrease if patients are treated with anticoagulation, what the risks of bleeding are or if patients will recover (quicker defervescence or shorter duration

of hospital stay) in comparison to those treated with antibiotics alone, preferably then treated with antibiotics and dosing strategies that likely reach higher levels within thrombi (221).

Thrombogenesis

It is evident that *F. necrophorum* is thrombogenic, though as reviewed earlier in this thesis, most laboratory research has identified more virulence factors potentially involved in thrombogenesis among *F. necrophorum* subsp. *necrophorum* (Table 2). When compared with other pathogens, thrombocytopenia is less commonly seen in other more common causes of bacteraemia than *F. necrophorum* (222), especially when presenting as Lemierre's syndrome where at least mild thrombocytopenia is seen in a majority of cases (21, 178, 223). Disseminated intravasal coagulation has been described to occur as part of the syndrome but is rarely seen (14, 224, 225, 226). A few case reports have described underlying thrombophilia as a potential cause of thrombogenesis in Lemierre's syndrome (195, 227, 228, 229, 230), but in a population-based evaluation by Holm et al. (21) no evidence for such a linkage was found. Given that infection arises adjacent to the internal jugular vein, where the vein has been described to be macroscopically inflamed (3, 231), it is likely that inflammatory cascades involving endothelial damage and exposure of tissue factor from these are involved in thrombogenesis, suggesting that the extrinsic pathway of coagulation is important (232). In vitro experiments by Holm et al. (97) have shown the contact system to be activated at the surface of *F. necrophorum* subsp. *funduliforme*, which subsequently could lead to the activation of the intrinsic pathway of coagulation. Given the common finding of thrombocytopenia in Lemierre's syndrome, it is plausible that platelet activation is involved in thrombogenesis. This would be in line with growing evidence suggesting an important role of platelet activation in the interplay between infection, inflammation, and thrombosis development, sometimes referred to as thromboinflammation (124, 233, 234). These links are not specific to *F. necrophorum* but could help form a framework for hypotheses on its pathogenesis. Acknowledging the complex interplay is nonetheless essential since most effects can have dual effects as well as cause reciprocal changes that could be both pro- and antithrombotic (124, 234).

Non-head and neck infections

The majority of infections with *F. necrophorum* arise from the head and neck region, but as previously described a subset of infections originate from gastrointestinal and urogenital sources (1, 36). These findings are commonly ascribed to an underlying locus minoris resistentiae (14, 21). A small proportion of

cases arising from skin infections have also been described (19). In addition, whereas head and neck infections are typically seen among adolescents and younger adults, non-head and neck infection occurs in an elderly population with the exception of rare obstetrical infections (18, 19, 21, 36, 235).

Aims and rationale

Aims

The papers of this thesis aimed to investigate:

I. Tonsillar carriage of *F. necrophorum* and its geographical differences

II. Associations of complications with *F. necrophorum* in pharyngotonsillitis

III. Incidence, clinical presentation, and prognosis of invasive *F. necrophorum* infections, focusing on Lemierre's syndrome

IV. The impact of the presence of jugular thrombosis in Lemierre's syndrome and the effects of anticoagulation therapy

V. Proteomic expression in plasma in Lemierre's syndrome to generate hypotheses on thrombogenesis

Rationale

Paper I

Defining what normal flora is, is not straightforward. *F. necrophorum* has historically been labelled normal flora, yet when literature is scrutinised, claims are largely unsubstantiated. Tonsillar carriage rates in healthy controls have varied from 0-21%, with the highest rates seen among adolescents and young adults in studies that originate from the United States, Sweden, Denmark, the United Kingdom, and Japan (52, 53, 75, 76, 77, 78, 79, 80, 81, 82). Due to suggestions of a transient pattern of carriage mainly among adolescents and young adults (11, 50), the rationale of this study was to investigate tonsillar carriage in this age group, prior to further studies investigating infections mainly occurring in the same age group. In addition, since only two previous studies have investigated the presence of *F. necrophorum* in Sub-Saharan Africa, both focusing on its role in noma (72, 74), no data was available on tonsillar carriage or infection in participants from Sub-Saharan Africa. Thus, a cross-sectional study in Sweden and Zambia was designed to investigate tonsillar carriage among asymptomatic participants, primarily aimed at 15–25-year-olds, with participants above 25 years old also recruited in Zambia given the lack of prior studies.

Paper II

Pharyngotonsillitis is one of the most common causes to attend primary health care and accounts for more than 10% of prescribed antibiotics in Sweden (186). Starting in the 1950s several studies investigated the effect of antibiotics in pharyngotonsillitis, or sore throat, and found that antibiotics decreased purulent complications, symptoms as well as non-purulent complications generally ascribed to as secondary to *S. pyogenes*, e.g., glomerulonephritis and rheumatic fever (144, 145, 146). However, since these studies were performed, the panorama of pharyngotonsillitis in high-income countries has changed. Numbers needed to treat to avoid purulent complications secondary to *S. pyogenes* have increased and no cases of non-purulent complications have been identified in study settings for more than half a century. Consequently, antibiotic therapy in *S. pyogenes* pharyngotonsillitis is now prescribed mainly to reduce symptom burden in high-income settings (144, 236). Given the findings of *F. necrophorum* as a common pathogen in pharyngotonsillitis in case-control and cross-sectional studies, diagnostics for *F. necrophorum* are increasingly being used despite no guidelines advocating its use (237, 238, 239). However, it has not been known whether a finding of *F. necrophorum* in pharyngotonsillitis is associated with the development of complications. Therefore, we designed a retrospective cohort study investigating the association of microbiological findings of *F. necrophorum*, or beta-haemolytic

streptococci, with the development of complications after an initial pharyngotonsillitis visit to either a primary health care centre or a hospital. All cases tested with both PCR for *F. necrophorum* and throat culture since the introduction of the *F. necrophorum* PCR in Region Skåne, Sweden were enrolled.

Paper III

Several investigations of invasive *F. necrophorum* infections have reported increasing incidence rates over the last decades (15, 16, 17, 18, 19, 20, 21, 149). In addition, a regional population-based study was recently performed in Region Skåne, Sweden, which highlighted a potential increase over time (21). While a few population-based studies have described the clinical presentation of invasive infections with *F. necrophorum* (18, 19, 20, 21), most literature is based on case reports and case series (14, 23, 37, 156, 178). To provide firm evidence of a potential increase in the incidence of invasive infections of *F. necrophorum* and an updated description of clinical features, hospitalisation, sequelae, and mortality, a nationwide, population-based study in Sweden from 2010-2017 was designed.

Paper IV

When caring for patients with Lemierre's syndrome, the most common question is whether to treat with anticoagulant therapy in the presence of jugular vein thrombosis. A few meta-analyses and reviews have assessed the available evidence, which primarily consists of case reports and series, with varying conclusions reached (14, 23, 24, 152). To produce data of better quality, we used data from the observational study above (Paper III) to investigate risks and benefits when therapeutic, prophylactic, or no anticoagulation was given to patients with Lemierre's syndrome with jugular vein thrombosis. We also investigated differences between patients with Lemierre's syndrome with jugular vein thrombosis as opposed to those with only indirect signs of septic thrombophlebitis, i.e., septic embolisation. This study represents the as of yet largest population-based data set available on Lemierre's syndrome.

Paper V

Then why do patients with Lemierre's syndrome develop thromboses? Evidence arising from studies on *F. necrophorum* subsp. *necrophorum* has suggested several pathogenic pathways, but these have often been less evident or not seen in studies of *F. necrophorum* subsp. *funduliforme* (Table 2) (106). However, *F. necrophorum* subsp. *funduliforme* can activate the contact pathway with potential subsequent activation of the intrinsic coagulation pathway (97) and given its predilection for venous inflammation and thrombosis, it is plausible that tissue factor exposure

following endothelial damage and activation of the extrinsic coagulation pathway could occur. Thrombocytopenia is similarly an important feature of Lemierre's syndrome (181), why platelets likely are involved in the crosstalk of inflammation and coagulation. Given its rarity, the few studies available focusing on thrombogenesis have been based on non-systematically enrolled isolates or purely in vitro experiments. We designed a prospective, population-based case-control study where all patients with Lemierre's syndrome were enrolled rapidly and compared to patients with other severe infections using mass spectrometry of plasma to characterise the plasma proteome of Lemierre's syndrome and compare it to other infectious presentations for hypothesis-generation on why septic thrombophlebitis develops.

Methods

Study design

	I	II	III	IV	V
Design	Prospective cross-sectional.	Retrospective regional cohort study.	Retrospective population-based nationwide descriptive study.	Retrospective population-based nationwide cohort study.	Prospective population-based (cases) case-control study.
Population	Healthy participants 15-25 years old in Lund, Sweden (n=100) and above 15 years old in Macha, Zambia (n=282).	Pharyngotonsillitis patients (ICD-10-code J02-3) tested for *F. necrophorum* (PCR) and beta-haemolytic streptococci (culture) in Region Skåne, Sweden (n=3700).	Invasive infections with *F. necrophorum* in Sweden 2010-17 (n=300).	Lemierre's syndrome patients who had been investigated for jugular venous thrombosis (n=82) and if present (n=51) treated with therapeutic, prophylactic, or no anticoagulation.	Lemierre's syndrome cases in Skåne 2017-21 (n=8), with age-matched controls (other severe infections) (n=15), other septic (n=3), or non-septic thromboses (n=3).
Data sources	Microbiology diagnostics and case report forms.	Regional microbiology and medical records, registries on antibiotic prescriptions, drug charts, health care visits, and diagnoses.	Nationwide microbiology and medical records from health care visits.	Nationwide microbiology and medical records from health care visits.	Regional microbiology diagnostics, case report forms, and plasma samples analysed by mass spectrometry.
Outcomes	Tonsillar carriage rate (PCR) with *F. necrophorum* by age, sex, and region.	Composite outcome of complications following a finding of *F. necrophorum,* GAS, or GCS/GGS with OR presented for associations with each microbiological finding.	Incidence and comparisons between 2010-13 vs. 2014-17. Clinical and laboratory presentation by disease category.	Clinical differences by the presence of jugular venous thrombosis. Complications by therapeutic, prophylactic, or no anticoagulation.	Differentially expressed proteins in cases vs. controls.

Overall methods

Setting and data

Investigations were performed in Lund, Sweden and Macha, Zambia (Paper I), in Region Skåne (Paper II and V), or nationwide in Sweden (Papers III, IV). Prospective enrolment of patients was performed in Papers I and V. In Paper I, a cross-sectional study design was used and in Paper V population-based enrolment was performed of cases, with mainly convenience sampling used in controls. Paper II was a regional registry-based observational analysis using medical, microbiological, and pharmacological registries. Papers III and IV were nationwide, population-based, observational studies based on microbiological data linked to medical records in Sweden.

Ethics

In prospective studies, informed consent was given by written consent, except for in illiterate participants, when thumbprints were used. In observational studies, ethical review boards waived the need for informed consent. Studies were approved by ethical review boards in Lund, Sweden (no. 2017/971, no. 2017/740), National Ethical Review Board in Sweden (no. 2019e03892), and the National Health Research Ethics Board in Zambia as well as the local Institutional Review Board (no. 2019.03) at Macha Research Trust, Zambia. Partnering studies used to enrol controls in Paper V were approved by Ethical Review Boards in Lund (no. 2015/143 and no. 2016/271).

Author contribution

For Paper I the ethical application process for Paper I was led by me. For Papers II-V, the ethical application process was initiated and led by Karin Holm. For Papers I-V I was involved in the design of the studies, with shared lead role with the senior author for Papers II and IV. For Papers I-V I had either a lead or a shared lead role concerning data curation, methodology, statistics, analysis, writing of original draft, review, and editing.

Specific methods

Paper I

Case definitions, microbiological diagnostics, and outcome

Healthy participants without signs of throat infection, prior antibiotics (4 weeks), and no history of tonsillectomy were enrolled in this cross-sectional study. Age and gender were noted. Tonsillar carriage of *F. necrophorum* was the measured outcome. The primary analysis focused on the tonsillar carriage rate and the geographical difference between Sweden and Zambia among 15–25-year-olds. A secondary analysis investigated the tonsillar carriage rate among participants aged above 25 years old in Zambia, and carriage rates by gender and age 15-19 years vs. 20-25 years were compared. Ct-values in positive findings were compared between countries. All samples were analysed with PCR for *F. necrophorum* at the Clinical Microbiology Laboratory at Skåne University Hospital, Sweden, and stored and shipped at -80 degrees Celsius prior to analysis (240). In brief, a real-time PCR was carried out on a CFX96 Real-Time PCR machine (BioRad). Primers amplified *rpoB* in *F. necrophorum* and used the housekeeping gene *RNaseP* (human) as amplification and inhibition control. The *rpoB*-probe was based mainly on Jensen et al. (53). If the amplification control for the human housekeeping gene *RNaseP* was negative, the samples were diluted 1:10 to reduce the impact of potential inhibitors. If still negative, an analysis of the *Beta-globin*-gene was performed. If still negative, the samples were excluded. Not part of our study, but previously performed, the sensitivity of the *F. necrophorum* real-time PCR was evaluated and the threshold for detection was found to be $10^3 – 10^4$ colony forming units/mL.

Statistics

Power calculation was performed *a priori*. Based on a previous meta-analysis (12) the tonsillar carriage rate was hypothesised to be 8%. The study was powered to detect a difference of 50% in either direction, with a power of 0.80 and a significance level at $p<0.05$. Due to the lack of data from Africa and Zambia, we wanted to include a larger number of participants here. Therefore, we opted to enrol participants in a 1:2 ratio. 99 individuals were required to be enrolled in Sweden, and 198 in Zambia to reach adequate power. We aimed at enrolling 100 participants in Sweden, 200 in Zambia (in the age group 15-25 years) with an additional 100 participants aged above 25 years in Zambia. Outcomes (tonsillar carriage) were compared using Fischer's exact test for binary variables and student's t-test for normally distributed continuous variables.

Paper II

Case definitions and microbiological diagnostics

Observational data were collected with the case definition being an episode of acute pharyngotonsillitis (ICD-10 code J02-03) in a patient where both an *F. necrophorum* PCR and a throat culture for beta-haemolytic streptococci had been taken. The study period was 2013-2020 and patients were included from all of Region Skåne, including both hospitals and primary health care centres. Patients were excluded if they had a previous (within 30 days) diagnosis of chronic tonsillitis, peritonsillar or other pharyngeal abscesses, sinusitis, otitis, sepsis or septic complications, including complications registered on the day of the index visit, or with previous (within 30 days) antibiotic therapy. This was defined as a beta-lactam antibiotic, clindamycin, or metronidazole. All microbiological results were collected from those performed as per the usual clinical routine. The real-time PCR was performed as described in Paper I above, with the exception that no *Beta-globin* control was performed in addition to the normal *RNaseP* control (since this is not part of the clinical routine). The clinical routine for throat culture includes tonsillar samples obtained using the ESwab test containing Liquid Amies medium and a regular flocked swab (Copan). 30 µL of the sample was plated on Columbia agar plates with sheep blood in the top layer, where the substrate was prepared using a Columbia II agar (BD2997596). The plates were incubated anaerobically in an Electrotek anaerobic workstation (Electrotek Scientific) for a minimum of 16 hours. Typical beta-haemolytic colonies were Lancefield classified using a Streptex Latex Agglutination Test, according to the manufacturer's instruction (Remel; Thermo Scientific R30950501) as GAS, GCS, or GGS. Further species identification of GCS/GGS (e.g., *S. dysgalactiae* supsp. *equisimilis* and *dysgalactiae*, *S. equi*, and *S. canis*) with MALDI-TOF MS was not routinely performed, since this was not part of the clinical routine for throat cultures in Region Skåne, Sweden.

Outcomes

The primary outcome of this registry-based observational study was a dichotomous composite outcome of complications occurring from day 1-30 from the index visit. A complication was defined as peritonsillar abscess, other pharyngeal abscesses, otitis, sinusitis, recurrence of pharyngotonsillitis (day 15–30), sepsis or septic complications, and hospitalisation. Hospitalisations with an orthopaedic, psychiatric, or surgical (e.g., trauma) primary diagnosis were excluded. Recurrence of pharyngotonsillitis was defined as a new diagnosis of acute pharyngotonsillitis after 15–30 days. The association between microbiological findings and the development of complications was investigated and compared.

Statistics

The number of cases during the study period determined our sample size. For the primary outcome, we performed crude and adjusted logistic regression analysis. The dependent variable was complication status within 30 days. Associations were investigated for *F. necrophorum*, GAS, and GCS/GGS with patients with negative findings used as reference category. Independent variables used to adjust regression analyses were age, gender, index test location (primary healthcare centre or hospital), and comorbidities (241).

We did not include antibiotics, since we considered it being an effect modifier with suspected indication bias (severely ill patients were suspected to be more likely to receive treatment). Instead, we stratified data into two groups (treated with antibiotics or not within one day of the index visit) and investigated associations within each stratum.

Subgroup analysis of cases with known RADT-negative pharyngotonsillitis was performed as well as sensitivity analyses, e.g., investigating co-infections separately, excluding cases with prior pharyngotonsillitis, and using different periods of inclusion.

Paper III

Case eligibility, microbiological diagnostics

A multicentric, nationwide, population-based observational study of all invasive infections with *F. necrophorum* in Sweden was performed from 2010-2017. A case was defined as a finding of *F. necrophorum* by culture or molecular methods (PCR or 16S rRNA) from normally sterile sites (blood, deep-lying abscesses (excluding peritonsillar abscesses), pleural, joint, cerebrospinal or pericardial fluid). When a case was identified, medical records were retrieved from the onset of the disease with follow-up for 6 months.

During the study period species identification from positive blood cultures changed to MALDI-TOF MS, which was routinely used at 17/23 laboratories in Sweden in early 2013. Prior to and parallel with its introduction, species identification was performed according to local routines at each laboratory, involving phenotypic characterisation and often 16S rRNA gene sequencing of isolates. In short, MALDI-TOF MS uses a laser beam to ionise bacteria that is placed on a target plate. On this target plate, a matrix is added, on which the bacteria are crystallised. When the laser beam hits the sample, protonated ions are generated. These are accelerated at a fixed potential, where they then separate according to their (and the bacterium's) mass-to-charge ratio. Almost all bacteria have a unique footprint of mass-to-charge ratios. Typically, in microbiological diagnostics, time of flight (TOF) is used to detect and

measure these mass-to-charge ratios, which rapidly and accurately identify the species of bacterium (242, 243).

Outcomes and definitions

The primary outcome was the incidence of *F. necrophorum* infection and investigation of a potential increase between 2010-2013 vs. 2014-2017. Secondary outcomes included sub-group categorisation as Lemierre's syndrome, head and neck infection without Lemierre's syndrome, and non-head and neck infections, with descriptions of each. Lemierre's syndrome was defined as an invasive infection due to *F. necrophorum* with a preceding oropharyngeal infection and the development of thrombosis or signs of septic embolisation. Head and neck-infection without Lemierre's syndrome comprised all head and neck infections not fulfilling the Lemierre's definition and invasive non-head and neck-infection comprised all other infections.

Statistics

No power calculation was performed, hence all cases diagnosed during the study period determined the sample size. When incidence increases were analysed and compared between the first four and the four last years of the study, the difference in incidence was analysed with student's t-test. Groupwise comparisons were performed using analysis of variance or Kruskal-Wallis's test according to distribution. Differences in dichotomous variables were analysed using the chi-square test. Post-hoc pairwise comparisons between groups separately were performed with the Bonferroni procedure to control for alpha error (Type 1).

Paper IV

Case eligibility

In Paper IV, the data collected during Paper III were investigated further, focusing on the importance of the presence of jugular vein thrombosis in Lemierre's syndrome and anticoagulation therapy. Hence, data collection was multicentric, nationwide, population-based, and observational. Invasive infection was similarly defined as in Paper III, but the case definition now required that radiological investigation of the presence of jugular vein thrombosis had occurred, in addition to cases fulfilling criteria for Lemierre's syndrome, i.e., invasive infection with *F. necrophorum*, oropharyngeal symptoms preceding presentation with invasive infection, and presence of septic thrombophlebitis, either as a radiologically visualised thrombosis or septic embolisation. Thus, patients who fulfilled Lemierre's syndrome criteria but were not investigated radiologically for jugular vein thrombosis by ultrasound, computerised tomography, or magnetic resonance imaging, were excluded.

Jugular vein thrombosis, exposure, outcomes

Patients were first grouped as Lemierre's syndrome with or without jugular vein thrombosis, and groups were compared. Then, patients with Lemierre's syndrome with jugular vein thrombosis were grouped according to exposure to anticoagulation and grouped by therapeutic dose, prophylactic dose, or none. Outcomes were defined as thrombosis progression or new occurrence of thrombosis, peripheral septic complication after diagnosis, peripheral septic complication after discharge, 30-day mortality, chronic major sequelae at 6 months, and major bleeding.

Statistical methods

As for Paper III, no power calculation was performed a priori, with the sample size limited to the number of cases identified in Sweden during the study period. Due to very low rates of missing data, a complete case analysis was performed. On analysis of normally distributed data, in pairwise comparisons student's t-test was used, and analysis of variance in multiple comparisons. Bonferroni correction to control for alpha error (Type 1) was used in pairwise comparisons. Non-normally distributed variables were compared using Kruskall-Wallis's test. Binary variables were compared using the Fischer-exact test due to the few participants in the separate groups.

Paper V

Case eligibility and study design

This was a case-control study, where the primary analysis involved:

1. Cases with Lemierre's syndrome enrolled during 2017-2021 following rapid notification when *F. necrophorum*-bacteraemia was identified at the Clinical Microbiology Laboratory, Skåne University Hospital, Lund.
2. Other severe infections in age-matched individuals (13-50 years) consisting of patients with *F. necrophorum*-bacteraemia without Lemierre's syndrome as well as cases enrolled by convenience sampling during clinical shifts and via a partnering sepsis alert study.

In secondary analyses, we enrolled age-matched patients with non-septic venous thromboembolism from a partner study at the Center of Thrombosis and Haemostasis, Skåne University Hospital, Malmö (244) and cases with other septic thromboses identified by convenience sampling.

Samples and mass spectrometry

Citrate plasma samples were drawn from all cases. The samples underwent routine preparation prior to mass spectrometry, including centrifugation, protein digestion, and desalting. Following the preparation of samples, high-performance liquid

chromatography was performed. In brief, this means that during liquid chromatography, individual components of a mixture (here a plasma sample) are separated. The sample is pushed through the liquid chromatography device by high pressure and becomes separated. It is the affinity of the individual components with a porous medium within the liquid chromatography device that determines the time it takes for the components to travel through the liquid chromatography device, separating each one at different times before they are injected into the mass spectrometer (245, 246, 247).

Mass spectrometry is an analytical technique based on the mass-to-charge ratio (m/z) of charged particles, i.e., ions. All mass spectrometers use electric or magnetic fields to determine the mass-to-charge ratio of the specific analytes. Step by step, the analytes introduced at different times following liquid chromatography become ionised. Ions are separated according to their masses by a mass analyser whereafter a detector measures the ions and calculates their respective abundances. In this way, specific peptides can be measured and their relative abundance in a sample determined, and the proteome can be described (245). A schematic of this process is shown in Figure 6.

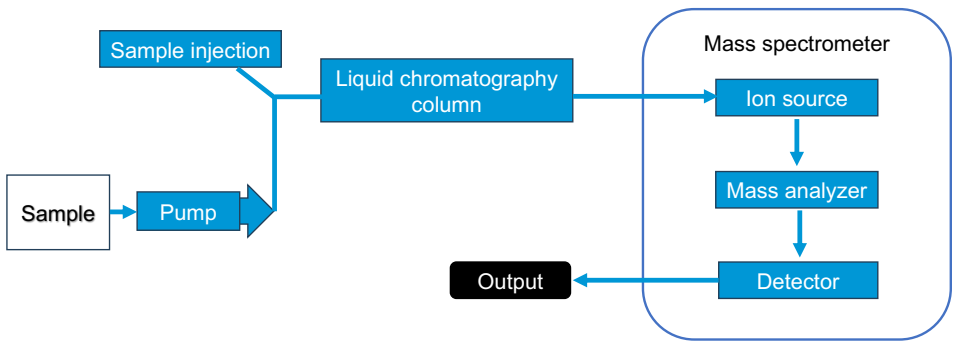

Figure 6: Schematic picture of liquid chomatography mass spectrometry
Schematic picture of when a sample, in our case plasma, is pumped with high pressure into the liquid chromatography column, where it is separated into individual components over time which are then ionised in the mass spectrometer. Hereafter, the mass-to-charge ratio is determined and ions detected according to this ratio as well as their relative abundance are measured. Finally, data are presented (output) whereafter data management is required, typically processed using commercial software, e.g., Spectronaut (248).

While these techniques are constantly developed, low-abundance peptides remain the hardest to detect and quantify. The three main strategies in mass spectrometry to detect peptides are selected reaction monitoring (targeted proteomics), date-dependent acquisition, and data-independent acquisition (non-targeted proteomics) (245, 247). In selected reaction monitoring (SRM) the analysis is focused on pre-defined proteins, which can be detected with high accuracy. This is a powerful

method but it is not useful when the aim of the experiment is discovery (249). In data-dependent acquisition (DDA), peptide signals that rise above noise are detected and matched to a spectrum in a database. This is very powerful, however, only picks out the peptides with the highest abundance and has lower resolution for low-abundance peptides. Data-independent acquisition (DIA) combines these two previous methods and is the most frequently used (245, 250). Its advantages include that it provides higher resolution within a defined mass-to-charge window, where typically a data library can be created, without the need for a previous library as in data-dependent acquisition or defined targets as in selected reaction monitoring. Data-independent acquisition thus provides higher resolution, including peptides of lower abundance, and is reproducible (250). In Paper V, we used DIA mass spectrometry to allow a richer proteomic description of the plasma samples.

Statistical analysis

Of the abovementioned targeted or non-targeted approaches for peptide identification, bioinformatics and statistical analysis are most complicated in DIA given the large amount of data generated and number of peptides detected (250, 251). Therefore, the output of mass spectrometry analyses from DIA is normally processed using one of several available and constantly updated software (248, 252, 253). In Paper V, Spectronaut was used. In short, data precursor and fragment ions were processed and correlated to identify and quantify peptides and proteins. Given the multiple comparisons performed, false discovery rates were reduced by setting q-values (false discovery rates) at <0.01. I.e., comparisons were punished to reduce statistical alpha or type 1 error (wrongful rejection of the null hypothesis). Following initial data analysis in Spectronaut, findings were exported. Hereafter, downstream analysis is more straightforward. Nevertheless, the least abundant proteins are likely to be missing in several individuals, and since several hundreds of proteins will have been detected further data management is essential.

This means that peptides with a certain range of missing data need to be discarded or imputed. We decided that if more than 30% of the data were missing for a specific peptide, this peptide was discarded. In short, data were then log2 transformed and normalised by median subtraction. If a peptide is abundant, it is easy to measure, why missing peptides are likely to be less abundant. Thus, most imputation approaches in mass spectrometry are left-censored, meaning that new imputed values will be positioned at the left range of the Gaussian curve (251, 254, 255). We used a standard method, which performs a downshift of 1.8 standard deviations on the Gaussian curve for the imputed value. Hereafter, the differential expression of proteins was investigated using student's t-test with Benjamini-Hochberg false discovery rate correction for multiple comparisons (256). Any protein with a significant log2 fold change of at least 1 was considered differentially expressed. These analyses were performed for the primary analysis, investigating differences between Lemierre's syndrome and other severe infections. The abundance of the

identified differentially expressed proteins where then also compared between Lemierre's syndrome and patients with venous thromboembolism as well as patients with other septic thromboses, again with multiple comparison correction using Benjamini-Hochberg (256). Proteins and their respective known functions were identified through Uniprot (257) and literature searches.

Results

Overall results

We described tonsillar carriage of *F. necrophorum* in one in five adolescents and young adults in Sweden, but it was rarely identified in Zambia. *F. necrophorum* was also shown to be the most prevalent finding among cases with pharyngotonsillitis tested for *F. necrophorum* and beta-haemolytic streptococci. Importantly, and despite the tonsillar carriage rate reported, a finding of *F.* necrophorum in pharyngotonsillitis was associated with increased rates of development of complications and at a similar rate as GAS. Interestingly, a finding of GCS/GGS had an inverse association with complications why its relevancy in pharyngotonsillitis is questioned. When invasive infection occurs, it either manifests as Lemierre's syndrome, other head and neck infections with bacteraemia, or other, often abdominal, infections. We describe that all invasive infections with *F. necrophorum* have increased in Sweden in recent years, for reasons unknown. Then we described anticoagulation therapy, where most patients with jugular vein thrombosis recovered well without therapeutic anticoagulation therapy, though adverse events were similarly rare in anticoagulated patients. Finally, in the prospective analysis of patients with Lemierre's syndrome using mass spectrometry of plasma, several proteins associated with thrombogenic pathways such as endothelial damage and platelet activation were differentially expressed which distinguished Lemierre's syndrome from other severe infections.

Paper I

Participants

A total of 382 participants were included, of whom 100 were 15-25 years old and recruited in Sweden, 201 were 15-25 years old and recruited in Zambia and 81 were above 25 years old and recruited in Zambia. Among Swedish participants (all 15-25 years old) 57% were female. Among Zambian participants of the same age, 73% were female. Among participants aged above 25 years in Zambia, 68% were female.

Primary outcomes

Overall, tonsillar carriage of *F. necrophorum* was seen in 8%. The tonsillar carriage rates in participants aged 15-25 years were 21 % in Sweden and 3% in Zambia. In Zambian participants aged above 25 years, the tonsillar carriage rate was 1%.

Secondary outcomes

The tonsillar carriage rates in 20–25-year-old participants were slightly higher when compared to 15–19-year-old participants, but the differences were not significant. Similarly, no significant difference was seen when compared by sex.

Paper II

Participants

A total of 3700 patients with pharyngotonsillitis who had been tested for both *F. necrophorum* (PCR) and beta-haemolytic streptococci (throat culture) were included. 54% had negative results in both investigations. *F. necrophorum* was the most prevalent finding (28%), followed by GCS/GGS (13%) and GAS (10%, either culture or RADT). Among all cases, 65% had a RADT performed and registered. In the subgroup of 13–30-year-olds prevalence of *F. necrophorum* was 38%. A slight female overrepresentation was seen in cases with pharyngotonsillitis who tested positive for *F. necrophorum* (58%). However, females were similarly overrepresented in the study (59% of all 3700 cases enrolled) why the risk of testing positive for *F. necrophorum* was similar regardless of sex.

Primary outcomes

In 20% of patients the primary outcome of any complication within 30 days occurred. The most common complications of the composite outcome were hospitalisation (9%), recurrence of pharyngotonsillitis (day 15-30) (9%), and peritonsillar abscess (5%). GAS had the highest complication rate of 28%, followed by 26% for *F. necrophorum*, 17% for negative results, and 16% for GCS/GGS. If co-infections with GCS/GGS and either GAS or *F. necrophorum* were disregarded, complication rates for GCS/GGS were even lower at 12%.

Association with the composite outcome of complications was seen for GAS (OR 1.9, 95% CI 1.5-2.5) and *F. necrophorum* (OR 1.8, 95% CI 1.5-2.1), whereas GCS/GGS were negatively associated (OR 0.7, 95% CI 0.5-0.98). After adjustment for age, sex, comorbidities, and site of inclusion, associations were similar. The timing of any complication and peritonsillar abscess is highlighted in Figure 7-8. In Figure 7, rates of *F. necrophorum* complications became equivalent to GAS after day 15, due to recurrence of pharyngotonsillitis more commonly seen for *F. necrophorum*. In Figure 8, the association between *F. necrophorum* and the development of peritonsillar abscess is highlighted specifically.

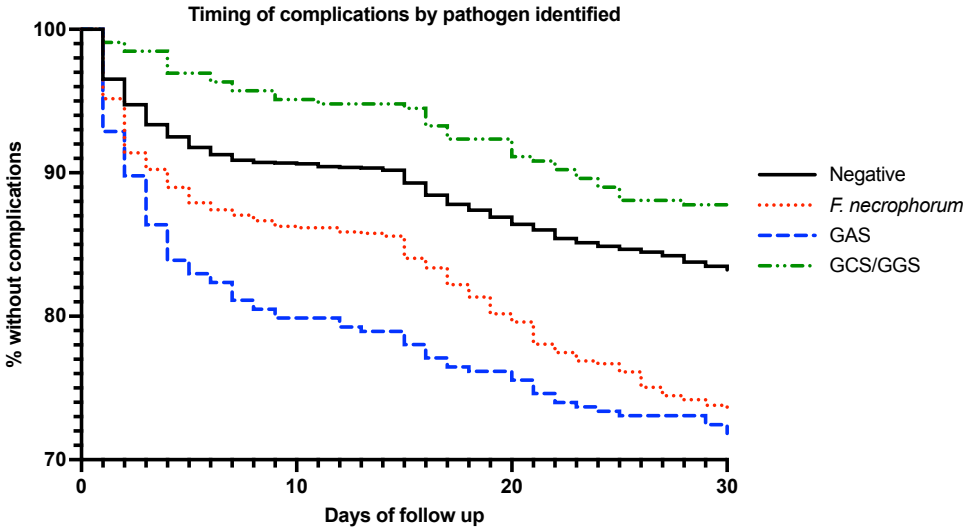

Figure 7: Timing of any complication of the composite outcome in patients with pharyngotonsillitis (n=3700) grouped by microbiological finding.

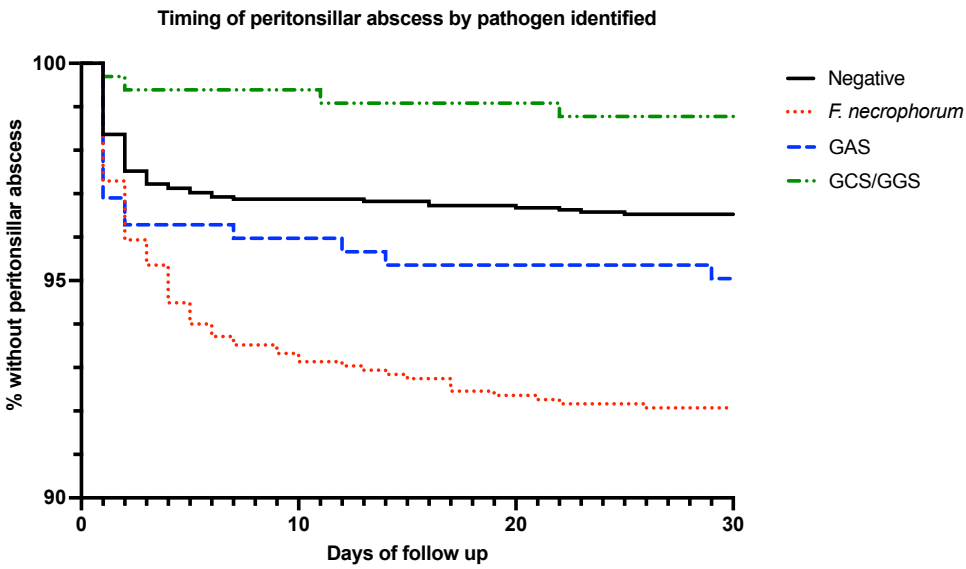

Figure 8: Timing of peritonsillar abscess in patients with pharyngotonsillitis (n=3700) grouped by microbiological finding.

Secondary outcomes

Most patients in the study received effective empiric antibiotics on the index visit, and we suspected that the distribution of antibiotic therapy would suffer from indication bias. This was evident when comparing patients with negative findings who were treated (n=1013) or not treated (n=1003). The complication rate among treated was 24% vs. 9% in non-treated, confirming the presence of indication bias.

Hence, the cohort was instead stratified into patients empirically treated vs. not treated. In each stratum, associations between complications and microbiological findings were evaluated. Among patients not empirically treated (n=1552) only *F. necrophorum* was associated with increased complication rates. Among non-treated cases, very few were RADT-positive for GAS, since treatment is then recommended (Figure 9A). Among patients empirically treated with antibiotics (n=2148), both GAS and *F. necrophorum* remained positively associated with increased complication rates (Figure 9B).

Several further subgroup and sensitivity analyses were performed. First, solely RADT-negative patients with pharyngotonsillitis were investigated (n=2292). Only *F. necrophorum* was then associated with increased complication rates (OR 2.0, 95% CI 1.5-2.5). Interestingly, GAS cases in this RADT-negative cohort were not associated with increased complication rates (OR 1.5, 95% CI 0.8-2.5), but numbers were low (n=97, 4%) and type 2 error possible.

Second, we investigated associations to complications in different age categories (<13, 13-30, and >30 years old) and found *F. necrophorum* to be associated with complications in patients 13-30 and >30 years old.

Third, co-infections were compared to monomicrobial infections in terms of associations with complications, and *F. necrophorum* had similar associations to complications regardless of co-infection with GAS/GCS/GGS or not. Overall, when identified *F. necrophorum* was the sole finding in 80%, with co-infections seen with GCS in 9%, with GAS in 5%, and GGS in 5%.

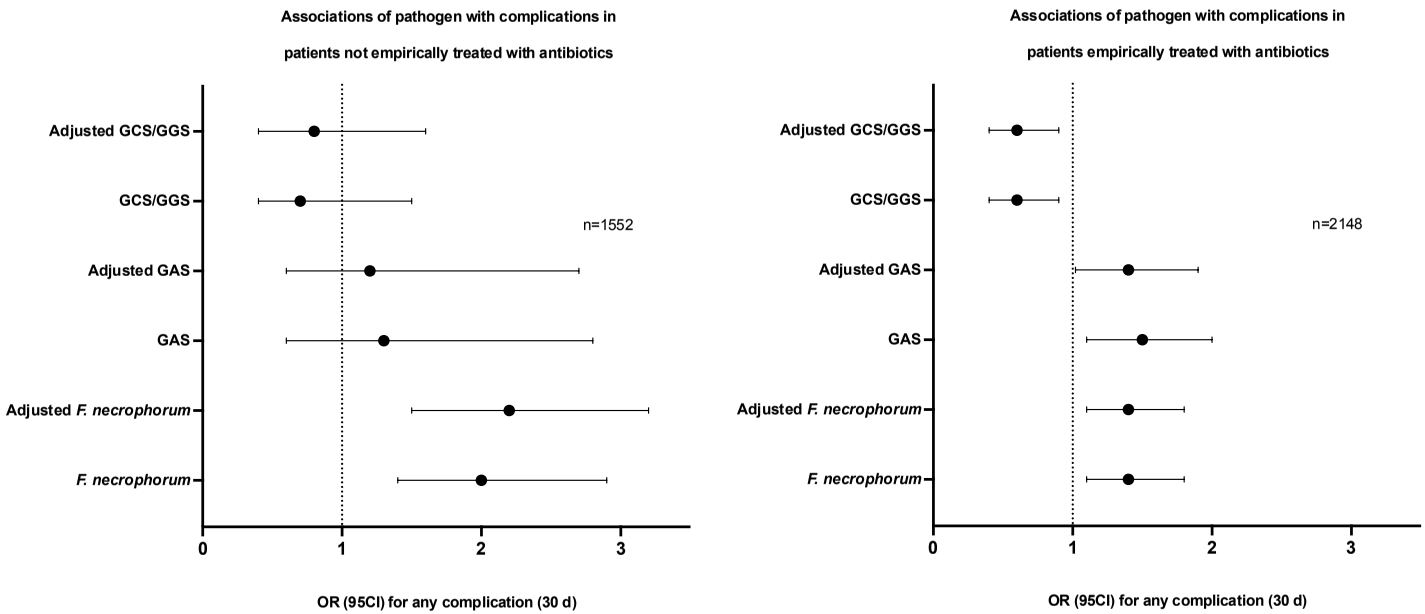

Figure 9A-B: Association of pathogen with complications in patients with pharyngotonsillitis stratified by empiric antibiotic therapy on day 1 (right-hand side) or not (left-hand side). Adjusted and crude analyses are shown for each microbiological finding. Patients with negative results were used as the reference category. Independent variables used to adjust regression analyses were age, gender, index test location (primary healthcare centre or hospital), and comorbidities.

Paper III

Participants

From 2010-2017 300 cases of invasive infection due to *F. necrophorum* were identified in Sweden, equating to an incidence of 3.9 cases/million/year. Cases were defined as Lemierre's syndrome (n=104), head and neck infection without Lemierre's syndrome (n=102), and invasive non-head and neck infection (n=94). 285/300 (95%) of cases were identified by bacteraemia, and the remaining 5% constituted culture, PCR, or 16S rRNA-positive samples from other normally sterile sites. Age distribution varied, with adolescents and young adults affected by head and neck infections and the elderly by non-head and neck infections (Figure 10). Lemierre's syndrome equally affected both sexes (52% female), whereas both other invasive head and neck infections and invasive non-head and neck infections were overrepresented in males (74% and 63% respectively).

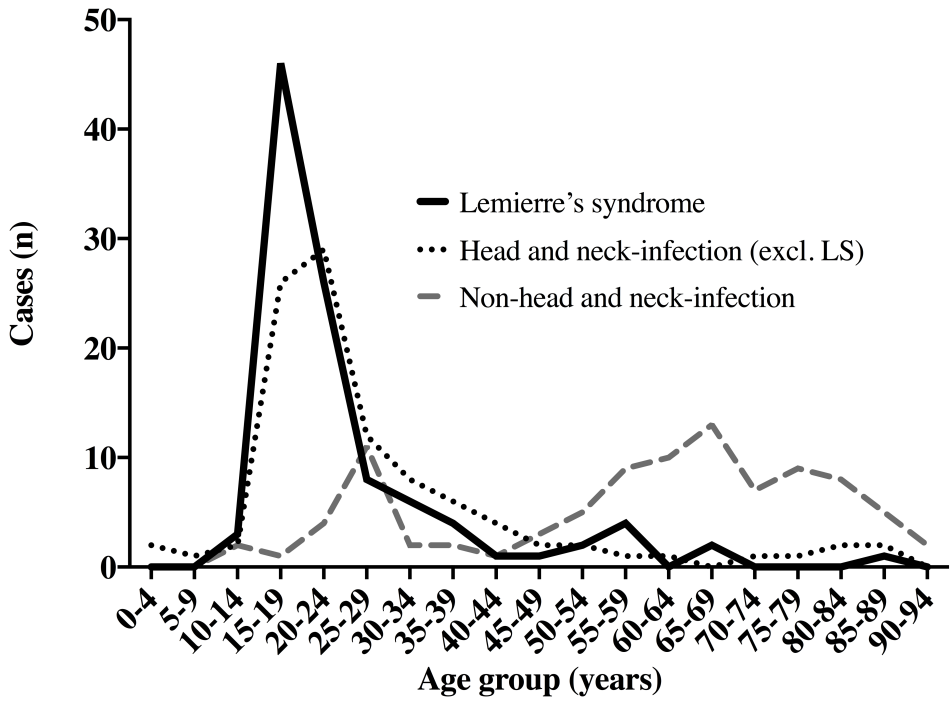

Figure 10: Age group distribution of invasive infections with *F. necrophorum* in Sweden 2010-2017 grouped as Lemierre's syndrome (LS), head and neck-infections without Lemierre's syndrome, and non-head and neck infections.

Primary analysis

When comparing 2010-2013 with 2014-2017, it was evident that infections had increased. This increase was seen for all three presentations (Figure 11). Overall, the incidence rates of invasive infections with *F. necrophorum* increased from 2.9 to 5.0 cases/million/year (95% CI 1.8-3.9 vs 4.4-5.6) when comparing the first with the last four years of the eight-year study period. Lemierre's syndrome increased from 1.0 to 1.7 cases/million/year (95% CI 0.79-1.2 vs. 1.2-2.2).

As implied from Figure 10, the peak incidence occurred in 15–19-year-olds. In this age group, the incidence of invasive infection increased from 10 to 23 cases/million/year (95% CI 3.8-17 vs. 18-28). The peak incidence of Lemierre's syndrome specifically in the age group 15-19-year-olds also increased from 5.6 to 16 cases/million/year (95% CI 2.6-8.6 vs. 9.1-22). Data on yearly incidence and by presentation are seen in Figure 11. There was no seasonal variation seen over the year (Table 6).

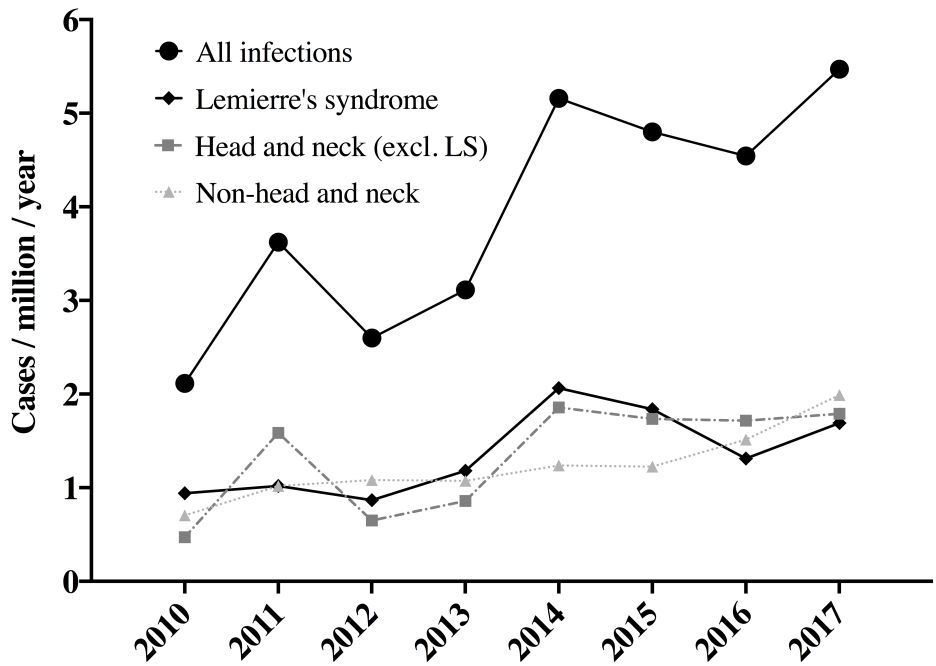

Figure 11: Yearly incidence of invasive infections with *F. necrophorum* in Sweden years 2010-2017 grouped as Lemierre's syndrome (LS), head and neck infections without Lemierre's syndrome, and non-head and neck infections.

Table 6 – Seasonal variation of invasive infections with *Fusobacterium necrophorum* in Sweden 2010-2017

Season	n/total (%)	Mean amount of cases per season per year (standard deviation)
January – March	72/300 (24%)	9 (6.6)
April – June	76/300 (25%)	9.5 (3.7)
July – September	71/300 (24%)	8.9 (3.3)
October – December	81/300 (27%)	10.1 (4.2)
p-value for seasonal difference (analyis of variance)	0.95	

Microbiology and antimicrobial susceptibility

Most infections were monomicrobial, yet in 18% of cases polymicrobial bacteraemia occurred. *S. anginosus* was the most common other species identified in co-infections (7%). Polymicrobial infection was most seen in non-head and neck infections (29%), followed by Lemierre's syndrome (17%), and head and neck infections without Lemierre's syndrome (9%). In patients with Lemierre's syndrome specifically, 98/104 were tested with culture from upper respiratory samples (nasopharyngeal or tonsillar). 6% grew GAS and 7% grew GCS. Findings consistent with acute EBV infection were found in 5/55 (9%) of those tested. Antimicrobial resistance was rare. In all *F. necrophorum* isolates, only two cases with reported resistance to penicillin were seen. No resistance to metronidazole or clindamycin was reported.

Lemierre's syndrome

The median age of the 104 patients with Lemierre's syndrome was 20 years (IQR 17-26) of which 61/104 (59%) reported a previous health care visit where only 6/104 (6%) received effective antibiotic therapy. 54/104 (52%) were female. Very few had less than 3 days from symptom onset when Lemierre's syndrome had developed, with a median of 5 days of symptoms (IQR 4-8) on presentation.

The vast majority had sepsis on presentation, and one in five presented with septic shock (Sepsis 3 (258)). Three quarters of patients had thrombocytopenia on admission. 57/82 (70%) of examined patients had direct signs of thrombosis, of which 62% were jugular vein thrombosis. Pulmonary complications were very common, mainly as multifocal pneumonia (91%) with specifically pulmonary septic emboli reported in half. Pleural empyema developed in 30%. Other complications such as arthritis were seen in 6%, whereas 2% had central nervous system infections. Very few patients with Lemierre's syndrome had a peritonsillar abscess (3%).

All received effective antibiotic therapy on admission, and the total duration of therapy was a median of 36 days (IQR 28-44) with intravenous therapy being given

for a median of 10 days (IQR 7-15), probably impacted by about a week until defervescence on median (IQR 3-13 days). ICU admission was required in 43%, with vasopressor therapy given in 28% and ventilator therapy in 26%. The duration of hospital stay was a median of 12 days (IQR 8-20). The 30-day mortality rate was 2%. Major sequelae at 180 days were seen in 7% of survivors. No case of relapse of invasive infection was seen.

Head and neck infections without Lemierre's syndrome

The median age of the 102 patients presenting with head and neck infections without Lemierre's syndrome was 23 years (IQR 18-33). A minority (26%) were female. 53% had a peritonsillar abscess and 33% had isolated pharyngotonsillitis with bacteraemia. In general, patients had a shorter duration of symptoms (median 3 days, IQR 2-6), were less commonly septic (26%), and less commonly thrombocytopenic (24%) compared to patients with Lemierre's syndrome. Like in Lemierre's syndrome, a majority (61/102 (61%)) reported a previous health care visit, however, few had received prior effective antibiotic therapy (16/102 (16%)). Given that peritonsillar abscess was the most common presentation, peritonsillar puncture or incision was commonly performed. Thrombosis was seen in one patient, who developed subdural empyema with secondary cerebral venous thrombosis, yet did not fulfil our definition of Lemierre's syndrome. Effective antibiotics were given on admission to 99% of patients. The total duration of antibiotics was a median of 13 days (IQR 10-14) and the time to defervescence was short (median 2, IQR 1-3 days). ICU admission was rare (6%). The length of hospital stay was a median of 3 days (IQR 1-4). 30-day mortality was 1% and major sequelae was seen in 3% of survivors at 180 days.

Non-head and neck infections

The median age of the 94 patients presenting with invasive non-head and neck infections was 64 years (IQR 46-75). 37% were female. These patients commonly had comorbidities (259), e.g., 31% had a known cancer diagnosis and 14% had disseminated malignancy. 50% had abdominal or urogenital foci. Other foci varied with pulmonary, gynaecological, skin, musculoskeletal, and peri- or endocarditis all described. Three patients had thrombosis, all in the portal vein. The 30-day mortality was the highest in this group (14%) which increased further at 180 days (24%).

Paper IV

Participants

Of the 104 patients in Paper III that fulfilled the criteria for Lemierre's syndrome, 82 had performed imaging of the jugular veins and were included in this study. First, patients were grouped by the presence of jugular vein thrombosis. No differences in baseline characteristics were seen between groups. Second, patients with jugular

vein thrombosis were grouped by exposure to therapeutic, prophylactic, or no anticoagulation. Neither here differences in baseline characteristics were seen between groups. No patient was treated with jugular ligation. No patient was treated with antiplatelet therapy.

Outcomes – impact of jugular vein thrombosis

When comparing patients with Lemierre's syndrome with (n=51) vs. without (n=31) jugular vein thrombosis, patients with jugular vein thrombosis had a trend for higher SOFA-score on admission (median 5 vs 4, p=0.05), had lower platelets (median 76 vs 112 x 10^9/L, p=0.04), more often had typical septic pulmonary emboli (69% vs 32%, p<0.01) and took longer to defervesce (12 vs 7 days, p=0.03). 30-day mortality and major sequelae at 6 months were similar between groups and rare.

Outcomes – anticoagulation therapy

Among the 51 patients with Lemierre's syndrome with the presence of jugular vein thrombosis, 20 received no anticoagulation, 14 received prophylactic dosing and 17 received therapeutic dosing. On presentation, baseline characteristics as well as SOFA scores were similar between patients. While patients were similarly ill on presentation, cases treated with either prophylactic or therapeutic anticoagulation more frequently had or developed pleural empyema, were admitted to the ICU to a larger extent, needed longer hospital stays, and took more days to defervesce. These factors were not considered as outcomes, since they could be considered as both cause and effect of anticoagulation treatment. To exemplify, prophylactic dosing anticoagulation therapy was associated with ventilator therapy, but ventilator therapy was the indication for anticoagulation to be given and not the inverse.

Instead, outcomes investigated were progression or new occurrence of thrombosis after diagnosis, peripheral complication after diagnosis, chronic major sequelae at 6 months, 30-day mortality, and major bleeding during hospitalisation. None of these differed between the groups, yet all outcomes were rare. Progression or new occurrence of thrombosis occurred in one patient without anticoagulation and in one treated with a therapeutic dose. Two patients developed peripheral septic complications after diagnosis, both presented with arthritis and were not treated with anticoagulation. Major sequelae were seen in one patient treated with prophylactic and one patient with therapeutic anticoagulation. 30-day mortality was seen in one patient without anticoagulation treatment, and none in other groups. One case of major bleeding was seen in a case treated with a therapeutic dose.

Paper V

Participants

Eight cases with Lemierre's syndrome and 15 with other severe infections were enrolled for the primary analysis as cases and controls. For secondary analyses, three patients with non-septic venous thromboembolism and three patients with other septic thromboses were enrolled. All cases with Lemierre's syndrome had *F. necrophorum* bacteraemia and represented all cases with Lemierre's syndrome in Region Skåne during the study period. Among patients with other severe infections used as controls, 8/15 (53%) had bacteraemia, in which *F. necrophorum* (n=3), *Escherichia coli* (n=2), *S. agalactiae* (n=1), *S. anginosus* (n=1) and polymicrobial bacteraemia (n=1) were identified. In terms of baseline characteristics, most notably, only 25% of cases with Lemierre's syndrome were female as compared to 47% of cases with other severe infections.

Primary analysis

23/454 proteins identified by mass spectrometry of plasma were differentially expressed in Lemierre's syndrome when compared to other severe infections (controls). Following a literature search, 16 of the 23 differentially expressed proteins were found to have known associations with thrombosis, either suggested as causal in function or implied as biomarkers. 14 of 16 had suggested prothrombotic effects in Lemierre's syndrome, and 2 of 16 had suggested antithrombotic effects. Four of the most interesting prothrombotic proteins identified were neutrophil defensin 1, intercellular cell adhesion molecule 1, CD44-antigen, and neutrophil gelatinase-associated lipocalin.

Of the two proteins with suggested antithrombotic effects differentially expressed in Lemierre's syndrome, the most interesting was Plastin-2.

Secondary analysis

The 23 differentially expressed proteins from the primary analysis were then compared against three patients with non-septic thromboembolism and three patients with other septic thromboses. These patients were age-matched and had few comorbidities, but patients with non-septic thromboembolism or other septic thromboses were more often started on anticoagulation therapy when sampled, and slight differences in age, sex, and sepsis were seen. Naturally, patients with non-septic thromboembolism did not have bacteraemia or sepsis. No proteins were differentially expressed between Lemierre's syndrome and other septic thromboses. 14/23 proteins were differentially expressed when compared with patients with non-septic thromboembolism, including neutrophil defensin 1, intercellular cell adhesion molecule 1, CD44-antigen, neutrophil gelatinase-associated lipocalin and Plastin-2 mentioned above.

Discussion

Taken together, Paper I-V study *F. necrophorum* from tonsillar carriage to Lemierre's syndrome. This rare but spectacular syndrome is at the centre of attention, yet the main findings of this thesis appear when findings are observed holistically. Despite tonsillar carriage with *F. necrophorum* being relatively common in adolescents and young adults, these individuals are evidently also affected by its most common presentation, namely pharyngotonsillitis. Its importance in pharyngotonsillitis is established when associations with common local complications are highlighted, particularly peritonsillar abscess and recurrence of pharyngotonsillitis. In addition, all its invasive infections are found to increase in Sweden and mainly arise from pharyngeal infections. Interestingly, an opportunity to prevent them is here highlighted. A majority of patients had prior health care visits before their septic presentation but were then rarely identified as having a potential pharyngeal *F. necrophorum* infection and were thus rarely tested or treated. Lemierre's syndrome is seen to have devastating individual effects but remains rare. Causes and treatment of septic thrombophlebitis in Lemierre's syndrome are investigated, and several thrombogenic pathways are highlighted. Whether patients benefit from anticoagulation therapy remains a debated topic.

Tonsillar carriage and geographical differences

In Paper I, the tonsillar carriage in adolescents and young adults in Sweden was seen to be as common as when described by Jensen et al. (53), but higher than in other studies (Table 1). Age and tonsillar carriage correlate, where children and the elderly appear to have the lowest rates (52). No apparent sex differences have been described in tonsillar carriage. Longitudinal studies of tonsillar carriage are few (50) but have indicated that carriage is transient. It remains possible that carriage varies over seasons, between settings and geographical areas. This is in line with the finding that in Zambian participants, the tonsillar carriage was very rare, regardless of age. Whereas tonsillar carriage rates have varied between 0-21% in studies from high-income countries (11, 52, 53, 76, 79, 80, 81), only two studies on the bacterium existed prior to this thesis from Sub-Saharan Africa and none investigated tonsillar carriage or infection (72, 74). Geographical differences have also been seen in other pharyngeal pathogens, e.g., *S. pyogenes* (260, 261), EBV (153, 262), and *N.*

meningitidis (140). Socioeconomic factors (263), animal exposures (36), and other yet unknown ecological factors could also impact the transmission, carriage, and infection of *F. necrophorum* and need to be studied.

While tonsillar carriage rates have implications in high-income countries on how test results from individuals with symptoms are interpreted, similar tonsillar carriage rates as described for *F. necrophorum* have also been described for *S. pyogenes* (79, 139), *S. dysgalactiae* subsp. *equisimilis* (GCS/GGS) (79, 264) and *N. meningitidis* (140, 265), which rarely are referred to as normal flora. My interpretation of the available evidence is that transient tonsillar carriage with *F. necrophorum* occurs during adolescence and young adulthood, and then seems to disappear. Given that this co-occurs with the age where most cases of local and invasive pharyngeal infections occur, a model of transmission in which *F. necrophorum* is exogenously acquired is appealing, where some go on to develop disease whilst others develop immunity and clear the bacteria from the tonsils. Finally, an important topic is raised, i.e., microbiological findings from high-income countries cannot always be extrapolated globally (236). Instead, efforts are necessary to make bacteriological investigations available in low to middle-income countries.

Pharyngotonsillitis and local complications

Guidelines on the management of pharyngotonsillitis vary globally but generally focus on GAS (236, 238, 266). With growing interest in non-streptococcal tonsillitis, and the emergence of *F. necrophorum* as a potentially important pathogen (11, 12), testing has become available (49, 52, 53, 59). In Sweden, guidelines recommend performing a RADT for GAS if patients have 3-4 Centor criteria (267) and treating them with penicillin for 10 days if the result is positive (237). Mainly, treatment is recommended to decrease symptom severity and duration, since the numbers needed to treat in high-income countries to avoid complications have increased (144). No guideline thus far recommends testing for *F. necrophorum* (147, 238, 266). Nevertheless, since the introduction of the PCR for *F. necrophorum* in clinical practice in Region Skåne, Sweden, it has increasingly been used. Recently, a small subset of patients with *F. necrophorum*-positive pharyngotonsillitis (n=29) was followed for 2 years and was not seen to develop more complications than controls (268). However, the same group also investigated a larger cohort retrospectively. In a selected cohort of patients with pharyngotonsillitis tested for *F. necrophorum*, 75/625 (12%) patients were positive. Among those positive, peritonsillar abscess developed in 18% when empirical treatment was not given, whereas only in 5% of empirically treated. Evidently, the complication rates were higher than normally expected (144), the difference did not reach statistical significance and the data are observational why cautious interpretation is necessary (269). Patients not empirically treated were more

commonly tested at an ear, nose, and throat-clinic (personal communication, Jon Pallon), why both selection and indication bias might have affected the findings.

In Paper II, we investigated 3700 patients with pharyngotonsillitis from all public primary health care facilities and hospitals in Region Skåne. While it is established that *F. necrophorum* is a prevalent finding in pharyngotonsillitis that mainly affects adolescents and young adults (13-30-year-olds) (78), our study also established a clear association with the development of complications, at a similar rate to GAS (270). Conversely to what has previously been suggested (11), we found no sex differences in relation to the positivity of a test for *F. necrophorum*. Given the presence of indication bias and lack of clinical data to adjust regression analyses, we could not adequately adjust analyses for antibiotics and determine their effect. Instead, we stratified patients by empiric treatment and found *F. necrophorum* to be associated with the development of complications in both cohorts (Figure 9A-B). Interestingly, our findings also suggest that there is a limited need for throat cultures in addition to RADT for GAS and PCR for *F. necrophorum* if the reason for identification is to prevent the development of complications. In fact, a finding of GCS/GGS was negatively associated with the development of complications, whereas if GAS grew in throat culture despite a negative RADT, this was not associated with the development of complications.

Importantly, sensitivity analyses investigated the impact of co-infections and found *F. necrophorum* to be similarly associated with complications regardless of the presence of another pathogen. While the study is large, its observational design selects a severely ill population, given the need for extended testing for the patients to be included. This explains the high rates of bacterial pathogens as well as the low rates of GAS found since most of these patients are identified by RADT in the Swedish setting and generally not tested further. It also explains the high complication frequency. Important to note, is that selection bias impacts *F. necrophorum* and GCS/GGS equally and we thus suggest that GCS/GGS might not need to be tested for despite reports of its potential to cause pharyngitis (138, 142, 143, 271, 272, 273). These findings should inform future guidelines on how to test beyond RADT for GAS. While awaiting future studies, guidelines on extended testing with PCR for *F. necrophorum* should target carefully selected 13–30-year-old RADT-negative pharyngotonsillitis patients with severe symptoms. Notably, the efficacy of antibiotic therapy in pharyngotonsillitis due to *F. necrophorum* has never been evaluated in an interventional trial. While efficacy of antibiotic therapy is plausible, until evaluated it remains speculative.

Invasive infection and opportunities for prevention

As described in Table 5, epidemiological studies of invasive infections with *F. necrophorum* suggest increasing incidence. This is likely partly due to the increased use of blood cultures and increased diagnostic sensitivity, but the perception of an increase perseveres. In Paper III, we presented data on increasing nationwide incidence rates in Sweden to the highest rates seen of invasive *F. necrophorum* infections (181). These infections were most common among adolescents and young adults in whom main presentations included either Lemierre's syndrome or pharyngeal infections (e.g., pharyngotonsillitis or peritonsillar abscess) with bacteraemia without septic thrombophlebitis. Interestingly, patients with pharyngeal infections yet not Lemierre's syndrome were less commonly seen to be thrombocytopenic and were less septic than Lemierre's syndrome patients who also seldomly developed a peritonsillar abscess. Non-head and neck infections represented a different entity of disease, with an elderly population with more comorbidities affected. Primary foci of infection were commonly abdominal, either gastrointestinal or genitourinary and locus minoris resistentiae and suspected bacterial translocation was commonly seen or suspected. Lemierre's syndrome was found to equally affect both sexes (52% female), similar to when described by Lemierre himself (1) and somewhat opposing recent meta-analyses (23, 24), whereas both other invasive head and neck infections and invasive non-head and neck infections were overrepresented among males (74% and 63% respectively), similar to what has previously been described (14, 21).

All types of invasive infections increased. One potential cause is diagnostic improvement due to the introduction of MALDI-TOF MS for species identification, but in most, including the largest, regions of Sweden, this shift had already occurred during the first years of the study. Interestingly, while far from proven as causal, antibiotic prescription of RADT-negative pharyngotonsillitis has decreased in line with the current guidelines in Sweden (186, 237) which coincide with the reported increase of primarily complications of pharyngeal infections. Similar patterns have been suggested in the United Kingdom (37).

Given the findings of Paper II, in which *F. necrophorum* is established as a cause of pharyngotonsillitis associated with complications, a noteworthy finding is that most patients investigated in Paper III with invasive pharyngeal infection had an initial health care visit due to this oropharyngeal infection, yet few received treatment prior to more severe presentation. Arguably, an opportunity to prevent these complications is missed in a majority of patients later identified to suffer invasive complications. Thus, the problem at hand is to identify who is at risk for complications among the vast number of patients attending primary health care due to uncomplicated sore throat. Testing criteria will need to be developed, for which studies are ongoing, and should focus primarily on adolescents and young adults.

Voices for improved guidance are continuously raised and guidelines are needed (147, 148).

Bank et al. performed a cost-effectiveness analysis, where early recognition and treatment in *F. necrophorum* was suggested to save money, should it reduce complications by 75%. Their approach involved analysing throat swabs for *F. necrophorum* in 15-25-year-olds to then guide therapy. If the reduction of complications were more modest (26%), early recognition would be similar in cost as compared to other life-saving medical interventions (274). It is impossible to perform a randomised controlled trial to investigate the impact on the incidence rate of Lemierre's syndrome, given its rarity. However, studies could focus on symptoms, peritonsillar abscess, and recurrence of pharyngotonsillitis as complications. Importantly, these studies, as well as current treatment guidelines of GAS in pharyngotonsillitis in areas with low risk of rheumatic fever need to consider that narrow-spectrum penicillin is perhaps not as benign in terms of ecological effects as previously suggested (275, 276).

Currently, to my knowledge, there is one study ongoing that focuses on the treatment effect in RADT-negative pharyngotonsillitis (ClinicalTrials.gov: NCT04083417). This study conducted in Sweden includes diagnostics for *F. necrophorum* with the potential for sub-group analysis. The study aims to enrol 260 patients and randomise antibiotic therapy (penicillin) 1:1. Given that this would be a cohort of mainly non-GAS-patients aged 15 years and above with high Centor scores (3-4) (267), it is likely that *F. necrophorum* positivity will be high given findings by Jensen et al. (53) and us (Paper II) (270) when using PCR. If a third of patients would be positive for *F. necrophorum*, then the sub-group analysis of *F. necrophorum* cases should involve at least 40 vs. 40 patients randomised to penicillin or no treatment. In Paper II, using a composite outcome, 26% of patients with *F. necrophorum*-pharyngotonsillitis developed complications. The complication rates were high due to the selection of severely ill given inclusion criteria that required extensive testing, why complication rates among prospective patients with Centor score 3-4 likely is lower. If we would assume a high rate of complications, e.g., 10% of cases among untreated, this study would not be powered to detect a reduction of complications in patients with *F. necrophorum*. At least 73 patients in each group would be required to detect a 100% decrease in complications (power of 80% and alpha 0.05), which is not realistic (144). Instead, potential findings of this study could address symptom reduction regardless of cause and potentially give new insights ahead of potential prospective studies focusing on *F. necrophorum* pharyngotonsillitis. There is already evidence available to suggest that symptom reduction occurs when patients with negative throat cultures for GAS are treated with antibiotics based on 3 studies from 1956 (146), 1997 (277), and 2000 (278), with a Cochrane review suggesting a risk ratio of 0.78 (95% CI 0.63-0.97) for symptom reduction on day 3 in this subgroup, primarily driven by the findings of these three studies. However, the Petersen et al. study (1997) (277) used

erythromycin as antibiotic therapy, which would have limited effect on *F. necrophorum*. None of these studies specifically investigated *F. necrophorum* as a cause of non-GAS pharyngotonsillitis.

Lemierre's syndrome – presentation including septic thrombophlebitis

Paper III provides an updated and population-based description of invasive infections, with a particular focus on Lemierre's syndrome. These patients often required ICU admission and lengthy hospitalisations with complications such as pleural empyema requiring surgical intervention being common. Luckily, given the continuous improvement of health care, the mortality rate was lower (2%) than previously described (14, 23). Among all patients in Sweden with invasive disease due to *F. necrophorum*, 108/300 (36%) had direct or indirect signs of venous thromboembolic disease as part of the presentation, a remarkably high number that greatly exceeds other infections which have been associated with thrombosis (279, 280, 281).

In Paper IV, we first focus on the impact of the presence of jugular vein thrombosis as part of Lemierre's syndrome. In clinical practice, this finding usually incites a discussion about anticoagulation. Here, we present data that its presence is associated with lower levels of thrombocytopenia and a longer duration to defervescence. Also, patients with a presence of jugular vein thrombosis more commonly had pulmonary septic emboli described. It is possible that in part, this might be due to classification bias as radiologists often were aware of the presence of septic thrombophlebitis. While not significantly different, patients with Lemierre's syndrome with jugular vein thrombosis also had higher SOFA-score (258), more often needed ICU admission, and spent longer time in the hospital (182). It appears, that on the continuous spectrum of Lemierre's syndrome, the presence of visible jugular vein thrombosis is associated with a more severe presentation, whereas patients without the presence of jugular vein thrombosis, either already had embolised vegetations or may have had smaller venous endothelial vegetations not necessarily seen on imaging. Despite these differences, mortality and major sequelae were similar.

Management of Lemierre's syndrome

In Paper III we highlight that all patients with Lemierre's syndrome in Sweden received effective antibiotic therapy on admission. Antimicrobial susceptibility

patterns of *F. necrophorum* remained favourable, where *F. necrophorum* was almost ubiquitously susceptible to beta-lactam antibiotics, clindamycin, and metronidazole.

Generally, combination therapy with a beta-lactam antibiotic and metronidazole or monotherapy with clindamycin is often suggested with a duration of (2) 4-6 weeks, with clindamycin advised as oral step-down therapy (14, 37, 62).

Based on opinion, I would suggest that patients with less severe presentations, e.g., isolated jugular vein thrombosis without embolisation or with pulmonary presentation solely involving a pulmonary septic embolus or less severe multifocal pneumonia without the development of empyema or abscesses, can be treated for two weeks. These patients constitute a minority, and two weeks of therapy requires that they have had a rapid initial response and stabilised quickly, and that source control is ascertained.

In patients with a more complicated disease, which is the most common presentation, e.g., including either the development of pleural empyema, pulmonary abscesses, slow response to therapy or initial ICU admission, I would suggest four weeks of therapy, with step-down to oral therapy after consideration of source control and when the fever has cleared. Given that Lemierre's syndrome could be considered an intravasal infection (in analogy to infective endocarditis) that is prone to develop abscesses and complications, a prolonged antibiotic duration is justified, yet not based on interventional trials. The duration of antibiotic therapy necessary to sterilise septic thromboses is uncertain. Given the reduced penetration of antibiotics into thrombi (221) and that vessel recanalisation often does not occur quickly (24), prolonged therapy to 4 weeks is reasonable in most cases.

In a subset of patients, a longer duration of therapy is warranted, e.g., including those who develop more complicated pleural empyema where surgical drainage or decortication have been deemed impossible or already unsuccessful, or patients with larger pulmonary abscesses or osteoarticular complications. This should be decided individually and based on clinical and radiological follow-up.

In terms of antibiotic choice, intravenous therapy with penicillin and metronidazole is suggested. This is based on the arguments raised above, i.e., to sterilise infected thrombi and abscesses where surgical drainage is not possible and simultaneously achieve a rapid bactericidal effect with a beta-lactam antibiotic. This is not based on interventional data, and it is possible that intravenous penicillin alone would suffice. However, case reports of failure on monotherapy with intravenous penicillin exist (282), and arguments that other co-infecting bacteria might produce beta-lactamases (283), low penetrance of beta-lactams into thrombi (221), and reduced efficacy in abscesses have been raised (284).

The choice of oral antibiotic should consider the duration of the therapy chosen. I would suggest combination therapy with oral penicillin and metronidazole in most

patients treated for a total of 2-4 weeks. Given anecdotic evidence of failure on both monotherapy with penicillin and clindamycin (14, 282), the poor bio-availability of penicillin when taken orally and the non-negligible presence of particularly streptococcal co-infections (14, 181), using a combination of penicillin and metronidazole is most appealing. Observational data support the use of metronidazole as efficient (14, 21), and as an oral drug, it has excellent bioavailability and could be considered for monotherapy in cases where other non-susceptible co-infections are considered unlikely. This is preferred to monotherapy with clindamycin due to its ecological and weaker, bacteriostatic effect (285, 286). An argument against oral penicillin is the reduced bioavailability when taken orally (287), why some prefer amoxicillin. Given the lack of clinical evidence of its impact, and when given in combination with metronidazole, using as narrow beta-lactams as possible is preferred, although an increased risk of *Clostridioides difficile* when comparing aminopenicillins with penicillin has not been seen (285).

For patients with a need for a longer duration of therapy than four weeks, I would taper intravenous therapy to monotherapy with clindamycin. In light of the increased risk for peripheral neuropathy with metronidazole after four weeks of therapy or a total dose exceeding 42 g, a longer duration than four weeks with metronidazole is not recommended since other alternatives exist (288).

In the typical presentation of Lemierre's syndrome, broader antibiotic therapy beyond intravenous penicillin and metronidazole is rarely indicated. Given that abdominal pain is a common feature initially and the syndromic diagnosis rarely is made in the emergency room, a broader beta-lactam antibiotic is commonly given empirically. In the case of polymicrobial infection, the most common bacterial findings are alpha- or beta-haemolytic streptococci and oral anaerobes, typically susceptible to a combination of penicillin and metronidazole (181). Exceptionally rare events involve polymicrobial infection with *Enterobacterales* and enterococci (14) which do not need to be covered empirically. It is important to acknowledge that clinicians cannot expect patients with Lemierre's syndrome to defervesce rapidly on antibiotics, likely due to a combination of pyrogenicity from slow clearance of bacteria in septic thrombophlebitis as well as complications such as lung abscesses and pleural empyema. In the absence of apparent septic deterioration, a persistent fever should trigger source control evaluation rather than a change of antimicrobial therapy. Multiple use of different antibiotics is commonly seen, but often likely unnecessary (23).

In Paper IV, we focused on anticoagulation therapy in an observational analysis. Patients were categorised according to no, prophylactic or therapeutic anticoagulation. Increasingly, anticoagulation therapy was used over time, yet the rates of adverse events due to Lemierre's syndrome or anticoagulation therapy were similar in all groups and events were rare in general. The main cause of the increased use of anticoagulation therapy was prophylactic anticoagulation therapy, mainly due to better adherence to guidelines of prophylaxis during ventilator therapy. It is

disheartening that we despite an eight-year nationwide study design are unable to conclude whether anticoagulation therapy is beneficial or not.

Conversely, one could also look at it from the opposite perspective. The mortality rate in Lemierre's syndrome is now reported as 2% (181), why this cannot be used as a study outcome should a prospective study be designed. When scrutinising the latest and largest meta-analyses of published cases, by Gore (24) and Valerio et al. (23) in relation to our data (Paper IV (182)), conclusions are heterogenic. This questions the presence of a large beneficial effect of anticoagulation therapy. On scrutiny of the analysis that shows a potential benefit of treatment, i.e., the meta-analysis by Valerio et al. (23), it includes a noteworthy amount of 712 cases defined as Lemierre's syndrome, of which 652 had in-hospital data. Here, early complications were defined as new or recurrent objectively diagnosed acute venous thromboembolism, new or worsening (peripheral) septic lesions, major bleeding, and death within 30 days or within the hospital and were found to be present in 112/652 (17%) patients. In terms of bleeding, antiplatelet agents are presented as one factor seen related to bleeding events. Presumably, they were less used in patients who were also on anticoagulation therapy, but data is not shown (23). Based on the data provided in supplementary tables and after excluding major bleeding events associated with antiplatelet agents, patients treated at any time with anticoagulation therapy had similar rates of major bleeding events (9/362 (2.5%)) as those without anticoagulation (6/290 (2.1%)). Thus, major bleeding events following anticoagulation therapy appear rare. These findings are similar to what we found in Paper IV. In this meta-analysis (23), the development of pleural empyema and lung abscess was considered a new event of "new or worsening (peripheral) septic lesions". Since pleural empyema and lung abscesses often develop from existing infiltrates, it can be debated whether the progression of an already existing pulmonary infiltrate, albeit due to embolisation, should be an outcome in evaluating anticoagulation therapy in septic thrombophlebitis. However, if prospectively evaluated this would be very interesting to investigate. Of course, septic pulmonary embolisation can cause de novo development of pulmonary infiltrates and secondary pleural empyema, yet the timing of events is hard to determine when not systematically investigated. In Paper IV we found the opposite pattern where pleural empyema was more common in patients on anticoagulation. We chose to not define this as an outcome since the timing and occurrence of events were hard to determine despite the availability of all medical records.

The timing of when the intervention starts in non-randomised observational studies is hard to define. This is important when evaluating events in relation to anticoagulation therapy. Many embolic events occur prior to the presentation to hospital, and naturally, it is first when patients have presented, clinicians can decide on whether to treat with anticoagulation or not. In the meta-analysis by Valerio et al (23), embolisation events that occurred after the definition of Lemierre's syndrome

had been fulfilled were registered as events. Since most patients will fulfil the criteria of Lemierre's syndrome on presentation (if microbiological identification is not mandatory), and many embolisation events that occur do so early, many will not have been started on anticoagulation when they occur. If these patients are considered as non-anticoagulated controls in observational analyses, rather than following a randomisation procedure at a specific time, potential bias is introduced with a risk that patients most prone to embolise are overrepresented at baseline among patients not anticoagulated. Anticoagulation therapy is not generally recommended why it often invokes a discussion and thus generally, treatment is not given as soon as Lemierre's syndrome is suspected or diagnosed (289). While hard to address in any observational analysis, several of the events reported in the meta-analysis occurred within the first three days which necessitates consideration when data is interpreted (23).

With these caveats, the remarkable summary of previously published cases provided in the meta-analysis by Valerio et al. (23) reports a trend that new venous thromboembolic events occur more commonly among patients not treated with anticoagulants at the time of the event (p=0.055). When the outcome "new or worsening (peripheral) septic lesions" is added, creating a composite outcome, the difference reaches statistical significance (p=0.035).

Based on the data provided in the supplement, 34 new thromboembolic events occurred in total. Of these, four occurred in patients where anticoagulation status were not specified (23). If these events are excluded, and the occurrence of new thromboembolic events are compared between those treated (13/343 (4%)) vs. not treated (17/309 (6%)) with anticoagulation at the time of the event as defined in the meta-analysis (23), the rates of new thromboembolic events are similar between groups. These rates are also similar to the data presented in Paper IV, where (1/20 (5%)) of not treated and 1/17 (6%) of treated patients developed progression or new occurrence of thrombosis after diagnosis. In Paper IV and here, a new thromboembolic event was used as an outcome instead of anticoagulation use by complication status, as presented by Valerio et al (23), and potential risk differences can then be easily compared between studies highlighting relatively similar findings. In work by Gore (24), in a meta-analysis of published cases with Lemierre's syndrome from 1980-2017, which overlaps the cases presented by Valerio et al. (23), only cases where serial imaging had been performed were enrolled to evaluate venous recanalisation by anticoagulation therapy. Venous recanalisation (i.e., thrombosis resolution) was then *less commonly* seen in patients treated (60%) compared to those not treated (71%) with anticoagulants, yet these differences were not significant and only 50 patients had performed serial imaging. However, a minority of patients were in the non-anticoagulated group and the time to follow-up imaging varied (24).

While efforts by Valerio et al. (23), Gore (24), and us (182) constitute the best available evidence, when assessing available data with the GRADE approach (290),

the risk of bias in all analyses is high, as is imprecision. Data reported are inconsistent, with varying conclusions and suggested effects. Findings are similarly indirect since the definition of a case does not necessitate the presence of jugular vein thrombosis in Valerio et al. (23), which it did in the analysis on vessel recanalisation by Gore (24) and in Paper IV (182), or requires a finding of *F. necrophorum*, which it does in Paper IV (182) but not in either meta-analysis. Both meta-analyses (23, 24) also suffer from publication bias. Thus, in essence, the quality of evidence is low at baseline and downgraded for reasons above to very low. Based on opinion and very low certainty of evidence, I reach the conclusion that prophylactic anticoagulation therapy should be used in patients when indicated (291) and the use of therapeutic anticoagulation should routinely solely be given in patients where thrombosis progression occurs despite adequate antibiotic therapy. Finally, jugular ligation or excision has not been proven to work and is not advised, whereas antiplatelet therapy has been described as a risk factor linked to major bleeding and should be avoided (23). Non-steroid anti-inflammatory drugs should similarly be very cautiously used given that thrombocytopenia is common, as well as acute kidney injury (181). Given that all retrospective data analysis will be affected by bias which we will not be able to adequately control, an interventional trial will be needed to identify a potential effect.

Thrombogenesis

While there is controversy on the role of anticoagulation therapy in Lemierre's syndrome, there is also uncertainty regarding why more than a third of patients with invasive disease due to *F. necrophorum* develop septic thrombophlebitis. Lately, there has been increasing interest in "thromboinflammation". This concept is broad and describes responses and mechanisms that are involved in both thrombosis and inflammation and have been discussed in varying infection-associated thrombotic diseases such as dengue, malaria, sepsis, and COVID-19 (124, 279, 292, 293, 294). Historically, thrombophilia in patients affected by Lemierre's syndrome has been suggested as a cause of septic thrombophlebitis (295), yet this has been shown to be rare in population-based studies (21). As shown in Table 2, most research that has identified virulence factors of *F. necrophorum*, have shown subsp. *necrophorum* (animal infections) to be more virulent. Yet, in *F. necrophorum* subsp. *funduliforme* activation of the contact system (97) has been described, linking inflammation to activation of the intrinsic pathway of coagulation (294, 296). Similarly, thrombocytopenia is commonly seen in Lemierre's syndrome. In sepsis in general, it is usually described to be due to either increased clearance or decreased production (294). Interestingly, among patients with Lemierre's syndrome in Paper III splenomegaly was a relatively common finding, for which causes are unknown

yet could be one of several explanations of why thrombocytopenia is seen (e.g., splenic sequestration).

In Paper V, using mass spectrometry to analyse acute plasma samples, patients with Lemierre's syndrome and other severe infections were compared. Interestingly, most proteins identified had previous associations with thrombosis or had been suggested as biomarkers of thromboembolic events. Acknowledging that thromboinflammation is complex and involves several cascades, all these findings are not necessarily causal or essential. Nonetheless, the results were noticeable. Among differentially expressed proteins with suggested prothrombotic effects in Lemierre's syndrome, intercellular cell adhesion molecule 1 has been shown to be a marker for endothelial injury (297, 298, 299), CD44-antigen has been seen to be expressed as a sign of platelet activation (300) and neutrophil defensin-1 has been described as a potential link between innate immunity and development of thrombosis (293, 301, 302). Interestingly, Plastin-2, known to inhibit platelet production, was also found to be elevated in patients with Lemierre's syndrome (303). Together with potential sequestration or clearance in the spleen, it is possible that the expression of Plastin-2 is a mechanism through which thrombocytopenia develops. Finally, a previously proposed biomarker of pulmonary (304) and septic embolism (305) (neutrophil gelatinase-associated lipocalin) was also found to be elevated in Lemierre's syndrome.

Secondary analyses in Paper V had limited power, yet interestingly found most proteins identified as differentially expressed in the main analysis to also differ when compared to non-septic venous thromboembolism. In line with the speculations about the importance of thromboinflammation, these findings fit and suggest that septic thrombophlebitis, perhaps not surprisingly, develops differently from non-septic thrombosis. Conversely, no proteins were found to be differentially expressed when comparing Lemierre's syndrome with other septic thromboses. The other septic thromboses were very similar in terms of presentation to Lemierre's syndrome, and if the microbiological definition used was less strict, two out of three would have been defined as Lemierre's syndrome, why similarities were expected. These findings are speculative and far from proven causal but provide clues to target for future studies investigating mainly endothelial injury, platelet activation, and inflammatory pathways as inducers of thrombogenesis in Lemierre's syndrome.

Study design, strengths, and limitations

The papers that form this thesis add to the literature on *F. necrophorum* in several aspects and involve several study designs. Paper I was a cross-sectional prospective study, Paper II an observational cohort study, Paper III a retrospective descriptive study, Paper IV a post hoc observational cohort study, and Paper V a prospective

case-control study. The main strengths are the nationwide and population-based data on invasive infections during several years in Paper III and IV, the largest study available on *F. necrophorum* pharyngotonsillitis establishing it as a pathogen associated with complications in Paper II, and the first experimental prospective population-based study investigating the pathogenesis of Lemierre's syndrome in Paper V, highlighting potential pathways through which septic thrombophlebitis develop. In Paper I, in addition to the main finding of the high tonsillar carriage rate found in Swedish 15-25-year-olds, the first data from tonsillar samples in Africa are presented which hopefully inspire future studies on this continent, which likely has the greatest burden of pharyngotonsillitis complications (261, 306).

However, limitations are several. Starting from Paper I, study participants were enrolled in the vicinity of hospital or student settings, which might impact the findings. Similarly, enrolment occurred in different months in Zambia and Sweden. While seasonal variation is not seen in invasive disease, it is not known whether this varies in terms of carriage (240).

In Paper II, analyses were based on a retrospective cohort of patients with pharyngotonsillitis who had been tested extensively to identify pathogens. Selection bias of more severely ill patients then occurs and needs to be considered when interpreting the results (270). This is the main explanation for why the complication rates described are much higher than normally seen in prospective studies on pharyngotonsillitis cases (144, 278, 307, 308, 309). Indication bias impacts analyses on antibiotic therapy, why conclusions about its effect are hard to draw.

In Paper III, the increased incidence described needs to be considered in the light of improved diagnostics (MALDI-TOF MS) as well as increasing use of blood cultures. However, Lemierre's syndrome and invasive infections requiring ICU admission were seen to increase during the study period, contradicting that less severe cases of bacteraemia were identified, which would be expected if the increase was due to widened indications for blood cultures (181).

In Paper IV, despite nationwide inclusion for 8 years to investigate the effect of anticoagulation therapy, patients and events investigated were rare why methods such as propensity-matched regression were not justified. Observational analysis of the impact of anticoagulation therapy has several other problems. First, serial and systematic evaluation of thrombosis progression or regression is not available, and the timing of events were hard to ascertain, given that silent pulmonary embolisation and thrombosis progression can occur. Pulmonary infiltrates can in themselves progress to cause pleural empyema or abscesses, without this necessarily being a thromboembolic event, whereas it is also possible that this arises *de novo* following new peripheral pulmonary septic embolisation. Thus, while the data presented are of the best quality yet, its main use is scrutiny of descriptive statistics and acknowledging that effect sizes of both benefits and harms from anticoagulation appear small.

In Paper V, the main difficulty was the design of a matched control group. Given that Lemierre's syndrome affects a young and previously healthy population, controls needed to be similar. Importantly, controls also needed to have an inflammatory response and suffer from severe infections, given that the main difference in Lemierre's syndrome from other infections is the addition of septic thrombophlebitis to a septic and inflammatory presentation. Thus, we enrolled patients from a partnering, and ongoing, sepsis-alert study as well as performed convenience sampling of young and previously healthy patients admitted with other severe infections, still cases and controls were not perfectly matched. Secondly, limitations mainly revolve around power. Because almost five years of prospective enrolment was required to identify eight cases of Lemierre's syndrome in a population of 1.4 million, a markedly prolonged study period would be needed. Since the aim was to generate hypotheses, we considered the study size sufficient and enrolled controls in a 1:2 ratio to increase the power of the primary analysis.

Conclusions

- Tonsillar carriage of *F. necrophorum* is seen in up to one in five adolescents and young adults and there may be considerable geographical differences.
- *F. necrophorum* is a common cause of pharyngotonsillitis and is associated with the development of complications in selected cohorts, particularly peritonsillar abscess and recurrent pharyngotonsillitis. The inverse association was seen for a finding of GCS/GGS, why relevancy of throat cultures beyond RADT for GAS is questioned.
- Invasive infection due to *F. necrophorum*, including Lemierre's syndrome, is increasing in Sweden. Mortality rates in Lemierre's syndrome have decreased to 2%, most likely due to improvements in health care, yet almost half of the patients require intensive care.
- Anticoagulation therapy of the jugular vein thrombosis in Lemierre's syndrome was neither associated with benefits nor with risks. While prophylactic anticoagulation therapy should be given when indicated, the efficacy of therapeutic anticoagulation is controversial, and its role will remain doubtful until investigated by an interventional study.
- Thrombogenesis in Lemierre's syndrome is hypothesised to occur second to endothelial damage, platelet activation, degranulation, and immunity-induced coagulation cascades.

Future perspectives

During the last decades, the knowledge about *F. necrophorum* has been raised beyond the association with Lemierre's syndrome. Papers that constitute milestones include studies on invasive diseases by Hagelskjaer et al. (18, 19) in Denmark and Jones et al. (149), and Brazier et al. (15) in the UK. These were followed by the development of the PCR for *F. necrophorum* by Aliyu et al. (52) and Jensen et al. (53), which helped to present it as a common finding in pharyngotonsillitis. The extensive reviews by Riordan (14) and Brazier (37) summarised the field, whereas Klug et al. highlighted its major importance in the development of peritonsillar abscesses (13). Since then Centor et al. (76, 147), Hedin et al. (81), Klug (12) and Kjaerulff et al. (82) and Holm et al. (11) have further put the spotlight on its role in pharyngotonsillitis. Recent impressive meta-analyses by Valerio et al. (23) and Gore (24) have renewed the focus on anticoagulation therapy in Lemierre's syndrome following large meta-analyses.

In the five papers of this thesis, we have trailed *F. necrophorum* from tonsillar carriage to Lemierre's syndrome, providing some new knowledge on the way. In Paper I and II we highlight the role of tonsillar carriage, yet establish that despite a relatively high carriage, a finding in patients with pharyngotonsillitis remains significant and causes complications at a similar rate to GAS. Future studies on pharyngotonsillitis should include the evaluation of Ct-values from PCR as a marker of an infection as opposed to a carriage, as well as a predictor of complications. Similarly, acute and convalescent serum analyses should be performed in pharyngotonsillitis, similar to what has been performed in peritonsillar abscess (130). Studies investigating transmission are also lacking and should investigate whether tonsillar carriage and infection rates are focal and occur in outbreaks and families. Testing criteria for *F. necrophorum* pharyngotonsillitis need to be developed and evaluated since current criteria (e.g., Centor criteria (267)) have been shown to have lower predictive potential than for GAS (12, 76). Importantly, differences in presentation of *F. necrophorum* pharyngotonsillitis as compared to GAS pharyngotonsillitis needs to continue to be evaluated (76, 80, 82). Furthermore, pharyngotonsillitis studies are needed in other geographical areas, including low- and middle-income countries, where the burden of pharyngotonsillitis complications is higher (261, 306). Most importantly, evaluation of efficacy in an interventional study using antibiotics to treat *F. necrophorum* pharyngotonsillitis (preferably penicillin or metronidazole) in reducing symptom

severity, symptom duration, and development of complications is needed. Luckily, studies investigating at least part of these objectives are ongoing or intended. Finally, the development of an antigen test for *F. necrophorum* would be an important tool in clinical practice in the subset of patients with pharyngotonsillitis where testing could be suggested.

In Paper III, an increasing incidence of invasive infections was reported. Similar population-based studies from other settings are needed, with data currently lacking from the US, most of Asia, Central- and South America, Oceania, and Africa. While many low-income countries lack diagnostic laboratories to perform blood cultures routinely, centres that do, have not prioritized anaerobic cultures (310). Following the WHO Global Antimicrobial Resistance and Use Surveillance System project (192), increasing amounts of data are gathered from blood cultures, and hopefully, future protocols following the soon to be presented FIEBRE study (311) will also include anaerobic cultures in the future. If there is a causal association between described increases in invasive disease due to decreased antibiotic prescriptions in RADT-negative pharyngotonsillitis, any changes in guidelines on the management of RADT-negative pharyngotonsillitis need to be followed by population-based incidence studies on Lemierre's syndrome.

To adequately study anticoagulation therapy, as intended in Paper IV, an interventional study would be needed. Given that mortality rates have decreased, other outcomes such as serial evaluation of thrombosis evolution, new septic pulmonary embolisation, distant manifestations (e.g., arthritis), time to defervescence, and duration of hospital stay should be investigated. Yet, this would require an immensely large multi-national prospective study to be adequately powered given an incidence of less than 2 cases per million per year, of which not all will have jugular vein thrombosis (181, 182). However, this would require substantial resources to study a therapy that will not prove to have a mortality benefit, given the low mortality rate currently seen (181). It appears reasonable that future research on the topic instead should aim at investigating ways to identify and treat pharyngotonsillitis patients prior to the development of complications.

Finally, in Paper V we designed a hypothesis-generating study that highlights potential thrombogenic pathways in septic thrombophlebitis. These mainly included endothelial injury, platelet activation, degranulation, and immunity-induced coagulation activation. Identified proteins and pathways should be studied in future targeted studies to confirm their importance and their interactions with *F. necrophorum*.

Populärvetenskaplig sammanfattning

Denna avhandling fokuserar på *Fusobacterium necrophorum,* en bakterie som blev känd efter att ha beskrivits i detalj under 1930-talet av bland annat André Lemierre, som ses på framsidan av denna avhandling. Bakterien har ibland beskrivits vara del av vår normalflora, det vill säga en del av de bakterier vi lever med snarare än de vi blir sjuka av, men få studier har undersökt om det faktiskt är så och var bärarskap då ses. Men framför allt är *F. necrophorum* känd som orsak till Lemierre's syndrom.

I flera arbeten beskrev André Lemierre hur tonåringar och unga vuxna i Paris först insjuknade med en halsinfektion. Därefter drabbades de av ett andra och septiskt insjuknande med svullnad på halsen på grund av blodproppsbildning i halsvener och tecken till spridd sjukdom i framför allt lungorna. Detta kallas idag Lemierre's syndrom. Efter att antibiotika blev tillgängligt, minskade rapporterna om denna sjukdom. Under de senaste decennierna har dock allt fler fall-rapporter och undersökningar visat på en tendens att syndromet ökar. I tillägg till detta, har allt fler studier visat att *F. necrophorum* är en viktig orsak till just halsinfektioner i sig, så väl som den vanligaste orsaken till komplikationen som kallas halsböld.

Eftersom många frågetecken kvarstod om hur vanligt det är med bärarskap utan sjukdom av bakterien, hur vanlig bakterien är vid halsinfektioner och hur ofta den ger upphov till mer komplicerade halsinfektioner som kräver specialistvård, så designade vi studierna I och II i denna avhandling.

I studie I undersökte vi hur vanligt det är att bära bakterien i halsen, dels i Sverige, dels i Zambia. I Zambia och närliggande länder i subsahariska Afrika är tillgången till bakteriediagnostik i sjukvården låg. Därför är kunskapen ofta mer bristfällig över vilka bakterier som sprids. Intressant nog, såg vi att en av fem unga vuxna och tonåringar i Sverige bar på bakterien, medan endast ett fåtal identifierades i Zambia, trots att vi letade hos hundratals och här också i spridda åldrar.

När det är vanligt med bärarskap i halsen blir diagnostik av bakterien vid sjukdom mer svårtolkad. Om vi har den när vi mår bra, hur vet vi då att den orsakar sjukdom om vi hittar den när vi mår dåligt? För att svara på detta undersökte vi i studie II hur vanligt det är att bakterien identifieras vid halsfluss och hur vanligt det är att man drabbas av komplikationer som halsböld när den väl har hittats. Intressant nog såg vi att *F. necrophorum* var lika starkt förknippad med utveckling av komplikationer som när Grupp A streptokocker hittades. Detta är den bakterie sjukvården historiskt fokuserat på och behandlat vid halsinfektioner. Dessa fynd är viktiga, då de är nya

och kan hjälpa oss att försöka förbättra handläggandet av patienter med halsinfektioner för att undvika allvarligare infektioner.

Men tillbaka till Lemierre's syndrom, denna fruktade komplikation till infektion med *F. necrophorum*. I studie III, undersökte vi om denna sjukdom, så väl som andra allvarliga infektioner av bakterien, ökat i Sverige under åren 2010 till 2017. Vi samlade ihop alla odlingar tagna från svårt sjuka patienter från alla mikrobiologiska laboratorier i Sverige och såg att fynden av *F. necrophorum* hade ökat. Denna ökning sågs oavsett om vi tittade på Lemierre's syndrom, andra halsinfektioner som halsböld med spridning av bakterier i blodet eller andra allvarliga till exempel bukinfektioner. Vi såg också att nästan hälften av de tonåringar och unga vuxna som drabbas av Lemierre's syndrom behövde vårdas på intensivvårdsavdelning men att dödligheten i sjukdomen sjunkit från 90%, då den beskrevs av André Lemierre, till 2% i Sverige idag.

Efter att ha visat att Lemierre's syndrom ökat i Sverige vände vi blicken mot det som starkast särskiljer den från andra infektioner, nämligen att patienterna drabbas av blodproppar i vener på halsen. Detta är det utmärkande fyndet i Lemierre's syndrom och dessa blodproppar tros bero på bakterien i sig. Det har länge funnits två åsikter vad det gäller behandlingen. Antingen förlitar man sig på antibiotika för att ha ihjäl bakterien som orsakar blodproppen, eller så vill man i tillägg till antibiotika också ge blodförtunnande medicin för att lösa upp blodpropparna. Vi jämförde hur det gick för patienter som behandlats respektive inte behandlats med blodförtunning i tillägg till antibiotika, och såg att det gick ungefär likadant för dem. Slutsatsen blir att det inte finns fog för att ge tillägg av blodförtunnande behandling.

Till sist undersökte vi patienter i Skåne som drabbats av Lemierre's syndrom. Hos dessa patienter tog vi akuta blodprover och jämförde sedan alla proteiner i blodet hos dem jämfört med patienter som insjuknat med andra allvarliga infektioner utan blodproppsbildning. Här sågs flera skillnader som kan hjälpa oss förstå varför just denna bakterie orsakar blodproppsbildning. Exempel är proteiner som vittnar om skada på venernas kärlvägg, blodplättars aktivering och signaler från immunsystemet som kan aktivera levring av blod.

Sammantaget visar dessa studier att *F. necrophorum* är en viktig och vanlig orsak till halsfluss trots att tonåringar och unga vuxna också kan bära på den utan att fara illa. Vi ser att den har ökat i betydelse och frekvens i sjukvården med mer allvarliga infektioner och att vi börjar kunna förstå varför patienterna utvecklar blodproppar. Trots att sjukdomen ökar, går det bättre för de patienter som drabbas av Lemierre's syndrom. Detta är troligen på grund av att sjukvården blir bättre och bättre. Tyvärr verkar det inte som att blodförtunnande behandling vid Lemierre's syndrom har en tydlig effekt, trots att patienterna drabbas av blodproppar, men vi behöver fortsätta studera varför det är så, varför infektionerna ökat i Sverige och hur vi kan förebygga att allvarliga infektioner utvecklas.

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About the author

David Nygren works as an Infectious Disease specialist at the Skåne University Hospital in Lund. His thesis focuses on *Fusobacterium necrophorum*, a bacterium mainly associated with Lemierre's syndrome. It affects adolescents and young adults and occurs after an initially benign pharyngeal infection, where patients then develop septic jugular vein thrombosis that embolises to the lungs and causes critical illness. Professor André Lemierre, pictured at the front of the thesis and after whom the syndrome is named, similarly has an avenue named after him in Paris, on which David is seen to the left.

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