# LUND UNIVERSITY

"A syndrome so characteristic" Molecular and clinical studies of Fusobacterium necrophorum and Lemierre's syndrome

Holm, Karin

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# "A syndrome so characteristic"

Molecular and clinical studies of *Fusobacterium necrophorum* and Lemierre's syndrome

**Karin Holm**
Institutionen för kliniska vetenskaper, Lund
Avdelningen för infektionsmedicin
Medicinska fakulteten

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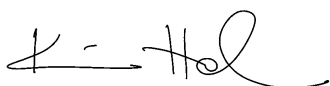
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Docent Peter Bergman
Centrum för Infektionsmedicin
Karolinska Institutet

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**Title and subtitle**

"A syndrome so charachteristic". Molecular and clinical studies of *Fusobacterium necrophorum* and Lemierre's syndrome.

**Abstract**

Lemierre's syndrome is caused by *Fusobacterium necrophorum* and involves tonsillitis, jugular vein thrombophlebitis and septic pulmonary emboli. The first studies underlying this thesis focused on bacterial interactions with coagulation. The contact system initiates the intrinsic pathway of coagulation and the release of the pro-inflammatory peptide bradykinin. We found that the contact system was activated at the surface of *F. necrophorum*, which may contribute the pathogenesis of thrombosis in Lemierre's syndrome. We also found that plasminogen of the fibrinolytic system may be recruited to and activated at the surface of *F. necrophorum*, which may be of importance for invasion and dissemination. To examine if host factors affecting coagulation and invasion could be important in Lemierre's syndrome we conducted a retrospective study of 65 patients with Lemierre's syndrome or other invasive infection with *F. necrophorum*. Patients with Lemierre's syndrome were screened for underlying thrombophilia and concomitant Epstein-Barr virus (EBV) infection and the clinical spectrum was described. On admittance, patients with Lemierre's syndrome were severely ill, in a majority fulfilling the criteria for severe sepsis. Underlying thrombophilia was not over-represented in patients with Lemierre's syndrome and concomitant EBV infection was uncommon. Finally, we found that the gene coding for leukotoxin, a well-known virulence factor for the animal subspecies, was present in human isolates and that there were three types of sequences, of which two were novel. Only a minority of the isolates had a leukotoxin gene of the previously described sequence type. This thesis provides new information about potential bacterial virulence factors, host factors and host-pathogen interactions that may be of importance for *F. necrophorum* infection and adds to the knowledge of the clinical spectrum of invasive infections with *F. necrophorum*.

Key words: *Fusobacterium necrophorum*, Lemierre's syndrome, contact system, fibrinolysis, thrombophilia, leukotoxin

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# "A syndrome so characteristic"

Molecular and clinical studies of *Fusobacterium necrophorum* and Lemierre's syndrome

Karin Holm

LUND
UNIVERSITY

Karin Holm
Division of Infection Medicine
Department of Clinical Sciences
Faculty of Medicine
Lund University
Biomedical Center, B14
Tornavägen 10
221 84 Lund
Sweden
Email: karin.holm@med.lu.se

Cover image by Mattias Karlén: Schematic representation of Lemierre's syndrome. Detail. ©Karin Holm

*"Ce tableau clinique est tellement caractéristique qu'un observateur prévenu ne s'y trompe pas."*

*-André Lemierre*

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# Preface

I believe every infectious diseases specialist has a relation to Lemierre's syndrome; a particularly interesting case, a severe complication, or a relation of when they made their first heroic diagnosis. This holds true also for my first encounters with patients with this infection. To hear, for the first time, about an anaerobe with the ill-fated, name *Fusobacterium necrophorum*, to read up on the diseases it may cause, and to realize that the young and very ill patient in front of you has every sign and symptom of the text-book description of Lemierre's syndrome is memorable. Physicians have re-discovered the diagnosis over and over again, and the excitement when realizing their discovery often results in the urge to publish case reports pointing out that this syndrome has been forgotten, but is now re-discovered (Table 1).

The many years of producing the studies underlying this thesis have led from molecular host-pathogen interactions to actually meeting almost all patients who have had Lemierre's syndrome in the Skåne region since the year 2000. I have had the great luck to be able to talk to the patients and share their insights into the patient perspective of a very serious condition. Sometimes I have also had the opportunity to help patients in understanding a disease that had not been fully explained or probably not fully understood at the time, since the awareness of Lemierre's syndrome has increased over the last 15 years. The combination of molecular and clinical studies has given me insights into the vast lack of knowledge about every aspect of *F. necrophorum* and the infections it causes, ranging from the lack of whole genome sequences, to the lack of evidence-based management of Lemierre's syndrome. There is so much more to do!

Karin Holm

Lund, October 2015

**Table 1**
Example from PubMed of "The forgotten disease"

| Title | Year | Author | Reference |
|---|---|---|---|
| Anaerobic septicemia following oropharyngeal infections (Lemierre's postanginal septicemia): a forgotten syndrome | 1983 | Hirschel | [1] |
| Necrobacillosis: a forgotten disease. | 1984 | Moore-Gillon | [2] |
| Lemierre syndrome: the forgotten disease. | 1993 | Weesner | [3] |
| Lemierre's syndrome: two cases of a forgotten disease. | 1994 | Carlson | [4] |
| Lemierre syndrome--a forgotten complication of acute tonsillitis. | 1995 | Koay | [5] |
| Lemierre syndrome: forgotten but not extinct--report of four cases. | 1999 | Screaton | [6] |
| Lemierre syndrome: remember the forgotten disease. | 2004 | McMullan | [7] |
| Lemierre's syndrome: a forgotten complication of oropharyngeal infection | 2005 | Shah | [8] |
| Lemierre syndrome: Three cases of "the forgotten" disease. | 2005 | Lundemose | [9] |
| Fusobacterium necrophorum meningitis: forgotten complication of a gingivitis. | 2007 | Duquesne | [10] |
| Lemierre's syndrome: a forgotten disease. | 2008 | Monteiro | [11] |
| "A forgotten disease": a case of Lemierre syndrome. | 2009 | Velagapudi | [12] |
| Lemierre's syndrome--the syndrome quite forgotten. | 2012 | Krishna | [13] |
| The 'forgotten disease' (or the never known). | 2013 | Murthy | [14] |
| The forgotten disease: Bilateral lemierre's disease with mycotic aneurysm of the vertebral artery. | 2014 | Gupta | [15] |
| Lemierre's syndrome: the forgotten disease. | 2014 | Johannesen | [16] |
| Lemierre's syndrome, the forgotten disease. | 2014 | Morariu | [17] |

# List of abbreviations

| | |
|---|---|
| α$_2$AP | alpha-2 antiplasmin |
| APTT | activated partial thromboplastin time |
| CNS | central nervous system |
| CRP | C-reactive protein |
| CT | Computed tomography |
| EBV | Epstein-Barr virus |
| HK | High molecular weight kininogen |
| ICU | Intensive care unit |
| MALDI-TOF MS | matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| PCR | Polymerase chain reaction |
| TF | tissue factor |
| tPA | tissue plasminogen activator |

# Summary

Every year, one or two previously healthy young people are diagnosed with Lemierre's syndrome in the Skåne Region. The strictly anaerobic Gram-negative rod *Fusobacterium necrophorum* is the pathogen isolated in the vast majority of cases. Tonsillar colonization by *F. necrophorum* is common in young people, which may in part explain why Lemierre's syndrome is confined to this age group. In some people the bacterium may cause local tonsillitis or a peritonsillar abscess, but in rare cases the bacterium may invade further into the parapharyngeal space and reach the jugular vein, resulting in septic thrombophlebitis. Clot material containing bacteria detaches from the thrombosis causing septic emboli to the lungs. This combination of tonsillitis, jugular vein thrombosis and septic pulmonary emboli constitutes Lemierre's syndrome. In the lungs, the septic emboli usually result in multi-focal pneumonia, very frequently complicated by pulmonary abscesses and empyema. The patients are usually severely ill and may require intensive care.

Since thrombosis is a striking phenomenon of Lemierre's syndrome, the studies underlying this thesis first focused on potential interactions between *F. necrophorum* and the human systems involved in coagulation. The contact system of human plasma initiates the intrinsic pathway of coagulation and the release of the pro-inflammatory peptide bradykinin. We found that the contact system binds to and is activated on the surface of *F. necrophorum*, which may contribute the pathogenesis of thrombosis in Lemierre's syndrome. We also found that plasminogen of the fibrinolytic system may be recruited to and activated at the surface of *F. necrophorum*, which may be of importance for invasion and dissemination.

In a retrospective study of 65 patients we described the clinical spectrum of Lemierre's syndrome or other invasive infections caused by *F. necrophorum* and found that patients with Lemierre's syndrome were young compared with patients with infections originating in the abdominal or urogenital tract. On admittance, patients with Lemierre's syndrome were severely ill, in a majority fulfilling the criteria for severe sepsis. To address if host factors could be important for the thrombosis and invasion in Lemierre's syndrome, patients who had Lemierre's syndrome were screened for underlying thrombophilia and for concomitant Epstein-Barr virus (EBV) infection. The results demonstrate that underlying thrombophilia was not over-represented in Lemierre's syndrome compared to the background prevalence and that concomitant Epstein-Barr virus (EBV) infection was uncommon.

Finally, we found that the gene coding for leukotoxin, a well known virulence factor in the animal pathogen *F. necrophorum* subspecies *necrophorum,* was present in human isolates of *F. necrophorum* and that there were three types of sequences, of which two were novel. In only a minority of the isolates, the sequence of the leukotoxin gene was of the previously described type.

In conclusion, this thesis provides new information about the interactions between *F. necrophorum* and the coagulation and fibrinolytic systems, about the clinical spectrum of invasive infection with *F. necrophorum*, the role of thrombophilia and EBV infection, and the presence of the leukotoxin gene in human isolates of *F. necrophorum.*

# List of papers included in this thesis

I. **Holm K**, Frick IM, Björck L, Rasmussen M (2011)
Activation of the Contact System at the Surface of *Fusobacterium necrophorum* Represents a Possible Virulence Mechanism in Lemierre's Syndrome.
Infect Immun 79 (8):3284-3290.

II. **Holm K**, Rasmussen M (2013)
Binding and activation of plasminogen at the surface of *Fusobacterium necrophorum*.
Microb Pathog 59-60:29-32.

III. **Holm K**, Svensson PJ, Rasmussen M (2015)
Invasive *Fusobacterium necrophorum* infections and Lemierre's syndrome: the role of thrombophilia and EBV.
Eur J Clin Microbiol Infect Dis. 34 (11):2199-2207

IV. **Holm K**, Collin M, Rasmussen M
Three variants of the leukotoxin gene in human isolates of *Fusobacterium necrophorum* subspecies *funduliforme*. Manuscript in preparation 2015

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Paper III is reprinted by permission from Springer.

# Chapter 1 Background

There are a few excellent review articles that I can recommend for further reading and they will be frequently cited in this thesis. Hagelskjaer and Prag have in depth clinical and bacteriological expertise in the field and their review article in Clinical Infectious diseases is very comprehensive [18]. Riordan wrote an impressively detailed review article in 2007, concerning every aspect possible of *F. necrophorum* infection, including a very ambitious review of published cases [19]. Furthermore, I can recommend what may be the only English publication by André Lemierre from 1938 describing the natural course of infection [20] and a review by Brazier from the Anaerobic Reference Laboratory in UK [21].

## Lemierre's syndrome

Lemierre's syndrome is a severe infection characterized by tonsillitis, thrombophlebitis of the jugular vein and septic pulmonary emboli leading to multifocal pneumonia and pulmonary abscesses. *Fusobacterium necrophorum* is the pathogen responsible for the vast majority of cases. In typical cases, the bacterium causes tonsillitis and gains entrance to the parapharygeal space. In early case reports, a peritonsillar abscess usually preceded invasion [20], but peritonsillar abscesses are now rare in combination with Lemierre's syndrome [22, 23]. The infection surrounds and invades the jugular vein, which becomes inflamed and in many cases a thrombosis is formed with subsequent shedding of microthrombi containing bacteria to the lungs (Figure 1).

If the infection is left untreated, distant sites may become infected, most commonly liver and joints, but also the meninges, skin, muscles and bone [20, 22, 23]. For unknown reasons, but probably related to immunity, anatomy and recent acquisition of the bacterium due to social behaviour, Lemierre's syndrome almost exclusively affects teenagers and young adults. Figure 2 describes the probable sequence of events leading to Lemierre's syndrome.

There is no definition generally agreed on of Lemierre's syndrome, but the following definition that has been widely spread stems from the review by Riordan [19], and emphasises the central role of the jugular vein thrombosis:

(1) History of anginal illness in the preceding 4 weeks or compatible clinical findings, (2) evidence of metastatic lesions in lungs and/or another remote site, and (3) evidence of internal jugular vein thrombophlebitis or isolation of *F. necrophorum* or *Fusobacterium species* from blood cultures or a normally sterile site.

In paper III of this thesis we have widened the definition somewhat to include not only infections originating in the throat, but also other infections originating in the head and neck, as long as the jugular vein is involved, and we have specified the radiological requirements of the lung lesions to pin-point the septic emboli:

A history of anginal illness or other primary focus in the head and neck in the preceding 4 weeks or compatible clinical findings, and at least two of the three following: (1) isolation of *F. necrophorum* from blood cultures or a normally sterile site; (2) evidence of internal jugular vein thrombophlebitis; (3) evidence of metastatic lesions in the lungs (multifocal pneumonia, or if only one lung infiltrate is present, a typical rounded consolidation, suggestive for septic embolization).



**Figure 1.**
CT scan of the thorax and neck of a 19 year-old woman with Lemierre's syndrome. Left: Multiple septic emboli in the right lung, one with a central cavity. Pleural fluid of the left lung. Right: Thrombosis of the right internal juguar vein visulized as a discontinuity of the contrast filling.
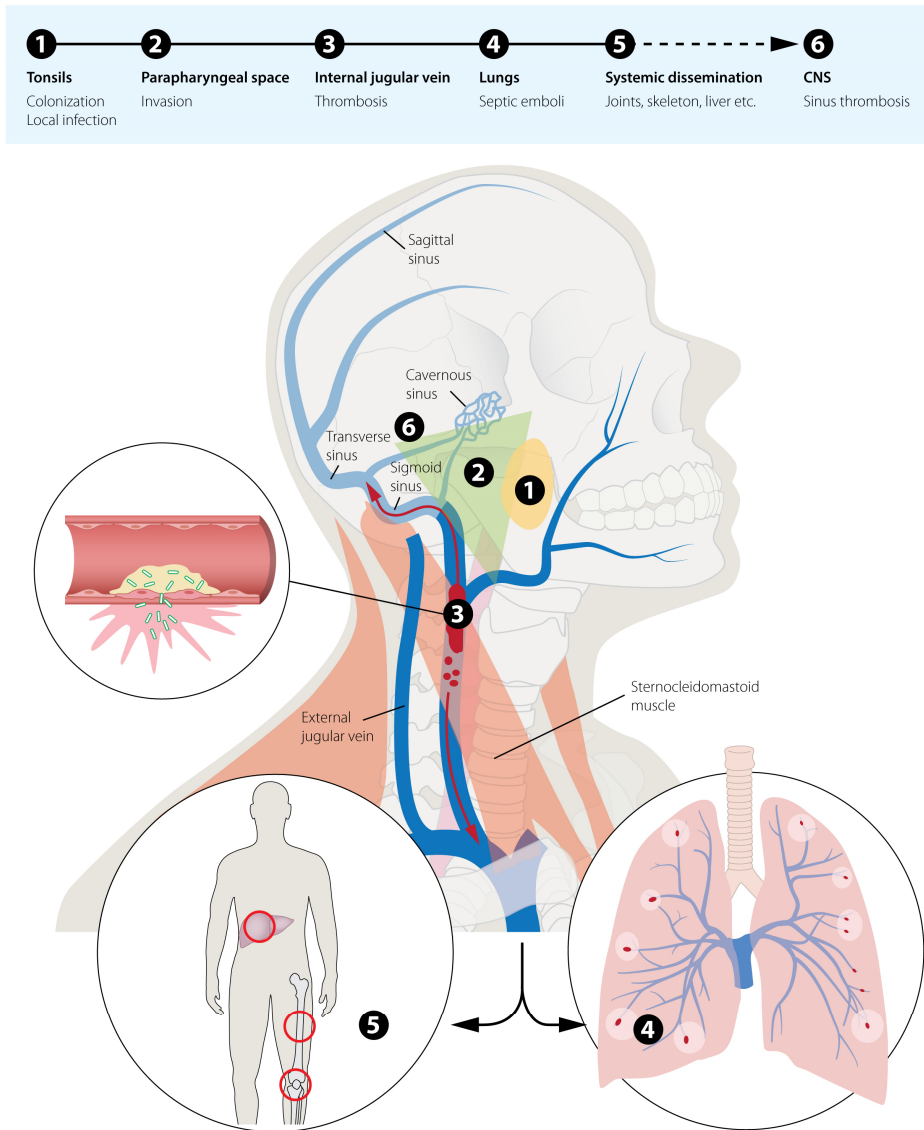
**Figure 2. Course of events leading to Lemierre's syndrome.**
**1.** Colonization of the tonsils by *F. necrophorum* leading to tonsillitis. **2.** Invasion into the parapharyngeal space. **3.** Invasion of the internal jugular vein, haematogenously via the tonsillar veins, by septic lymph or through the tissue, leading to intense inflammation and thrombosis. **4.** Detachment of clot material containing bacteria leading to septic pulmonary emboli. **5.** Systemic haematogenous dissemination and distant manifestations from joints, skin, skeleton, muscles, liver and meninges. **6.** In rare cases, retrograde progression of the internal jugular vein thrombosis may resulting in intracranial sinus thrombosis. (Image Mattias Karlén)

# Historical overview

Publications of cases and case series of what was then called necrobacillosis (due to the necrotic abscesses caused by the bacteria), date back to the late 19th century, mostly in French and German journals, but it was not until 1980 that the name "Lemierre" was used in a title to refer to the syndrome as "Lemierre's post-anginal septicaemia" [7], and until 1983 for the eponym "Lemierre's syndrome" [8].

André Lemierre (1875-1956) was a French physician and professor of microbiology at the Hospital Claude Bernard in Paris. He was known to be a brilliant clinician with the ability to draw conclusions from thorough examination and history taking [9]. In a time when that was common practice, he had the opportunity to follow his patients clinically, to participate in the microbiology work-up, and in many cases with fatal outcome, to participate in the post-mortem examination [3]. His contribution to the understanding of the syndrome that bears his name was neither the first case-description, nor the discovery of *F. necrophorum*, and not even the conclusion about the linkage between the pathogen and the disease. Instead, it was the numerous publications with comprehensive case-descriptions, pin-pointing the particulars of the syndrome that rendered him famous [2]. Most of his publications were in French, but he wrote one English report in 1936 in the Lancet that describes several typical cases caused by "*Bacillus funduliformis*", later *Fusobacterium necrophorum* [3]. Lemierre claims that the syndrome is very easily distinguished, and the following passage from that publication has been frequently cited:

> "To anyone instructed as to the nature of these septicemias 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"

**Figure 3.**
Professor André Lemierre (1875-1956), Paris, November 1940. Copyright: © LAPI / Roger-Viollet

In most of his publications about "post-anginal septicaemia" as he named the syndrome, Lemierre meticulously describes the natural course of the infection in the pre-antibiotic era, in a devastating majority leading to fatal outcome of young and previously healthy patients. He points out the need for early surgical intervention such as drainage and describes attempts to ligate the jugular vein to prevent dissemination of the infection [24]. At the end of his carrier he was able to watch the complete reversal of the poor prognosis by the arrival of antibiotics [25-27].

# Microbiology

The genus *Fusobacterium* (*Fusobacteriaceae* family, order *Fusobacteriales* phylum *Fusobacteria*) currently includes 13 species [28], with *Fusobacterium necrophorum* and *Fusobacterium nucleatum* being the most important in human disease. Fusobacteria are continuously re-classified as new sequencing data is available. Figure 3 shows the most recently published phylogenetic tree [29] of seven species of *Fusobacterium* and similar results were also found in an earlier study [30]. Table 2 lists the current status, pathology and origin of the species within the genus *Fusobacterium*.
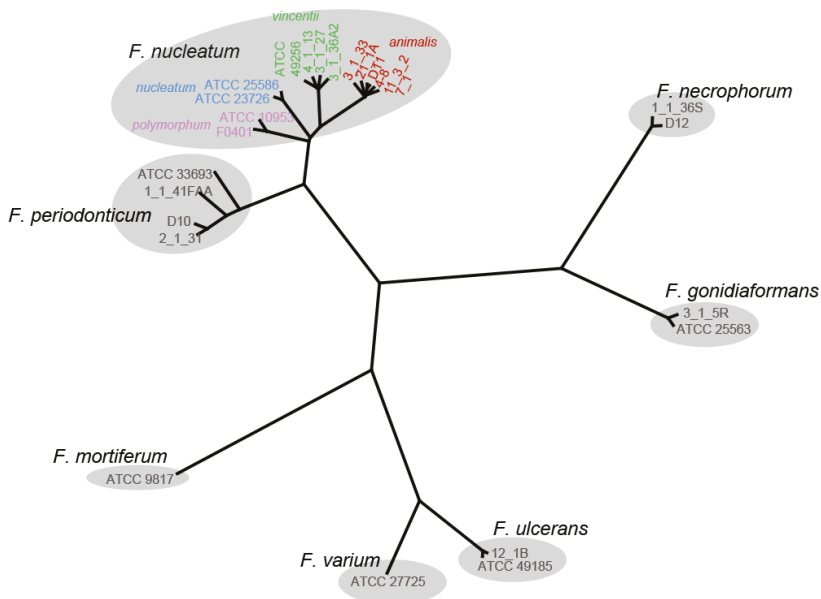


**Figure 4.**
Phylogenetic tree based on single copy core orthogroups, including sequences from the human microbiome project and whole genomes deposited in GenBank. Published with the permission from [29].

**Table 2.**
Origin and main pathology in humans of the 13 species currently included in the genus *Fusobacterium*

| Species | Host | Isolated from | Associated diagnosis in humans |
|---|---|---|---|
| *F. necrophorum* subspecies *funduliforme* | Human<br><br>Animal | Tonsils. Gastrointestinal tract | Lemierre's syndrome, tonsillitis, peritonsillar abscess [31], noma? [32, 33], appendicitis [34], urogenital and abdominal opportunistic infections |
| *F. necrophorum* subspecies *necrophorum* | Animal | | Lemierre's syndrome and other necrobacillosis in people working with animals (rare) [22] |
| *F. nucleatum* | Human | Oral cavity (some subspecies). Gastrointestinal tract (some subspecies) | Periodontal disease [35], adverse pregnancy outcomes, appendicitis, colorectal cancer, inflammatory bowel disease, appendicitis (reviewed in [36])<br>Lung and brain abscesses with oral flora |
| *F. varium* | Human | Gastrointestinal tract | Rare intraabdominal infections [37] |
| *F. mortiferum* | Human | Oral cavity? | Periodontal disease (rare) [38] |
| *F. periodonticum* | Human | Oral cavity, gatrointestinal tract [39] | Periodontal disease [40] |
| *F. ulcerans* | Human | Tropical ulcers | Tropical ulcers [41] |
| *F. gonidiaformans* | Human | Gastrointestinal tract | Rare opportunistic infections of gastrointestinal or urogenital origin [28, 42] |
| *F. naviforme* | | Oral cavity | Periodontal disease [35] |
| *F. necrogenes* | Chicken | N/A | N/A |
| *F. perfoetans* | Human? | Gastrointestinal tract [28] | ? |
| *F. simae* | Monkey | N/A | N/A |
| *F. russii* | Cats and dogs | N/A | Infected cat and dog bites [43, 44] |
| *F. equinum* | Horse | N/A | N/A |

*Fusobacterium necrophorum* is a non-motile, strictly anaerobic, non spore-forming gram-negative rod. The first description of a human isolate of what we now call *Fusobacterium necrophorum* was from a thesis about the female genital tract flora by Hallé in 1898 [45]. The bacterium was later described independently several times, and given different names by different investigators, such as *Bacillus necrophorus*, *Bacillus funduliformis*, *Bacillus symbiophiles*, *Actinomyces necrophorus* and *Sphaerophorus funduliformis* [19]. According to the extensive review by Riordan, early French work

recognised two different phenotypes of *F. necrophorum* isolates of animal or human origin. This was later confirmed [46], and in 1963 a division into biovar A and B was suggested [47], where biovar A was of animal origin and biovar B was of human origin. In addition, a biovar C was suggested, but was later reclassified to the species *F. pseudonecrophorum* [48], which, in turn, is now included in the species *F. varium* [28]. Bacteria with biovar B characteristics were later also isolated from animals, where they seemed to be primarily commensals, whereas biovar A was isolated from animal necrotic infections. Later the animal biovar A and B were re-named as subspecies *necrophorum* and *funduliforme* respectively [49] and the division into subspecies was supported for animal isolates by sequencing of the 16S-rRNA gene [50], and the 16S-23SrDNA spacer sequence [30]. Human isolates were also considered to belong to the subspecies *funduliforme*. During the completion of this thesis it has been difficult to find evidence that human isolates are actually equivalent with subspecies *funduliforme* (former biovar B) of animals. One study from 1997 comparing animal biovar A and B and human isolates points out several anomalous strains among the human isolates that did not fit the groupings and calls for comparative DNA-studies of multiple human and animal subspecies *funduliforme* isolates, but such studies have not yet been performed [51]. However, according to an ongoing whole genome-sequencing project, human isolates do not seem to be different from animal *funduliforme*, though there are distinct clusters among the human isolates (Jensen, personal communication). Phenotypic differences between subspecies *funduliforme* and *necrophorum* are listed in Table 3.

**Table 3.**
Phenotypic differences between subspecies *necrophorum* and *funduliforme*

|  | *F. necrophorum* subspecies *funduliforme* | *F. necrophorum* subspecies *necrophorum* | Reference |
|---|---|---|---|
| **Mainly isolated from** | Human infections or gastrointestinal content of animals | Animal infections |  |
| **Colony morphology** | Rounded, shiny, creamy | Umbonate, dull, waxy | [52] [47] |
| **Gram stain appearance** | Short, cocco-bacillary | Rods | [52, 53] [47] |
| **Haemagglutinin activity (chicken erythrocytes)** | - | + | [46] |
| **Platelet aggregation** | - | + | [54, 55] |
| **Leukotoxin activity** | + | ++ | [56] |
| **Virulence in rabbit and mouse model** | - | + | [46, 57] |
| **LPS activity** | + | ++ | [58] |
| **Hemolysin activity** | + | ++ | [59] [60] |
| **Adherence to entothelial cells** | ? | + | [61] |

*F. necrophorum* subspecies *necrophorum* causes a variety of necrotic infections in domestic and wild animals such as rumenitis (inflammation of the first chamber of the alimentary tract of cattle), hepatic abscesses, foot rot, calf diphteria and necrotic stomatitis [62]. Of these, bovine liver abscesses and foot rot are of significant concern to the cattle industry, and therefore most studies of the pathogenesis have been performed on subspecies *necrophorum*. The fact that subspecies *funduliforme* is distinctly different renders these studies of questionable importance to the understanding of human infection.

The routine identification of *F. necrophorum* is based on colony morphology and gram stain appearance. Sequencing of the 16S-rDNA gene could be used in selected cases, but the AP1-system is unable to discriminate between different *fusobacterium* species due to the fact that only very few reactions are positive [52]. Jensen *et al.* proposed the following minimum criteria for the identification of *F. necrophorum* that were reproducible for their collection of more than 300 isolates: Typical pleomorphic, long and short rods sometimes with curling and tangling (Figure 5), typical colony morphology, susceptibility to kanamycin and metronidazole, smell of butyric acid, chartreuse colour fluorescence, and β- haemolysis on horse blood agar [52]. This should still be sufficient for clinical microbiological routine work, but MALDI-TOF MS has become the method of choice, though not yet fully evaluated for sensitivity and specificity in discriminating between different *fusobacterium* species. Molecular detection of *F. necrophorum* from clinical samples by real-time PCR of the *rpoB* gene was developed by Aliyu [63], and has become a common tool in the clinical microbiology routine, but subspecies determination is not possible with that method. Sequencing of the 16S-rDNA gene can discriminate between human *funduliforme* and animal *necrophorum*, but is less sensitive for direct molecular detection. Instead, a real-time PCR assay of the *gyrB* gene was developed, that could be used for molecular detection of *F. necrophorum* from clinical samples, and could discriminate between the two subtypes by using subspecies-specific probes [64].
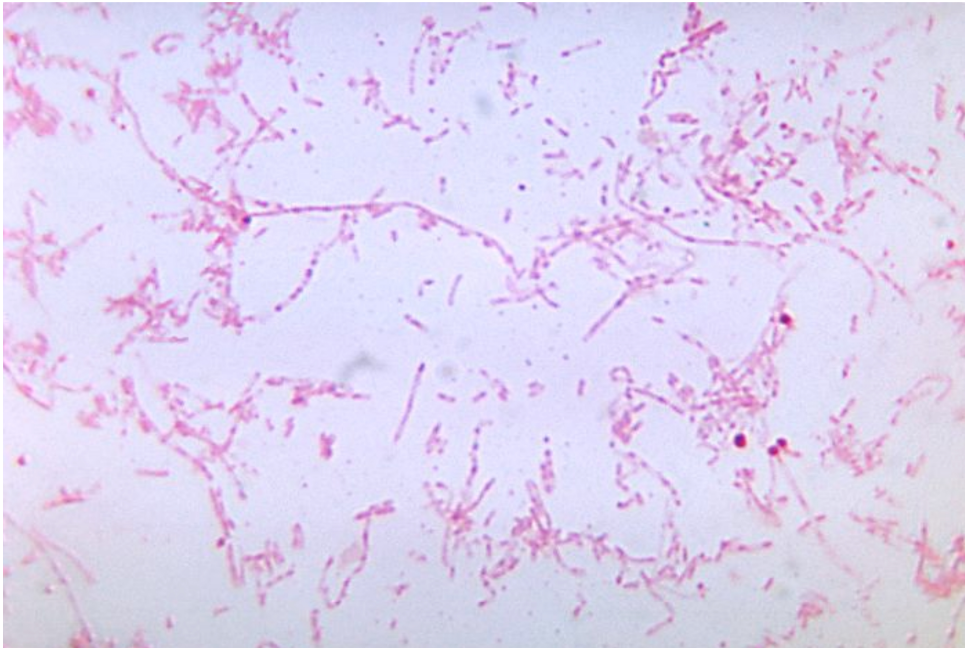
**Figure 5.**
Gram stain of *F. necrophorum* with typical pleomorphic rods.

# Chapter 2 Tonsillar colonization and invasion

The mechanisms and theories behind bacterial colonization and invasion are complex. Many bacteria can act both as commensals and pathogens, where, from an evolutionary point of view, bacterial adaption to the host to achieve the highest possible transmission and persistence involves asymptomatic colonization and local infections. For most bacteria, invasion of blood or another sterile site will result in a dead end of transmission. Therefore, bacterial virulence factors should primarily be viewed as tools to provide nutrients and optimize proliferation in local infections and colonization. Nevertheless, when invasive disease does occur the virulence factors may play a crucial role in propagating the invasion and causing overwhelming symptoms [65].

This chapter discusses the frequency of, and possible factors involved in, colonization, local tonsillar infections and further invasion caused by *F. necrophorum*.

Such factors could include:

1. Recent acquisition of the bacterium.

2. Local and systemic immunity

3. Break in mucosal integrity caused by trauma or a viral or bacterial co-infection.

4. Bacterial virulence factors or the acquisition of host factors used for invasion.

# Studies of colonization, local infection and further invasion

## Colonization

*F. necrophorum* is often described as part of the normal oro-pharyngal flora. However, several studies over the last 10 years have instead pointed out that there seems to be a transient carriage of the bacterium in a restricted age group, and that this carriage is associated with local and sometimes invasive infections. In a recent Swedish study of patients undergoing tonsillectomy due to chronic or recurrent tonsillitis, repeated follow-up cultures revealed that, on an individual basis, the colonization varied over time, indicating a transient colonization [66]. Three very recent studies have detected *F. necrophorum* in throat swab cultures from 2-10% of healthy controls. The study with the lowest median age (19 years) had the highest detection rate [67-69]. Culturing of anaerobes from the tonsils may be problematic since the living bacteria are hidden in the tonsillar crypts. An alternative approach is molecular detection with PCR. Aliyu *et al.* analysed 100 tonsillar swabs from healthy adult subjects (mean age of 40) by using real-time PCR of the *rpoB*-gene and failed to detect *F. necrophorum* in any of the samples [63]. However, using the same method, Ludlam *et al.* found *F. necrophorum* carriage in 7% of asymptomatic students [70], and Jensen *et al.* found *F. necrophorum* in 20% of healthy nurse students and recruits by real-time PCR of the *gyrB*-gene [64]. Both these last studies that had a low median age (20 and 22) point out that the age is most likely an important factor, and conclude that asymptomatic carriage is rare in older adults, but more frequent in adolescents and young adults. The route of acquisition is also discussed, and Ludlam, investigating a number of possible risk factors, found that those who were colonized with *F. necrophorum* were significantly more likely to have had lip-to-lip kissing contacts with one or more partners in the previous 4 weeks. The authors postulate that the route of transmission is probably by close oral contact, similar to the acquisition of Epstein-Barr virus (EBV) and meningococci. EBV and beta-haemolytic streptococci were also more common in those who were positive for *F. necrophorum*, indicating increased transmission of all throat pathogens in this group [70]. In Table 4 the detection rates of *F. necrophorum* in asymptomatic controls for the six studies mentioned above are listed together with data from tonsillitis patients.

Whether or not the carriage rate of 7-20% in a restricted age group qualifies as "normal flora" is a matter of definition. The percentage of carriers of *F. necrophorum* in the gut or vaginal flora is even less studied. *F. necrophorum* is clearly associated with

infections of abdominal or urogenital origin [22, 34, 71-73], but recent acquisition of the bacteria can not be ruled out. For older or chronically ill patient with typical opportunistic infections caused by *F. necrophorum* due to translocation of bacteria through a damaged gut epithelial lining, it is probably more likely that a long time carriage preceded the invasion.

## Local tonsillar infection

### Tonsillitis

There is now enough evidence to support that *F. necrophorum* is a primary pathogen causing tonsillitis.

In three studies from clinical microbiology departments the number of throat swabs that were positive for *F. necrophorum* varied from 4.9-9.7% [74-76]. A summary of the clinical studies published of the association between *F. necrophorum* and tonsillitis is made in Table 4 excluding studies that lack healthy controls. Five of the six studies comparing patients with tonsillitis with healthy controls found that *F. necrophorum* was significantly more common in the tonsillitis group, and was the only pathogen found in a significant number of cases [63, 64, 67-70]. In addition to an association between *F. necrophorum* and acute tonsillitis, one study found an even stronger association with recurrent or chronic tonsillitis [68].

### Peritonsillar and parapharyngeal abscess

A peritonsillar abscess, a collection of pus between the tonsillar capsule and the constrictor muscle surrounding the tonsil laterally and posteriorly, is the most frequent complication to tonsillitis. Group A streptococci, *F. necrophorum* and group G and C streptococci are commonly found in cultures from such abscesses. A five-year retrospective study of all peritonsillar abscesses at a tertiary hospital in Denmark found that *F. necrophorum* was the most prevalent pathogen, found in 23% of the abscesses. Of these, 81% were in pure culture. Group A streptococci were found in 17% and group C and G in 5% together with *F. necrophorum* [77]. In a later study, Patients with peritonsillar abscesses with *F. necrophorum* were found to develop antibodies to *F. necrophorum,* indicating that the bacterium is a significant pathogen in peritonsillar abscesses [78].

If an abscess develops laterally or posteriorly to the constrictor muscle, they are called parapharyngeal or retropharyngeal abscesses respectively.  These abscesses are serious due to the fact that they can progress *per continuum* to the mediastinum. A study of sixty-one patients with parapharyngeal abscesses found that 12% were positive for *F. necrophorum* [77]. A parapharyngeal abscess is located lateral of the constrictor

muscle, but does not necessarily occupy the space adjacent to the carotid sheath where the jugular vein is located. This may be one reason why a parapharyngeal abscess is not normally preceding invasion of the jugular vein and Lemierre's syndrome, but is usually a different disease in an older age group.

## Further invasion

The duration of tonsillar colonization, the percentage of carriers who will eventually develop *F. necrophorum* tonsillitis, and the risk of further invasion and Lemerre's syndrome are not known but attempts have been made to calculate the risk. Centor [79], by collecting data from other studies and US health care statistics, estimated that *F. necrophorum* accounts for approximately 10% of tonsillitis in adolescents and young adults (age 15-24) and that, based on the incidence of Lemierre's syndrome from the only published prospective study [22], approximately 1 in 400 cases of *F. necrophorum* tonsillitis will result in Lemierre's syndrome. Albeit a rough estimation, this risk by far exceeds that of rheumatic fever after *S. pyogenes* tonsillitis [79]. One study estimating the cost-effectiveness of treating *F. necrophorum* tonsillitis found that a reduction of the incidence of Lemierre's syndrome and peritonsillar abscess of only 20-25% would be cost-effective [80]. There are certainly more frequent case reports of Lemierre's syndrome over the last 15 years than before that, and some retrospective studies find an increased incidence of Lemierre's syndrome and invasive infection caused by *F. necrophorum* over the years studied [81-83]. The decreased use of antibiotic treatment for tonsillitis may be to blame for that, but better diagnostic methods and increased awareness may also be important.

In paper III of this thesis we found that 28% of the cases of Lemierre's syndrome was diagnosed in the first 8 years of the study and 72% in the last 7.5 years

**Table 4. Clinical studies of the association between *F. necrophorum* and tonsillitis.**

| Author | Prospective? | Case definition | Age (mean) | Control | Age (mean) | Method | No of cases/ controls | Fn[2] pos case/ control (%) | As only pathogen (% of Fn pos) | Significance? |
|---|---|---|---|---|---|---|---|---|---|---|
| Aliuy [63] | no | "Sore throat" swabs received by clin. microbiology | 0.5-79 (25) | "Healthy adults" | 22-64 (40) | PCR | 100/100 | 10/0 | 80[6] | Yes |
| Jensen [64] | no | "Sore throat" swabs received by clin. microbiology. Fever, enlarged lymph nodes or high CRP. Neg EBV and streptococci | 18-32 | Recruits and nurse students | 18-32 (22) | PCR | 61/92 | 48/21 | NA | Yes |
| Ludlam [70] | yes | "Sore throat" at GP | 2-77 (26 median) | Students | 18-39 (20 median) | PCR | 103/397 | 19/7 | 50[5] | Yes |
| Jensen [68] | no | "Sore throat" swabs received by clin. microbiology. 3/4 Centor criteria. | 19 median | Same as cases, but not fulfilling 3/4 Centor criteria. Swabs for screening for MRSA. | 22 median | Culture | 212/176 | 27/6 | 44[3] | Yes |
| Kjaerulff [69] | yes | "Sore throat". 1/4 Centor criteria. No recurrent tonsillitis. | 15-40 (28) | GP[1] non-infectious reasons | 15-40 (29) | Culture | 100/100 | 16/9 | 75[3] | No |
| Hedin [67] | yes | "Sore throat" at GP. 1/4 Centor criteria. | 15-48 (33) | GP non-infectious reasons | 16-46 (31) | Culture | 220/128 | 15/2 | 33[4] | Yes |

[1] General practitioner. [2] *F. necrophorum*. [3] Only examined for beta-haemolytic streptococci. [4] Examined for beta-haemolytic streptococci, RS-, *Chlamydophila pneumonia*, *Mycoplasma pneumonia*, Adeno-, Boca-, Corona, Epstein-Barr-, Entero-, Influenza A-, Influenza B-, Metapneumo-, Parainfluenza- and Rhinovirus. [5] Examined for EBV and beta-haemolytic streptococci. [6] Examined for beta-haemolytic streptococci and *Arcanobacterium haemolyticum*.

# Factors that may affect colonization and invasion

## Innate and adaptive immunity

The rapid and non-specific response of the innate immunity stands for the first line defence against invading microorganisms. A few studies have investigated innate immunity to *F. necrophorum* and possible evasion mechanisms by the bacterium.

### Complement

The complement system can be activated by the adaptive immune system (via the classical pathway) or by bacterial molecules (via the alternative or lectin pathway). Complement activation results in the production of C5a and C3a of importance for the recruitment of neutrophils and local inflammation, C3b which opsonises the pathogen for phagocytosis, and by the formation of the pore-forming membrane attack complex, MAC. A central enzyme complex of the cascade is the C3-convertase. Factor B promotes the formation and stability of the C3 convertase on bacterial surfaces. However, some bacteria instead of binding factor B have acquired a capacity of binding factor H, which occupies the binding site for factor B, resulting in degradation of the C3 convertase, thereby inhibiting the alternative pathway of complement. Factor H binding and complement evasion in a plasma environment has been shown for *F. necrophorum* subspecies *funduliforme*, and the binding capacity of the respective isolate correlated with the severity of disease [84].

### Leukotoxin

Early animal studies showed that a subcutaneous injection of cultures or culture supernatants of *F. necrophorum* subspecies *necrophorum* in rabbits and guinea-pigs resulted in a strong inflammatory response, but the neutrophils recruited to the area died within a certain distance from the infected area and the extravasation from the local capillaries was haltered [85]. This was the first indication that *F. necrophorum* may possess leukotoxic activity. Since then the leukotoxin has been thoroughly investigated and found to mainly affect neutrophils, but also hepatocytes and ruminal epithelial cells (lining the rumen, i.e. the first chamber of the bovine alimentary tract) [86]. Low concentrations of leukotoxin result in the activation of neutrophils and changes characteristic of apoptosis, and high concentrations result in necrotic cell death of neutrophils [87].

Leukotoxins are exotoxins that are primarily toxic to leukocytes, especially neutrophils. The term is not strictly defined, and sometimes the term leucocidin is

used interchangeably, though it usually refers to the Panton-Valentine leukotoxin produced by *Staphylococcus areus*. Well characterized leukotoxins from the animal pathogen *Mannheimia (Pasteurella) haemolytica* and the human periodontal and endocarditis pathogen *Actinobacillus actinomycetemcomitans* [88] belong to the RTX family of toxins produced by Gram-negative bacteria. These toxins are pore-forming and receptor specific.

In 2001, the leukotoxin (*lkt*) gene of *F. necrophorum* subspecies *necrophorum* was discovered by screening of a genomic library for leukotoxic activity, and the *lktA* gene was subsequently cloned and expressed in *E. coli* [89]. The corresponding protein sequence bears no resemblance to any other known protein. The *lktA* gene, encoding a protein of approximately 336 kDa, is part of a putative *lktBAC* tricistronic operon. The leukotoxin gene of bovine isolates of subspecies *funduliforme* has also been sequenced and found to be highly similar, but not completely identical to that of subspecies *necrophorum* [90]. The putative promoter regions of bovine isolates of subspecies *necrophorum* and *funduliforme* have been sequenced and found to be distinct from each other, and the subspecies *necrophorum* promoter was stronger when tested in an *E. coli* system [91]. This may be one reason why subspecies *necrophorum* has higher leukotoxin activity than subspecies *funduliforme* [90]. The leukotoxin operon is not present in most other species of *Fusobacterium,* but has been detected in *Fusobacterium equinum*, a recently discovered *Fusobacterium* species isolated from horses [92]. The leukotoxin gene was detected in four human isolates of *F. necrophorum* subspecies *funduliforme* by Southern blot of digested genomic DNA, and the isolates were shown to exhibit leukotoxic activity at about the same level as bovine isolates of subspecies *funduliforme* [56]. In contrast, Ludlam *et al.* [93], using three primer pairs designed from different parts of the *lktA* gene from subspecies *necrophorum*, found the *lktA* gene in only a minority (2/21) of human invasive *funduliforme* isolates. Thus, the presence of the leukotoxin gene in human isolates of *F. necrophorum* has been under debate, and the role of leukotoxin in human infection even less uncertain.

In paper IV of the this thesis, we find that the leukotoxin gene is present in all 81 human isolates examined, and sequencing of the gene reveals three different variants, of which two have not been described before.

Leukotoxin is considered a major virulence factor in animal disease with *F. necrophorum* and there are a few studies supporting this assumption, though some evidence can be questioned. One frequently cited study compared a highly leukotoxin producing isolate with a low producing strain after inoculation in mice, and found that the isolate producing low amounts of leukotoxin did not cause significant

infection. However, it is not clear whether the leukotoxin production was the only difference between the isolates [94]. Cattle immunised with a cell free supernatant of a highly leukotoxin producing strain of *F. necrophorum* subspecies *necrophorum* developed liver abscesses to less extent after intra-peritoneal injection of *F. necrophorum,* and those with lower antibody titres to leukotoxin were more susceptible to developing liver abscess, suggesting that the antibody response to leukotoxin was protective [95-97]. Injections of truncated recombinantly expressed leukotoxin resulted in leukotoxin neutralizing antibodies in rabbits [89], and in lower counts of *F. necrophorum* in the livers of mice injected intraperitoneally with *F. necrophorum* [98].

*Adaptive immunity*

There are several animal studies examining antibody response and protective immunity [86, 97-102]. No human study has evaluated immunity to *F. necrophorum*, but one study mentioned above demonstrated that antibodies to whole cell preparations of *F. necrophorum* develop following peritonsillar abscesses that are culture positive for *F. necrophorum* [31]. Studies of other pathogens with similar age distribution such as meningococci reveal sero-conversion at a group level over time [103, 104] without invasive infection. A similar study involving sera from a large population at different ages, sera from patients colonized with *F. necrophorum* and pre-immune and immune sera from patients with *F. necrophorum* infections would shed light on the possible role for adaptive immunity on the typical age distribution of *F. necrophorum* colonization and infection.

Most patients with *F. necrophorum* tonsillitis and Lemierre's syndrome seem to be previously healthy, and there are no indications that they would be more prone to infections [19, 22]. Therefore, it is unlikely that immune deficiencies are over-represented in patients with tonsillar colonization or infection with *F. necrophorum*.

EBV is believed to impair local immunity [105], and a correlation between current EBV infection and tonsillar colonization with *F. necrophorum* has been suggested (see below).

## Viral and bacterial co-infections

It is possible that, in addition to impairing local immunity, EBV could also cause mucosal damage and facilitate invasion of *F. necrophorum*. EBV infection concomitant with Lemierre's syndrome has been described in several case reports [106-108]. In a series of six cases, three had evidence of acute EBV infection and the

severity of Lemierre's syndrome seemed to be worse in patients with concomitant mononucleosis [109]. However, the true prevalence of EBV infection in patients with Lemierre's syndrome is unknown.

We address this question in paper III of this thesis, where we find serological evidence of an ongoing EBV infection in only one of 22 tested patients. Thus, EBV infection seems to be less important than expected from previous reports.

Any respiratory virus or bacterium could theoretically increase the risk for invasion of *F. necrophorum*. A recent Swedish study included patients with sore throats prospectively and examined throat swabs for a wide range of viruses and bacteria (See Table 4), not excluding patients with a positive strepA test. Interestingly, in two thirds of the *F. necrophorum* positive tonsillitis patients, another bacterium or virus was also detected; most commonly group A streptococci (11 of 33 patients). Most other studies have not reported an association between group A streptococci and *F. necrophorum*, since they have usually only included strepA negative patients. Viral infection was found in 7 of 33 patients (one influenza B virus, one coronavirus, two rhinovirus, one metapneumovirus, and two Eptein-Barr virus). The co-infections may have contributed to the tonsillitis in these patients, but if further invasion occurred was not evaluated in that study [67].

## Bacterial proteolytic activity and recruitment of plasminogen

Tissue invasion is thought to involve proteolytic enzymes, either secreted by the bacteria or by the recruitment of host proteases. *F. necrophorum* subspecies *necrophorum* has been shown to secrete proteases [110], but no proteases of subspecies *funduliforme* have been described. Recruitment of host proteases is an alternative way for the bacterium to acquire protease activity. Plasminogen acquisition is a well-known example of this, and has been shown to be important for bacterial invasion [111, 112].

Plasminogen is a 90 kDa proenzyme that is converted to the serine protease plasmin by tissue plasminogen activator (tPA) present in plasma. Plasmin cleaves fibrin but also a broad range of extracellular matrix and basal membrane substrates.

Bacteria have developed different methods for activating plasminogen. *Staphylococcus aureus* and *Streptococcus pyogenes* have surface proteins that bind to, and change the conformation of plasminogen, making the enhancement-loop of plasmin cleavage of plasminogen more efficient [113, 114]. *Yersinia pestis* has a surface protein that activates plasminogen in a similar way as tPA [115]. Many other bacteria have surface receptors for plasminogen, but rely on host factors, such as tPA, for plasminogen

activation. Binding of plasminogen to bacteria typically occurs via the kringle region of plasminogen to lysine-rich regions of the receptor and leads to a conformational change [116] that renders plasminogen more susceptible to cleavage by plasminogen activators [111]. The plasminogen/plasmin kringle domain is also the binding site for the plasmin inhibitor $\alpha_2$-antiplasmin ($\alpha_2$AP), and binding of plasmin to bacteria, via the kringle domain, protects it from $\alpha_2$AP.

Acquisition of plasminogen activity has been demonstrated for *Fusobacterium nucleatum* [117], but not for other *Fusobacteria*.

We address this question in paper II of this thesis, where we find that *F. necrophorum* binds to plasminogen, which can be activated to plasmin at the bacterial surface, and that plasmin bound to the surface is protected from degradation by $\alpha_2$AP.

*Other virulence factors*

Other possible virulence factors of *F. necrophorum* (mainly subspecies *necrophorum*) have also been described and sometimes characterized, such as haemolysin and adhesins [61, 118-121].

# Conclusion

It is likely that a recent acquisition of *F. necrophorum* and the lack of immunity are responsible for the colonization and local infections. A bacterial or viral co-infection may cause mucosal damage and increase the risk of invasion. Evasion of innate immunity due to leukotoxin production and complement inhibition may contribute.

# Chapter 3 Thrombosis of the jugular vein

Thrombosis is a striking phenomenon of invasive infection with *F. necrophorum*. Most commonly it is described in Lemierre's syndrome, but pelvic thrombosis due to genital infections [122], portal and mesenteric vein thrombosis due to liver abscesses or infections of intestinal origin, [123-125] and intracranial sinus thrombosis due to invasive otogenic infections [126-139] have also been described. Deep vein thrombosis of the leg is also described in association with local infection, leading to a Lemierre-like condition with septic pulmonary embolism [140].

In paper III of this thesis, we describe two patients with thrombosis of the deep veins of the leg associated with local infections or trauma to the leg and *F. necrophorum* bacteraemia.

Local thrombosis is also described in association with other bacteria, but not so frequently and consistently as for *F. necrophorum*. What could possibly be the reason for this association between *F. necrophorum* and thrombosis? That was one of the earliest and most obvious questions leading to the thesis. Since other bacteria do not cause thrombosis to this extent, there must be specific pro-coagulant conditions in invasive cases of *F. necrophorum* infections. Such conditions could include:

1. Expression of directly pro-coagulant secreted or surface-bound molecules by *F. necrophorum*.

2. Recruitment of plasma coagulation factors to the bacterial surface, and subsequent activation, either directly by bacterial proteases, or indirectly by providing favourable conditions for activation by plasma proteases

3. Direct bacterial effects on tissue factor expression by endothelial cells and monocytes.

4. Factors that direct the infection to involve blood vessels, to infiltration of bacteria in the perivascular area and subsequent massive inflammation. Inflammation and endothelial damage could lead to exposure of sub-

endothelial pro-coagulant molecules or up-regulation of pro-coagulant molecules on the endothelium or other vascular cells.

5.  Host factors could also play a role, and underlying thrombophilia could increase the risk for invasive infection with *F. necrophorum*, with a thrombosis providing shelter from the toxic aerobic environment.

This chapter reviews the knowledge of the nature of the jugular vein thrombosis in Lemierre's syndrome and the background of the different hypotheses mentioned above.


# Prevalence and characteristics of the thrombosis

The jugular vein is the most common vessel affected in Lemierre's syndrome, most likely due to its location in the parapharyngeal space adjacent to the tonsils (Figure 6). Invasion from the tonsils to the jugular vein could be haematogenous via thrombosis of the tonsillar vein, lymphatic or continuous through the loose connective tissue of the parapharyngeal space. Thrombosis of the tonsillar vein was sometimes observed in the patient data of paper III of this thesis, supporting the first hypothesis, but more frequently, the jugular vein inflammation and thrombosis were located more distant, and sometimes the external jugular vein was affected instead (unpublished observations). This makes it likely that the infection spreads through the tissues or via the lymphatic system, subsequently involving the perivascular space, leading to inflammation of the vessel wall, thrombosis and invasion by the bacteria.

Evidence of jugular vein involvement is included in most definitions of Lemierre's syndrome. Therefore, microthrombi or vegetations are in theory always present on the inside of the vessel wall, but the incidence of visible jugular vein thrombosis in Lemierre's syndrome is not known. It is very difficult to draw any certain conclusions from the literature since the definition varies. Some authors require direct radiologic evidence of jugular vein thrombosis, whereas others do not. Moreover, in many published cases, relevant radiology is not even performed. There could also be a publication bias for cases with evidence of jugular vein thrombosis, since this may have helped in diagnosing the condition. Riordan, in his very extensive overview of Lemierre's syndrome found that jugular vein thrombosis was reported in 59 of 222 included cases from published case reports or case series [19].

In paper III of this thesis, we find radiological evidence of jugular vein thrombosis in 14 of 23 examined patients.
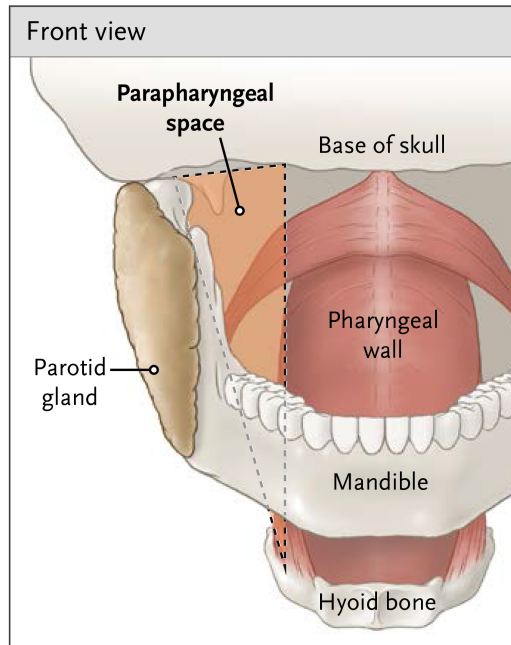
**Figure 6.**
Parapharyngeal space containing the carotid sheath with the internal jugular vein, common and internal carotid artery and cranial nerves IX, X and XII. Detail reproduced with permission from [141], ©Massachusetts Medical Society.

The thrombosis in Lemierre's syndrome is usually referred to as septic thrombophlebitis, sometimes with the addition of suppurative, implying that there is pus in, or surrounding the thrombosis. There is plenty of clinical evidence of pus collections surrounding the jugular vein [20, 24, 142], and the septic embolization proves that the thrombus contains bacteria, which may eventually lead to pus within the thrombus. However, database searches in PubMed only revealed three reports of the macroscopic or microscopic appearance of the thrombosis in Lemierre's syndrome, either from excised jugular veins, or on post-mortem examination. Two reports describe pus collections within the thrombi [143, 144], and one a macroscopic organizing thrombosis without pus [145].

# Bacterial factors affecting coagulation

## Directly pro-coagulant bacterial factors

### Haemagglutinin and platelet aggregation

The ability to agglutinate chicken and sheep erythrocytes is one of the means of distinguishing the two subspecies of *F. necrophorum*. Using a qualitative glass slide method [46], several studies show that subspecies *necrophorum* agglutinates chicken and sheep erythrocytes whereas subspecies *funduliforme* does not [47, 50-52]. A medium supernatant of subspecies *necrophorum* containing the haemagglutinin activity caused thrombosis of guinea pig mesenteric circulation [146]. Using a quantitative method, subspecies *funduliforme* was shown to possess a weak heamagglutinin activity [147], but the relevance to the pathogenesis of human infection is unknown. Similarly to the ability to agglutinate red blood cells, subspecies *necrophorum*, but not *funduliforme*, can aggregate platelets [54, 55, 148], and there are indications that erythrocyte agglutination and platelet aggregation may be caused by the same bacterial molecule [55] though not yet characterized to the gene level.

## Recruitment and activation of plasma coagulation factors

Blood coagulation is mediated and regulated by several systems involving proteolytic cascades. The most important system for haemostasis (stopping of bleeding) is the extrinsic pathway whereas the intrinsic pathway may have implications for thrombosis.

### The contact system

The contact system is involved in inflammation and coagulation by generating the pro-inflammatory peptide bradykinin and by activating the intrinsic pathway of coagulation. Bradykinin has many effects, involving increased vascular permeability and plasma leakage, lowering of blood pressure, and recruitment of neutrophils to the infection site [149-152]. Mice and humans deficient in contact factors have normal bleeding phenotypes, but mice deficient in factor XII are resistant to experimentally induced thrombosis. Therefore, the intrinsic pathway of coagulation seems to be of little importance for haemostasis, but of importance for the pathogenesis of thrombosis [150]. The contact system comprises the three serine proteases factor XII (FXII), plasma prekallikrein (PK) and factor XI (FXI), the non-enzymatic co-factor high molecular weight kininogen (HK) and the main inhibitor complement factor 1

inhibitor (C1INH). Activation can take place *ex vivo* on various synthetic negatively charged surfaces such as kaolin, which is used in the APTT assay. Several biological surfaces and compounds have also been shown to assemble and initiate the contact system, involving collagen, glycosaminoglycans, and importantly, polyphosphates released from activated platelets [153]. Also, several bacteria have been shown to activate the contact system.

The contact system is initiated when FXII binds to an activating surface and, by mechanisms that are not completely understood, undergoes conformational changes leading to limited proteolytic activity. Activated FXII initiates the two arms of the contact system. In the first arm it cleaves HK-bound pre-kallikrein, leading to the active serine protease PK, which in turn can activate more FXII and also cleave HK, leading to the release of bradykinin (Figure 7). Bradykinin is a short-lived nona-peptide that binds to the G-coupled receptors 1 or 2 (B1R and B2R) at the endothelium, leading to vasodilatation, increased vascular permeability and plasma leakage. B2R is constitutively expressed and is stimulated by bradykinin, whereas B1R is up-regulated by pro-inflammatory molecules. In the second arm of the contact system, active FXII cleaves HK-bound FXI to the active serine protease FXIa, which initiates the intrinsic pathway of coagulation. The role of FXII-initiated coagulation has been questioned since there is no bleeding phenotype in individuals deficient in any of the contact factors. However, over the last ten years, a role for the contact system in thrombus propagation rather than haemostasis has been suggested. This may in part be due to activation of the contact system by polyphosphates released from activated platelets in the developing thrombus [151, 154].
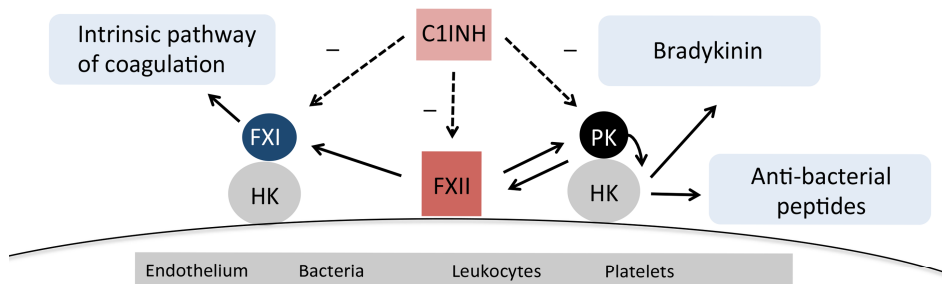
**Figure 7**

Schematic model of the assembly, activation and inhibition of the contact system. Factor XII (FXII) binds to a negatively charged surface and undergoes limited autoactivation. Next, it cleaves and activates plasma pre-kallikrein (PK) and factor XI (FXI) that are bound to the bacterial surface via the non-enzymatic co-factor high molecular weight kininogen (HK). PK can in turn activate more FXII, and cleaves HK to generate bradykinin. Further processing of HK may also generate antibacterial peptides. Active FXI initiates the intrinsic pathway of coagulation. The serine proteases of the contact system can all be inhibited by the complement factor 1 inhibitor (C1INH).

Contact factors have shown to assemble and be activated on various bacterial surfaces by different mechanisms. Some bacteria possess proteases that can directly activate factors of the contact system or cleave HK to generate bradykinin [155-159]. Polyphosphates have also been shown to be released by several bacteria and to activate FXII [160], and could therefore play a role in bacterial activation of the contact system. For most other bacteria studied, contact activation takes place on bacterial surfaces using the host proteases. In some cases, the molecules responsible for binding of the contact factors to the bacterial surface have been identified, for example the M-protein of *Streptococcus pyogenes* [161] curli of *Escherichia coli* [162], gingipains of *Porphyromonas gingivalis* [163] and lipopolysaccharides (LPS) [160, 164].

HK has six domains and binds to the surface of bacteria and other negatively charged surfaces via the poly-lysin rich domain 5 [161, 165]. Domain 3 and domain 4 have been shown to be involved in binding to platelets and endothelial cells [166-168], whereas bradykinin is released from domain 4, and domain 6 mediates binding to plasma PK and FXI [169]. In addition to the activation of the intrinsic pathway of coagulation and the generation of bradykinin, the contact system seems to be involved in innate immunity. The generation of antibacterial peptides from domain 3 of HK has been demonstrated [170] and blocking the contact system promotes growth and dissemination of *S. pyogenes* in plasma [170]. Moreover, FXII and PK

have been shown to activate complement, but the role for the contact system in complement activation *in vivo* is uncertain [171, 172].

There seems to be a dual role for the contact system in bacterial infection [160, 164, 173, 174]. Activation of the contact system may be beneficial for the host due to the contribution to the host defence such as complement activation and recruitment of cells of the innate immune system [171, 175, 176]. However, overwhelming infection resulting in massive contact system activation may lead to hypotension and vascular leak [164]. Inhibition of the contact system in patients with sepsis and animal models of sepsis has resulted in increased survival and less severe lesions [177, 178].

In paper I of this thesis we examine the interactions between the contact system and *F. necrophorum*, and find that factors of the contact system bind to the bacterial surface, that bradykinin is released and that the intrinsic pathway of coagulation is activated.

*Activation of the extrinsic pathway of coagulation and adherence of F. necrophorum to endothelial cells*

Another possible route for initiation of coagulation is by the extrinsic pathway of coagulation, which is activated by tissue factor (TF) on subendothelial structures that become exposed on endothelial damage. TF in turn activates FVII that in turn activates FX leading to the conversion of prothrombin to thrombin. TF can also be upregulated in the circulation predominantly on monocytes by pro-inflammatory molecules, and there are reports of induction of TF on endothelial cells though this is debateable [179]. It is possible that endothelial damage due to the invading bacteria in Lemierre's syndrome could result in the exposure of subendothelial TF. Upregulation of TF on monocytes and possibly on endothelial cells by the massive inflammation could also contribute. Bacterial molecules such as M-protein of *Streptococcus pyogenes*, peptidoglycan of *S. aureus* and LPS of gram-negative bacteria have previously been shown to induce TF on monocytes [180-182].

In a series of unpublished experiments, we pre-incubated whole citrated blood with *F. necrophorum*. The cell suspension was added to citrated plasma and the clotting time measured after the addition of an excess of $Ca^{2+}$. The clotting time was significantly reduced, indicating that TF had been upregulated on monocytes. The addition of bacteria to cell free plasma only in the clotting assay did not result in a decrease in clotting time, demonstrating that there are no direct effects of the bacteria on the extrinsic pathway of coagulation. Upregulation of TF on monocytes by *F. necrophorum* may be mediated by LPS or other surface molecules.

A recent study demonstrates that *F. necrophorum* subspecies *necrophorum* can adhere to bovine endothelial cells, and a bacterial outer membrane protein was identified which mediates the binding. The protein sequence was highly similar to the outer membrane protein FomA of *F. nucleatum*, and the gene was identified in several isolates of subspecies *necrophorum* and human and bovine isolates of subspecies *funduliforme* [61]. Adherence of *F. necrophorum* to endothelial cells could possibly direct the infection to involve blood vessels, thereby creating the massive inflammation that could lead to thrombosis. However, since the protein does not seem to be unique for *F. necrophorum*, other factors that direct the bacteria to the blood vessel may be involved.

# Host risk factors for thrombosis

*Underlying thrombophilia*

Thrombophilia, meaning hypercoagulability, is caused by a number of genetic or acquired abnormalities of the coagulation system. Thrombophilia is common, with factor V-Leiden mutation leading to APC-resistance as the most common abnormality, found in 5-15% of healthy individuals [183]. Most types of thrombophilia have only mild effects on the risk of developing venous thrombosis. Screening is not recommended in the general population, and not even after a single episode of deep vein thrombosis. However, it is usually recommended for unprovoked thromboembolic disease in young people, and when the location of the thrombosis is unusual. Screening for thrombophilia could involve different analyses, but the protocol in the Skåne Region involves the most common types of thrombophilia of clinical relevance and consists of G20210A prothrombin- and factor V-Leiden mutations, protein C, S and antithrombin III deficiencies, cardiolipin antibodies and lupus anticoagulants.

It is possible that underlying thrombophilia could be a risk factor for Lemierre's syndrome by increasing the probability of clot formation surrounding the bacteria that may in this way be protected.

In paper III of this thesis we address this question by performing a coagulation screening of patients with Lemierre's syndrome. The results indicate that thrombophilia is not more common in patients with Lemierre's syndrome than in the general population.

# Conclusion

The thrombi in Lemierre's syndrome are composed of fibrin clots together with bacteria, and the vein is intensely inflamed. The question why *F. necrophorum* is associated with thrombi is not solved, but the inflammation is most likely of importance, and interactions with the intrinsic pathway of coagulation, endothelial cells and tissue factor expression have been demonstrated for human isolates of *F. necrophorum*, whereas only subspecies *necrophorum* can aggregate platelets and has a strong haemagglutinin activity. Underlying thrombophilia of the host does not seem to be important.

# Chapter 4 Embolic and systemic complications and clinical management

## Retrograde progression of the thrombosis

Once an occlusive thrombosis is formed in the jugular vein, there is a risk of retrograde progression that will first occlude the entire jugular vein, and may subsequently progress through the jugular foramen into the intracranial sigmoid sinus and sometimes to the cavernous sinus. Progression may be due to stagnation of venous blood flow, bacterial growth or the intense inflammation of the vessel, and most probably a combination of the three. The nature of the sinus thrombosis, whether it consists of thrombotic material or if it, like the local jugular vein thrombosis, contains bacteria and pus, is not known. Intracranial sinus thrombosis due to Lemierre's syndrome has been described in several case reports, but the risk has not been calculated. It appears that sinus thrombosis is rare in Lemierre's syndrome originating in the throat, but common in patients with Lemierre-like syndromes of otogenic origin. The otogenic infections usually begin as a media otitis which progresses to mastoiditis, intracranial abscess and subsequent sinus thrombosis, much like the progression from tonsillitis to a jugular vein thrombosis. In the extensive review by Riordan, 2 of 179 cases of Lemierre's syndrome secondary to a throat infection had a sinus thrombosis, and 15 of 33 cases with otogenic origin [19]. In the prospective study by Hagelskjaer, none of 58 patients with Lemierre's or Lemierre-like syndrome had a sinus thrombosis, including 5 patients with otogenic origin of infection [22]. A literature search in PubMed using the terms "sinus thrombosis" and "Lemierre's syndrome" reveals 13 cases with Lemierre's syndrome with a throat origin and a sinus thrombosis (cavernous or sigmoid). All had a jugular vein thrombosis and in 10 of the 11 cases that I could evaluate, the jugular vein was totally occluded [184-193].

In paper III of this thesis we report that 1 of 33 patients with Lemierre's syndrome had a sinus thrombosis.

The question of whether or not to administer anti-coagulant therapy to patients with jugular vein thrombosis to prevent retrograde progression or possibly, to decrease the risk of septic embolization to the lungs and shorten the duration of symptoms, is controversial. On the one hand, intracranial sinus thrombosis is a serious complication associated with seizures, increased intracranial pressure and nerve palsies carrying up to 15% mortality [194]. On the other hand, there is no solid data on the effect of anticoagulants on septic thrombosis that may contain infectious material rather than just fibrin clots. A small, randomized study of septic pelvic thrombophlebitis did not show any shortening of symptoms in the anti-coagulated group compared to the placebo group [195, 196], but pelvic thrombosis shares no analogy to the intracranial retrograde progression, making comparisons difficult. A Cochrane analysis evaluated two small, randomized clinical trials of heparin or low molecular weight heparin treatment for sinus thrombosis of all causes, including infections [197, 198]. The author stated that "based upon the limited evidence available, anticoagulant treatment for cerebral venous sinus thrombosis appeared to be safe and was associated with a potentially important reduction in the risk of death or dependency which did not reach statistical significance". The analysis concludes that most clinicians will prescribe anticoagulant therapy to patients with cerebral venous thrombosis [154].

It will probably never be possible to perform a clinical study that can answer the question of whether or not anti-coagulant therapy should be administered, and therefore, the decision has to be based on theoretical arguments. It seems reasonable to prescribe anti-coagulant therapy to patients who develop sinus thrombosis in analogy with patients with sinus thrombosis of non-infectious origin, and to consider anticoagulation in patients with occlusive thrombosis of the jugular vein, in the absence of contraindications. In the light of the finding above that jugular vein thrombosis is almost always totally occlusive in patients with sinus thrombosis, it does not seem reasonable to administer anti-coagulant therapy to all patients with small, non-occlusive thrombi or thrombi affecting the external or anterior jugular vein. If the decision is not to give anti-coagulants, physicians may consider a follow-up ultrasound after a few days to evaluate if there is progression of the thrombosis.

Long-term recanalization of the jugular vein thrombosis may not be important. In a case series of nine children with jugular vein thrombosis, the thrombosis failed to resolve at three to six months in four of five children with an occlusive thrombosis, but the children were asymptomatic. Three of the children without recanalization had

received anticoagulant therapy [199]. The dual venous drainage of the brain makes it less likely that an occlusive jugular vein thrombosis will cause symptoms from the central nervous system due to stagnation of venous flow. Therefore, long-term follow up with ultrasound to reveal possible re-canalization of the thrombosis in patients without symptoms is probably not necessary.

# Septic pulmonary embolism

In the published case reports and case series, septic pulmonary embolism is almost invariably present. The emboli are usually small, and do not normally cause symptoms of decreased blood flow to the lungs. Instead they cause symptoms due to the peripheral nature of the emboli, with mainly pleuritic pain. Subsequently, multifocal pneumonia develops with very dense, rounded consolidations that will in a high percentage form lung abscesses [18, 19, 21]. In the prospective study by Hagelskjaer *et al.* [22], 21 of 26 patients with Lemierre's syndrome originating in the throat with metastatic infections had pleuropulmonary manifestations and 12 of 15 patients in the retrospective study by the same authors [82]. Eykyn reported chest pain, dyspnoea and haemoptysis in 23 of 29 patients with Lemierre's syndrome [200]. Pleural fluid developed in 8 of 26 and 4 of 15 patients respectively in the studies described above [22, 82], first as aseptic plural effusions, which may develop into empyema if left untreated. In the case review by Riordan, 77% of the cases reported lung lesions, and 15% empyema [19].

In paper III of this thesis, of 33 patients with Lemierre's syndrome, all had pulmonary involvement. Seventeen had radiological evidence of pulmonary abscesses and 25 of pleural effusion. Ten patients received a chest drain. Two patients had verified empyema.

# Systemic dissemination

The septic emboli containing bacteria will end up in the pulmonary vascular tree, and not in the systemic circulation. At the time of admission, blood cultures are positive for *F. necrophorum* in a majority of cases. This means that in addition to the septic pulmonary emboli, there is also systemic dissemination through the blood stream. If left untreated, the bacteria can end up at distant sites, such as joints, skin, skeleton, muscles, meninges and liver. In a retrospective study including 15 patients with

Lemierre's syndrome of throat origin, Hagelskjaer *et al.* found no cases of dissemination to distant sites [82]. In a later prospective study including 26 cases of Lemierre's syndrome of throat origin, the same authors reported dissemination to the meninges in four cases and to subcutaneous tissue in five cases, but no other distant manifestations [22]. In the review by Riordan, distant manifestations were very common and were reported from joints 11%, muscles 7%, skeleton 5%, liver 4%, skin 3% and CNS 1-2% [19]. Since that review is a collection of case reports, and more severe cases are likely to be overrepresented, these numbers are probably overestimating the actual prevalence of distant manifestations. Lemierre did not report any exact numbers in his English article but claimed that joint manifestations were "extremely common" [20]. In the pre-antibiotic era, distant manifestations probably developed eventually in almost all patients if they survived long enough.

> In paper III of this thesis, we find septic dissemination to distant sites in four of 33 patients with Lemierre's syndrome: One patient with meningitis, one patient with meningitis and multiple brain abscesses, one patient with mediastinitis and one patient with abscess of the iliopsoas muscle and osteitis of the iliac bone. These patients had significantly longer duration of symptoms prior to treatment compared to the patients without distant manifestations.

# Clinical management

### Diagnosis

*Symptoms, signs and laboratory findings on admission*

With a high level of suspicion among physicians who are well aware of Lemierre's syndrome, the diagnosis should be among the differential diagnoses after a careful examination and history taking. A young, previously healthy patient presenting with a sore throat or a history of a sore throat, unilateral tenderness along the sternocleidomastoid muscle, fevers and rigors, and signs and symptoms of severe sepsis and pneumonia should lead the clinician to a preliminary diagnosis of Lemierre's syndrome. However, diagnostic help from radiology, clinical chemistry and clinical microbiology is usually needed to provide the diagnosis with certainty.

Most patients have an initial sore throat, which is usually bilateral, though it may be more pronounced on one side. The findings of the throat examination can vary from completely normal to only mild inflammation to severe exudative tonsillitis. The

tonsillitis may in fact have resolved at the onset of septic symptoms which, according to Riordan [19] lead to the following dark warning by Hall in 1939 [201]:

> "Be not deceived by a comparatively innocent appearing pharynx as the veins of the tonsils may be carrying the death sentence of your patient"

A peritonsillar abscess was very common in combination with Lemierre's syndrome in the original reports by Lemierre, but now seems to be uncommon [22], probably due to early recognition and treatment. Tenderness along the inflamed and sometimes thrombotised internal jugular vein is also a typical finding that should lead the clinician to suspect Lemierre's syndrome, reported in over 60% of the cases in the prospective Danish study [22]. The thrombophlebitis can be difficult to distinguish from lymphadenopathy unless carefully examined, and sometimes both are present. The pain from the thrombophlebitis may be severe, and sometimes there are signs of subcutaneous infection with oedema and diffuse erythema, and local suppuration and abscess formation may follow.

The peripheral pulmonary emboli typically cause pleuritic chest pain, whereas shortness of breath due to embolism is uncommon. However, subsequent multifocal pneumonia, pleural fluid and abscesses may lead to respiratory failure in many patients. Since the pneumonia is secondary to septic emboli and not to bronchitis, cough may not be present on admittance, but eventually develops in almost all patients.

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In paper III of this thesis we find that 1 of 33 patients with Lemierre's syndrome had a concomitant peritonsillar abscess. Unilateral tenderness of the neck was documented on admittance or later for 22 of the patients and 10 patients complained of cough. We list the frequency of a number of symptoms, signs and blood chemistry findings on admission.

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The systemic inflammation is usually severe due to dissemination, multiple necrotic infections and abscesses, with high fever and rigors. The frequency of severe sepsis has, to my knowledge, not been investigated in previous studies. ICU care was required for 11 of 37 cases in the prospective study by Hagelskjaer [22]. Typical blood chemistry findings in Lemierre's syndrome include very high CRP (often more than 200 mg/L), low platelet counts and elevated creatinine and bilirubin.

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In paper III of this thesis we find that 26 of 33 patients fulfilled the surviving sepsis campaign criteria for severe sepsis [202] on admittance and 29 of 33 within the first 24 hours. Twelve patients developed septic shock. Thrombocytopenia, renal failure and elevated bilirubin were the most common abnormal laboratory findings defining severe sepsis. 12 patients required ICU care.

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*Clinical microbiology*

Blood culture prior to antibiotic administration is crucial since the diagnosis is very often based on the findings of *F. necrophorum* in blood. Blood culture is especially important for diagnosis if the radiological findings are atypical, or before they have developed, and in patients in whom a jugular vein thrombosis is not detected. Many clinical microbiology laboratories now have a specific PCR for *F. necrophorum* that is very useful for detection of *F. necrophorum* at the tonsils [63], especially if the patient has received antibiotics. Culture from the tonsils could also be attempted, but may yield fewer positive results [64]. Detection of *F. necrophorum* in blood culture, or at the tonsils should direct the suspicions to Lemierre's syndrome. However, in some cases with tonsillitis and positive blood cultures for *F. necrophorum*, the criteria for Lemierre's syndrome are not fulfilled, due to the lack of jugular vein thrombosis and/or pulmonary septic emboli. This situation could either represent an early stage of Lemierre's syndrome, or another disease. In the prospective study by Hagelskjaer, 11 of 37 patients with a throat focus had a positive blood culture as the only sign of dissemination [22], which would not have fulfilled the criteria we used in paper III of this thesis (modified from the suggestion by Riordan [19]).

The detection rates of *F. necrophorum* in blood cultures vary between different studies. All the large retrospective and prospective studies have relied on the detection of *F. necrophorum*. There is no ICD-10 code for Lemierre's syndrome, meaning that finding culture negative Lemierre's syndrome cases needs to rely on other approaches. Therefore, it is very difficult to determine the actual percentage of culture negative Lemierre's syndrome, or Lemierre's syndrome with another pathogen than *F. necrophorum*. One study using an unusual approach, identified nine children with Lemierre's syndrome from a large American cohort of paediatric thrombosis [199]. Only two of the children had a positive culture for *F. necrophorum*. It is not clear if all the patients had a blood culture taken, and there is no data on possible antibiotic treatment prior to blood culture. Moreover, there are numerous case reports describing other pathogens than *F. necrophorum* in association with typical Lemierre's syndrome. Usually, these bacteria are the same bacteria that are found in co-infections with *F. necrophorum*, such as oral streptococci and oral anaerobes, raising the suspicion that *F. necrophorum* may have gone undetected in those infections. There may also be a publication bias. In the case series by Riordan 22/168 blood cultures were mixed whereas all 16 cultures from liver abscess, joint fluid, or bone yielded pure growths of *F. necrophorum* [19]. Therefore, the growth of another oral bacterium in blood cultures and a typical clinical picture should still direct the suspicions to Lemierre's syndrome.

In paper III of this thesis cultures from blood or another sterile site were positive for *F. necrophorum* in 30 of 33 patients with Lemierre's syndrome. Two patients had a positive PCR from throat swabs for *F. necrophorum*. *F. necrophorum* in mono-culture was found in 20 of 33 patients. Among the co-pathogens, viridans streptococci were most commonly detected. In one patient, *Archanobacterium haemolyticum* and viridans streptococci grew in blood culture. *F. necrophorum* was not detected in that patient, but PCR from throat swabs was not performed.

*Radiology*

The initial x-ray may be normal, and needs to be repeated if there is still a suspicion of Lemierre's syndrome. This is very important in the case of mild to moderate severity, when the diagnosis may otherwise be missed. CT scan of the lungs and neck is a practical approach once the diagnosis is suspected. CT with intravenous contrast may be more sensitive for the detection of jugular vein thrombosis than ultrasound, but repeated CT scans should be avoided in this young age group. Repeated chest x-rays and repeated ultrasounds of the jugular vein should probably be performed more often to follow the development of the syndrome and detect complications.

## Treatment

*Antibiotic treatment*

The best antibiotic regimen has not been proven in a randomized study, and it will probably never be possible to perform such studies. Instead, we have to rely on *in vitro* data on antibiotic susceptibility and on retrospective studies and case reports. *F. necrophorum* is sensitive to most antibiotics. Beta-lactams, metronidazole and clindamycin all have very low minimal inhibitory concentrations (MIC) to *F. necrophorum*, and resistance does not seem to be a problem. However, one frequently cited study reported beta-lactamase production from 22% of clinical isolates of *F. necrophorum* collected from 28 different geographical sites in the US [203]. This finding has not been repeated, and the question is if there may have been some mistake in the species determination. Antibiotic resistance to erythromycin is common, and *Fusobacteria*, as other anaerobes, are intrinsically resistant to aminoglycosides. In a survey of 100 blood cultures positive for *F. necrophorum* in England and Wales from 1990-2000, no resistance was recorded to metronidazole, amoxicillin/clavulanate, cefoxitin, chloramphenicol, clindamycin or imipenem. 15 % of the isolates showed either resistance or reduced sensitivity to erythromycin, 2% to penicillin and 1% to tetracycline [83]. A study of 40 clinical isolates from Denmark

revealed no resistance to penicillin, cephalosporin, clindamycin or metronidazole [52].

In paper III of this thesis, no resistance to penicillin, imipenem, cefotaxime, clindamycin or metronidazole was reported from the clinical microbiology department.

Contrary to the low resistance to penicillin by *F. necrophorum*, beta-lactamase production by *F. nucleatum* is reported in several studies [204, 205].

Intravenous penicillin would, based on theory, be an excellent choice as single therapy of Lemierre's syndrome, but there are case reports with failure on single therapy with penicillin G [206-208]. However, the definition of failure in the case of Lemierre's syndrome is complicated, since the duration of fever is very often prolonged, probably due to multiple abscesses, continuous embolization, and pleural fluid. There is a risk that in the studies reporting failures, the change of therapy has coincided with the time when the fever would have subsided anyway. Since resistance is usually not a problem, the theories behind failure of penicillin therapy involve beta-lactamase production by other bacteria in a tonsillar co-infection, the low penetrance of beta-lactams inside a thrombosis [209] and possibly, the low penetrance of beta-lactams into an abscess. One rational would be to switch from a beta-lactam to monotherapy with clindamycin or metronidazole. However, there are also case reports describing failure with mono-therapy with clindamycin [210]. Moreover, due to the severe septic symptoms of the patients, many physicians would prefer the bacteriocidal beta-lactam antibiotic to the bacteriostatic clindamycin. Therefore, most patients receive a combination of a beta-lactam antibiotic and either metronidazole or clindamycin.

In my opinion, unless a resistant bacterium has been isolated from blood cultures, there is no need to use any other beta-lactam antibiotic than penicillin. Until more data is available, combination therapy with clindamycin or metronidazole may be advised, though the data supporting that advice is lacking. The duration of antibiotic therapy needs to be adjusted to the individual patients. Prolonged antibiotic therapy is needed for patients with multiple abscesses and empyema, and even the infected blood clot may theoretically harbour live bacteria for extended periods of time. Most authors seem to agree on a minimum of 14 days of antibiotic therapy, but four to six weeks is not uncommon, and careful examination for the need of drainage also after an initial improvement is necessary. For oral treatment after discharge, many authors prefer amoxicillin to penicillin due to the more stable and predictable uptake, taking in account that penetration of blood clots, abscesses and into the pleural cavity may be needed. Clindamycin, or in case of a short antibiotic course, metronidazole, is also a good choice.

## Course of infection and prognosis

The outcome of Lemierre's syndrome is usually good upon antibiotic treatment and appropriate drainage. Fever may be prolonged despite adequate antibiotic therapy, sometimes leading the clinician to suspect antibiotic failure and to change therapy. Instead, the clinician should focus on finding alternative causes of prolonged fever that may be treated. A chest x-ray should be repeated to evaluate the development of pleural fluid and pulmonary abscesses. Pleural fluid often develops late in the course of disease, and may also be purulent, in which case it should be drained. Massive amounts of pleural fluid, even when non-purulent, should also be drained. Multiple pulmonary abscesses will result in prolonged fever despite of correct antibiotic treatment, and patience is recommended, as long as the patient's condition does not deteriorate.

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In paper III of this thesis, the median time to defervescence was 5 days (range 1-16 days)

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For very large, peripheral abscesses, percutaneous drainage may be considered, bearing in mind the risk of broncho-pleural fistulas. Open thoracic surgery may be needed. Most metastatic infections to distant sites will be manifested with obvious symptoms that should be addressed with the appropriate diagnostic method, such as aspiration from infected joints. Liver abscesses may occur, and CT scan or ultrasound of the abdomen may be informative, though liver abscesses can usually be treated with antibiotics only. If the patient's condition deteriorates, intracerebral complications have to be suspected, such as sinus thrombosis, intracerebral abscesses, or meningitis. For cases that are initially mild, the treating physician needs to bear in mind that close follow up is required if the patient is discharged early, since abscesses and pleural fluid may form later, requiring drainage and possibly intravenous antibiotics.

# Chapter 5 Present investigation

## Aims of this thesis

This thesis has been produced in the intersection between molecular bacterial pathogenesis research and the clinical infectious diseases specialty. The overall aims have been to shed light on Lemierre's syndrome and other infections caused by *F. necrophorum*, with a special focus on the pathogenesis and clinical manifestations of the jugular vein thrombosis.

The specific aims were

- To examine interactions between *F. necrophorum* and the plasma pro-inflammatory, pro-coagulant and fibrinolytic systems.

- To systematically describe the clinical spectrum of Lemierre's syndrome and other invasive infections caused by *F. necrophorum*.

- To investigate if concomitant Epstein-Barr virus infection is common in Lemierre's syndrome.

- To investigate if patients with Lemierre's syndrome have an underlying condition making them more prone to develop thrombosis, so called thrombophilia.

- To examine if the most well-known virulence factor of the animal subspecies is also present in human isolates of *F. necrophorum*.
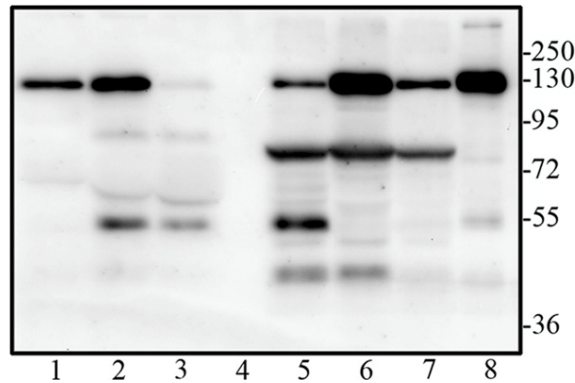
# Paper I



**Figure 8.**
*F. necrophorum* was incubated with fresh plasma (Lane 5) or buffer (Lane 4) for 30 min. After washing, proteins bound to the bacterial surface were eluted and analyzed by Western blotting using a polyclonal anti-HK antibody. The FXII and plasma kallikrein inhibitor H-D-Pro-Phe-Arg-CMK was added during the incubation step in lane 6, and the domain D5 peptide HKH20 in lane 7. Untreated plasma (lane1), plasma treated with kaolin for 30 or 60 sec (lanes 2 and 3), or pure HK added to the bacteria (lane 8), served as controls.

The contact system is a pro-inflammatory and pro-coagulant plasma protease cascade system. Early in the experiments of this thesis I found that high molecular weigh kininogen (HK), a central protein in the assembly and activation of the contact system bound significantly to the surface of several invasive *F. necrophorum* isolates. I also demonstrated that HK binds to the surface of *F. necrophorum* via its domain 5. Despite considerable efforts I was unable to find the bacterial molecule responsible for the binding to HK, but treatment of the bacteria with trypsin, which breaks down surface proteins, reduced the binding capacity for HK. This indicates that a bacterial surface protein may be responsible for the binding of HK. When contact activation takes place, HK is cleaved to generate bradykinin, a potent pro-inflammatory molecule. In two different sets of experiments I could demonstrate that such activation takes place at the surface of *F. necrophorum*. First, I incubated the bacteria with plasma and, after washing, the HK that had bound to the bacteria was examined by gel electrophoresis and Western blot with an anti-HK antibody. The breakdown pattern of HK was in accordance with the pattern generated when HK is cleaved to release bradykinin (Figure 8). In addition, bradykinin was detected in the supernatant by enzyme-linked immunosorbent assay, ELISA, using an anti-bradykinin antibody. Next, I examined the other branch of the contact system, the intrinsic pathway of coagulation, which plays a role in the propagation of thrombosis. I showed that one

of the first components of the intrinsic pathway of coagulation, factor XI (FXI) binds via HK to *F. necrophorum* and that the bound FXI reconstitutes the activated partial thromboplastin time (APTT) of FXI-deficient plasma. Activity of the end protease of the coagulation cascade, thrombin, was detected at the surface of the bacteria following incubation with plasma. This activity was completely blocked by inhibitors of the contact system. The combined results illustrate that the contact system is activated at the surface of *F. necrophorum*, suggesting a pathogenic role for this system in Lemierre's syndrome. As described earlier in this thesis, contact activation has been demonstrated at the surfaces of several bacterial species not known to induce thrombosis, suggesting that it is not the only mechanism behind the pathological coagulation in Lemierre's syndrome. In Lemierre's syndrome, the bacteria may migrate through the vessel wall of the internal jugular vein, and cause inflammation as well as initiation of the extrinsic pathway of coagulation. By binding contact factors to its surface and activating the intrinsic pathway*, F. necrophorum* may further enhance local coagulation.

# Paper II

Plasmin is a serine protease that degrades fibrin clots. In addition it has activity against extracellular matrix proteins. The acquisition of plasmin activity has been shown to be important for invasion and virulence of several pathogenic bacteria. In paper II, I examined the interactions between *F. necrophorum* and plasminogen, the inactive precursor of plasmin.

First, I showed that *F. necrophorum* binds significant amounts of radiolabelled plasminogen. This binding could be inhibited by a lysine analogue, which indicates that the poly-lysine binding kringle domain of the plasminogen heavy chain is responsible for binding to the bacteria. Next, I examined if the bacteria-bound plasminogen could be activated by tPA, a plasminogen activator normally present in plasma. I used two strategies to address this. First, S-2251, a chromogenic substrate that changes colour in the presence of plasmin was used to analyse the different combinations of tPA, bacteria and plasminogen. Only the combination of the three components resulted in a change of colour indicating that plasminogen had been activated at the surface of the bacteria by tPA. Secondly, plasminogen was incubated with or without bacteria or tPA, followed by Western blot analysis using anti-plasmin/plasminogen antibodies. For the combination of tPA, plasminogen and bacteria, a product with a molecular weight similar to plasmin was generated, in addition to plasminogen. For the combination of plasminogen and tPA without

bacteria, no plasmin could be detected. These two experiments demonstrated that plasminogen bound to the surface of *F. necrophorum* is more readily activated by tPA than plasminogen in plasma, probably due to a conformational change of plasminogen when it binds to the bacterial surface.

Next, I examined if active plasmin at the bacterial surface was protected from inhibition by the plasmin inhibitor alpha2-antiplasmin ($\alpha_2$AP). In an experiment analogous to the plasminogen activation experiment above, different combinations of bacteria, plasmin and $\alpha_2$AP were incubated and analysed for plasmin activity with the chromogenic substrate for plasmin. For plasmin bound to the bacteria, the addition of $\alpha_2$AP did not significantly reduce the plasmin activity, whereas plasmin in buffer was readily inactivated by $\alpha_2$AP. This demonstrated that plasmin bound to the surface of *F. necrophorum* is protected from inactivation by $\alpha_2$AP, probably because the cleavage site for $\alpha_2$AP is located inwards towards the cell surface.

Plasmin activity at the bacterial surface could be important for the progression from localized tonsillitis to invasion through the vessel wall. Plasmin activity may also facilitate micro-embolism and systemic dissemination of the infection.

# Paper III

In the first two papers I focused on the interactions between the bacteria and the plasma pro-coagulant and fibrinolytic systems of the host. Next, I was interested in the possibility that host pro-thrombotic factors could be important for the thrombosis in Lemierre's syndrome. I initiated a retrospective study dating from the year 2000 in the Skåne region, of all patients with a positive culture for *F. necrophorum* from blood or another sterile site. In the study, underlying thrombophilia in patients with Lemierre's syndrome was investigated by collecting blood samples for the analysis of the most common causes of genetic or acquired thrombophilia: factor V Leiden and prothrombin 20210 G to A mutation, protein S-, protein C-, and antithrombin deficiencies, cardiolipin antibodies and lupus anticoagulants. At the same time I also wished to describe the clinical spectrum and thrombotic complications in Lemierre's syndrome and other invasive infections caused by *F. necrophorum*. By collecting paired sera from prospectively included patients the frequency of concomitant mononucleosis caused by EBV in Lemierre's syndrome was also investigated.

Sixty-five patients were identified either prospectively or retrospectively from the year 2000 to 2015. Of the 65 patients included, 33 had Lemierre's syndrome. The patients with Lemierre's syndrome were young with a median age of 19 (15-37) as

were ten patients with other infections originating at the tonsils, most commonly tonsillitis or peritonsillar abscess. The median age was significantly higher in the remaining 22 patients (60-63 years) among whom abdominal, urogenital or pleuropulmonary infections were most common.

On admittance 26 of 33 patients with Lemierre's syndrome had severe sepsis, most commonly defined by low platelet count or high serum creatinine. Intensive care was required for 12 patients. The median time of hospital stay was 14 days (range 5-37 days) and the median duration of fever was five days (1-16). Jugular vein thrombosis was verified in 14 patients of whom one developed a cavernous sinus thrombosis and one a permanent hypoglossal nerve palsy. Four patients developed distant metastatic infections: Two cases of meningitis, one mediastinitis and one abscess of the ileopsoas muscle. These four patients had significantly longer duration of symptoms prior to treatment than patients without distant manifestations.

Of 26 patients tested, three had factor V-Leiden mutation, which is not different from the background prevalence, indicating that underlying thrombophilia is probably not over-represented in Lemierre's syndrome. One patient was also positive for lupus anticoagulants. Concomitant mononucleosis was verified in one of 23 patients tested.

The study confirmed earlier studies of the clinical spectrum caused by *F. necrophorum*. The time to treatment seemed to be important for the risk of severe disease. Among the patients with Lemierre's syndrome, concomitant EBV infection or underlying thrombophilia appears to be uncommon.


# Paper IV

Next, I returned to molecular pathogenesis. *F. necrophorum* subspecies *necrophorum*, an important animal pathogen that causes significant economical impact for farmers possesses a leukotoxin that seems to be important for virulence. There have been some controversies as due to the presence or absence of this leukotoxin in human isolates of *F. necrophorum* subspecies *funduliforme*. Out of that reason, I screened my collection of *F. necrophorum* for the leukotoxin gene, using primers designed from the subspecies *necrophorum* sequence. I could detect at least parts of the gene in all isolates tested. Next, the entire gene was sequenced in ten invasive isolates from Lemierre- and non-Lemierre patients using overlapping primers. The organisation of the gene was identical to previous reports with an *lktBAC* tricistronic putative operon, in which the *lktA* gene codes for the active leukotoxin (Figure 9). The sequencing revealed three types of leukotoxin genes of which two have not been previously

described. One of these novel gene types with an otherwise almost complete identity to previously published sequences had a two-nucleotide frame-shifting deletion (*lkt type 1b*). Upon translation, this deletion would result in a truncated protein with a molecular mass of about 140 kDa instead of 337 kDa. The other novel sequence was markedly different from the previous published sequence of subspecies *funduliforme*, with only a 78% sequence identity (*lkt type 2*).
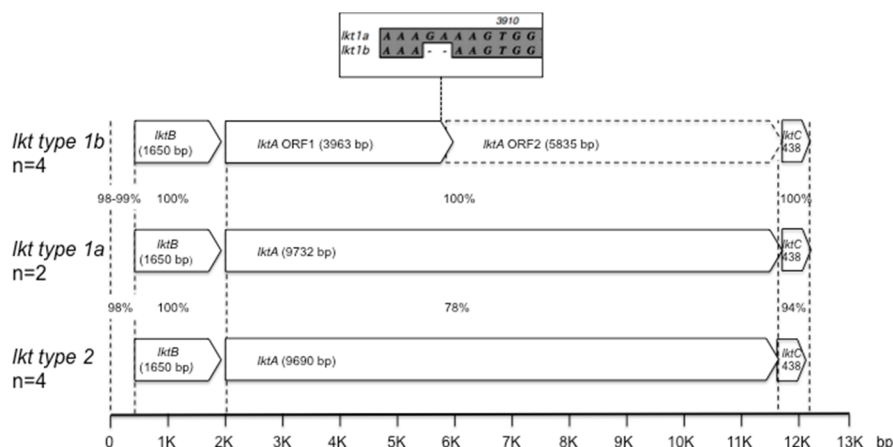


**Figure 9.**
Comparison of ten sequenced putative lkt operons. Nucleotide sequence identity after ClustalW alignment is indicated as percentage of the lkt type 1a sequence.

Next, I used the different sequences to design primers for screening of our entire collection of invasive *F. necrophorum* isolates to reveal the prevalence of different sequence types. Of 81 isolates tested, 49 belonged to the *lkt type 2* sequence, and of the remaining 32 isolates, 21 had the frame-shifting deletion Thus, only 11 of 81 isolates had a leukotoxin gene with the previously published sequence. No specific sequence type could be attributed to the different sources of invasive *F. necrophorum* infections, but of the ten type 1 sequences in isolates from patients with Lemierre's syndrome, nine were of the type 1b sequence (with deletion).

The first part of the *lkt type 1b* gene was cloned and expressed in *E. coli*, and the expressed protein was used to test paired sera from patients with Lemierre's syndrome. No sero-conversion to the leukotoxin could be detected in Western blot with paired sera from three different patients. This indicates that either, leukotoxin is not expressed by *F. necrophorum* in Lemierre's syndrome, or there is an impaired immune response to the leukotoxin in these patients.

# Chapter 6 Future perspectives

The knowledge of the molecular pathogenesis and the optimal clinical management of Lemierre's syndrome and other infections caused by *F. necrophorum* is still limited. There is a need for laboratory environments where the bacteria are studied by advanced molecular methods. In addition there is a need for interested clinicians who are willing to bring the molecular discoveries into the clinic and who are able to conduct large-scale clinical studies and bring back clinical samples to the laboratories. Below are some areas that may be the focus of future research.

*Clinical importance of F. necrophorum and its role in the normal flora*

Recently, a number of studies describing colonization and local tonsillar infection with *F. necrophorum* have been published. However, there are still many unsolved questions about the role for *F. necrophorum* in the normal flora. For how long are the tonsils colonized in symptomatic or asymptomatic carriers, and what happens next? Can the bacteria be detected in faeces, and is oral colonization preceding gastrointestinal colonization? The identification of a robust serologic marker for *F. necrophorum* to use for serologic studies in different age groups and different groups of patients would add information about the route of acquisition and immunity. Ongoing whole-genome sequencing projects will hopefully be helpful in identifying potential serological markers. Studies of the gut microbiota have become very popular, but *Fusobacteria* are usually described on a genus level. Specific focus on *F. necrophorum* would shed light on its role in the normal gut flora and also shed light on its association to health and disease.

Though there is no evidence, it is likely that the route of acquisition of *F. necrophorum* in Lemierre's syndrome is the same as for asymptomatic colonization and local tonsillitis. A recent acquisition of the bacterium that coincides with other factors that may favour invasion, such as mucosal damage due to a concomitant viral or bacterial infection could in rare cases lead to invasive disease and Lemierre's syndrome. Means of addressing this hypothesis could be extensive microbiological testing for co-infections and serological testing to reveal if the patients with Lemierre's syndrome are sero-negative to *F. necrophorum* at the time of diagnosis. Screening of

close contacts for *F. necrophorum* colonization, using genetic typing in positive cases could shed light on the route of transmission.

Whereas tonsillar colonization, tonsillar local infections and Lemierre's syndrome are likely to be part of a continuum, opportunistic infections from the urogenital and abdominal tracts generally affect a completely different age group in whom chronic underlying diseases are much more common. Are these disease entities caused by the same types of *F. necrophorum* or could there be genetic differences? A whole-genome sequencing project of different *Fusobacterium* species has been performed recently [29] and another large-scale whole-genome sequencing project of clinical isolates of *F. necrophorum* is ongoing, and should shed light on such differences (Jensen personal communication).

What is the appropriate treatment for Lemierre's syndrome? How should we study a condition that is associated with a considerable morbidity and at the same time is so rare, since the really serious complications, such as sinus thrombosis are even less common? Is it at all possible to perform a prospective study or could a multi-centre retrospective case-control study be designed to try to answer the questions about anti-coagulation and the appropriate antibiotic treatment? Even though a prospective study will probably not have enough statistical power to answer questions about treatment, a prospective approach could still be valuable since it offers the possibility for systematic collection of data.

In the clinical study of invasive *F. necrophorum* infection in this thesis, not a single child was identified from the databases of clinical microbiology. *F. necrophorum* is a well known cause of complicated media otitis in small children, frequently associated with mastoiditis, intracranial abscess and sinus thrombosis. Insufficient microbiological sampling, antibiotic treatment prior to culturing and the use of aerobic pediatric blood culture bottles are probably responsible for the lack of positive cultures in these children in Skåne. An ongoing prospective study of mastoiditis using molecular approaches in combination with culture will hopefully add knowledge about the prevalence of *F. necrophorum* in such infections (Enoksson, personal communication).

The use of molecular detection of *F. necrophorum* from throat swabs has now become widespread in clinical microbiology and may have implications on the treatment tradition of StrepA negative tonsillitis. *F. necrophorum* may be found in up to 20% of asymptomatic patients in certain age groups, making the interpretation of positive results difficult, and there are no studies evaluating the natural course of *F. necrophorum* tonsillitis and the effects of antibiotic treatment. A Danish prospective study addressing the effects on the duration of symptoms of antibiotics in *F. necrophorum* tonsillitis is planned (Ehlers Klug, personal communication), but the

study will not be able to address the possible risk reduction for peritonsillar abscess and Lemierre's syndrome. Studies that are able to answer those questions may be very difficult to conduct, since the number of patients that needs to be included to achieve enough statistical power is very high. We may therefore have to rely on large-scale epidemiological studies associating the trends in Lemierre's syndrome and peritonsillar abscesses to the detection of *F. necrophorum* from throat swabs and the possible treatment the detection resulted in.

*Molecular pathogenesis and host-pathogen interactions in F. necrophorum infection*

In my thesis I have addressed the question of why *F. necrophorum* causes thrombosis by identifying interactions between *F. necrophorum* and the pro-coagulant and pro-inflammatory contact system and the up-regulation of tissue factor on monocytes. These are factors that may contribute to the pathogenesis of Lemierre's syndrome, but they are by no means unique for *F. necrophorum*. Further studies may identify additional interactions between *F. necrophorum* and the host systems for coagulation. Interactions between the bacteria and endothelium or other cells or structures associated with blood vessels may identify adhesins or other factors that may direct the bacteria to the blood vessels and result in thrombosis. Moreover, the route of invasion to form the jugular vein thrombosis is not known, and histological studies of tonsils from patients colonized with *F. necrophorum* could reveal where the bacteria are localized and the vicinity to the tonsillar veins.

The natural habitat of *F. necrophorum* in humans is probably the gastrointestinal tract and to some extent, the oral cavity. Host-pathogen interactions should therefore not only be studied in plasma, but also for saliva and epithelial cells of oral or gastrointestinal origin. Information about the surface proteome of *F. necrophorum* is also almost completely lacking, and studies of host-pathogen interactions and whole genome sequencing projects should help identifying such proteins.

# Populärvetenskaplig sammanfattning

Varje år drabbas en till två tidigare helt friska ungdomar i Skåne av Lemierres syndrom. Det är en sjukdom som orsakas av bakterien *Fusobacterium necrophorum*, där första delen av namnet beskriver att bakterien är spolformad när man tittar på den i mikroskop och den andra delen antyder att den kan orsaka nekros, dvs. vävnadsdöd i kroppen. Tidigare studier har visat att framförallt ungdomar kan bära på bakterien i halsen. Hos vissa ger bakterierna en lokal infektion som halsfluss eller halsböld men i sällsynta fall kan bakterien ta sig in ytterligare på djupet och nå fram till blodkärlen på halsen. Via okända mekanismer kan den sedan ta sig in i blodkärlet där det bildas blodproppar. Små bitar av dessa proppar, fulla med bakterier, kan lossna och ta sig via blodflödet till lungorna där de fastnar längst ute i de små blodkärlen. Denna sjukdomsbild med halsfluss, proppar i halskärlen och spridning till lungan kallas Lemierres syndrom. I lungan kan bakterierna växa till och orsaka lunginflammation på flera ställen samtidigt. Dessa lunginflammationer blir ofta väldigt irriterade och täta, och i mitten kan lungvävnaden falla samman till hålrum med varbildning, så kallade lungabscesser. Om behandling inte sätts in kan bakterier också transporteras med blodet till leder, muskler, skelett och hjärnhinnor. Proppen i halskärlen kan i sällsynta fall växa till uppåt och ge proppar i blodkärlen i hjärnan. De patienter som drabbas av Lemierres syndrom brukar bli väldigt sjuka, många behöver intensivvård med respiratorbehandling, och det är inte ovanligt att de behöver vara inlagda i flera veckor. Långvarig antibiotikabehandling brukar bota de flesta, men ofta behöver man också tömma varansamlingar från lungsäcken eller från halsen.

Det finns egentligen ingen annan bakteriesjukdom som så tydligt är förknippad med blodproppar som Lemierres syndrom, och det blev därför en central frågeställning i mina studier. Jag ville ta reda på om det finns någon faktor hos bakterien som ger upphov till blodproppar eller om de som drabbas har en bakomliggande ökad risk för blodproppar. Då sjukdomen är ovanlig ville jag också beskriva så många patientfall som möjligt för att kunna dra slutsatser om hur sjukdomen normalt beter sig och vilka komplikationer som kan uppstå.

Koagulation är centralt för att stilla blödning efter en skada. Det finns flera system som reglerar blodkoagulationen. Ett yttre system aktiveras när molekyler som ligger utanför ett blodkärl kommer i kontakt med blodet vid en skada och kan snabbt stilla en blödning. Det finns också ett inre system, som inte är inblandat i att stilla blödningar, men som har visat sig vara betydelsefullt för blodproppsbildning inne i

kärlen. Mina resultat visar att *Fusobacterium necrophorum* kan binda till sig faktorer från detta inre system och att dessa faktorer blir aktiva på bakterieytan. Det är tänkbart att en blodpropp bildas när blodkärlet skadas av den kraftiga inflammationen som bildas när bakterierna tar sig in. Det inre systemet som aktiveras på bakteriernas yta när den kommer i kontakt med blodet kan vara av betydelse för att stabilisera och bygga vidare på blodproppen. I samband med att det inre systemet aktiveras frisätts också en inflammationsmolekyl, bradykinin, som kan bidra till den kraftiga inflammationen vid Lemierres syndrom.

Proteaser är molekyler med förmåga att bryta ned proteiner. Ett exempel är plasmin, som bryter ned fibrin, de trådar som binder ihop en blodpropp. Plasmin kan också bryta ned flera proteiner som finns i vävnaderna i kroppen. För bakterier som kan binda till sig plasmin kan det vara betydelsefullt för deras förmåga att ta sig in i kroppen. Mina resultat visar att *Fusobacterium necrophorum* kan binda till sig plasminogen som är den inaktiva föregångaren till plasmin, och att plasminogenet på bakterieytan lättare kan omvandlas till aktivt plasmin med hjälp av ett annat proteas i blodet. Det plasmin som sitter bundet till bakterien skyddas från inaktivering. Aktivt plasmin på ytan av fusobakterierna kan vara av betydelse för förmågan att invadera vävnaderna kring halsmandlarna, att ta sig in i blodkärlen, och för att lösa upp bitar av blodproppar som då kan sprida infektionen i kroppen.

Mina första två arbeten handlade om bakteriernas interaktioner med kroppens system som reglerar blodkoagulationen. I mitt tredje arbete undersökte jag om det kan finnas värdfaktorer som bidrar till att det bildas blodproppar i samband med Lemierres sjukdom, och i samband med detta även göra en journalstudie för att beskriva sjukdomsförloppet. Jag tog kontakt med de patienter som haft Lemierres syndrom eller annan sjukdom med *Fusobacterium necrophorum* i blodet i Region Skåne sedan år 2000 och fick blodprover från dem. Dessa blodprover analyserades för de vanligaste medfödda eller förvärvade tillstånden som kan ge ökad risk för blodproppar. Då patienter med Lemierres sjukdom är i samma ålder som de som insjuknar med körtelfeber har det spekulerats i om samtidig körtelfeber skulle kunna göra att fusobakterierna lättare kan orsaka sjukdom. Blodproven från patienterna med Lemierres syndrom undersöktes därför också för antikroppar mot Epstein-Barr-virus, det virus som orsakar körtelfeber. 65 patienter inkluderades i studien varav 33 hade Lemierres syndrom. Tre patienter med Lemierres syndrom hade den vanligaste typen av bakomliggande ökad risk för blodpropp, vilket inte är högre förekomst än hos normalbefolkningen. Endast hos en patient fann vi tecken till samtidig körtelfeber. Resultaten visar att en bakomliggande ökad benägenhet att bilda blodproppar inte är vanligare hos patienter med Lemierres syndrom jämfört med friska kontroller. Jag fann vidare att patienterna med Lemierres syndrom var mellan 15-37 år, att de var svårt sjuka vid ankomst till sjukhuset och att 12 av 33 behövde intensivvård. Fjorton patienter hade en blodpropp i halskärlen som syntes på röntgenundersökning, och i ett fall hade denna byggt på sig uppåt och orsakat en propp i hjärnans vener. Hos de

patienter som inte hade Lemierres syndrom var de vanligaste diagnoserna hos friska personer halsfluss eller halsböld, medan personer med kroniska sjukdomar som spridd cancer hade infektioner som utgick från tarmen eller de gynekologiska organen samt lunginflammation.

Hos en närbesläktad bakterie hos djur, framförallt nötboskap, finns ett toxin, ett så kallat leukotoxin, med effekt på vita blodkroppar. Toxinet har visat sig vara betydelsefullt för bakteriernas förmåga att orsaka sjukdom hos djur. Det har varit oklart om leukotoxinet finns hos de bakterier som orsakar sjukdom hos människor. I mina studier har jag kartlagt leukotoxingenen och visat att den finns hos samtliga bakteriestammar som jag har undersökt. Jag har även kartlagt sekvensen och funnit att genen finns i tre olika varianter, varav två är tidigare okända. De två sekvenstyper som tidigare inte har beskrivits är mycket vanligare än den tidigare beskrivna varianten hos de bakterier som isolerats från patienter med Lemierres syndrom. Det verkar inte som om de patienter som har haft Lemierres syndrom har bildat antikroppar mot leukotoxinet så det är oklart om det är betydelsefullt.

Jag planerar fortsatt undersökningar av leukotoxinets eventuella betydelse för sjukdom hos människa, dess effekt på mänskliga celltyper och förekomsten av eventuell immunitet mot toxinet hos vissa patientgrupper. Patientstudien gav upphov till flera nya frågor. Med utgångspunkt i patientstudien vill jag lägga upp en ny, nationell studie där patienterna inkluderas redan när de läggs in på sjukhus. Det ger möjlighet att samla in uppgifter på ett mer strukturerat sätt än att läsa patientjournaler i efterhand. För närvarande pågår i Danmark ett projekt för att sekvensera DNA från många stammar av *Fusobacterium necrophorum*. Detta kommer att ge upphov till nya möjligheter att studera bakterierna och de sjukdomar de orsakar.

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# Appendix: Paper I-IV