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**INFECTIOUS ENDOCARDITIS, ASPECTS ON  
PATHOGENESIS, DIAGNOSIS AND PROGNOSIS.**

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## ABSTRACT

The incidence of infectious endocarditis (IE) is estimated to 5.9/100.000 inhabitants and year. In recent years there has been an improvement in prognosis mainly due to more sensitive echocardiographic methods and surgery in the acute phase of the infection. However the mortality rate is still between 10-20% with an even higher mortality in IE caused by *Staphylococcus aureus* (SA). In this thesis some aspects of pathogenesis, diagnosis and prognosis have been studied.

In paper I, a retrospective study started 1994 the Duke criteria were applied to 83 patients who in 88 episodes had been examined by transesophageal echocardiography (TEE) for IE. In 49 episodes no treatment was given, all these episodes were classified as rejected. In 39 episodes the patients were treated, 26 definite, 11 possible and 2 rejected episodes. The Duke criteria were well adapted to use in clinical routine and valuable both in excluding patients without IE and identifying patients with IE.

In paper II thirty-four patients with 35 episodes of IE were followed prospectively with repeated TEE examinations (at diagnosis, discharge and follow-up 5 months later). The use of TEE for the diagnosis was found to be valuable as the high sensitivity and resolution enabled the diagnosis of small vegetations (< 5 mm) in 9/35 episodes and the identification of indications for surgery in 8/35 at the first TEE. The size of the vegetations decreased significantly during treatment. The repeated TEE examinations did not detect any previously unknown complications or influence the treatment.

In paper III, a retrospective study of the period from 1994 to 2000 the in-hospital and long-term mortality of injecting drug-users (IDU) and non-IDU patients was compared. In this study 195 IE episodes, 60 in IDUs and 135 non-IDUs were included. The episodes were classified by the Duke criteria and 145 definite episodes were analysed in detail. The favourable prognosis in right-sided IE was confirmed with no in-hospital mortality in 29 episodes in IDUs, and long-term mortality rate as IDUs in general. The in-hospital mortality did not differ between IDUs and non-IDUs but IDUs with left-sided IE had a higher long-term mortality rate than non-IDUs with left-sided IE and IDUs with right-sided IE. This excess mortality was explained by the poor long-term survival of operated IDUs.

In paper IV the internalization of *S aureus* in endothelial cells was studied as this might be one explanation for the difficulty in treating IE caused by SA. In an experimental model the rate of internalization of *S aureus* in cultured endothelial cells was compared to the rate in human heart valve biopsies and umbilical cord veins. The internalization rate into biopsies was significantly diminished by a factor 300 – 1000 compared to cultured cells. Furthermore we studied the role of Fibronectin Binding Protein (FnBP) on internalization into biopsies. In cultured endothelial cells we could confirm the vital role of FnBP for internalization but not so in the biopsies. This raises the question if internalization is of less importance in vivo than in vitro.

Keywords: endocarditis, diagnosis, prognosis, outcome, criteria, echocardiography, TEE, injecting druguse, *S aureus*, internalization, FnBP, endothelial cells

## LIST OF PUBLICATIONS

- I. Thalme A, Nygren A T, Julander I and Freyschuss U: Classification of infective endocarditis by Duke's criteria and transesophageal echocardiography: a 1-year retrospective analysis. *Scand J Infect Dis* 1996;28(4):p407-10
- II. Thalme A, Nygren A T, Julander I and Freyschuss U: Endocarditis: clinical outcome and benefit of trans-oesophageal echocardiography. *Scand J Infect Dis* 2000;32(3):p303-7
- III. Thalme A, Westling K, Julander I: Infective endocarditis, short and long-term mortality in injecting drug-users compared to non drug-users. Manuscript
- IV. Rennermalm A, Thalme A and Flock J-I: *Staphylococcus aureus* internalization into endothelial cells in cell culture and human tissues; differences in internalization efficiency. Submitted

Illustrations in this thesis previously published in paper I and II and the papers are reprinted with permission from Taylor & Francis, publishers of *Scandinavian Journal of Infectious Diseases*

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## LIST OF ABBREVIATIONS

BC	Blood Culture
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
CI	Confidence Interval
Clf	Clumping Factor
CNS	<i>Coagulase Negative Staphylococci</i>
CO <sub>2</sub>	Carbon Dioxide
CRP	C-Reactive Protein
Def.	Definite
DF	Degrees of Freedom
ECG	ElectroCardioGram
Em	Erythromycin
ESR	Erythrocyte Sedimentation Rate
Fn	Fibronectin
FnBP	Fibronectin Binding Protein
GST	Glutathione-S-Transferase
HACEK	<i>Haemophilus spp, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens, Kingella kingae</i>
HAEC	Human Aortic Endothelial Cells
HIV	Human Immunodeficiency Virus
HUVEC	Human Umbilical Cord Endothelial Cells
IDU	Injecting Drug User
IE	Infectious Endocarditis
LA	Levinthal's agar
LSGS	Low Serum Growth Supplement
mg	Milligram
ml	Millilitre
mm	Millimetre
MRI	Magnetic Resonance Imaging
non-IDU	Non Injecting Drug User
NVE	Native Valve Endocarditis
NYHA	New York Heart Association
OD	Optical Density
Op	Operation
PAD	Pathological Anatomical Diagnosis
PBS	Physiologic Buffered Saline
PBST	Physiologic Buffered Saline + Tween
PCR	Polymerase Chain reaction
Poss.	Possible
PTS	Patients
PVE	Prosthetic Valve Endocarditis
Rej.	Rejected
SA	<i>Staphylococcus aureus</i>

SE	Standard Error of the Mean
Tc	Tetracycline
TEE	Trans Esophageal Echocardiography
TTE	Trans Thoracic Echocardiography
VAS	Visual Analogue Scale
VGS	<i>Viridans Group Streptococci</i>

# 1 INTRODUCTION

Infectious endocarditis is an “old” disease. The first known case presentation including both a clinical history and autopsy findings dates back to 1646 and was recorded by Lazarus Rivière (64). It has later been referred to in the historical review by Major in 1945 (48). The diagnosis of infectious endocarditis (IE) is based on recognition of the clinical signs of a bacterial systemic infection with the associated inflammatory process, the identification of the causative microorganisms and the diagnosis of the intracardiac lesions, the classical being a vegetation on a previously damaged heart valve. However the diagnostic tools for observing the signs and symptoms of IE have been available to physicians for less than 200 years.

Observation and registration of the inflammatory process in medical practice began with the medical use of thermometry that started in earnest after Wunderlich in 1868 published “Das Verhalten der Eigenvärme in Krankheiten”. In this work based on 1 million observations in 25.000 people he established the normal body temperature range and laid the foundation for registration of fever. The laboratory diagnosis of inflammation became possible in 1897 after the discovery of the increased erythrocyte sedimentation rate in infections by the Polish pathologist Biernacki. This was later developed into a clinical useful test by the Swedes Fåhræus (1918) and Westergren (1921). Thus from the beginning of the 20<sup>th</sup> century a quantitative diagnosis of inflammation was possible.

The observations that the vegetations in IE contain microorganisms stem from the second half of the 19<sup>th</sup> century when Virchow (69), Winge (75) and Heiberg (33) observed bacteria in endocarditic vegetations and emboli in autopsy specimens. At the end of the 19<sup>th</sup> century culture of bacteria from the blood of living persons became possible and in two case series published in 1909 by Horder (37) and 1910 by Libman (46) blood cultures were part of the examinations performed and thus a certain diagnosis of infectious endocarditis was possible. The use of blood cultures has been and is the mainstay in identifying the causative bacteria in IE cases. The blood culture methods are much improved since then and hard to culture bacteria like the HACEK group are readily diagnosed in automated systems that alerts the microbiologist to growth in the cultures. Only in few cases are other methods like serology used. In recent years PCR has been used to examine excised heart valves in operated patients and development for using it on blood is ongoing but it is not yet a routine method.

The diagnosis of the actual lesions, vegetations, in living patients has until the development of medical ultrasound technologies been based on phenomena secondary to the lesions. In the course of growing a vegetation on a heart valve will cause a disturbance in the normal laminar blood flow and in some cases damage the valve and cause valvular insufficiency. This disturbance in the blood flow over the valves causes the heart murmurs that medical students are taught to recognize early in their training. The basic tool for diagnosing heart murmurs is the stethoscope but it was invented as late as 1816 by Laënnec. After the invention of the stethoscope it was possible to recognize the secondary effects (murmurs) of the valvular changes. With the

development of the electrocardiogram (ECG) at the end of the 19<sup>th</sup> century diagnosis of complications like conduction abnormalities due to invasive infection became possible. The phonocardiogram can be viewed as a tool to visualize murmurs but does not record anything else than the heart sounds that can be listened to in the stethoscope. Radiology techniques like angiography with cardiac catheterization show valvular insufficiencies but is a poor tool for observing vegetations (52). It was not until the invention of medical ultrasound and echocardiography in the 1970s that direct observation of the intra cardiac changes in patients secondary to IE became possible. The development of echocardiography has been rapid since the early M-mode recordings that demonstrated vegetations in 1973 (18) followed by two-dimensional echocardiography visualizing vegetations in 1977 (28). One limitation of transthoracic echocardiography, TTE, is the dependency of sound transmission through the thoracic wall. In the 1980ies transesophageal echocardiography, TEE, was introduced and with a probe in the esophagus the problem with poor transmission through the thoracic wall was solved and examinations with high resolution and observations of small vegetations was possible (16, 20).

To diagnose IE in systematic way various diagnostic criteria have been used, the most widespread have been the von Reyn criteria presented 1981 (70) and the Duke criteria presented in 1994 (21). The von Reyn criteria grouped IE cases in “definite, probable, possible and rejected” using clinical signs and microbiological and histological findings but did not include echocardiography findings. To classify a case as definite a histological or bacteriological examination of a vegetation or peripheral embolus was necessary and the group of rejected IE included cases classified as “rejected, but empiric antibiotic therapy warranted”, this made the criteria hard to use in everyday clinical practice. The Duke criteria presented 13 years later included echocardiography and classifies cases as definite, possible or rejected. To adapt the criteria to clinical practice a category of clinical definite cases was introduced where cases with positive blood-cultures and valvular vegetations could be classified as definite IE.

The spectrum of bacteria causing IE has changed with improved health in the general population, pneumococci as cause of IE is now rarely seen but was not uncommon at the beginning of the 20<sup>th</sup> century (37), the viridans group streptococci has been the dominant organism for most of the 20<sup>th</sup> century but in recent years there has been a shift towards staphylococcal etiology. In some materials published recently IE caused by viridans group streptococci (VGS) and staphylococci are equally common (12, 68) and in others staphylococci dominate (7, 10).

An effective treatment for infective endocarditis was not available until penicillin was tried in 1943 successfully in seven patients (47). Thus from the end of the 19<sup>th</sup> century to the 1940ies a diagnosis based on clinical symptoms and blood culture findings was possible without any effective treatment options being present. In fact the mortality in series on blood culture positive IE from the pre-penicillin era is close to 100% (6, 41). The psychological impact of an endocarditis diagnosis in 1931 is well described in the diary of the medical student A. S. R. (73). He describes his reaction to hearing that his

blood culture was positive for viridans streptococci as "the *dictum ultimum* from which there was no escape.....this message from the angel of death".

With the introduction of penicillin therapy a previously fatal disease became possible to cure, however the mortality remained high. Cardiac surgery with valve replacement in active infection was first described in 1965 (72). The mortality remained high, (30%), in the 1970s and 1980s and it was only with increasing use of cardiac surgery with valve replacement in patients with active infection that mortality decreased further (59). In IE caused by *Viridans group streptococci* (VGS) the mortality has been greatly reduced since the introduction of penicillin in the 1940s and the in-hospital mortality is now 5-6% (56), (57) in recent series. In IE caused by *Staphylococcus aureus* (SA) the mortality remains high with a 20 % (10) in-hospital mortality despite effective antibiotics used for long periods and the use of cardiac surgery in many patients.

Infections in injecting drugusers, IDU, including IE are a common problem in many urban areas. In this group SA is the dominant pathogen but in many cases the infection is localized to the right side of the heart and associated with a much lower mortality, 7% (32), than left-sided IE caused by SA. Treating IDUs poses special problems with poor adherence to treatment regimes unless drug withdrawal symptoms and other problems related to the use of illicit drugs are taken care of. In our hospital the solution to this has been the establishment of a ward specialized in treating IDUs with infections.

At the time when the present studies were initiated the diagnostic possibilities were expanding with TEE becoming available in many institutions, new diagnostic criteria had recently been presented, cardiac surgery in active infection was possible and special care for IDUs were available. With these new diagnostic and therapeutic tools in place studies on the outcome when applying this seemed appropriate.

## 2 AIMS OF THE STUDY

The main aim of the study was to evaluate the clinical value of: new diagnostic criteria; new diagnostic methods like TEE, using specialized care in treating injecting drugusers. As a secondary aim to study if the difficulties in treating Staphylococcal IE could be caused by internalized bacteria.

1. To evaluate the usefulness of Duke's criteria for diagnosis in patients with IE examined by TEE. Paper I
2. To evaluate the clinical value of TEE for diagnosis and outcome of IE by repeated TEE examinations. Paper II
3. To analyze in-hospital and long-term mortality in patients with IE and to compare mortality between IDU and Non-IDU. Paper III
4. To study internalization of *S. aureus* in endothelial cells as a model of pathogenesis of IE. Paper IV

## **3 MATERIALS AND METHODS**

### **3.1 PATIENTS AND MATERIALS**

#### **3.1.1 Paper I**

Patients referred from the Department of Infectious Diseases for transesophageal echocardiography (TEE) on the suspicion of IE between Sept. 1992 – Sept. 1993 were included. Eighty-three patients (48 men, 35 women) were included and their medical records were examined; episodes of IE were classified retrospectively using the Duke criteria.

#### **3.1.2 Paper II**

Patients with cardiac vegetations diagnosed by transesophageal echocardiography (TEE) between March 1994 to February 1997 were reported from the Department of Clinical Physiology and included in a prospective study. During this period 38 patients were asked to participate in the study, 4 were excluded and the remaining 34 patients were included. The patients were followed clinically and with TEE at diagnosis, discharge and the follow-up visit. At admission clinical parameters were recorded, among them temperature, NYHA classification and serum level of C-reactive protein (CRP). To evaluate the patients' subjective opinion of their state of health at admission, discharge and follow-up they were asked to complete a small questionnaire including a visual analogue scale. To control for patients not included in the prospective study all medical records of patients examined by TEE and treated for IE at the Department of Infectious Diseases in the same period were analyzed in retrospect, thus 32 patients were identified and included in a retrospective part of the study. Episodes were classified using the Duke criteria, TEE was performed using a biplane probe. All examination were recorded on S-VHS and evaluated in retrospect by one of the authors (A.N.).

#### **3.1.3 Paper III**

Patients treated for IE at the Department of Infectious Diseases between 1995 and 2000 were included in a retrospective study comparing mortality between patients injecting and not injecting illegal drugs. 177 patients with 195 episodes of IE were included. All episodes were classified using the Duke criteria, clinical data, results of echocardiographic examinations and microbiological analyses were retrieved from the medical records. Survival time after discharge up to the end of the study period was obtained from the Swedish central population registry

#### **3.1.4 Paper IV**

In an experimental work the internalization of various *S. aureus* strains in endothelial cells from fresh human biopsies was compared with internalization in cultured endothelial cells. Fresh human heart valves were obtained from patients undergoing elective heart valve replacement, fresh human umbilical cords were obtained from

mothers giving birth at the hospital. For the experiments with human biopsies heart valves from 5 patients and more than 20 umbilical cords were used. Internalization was studied using three different SA strains with different efficiency in internalization and different expression of Fibronectin Binding Protein.

### 3.1.5 Patients - summary

Patients in paper I, II and III were recruited among patients evaluated for or treated for IE at the Department of Infectious Diseases at Huddinge University Hospital. In paper I all patients who were examined by transesophageal echocardiography on suspicion of IE between Sept. 1992 – Sept. 1993 were included. In paper II patients all patients treated for IE between March 1994 and February 1997 were included. In paper III all patients treated for IE between 1995 and 2000 were included, thus some patients were included both in paper II and III.

## 3.2 METHODS

### 3.2.1 Definitions

*Case definition:* All episodes of IE were classified using the Duke criteria published 1994, by Durack et al at Duke's university (21).

*An episode of IE* was defined as a case of IE admitted for treatment regardless of the time between this admission and any previous episode.

*Treatment period* was defined as the period when the patient receives intravenous antibiotics.

*Acute surgery* was defined as cardiac surgery performed in the treatment period.

*In-hospital mortality* was defined as death of any cause before discharge.

*Follow-up time* was defined as time from admission to either death or to the end of the follow-up period.

*Native valve endocarditis* (NVE) was defined as IE in a native valve.

*Prosthetic valve endocarditis* (PVE) was defined as IE in a mechanical prosthesis, a bioprosthesis or a homograft.

*Intravenous drug use* (IVDU) and *injecting drug use* (IDU) was defined as injecting drug use noted in the medical records as admitted by the patient or from physical findings (needle marks)

*Left-sided IE* was defined as any involvement of the aortic or mitral valve as observed by echocardiography

*Right-sided IE* was defined as the involvement of the tricuspid or pulmonic valve without the involvement of the aortic or mitral valve as observed by echocardiography.

*Vegetation length* was defined as the longest measure observed in the echocardiographic examination and recorded in millimetre.

*Valvular insufficiency* was measured using Doppler echocardiography and graded using a numerical scale between 0 and 4 with no insufficiency (0), mild (1-2), moderate (3), and severe insufficiency (4).

### 3.2.2 Diagnostic criteria

In paper I, II and III the unmodified Duke criteria (21) were used. The Duke criteria classify IE as definite, possible or rejected on basis of a combination of major and minor criteria. For classifying IE cases information from clinical findings, microbiology examinations, and echocardiography and in operated patients histological and microbiological examination of excised material is used. On basis of this cases can be classified as clinical definite IE without use histology. When the first study started the clinical practice in our Department on diagnosing IE included echocardiography in all patients. Thus using the previously commonly used von Reyn criteria (70) that do not include echocardiography would be to exclude relevant information from the diagnosis. In paper I the medical records of all patients examined by TEE on suspicion of IE were reviewed. When the records were incomplete we tried to collect the missing information from other sources like the microbiology laboratory, pathology department and records from other departments. All episodes were classified in retrospect by one of the authors (A.T.), ambiguous results were discussed within the group of authors.

In paper II the IE episodes in patients followed prospectively were classified within the first week of admission, some episodes were reclassified after additional information was available (i.e. culture or histology examination of excised valves).

After the Duke criteria had been used for a couple of years one drawback with the “possible” group that including cases with very different probability of IE. Thus modifications to the original criteria have been suggested from Duke University in 2000 (45).

In paper III the unmodified Duke criteria were used for the analysis but a comparison with the modified criteria proposed by Li et al (45) was also made. All episodes were classified in retrospect using the information in the records.

### 3.2.3 The von Reyn criteria (70).

#### **Definite:**

Direct evidence of infective endocarditis based on histology from surgery or autopsy or on bacteriology (Gram's stain or culture) of valvular vegetation or peripheral embolus.

#### **Probable:**

**A** Persistently positive blood cultures\*, plus one of the following:

- 1 new regurgitant murmur OR
- 2 predisposing heart disease\*\* and vascular phenomena‡

**B** Negative or intermittently positive blood cultures§ plus three of the following:

1. Fever
2. New regurgitant murmur, AND
3. Vascular phenomena

#### **Possible:**

**A** Persistently positive blood cultures plus one of the following:

1. Predisposing heart disease OR
2. Vascular phenomena.

**B.** Negative or intermittently positive blood cultures with all three of the following:

1. Fever
2. Predisposing heart disease, AND
3. Vascular phenomena

**C.** For viridans streptococcal cases only- at least two positive blood cultures without an extra-cardiac source and fever

#### **Rejected:**

**A.** Endocarditis unlikely- alternative diagnosis already apparent.

**B.** Endocarditis likely, empiric antibiotic therapy warranted.

**C.** Culture negative endocarditis diagnosed clinically, but excluded by post-mortem

\* At least two blood cultures obtained, with two of two positive, three of three positive or at least 70% of cultures positive if four or more cultures obtained.

\*\* Definite valvular or congenital heart disease, or a cardiac prosthesis (excluding permanent pacemakers)

‡ Petechiae, splinter haemorrhages, conjunctival haemorrhages, Roth spots, Osler's nodes, Janeway lesions, aseptic meningitis, glomerulonephritis and pulmonary, central nervous system, coronary or peripheral emboli.

§ any rate of blood culture positivity that does not meet the definition of persistently positive

### 3.2.4 The Duke criteria (21)

#### **Definite Infective Endocarditis**

##### Pathologic criteria

Microrganisms: demonstrated by culture or histology in a vegetation, or in a vegetation that has embolized, or in an intracardiac abscess, or

Pathologic lesions: vegetation or intracardiac abscess present. confirmed by histology showing active endocarditis

##### Clinical criteria, using specific definitions listed below

2 major criteria, or

1 major and 3 minor criteria, or

5 minor criteria

#### **Possible Infective Endocarditis**

Findings consistent with infective endocarditis that falls short of “Definite,” but not “rejected.”

#### **Rejected**

Firm alternate diagnosis for manifestations of endocarditis, or

Resolution of manifestations of endocarditis, with antibiotic therapy for 4 days or less, or

No pathologic evidence of infective endocarditis at surgery or autopsy, after antibiotic therapy for 4 days or less

#### **Definitions of Terminology**

##### **Major Criteria**

##### Positive blood culture for infective endocarditis

Typical microorganism for infective endocarditis from two separate blood cultures

Viridans streptococci, \* Streptococcus bovis, HACEK group, or

Community-acquired Staphylococcus aureus or enterococci, in the absence of a primary focus, or

Persistently positive blood culture, defined as recovery of a microorganism consistent with infective endocarditis from:

- (i) Blood cultures drawn more than 12 hours apart, or
- (ii) All of three or a majority of four or more separate blood cultures, with first and last drawn at least 1 hour apart

##### Evidence of endocardial involvement

##### Positive echocardiogram for infective endocarditis

- (i) Oscillating intracardiac mass, on valve or supporting structures, or in the path of regurgitant jets, or on implanted material, In the absence of an alternative anatomic explanation, or
- (ii) Abscess, or
- (iii) New partial dehiscence of prosthetic valve, or

New valvular regurgitation (increase or change in pre-existing murmur not sufficient)

## **Minor Criteria**

### Predisposition: predisposing heart condition or intravenous drug use

- (i) Fever:  $\geq 38.0^{\circ}\text{C}$  ( $100.4^{\circ}\text{F}$ )
- (ii) Vascular phenomena: major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial hemorrhage, conjunctival hemorrhages, Janeway lesions
- (iii) Immunologic phenomena: glomerulonephritis, Osler's nodes, Roth spots, rheumatoid factor
- (iv) Microbiologic evidence: positive blood culture but not meeting major criterion as noted previously+ or serologic evidence of active infection with organism consistent with infective endocarditis
- (v) Echocardiogram: consistent with infective endocarditis but not meeting major criterion as noted previously

\*including nutritional variant strains.

+Excluding single positive cultures for coagulase-negative staphylococci and organisms that do not cause endocarditis.

### 3.2.5 The modified Duke criteria (45)

Definition of infective endocarditis according to the proposed modified Duke criteria, with modifications shown in boldface.

#### Definite infective endocarditis

##### Pathologic criteria

- (1) Microorganisms demonstrated by culture or histologic examination of a vegetation, a vegetation that has embolized, or an intracardiac abscess specimen; or
- (2) Pathologic lesions; vegetation or intracardiac abscess confirmed by histologic examination showing active endocarditis

##### Clinical criteria

- (1) 2 major criteria; or
- (2) 1 major criterion and 3 minor criteria; or
- (3) 5 minor criteria

#### Possible infective endocarditis

- (1) 1 major criterion and 1 minor criterion; or**
- (2) 3 minor criteria**

#### Rejected

- (1) Firm alternate diagnosis explaining evidence of infective endocarditis; or
- (2) Resolution of infective endocarditis syndrome with antibiotic therapy for <4 days; or
- (3) No pathologic evidence of infective endocarditis at surgery or autopsy, with antibiotic therapy for <4 days; or
- (4) Does not meet criteria for possible infective endocarditis, as above

Definition of terms used in the proposed modified Duke criteria for the diagnosis of infective endocarditis (IE), with modifications shown in boldface.

### Major criteria

Blood culture positive for IE

Typical microorganisms consistent with IE from 2 separate blood cultures:

Viridans streptococci, Streptococcus bovis, HACEK group, Staphylococcus aureus; or Community-acquired enterococci, in the absence of a primary focus; or

Microorganisms consistent with IE from persistently positive blood cultures, defined as follows:

At least 2 positive cultures of blood samples drawn 12 h apart; or

All of 3 or a majority of >4 separate cultures of blood (with first and last sample drawn at least 1 h apart)

**Single positive blood culture for Coxiella burnetii or antiphase I IgG antibody titer 1:800**

Evidence of endocardial involvement

Echocardiogram positive for IE

**(TEE recommended in patients with prosthetic valves, rated at least “possible IE” by clinical criteria, or complicated IE [paravalvular abscess]; TTE as first test in other patients),** defined as follows:

Oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation; or

Abscess; or

New partial dehiscence of prosthetic valve

New valvular regurgitation (worsening or changing of pre-existing murmur not sufficient)

### Minor criteria

- Predisposition, predisposing heart condition or injection drug use
- Fever, temperature >38.0°C
- Vascular phenomena, major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial hemorrhage, conjunctival hemorrhages, and Janeway’s lesions
- Immunologic phenomena: glomerulonephritis, Osler’s nodes, Roth’s spots, and rheumatoid factor
- Microbiological evidence: positive blood culture but does not meet a major criterion as noted above or serological evidence of active infection with organism consistent with IE
- **Echocardiographic minor criteria eliminated**

### 3.2.6 Echocardiography

All examinations were performed in the Department of Clinical Physiology, Huddinge University Hospital. The examinations, both TEE and TTE, were done in the usual clinical routine setting and thus performed by different physicians. All TEE examinations in paper I and II were saved on S-VHS and analyzed both at the time of examination and in retrospect by one of the authors (A.N.). Vegetation size was measured in mm and the longest measure recorded. Valvular insufficiency was graded using a numerical scale between 0 and 4 with no insufficiency (0), mild (1-2), moderate (3), and severe insufficiency (4). For paper III the results from the routine examinations were used unless the patient had been included in any of the previous studies, if so the results from the analysis in these studies were used.

### 3.2.7 Blood cultures

All blood cultures were performed according to the local guidelines of the Department of Infectious Diseases. The general recommendation in the study period was to obtain at least 2 sets of blood cultures in patients with suspected sepsis and 3 sets if IE was suspected. Each set contained one aerobic and one anaerobic bottle with 10 ml blood each. The blood cultures were analyzed in the Clinical Microbiology laboratory at Huddinge University Hospital, cultures were kept for 5-7 days, longer if IE was suspected. From the start of the study period up to March 1996 the Bactec 860 system was used, from April 1996 to April 2001 the VITAL system was used.

### 3.2.8 Questionnaire

In paper number II patients followed prospectively were asked to complete a questionnaire with two questions on their subjective opinion on their health at admission, discharge and follow-up. The questions were; "How is your general health today?" with the VAS scale ranging from "has never been worse" (0 on VAS) to "has never been better" (10 on VAS) and "How is your physical strength today?" with the VAS scale ranging from "have never felt weaker" (0 on VAS) to "have never felt stronger" (10 on VAS).

### 3.2.9 NYHA classification

Patients in the prospective part of paper II were examined by one of the authors (A.T.) and their condition was classified according to the NYHA scale (1-4).

### 3.2.10 C-reactive protein

C-reactive protein was measured as a part of the clinical routine in all patients with IE and recorded for patients in the prospective part of paper II at admission, at least once weekly during treatment, at discharge and follow-up. Reference value, upper normal limit: < 10 mg/l

### 3.2.11 Statistics

All statistic calculations were performed in either JMP or JMP IN software from the SAS institute.

In paper II one-way ANOVA was used to compare differences in vegetation size and valvular insufficiency.

In paper III for differences in proportions the  $X^2$  test or Fischers exact test were used, for survival analysis product-limit (Kaplan-Meier) estimates were used with log-rank test used to test homogeneity between groups.

In paper IV the Wilcoxon sum rank test was used to compare differences in internalization between endothelial cellcultures and biopsies using the same *S. aureus* strain, to analyze differences in multiplicity using different inoculum doses in umbilical cords the paired T-test was used. To show differences in internalization efficiency between different *S. aureus* strains in biopsies and cellcultures 95% confidence intervals were used.

## 3.3 MICROBIOLOGY METHODS PAPER IV

### 3.3.1 Bacterial culture.

The following strains were used: Cowan 1 for its potent ability to internalize into cultured cells, 8325-4 and its isogenic double mutant DU5883 (*fnbA*::Tc<sup>R</sup>, *fnbB*::Em<sup>R</sup>) (30), a gift from Prof. T.J. Foster, Trinity College, Dublin, Ireland) to investigate the role of fibronectin binding protein (FnBP). The strains were cultured at 37°C under mild shaking in Luria-Bertani broth over night, reinoculated 1:20 in fresh broth and further cultured for 1.5 h for maximal expression of FnBP. The bacteria were adjusted to OD<sub>550</sub>=1 and kept at -70°C until use. Fibronectin binding was tested by addition of bacteria to microtiter plates (Costar) pre-coated with Fn 20µg/ml and blocked with 2% BSA (Sigma). The plates were incubated 1 h at 37° and washed with PBST. Residual bacteria were detached with 1M NaCl and spread on blood agar plates for viable count.

### 3.3.2 Endothelial cell culture.

The following cells were used: primary human umbilical cord vein endothelial cells, HUVEC, (passage 2-3, prepared as described (60)), and human aortic endothelial cells, HAEC (Passage<15, Cascade Biologics). The cells were maintained in gelatine coated flasks in Medium 200 + low serum growth supplement, LSGS, (Cascade Biologics) in 5% CO<sub>2</sub> and were grown to confluency according to the suppliers' instructions.

### 3.3.3 Cell culture internalization assays.

Cells were grown to confluence in 24-well plates (Costar). The number of cells was counted in Bürker chambers. Viability was tested with trypan blue. Bacteria were added to concentrations of 10<sup>5</sup>-10<sup>8</sup> bacteria/well and incubated for 2 hours at 37°C 5% CO<sub>2</sub>. Plates were washed with PBS. Lysostaphin (Sigma), 20µg/ml in PBS, was added

for 20 minutes to kill extracellular bacteria. Plates were washed with PBS. Cells and bacteria were detached with trypsin (Sigma) for 10 minutes at room temperature. Sterile de-ionized water was added to lyse endothelial cells. The contents of the wells were serially diluted and plated onto blood agar plates. In competition assays, DU5883 and 8325-4 cells were mixed before addition to the cells. To determine the ratio of DU5883 (*fnbA*::Tc<sup>R</sup>, *fnbB*::Em<sup>R</sup>)/ 8325-4, single colonies were randomly picked from blood agar plates and spread on both LA agar plates and LA agar with 5 µg/ml erythromycin. At least 56 colonies were picked per sample. The relative ratio of DU5883/(DU5883+8325-4) in the recovered samples compared to the inoculum was defined as ((DU5883/(DU5883+8325-4)) in the sample / (DU5883/(DU5883+8325-4)) in the inoculum. In blocking assays, the D1-D3 fibronectin binding domains of FnBP A (provided by C. Signäs, Swedish University of agricultural sciences, Uppsala, Sweden) was added (40µg/ml) and incubated with the cells 30 min prior to addition of bacteria. As a negative control, an irrelevant polypeptide, Glutathione-S-transferase (GST, purified according to manufacturers instructions, Pharmacia), was added in equimolar concentration.

### 3.3.4 Human tissue sampling.

Fresh human umbilical cord veins were obtained from the maternity ward at Karolinska University Hospital, Huddinge, Sweden. The cords were taken from healthy mothers. The umbilical cords were stored at 4-8°C in PBS with penicillin G and streptomycin added and used within 3-4 hours. Fresh heart valve biopsies were obtained immediately after surgery of volunteering patients undergoing heart valve replacement. Washing with Mod. Eagles medium (Dulbeco) ensured that no residual antibiotics were left on the biopsies. After gross examination, intact parts of the valves were chosen for further studies.

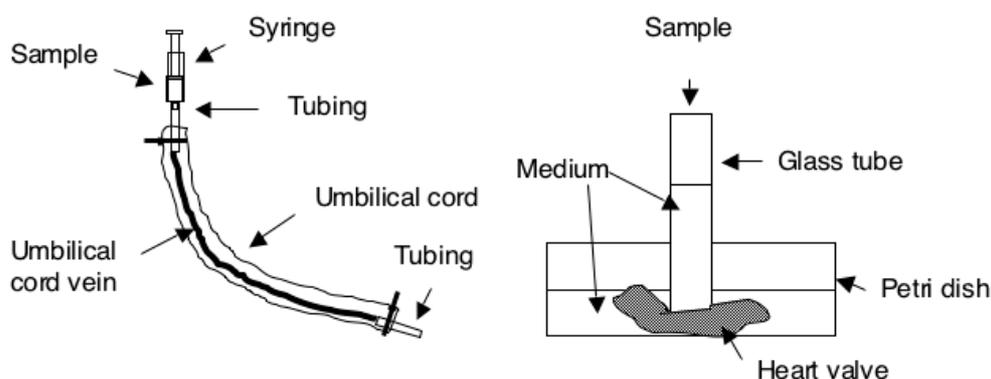
### 3.3.5 Human tissue examination.

Sample pieces of tissues were stained for endothelial cell markers and examined by fluorescence microscopy. The tissue pieces were fixed with formalin 2%. Unspecific binding sites were blocked with 2% heat inactivated fetal bovine serum in a 0.2% saponin solution with HEPES-buffer (PBS-sap) and subsequently with biotin/avidin according to manufacturers instruction (Vectastain) followed by another blocking with blocking buffer PBS-sap with 0.1% BSA-c (Aurium). Mouse anti-human von Willebrand factor (DAKO) was diluted 1:25 in blocking buffer + 0.002% NaN<sub>3</sub> and incubated with the sample over night at room temperature. Samples were incubated with Goat anti-mouse IgG1 1:300 in blocking buffer 1h room temperature, washed and incubated with Alexa 546 (Molecular Probes) 1:700 in blocking buffer 1h room temperature. The samples were washed with PBS-sap and the nuclei stained with Sytox green according to manufacturers instruction (Molecular Probes). The samples were sliced and mounted on glass slides that were examined by fluorescence microscopy (Zeiss).

### 3.3.6 Human tissue internalization assays.

The tissues were kept intact throughout the assays. Isolated chambers were made by placing a glass cylinder (6 mm inner diameter, 1.5 ml) vertically on top of the valve surface, thus ensuring exposure only to the valvular endothelial cells (Figure 1). For umbilical cords, catheters were inserted 3 cm into the umbilical vein and sealed with plastic ties (Figure 1). The vein was rinsed with PBS until no traces of blood was visible, minimum 20 ml/vein thus ensuring removal of residual antibiotics. The tissues were washed with Mod. Eagles medium (Dulbeco). Cowan 1 or a mix of 8325-4 and its isogenic FnBP-depleted mutant DU5883 in cell medium (umbilical cords HBSS) were added to a final concentration of  $10^5$ - $10^8$  CFU/ml. After 2 hrs incubation at 37°C, the tissues were washed and trypsinated for 10-15 minutes at 37°C. Cells were counted and viability was tested with trypan blue. Sterile de-ionized water was added to lyse the cells. The samples were serially diluted and plated onto blood agar plates. To determine the exact ratio of DU5883 (*fnbA*::Tc<sup>R</sup>, *fnbB*::Em<sup>R</sup>)/ 8325-4 in the inoculum and in the sample recovered, single colonies were randomly picked from the blood agar plates and spread both on LA agar plates and LA agar with 5 µg/ml erythromycin. At least 84 colonies were picked per sample. The relative ratio of DU5883/(DU5883+8325-4) in the sample compared to the inoculum was defined as ((DU5883/(DU5883+8325-4) in the sample / DU5883/(DU5883+8325-4)) in the inoculum. To investigate the role of Fn, some experiments were conducted in absence of Fn, others in the presence of extra-added Fn, 0.2 mg/ml.

Figure 1. Experimental set up for assessment of internalization into tissue biopsies.



### 3.3.7 Statistical analysis – microbiology paper.

The experiments on the tissues were analyzed with paired t-test. Analysis of difference in ratio of internalized bacteria between cultured cells and tissue were performed using the Wilcoxon sum rank test. Differences in relative ratio of DU5883 / (DU5883+8325-4) in internalization assays were visualized by showing 95% CI.

### **3.4 ETHICS COMMITTEE APPROVAL**

The studies included in this thesis were approved by the ethics committee at the Karolinska Institute, Huddinge University Hospital. Permission Number: 152/01; 57/94; 101/99; 489/00; 281/01; 258/02

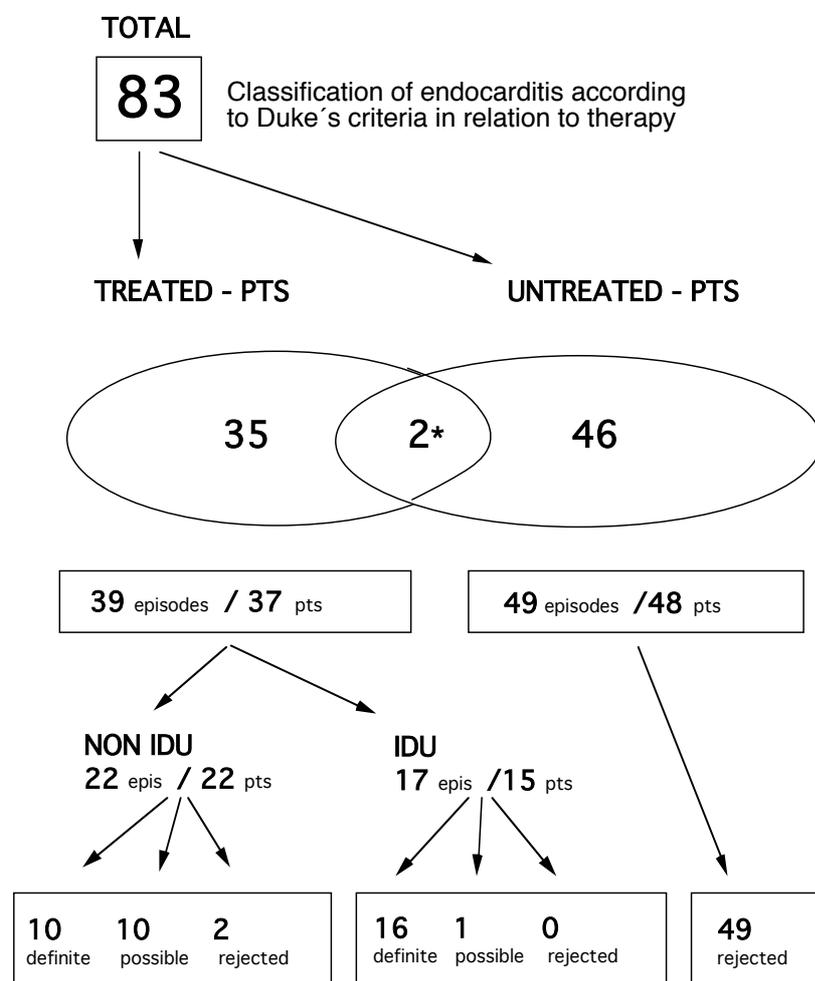
## 4 RESULTS AND DISCUSSION

### 4.1 PAPER I

In this retrospective study where data was collected and analyzed in 1994 –1995 the medical records of patients examined by TEE on suspicion of IE between September 1992 – September 1993 were studied. Thus the interval between the patients suspected IE episode and the extraction of data from the records was at least one year.

Eighty-three patients were included, in the material there were 35 treated patients, 46 untreated patients and 2 patients with both treated and untreated episodes (1 patient with 2 episodes of treated definite IE and 1 untreated episode, 1 patient with 1 treated episode of definite IE and 1 untreated episode). The distribution of patients with regard to treated /untreated episodes and IDU/non-IDU is shown in Figure 2.

Figure 2



\* 2 patients with both untreated and treated episodes included

#### Treated patients

Thirty-seven patients with 39 episodes were treated, the episodes were classified on basis of the information in the records and the re-evaluated TEE examinations. The

treated patients were in the analysis divided in patients injecting illicit drugs, injecting drugusers (IDU), and patients not injecting drugs (non-IDU). There were 22 non-IDU with an age range of 30-86 years, 15 were men and 7 women. The 22 episodes in the non-IDU were classified as; definite 10, possible 10 and rejected 2. The IDUs were younger with an age range of 30-62 years, 7 were men and 8 were women and one was HIV-positive. The 17 episodes in the IDUs were classified as; definite 16 and possible 1. The criteria used in classifying the episodes are shown in table 1 and 2

Table 1. Non IDU treated for endocarditis, criteria recorded

Patient	Major criteria		Minor criteria					
	Micro-biology	Endocardial involvement	Predisposition	Fever	Vascular phen*	Immunol .phen*	Echocardiogram	Micro-biology
Definite Pathologic IE								
AK (26)	-	+	-	+	-	-	-	+
Definite Clinical IE								
SK (01)	+	+	-	+	-	-	-	-
AB (06)	-	+	-	+	-	-	+	+
AF (16)	+	+	-	+	-	-	-	-
KG (18)	+	+	-	-	-	-	-	-
VG (23)	-	+	+	+	-	-	-	+
GF (24)	+	+	+	+	-	-	-	-
BÅ (27)	+	+	-	-	-	-	+	-
KT (28)	+	+	-	+	-	-	-	-
HH (32)	-	+	+	+	-	-	-	+
Possible IE								
EW (03)	-	-	+	-	-	-	+	-
LS (05)	+	-	-	+	-	-	+	-
AH (10)	-	+	-	-	-	-	-	-
SB (15)	-	-	-	-	-	+	+	-
GL (17)	-	-	-	+	-	-	+	-
GuL(19)	-	+	-	-	-	-	-	-
LR (20)	-	+	-	+	-	-	-	-
AM (21)	-	+	+	-	-	-	-	-
HH (22)	-	-	+	-	-	-	+	-
TG (25)	-	+	+	-	-	-	-	-
Rejected IE								
AJ (13)	-	-	-	+	-	+	-	-
LJ (04)	-	-	+	-	-	-	+	-

\*Vascular and immunologic phenomena were rarely recorded in the files, negative includes both missing data and negative findings.

Table 2. IDU treated for endocarditis, criteria recorded

Patient	Major criteria		Minor criteria					
	Micro-biology	Endocardial involv	Predispo-sition	Fever	Vascular phen*	Immunol .phen*	Echocardiogram	Micro-biology
<b>Definite Clinical IE</b>								
MK (02)	+	-	+	+	+	-	+	-
JH (07)	-	+	+	+	-	-	-	+
JE (08)	+	+	+	+	-	-	-	-
DG (09)	+	+	+	+	+	-	-	-
EA (11)	+	+	+	+	-	-	-	-
AL (12)	+	+	+	+	+	-	-	-
EN (29)	+	-	+	+	+	-	-	-
AE (30)	+	+	+	-	-	-	-	-
CP (31)	+	+	+	+	-	-	-	-
LP (35)	+	+	+	+	+	-	-	-
AL (36)	+	+	+	+	+	-	-	-
AL (37)	-	+	+	+	+	-	-	+
AR (38)	-	+	+	+	+	-	-	+
AR (39)	+	+	+	-	-	-	-	-
PW (40)	-	+	+	+	+	-	-	+
AM (41)	+	+	+	+	-	-	-	-
<b>Possible IE</b>								
SJ (14)	+	-	+	+	-	-	-	-

Vascular and immunologic phenomena were rarely recorded in the files, negative includes both missing data and negative findings.

As described in other materials (43) (13) the episodes classified as possible IE according to the original Duke criteria (21) includes patients with a very varied probability of IE, in this small material of 11 possible episodes there are patients with only 2 minor criteria and no major criteria and others with 1 major criterion and 2 minor. This heterogeneity has later led to the suggestion that the Duke criteria should be modified and to increase the specificity of the possible group (45).

#### 4.1.1 Blood cultures

In all episodes of definite IE the blood cultures were positive but only two of the possible episodes were BC positive, one among IDUs and one in the non IDU group. The reason for the low number of BC positive episodes in the possible group is unclear but in 4/9 BC negative episodes the patients had received antibiotics prior to admission. The etiology in the 28 BC positive episodes (26 definite and 2 possible) is described in Table 3.

Table 3. Etiology in 28 BC positive episodes

Etiology	Non-IDU	IDU	Total
<i>S. aureus</i>	2	16	18
<i>Viridans group streptococci</i> (VGS)	4	0	4
<i><math>\beta</math>-hemolytic streptococci gr G</i>	2	0	2
<i>Enterococci</i>	1	1	2
<i>Brucella</i>	1	0	1
<i>Proteus mir.</i> + <i>Enterococci</i>	1	0	1
	11	17	28

#### 4.1.2 Echocardiography

In this study with TEE was used in all patients and the TEE findings in treated patients were classified as major 27, minor 9 and negative 3. Two of three negative TEE examinations occurred in IDUs, 1 definite episode (*S. aureus*, septic pulmonary infarctions), 1 possible episode (*S. aureus*). One rejected episode with negative TEE was in a patient, non-IDU with rheumatic arthritis. In the untreated patients 36/49 TEEs were normal and the changes detected in the remaining 13 showed mostly aortic valve sclerosis and in 2 episodes old vegetations remaining from previous IE episodes. No TEE examinations in the untreated group fulfilled the major criteria for echocardiographic findings.

#### 4.1.3 Untreated patients

In the material there were 49 untreated episodes in 48 patients, all of which were classified as rejected IE using the at that time new Duke criteria. The TEE examinations in this group were normal in 36 episodes, 13 examinations showed valvular changes, mostly aortic valve sclerosis, but in two patients old vegetations from a previous IE episode were seen. As this study lacks a control group or a golden standard to compare with no calculation of the negative predictive value was possible but the fact that none of the untreated patients classified as rejected IE were readmitted with IE in the interval until the start of the study suggest a high negative predictive value. The high negative predictive value of the Duke criteria has later been shown in other studies (3, 19, 44).

#### 4.1.4 Discussion - usefulness of the Duke criteria

In this material the Duke criteria were easy to use which reflects the clinical practice in our department at that time. In the study no comparison was made with the von Reyn criteria (70), as the supportive clinical findings used in them were not consistently recorded, especially not when both a TEE finding and a positive blood culture were present. The necessity to combine clinical and echocardiographic criteria for the diagnosis of IE was obvious with 13 of the untreated patients having valvular changes and despite this being classified as rejected IE. The Duke criteria were valuable both in excluding patients that did not have IE and identifying patients with IE and were easy to apply in a clinical routine setting.

## 4.2 PAPER II

In this study that consists of two parts, a prospective part (labelled group A) and a retrospective part (group B). The patients in the prospective part were included from March 1994 to February 1997. Data for the retrospective part was collected in the year after the end of the prospective part, the period studied was identical.

### 4.2.1 Patients

During the study period 38 patients with 39 episodes of IE were initially included in the prospective part of the study. Two patients wished to be excluded, another 1 was excluded due to poor compliance and another 1 was excluded when by mistake a TTE was performed instead of a TEE, thus 34 patients (23 males, 11 females) with 35 episodes remained in group A.

In the retrospective part (group B) 32 patients (19 males, 13 females) with 34 episodes were included. The patient characteristics are summarized in Table 4.

Table 4. Patient characteristics

	Group A	Group B	Total
Age (median, range)	50 (27-85)	54 (26-85)	50 (26-85)
IDU (pats, episodes)	13 (14)	6 (6)	19 (20)
Pats with 1 episode, (n, n episodes)	33 (33)	30 (30)	63 (63)
Pats with 2 episodes, (n, n episodes)	1 (2)	2 (4)	3 (6)
Episodes of PVE (n, %)	4 (11%)	7 (20%)	11 (16%)
Surgery (n, %)	8 (23%)	7 (21%)	15 (22%)
Blood culture positive (n, %)	28 (80%)	26 (76%)	54 (78%)
Definite episodes	28	21	49
Total episodes	35	34	69

### 4.2.2 Echocardiography in prospective part, group A

#### 4.2.2.1 Vegetations

At the first, diagnostic TEE vegetations were found in 30 episodes and suspected vegetations in 5 episodes. The vegetations were small (<5mm) in 9 episodes. Changes in vegetation size were calculated on all examinations in 27 patients not operated. The size (mean +/- SE) at diagnosis was 7.8mm +/- 0.9mm, at discharge 3.6mm +/- 1.1mm and at follow-up 1.2mm +/-0.5mm. In the publication the change in size between the first and second examination was found statistically significant ( $p<0.001$ ) using ANOVA. However a reevaluation with another method, a T-test using matched pairs, gave the p-value for the change between examination 1 and 2 as 0.0002, for the change between 2 and 3 the p-value was 0.07.

As shown in Figure 3 the majority of vegetations (18) were smaller at discharge, 2 were larger (2-3mm) and 6 remained unchanged (change  $\leq 1$ mm).

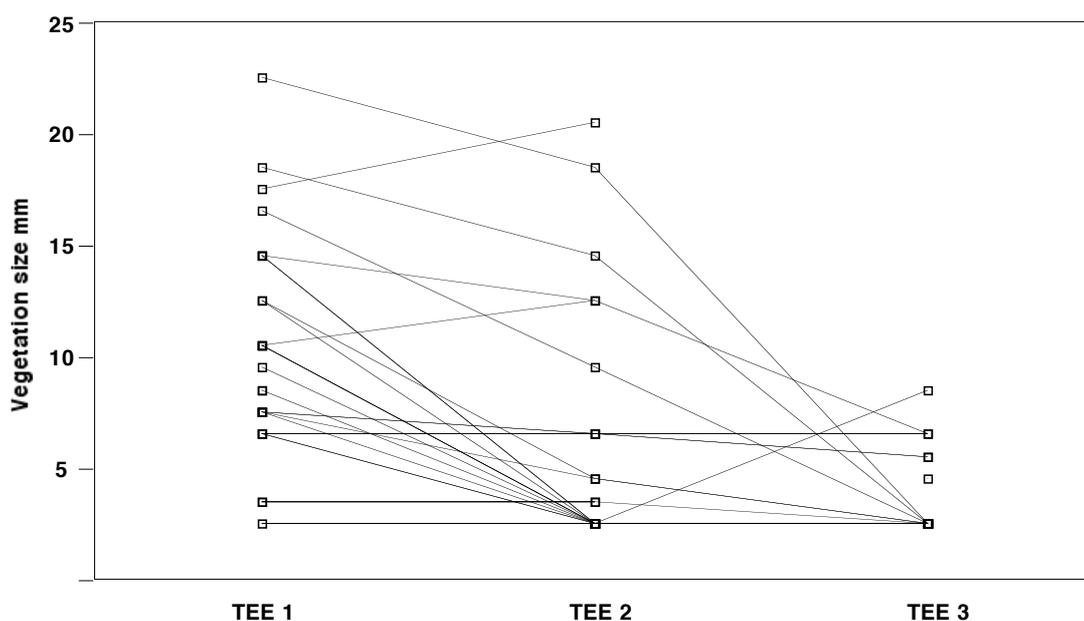


Figure 3, change in vegetation size between admission, discharge and follow-up. The individual vegetations are connected by lines

#### 4.2.2.2 Valvular insufficiency

The valvular insufficiency was graded 0-4, changes in grading between the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> TEE were analyzed in the 27 episodes with patients not operated. It was graded 2.3  $\pm$  0.2 at diagnosis, 2.2  $\pm$  0.2 at discharge and 1.9  $\pm$  0.3 at follow-up. The changes were non-significant.

#### 4.2.3 Prospective part, unpublished data.

##### 4.2.3.1 C-reactive protein group A

At admission the CRP level was recorded in all 35 episodes in group A, the levels mean (25-75 percentile) are shown grouped for different etiologies in Table 5, only etiologies with more than 5 episodes are shown separately.

Table 5. CRP-levels at admission

	Mean (mg/l)	Median (mg/l)	25-75% (mg/l)
<i>S. aureus</i> (n=11)	211*	200	160-271
<i>Viridans group streptococci</i> (n=11)	106*	93	49-170
Other BC positive episodes (n=6)	126	84	42-183
BC negative (n=7)	98*	106	42-171

\*p<0.05, T-test

The patients in group A were followed with CRP at least once weekly during the treatment period. In Figure 4 the mean CRP levels for week 1 through four are shown for 26 patients with IE caused by *S. aureus* (n=9), *Viridans group streptococci* (n=8), other blood culture positive cases (n=4) and blood culture negative IE (n=6). The 8 patients operated during the treatment period are excluded.

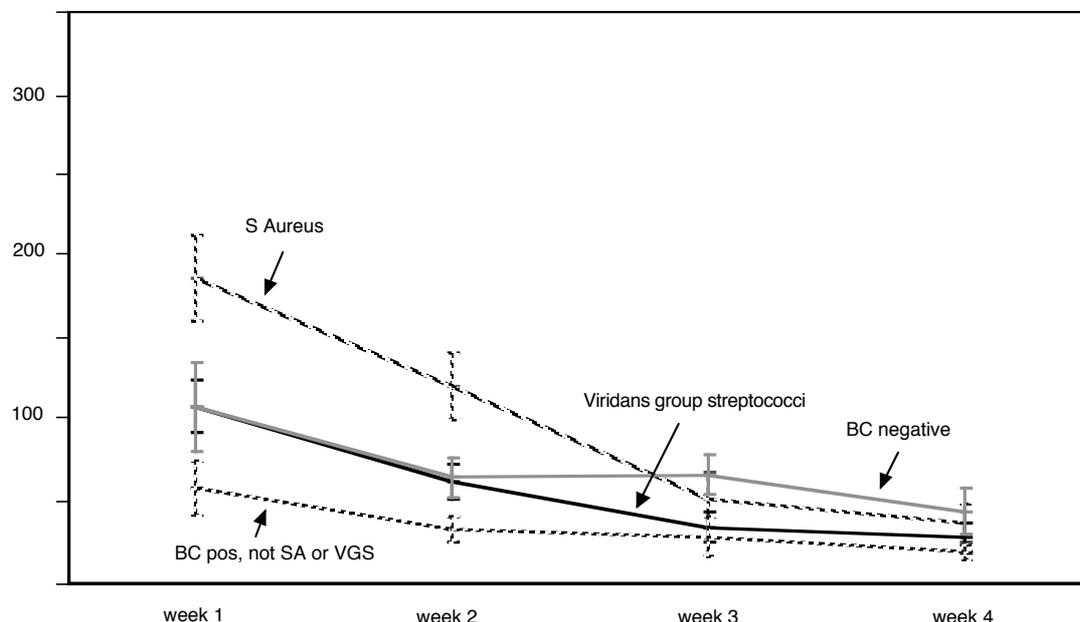


Figure 4. Mean CRP levels for week 1-4, error bars show +/- SE in 26 episodes, SA (9), VGS (8), BC negative (6) and other BC positive (4).

From the figure it is obvious that elevated CRP-levels persist well in to week 2 and 3 of the treatment without this being a sign of treatment failure

#### 4.2.3.2 Subjective health

Subjective opinion of health at admission, discharge and follow-up. This was recorded using a visual analogue scale (VAS) with two questions; How is your general health today (0= have never been worse, 100 have never been better), How is your physical strength today (0= have never been weaker, 100 have never been stronger). There were no significant differences regarding subjective opinion of health between en different etiologies at any point but IDU patients felt worse at admission. This difference could not be observed at discharge or follow-up. The details are shown in Table 6

Table 6. Mean VAS levels recorded

Group A	VAS 1		VAS 2		VAS 3	
	health	strength	health	strength	health	strength
Non-IDU n=16	54	36*	59	55	64	55
IDU n=9	36	15*	58	35	72	64

P<0.05 T-test

#### 4.2.3.3 NYHA grading

At the same time that the patients recorded their subjective health a grading of their condition by using the NYHA scale was done. This scale grades the functional impairment of heart disease from 1 – 4 with 1 being no impairment and 4 symptoms present at rest. The same tendency as in the subjective health assessment was found with a higher mean value (2.2) in the IDUs than in the non-IDUs (1.3) at admission. This difference did not reach statistical significance and disappeared during the treatment.

#### 4.2.4 Group A and B

##### 4.2.4.1 Classification

The 35 episodes in group A were classified during the treatment period, 34 episodes in group B were classified after the study period had ended. Duke's unmodified criteria were used, the results are summarized in Table 7

Table 7. Classification of 69 episodes using the Duke criteria

Duke classification	Group A	Group B	Total
Definite IE, PAD	6	2	8
Definite IE, clinical	22	19	41
Possible IE	7	13	20
Total	35	34	69

##### 4.2.4.2 Etiology

In the 49 definite episodes all but one were culture positive. The culture negative definite episode was confirmed by histology but no etiological diagnosis was made. In the 20 possible episodes only 6 were culture positive. The findings from blood-cultures and valvular cultures are detailed in Table 8

Table 8. Etiology in 69 episodes of endocarditis

Etiology	Group	A	Group	B	Total
	Non IDU	IDU	Non IDU	IDU	
<i>Staphylococcus aureus</i>	1	10	6	6	23
<i>Viridans group streptococci</i>	9	2	4	0	15
<i>Enterococci</i>	2	1	3	0	6
<i>HACEK</i>	1	0	4	0	5
<i>Coagulase neg staphylococci</i>	1	0	1	0	2
<i>β-hemolytic streptococci gr B</i>	0	0	1	0	1
<i>Pneumococci</i>	1	0	0	0	1
<i>Corynebacterium sp</i>	0	0	1	0	1
<i>Culture neg</i>	7	0	8	0	15
Total	22	13	28	6	69

##### 4.2.4.3 Echocardiography

Group A: Three TEE were planned for each patient, in 24 patients all 3 were carried out, in 8 patients only 2 were done and in 3 patients only one TEE was done. Of these 3

patients 2 had undergone surgery and refused further TEEs, one died after only 10 days of treatment.

Group B: In 31 of 34 episodes TEE was used for diagnosis, 3 episodes TTE was used. No record was made of further examinations during and after completed treatment.

#### 4.2.4.4 Valves involved

In non-IDUs there were 47 episodes of left-sided IE, 1 episode of right-sided IE and 2 episodes where no valve was identified. In IDUs there were 7 episodes of left-sided IE, 10 episodes of right-sided IE and 2 episodes where no valve was identified. The exact details of the valves involved is shown in Table 9

Table 9. Affected valves in 69 episodes of endocarditis

	Group Non IDU	A IDU	Group Non IDU	B IDU	Total
Aortic	10	2	15	1	28
Mitral	10	2	9	0	21
Tricuspid	0	7	0	1	8
Pulmonic	1	0	0	1	2
Aortic + mitral	1	1	2	1	5
Tricuspid + pulmonic	0	1	0	0	1
No valve	0	0	2a	2b	4
Total	22	13	28	6	69

Patients with no valve identified:

- a) 1. Male, 27 years, *S. aureus* in blood cultures, aortic and mitral valve prosthesis, "clinical definite IE", 2. Female, 36 years, *Actinobacillus actinomycetem comitans* in blood cultures, "possible IE"
- b) 1. Female, 32 years, *S. aureus* in blood cultures, "clinical definite IE", 2. Male, 26 years, *S. aureus* in blood cultures, "possible IE"

#### 4.2.4.5 Clinical outcome

No previously undetected complications were discovered in the discharge or follow-up TEE examinations in group A but in one patient the need for valve replacement due to cardiac failure was confirmed.

#### 4.2.4.6 Surgical therapy

In total 15 patients were operated in the period of antibiotic treatment, the surgery was done early in the treatment course, median 7 days, range 0-18 days. The indication for surgery in patients with NVE was heart failure (6 patients) and a large vegetation and risk for embolism (7 patients). In two patients with PVE the indication was dehiscence of an aortic valve prosthesis in one and *S. aureus* PVE not responding to conservative therapy in the other.

Another 3 patients were operated after cured IE because of heart failure.

#### 4.2.4.7 Prosthetic valve endocarditis

There were 11 episodes of PVE, 6 classified as definite IE and 5 as possible IE. The details are shown in Table 10

Table 10. PVE – details of 11 episodes

Patient	IE Class	Valve	Etiology	Surgery	Outcome
EC, woman 78 years	Definite	Aortic	VGS*	No	Cure
GS man 84 years	Definite	Mitral	VGS*	No	Cure
MT man 36 years	Definite	Aortic	VGS*	No	Death**
DAY man 27 years	Definite	No valve ID	<i>S. aureus</i>	No	Relapse#
DAY man 27 years	Definite	Mitral	<i>S. aureus</i>	Yes	Cure
KEV man 69 years	Definite	Aortic	<i>Enterococci</i>	Yes	Cure
KA woman 84 years	Possible	Mitral	<i>BC negative</i>	No	Cure
MS woman 66 years	Possible	Aortic	<i>BC negative</i>	No	Cure
NG man 80 years	Possible	Mitral	<i>BC negative</i>	No	Cure
RS man 64 years	Possible	Aortic	<i>HACEK</i>	No	Cure
KGK man 47 years	Possible	Aortic	<i>BC negative</i>	Yes	Cure

\* VGS = *Viridans group streptococci*

\*\* IDU patient with aortic and mitral valve prosthesis after previous IE 2 years earlier, succumbed after a septic myocardial infarction.

# non-IDU patient, relapsed after receiving 4 weeks treatment for *S. aureus* IE were no vegetations were found on TEE in the first or second episode. A vegetation was later identified on the mitral valve at surgery.

#### 4.2.4.8 Mortality

Only two patients died, one the 36 year old IDU with PVE and the other an 82 year old non-IDU with aortic NVE caused by *S. aureus*.

#### 4.2.5 Discussion

In this study the in-hospital mortality was low, lower than in many other series (2, 5, 11). The reason for this is unclear but as the study is small it can be due to chance. This seems to be the most likely explanation as the mortality in our own larger series in paper III is 10% of definite episodes. Still the inclusion of many patients with small vegetations could imply that many “mild” IE cases were included. The diagnostic value of the TEE was obvious both by correctly diagnosing patients with small vegetations (<5mm) that might have been overlooked on TTE and by identifying patients with the need for surgery. The indication for surgery was in this series consistently evident at the first TEE. The clinical value of the examinations at discharge and follow-up were low, however the series was small and consisted of patients with NVE except for 4 episodes. In short no benefit was observed from TEE repeated at discharge and the follow-up visit in patients with un-complicated NVE. In the health assessment the IDUs felt worse and also had a higher mean score on the NYHA scale, why this was so is unclear. The IDUs were more often infected with *S. aureus* and a more septic condition at admission could be expected but no correlation with the infecting organism was found.

### 4.3 PAPER III

In this retrospective study the patients included in the study had been treated for IE between March 1994 and December 2000 at the Department of Infectious Diseases Huddinge University Hospital. Thus most of the patients in paper II are included in this material as well. Some of the data used in the present study was collected in the course of the previous study, the rest of the data was retrieved from the medical records during 2002 and 2003. Survival data was collected up to September 16<sup>th</sup> 2003.

#### 4.3.1 Patients

All patients treated for IE in the study period that were alive were asked for written consent, dead patients and patients with unknown address were included without consent by approval of the ethics committee. In all 183 patients with 201 episodes of IE had been treated in the period. After six patients declined to participate 177 patients with 195 episodes remained and were included the study. The patient characteristics are summarized in Table 11

Table 11. Patient characteristics

	Non-IDU	IDU	Total
Male (n, %)	76 (59)	31 (63)	107 (61%)
Age (median, range)	64 (21-91)	39 (24-52)	56 (21-91)
Pats with 1 episode, (n, n episodes)	121 (121)	38 (38)	159 (159)
Pats with 2 episodes, (n, n episodes)	7 (14)	11 (22)	18 (36)
Episodes of PVE	27	2	29
HIV positive (n, n episodes)	0	9 (11)	9 (11)
Follow-up episode 1 (median, range) years	4.7 (0-9.5)	3.9 (0-9.5)	4.5 (0-9.5)
Definite episodes	92	53	145
Total episodes	135	60	195

The patients were divided into two groups, injecting drug-users (IDU) or non-injecting drug-users (non-IDU) including both patients without any use of illicit drugs and patients using drugs orally or inhaling. As expected the two groups were different with the IDUs being younger, some being HIV-positive, having a lower frequency of PVE and 2 episodes being more common, these differences were significant at a level of  $p < 0.01$  or below. The only differences shown in the table that not were significant were sex and follow-up time for episode 1.

#### 4.3.2 Microbiology

Data on the number of blood cultures taken were available for 181 of 195 episodes (median 2, mean 2.4, range 1-6) and is shown in Table 12.

Table 12. Etiology in 195 episodes

Etiology	Non-IDU	IDU	Total
<i>S. aureus</i>	24	45	69
<i>VGS</i>	41	4	45
Culture neg	29	1	30
<i>Enterococci</i>	13	7	20
<i>Other aerobic sp*</i>	7	2	9
<i>CNS</i>	8	0	8
<i>HACEK</i>	5	0	5
<i>S pneumoniae</i>	4	0	4
Mixed etiology	2	1	3
$\beta$ -hemolytic str gr A	1	0	1
<i>Fungi</i>	1	0	1
Total episodes	135	60	195

\*other aerobic sp: IDU *Actinomyces turensis*, *Enterobacter cloacae*, non-IDU  $\beta$ -hemolytic streptococci gr B, gr C, *Corynebacterium xerosis*, *Lactobacillus sp*, untyped grampositive rods

The dominant pathogens were as expected *S. aureus*, *Viridans group streptococci* and *Enterococci*. There were no statistically significant differences in either in-hospital mortality or long-term mortality for different etiologies in either IDUs or non-IDUs with definite IE. (data not shown).

#### 4.3.3 Echocardiography

In 41 episodes (32 non-IDU, 9 IDU) both TEE and TTE was performed, in 125 episodes only TEE was done (91 non-IDU, 34 IDU) and in 29 episodes only TTE was done (12 non-IDU, 17 IDU). There were no statistically significant differences in classification of episodes in regard to what type of echocardiographic examination (TTE or TEE) that was performed (data not shown).

#### 4.3.4 Classification of IE episodes

In this study the 195 episodes of IE were classified using the original Duke criteria (21). As modifications have been suggested to increase the specificity of the criteria the modifications suggested by Li et al in 2000 (45) were also applied. In contrast the modifications suggested by Lamas et al (43) were not tested, mainly because the addition of CRP > 100 mg/l and a high ESR were felt to add too much weight to the inflammatory process which already is included in the fever criterion. The results of using both the original and the modified criteria are shown in Table 13.

Table 13. Comparison of the original and the modified (Li) Duke criteria

Classification	Original criteria			Modified criteria		
	Non-IDU	IDU	All patients	Non-IDU	IDU	All patients
Definite	92	53	145	75	52	127
Possible	40	7	47	46	7	53
Rejected	3	0	3	14	1	15

The use of the modified criteria had little impact on classification of IE in IDUs, one definite episode was reclassified as possible and one possible episode was changed to rejected. In non-IDUs 17 (18%) definite episodes were reclassified as possible, 11 possible episodes were changed to rejected. In the 15 episodes classified as rejected by the modified criteria all patients had been examined by TEE and there was no in-hospital mortality. Two patients had vegetations constituting a major criterion but fulfilled no other criteria. Three patients were blood culture positive but had only one taken set of cultures taken. One was a 49 year old man, IDU with spondylitis and *S. aureus* in the blood culture, one was a 79 year old man with cancer of the colon and *Streptococcus sanguis* and *Pseudomonas* in the blood culture and the last was a 66 year old man with *Coagulase Negative Staphylococci* in the blood culture.

The change in classification from definite to possible was in 18/18 changed cases caused by elimination of echocardiography as a minor criterion. The change of 12 possible episodes to rejected were in 6 cases caused by eliminating the echocardiographic minor criterion, in 6 cases by raising the floor for possible IE to either 1 major and 1 minor or 3 minor.

### 4.3.5 Valves affected

The details of affected valves in all 195 episodes of IE separated with respect to classification of the episode and presence of IDU is shown in Table 14.

Table 14. Affected valves in 195 episodes

	Non-IDU				IDU			All patients			
	Def	Poss	Rej	Total	Def	Poss	Total	Def	Poss	Rej	Total
<b>Single valve</b>											
Aortic	42	16	0	58	8	0	8	50	16	0	66
Mitral	38	17	0	55	6	0	6	44	17	0	61
Pulmonic	0	1	0	1	0	0	0	0	1	0	1
Tricuspid	0	2	0	2	28	4	32	28	6	0	34
<b>Multiple valves</b>											
Aortic + Mitral	9	1	0	10	4	0	4	13	1	0	14
Aortic + Tricuspid	0	0	0	0	2	0	2	2	0	0	2
Aortic + Mitral + Tricuspid	0	0	0	0	1	0	1	1	0	0	1
Mitral + Pulmonic	0	0	0	0	1	0	1	1	0	0	1
Mitral + Tricuspid	0	0	0	0	1	0	1	1	0	0	1
Tricuspid + Pulmonic	0	0	0	0	1	0	1	1	0	0	1
<b>No valve identified</b>	3	3	3	9	1	3	4	4	6	3	13
<b>Total</b>	<b>92</b>	<b>40</b>	<b>3</b>	<b>135</b>	<b>53</b>	<b>7</b>	<b>60</b>	<b>145</b>	<b>47</b>	<b>3</b>	<b>195</b>

### 4.3.6 Treatment

The treatment time was in left sided IE median 33 days (25-75% interval 28-43 days), non-IDUs 32 days and IDUs 42 days. In right-sided IE the treatment time was median 28 days, (25-75% interval 25-39 days) non-IDUs 36 days and IDUs 28 days.

### 4.3.7 Surgery

Data regarding surgery was available for 194 episodes, missing in one patient who was transferred to another hospital. No patients with right-sided IE were operated. In non-IDU patients 32/134 episodes were treated surgically including 3 episodes classified as possible. The 3 possible episodes included; two patients with NVE where vegetations were found at surgery (no pathological examination performed, valve cultures negative), the third patient had PVE with a vegetation on echo and a pseudoaneurysm. In IDU patients 5/60 episodes were operated. The median time from start of antibiotic treatment to surgery was 8 days (25-75% interval 3-15 days).

### 4.3.8 Mortality

#### In-hospital mortality

In the complete material, 195 episodes, there were 16 deaths during hospitalisation, 2 deaths occurred in 47 possible episodes, 14 deaths in 145 definite episodes. All deaths in the definite episodes occurred in left-sided IE. The 2 deaths in the patients with an IE episodes classified as possible were both caused by *S. aureus*, in both cases only one set of blood cultures were drawn. One was a 73 year old woman with rheumatoid arthritis admitted in septic chock with a tricuspid valve endocarditis who died after 17 days, the other was a 48 year old woman in hemodialysis and a previous liver transplant who died after 21 days in another hospital. The details of the in hospital mortality in the 144 definite episodes with information available on surgery is shown in Table 15. One definite episode excluded due to lack of information about surgery.

Table 15. In-hospital mortality with regard to surgery

Outcome	No Operation			Operation		
	Non-IDU	IDU	All patients	Non-IDU	IDU	All patients
Discharge alive	55	44	99	26	5	31
Death	7	4	11	3	0	3
Total	62	48	110	29	5	34

### 4.3.9 Long-term follow-up

To analyse mortality rate and survival only the first episode of definite IE was used in patients having more than one episode during the study period. The patients were followed from admission until September 16<sup>th</sup> 2003. The mean follow-up time was 4.47 years with a range from 1 day to 9.5 years. The patient followed only for 1 day was an 81 year old woman with aortic valve IE who died in septic chock within 24 hours of admission.

The mortality rate was calculated in all 129 first episodes of definite IE. As seen in Table 16 the only big difference in mortality rate was observed in IDUs with left-sided IE.

Table 16. Mortality rate in long-term follow-up, 129 episodes

	n	Deaths	Follow-up mean	Mortality rate
Left-sided non-IDU	84	29	4.5 years	0.076/year
Left-sided IDU	16	9	3.3 years	0.17/year
Right-sided IDU	26	9	5.1 years	0.067/year
No valve id non-IDU	3	1	5.7 years	0.058/year
No valve id IDU	1	0	7.2 years	0
All episodes	129	48	4.5 years	0.081/year

The long-term survival was analysed in the 124 definite episodes where the infected valve was identified and data regarding surgery was available. Thus 5 definite episodes were excluded, 4 as no valve was identified and one because of lacking information on surgery. The survival is shown in Figure 5.

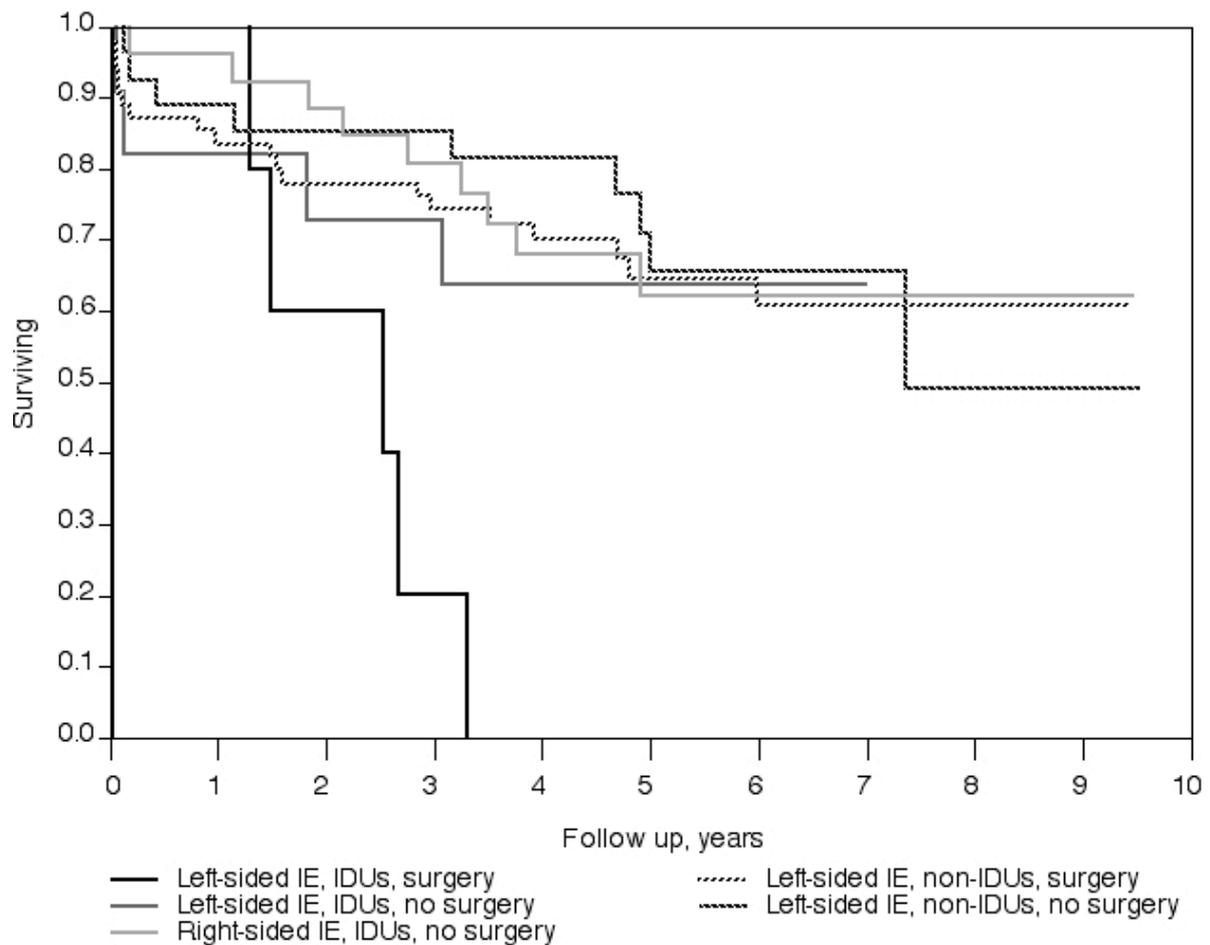


Figure 5. Kaplan-Meier curve on long-term survival in 124 definite first episodes with identified valve and information on surgery. Results shown grouped by left or right-sided IE and IDU or non-IDU. Test for homogeneity between groups: chi-square 14.0, DF 4,  $p > \text{chi-square } 0,007$ .

In the group of non-IDU there is no difference in long-term survival between patients operated and not operated. The only big difference in long-term survival observed in this material is between operated and not operated IDUs with left-sided IE. Of the very small number of IDUs operated ( $n=5$ ) no one lived more than 3.3 years. The cause of death is not known in all patients but at least 3/5 operated died in verified PVE or sepsis. This in contrast to the good short time results with no in-hospital mortality among operated IDUs. The short survival in this group explains in full the lack of homogeneity between the groups, when removed from the analysis no significant difference remains. The long-term survival in IDUs with left-sided IE is shown in better detail in table 17.

Table 17. Long-term survival of IDUs

	n	Discharge		Follow-up	
		Dead	Alive	Dead	Alive
No operation	18	4	14	8	10
Operation	5	0	5	5	0
Total	23	4	19	13	10

The difference between groups is highly significant, 0,009 using the Chi-Square test.

#### 4.3.10 Discussion

In this retrospective study the patients included reflect the urban population with a high percentage (28%) of IDUs among the patients. This ratio of IDUs is much higher than in a Swedish epidemiological study that included 7% IDUs (36) and estimated the incidence of IE as 5.9/100.000 inhabitants per year. A selection bias for IDUs with a severe addiction is likely as the only ward in the Stockholm County specialized in treating IDUs with infections is located in our department. There may also be a selection bias for IE patients requiring surgery as Huddinge University Hospital was one of two hospitals in Stockholm with a department for thoracic surgery during the study period.

The dominant infecting organisms are as expected *S. aureus* (SA), *VGS* and *Enterococci* with SA dominating in IDUs and VGS in non-IDUs. The distribution of different causative organisms is roughly the same as seen in other series.(5, 36, 42)

In the complete material of 195 episodes there are 15% of blood culture negative cases while there are no BC negative among the 146 definite episodes. This percentage of BC negative IE is in the same range as a recent Swedish study with 20 % BC negative cases (74). The absence of BC negative cases among the definite episodes can be explained by the construction of the diagnostic criteria where IE patients with negative BC or those who only have one BC done tend to be classified as possible. The same tendency to classify BC negative cases as possible IE is seen in the study mentioned above.

When comparing the original Duke criteria to the modified criteria there was little difference in classification of episodes among IDUs, only 2 of 60 episodes had a change in classification. In non-IDUs the situation was different, 28 of 135 episodes (21%) were classified differently. The change, downgrading, in classification was in a large majority of episodes caused by the removal of the unspecific echocardiographic findings as a minor criterion and only to lesser extent caused by increasing the number of criteria needed for classification as possible IE. The 15 episodes classified as rejected by the modified criteria in this material seem to only include patients with low risk of IE.

A large part of the patients included are IDUs, among them there is a slight dominance for right-sided IE (in all cases tricuspid valve IE), with 55% of the definite episodes. The prognosis for right-sided IE is known to be better than in left-sided IE with an in-hospital mortality rate below 10%; 7% in a retrospective study with 98 evaluable

episodes (32). In previous materials the proportion of right-sided IE among IDUs has varied a lot from about 1/3 of the patients (32% (38), 34% (9) 46% (51) up to more than 2/3 of cases (74% (53), 86% (54)). In clinical practice this means that despite the emphasis on right-sided IE in earlier reports there is significant risk (43% in our material) that the IDU patient with suspected IE suffers from left-sided IE with a worse prognosis. This risk combined with the dominance of SA as etiology with the commonly seen aggressive course makes the quality of the echocardiographic examination of the mitral and aortic valves important and necessitates the use of TEE in many IDU patients.

In the present study there was no significant differences regarding in-hospital mortality between groups. In right-sided IE there was no in-hospital mortality and there were no relapses of IE in the IDUs. This may reflect the tradition in our department to treat right-sided IE for 4 weeks as standard and no patients with definite right-sided IE were treated for less than 3 weeks.

In left-sided IE the in-hospital mortality is still significant (13%) despite the use of early surgery in 30% of the patients with definite IE. There is no difference in long-term survival between operated and not operated non-IDU patients. As the patients selected for surgery were the most severely ill this implies that the prognosis for these patients was improved by the use of early surgery. This is in accordance with earlier studies that have showed reduced mortality in operated patients. (15, 63) One prospective study from Sweden 1996 showed both decreased in-hospital mortality and increased 5 year survival for IE patients with cardiac decompensation who were surgically treated in the acute phase.(59)

The long-term mortality rate for IDUs with right-sided IE is though high in the same range as the rate in other materials for a general IDU population (9,6/1000 Glasgow 1985 (27), 33/1000 USA 1998 HIV-neg IDU (29), 64 Milan 1992 (4)). In a Swedish material collected 1980-81 (71) the 5 year survival was above 90%, however in that cohort with 38% opiate, 30% central stimulants and 24% mixed drugs abuse the proportion of injecting drugusers is not defined. The injecting drugusers with left-sided IE in our study have a high mortality rate even when compared with general population of IDU.

The explanation for the very high mortality rate among IDU with left-sided IE who were operated can't be established with certainty in this retrospective study. A striking observation is that there were no in-hospital deaths but a number of late deaths due to infectious complications. Earlier studies have shown divergent data on the survival after discharge of IDUs treated surgically. There have been reports on both low, 40%, 3-year survival (mean time to death 13 months) (38) and high, 74%, 3-year survival (50) after surgical treatment.

The risk for complicated infections in implanted prosthetic heart valves is well known with the incidence of late PVE being 0,42% in non-IDUs (14) and a 50% risk of developing PVE in the presence of SA bacteremia (23). This underscores that the

indication for cardiac surgery with valve replacement in IDUs who expose themselves to repeated bacteremias need to be strict.

#### 4.3.11 Conclusion

In this study both the short and long-term results on treating IDUs with right-sided IE are encouraging. There were no in-hospital deaths or relapses for right-sided IE and the long-term prognosis does not differ from the general population of IDUs. In left-sided IE the in-hospital mortality in IDUs not worse than for patients without drug addiction but the long-term prognosis for IDU patients with left-sided IE is worse. This increased long-term mortality can be completely explained by the poor prognosis for the surgically treated IDUs that is much worse than for patients without drug addiction. The reason for this cannot be ascertained in this study but it seems likely to be related to drug abuse and an increased risk for infection in prosthetic valves in operated IDUs. If this is the case the inclusion of operated IDUs in a drug addiction treatment program before discharge from hospital seems prudent.

Despite the risk for late infections and death after surgery for IE in IDUs our conclusion is that surgery improves the prognosis and should be part of the treatment when necessary. This as the prognosis without surgery is very poor.

## 4.4 PAPER IV

This experimental study was started in year 2000 to look at internalization of *Staphylococcus aureus* in endothelial cells as a possible explanation to the difficulties observed in treating IE caused by SA. To study the internalization in endothelial cells not cultured but still attached to their normal matrix fresh human heart valves and umbilical cords were used. To analyze the role of fibronectin binding protein two isogenic strains of SA, one which is FnBP deficient were used.

### 4.4.1 Internalization of *S. aureus* into endothelial cells

The *S aureus* strain Cowan 1 was used to determine internalization into endothelial cells, both tissue biopsies and primary cultures of endothelial cells.

#### 4.4.1.1 Umbilical cords and cultured endothelial cells from the umbilical vein (HUVEC).

To study the dose response of an inoculum that varied in concentration from  $1 \times 10^4$  to  $1 \times 10^8$  cfu/ml human umbilical cord veins were compared with HUVEC. Internalization of bacteria into HUVEC compared to umbilical cord veins was significantly higher at all inoculum doses ( $p < 0.05$ ). The number of intracellular bacteria increased with higher multiplicity of infection in both cultured cells and biopsies. The dose response for internalization is shown in Figure 6.

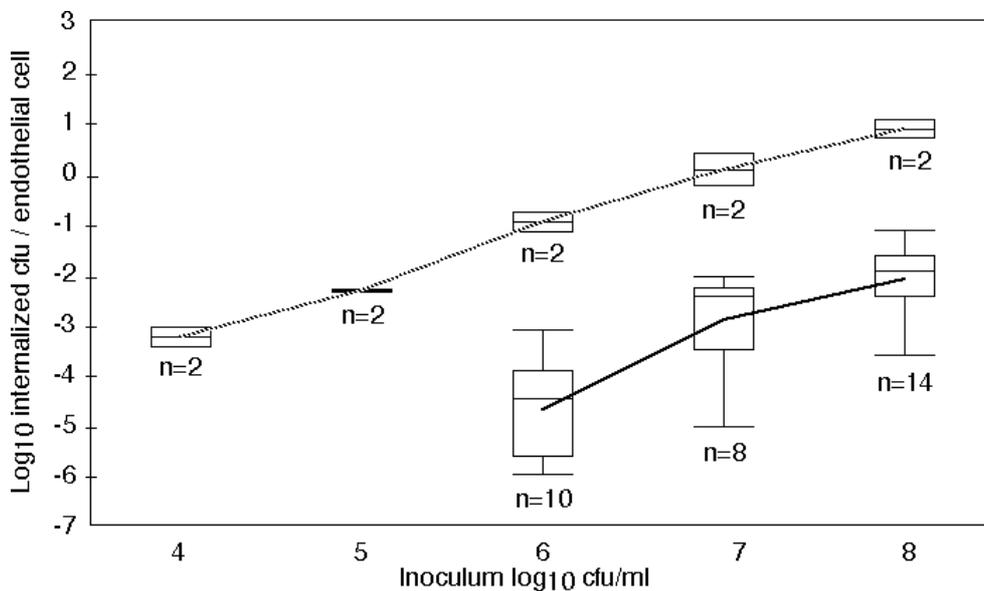


Figure 6

Internalization into umbilical cord vein endothelial cells (solid line) and HUVEC (dotted line). The log number of bacteria per cell is plotted as a function of inoculum size. Median values, 25/75 percentiles and outliers are shown.

#### 4.4.1.2 Heart valves and cultured human aortic endothelial cells (HAEC).

The dose response was studied with an inoculum that varied in concentration from  $1 \times 10^5$  to  $1 \times 10^8$  cfu/ml in HAEC. Heart valve biopsies were only studied with an inoculum of  $10^7$  cfu/ml due to the difficulty to obtain biopsies. As with HUVEC and umbilical cord veins the number of intracellular bacteria increased with higher multiplicity of infection in HAEC. Internalization into HAEC was significantly higher compared to internalization into heart valve endothelial cells ( $p < 0.05$ ) as shown in Figure 7.

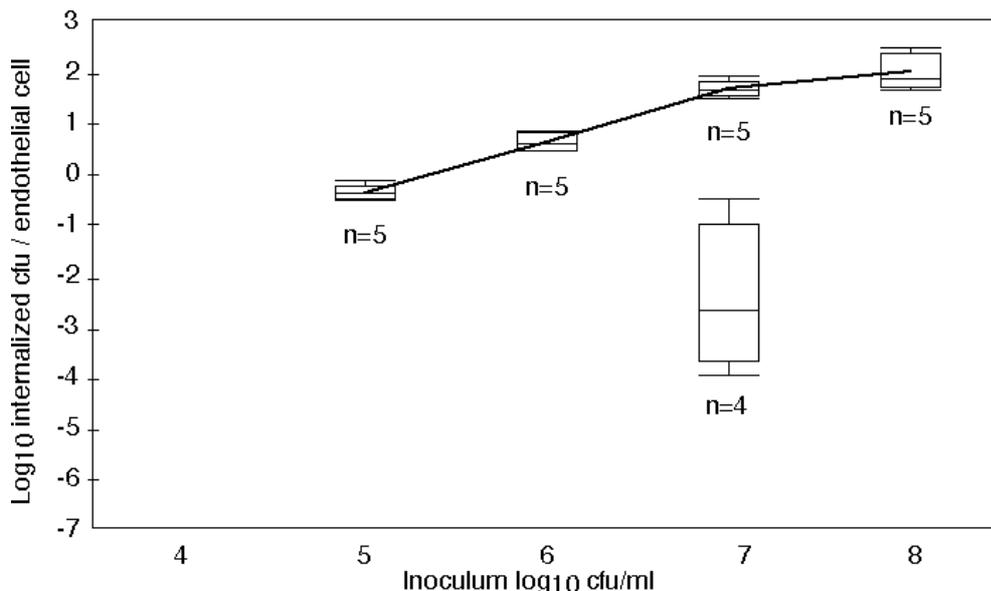


Figure 7

Internalization into heart valve endothelial cells (single box plot) and HAEC (solid line). The log number of bacteria per cell is plotted as a function of inoculum size. Median values, 25/75 percentiles and outliers are shown.

#### 4.4.2 Role of Fibronectin binding protein (FnBP) on internalization of *S. aureus* into endothelial cell biopsies.

##### 4.4.2.1 Binding to fibronectin.

FnBP is important for internalization of *S. aureus* into cultured endothelial cells. All the *S. aureus* strains Cowan 1, 8325-4 and DU5883 bound to immobilized fibronectin. The binding of the FnBP depleted strain DU5883 was however significantly reduced compared to its parent strain 8325-4 which is in accordance with previous findings. The adhesion to fibronectin is shown in Figure 8.

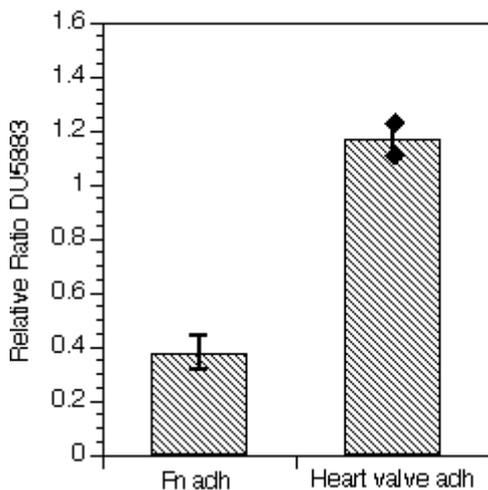


Figure 8

Results for adhesion to immobilized Fn (n=12) is shown as mean and 95% CI (left bar). Results for adhesion to heart valves (n=2), show mean and individual results (diamonds, right bar).

#### 4.4.3 Internalization

Using cultured cells our experiments confirmed previously published data, the internalization is dependent on strain and cell type. Cowan 1 is a good internalizer and could lead to as many as 10 CFU/cell in HUVEC and 300 CFU/cell in HAEC. When comparing internalization of Cowan 1 and 8325-4 in HAEC, the 8325-4 strain could be found intracellularly in a 10 fold lower frequency (data not shown). To compare 8325-4 and the FnBP depleted double mutant DU5883, these strains were mixed before adding them to the cultured cells or the biopsies. The proportion of erythromycin resistant *S. aureus* recovered from intracellular localization was compared with the proportion in the inoculum. A decreased proportion (from 38% to 4%) recovered from HAEC shows that DU5883 is a poorer internalizer than 8325-4 and that it is only found intracellularly in a very low frequency that is shown in Figure 9. This confirms the important role for FnBP in internalization in cultured cells. To confirm this a blocking assay was done where D1-D3 from the binding region of FnBPA was added to the cells prior to inoculation. In this assay the internalization of 8325-4 decreased > 1000 fold whereas DU5883 did not. An irrelevant peptide Glutathione-S-Transferase (GST) had no effect on internalization (data not shown).

To make a comparison with biopsies both umbilical cord veins and heart valves were studied in the same way. A mixture of 8325-4 and DU5883 was added to the biopsies and the proportion erythromycin resistant *S. aureus* recovered from the intracellular was compared to the inoculum. In this case the proportions were roughly equal which contrasts with the results from the cultured cells. The results are shown in Figure 9. Extra fibronectin (0.2 mg/ml) were added to the umbilical cord veins to ensure that lack of Fn was not the cause of the discrepancy observed between cultured cells and tissue.

Addition of Fn did not enhance internalization of *S. aureus* into the endothelial cells of umbilical cord veins (data not shown)

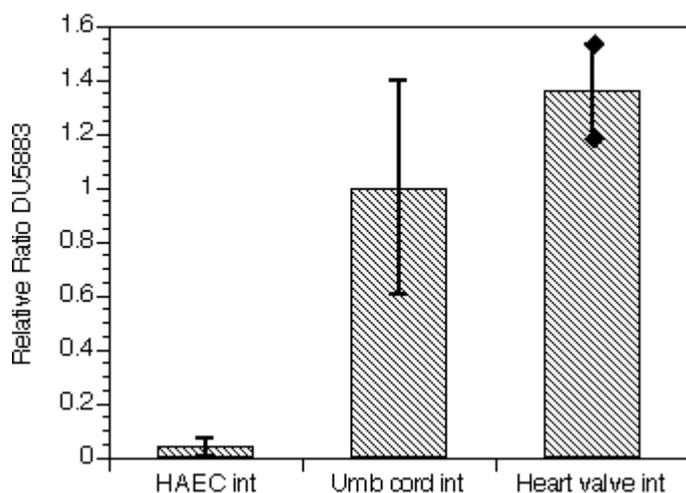


Figure 9

Internalization into cells. HAEC, n=9, umbilical cords, n=3 run in duplicates shown as mean and 95% CI. Results for internalization in heart valves (n=2), show mean and individual results (diamonds, right bar).

#### 4.4.4 Discussion

Internalized *Staphylococcus aureus* may account for the persistence of staphylococcal infections like furunculosis where the disease usually is of long duration with frequent relapses despite antibiotic treatment. The internalized bacteria may also explain the need for longer duration of antibiotic treatment in infective endocarditis caused by *S. aureus* than in streptococcal endocarditis. We here demonstrate the ability of *S. aureus* to invade human endothelial cells in tissue although to a markedly lower extent than in cell culture.

*S. aureus* has a wide range of extra cellular matrix binding proteins (24, 61). The fibrinogen binding protein Clumping factor A, Clf, is of importance for the infection to progress, and vaccination with Clf was protective in vaccination trials (25, 40). Immunization with FnBP D-peptide from the binding region of the protein ameliorated infection with the wild type strain, as did passive administration of anti-FnBP antibodies (62, 66). Other vaccination trials confirm that FnBP is a good target for vaccination (49). However, FnBP is not crucial for infection of endocardiac vegetation in a rat infection model, as the mutant strain DU5883 resulted in as much endocarditis as did the parental strain 8325-4 (25).

Both FnBP and Eap have been shown to be of vital importance for internalization into cells in vitro (22, 31, 67). In this study, we could see no pronounced difference in internalization between the 8325-4 and its isogenic mutant DU5883 into human

endothelial tissue from biopsies. Hence there is no direct importance of FnBP for internalization into endothelial cells in human biopsies. In a mouse mastitis study, the amount of the FnBP deficient mutant DU5883 in the mammary glands was reduced upon suckling, perhaps due to increased flow compared to the parent strain 8325-4 (8). The difference in internalization of *S. aureus* into cultured cells compared to tissues could be a reflection of limitations of cultured cells. The issue with model-dependent results have previously been addressed by Darouiche et al (17). In a rabbit osteomyelitis model they were unable to confirm decreased virulence in a mutant of *S. aureus* lacking FnBP A, FnBP B, Clf and the collagen binding protein CNA. These proteins have been proven important for virulence in other studies. Several studies have pointed out the fibronectin-binding  $\alpha 5\beta 1$ -integrin on the human cell as a target for FnBP-mediated internalization (1, 26, 55, 67). However, the expression of this integrin in biopsies is not determined in relation to in vitro grown cells.

This work has demonstrated that *S. aureus* internalize into human endothelial tissue, but to a much lesser extent compared with cultured endothelial cells. The role of FnBP for internalization into endothelial cells in biopsies is not as pronounced as for the corresponding cultured cells.

## 4.5 GENERAL SUMMARY AND DISCUSSION

When the first study was started the Duke criteria (21) had recently been published and were only validated by the same group that proposed them. Both the sensibility and specificity were soon studied by external groups. In 1995 Hoen et al (35) published a study that confirmed the high sensitivity of the Duke criteria compared to the von Reyn criteria (70) and in 1996 (34) another study confirming the specificity. The high negative predictive value was shown in a study published 1996 by Dodds (19). In our study (paper I) the Duke criteria were tested in a retrospective study and found suitable for use in clinical practice. The new criteria were both easy to use and valuable in both excluding and diagnosing IE. No comparison was made to the von Reyn criteria as the medical records did not include all information necessary for using them whereas the important information for using the Duke was available in the records. In fact the Duke criteria reflected how the clinical practice in diagnosing IE had evolved with an emphasis on echocardiography and blood culture findings for the diagnosis.

In the second paper the main aim was to evaluate the clinical value of repeated TEE examinations for the diagnosis and outcome of IE. In this small study the initial TEE proved to be important with a high diagnostic value. In several cases patients with small vegetations were correctly diagnosed with IE because of the high resolution and high sensitivity of TEE (39). The indication for surgery in patients needing operation during the treatment period was in all cases evident on the first TEE, the following examinations did not show any undiagnosed complications or change the follow-up of the patients. The vegetation size decreased during therapy in all but 2 patients, this in contrast to an earlier study (65) where 35% of patients had vegetations that remained constant or increased in size. In the analysis of outcome a low in-hospital mortality was found, only 2 of 49 patients with definite IE died. The frequency of surgery was 22%. The low mortality rate might be explained by the inclusion of patients with right-sided IE, patients with mild IE (small vegetations) and a high surgical frequency. The biggest limitation of this study is the small size, only 23 patients with NVE not operated were followed in the prospective part. Because of this only limited conclusions can be drawn but no benefit was demonstrated with repeated TEE in uncomplicated NVE.

In the third paper we looked at the results of treating IDUs with IE compared to non-IDUs. There was no significant difference in-hospital mortality between the two groups and in left-sided IE the length of treatment was similar in both groups signifying that it was possible to motivate the IDUs to stay for the full treatment period. In right-sided IE in IDUs there was no in-hospital mortality and no patients were treated surgically. The mortality rate in the long-term follow-up was the same as in the general population of IDUs (4, 27, 29). In contrast the mortality rate in IDUs with left-sided IE was higher than in all other groups, when analyzing this in detail the cause was the poor survival of operated IDUs. This high mortality in IDUs with left-sided IE makes a correct diagnosis important and in this material the percentage of left-sided IE among IDUs was quite high, 43%, of definite episodes. This will make the use of TEE necessary in many IDUs as well as make short treatment impossible for many of those patients. The surgical treatment of IE with implantation of valvular prostheses is common in treatment of IE today, 25-30% of patients are operated in the acute phase (58). The

operated IDUs with left-sided IE form a special risk group with regard to the risk for late PVE. In some materials the long-term survival has been quite good (50), the poor outcome for the few operated IDUs in our material form a contrast.

In the fourth paper the aim was to study if *S. aureus* did internalize in endothelial cells in biopsies to the same extent as noted in studies on cultured cells. This as the intracellular location can be viewed as a sanctuary with regard to antibiotic treatment. To study this we developed a model in which we exposed the endothelial cells from fresh biopsies of umbilical veins and heart valves to various concentrations of *S. aureus*. In the results in this model we did not see the same rate of internalization in biopsies as in cultured cells, in fact at a rate of 1/300 to 1/1000 when compared with cultured endothelial cells. The vital role of Fibronectin Binding Protein in internalization of *S. aureus* in cultured cells is well documented (22, 67) but in our model with biopsies the role of FnBP was not pronounced. This lower rate of internalization and diminished role of FnBP raises again the question about model dependent results from internalization studies on cultured cells (17). It can also be interpreted as a note of caution in transferring in vitro data to in vivo situations and can imply that internalization is less of a problem in vivo than previously inferred from experimental data.

## **4.6 CONCLUSIONS**

The Duke criteria for diagnosis of IE were useful in patients examined by TEE but also to rule out IE.

In patients with IE followed by three TEE examinations, the first one gave the important information but the following ones did not add any data for clinical decisions.

In patients with IE the in-hospital mortality was similar in non-IDU and IDU but the IDU with left-sided IE had a worse long-term prognosis

Internalization of *S aureus* into biopsies could be demonstrated but to a lesser extent than in cell cultures. The importance of FnBP for internalization into cultured cells was verified but could not be observed for biopsies.

## **4.7 ASPECTS FOR THE FUTURE**

Although improvement of outcome of IE has been demonstrated, the mortality is still too high and it is important:

To improve the diagnosis of IE, i.e. find a specific marker for endovascular infections

To improve the methods for a rapid and sensitive diagnosis of etiological agents in IE

To improve early diagnosis of intracardiac complications like dissemination beyond the valves, “new” technologies like MRI need to be adapted.

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