Neoehrlichiosis - Latent infection of endothelium and immune defense

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Till Britta och Inga

Två inspirerande kvinnor som, på den tiden det var allt annat än självklart, vågade ta sig till bättre platser och skapa sig en bättre framtid. De arbetade stenhårt med en sensationell arbetsmoral och framstod allt jämt som outtröttliga. Gemensamt för de båda var den kärlek för kunskap och böcker de delade. Därför var det föga förvånande att de snabbt blev så goda vänner, trots att de träffades på ålderns höst. Båda var alltid positiva och glada, och ingen skulle någonsin gissat att deras liv varit allt annat än enkla. De och den generation de representerade kommer för alltid vara en inspiration för mig. De är kvinnorna som gjort oss till de vi är och de vilka vi aspirerar att vara.

Aldrig stressa, bara skynda

- Inga Bergström (1930-2022)

Långa benet före!

- Britta Billman (1931-2022)

Neoehrlichiosis - Latent infection of endothelium and immune defense

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ABSTRACT

This thesis is a study of the tick-borne pathogen *Neoehrlichia (N.) mikurensis* that causes the infectious disease neoehrlichiosis in humans. The disease affects both individuals with competent and suppressed immune defense with the majority of the affected being immunosuppressed. The most common symptoms of neoehrlichiosis are recurrent fever, nightly sweats as well as muscle and joint pain. The most prominent clinical features are vascular events with thrombosis and arteritis. The aims of this thesis were to identify the target cells of the infection and get a deeper understanding of the immune responses evoked by *N. mikurensis*. In the first paper, it is described how some patients diagnosed with neoehrlichiosis also have antibodies targeted against another member of the *Anaplasmataceae* family, *Anaplasma phagocytophilum*. This can lead to patients with neoehrlichiosis mistakenly being diagnosed with anaplasmosis. In the second paper we describe for the first time that *N. mikurensis* can be cultivated and propagated both in tick cells and human endothelial cells. *N. mikurensis* was also demonstrated inside of circulating endothelial cells isolated from the blood of patients with neoehrlichiosis. These findings indicate that endothelial cells are a target cell of the infection, which could also explain the many vascular events affecting patients. In paper III we show that immunosuppressed patients with *N. mikurensis* infection had increased serum levels of cytokines involved in B-cell responses (CXCL13 and B-cell activating factor), the pro-inflammatory cytokine CXCL10 as well as cytokines associated with growth of blood vessels (vascular endothelial growth factor and fibroblast growth factor basic). We also show the

importance of confirming results obtained with multiplex cytokine assays with single plex tests. In the last paper IV, we lend support to the hypothesis that *N. mikurensis* infections in humans are latent infections that can reactivate when B-cell responses are suppressed in patients with B-cell lymphomas. Out of 97 patients treated with anti-CD20 antibody therapy, 8 % had a latent *N. mikurensis* infection, it was only the patients with latent infection that had T cells specific for *N. mikurensis*. These T cells were of the γδ, T-helper 1 and cytotoxic T lymphocyte types and expressed the cytolytic protein perforin. This work and future *N. mikurensis* projects will lead to a greater understanding of the infectious disease neoehrlichiosis.

Keywords: *Neoehrlichia mikurensis*, neoehrlichiosis, human, infectious disease, endothelial cells, T cells

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SAMMANFATTNING PÅ SVENSKA

Den fästingburna bakterien *Neoehrlichia (N.) mikurensis* orsakar infektionssjukdomen neoehrlichios hos människor och hundar. Första fallet rapporterades år 2010 vid Klinisk mikrobiologi, Sahlgrenska universitetssjukhuset i Göteborg. Efter fyndet har sjukdomen rapporterats från många länder i Europa samt Kina. Sjukdomen kan drabba individer som har ett fungerande immunförsvar, som är immunfriska. Desto vanligare är dock att patienter med en underliggande sjukdom så som blodcancer eller multipel skleros drabbas av infektionen. De vanligaste symptomen för neoehrlichios är feber, nattliga svettningar samt muskel- och ledsmärta. Utmärkande kliniska fynd för denna infektion är kärlpåverkan med risk för proppbildning. Bakterien är intracellulär, den behöver en värdcell för att överleva och föröka sig. I dagsläget kan diagnos endast ställas med mikrobiologisk diagnostik i form av PCR (polymerase chain reaction) som utförs på blodprov. I denna avhandling beskrivs i delarbete I hur vissa av patienterna diagnostiserade med neoehrlichios även kan ha antikroppar riktade mot en släkting till *N. mikurensis, Anaplasma phagocytophilum*. Det viktigaste fyndet i studien är att neoehrlichiospatienter kan misstolkas för den mer erkända infektionen anaplasmos. I delarbete II visas hur denna sjukdomsalstrande bakterie för första gången kan odlas i celler från fästingar samt kärlväggsceller, endotelceller, härstammade från människa. Vidare kunde vi påvisa bakterien i endotelceller isolerade från patienter diagnostiserade med neoehrlichios. Detta visar att en trolig målcell för *N. mikurensis* är endotelceller, vilket kan förklara de kärlkomplikationer som drabbar flertalet av patienterna. Vi påvisade i delarbete III att den immunnedsatta gruppen av patienter hade förhöjda nivåer av cytokiner inblandade i det specifika immunförsvaret som involverar B-celler. Både immunfriska- och immunnedsatta patienter med neoehrlichios hade även förhöjda värden av inflammationscytokiner. Ett viktigt fynd i denna avhandling är att infektionen är av latent typ, infektionen vilar i kroppen utan att ge symptom fram tills immunförsvaret av någon anledning blir nedsatt. I delarbete IV utvecklades en immunologisk metod där immunförsvarets T lymfocyter, en vit blodkropp som är specialiserad på att känna igen infekterade celler analyserades. Resultaten från dessa studier och framtida projekt om *N. mikurensis* leder förhoppningsvis till fortsatt förståelse för sjukdomen. Kunskapsspridning bland hälsoprofessioner och allmänheten är en viktig del i arbetet för en snabbare diagnos av neoehrlichios!

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Wass L**, Grankvist A, Mattsson M, Gustafsson H, Krogfelt K, Olsen B, Nilsson K, Mårtensson A, Quarsten H, Henningsson AJ, Wennerås C. Serological reactivity to *Anaplasma phagocytophilum* in neoehrlichiosis patients. Eur J Clin Microbiol Infect Dis. 2018 Sep;37(9):1673-1678. doi: 10.1007/s10096-018-3298-3.

II. **Wass L***, Grankvist A*, Bell-Sakyi L, Bergström M, Ulfhammer E, Lingblom C, Wennerås C. Cultivation of the causative agent of human neoehrlichiosis from clinical isolates identifies vascular endothelium as a target of infection. Emerg Microbes Infect. 2019;8(1):413-425. doi: 10.1080/22221751.2019.1584017.

*The authors contributed equally

III. **Wass L**, Quarsten H, Lindgren PE, Forsberg P, Skoog E, Nilsson K, Lingblom C, Wennerås C. Cytokine responses of immunosuppressed and immunocompetent patients with *Neoehrlichia mikurensis* infection. Med Microbiol Immunol. 2022 Jun;211(2-3):133-141. doi: 10.1007/s00430-022-00737-6.

IV. **Wass L**, Lewerin C, Jaén-Luchoro D, Brundin S, Lingblom C, Wennerås C. *Neoehrlichia mikurensis* causes latent infections that reactivate when B cells are suppressed in patients with B-cell lymphomas (in manuscript).

PUBLICATIONS NOT INCLUDED IN THE THESIS

Wennerås C, Goldblatt D, Zancolli M, Mattsson M, **Wass L**, Hörkkö S, Rosén A. Natural IgM antibodies in the immune defence against neoehrlichiosis. Infect Dis (Lond). 2017 Nov-Dec;49(11-12):809-816. doi: 10.1080/23744235.2017.1347815.

Lager M, Dessau RB, Wilhelmsson P, Nyman D, Jensen GF, Matussek A, Lindgren PE, Henningsson AJ; ScandTick Biobank Study Group; Baqir H, Serrander L, Johansson M, Tjernberg I, Skarstein I, Ulvestad E, Grude N, Pedersen AB, Bredberg A, Veflingstad R, **Wass L**, Aleke J, Nordberg M, Nyberg C, Perander L, Bojesson C, Sjöberg E, Lorentzen ÅR, Eikeland R, Noraas S, Henriksson GA, Petrányi G. Serological diagnostics of Lyme borreliosis: comparison of assays in twelve clinical laboratories in Northern Europe. Eur J Clin Microbiol Infect Dis. 2019 Oct;38(10):1933-1945. doi: 10.1007/s10096-019-03631-x.

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Wennerås C, Aranburu A, **Wass L**, Grankvist A, Staffas A, Soboli A, Mårtensson IL, Fogelstrand L, Lewerin C. Infection with *Neoehrlichia mikurensis* promotes the development of malignant B-cell lymphomas. Br J Haematol. 2023 Jan 17. doi: 10.1111/bjh.18652.

CONTENTS

ABBREVATIONS

BAFF	B-cell activating factor
CF	Cystic fibrosis
CLL	Chronic lymphocytic leukaemia
CM	Central memory T cell
CMV	Cytomegalovirus
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CTL	Cytotoxic T cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immune absorbent assay
EM	Effector memory T cell
ESR	Erythrocyte sedimentation rate
FAM	Fluorescent label fluorescein
FITC	Fluorescein isothiocyanate
HGA	Human granulocytic anaplasmosis
HIV	Human immunodeficiency virus
HLH	Hemophagocytic lymphohistiocytosis
IFA	Immunofluorescent antibody assay
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide

mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK	Natural killer
NO	Nitric oxide
OD	Optical density
OPLS-DA	Orthogonal projection to latent structures by means of partial least squares-discriminant analysis
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohemagglutinin
RA	Rheumatoid arteritis
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SLE	Systemic lupus erythematosus
TCR	T-cell receptor
TE	Terminal effector T cell
Th	T helper
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
γδ T cells	Gamma delta T cells

INTRODUCTION

Neoehrlichia (N.) mikurensis is an emerging tick-borne pathogen in Europe and Asia which has rodents as its natural hosts. This intracellular bacterium causes the infectious disease neoehrlichiosis. Both immunocompetent and immunosuppressed patients are affected. Both groups may contract a severe form of neoehrlichiosis with fever and vascular/thromboembolic events [1]. As the pathogen is not detected with routine culture-based methods, neoehrlichiosis is underdiagnosed. This thesis suggests that the infection in some individuals exists in a resting, latent state, not causing any noticeable symptoms, which can last a long period of time. In these cases, the bacteria remain quiescent until the patient's immune defense is challenged.

Another important finding in this thesis is that endothelial cells are a target cell for this bacterium (Figure 1). This leads to damage and vascular events in the endothelium.

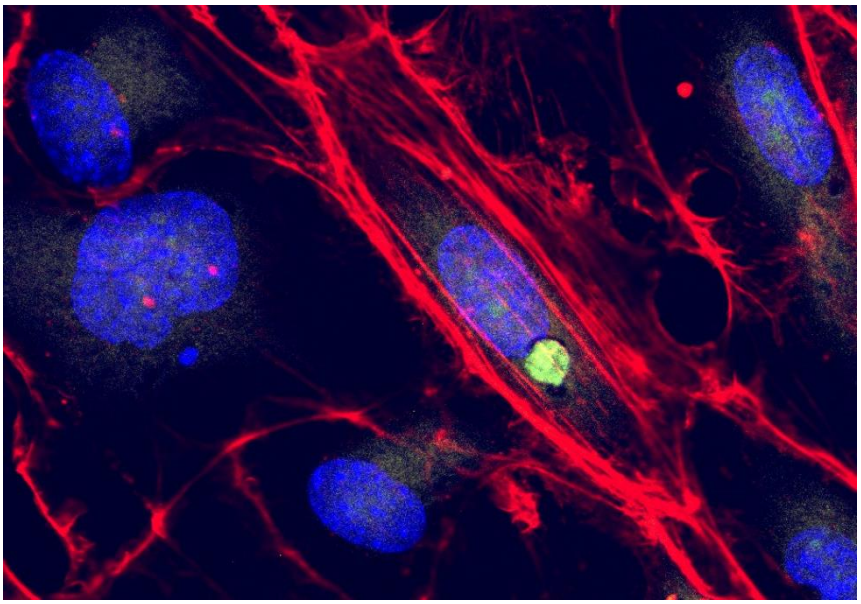

Figure 1. Confocal image of an endothelial cell infected with N. mikurensis shown as a microcolony vacuole close to the nucleus. *Blue = Hoechst nuclei stain, green = specific-N. mikurensis DNA probe and a Eubacteria (EUB339) probe (merged), red = Phalloidin staining cell membranes. Courtesy of Dr. Morsing, Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden.*

TICK-BORNE DISEASES

Ticks, blood-feeding ectoparasites, have been around since dinosaurs walked the earth making them extraordinary survivors. A tick has been found in a 99-million-year-old amber stone, showing that ticks fed on feathered dinosaurs (Figure 2) [2]. Fast forward millions of years to 2018, the forest fires in middle Sweden showed ticks' outstanding survival skills when they recovered in the same area after only two years [3]. Because of the viability of ticks, it can be assumed that tick-borne pathogens and diseases will continue to be a health concern. The health concern are of a special contemporary consideration because the climate change contributes to an increase in numbers of ticks and their tick pathogen reservoir hosts [4]. Thereby, leading to an increase in exposure for us humans.

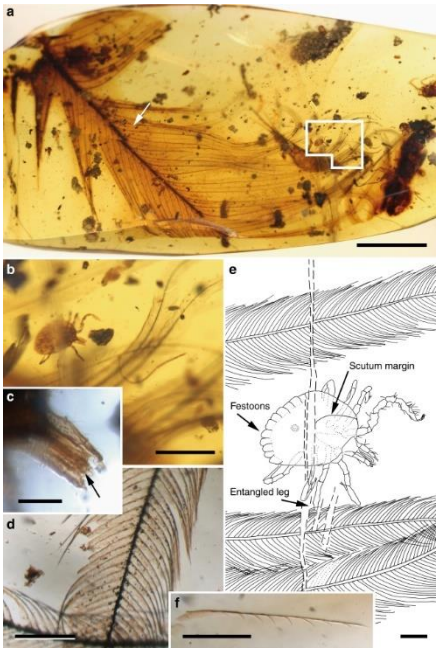

Figure 2. A tick entangled in a feather on the inside of an amber stone fossil. *[Reprinted with permission from Nature Communications, Peñalver E et al, Ticks parasitised feathered dinosaurs as revealed by Cretaceous amber assemblages. Copyright (2017), with permission from Springer Nature]*

In Sweden, the most widespread tick species is the *Ixodes (I.) ricinus*, whose life span varies from several months to 2-3 years. Ticks can live for six years with the right conditions for survival, important factors are access to blood hosts, temperature, and humidity [5]. The tick life cycle is shown in Figure 3. The last decade the geographic distribution of ticks has spread in both

longitude and latitude, one important reason for this is climate change [6]. A warmer climate, shorter and milder winters is beneficial for both ticks and tick hosts such as roe deer. This contributes to increased tick geographic range due to increased tick survival, abundance, and seasonal activity [7, 8]. The Public Health Agency of Sweden have listed tick-borne diseases together with heat waves as the two greatest threats to health caused by climate change in Sweden [8]. In addition, new tick species have been found in Sweden, such as *Hyalomma marginatum*, which could lead to new tick-borne infectious diseases [9].

Tick-borne infections are the dominant vector-borne diseases in Europe and the *I. ricinus* ticks are the main vectors [10]. The most recognized tick-borne pathogens of humans in Sweden (Tabel 1) are Borrelia species of the *Borrelia burgdorferi sensu lato* complex and tick-borne encephalitis virus (TBEV) according to the European Centre for Disease Prevention and Control (ECDC) [11]. *Borrelia species of the burgdorferi* sensu lato *complex*-are reported to be both the most common pathogen in ticks and the tick-borne microorganism with most reported cases of illness, causing borreliosis [12, 13]. Over the last years new tick-borne pathogens have been reported such as *Spiroplasma (S.) ixodetis* and the subject of this thesis, *N. mikurensis* (Table 1) [14-17]. *S. ixodetis* are intracellular bacteria that belong to the family of *Spiroplasmataceae*. Two cases of *Spiroplasma*-infection have been reported, one in an immunocompetent patient and one in an immunocompromised patient after tick exposure in Sweden. The patients had fever, a low platelet count and increased liver enzymes [14].

During the last decades, molecular diagnostic tools have had an immense development, resulting in the findings of new pathogens such as *S. ixodetis* and *N. mikurensis*, thereby enabling correct diagnoses on infectious diseases, which also contributes to the rising number of cases.

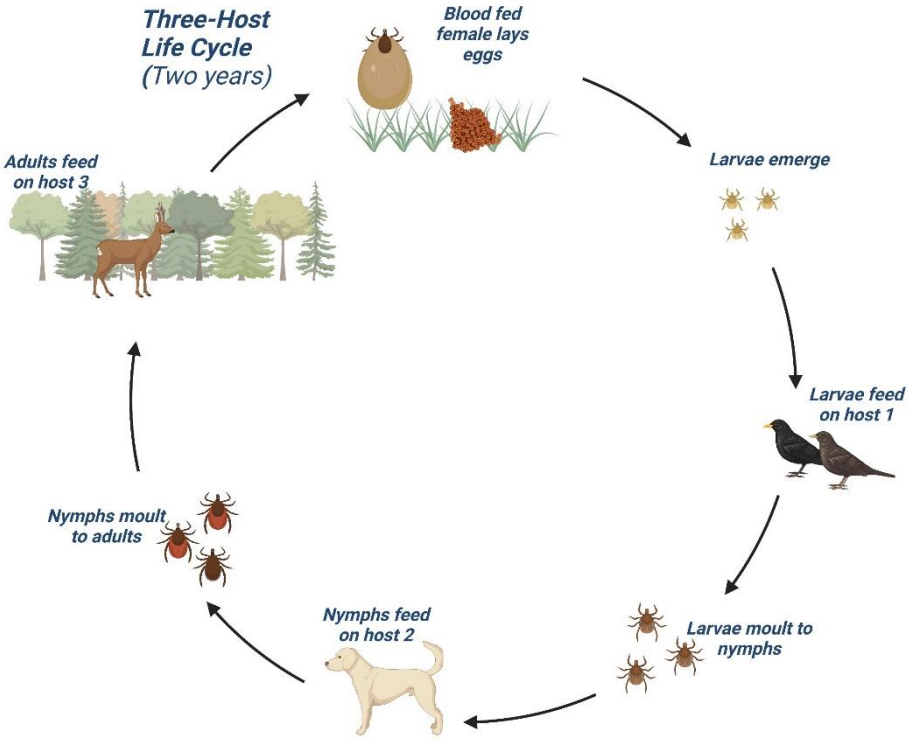

Figure 3. The developmental cycle of a tick. *An adult female tick lays thousands of eggs in the vegetation and the eggs hatch to larvae, which have their first blood meal. In the next step, the larvae moult to the nymph stage and have the second blood meal, preferred on a medium-sized host this time. Nymphs moult to adult females- or males after which the tick is ready to reproduce. The female has her last blood meal, falls to the ground and lays her eggs and dies. The male tick dies after the reproduction is consummated. [Created with BioRender.com]*

Table 1. Tick-borne diseases reported in Sweden [18].

Pathogen	Name of disease	Reference
Anaplasma phagocytophilum	Anaplasmosis	[18]
Babesia *spp*	Babesiosis	[19, 20]
Borrelia miyamotoi	Borrelia miyamotoi disease	[21, 22]
Borrelia burgdorferi sensu lato complex	Borreliosis	[23]
Francisella tularensis	Tularemia	[24]
Tick-borne encephalitis virus (TBEV)	Tick-borne encephalitis	[25]
Neoehrlichia mikurensis	Neoehrlichiosis	[15, 26, 27]
Rickettsia spp	Rickettsiosis	[28]
Spiroplasma ixodetis	Spiroplasmosis	[14]

THE FAMILY *ANAPLASMATACEAE*

The family *Anaplasmataceae* is in the order of *Rickettsiales,* and there are five genera within the family: *Ehrlichia, Anaplasma, Wolbachia, Neorickettsia* and *Neoehrlichia* (Figure 4) [29]. Many of the members have been known in veterinary medicine for a long time before being recognized as human pathogens [30]. All species of this family are small gram-negative pleomorphic cocci and obligatory intracellular bacteria. Some species have shifted from the family *Rickettsiaceae*, with the biggest difference being that bacteria belonging to *Anaplasmataceae* are enclosed within the membrane-bound compartments in the host cytoplasm in contrast to *Rickettsiaceae* where bacteria are free in the cytoplasm [31, 32].

The intracellular bacteria of this family need both to escape the immune system during their extracellular and infective stages and to be able to survive and replicate intracellularly.

Within the genus *Anaplasma, Anaplasma (A.) phagocytophilum* causes human granulocytic anaplasmosis (HGA), commonly called anaplasmosis. This tick-borne pathogen is discussed in paper I. As its name implies, the bacteria have a partiality for phagocytic cells and is one of very few bacterial

species known to survive and replicate within neutrophil granulocytes [33]. Infection with *A. phagocytophilum* has been reported in humans and a variety of domestic and wild animals, with fatal cases having been reported in humans, horses, dogs, sheep, cattle, reindeer, roe deer, and moose [34]. Based on molecular detection in questing ticks, *A. phagocytophilum* seem to exist in all of Europe.

Clinical symptoms of human anaplasmosis are fever, myalgia, malaise, fatigue, and headache. Blood parameters are leukopenia, thrombocytopenia and elevated levels of hepatic enzymes [35].

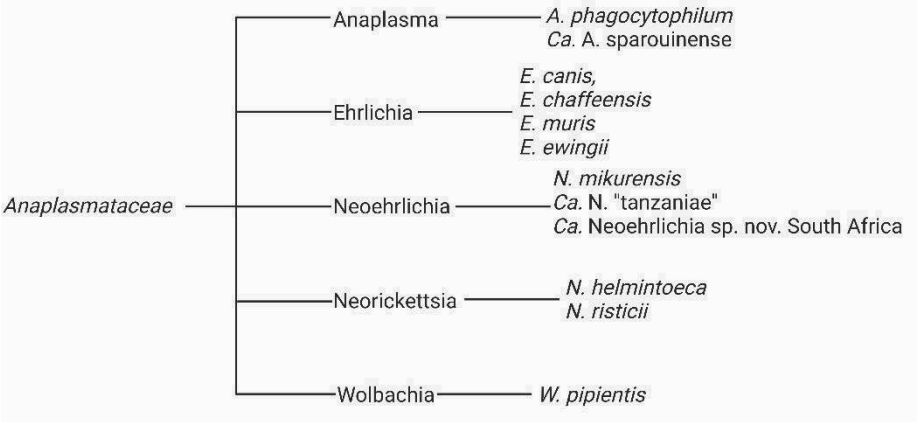

Figure 4. The bacterial species belonging to the Anaplasmataceae family. *Only bacteria causing human disease are shown.* [*Created with BioRender.com*][36, 37]

NEOEHRLICHIA GENUS

Apart from *N. mikurensis*, the "Neoehrlichia" genus includes at least six other members; *N. lotoris* which have been detected in raccoons from North America [38], the close relative to *N. lotoris*, '*Candidatus (Ca.)* Neoehrlichia' sp. FU98, which have been found in red foxes, a badger and one *I. rugicollis* tick from Europe [39, 40]. Furthermore, *Ca.* N. australis and *Ca.* N. arcana have been described in questing *I. holocyclus* ticks from Australia [41].

The Neoehrlichia species that have been involved as a human pathogen are *N. mikurensis*, *Ca.* N. sp. nov. South Africa and an agent named Ca. N. "tanzaniae" [42]. This last infection was described in Austria where an otherwise healthy woman with no underlying hematologic or autoimmune disorders developed a symptomatic Neoehrlichia infection. She had returned from a 28-day vacation in Tanzania and had developed high fevers, chills, night sweats, headache, muscle pain and malaise. In this case report splenomegaly and a low platelet count was also described [42].

NEOEHRLICHIA MIKURENSIS

N. mikurensis was first found 1999 in the Netherlands in ticks and was given its name in 2004 when it was found in ticks and rodents in the Japanese island of Mikura [29]. It was shown in rat spleens from this island and in questing *I. ovatus* ticks in Hokkaido, located in northern Japan. With that publication it got its name *Candidatus* Neoehrlichia mikurensis, *Candidatus* because it was an uncultivated bacterial species [29]. This nomenclature is used to name procaryotic species that have been identified by molecular methods but not yet isolated by culture. "Candidatus" was dropped from *N. mikurensis* by the scientific community after the year 2021 when it had both been cultured and whole-genome sequenced by our research group.

N. mikurensis had already had many different names since it was first found. It was called "Ehrlichia-like species Schotti variant" when it was discovered in questing *I. ricinus* ticks in the Netherlands and Russia [43, 44], "Ehrlichia-like species *Rattus* variant" in *Rattus norvegicus* rats in China [45] and *Ca.* Ehrlichia "walkerii" in *I. ricinus* ticks in Italy [46, 47].

It has been shown in electron microscopy pictures that the bacteria presumably are located in the salivary glands in a female tick [48]. Nevertheless, all bacteria and viruses that ticks carry will eventually be present in the salivary glands, even if first localized in the mid-gut section.

MICROBIOLOGY

Some bacterial species related to *N. mikurensis*, e.g., *Ehrlichia (E.) chaffeensis*, are known to have different morphology in mammalian and tick cells [49]. The *N. mikurensis* visualized in the salivary glands of an *I. ricinus* tick previously mentioned were of coccoid shape between 0.5 – 0.8 μm surrounded by a double-layer membrane composed of an inner cytoplasmic membrane and a rippled outer membrane separated by an irregular periplasmic space [48]. In paper II, where we cultivate the bacteria in two tick cell lines, one from *I. ricinus* and one derived from *I. scapularis* we describe aggregates of *N. mikurensis* tightly surrounding the tick cell nucleus using a labelled *N. mikurensis*-specific DNA probe. Presumably, these bacteria were packed in intravacuolar microcolonies, formations of numerous organisms typical of related intracellular bacteria, e. g., *N. lotoris* [38, 50], *Ca.* Ehrlichia khabarensis [51] and *A. phagocytophilum* [52].

EPIDEMIOLOGY

N. mikurensis in *I. ricinus* ticks

N. mikurensis has been found in *I. ricinus* ticks collected from the vegetation throughout 17 European countries. The prevalence differs widely from 0.1 % in Denmark to 24 % in Hungary [53-56]. Countries are listed in Table 2.

Table 2. *Prevalence of N. mikurensis in I. ricinus ticks in European countries.*

Country	Prevalence (%)	Reference
Belgium	0.4	[57]
Czech Republic	0.4–10	[58-61]
Denmark	0.1–0.9	[53, 54]
Estonia	1.3	[62]
France	0.2–1.7	[54, 59]
Germany	2.2–24.2	[59, 63, 64]
Hungary	8.8–24.3	[55, 56]
Italy	10.5	[65]
Moldova	0.8	[66]
The Netherlands	2.4–11.7	[67-70]
Norway	11	[71]
Poland	0.3	[72]
Romania	5.3–14.6	[67, 73]
Serbia	4.2	[74]
Slovakia	1.1–11.6	[59, 68, 75, 76]
Sweden	6-15	[77, 78]
Switzerland	5.6-6.4	[79, 80]

N. mikurensis in individuals bitten by *I. ricinus* ticks

Several countries in Europe and also China have performed prospective and retrospective studies on the prevalence of *N. mikurensis* DNA in serum/blood samples from tick-bitten individuals (Figure 3).

Table 3. *Prevalence of N. mikurensis DNA in the blood of tick-bitten individuals.*

Country	No. PCR+ / No. blood samples	%	Reference
Austria	11/489	2.3	[81]
China	7/622	1.1	[82]
France	4/934	0.4	[83]
The Netherlands	7/626	1.1	[84]
Norway	7/70	10	[85]
Poland	5/316	1.6	[86]
Sweden	2/102	1.9	[87]

VECTORS, RESERVOIRS AND TRANSMISSION

The tick *I. ricinus* is the most frequent tick species involved in transmission of infection agents to humans in northern Europe. This includes *N. mikurensis*, where it is believed to be the main vector, presumably transmitting the pathogen transstadial, meaning that the pathogen follows the vector between the different life stages [47, 88]. The bacteria have been found in all four stages of *I. ricinus* life cycle although transovarial transmission is reported to not occur [58, 89].

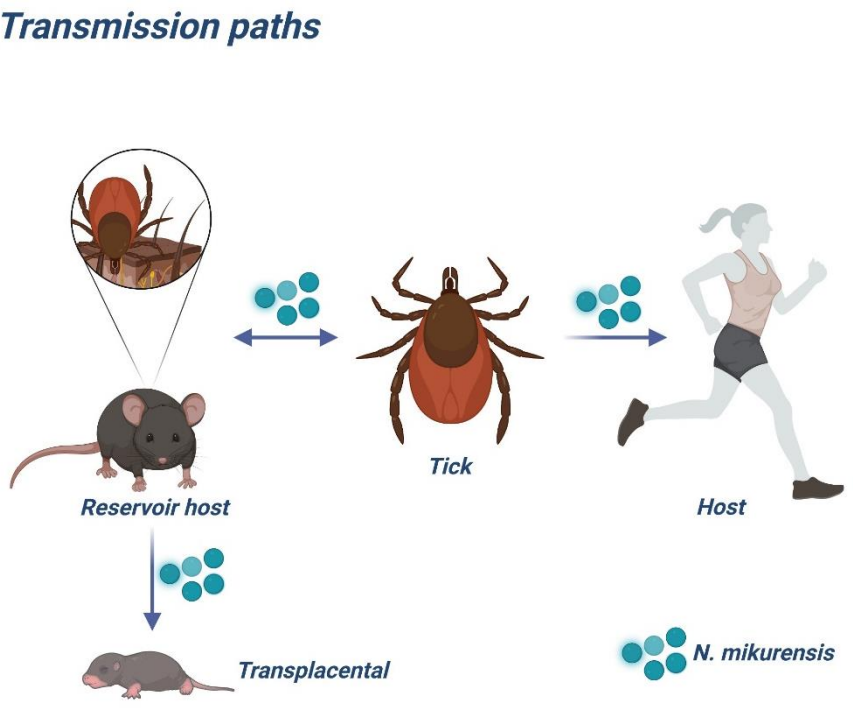

Figure 5. *Possible transmission paths of N. mikurensis between the tick, the reservoir host, and the host. [Created with BioRender.com]*

Wild rodents are reservoirs of the infection, and the prevalence of *N. mikurensis* carriage is known to be higher in ticks collected from wild rodents compared to questing ticks [64]. This makes wild rodents an important part for the survival of *N. mikurensis* particularly if transovarial transmission in ticks does not occur [63]. Wild rodents have been observed to have

transplacental transmission of the pathogen (Figure 5) [64]. The rodents are asymptomatic carriers of the disease and can clear the infection by themselves [77]. Interestingly, the only known animal beside humans that develops symptomatic *N. mikurensis* infection is the dog [90, 91].

The transmission of *N. mikurensis* to humans occurs mainly via tick bites and the bacteria are inoculated through the skin during the tick bite. Many patients with neoehrlichiosis do remember one or several tick bites, but not always, it has been shown that about 50 % of patients with tick-borne diseases don't recall a tick bite [92]. Other family members within the *Anaplasmataceae,* also have blood transfusion as a mode of transmission [93, 94], which now also have been found possible for *N. mikurensis* [95]. In this first article reporting blood transfusion as a potential transmission path of *N. mikurensis* infection, 1006 blood donors in South-East of Sweden was screened for *N. mikurensis* with PCR. The bacteria were found in the blood of 7 donors (0.7 %), nevertheless no transmission to the blood recipients was identified [95]. This is an important subject of future research.

NEOEHRLICHIOSIS IN HUMANS

Humans are beside canines the only animal that becomes sick when infected with *N. mikurensis* [90, 91]. Furthermore, children with the disease have never been found. The first human case described in Sweden is a story that started with a kayaking trip and ended with the finding of a new tick-borne disease. A sporty, elderly man with chronic lymphatic leukaemia (CLL) became ill when kayaking in Sweden. After being hospitalized and treated for suspected sepsis he was discharged with two diagnoses: deep vein thrombosis and pulmonary embolism. The next four months his fever returned repeatedly, and a widened microbiologic diagnostic investigation was initiated together with treatment with a several types of broad-spectrum antibiotics. The man also suffered a transitory ischemic attack while treated with the anticoagulant Warfarin. Eventually, he was diagnosed with the, at that time unheard of, human bacterial pathogen *Ca.* N. mikurensis! This was based on the results of pan-bacterial PCR performed on blood samples with subsequent Sanger sequencing [15]. Since this first case report, human *N. mikurensis* infection has been reported from many European countries together with China (Table 4).

As of today (October 9th, 2023) there are $N = 141$ patients that have been diagnosed with neoehrlichiosis in Sweden (data not published).

Table 4. Countries reporting human cases of infection with N. mikurensis.

Country	Ref.
Austria	[81]
China	[82]
Czech Republic	[96]
Denmark	[97]
France	[83]
Germany	[17]
The Netherlands	[84]
Norway	[98]
Poland	[86]
Slovenia	[99]
Spain	[100]
Sweden	[15]
Switzerland	[16, 101]

CLINICAL FEATURES

The most common clinical features patients describe are high and recurring fever, often along with chills and night sweat [102]. Other commonly reported symptoms are migrating or localized pain in the neck or the joints, weight loss, abdominal pain, cough and diarrhoea [26, 102]. Many patients also present different types of skin rashes such as erythema nodosum or erysipelas skin lesions corresponding to inflamed subcutaneous veins [96]. The most severe symptoms arise from vascular and thromboembolic events [102]. It is not known if these events are caused by damage attributed to infection of the endothelial cells and/or the inflammatory response evoked by the infection. Most common are the venous events causing inflammation of superficial veins, thrombophlebitis, or deep vein thrombosis in legs or arms [15, 26, 103]. Other vascular events are arterial inflammation, arteritis, arterial aneurysms, or transitory ischemic attacks [15, 26, 103].

In a study from our research group, 40 Swedish patients and their vascular events were investigated, it was identified that most of the patients (60 %) developed vascular events ranging from repeated thrombophlebitis, deep vein thrombosis, pulmonary embolism, transitory ischemic attacks, to

arteritis. It was also found that younger age was a risk factor for vascular complications, but there was no difference in the incidence of vascular events between immunosuppressed and immunocompetent patients [1].

Both immunosuppressed and immunocompetent individuals can get neoehrlichiosis, but the clinical picture differs between the two patient groups. Initially it was only reported about cases affecting immune suppressed patients such as patients with haematological, autoimmune, and rheumatic diseases. The last years, reports of immune competent individuals have been increasing, showing both asymptomatic cases and systemic infection with both fever and vascular complications [81-83, 86, 87]. It should be noted that the asymptomatic cases have also been discovered among immunosuppressed patients [86, 104]. Moreover, immunocompromised patients with undiagnosed neoehrlichiosis may have symptomatic disease for several months to years before diagnosis [83, 103, 105]. There have been no reports of deaths among immunocompromised patients with this infection, the only fatal cases that may be attributed to *N. mikurensis* have occurred in immunocompetent individuals [17].

A recent publication has reported the first case of hemophagocytic lymphohistiocytosis (HLH) caused by *N. mikurensis* [106]. HLH is a disorder causing hyperinflammation with deadly outcome in up to 88 % in adults [107]. The HLH syndromes different criteria's were fulfilled by the 67-year-old woman described in the article, she was affected with fever, anaemia, hemophagocytosis (macrophages and dendritic cells phagocyte red blood cells, leukocytes and platelets in the bone marrow and/or tissue), enlarged spleen, high levels of ferritin, triglycerides and interleukin (IL) -2 due to activation of the immune defense [106]. *N. mikurensis* is the second tick-borne pathogen reported to be able to cause hemophagocytosis; Babesia spp. have previously been shown to trigger this condition [20]. It has also lately been suggested by our research group that infection with *N. mikurensis* contributes to the development of malignant B-cell lymphomas [108]. In this publication we discuss the possibility of *N. mikurensis* stimulating B cells over a long time with the possible result of B-cell transformation and the development of malignant B-cell lymphomas.

Laboratory findings in patients with febrile neoehrlichiosis are increased inflammatory markers such as C-reactive protein (CRP), procalcitonin and erythrocyte sedimentation rate (ESR) and elevated white blood cell counts with neutrophilia. Other blood work indicators are lymphopenia, anaemia and hyponatremia. Slightly elevated levels of hepatic transaminases and/or

lactate dehydrogenases can also be an indication of infection [82, 102]. Platelet levels range from normal to modestly or highly increased. This differs from the infections of anaplasmosis and spiroplasmosis where platelet counts are low in comparison [14, 106].

BLOOD VESSEL ENDOTHELIUM

A characteristic of infection with neoehrlichiosis is its common association with vascular events, mainly affecting the venous side of the circulation. In paper II, we report that *N. mikurensis* was found to be cultivatable in two human endothelial cell lines, one derived from primary dermal microvascular endothelial cells and the other one derived from primary pulmonary artery endothelial cells [78].

Although most of the patients diagnosed to date with neoehrlichiosis have been immunosuppressed, none of them have developed arteritis, instead they have almost exclusively been afflicted by venous thromboembolic events such as deep vein thrombosis [1]. In contrast, arthritis and other arterial events have only been reported to affect immunocompetent patients (Figure 6) [1].

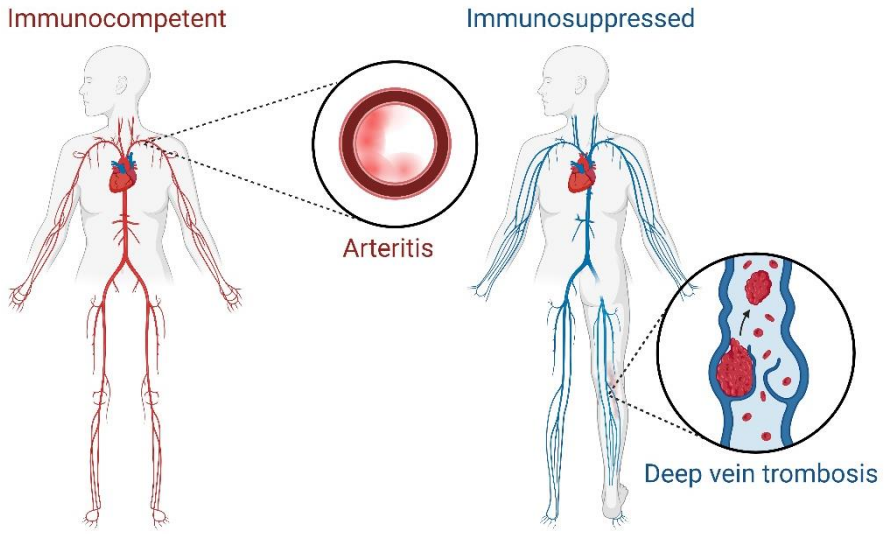

Figure 6. Most common vascular event in respective patient group. *[Created with BioRender.com]*

Since most of the immunosuppressed patients have had compromised B-cell defenses, it might be inferred that B cells and *de novo* synthesis of antibodies are needed for the development of *N. mikurensis* arteritis.

N. mikurensis is not the first *Anaplasmataceae* member to be associated with vasculitis, *E. chaffeensis* has also been associated to cutaneous small-vessel vasculitis in some cases, based on serologic findings. A study ran serology on serum from 55 patients that had been discharged from hospital with the diagnosis of vasculitis and found *E. chaffeensis* antibodies in 5.5 % of the individuals [109]. Other intracellular pathogens that have been connected to different types of vasculitis are *Mycoplasma pneumoniae* (giant cell arteritis) [110] and Hepatitis B virus (polyarteritis nodosa) [111]. Actually, infections are the most common cause of secondary vasculitis [112]. The infectious agent can either affect the vascular wall directly by the inflammatory response that is triggered by damaged endothelium or by the infectious agents stimulating an immune response against blood vessels [112].

Pathogenic microorganisms that can trigger vasculitis by infecting, and causing tissue damage of vascular endothelium are for example *Rickettsia spp* and Cytomegalovirus (CMV) [113]. Another group of microbial agents, that can damage the vasculature is Hepatitis C Virus through the production of cryoglobulins that attack the endothelium [114].

PATHOGENESIS

Paper II suggests that the main target of *N. mikurensis* infection is the vascular endothelium, we demonstrate *N. mikurensis* inside of the so called "circulating endothelial cells", isolated from the blood of neoehrlichiosis patients. The bacteria are also able to propagate inside endothelial cell lines and an electron microscopy picture of a rat's spleen also suggests that *N. mikurensis* is located inside the endothelial cells of the spleen; a caveat is that splenic cells also function as phagocytic cells [29]. It has also been suggested that granulocytes are a target cell of infection, although this is more likely to reflect normal phagocytosis of bacteria by professional phagocytes [96].

Figure 7 illustrates possible inflammation mechanisms in the vessels behind the vascular events associated with *N. mikurensis* infections.

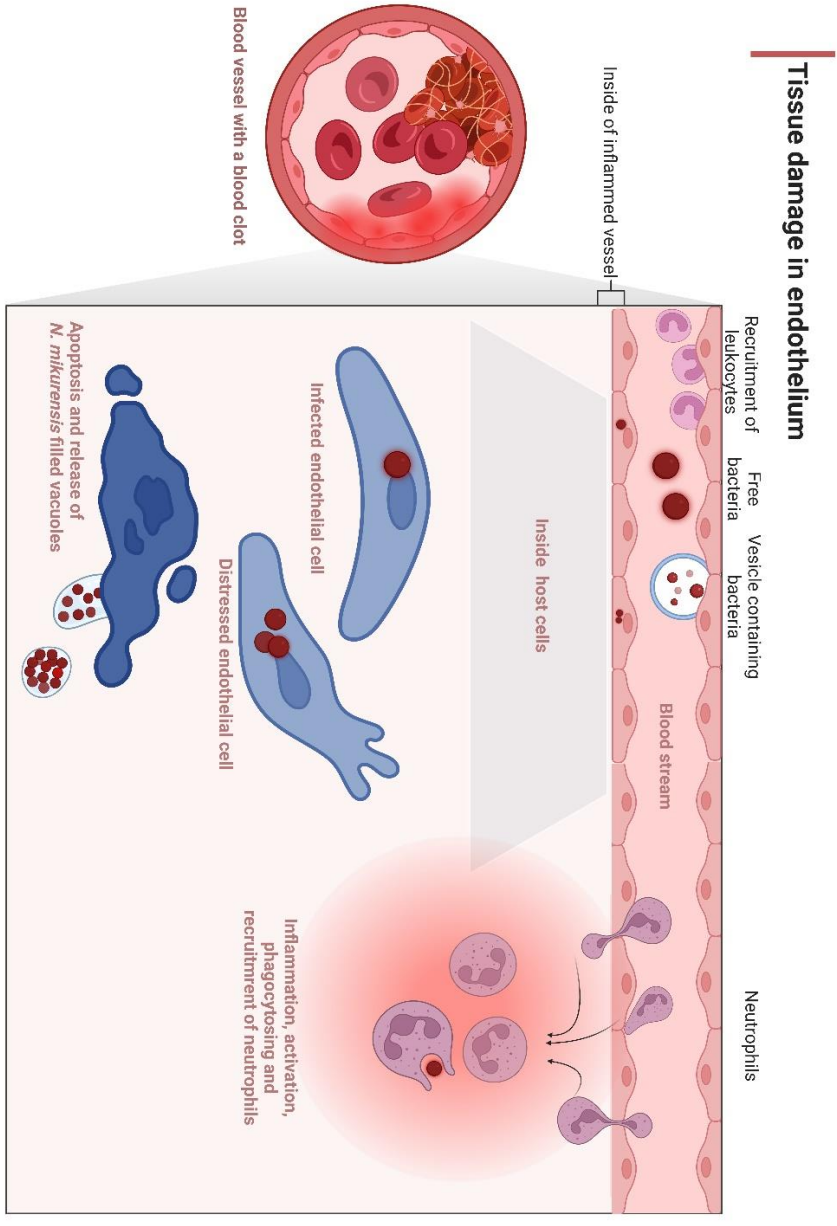

Figure 7. A possible model for tissue damage associated with N. mikurensis infection. *N. mikurensis infects endothelial cells of a blood vessel and causes inflammation and damage by phagocytosis and apoptosis of endothelial cells. Inside the blood vessel there is recruitment and activation of neutrophils as a response to bacteria in the tissue. [Created with BioRender.com]*

PERSISTENT INFECTIONS

Patients are diagnosed with *N. mikurensis* all year around, even during months without tick activity (Figure 8). Since the Clinical Microbiology Laboratory in Gothenburg is the only Swedish laboratory performing *N. mikurensis*-specific PCR to diagnose human cases of infection this data represents all diagnosed Swedish patients. The incubation time is unknown. However, studies have reported time from when patients first had symptoms until time of diagnosis between 26 days and 1.5 years [26, 105]. This wide time range may be more due to delay of diagnosis rather than the time of incubation being very long. The finding of human *N. mikurensis* infections being diagnosed during the winter months when ticks are not active together with several reports of suspected long-time carriage of the infection in asymptomatic immunocompetent individuals led to the hypothesis of *N. mikurensis* being able to cause latent infections [81, 83, 86, 87].

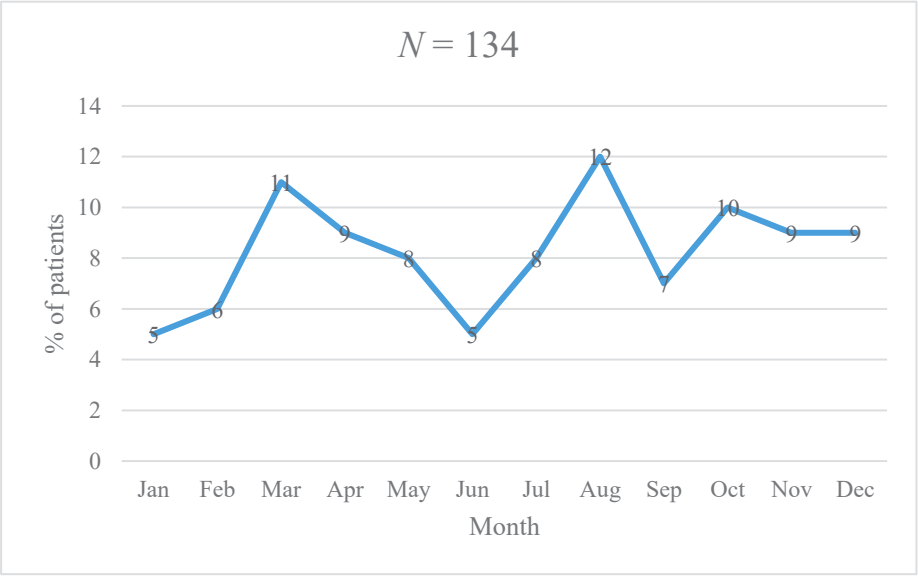

Figure 8. Monthly distribution of N. mikurensis cases diagnosed in Sweden (2009-2023) presented as percentage. *The month corresponds to when the case was diagnosed by PCR (data not published).*

Persistent infections are infections that are not cleared from the host following primary infection but remain associated with specific cells. The infections can be subdivided into three types of infection: Latent, chronic and slow infection (1). These infections may involve phases of both resting and active infection without killing or even causing severe damage to the host

cells [115]. Chronic infections are characterized by the presence of the infectious agent following the primary infection. An example of a chronic infection is hepatitis B where patients get a long-lasting infection that cannot be eradicated [116]. Slow infections on the other hand have a prolonged incubation period followed by progressive disease as its signature, an example of this is acquired immunodeficiency syndrome (AIDS) caused by HIV-1 and HIV-2 [115, 117].

Lastly, latent infections are defined by microorganisms that have entered a resting phase and don't give rise to symptoms or signs of infection [118]. Due to this the latent variant is most likely to be involved in the *N. mikurensis* infection if compared the findings in immunocompetent individuals without clinical neoehrlichiosis features. The most "famous" latent intracellular pathogen must be *Mycobacterium tuberculosis* causing latent tuberculosis. In tuberculosis the bacteria remain latent until the immune defence declines and then cause active tuberculosis [117].

In the *Anaplasmataceae* family, it has been suggested that *A. phagocytophilum* and *A. marginale* use endothelial cells as a residence and are able to avoid host immune effectors within the blood vessels [119]. In that article, the authors speculate about the two pathogens using the endothelial cells for interference with the rolling and transmigration process of granulocytes. Partly by transferring the infection to them and also stop granulocytes from leaving the blood stream and with that escape from phagocytosis [119]. Their usual target cells are neutrophilic granulocytes and erythrocytes, respectively, and endothelial cells could provide a safe zone that allows the pathogens to persist in the host for long periods. The authors also theorize that the longer-lived endothelial cells are a perfect host cell for persistent, latent infection in the case of *A. phagocytophilum* and *A. marginale* since granulocytes only live a few hours, and erythrocytes provides little space for bacteria.

RISK FACTORS

The severe form of neoehrlichiosis is characterized by high fever together with vascular events [1]. The more severe forms of neoehrlichiosis tend to affect splenectomized individuals, patients with malignant or autoimmune clonal B-cell diseases and/or treatment with anti-B cell therapy. Examples of clonal B cell diseases are haematological diseases such as such as CLL, malignant B-cell lymphomas and autoimmune/rheumatic disorders such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and multiple sclerosis (MS) [102]. It is common for these conditions to be treated with the monoclonal antibody rituximab directed against CD20 on B cells, a therapy that has been shown to be a strong risk factor for severe neoehrlichiosis [102]. All B cell stages except for plasma cells express CD20; hence, rituximab affects both the generation of naïve B cells and the levels of memory B cells, which remain low for about 2-6 months after termination of treatment and normalize after 12 months [120]. Although, there is no serology available to diagnose *N. mikurensis* at present, it is likely that reduced levels of specific antibodies explains why rituximab is an important risk factor for neoehrlichiosis. It has been suggested that the spleen contributes to the defense of neoehrlichiosis through production of immunoglobulins, IgM and IgG, putting splenectomized individuals at extra risk [121]. This is believed to be because of the absence of *N. mikurensis*-specific B-cell responses that should have been produced in the spleen [121]. We have though shown that natural IgM antibodies are not essential in the innate part of the immune defense [121].

The fact that clonal B-cell diseases, rituximab and splenectomy are important risk factors implies that the B-cell defense is crucial in the protection against this infectious agent.

Beside underlying diseases and their treatments, high age and recent chemotherapy are additional risk factors for neoehrlichiosis [102].

IMMUNE RESPONSE

Innate immunity

The innate cellular immune responses, performed by neutrophils, macrophages and natural killer (NK) cells are involved in the defense against intracellular pathogens, although innate immune responses often fail to eradicate infections since intracellular bacteria are good at escaping phagocytic cells [122]. A typical feature of neoehrlichiosis is raised levels of neutrophils [26], indicating their contribution to the defense against *N. mikurensis,* probably both by their direct phagocyting characteristics and also by their indirect cleaning of dead cells and bacteria. Otherwise, it is mainly NK cells and macrophages that mediate the innate immune response to intracellular pathogens. The presence of intracellular bacteria in the tissues activates dendritic cells and macrophages to produce the cytokine IL-12, which in turn activates NK cells to produce interferon (IFN)-γ. The cytokine IFN-γ activates macrophages to become even better at phagocytosing and killing bacteria. IFN-γ is the key cytokine in intracellular infections: the production of IFN-γ also initiates the production of "interferon gamma-induced protein 10" (IP-10), also known as CXCL10, which is secreted by several cell types such as such as endothelial cells, fibroblasts, epithelial cells, monocytes, activated neutrophils and T cells. CXCL10 are involved in the chemotaxis of cells such as monocytes, macrophages, dendritic cells, activated T cells and NK-cells towards inflammatory regions [123].

Adaptive cellular immune response

The adaptive cellular immune responses evoked by *N. mikurensis* are not yet elucidated, so the following text about immune responses are speculations based on other intracellular bacteria. Since the innate immune defense rarely succeeds to definitely eradicate infections with intracellular bacteria help is also needed from the adaptive immune defense [122]. Dendritic cell present antigen from bacteria that activates Th 1 cells (T-helper) that produce IFN-γ that activates macrophages to produce tumour necrosis factor (TNF), reactive oxygen species (ROS), and nitric oxide (NO), all toxic to microbes [124]. Th 1 cells are activated CD4+ positive T cells whose IFN-γ production also stimulates the activation of cytotoxic T cells (CTL) that can kill infected host cells by inducing the cells to undergo

programmed cell death or via the release of cytotoxic granules containing lytic proteins that trigger apoptosis [125]. Th 1 and CTL are cardinal features of immunity to intracellular pathogens.

Other important immune responses for defeating intracellular pathogens are T-cell populations that express perforin and CXCL10 [126, 127]. Perforin is a glycoprotein that forms pores in cell membranes of target cells. NK cells and CTL (CD8+) cells are the main source of perforin [127].

Another T-cell population that is important in host defense against persistent intracellular infection and serves as a bridge between the innate and the adaptive immune defense are the gamma delta T cells (γδ T cells). They present antigens to CD4+ T cells on major histocompatibility complex (MHC) class II and are even capable of phagocytosis [128].

As already discussed, it is proposed that B cells are important to fight *N. mikurensis* infections since being splectomized and undergoing treatment with B-cell depleting drugs are risk factors for severe infectious disease. Also already mentioned, the importance of the spleen in the immune defense against *N. mikurensis* probably lies in its capacity to generate or maintain specific antibodies. Since intracellular microorganisms are out of reach for protective circulating antibodies produced by B cells the adaptive humoral immune response is not the first line defense against pathogens hidden inside cells [129]. Even so, it has been shown that *E. chaffeensis* are found both inside and outside of cells during active infection, making them a target for humoral immunity [130]. This is probably the case also for *N. mikurensis*, in Figure 9, B cells are shown with bacteria attached to the surface, showing that circulating bacteria, outside of host cells are targeted by B cells.

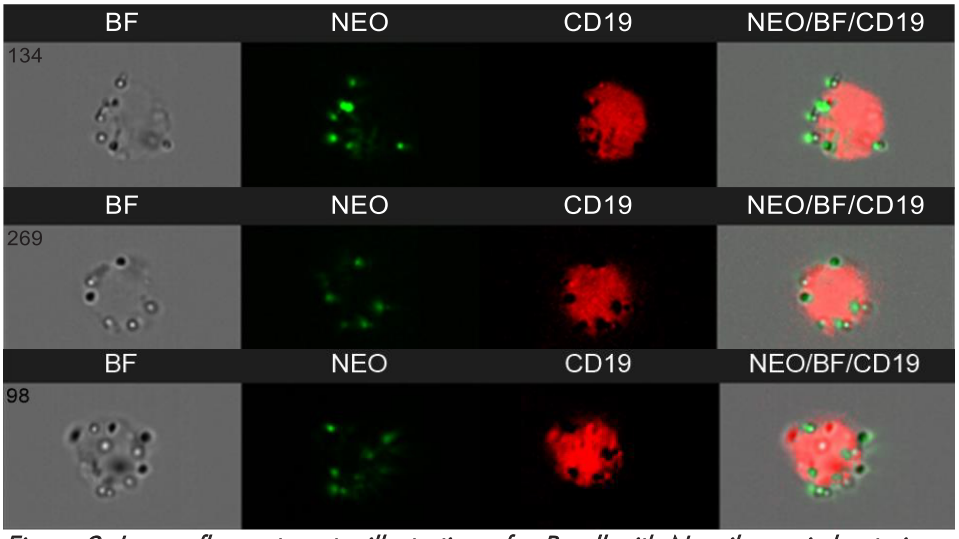

Figure 9. Image flow cytometry illustration of a B cell with N. mikurensis bacteria attached to the surface. *Blood from a B-cell CLL patient treated with rituximab with neoehrlichiosis was labelled using B-cell lymphocyte antigen (CD19) and N. mikurensis-specific DNA probe (NEO). Brightfield (BF) shows the contours of the cell and bacteria. The overlay image merges all pictures. Courtesy of Dr. Grankvist and Ass. Prof. Lingblom, Department of Infectious Diseases, Institution of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden.*

MICROBIOLOGIC DIAGNOSIS

Due to *N. mikurensi's* intracellular nature, it is not detected with routine microbial culture methods based on cell-free media. Only PCR is available as a diagnostic tool. A specific PCR directed against the housekeeping gene *GroEL* or 16S-rRNA of *N. mikurensis* are the most common and widely available [102]. To enable calculation of bacterial burden of *N. mikurensis* in samples a positive control plasmid with a known DNA concentration can be used, and this has shown that immunosuppressed patients have a higher number of bacteria in their blood than immunocompetent patients [26, 80].

N. mikurensis DNA has been recovered in plasma, serum, whole blood, blood culture bottle contents, bone marrow and a skin biopsy but never from cerebrospinal fluid (CSF) [26, 80, 131].

TREATMENT

Recommended treatment is an antibiotic targeting intracellular bacteria, oral doxycycline, 100 mg x 2 for three weeks [26]. This gives effect for most patients, and they improve rapidly. In paper I we report treatment failure of a patient when a too low dose of doxycycline was given. If doxycycline is contraindicated, oral rifampicin 300 mg x 2 for three weeks is recommended instead [87, 102]. Before diagnosis with neoehrlichiosis patients often receive treatment with broad-spectrum antibiotics that may temporarily relieve symptoms but do not clear the infection [26].

AIMS

Overall aim

The aims of this thesis were to explore the tick-borne bacterium *N. mikurensis*, focusing on human infection with regard to pathogenesis and clinical picture of this emerging pathogen.

To do so, the following hypotheses were stated:

- Serological cross-reactivity occurs between *N. mikurensis* and *A. phagocytophilum* and neoehrlichiosis patients can be discovered among the group of patients with suspected anaplasmosis.
- *N. mikurensis*-associated vascular events are due to endothelial cells being the target of infection.
- Immunocompetent and immunosuppressed patients with *N. mikurensis* infection have shared and unique cytokine profiles.
- *N. mikurensis* causes latent infections that reactivate when B-cell defense is suppressed.
- *N. mikurensis* infection trigger T cell responses of the Th1 and CTL types.

Specific aims

The specific aims to test the hypotheses were:

- Develop an *A. phagocytophilum*-specific PCR to determine if patients screened for fever of unknown cause who have antibodies to *A. phagocytophilum* have a current infection with *A. phagocytophilum* and/or *N. mikurensis.*
- To investigate if it is possible to infect tick cell lines with *N. mikurensis* and transfer the infection to human endothelial cell lines, and to develop methods for demonstration of the bacteria inside of cells.
- Perform multi- and single plex cytokine assays using stored plasma and serum samples to compare cytokine profiles in immunosuppressed versus immunocompetent patients with *N. mikurensis* infection.

- To develop T-cell assays specific for *N. mikurensis* inspired by the T cell-based assays used to diagnose latent tuberculosis.
- To determine the incidence of *N. mikurensis* reactivation in patients with malignant B-cell lymphomas treated with anti-CD20 antibody therapy.
- To show that only patients who reactivate a latent *N. mikurensis* infection have T cells specific for *N. mikurensis.*
- To elucidate the types of T cells that are evoked by *N. mikurensis* infection by multiparameter phenotyping of T cells using CyTOF.

MATERIALS AND METHODS

STUDY SUBJECTS AND SAMPLE TYPES

In all papers included in this thesis infected human material (whole blood, plasma, serum, peripheral blood mononuclear cells (PBMC), and buffy coat) have been used, that were collected from patients who tested positive for *N. mikurensis* by real-time PCR (polymerase chain reaction). Their blood components were also analysed with 16S rRNA (ribosomal ribonucleic acid) PCR and sequenced to ensure there were no multiple infections and to confirm the *N. mikurensis* finding. *N. mikurensis*-specific PCR was also performed on ticks (tick haemolymph and tick homogenate). Samples from humans used in the different papers are described in Table 5. Sample types not directly derived from humans like supernatants, cell lines etcetera is instead described under the specific method used for analysis.

Table 5. Study populations in paper I-IV.

Paper	Individual	Sample type	*N*	Analysis
I	NEO+	Serum	18	AP serology (IFA)
I	Queried for AP	Serum	101	AP-PCR, NEO-PCR
II	NEO+	Whole blood	7	Inoculation of cell cultures, IMX
II	NEO+	Buffy coat + plasma	7	Inoculation of cell cultures
II	NEO+	Serum	1	NEO serology
III	NEO+	Plasma + serum	46	Multiplex: Bioplex, Uniplex: ELISA
III	NEO- Age/gender matched Ctrl	Plasma + serum	14	Multiplex: Bioplex, Uniplex: ELISA
IV	Malignant B-cell lymphoma patients	Plasma	97	NEO-PCR
IV	NEO+ Malignant B-cell lymphoma patients	PBMC	8	Flow cytometry, Helios mass cytometry/CyTOF
IV	NEO- Malignant B-cell lymphoma patients, Ctrl	PBMC	8	Flow cytometry, Helios mass cytometry/CyTOF

NEO = *N. mikurensis*, NEO+ = *N. mikurensis* PCR positive sample, NEO- = *N. mikurensis* PCR negative sample, AP = *A. phagocytophilum*, IMX = Imaging flow cytometry, Ctrl = controls, PBMC = peripheral blood mononuclear cells

DIAGNOSTIC METHODS

POLYMERASE CHAIN REACTION (PCR) (PAPER I-IV)

The neoehrlichiosis diagnosis was based on PCR analysis of blood samples in all four studies. PCR is an *in vitro* technique for amplifying a targeted DNA sequence to billions of copies in a short time. A PCR cycle contains three steps: denaturation of the double-stranded DNA by high temperature, the annealing step which serves to cool the reaction, and primers attach to the specific location on the now single-stranded template DNA, and lastly, the extension phase where the temperature again is raised and a new strand of DNA if made by Taq polymerase enzyme. These stages are then usually repeated around 30 times, sometimes as high as 45 times, each time the number of DNA copies is doubled [132]. In real-time PCR, the method used in our studies, the DNA-product is detected by measuring of fluorescence during the PCR cycles. This enables the usage of a threshold (an amount of fluorescence) called the cycle threshold (Ct).

All blood samples from Swedish patients have had their EDTA-blood analysed at the laboratory at the Department of Clinical Microbiology, Sahlgrenska University Hospital, according to the following: plasma from EDTA blood was concentrated by centrifuging at 16000 x g for 2 minutes, DNA was extracted using MagnaPure compact extraction robot (Roche, Basel, Switzerland) according to the manufacturer's protocol with isolation kits (Nucleic acid isolation kit I, Roche), followed by the PCR targeting a 169-bp segment of the house keeping gene *GroEL* used with a TaqMan probe with the fluorescent label fluorescein (FAM) and a synthetic plasmid containing the 169-bp sequence of the GroEL gene cloned into a pUC57 vector (Genescript, Piscataway, NJ, USA). All positive samples were confirmed with 16S-rRNA PCR and sequencing [26].

In paper I an *A. phagocytophilum* PCR was developed. DNA from samples were extracted as above and analysed with two newly developed real-time PCR-methods. One method targeted the Msp2 gene and the other the GroEL gene. A synthetic plasmid that was established was also used with these PCR assays.

CELL CULTURES

TICK CELLS (PAPER II)

For culturing experiments in tick cell lines, two different cell lines were used, ISE-6 derived from an *I. scapularis* tick and IRE/CTVM20 with the *I. ricinus* tick as source. The two cell lines were selected by Dr. Lesley Bell-Sakyi, who is responsible for the tick cell biobank at the University of Liverpool, UK. The ISE-6 tick cell line had been successful in propagating of related bacterial species such as *A. marginale, A. phagocytophilum, E. chaffeensis and E. canis* [119, 133] and IRE/CTVM was a comparatively newly established cell line, so far only used for successful cultivation of *A. phagocytophilum* [134].

Tick cell lines differ a lot from traditional human cell lines, by often having been established from embryonic cells without attempts made to select tissue types for the primary cell culture. This is why tick cell lines generally consist of two or more cell types and never are single cell lines. The mixture of cells are believed to be necessary for the survival of the tick cell line since attempts to clone tick cells have never worked [135].

These cell lines grow in mammalian culture media containing mammalian serum, at incubation temperatures of around 28°C up to 37°C, no CO_2, and only require cell media change once a week. Like ticks, they are also extremely long-lived, surviving for several years with regular medium changes and occasional subcultures. Cells do not need contact with other cells, and most grow in three dimensions as an incomplete monolayer, not adherent, and loose in suspension. They also divide relatively slowly, don't mind living crowded, and not mind being without subculturing, making them especially suited for cultivation of slow-growing bacteria and viruses. Cryopreservation should generally be avoided, as frozen tick cells can be difficult to resuscitate, it is better to let them grow year after year in their culture tube [136]. A possible negative thing working with tick cell lines is the complicated and time-consuming manufacturing of their cell media.

Infection of tick cell lines was monitored with loupe microscope, Giemsa-stained cell smears and *N. mikurensis*-specific PCR.

When infection was stable in tick cells, *N. mikurensis* was harvested by destroying the cells so bacteria is released by passage of the cell suspension

through a small needle and centrifugation in several steps. The product of this was a bacterial pellet used to infect endothelial cells.

ENDOTHELIAL CELLS (PAPER II)

For the experiments with endothelial cell lines, two types were used: a primary dermal microvascular, HDMVECn (human dermal microvascular endothelial cells, neonatal), line (ATCC® PCS-110-010) and a primary pulmonary artery, HPAEC (human pulmonary artery endothelial cells), line (ATCC® PCS-100-022) (ATCC®, Manassas, VA, USA). The choice of cell line types was done based on the finding of neoehrlichiosis patients with vascular events encompassing the large arteries of the lungs and thrombophlebitis of the smaller vessels in the skin. Both cell lines were adherent and formed a single cell layer on the surface of culture bottles. The cells received regular medium changes every third day with subculturing approximately once a week.

When the human cell cultures had been infected with *N. mikurensis* from tick cell lines, the medium changes were done more seldom and further apart to keep the bacterial load as high as possible for a longer period. Cells from the culture were instead harvested and analysed just when the cell cultures had reached a level of stress caused by the infection resulting in pH-change and loose dead cells in the medium.

CELL CULTURE MEDIUM (PAPER II)

Tick cell lines

All cell culture media were freshly prepared weekly, stored at 4°C and used within a week. The tick cell line ISE-6 was given the medium L-15B [137] and the IRE/CTVM cells were given a mixture (50/50 %) of L-15 (Leibovitz) and L-15B. The L-15 medium was supplemented with 20 % FBS, 10 % tryptose phosphate broth (TPB), 2 mM l-glutamine and antibiotics penicillin (100 U/mL) and streptomycin (100 μg/mL).

The L-15B medium was prepared by dissolving L-15 powder (Invitrogen, Waltham, MA, USA) in 900 mL distilled water together with 299 mg aspartic acid, 500 mg glutamic acid, 300 mg proline, 299 mg, α-ketoglutaric acid and 1 mL each of "trace mineral stock solution D" and "vitamin stock" (see Table 6). The volume was then brought to 1 L and the medium was sterilized

by filtration (0.22 μm) and stored in 4°C up to four months. The prepared L-15B medium were finally supplemented on the day of use as above for the L-15 medium, with the addition of 0.1 % bovine lipoprotein (MP Biomedicals, Santa Ana, CA, USA) and being pH-corrected to an orange colour with the use of sterile 1N sodium hydroxide.

Table 6. Ingredients for mineral and vitamin stock solutions used in L-15B medium.

Ingredient	Weight (mg/100 mL)
Stock solution A	
Cobaltous chloride hexahydrate	20
Copper sulphate pentahydrate	20
Manganese sulphate	160
Zinc sulphate heptahydrate	200
Stock solution B	
Sodium molybdate	20
Stock solution C	
Sodium selenite pentahydrate	20
Stock solution D	
Glutathione (reduced)	1000
Ascorbic acid	1000
Iron(II) sulphate heptahydrate	50
Stock solution A	1 mL
Stock solution B	1 mL
Stock solution C	1 mL
Vitamin stock solution	
p-aminobenzoic acid	100
Cyanocobalamine	50
d-biotin	10

Endothelial cell lines

For both endothelial cell lines a vascular cell basal medium was used with the addition of a cell growth kit used for microvascular endothelial cells (ATCC® PCS-100-030, ATCC® PCS-110-040). The vascular cell basal medium was a sterile phenol-red free liquid tissue culture medium containing essential- and non-essential amino acids, vitamins, trace minerals and inorganic salts. The growth kit added to the medium contained 0.2 % bovine brain extract, 5 ng/mL vascular endothelial growth factor, 5 ng/mL epidermal growth factor, 5 ng/mL fibroblast growth factor, 15 ng/mL insulin-like growth factor, 10 mM L-glutamine, 0.75 U/mL heparin sulphate, 1 µg/mL hydrocortisone hemisuccinate, 2% FBS and 50 µg/ml ascorbic acid. No antibiotics were used in the endothelial cell medium.

IMMUNE ASSAYS

ENZYME-LINKED IMMUNOSORBENT ASSAY (PAPER III, IV)

Solid phase capture/sandwich enzyme-linked immunosorbent assay (ELISA) was performed on serum- and plasma samples and cell culture supernatants from stimulation experiments (Figure 10). A sandwich ELISA measures the amount of targeted antigen between two layers of antibodies, capture- and detection antibody. One of the antibodies is coated on the surface of the well in a microtitre plate and functions as a capture antibody to better display the antigen to be detected, in this case cytokines antigens. The other antibody, the detection antibody is conjugated to facilitate the detection of the antigen. After the addition of a substrate that induces a colour reaction if any antigen present, the colour intensity can be measured spectrophotometrically as optical density (OD). The amount of antigen in the sample is correlating to the OD-value [138]. The commercial ELISA kits used in the papers were performed as the manufacturer recommended and samples were diluted and analysed in a 96-well half area microplate.

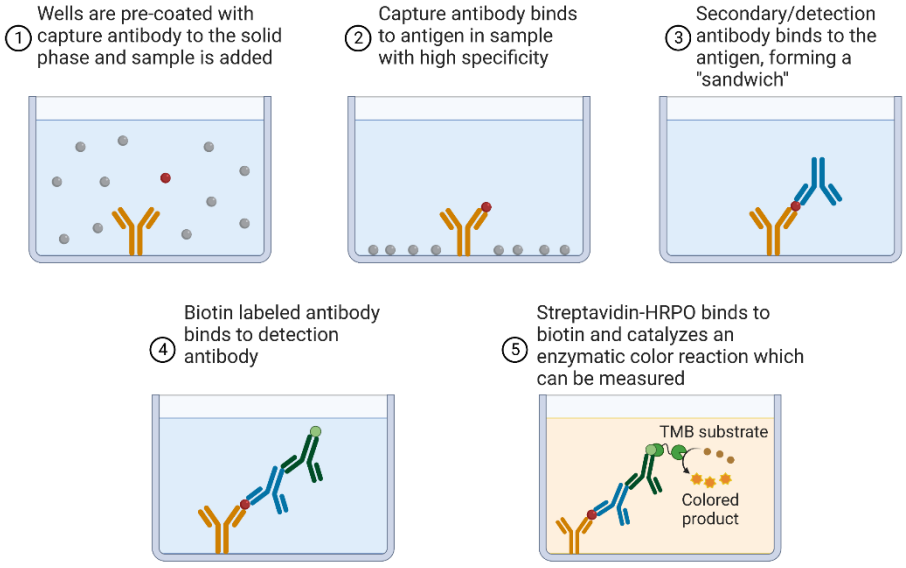

Figure 10. Principle for capture/sandwich ELISA assay. [Created with BioRender.com]

IMMUNOFLUORESCENT ANTIBODY ASSAY (IFA) (PAPER I)

A. phagocytophilum IgG and IgM antibodies were examined using a commercial semiquantitative, direct IFA kit from Focus Diagnostics (Cypress, CA, USA) according to the manufacturer's recommendation. Microscopic glass slides had been coated with a human isolate of *A. phagocytophilum* in HL60 cells, (promyeoloblast cell line) already fixated and permeabilizated. Patients' serum samples were then added to the glass, where antibodies can bind to the *A. phagocytophilum* antigens. In the next step, antibodies against human IgG labelled with a fluorochrome, fluorescein isothiocyanate (FITC), were bound to the patients' antibodies. Antibodies were then detected by fluorescence microscopy. See Figure 11 for a picture from a fluorescent microscope.

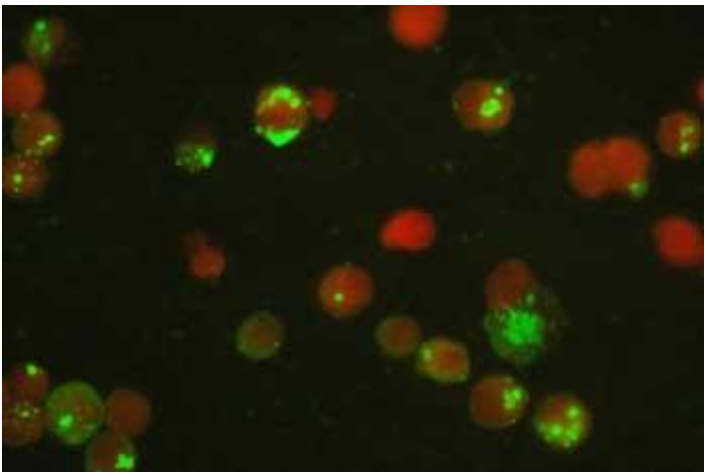

Figure 11. A. phagocytophilum antibodies detected by IFA. *Antibodies binding to intracellularly located bacteria are seen in green. Red indicates nuclei of HL60 cells. [Copyright © 2002 GENTAUR Molecular Products] Used with permission.*

MULTIPLEX CYTOKINE ASSAY (PAPER III)

Luminex technology was used to perform multiplex assays of cytokines in blood samples together with a Bioplex Pro™ human cytokine standard 27-plex panel (Bio-Rad Laboratory, Hercules, CA, USA). Luminex technology is based on magnetic polystyrene beads that are dyed as per colour- code and coated with capture antibodies against the cytokines of interest. Samples and anti-human biotinylated (phycoerythrin (PE)-conjugated streptavidin) detection antibodies specific for the cytokines of interest were added to wells containing the beads where a cytokine-capture antibody-detection antibody complex is formed (Figure 12). The samples were then analysed in a Luminex instrument (Bio-Rad) that contains two lasers, one laser classifies the beads and the corresponding cytokine and one that measures the magnitude of the PE-signal. The signal is proportional to the quantity of bound cytokine [139].

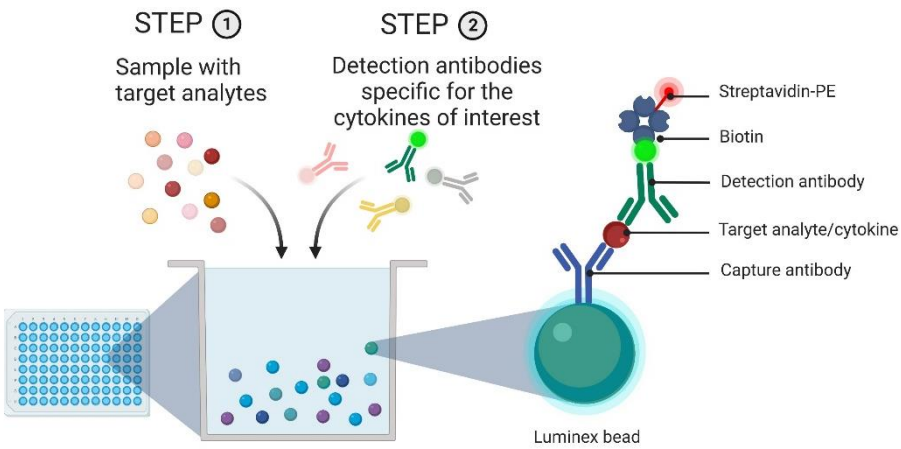

Figure 12. Principle for Luminex technology. [Created with BioRender.com]

FLOW CYTOMETRY (PAPER IV)

Flow cytometry was one method used for analysis of T cells. The principle behind the technique is that lasers in the machine produce both scattered and fluorescent light signals that are read by detectors. The cell populations analysed are stained with fluorescently conjugated antibodies and can therefor then be identified based on their fluorescent or light scattering characteristics. The signals the lasers detect from each cell are converted into electronic signals that are analysed by a computer and written to a standardized format data file [140].

Flow cytometry was used to determine the percentage of T cells that proliferated after stimulation with peptides. This was measured by the usage of CellTrace™ Violet (Invitrogen), a fluorescent dye that works by the principle of labelling cells to trace cell division using dye dilution (as viable cells divide, fluorescence intensity is halved per generation of daughter cells) with flow cytometry [141]. Phytohemagglutinin (PHA), a type of lectin protein, was used as a positive control to recognize if patients T cells were able to proliferate. PHA works by cross-linking the T-cell receptor (TCR) in an antigen-independent way giving rise to polyclonal T-cell activation and proliferation [142].

In paper IV, T cells (PBMC) were first incubated with human FC-block (BD Bioscience, Franklin Lakes, NJ, USA) for 10 minutes before incubation with fluorochrome-conjugated mABs (listed in Table 7) for 20 minutes, in the dark at 4°C. Cells were washed and resuspended in MACS-buffer (Ca^{2+}-free Krebs-Ringer glucose, 2.5 mM EDTA, 0.5 % BSA, pH 7.3) Approximately 10 minutes before analysing samples, 7-AAD (staining dead cells) was added to the cells, which were analysed using FACSCanto II Flow Cytometer with FACSDiva Software 6.0 (BD Biosciences).

The data was analysed using the FlowJo software version 10.7.1 (Three Star, Ashland, OR, USA) and T cells were identified based on low side-scatter and high CD3 levels, CD4+ and CD8+ T cells were then identified among the CD3+ T cells. Proliferation data were expressed as percentage of cells expressing a certain marker.

Table 7. Antibodies used for fluorescence-based analyses of cells.

Antibody target	Cell type	Clone	Isotype	Conjugate	Manufacturer
CD3	PBMC	UCHT-1	Mouse IgG1, κ	FITC	BD Biosciences
CD4	PBMC	SK3	Mouse (BALB/c) IgG1, κ	APC	BD Biosciences
CD8	PBMC	RPA-T8	Mouse IgG1, κ	PE-Cy7	BD Biosciences
CD19	PBMC	SJ25C1	Mouse (BALB/c) IgG1, κ	R718	BD Biosciences
CD38	PBMC	HB7	Mouse IgG1, κ	PE	BD Biosciences

IMAGING FLOW CYTOMERY (PAPER II)

Imaging flow cytometers combine traditional flow cytometry with fluorescence microscopy which gives high-resolution images of every cell directly in flow. This method was used in paper II to visualize *N. mikurensis* inside of different eukaryotic cell types stained with fluorescent probes. Tick cells were harvested, fixed and permeabilized with BD cytofix and cytoperm kit (Becon Dickinson, San Jose, CA, USA) and hybridized with antibacterial DNA-probes as seen in Table 8. All probes were used with a hybridization buffer containing 3.6 M NaCl, 80 mM Tris-HCl at pH 4.7 with 30 % formamide and 5 % SDS. Hybridization of cells was performed overnight at 42°C with a concentration of each probe of 10 ng/μL. Next day, the cells were washed two times, once with pre-heated hybridization buffer without formamide and SDS (sodium dodecyl sulfate) and once with room-tempered PBS. DRAQ5 (1 μM) was added 10 minutes before analyses to stain the nuclei of eukaryotic cells.

Endothelial cells from cultures were harvested by flushing with EDTA (0.5 mM) and 2 % trypsin, hybridized as described for the tick cells, but using other DNA probes, seen in Table 8. For the experiments with endothelial

cells and immune sera, a FITC-labelled antibody was used instead of DNA-probes. Circulating endothelial cells isolated from EDTA-anticoagulated blood samples derived from two neoehrlichiosis patients were stained with both monoclonal antibodies and DNA probes but beside that analysed as described above.

Analyses were performed using an ImageStream X Mk II imaging flow cytometer (Amnis, Seattle, WA, USA) and IDEAS software v. 6.0, where 1000 cells were collected from each sample and a custom-made data analysis strategy was developed to determine the proportion of infected cells in the cell suspension. This analysis strategy was performed by creating a mask around cells cytosol zone. Next, the area of were the *N. mikurensis*-specific DNA probe had bound was divided by the cytosol zone and multiplied by 100. The values were then presented as percent of infected cells per 1000 cells.

Table 8. Antibodies and DNA probes used for fluorescence-based analyses of cells infected with N. mikurensis.

Antibody target	Cell type	Clone	Isotype	Conjugate	Manufacturer
CD146	EC	541-10B2	Mouse IgG1, κ	PE	Miltenyi Biotech
CD146	EC, CEC	541-10B2	Mouse IgG1, κ	APC	Miltenyi Biotech
IgG	EC	-	Goat IgG1	FITC	Focus Diagnostics
vWF	EC, CEC	3E2D10 + VWF635	Mouse IgG1, κ	AF405	R&D Systems
Probes	**Cell type**			**Conjugate**	**Manufacturer**
EUB338	TC, EC			AF488	Eurofins Genomics
Neo-spec	TC, EC			AF555	Eurofins Genomics
Non-EUB	TC, EC			AF488	Eurofins Genomics
Non-Neo	TC, EC			AF555	Eurofins Genomics
DRAQ5	TC, EC, CEC				Affymetrix eBioscience
DAPI	EC, CEC				Thermo Fisher Scientific

TC = tick cells, EC = endothelial cells, CEC = circulating endothelial cells

CYTOMETRY BY TIME-OF-FLIGHT (PAPER IV)

Phenotyping and in-depth analyses of T cells was performed with cytometry by time-of-flight (CyTOF) of PBMC samples that had been stimulated with *N. mikurensis*-specific peptides. This method uses time-of-flight mass spectrometry together with metal-labelled antibodies. CyTOF enables deep cell studies such as: examination of cellular phenotypes, function, and cell signalling with the ability of staining with more than 40 markers. One big advantage of CyTOF is that no fluorescent overlap or autofluorescence is affecting the data as in regular flow cytometry. Instead metal contamination of samples is something to consider during laboratory work, therefor all reagents need to be metal-free [140]. The analysis principle of this method is described in Figure 13.

Staining protocol

Cells were washed with conventional PBS and Maxpar PBS (Fluidigm, South San Francisco, CA, USA) and stained with Cell-ID® Cisplatin (Fluidigm), which binds to dead cells, for 5 minutes at room temperature (RT). This was followed by two washes with Maxpar cell staining buffer (CSB, Fluidigm) and staining the cells with a panel of antibodies directed against cell surface markers (Table 9) for 30 min, RT.

The cells were washed again with CSB and then fixated with a 1.6 % formaldehyde solution, 10 min, RT. The supernatant was then discarded after centrifugation and cells were resuspended in fixation and permeabilization buffer eBioscience Foxp3/Transcription Factor Staining buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h, RT. Samples were further washed with CSB and permeabilization buffer (included in eBioscience kit above) and incubated with the antibody cocktail directed against intracellular markers (Table 9) for 1.5 h, RT and washed with permeabilization buffer and CSB.

Next the samples were incubated with intercalation solution (Maxpar Fix and Perm Buffer mixed with Cell-ID® Intercalator-Ir, Fluidigm), for 45 min, RT. This step has the function of discrimination single cells from doublets. After this the cells were centrifuged and resuspended in about 100 μL residual volume and stored at -80°C until samples were analysed using the CyTOF instrument Helios (Fluidigm).

On the day of analysis, cells were thawed by holding the tube in your hand for 1 minute followed by washing cells with CSB one time and in Maxpar PBS one time. Prior to Helios analysis, cells were resuspended in Maxpar cell acquisition solution (CAS, Fluidigm) together with 0.1X EQ Four Element Calibration Beads (Fluidigm).

The Helios instrument was used together with CyTOF Software v7.0. (Fluidigm) and gating of cells was performed using FlowJo software (Three Star).

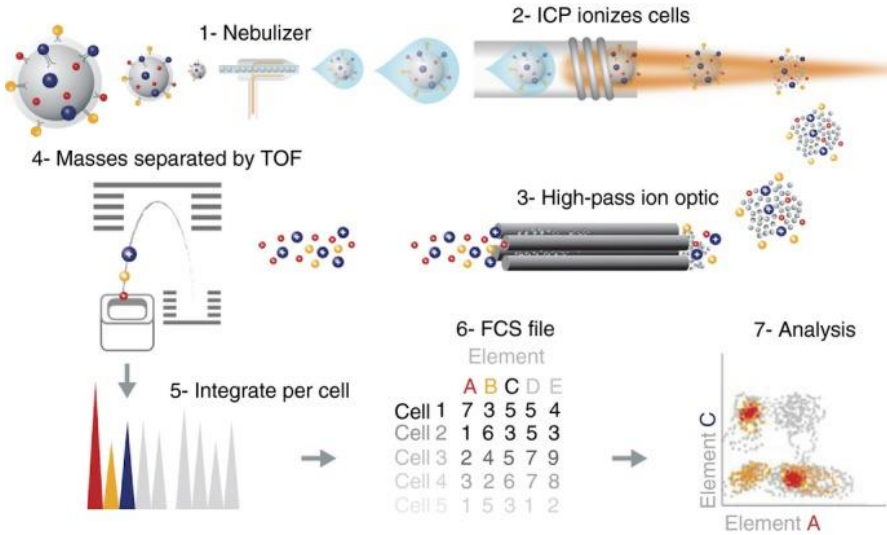

Figure 13. Schematic representation of basic principles of cytometry by time-of-flight (CyTOF). *(1) Cells in single cell suspension labelled with a panel of heavy-metal-conjugated probes is run through a nebulizer which aerosolizes the cells. (2) Sample runs through the heated spray chamber where all water is removed so it can be ionized in the plasma. (3) The now aerosol droplets from the cell exit the spray chamber and moves on to the inductively coupled plasma (ICP) source where they are vaporized, atomized, ionized and overly abundant ions are removed. (4) In the plasma step the mass analysis is performed by separating masses after time of flight (TOF) based on their mass-to-charge ratio. (5) The ion clouds leave the plasma at 13-microsecond pushes (pulses), lightest probes reach the detector first, followed by heavier masses reaching the detector later. (6) Signals corresponding to each metal label are correlated to the different original cells. (7) Samples are analysed using conventional cytometry software programs. [Reprinted with permission from Immunology, David, B.A., et al, Isolation and high-dimensional phenotyping of gastrointestinal immune cells. Copyright (2017), with permission from John Wiley and Sons]*

Table 9. Antibodies used for CyTOF experiments.

Specificity	Metal label	Clone	Source
CD45	Y89	HI30	AH Diagnostics
CD107a	Cd106	H4A3	AH Diagnostics
CD69	Cd113	FN50	AH Diagnostics
IFN-γ*	Cd116	B27	AH Diagnostics
CD196/CCR6	Pr141	G034E3	AH Diagnostics
CD19	Nd142	HIB19	AH Diagnostics
CD5	Nd143	UCHT2	AH Diagnostics
CD31	Nd144	WM59	AH Diagnostics
CD4	Nd145	RPA-T4	AH Diagnostics
CD8	Nd146	RPA-T8	AH Diagnostics
CD20	Sm147	2H7	AH Diagnostics
CD274/PD-L1	Nd148	29E.2A3	AH Diagnostics
CD25	Sm149	2A3	AH Diagnostics
CD134/OX40	Nd150	ACT35	AH Diagnostics
CD123/IL-3R	Eu151	6H6	AH Diagnostics
TCRγδ	Sm152	11F2	AH Diagnostics
CD185/CXCR5	Eu153	RF8B2	AH Diagnostics
CD3	Sm154	UCHT1	AH Diagnostics
CD45RA	Gd155	HI100	AH Diagnostics
CD14	Gd156	HCD14	AH Diagnostics
CD194/CCR4	Gd158	L291H4	AH Diagnostics
CD161	Tb159	HP-3G10	AH Diagnostics
CD28	Gd160	CD28.2	AH Diagnostics
Ki-67*	Dy161	B56	AH Diagnostics
CD66b	Dy162	80H3	AH Diagnostics
CD183/CXCR3	Dy163	G025H7	AH Diagnostics
CD45RO	Dy164	UCHL1	AH Diagnostics
CD279/PD-1	Ho165	EH12.2H7	AH Diagnostics
CD197/CCR7	Er167	G043H7	AH Diagnostics

CD127/IL-7Ra	Er168	A019D5	AH Diagnostics
CD278/ICOS	Tm169	C398.4A	AH Diagnostics
CD152/CTLA-4*	Er170	14D3	AH Diagnostics
CD38	Yb172	HIT2	AH Diagnostics
HLA-DR	Tb173	L243	AH Diagnostics
CXCL10*	Yb174	J034D6	In-house
Perforin*	Lu175	B-D48	AH Diagnostics
CD56	Yb176	NCAM16.2	AH Diagnostics
Cell-ID Cisplatin	Pt195	-	AH Diagnostics
CD16	Bi209	3G8	AH Diagnostics
DNA1	191Ir	-	AH Diagnostics
DNA2	193Ir	-	AH Diagnostics

*Intracellular markers

T-CELL RESPONSES *IN VITRO* (PAPER IV)

Antigen-specific stimulation of T cells within PBMC was employed to detect T cells specific for *N. mikurensis*. This was used in paper IV where PBMC from the study patients were cultured with *N. mikurensis* peptides used as antigens. PBMC, containing T cells from individuals previously exposed to an antigen proliferate *in vitro* when stimulated with the specific antigen [142]. Peptides bind to MHC molecules that bind to the TCR on the T cells to activate them [143]. T-cell activation results in cytokine production, cytokine receptor expression, and proliferation of the activated T cells, all of which can be measured. A common T-cell expressed cytokine of interest in immune responses is IFN-γ, which activates macrophages to kill intracellular pathogens. The main producers of IFN-γ are CD4+, CD8+ T cells and NK cells [117].

PEPTIDES (PAPER IV)

To stimulate the T cells of *N. mikurensis*-infected individuals, a pool of recombinant peptides (Peptides & Elephants, Hennigsdorf, Germany) believed to be specific for *N. mikurensis* was used. The 41 peptides originated from a so far unnamed outer membrane protein found in reference genome of *N. mikurensis* from three Swedish patients [144]. The

protein which the peptides originate from has the size of 19 kDa and belongs to the P44/Msp2 family of proteins which is a known inducer of immune response in related bacteria such as *A. phagocytophilum* and *Wolbachia pipientis* [145, 146]. The peptides were 15 amino acid long, which is documented to be stimulatory for both CD8+ and CD4+ T cells [143].

IN VITRO T CELL STIMULATION (PAPER IV)

For the cell stimulation experiments described in paper IV PBMC had been frozen upon arrival and placed at -140°C until analysis. On the day of the experiment, PBMCs were thawed in a 37°C water bath, allowed to just thaw before being mixed with pre-heated X-vivo medium containing gentamicin (Lonza, Basel, Schweiz) with nuclease added dropwise. Cells were then washed to remove all components from the freeze medium and resuspended in PBS, since cells were to be stained with CellTrace™ Violet (Invitrogen) that binds to cellular proteins, including amines in medium components. Cells were stained with CellTrace™ Violet for 20 minutes, kept in the dark at room temperature, diluted with some more medium, incubated again for 5 minutes and then centrifuged and let to rest for 10 minutes before seeding them in a 96-well culture plate. PBMC were then mixed with *N. mikurensis* peptides, cell media as a negative control and PHA as positive control. During the development of the method other stimuli were tested for activating *N. mikurensis* immune responses, such as *N. mikurensis* PCR-positive plasma samples from patients with low PCR CT-values or cell suspensions, as well as supernatants derived from tick cell lines and endothelial cell lines infected with *N. mikurensis*.

Cells were then incubated at 37°C and 5 % CO_2 and harvested after five days, cell suspension supernatants were saved for later cytokine analysis with ELISA and the cells were ready for the flow cytometry protocol with antibody-staining.

STATISTICS

UNIVARIATE ANALYSIS (PAPER I, III)

For comparison of two groups, the Wilcoxon matched-pairs signed rank test (paired groups) and Mann-Whitney *U* test (unpaired groups) were used. Kruskal-Wallis test was used to compare groups of three. *P*-values ≤ 0.05 were considered significantly significant. The GraphPad Prism software (version 8.4.2 and 9.0.2, GraphPad, San Diego, CA, USA) was used for statistical calculations.

MULTIVARIATE ANALYSES (PAPER III)

The multivariate method, "Orthogonal projection to latent structures by means of partial least squares-discriminant analysis" (OPLS-DA) is a pattern recognition method using the relationship between a Y-variable and multiple X-variables [147]. In paper III, the outcome variable Y was set to "immune status" of the study patients and X-variables were the levels of cytokines.

The method was employed using the SIMCA statistical package version 15.0.2 (MKS Data Analytics Solutions, Malmö, Sweden).

CLUSTER ANALYSIS (PAPER IV)

The clustering algorithm X-shift of the VorteX software was used to identify cell subpopulations in T cell populations identified by CyTOF in paper IV. Gated CD3+ cells sorted with FlowJo (Three Star) were analysed with cluster analysis. The software principle is based on weighted k-nearest-neighbour density calculation [148]. The density estimated for each data point is evaluated and the density maximum in the nearest-neighbour graph is identified and converted to clusters. The results can then be represented as minimum spanning trees, module maps or as single cells contributing to the specific cluster [149, 150].

RESULTS AND DISCUSSION

PAPER I

In paper I, samples from 18 patients diagnosed with neoehrlichiosis were investigated with serological assay against *A. phagocytophilum* to evaluate if they had cross-reactive antibodies. We also analysed 101 samples from suspected anaplasmosis patients with two separate PCR:s targeting *N. mikurensis* and *A. phagocytophilum*, respectively.

A. phagocytophilum antibodies detected among patients diagnosed with neoehrlichiosis

When investigating the presence of *A. phagocytophilum* antibodies in serum samples from immunosuppressed patients diagnosed with neoehrlichiosis, four of them (22 %) had measurable levels of serum antibodies to *A. phagocytophilum* as determined by using a commercial IFA based on *A. phagocytophilum*-infected HL60 cells. Three of them were immunosuppressed and the only examined immunocompetent individual was also positive; all four patients had relatively low titers (1/80-1/320).

Neoehrlichiosis patients found among patients with suspected anaplasmosis

The PCR targeting *A. phagocytophilum* gave no positive findings in the study population of the 101 samples from suspected anaplasmosis patients. Despite this, two new individuals positive for *N. mikurensis* in their blood were found in the group of patients with suspected anaplasmosis when performing *N. mikurensis*-specific PCR analysis of stored serum samples.

Discussion

The findings of this study indicate that Swedish patients with fever of uncertain origin should be queried for *N. mikurensis* infection by PCR. Moreover, clinical laboratories should recommend testing for *N. mikurensis* in addition to *A. phagocytophilum* for such patients, and be aware of the possibility that a positive *A. phagocytophilum* antibody titer could indicate an underlying *N. mikurensis* infection. In view of our inability to detect *A. phagocytophilum* DNA in samples testing positive for *A. phagocytophilum*

antibodies, this raises the question if there are more cases of neoehrlichiosis than anaplasmosis in Sweden.

The two infections resemble each other regarding the clinical picture, e.g., fever, nightly sweats and myalgia but differ with respect to the vascular events associated with neoehrlichiosis but not anaplasmosis. Furthermore, thrombocytopenia is commonly seen in anaplasmosis, but more rarely in neoehrlichiosis.

The prevalence of *A. phagocytophilum* in field-collected ticks is estimated to be 1.3-15 % in Sweden depending on the geographical area [151] and the seroprevalence in Sweden is between 8-21 % [152-155]. Despite the high prevalence of *A. phagocytophilum* in the environment there are very few reports of human anaplasmosis in Sweden [18]. However, since the PCR described in paper I was established in 2019 at the Clinical Microbiology Laboratory at Sahlgrenska University Hospital in Gothenburg, Sweden, four PCR-positive patients have been detected (Oct 2023, data not published). This indicates that the European strains of *A. phagocytophilum* perhaps isn't that virulent, not causing severe infections in humans. The PCR method is a useful complement to antibody detection, serology, with the use of an IFA to prove active infection.

The findings of positive *A. phagocytophilum* serology within the neoehrlichiosis group could imply that patients with neoehrlichiosis also have been exposed to *A. phagocytophilum and/*or antibody cross-reactivity due to shared antigenic epitopes. The two pathogens belong to the same family of *Anaplasmataceae*. However, since paper I was published, we have succeeded to whole-genome sequence *N. mikurensis* and discovered a more pronounce difference between *N. mikurensis* and *A. phagocytophilum* regarding their protein profiles than what was believed earlier. The genome article showed that 109 proteins were shared by *N. mikurensis* and *A. phagocytophilum,* as well as with the other *Anaplasmataceae* family members*,* but no proteins were shared solely between *N. mikurensis* and *A. phagocytophilum*; the latter supports their more distant relatedness compared with other species in the family of *Anaplasmataceae* [144].

A possible bias with the study was that the *A. phagocytophilum* DNA analysis was performed on serum- and plasma samples. Since the host cells for *A. phagocytophilum* are the neutrophilic granulocytes, whole blood would

have been the optimal blood sample type. This could have led to the loss of sensitivity for the assay.

PAPER II

In paper II, we wanted to 1) cultivate *N. mikurensis*, and 2) identify the target cells of *N. mikurensis* infection. Since many neoehrlichiosis patients had presented with vascular events, a tropism of *N. mikurensis* for vascular endothelium was hypothesised.

N. mikurensis cultivated in tick cells and human endothelial cells

The strategy to first attempt cultivation of infected blood derived from neoehrlichiosis patients using two different tick cell lines and then transferring the infection to two different primary human endothelial cell lines as successful. We found that small coccoid bacteria labelled either with a DNA probe or immune serum localized to cytoplasmic inclusions close to the cell nucleus both in endothelial cells and in tick cells. This was very similar to the infection pattern of *N. mikurensis´* closest relative, *N. lotoris* [38].

N. mikurensis detected in circulating endothelial cells isolated from patients diagnosed with neoehrlichiosis

After we managed to show that *N. mikurensis* can grow and propagate in primary human endothelial cell lines, we attempted to perform *in vivo*-like detection of the bacteria in endothelial cells isolated from patient blood of patients with active neoehrlichiosis. This was achieved by double labelling of circulating endothelial cells and *N. mikurensis*. Circulating endothelial cells are rare large cells in the blood that have shed from the blood vessel wall. The infected circulating endothelial cells appeared damaged and to be affected by the infection.

Discussion

The highlight of this study was to finally manage to cultivate *N. mikurensis* and to identify vascular endothelium as a target of *N. mikurensis* infection. We also visualized the bacteria inside of cells.

Regarding the morphology and ultrastructure of *N. mikurensis* in mammalian cells, it has been presumedly been shown in endothelial cells in the spleen of rodents [29] and human granulocytes [96]. Interestingly, the

pictures from neutrophils and salivary glands shows individual bacteria, in contrast, in the tick cells and endothelial cells we describe in paper II, both patterns of only a few rounded bacteria-like structures to more tightly, dense packed inclusions or so called microcolonies [29, 48, 96].

Many attempts of cultivation had been performed before this first successful one, using different endothelial cell lines, leukocytes, and *in vivo* experiments in rats. *N. mikurensis* infection was in these attempts introduced as cell suspension from the spleen of infected voles, tick homogenate or haemolymph (tick blood) of *N. mikurensis* PCR-positive ticks and blood components from naturally infected humans.

The strength of this publication is the usage of *N. mikurensis*-specific DNA probes to label the bacteria since earlier studies have been morphologic studies with no attempts at specific labelling of the bacteria. Beside the electron microscopy picture suggesting the presence of *N. mikurensis* in endothelial cells in the spleen of a rat [29], only granulocytes have been suggested as target cell [96]. The human neutrophilic granulocytes isolated from a patient is an intriguing finding but since it has never been reproduced probably shows a phagocytosed bacteria rather than the bacteria inside of its host cell.

Now that we know how to cultivate *N. mikurensis*, we will have access to bacterial antigens, which should facilitate future work to increase the understanding of *N. mikurensis* pathogenesis and for the development of a serological methods.

These results, considering endothelial cells being relatively long-lived, made us wonder if the infection could be present in the cells for a longer time causing latent infection [156]. This was examined in paper IV.

PAPER III

Considering the difference in how neoehrlichiosis disease expresses itself between immunocompromised and immunosuppressed individuals, the question rose if cytokine responses evoked by *N. mikurensis* infection differed depending on immune status. Our research group had observed that the patient group with underlying diseases causing immunosuppression had more severe neoehrlichiosis infection than the immune competent group.

Cytokine analyses were performed on 30 stored blood samples from immunosuppressed patients and 16 blood samples from the immunocompetent group, all infected with *N. mikurensis*. All samples were analysed with both a multiplex cytokine assay and single plex ELISA cytokine assays. Positive findings from multiplex 27-plex cytokine analysis were confirmed with single plex ELISA for each cytokine.

Neoehrlichiosis patients with underlying immunosuppressive diseases had an increased production of cytokines involved in the B cell-mediated immune defense.

The immunosuppressed group had higher cytokine concentrations of CXCL13 (B-cell attracting chemokine 1) and B-cell activating factor (BAFF) than the immunocompetent group. CXCL10 was also higher in the immunosuppressed group.

Both groups of patients had elevated levels of IFN-γ and of cytokines associated with blood vessel growth

For all patients with neoehrlichiosis, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF2) was increased in comparison with the healthy control group. IFN-γ was also slightly elevated in serum samples from patients with neoehrlichiosis.

Discussion

Many of the patients in the immunosuppressed group had underlying treatment with B-lymphocyte-depleting agent rituximab (67 %), a monoclonal antibody directed against CD20, which is expressed on the

surface of most maturational stages of B cells. This gave expected results regarding the B-cell associated cytokines CXCL13 and BAFF being overly produced to compensate for the loss of B cells [157, 158].

In a perfect world, it would have been desirable that all patients had the exact same blood sampling conditions. Blood samples travel different periods of time and are stored at different temperatures before arrival at the diagnostic laboratory. This could have led to cytokine leakage from cells in the blood after blood sampling.

An important learning this study gave was the importance of verifying results from so called multiplex analyses, where many targets are analysed at the same time. On the other hand, the multiplex method is a much-appreciated method for screening and especially in this case where little was known beforehand. This together with the multivariate analysis performed in the article gave the opportunity to compare immunosuppressed with immunocompetent neoehrlichiosis patients and their cytokine patterns.

The novel finding of CXCL10 being strongly associated with the immunosuppressed group of neoehrlichiosis patients in this paper led us to study it more closely in paper IV, together with IFN-γ. For IFN-γ, a trend was noted in this study with slightly increased levels in the group of neoehrlichiosis patients, but it didn't reach statistical significance when compared with healthy controls.

IFN-γ is an important mediator of many antimicrobial effector mechanisms, with the activation of the bactericidal function of macrophages as the most prominent one. It is substantial in the early phase of infections with intracellular microorganisms activating defense mechanisms in both "infected" phagocytic and non-phagocytic cells [124]. The cytokine IFN-γ has also been reported to inhibit the infection of closely related bacteria *E. chaffeensis* [159]. *E. ruminantium*, a bacterial species closely related to *N. mikurensis* that gives rise to severe infections in ruminants, has tropism for endothelial cells just like *N. mikurensis*. IFN-γ is a powerful inhibitor of both *E. ruminantium* infection *in vivo* and *ex vivo* infection of endothelial cells [160]. The IFN-γ induced chemokine CXCL10 is able to directly inactivate bacteria through disruption of the microbial cell membrane and metabolic interference, mimicking the function of anti-microbial defensins [161].

PAPER IV

The last paper, IV, is a study performed on a cohort (N = 97) of B-cell lymphoma patients that were scheduled to start treatment with B-lymphocyte-depleting agent rituximab. Patients were monitored for the presence of *N. mikurensis* before, during and after finished treatment with rituximab. All patients also had their PBMC frozen from blood samples collected before the first rituximab dose. All *N. mikurensis*-positive lymphoma patients found in the study (N = 8) had their PBMC samples analysed with extended T cell analysis. T cells were stimulated with *N. mikurensis*-specific peptides, proliferation and upregulation of proteins were then measured.

The study was initiated to examine the hypothesis of neoehrlichiosis being a latent infection based on the frequent reports of healthy *N. mikurensis* carriers and unexpectedly common cases of *N. mikurensis* infection diagnosed during the winter months in Sweden.

Lymphoma patients with latent infection with *N. mikurensis* that reactivated after treatment with rituximab

Apparent reactivation of a latent *N. mikurensis* infection disease was identified in four patients (4 %). Unexpectedly, an additional four patients (4 %) carrying asymptomatic *N. mikurensis* infection were identified before starting medication with rituximab. In all eight individuals an *N. mikurensis*-specific T-cell response was observed with proliferation of CD3+ T cells and an upregulation of CXCL10 and IFN-γ expression by T cells in response to stimulation with bacterial peptides. We also discovered that all *N. mikurensis*-specific T cells expressed the cytotoxic protein perforin.

Identified subpopulations of T cells among the study group of lymphoma patients with latent *N. mikurensis* infection

Several subgroups of T cells in the *N. mikurensis* population group were identified in this study. Subgroups were, again, perforin- expressing CD4+ Th 1 cells and effector memory (EM) T cells, CD8+ EM, central memory (CM) and terminal effector (TE) T cells. In addition, subpopulations of γδ T cells were associated with the latent neoehrlichiosis patients.

Discussion

The T cell subpopulations that we identified are the types of T cells that are important in the defense against intracellular infections: CD4+ Th 1 cells contribute by enhancing the bactericidal capacity of macrophages via the secretion of IFN-γ, and the CD8+ cytotoxic T cells which have the ability to kill bacteria after being activated by CD4+ Th 1 cells [118]. With respect to another intracellular bacterial infection, tuberculosis, T cells have been suggested to control the *M. tuberculosis* infection and that the Th 1 cells are especially important for protection [117].

Based on the cytokine findings from paper III, we chose to manufacture an in-house metal-conjugated antibody targeted against CXCL10 for multiparameter CyTOF analysis of *N. mikurensis*-specific T cells. Consistent with our findings from paper III, there was increased expression of both IFN-γ and CXCL10 by T cells derived from the immunosuppressed lymphoma patient group with *N. mikurensis* infection.

An important aspect of this study was the matched control group of lymphoma patients, where we were successful in matching *N. mikurensis*-positive patients with *N. mikurensis*-negative lymphoma patients with respect to lymphoma type, sex and age were matched to a great extent. The paired patient T cell samples were always analysed together, to avoid run-to-run differences.

This study presents a prevalence figure of 8 % (8/97) of *N. mikurensis-infection* in B-cell lymphoma patients, much similar to earlier findings in this group of patients [162]. All PCR-positive patients received treatment with doxycycline and were tested by PCR to ensure successful eradication of the infection, which was true for all eight patients. This was done despite the lack of symptoms of infection. We argue that it is not defensible to await active infection with the risk of damage of the blood vessels.

Many questions about the latency of *N. mikurensis* remain unanswered: What regulates the transition from initial infection to persistence? What are the required factors of the bacteria for persistent infection? What is the state of *N. mikurensis* during latent infection, are the bacteria replicating or not?

CONCLUSIONS

In this thesis the infectious disease neoehrlichiosis is studied to learn more about this emerging tick-borne bacterial infection. We show that a target cell of infection are vascular endothelial cells. We have confidence in the cell type being the replication host cell for the bacteria and possibly also being a permissive cell for the bacteria's less active stages. In Figure 14 a cross-section of infected endothelium is shown with infected endothelial cells in the wall of the vessel. It is also shown in so called, circulating endothelial cells, cells that have been released from the blood vessel wall due to damage or stress from the infection and are now circulating in the blood stream.

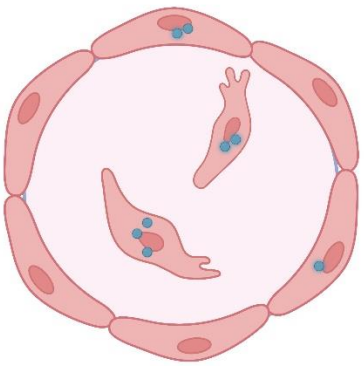

Figure 14. Cross-section of an infected blood vessel. *Infected endothelial cells are seen in the blood vessel wall and circulating endothelial cells in the blood stream. [Created with BioRender.com]*

It is also shown that *N. mikurensis* lay dormant in a latent state giving rise to no symptoms in the infected person until the B-cell defense is downregulated due to biological drug treatment. This conclusion was reached by using the same strategy used to detect latent cases of tuberculosis, namely identification of specific T cells in latently infected individuals.

In Figure 15, a proposal of how the innate and adaptive immunity works in infection with *N. mikurensis* presented based on the findings of this thesis and previous work. Important defense mechanisms of innate immunity are phagocytosis by neutrophils and macrophages and production of cytokines. γδ T cells are generated in response to IFN-γ and CXCL10 produced by innate immune cells. Th 1 cells activate CTL cells and give help to antibody-producing B cells. Several T cell types produce cytotoxic perforin to destroy infected cells.

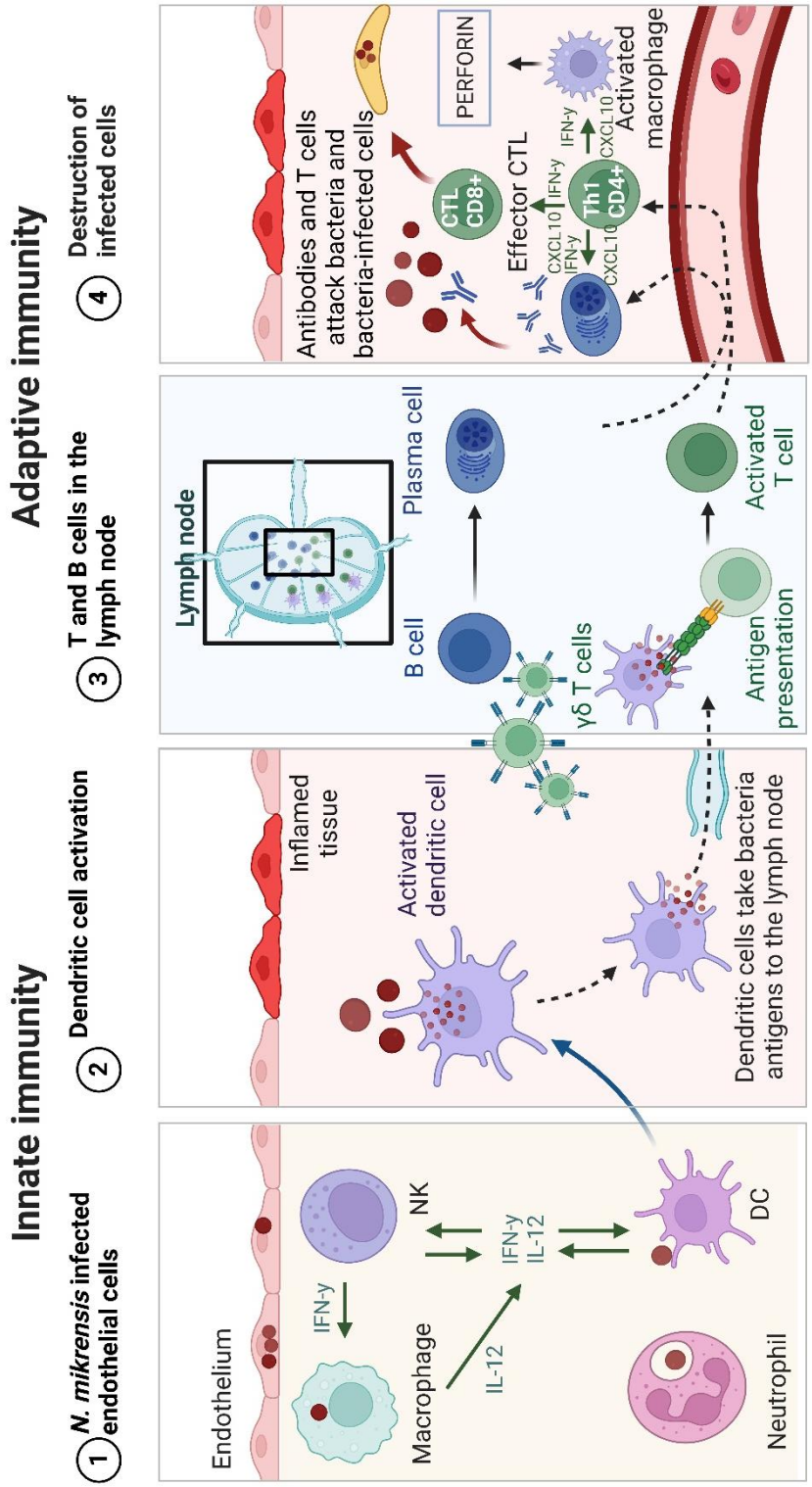
Innate immunity
Adaptive immunity
1 N. mikrensis infected endothelial cells
2 Dendritic cell activation
3 T and B cells in the lymph node
4 Destruction of infected cells
Endothelium
Macrophage
IFN-γ
IL-12
NK
IFN-γ
IL-12
DC
Neutrophil
Inflamed tissue
Activated dendritic cell
Dendritic cells take bacteria antigens to the lymph node
Lymph node
B cell
Plasma cell
γδ T cells
Antigen presentation
Activated T cell
Antibodies and T cells attack bacteria and bacteria-infected cells
PERFORIN
CTL CD8+
Effector CTL
CXCL10
IFN-γ
Th1 CD4+
IFN-γ
CXCL10
Activated macrophage

Figure 15. Immune responses directed against N. mikurensis infecting the endothelium. *The innate immunity with phagocytic cells and dendritic cell activation. For full eradication the adaptive immune system is also needed with adaptive cell-mediated T-cell responses and plasma cells that produce antibodies. (1) Macrophages and neutrophils phagocyte N. mikurensis and (2) antigen presenting dendritic cells are activated through cytokine stimulation and the presence of bacteria. (3) Dendritic cells present antigens in the lymph node and activates T cells that matures to Th 1 effector cells. Th 1 cells activates B cells and enhances antibody production. γδ T cells are present and serve as a bridge between the innate and adapted immune defense. (4) IFN-γ is produced by Th1 cells, Th 1 cells, which activates CTL to attack infected cells. Perforin and CXCL10 are expressed by all T cells. EC = endothelial cell, NK = natural killer cell, DC = dendritic cell, IL = interleukin, IFN = interferon, Th = T-helper cell, CTL = cytotoxic T cell [Created with BioRender.com]*

FUTURE PERSPECITIVES

The two most important findings in this thesis, the target cell of *N. mikurensis* infection being endothelium and the infection presenting asymptomatically as a latent disease that can reactivate when B-cell defense is suppressed have led to new knowledge and new hypothesis to answer: Are latent *N. mikurensis* infections life-long? Are there other factors which may trigger reactivation of the infection besides anti-B-cell treatment? Will it be necessary to screen patients scheduled to start treatment with biological B-cell for *N. mikurensis*-specific T cells similar to what is done to detect latent tuberculosis in patients starting anti-TNF treatment?

The opportunity of producing bacteria in tick cell lines and endothelial cell lines is important for the future work of developing a serology method and to understand the disease pathogenesis.

Neoehrlichiosis is still an underdiagnosed infection. This is due to several reasons but the biggest challenge till this day is to spread information about this pathogen to health care workers meeting the patients. Since *N. mikurensis* does not grow in cell-free media and microbiologic diagnosis relies on molecular diagnostics with specific PCR it increases the demand on clinicians examining the patients. This together with the fact that patients often have underlying diseases with symptoms that can mimic neoehrlichiosis is a big challenge for physicians! An important take-away message is that neoehrlichiosis is an important differential diagnosis in patients with fever of uncertain origin and unexpected vascular and thromboembolic events.

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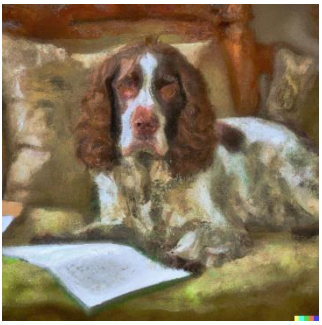

Till de många som delat med sig av sin kunskap, hjälpt mig genom att utmana min föreställning av vad jag klarar av tillika allt stöd i att skriva denna avhandling. För den tid och energi ni investerat i mig har ni för evigt min tacksamhet.

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-Rihanna

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