”*Candidatus* Neoehrlichia mikurensis” – a new agent of tick-borne infectious disease

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The expert in anything was one time a beginner

-Unknown

Abstract

"*Candidatus* Neoehrlichia mikurensis" (*Ca*. N. mikurensis) is a tick-borne bacterial pathogen that can cause disease particularly among immune compromised persons. This new infectious disease is called neoehrlichiosis. The clinical picture of neoehrlichios is characterized by fever, migrating pain, and vascular/thromboembolic complications. The bacterium received its name in 2004, after its discovery in ticks and rodents on the Japanese island of Mikura. This thesis have four main aims 1) Map this new infectious disease with respect to what types of patients that are afflicted, the clinical picture displayed by the patient categories, and the pattern of laboratory findings seen in infected patients. This is described in paper I; where clinical data of six patients participating in the "NEO-VÄST study" are described together with additional cases from Europe. 2) Determine if *Ca.* N. mikurensis is an opportunist that only afflicts immune compromised patients? In paper II we describe two immune competent patients who had raised levels of *Ca.* N. mikurensis DNA in the blood accompanied by a cytokine response for several months. The patients were diagnosed after PCR screening of plasma samples from 102 tick-bitten persons in Sweden who participated in the Tick-Borne Disease Study called STING. A PCR assay for clinical use was developed in this study and the cytokine levels were measured with multiplex technology. 3) Establish if *Ca*. N. mikurensis strains in Europe vary genetically. Paper III describes the development and use of a multilocus sequence analysis (MLSA) protocol to investigate the genetic diversity of clinical *Ca.* N. mikurensis strains in Europe. A low genetic diversity was seen among the strains, all of which were derived from immune compromised patients. Unexpectedly, *Ehrlichia ruminantium* was found to be the closest relative of *Ca.* N. mikurensis within the family of *Anaplasmataceae*. 4) Perform *de novo* whole-genome sequencing of *Ca.* N. mikurensis to characterize the bacterium. In paper IV we determined the complete reference genome sequence of *Ca.* N. mikurensis, sequenced directly from the blood of three immune suppressed patients. We also compared these sequences with those of other whole-genome sequenced relatives of *Ca.* N. mikurensis. The sequencing strategy relied on library preparation using a new type of technology called 10X Chromium followed by Hiseq Illumina sequencing, sequence assembly and *de novo* annotation. Our studies have yielded more knowledge about this anonymous emerging pathogen but much remains to be resolved, the work continues!

Keywords

Tick-borne disease, *Candidatus* Neoehrlichia mikurensis, infectious disease, human, neoehrlichiosis

Sammanfattning på svenska

Candidatus Neoehrlichia mikurensis (*Ca*. N. mikurensis) är en fästingburen bakterie som kan orsaka sjukdom hos människa. Det är främst patienter med nedsatt immunförsvar som drabbas av denna nya infektionssjukdom som kallas ”neoehrlichios”. Symtombilden vid neoehrlichios kan se väldigt olika ut men de vanligaste kännetecknen är feber, migrerande muskelvärk, huvudvärk, nackstelhet och ledvärk. De allvarligaste konsekvenserna av infektionen är de tromboemboliska och vaskulära komplikationer som över hälften av svenska patienter har drabbats utav. De första fallen av neoehrlichios hos människa publicerades 2010 men bakterien namngavs redan 2004 då den hittades hos fästingar och råttor på ön Mikura i Japan. Denna avhandling innehåller fyra delarbeten där det huvudsakliga målet har varit att ta reda på mer om denna infektionssjukdom samt förbättra diagnostiken så att flera patienter kan identifieras, behandlas, slippa långvarigt lidande och svåra komplikationer. I det första arbetet beskrivs symptom och fynd hos svenska, tyska, schweiziska och tjeckiska patienter som hade diagnostiserats med neoehrlichios och vars fallrapporter publicerats fram till 2013. I delarbete II undersöks om *Ca.* N. mikurensis kan ge upphov till sjukdom hos patienter med normalt immunförsvar. Mikrobiologisk diagnostik i form av en PCR (polymeras chain reaction) specifik för *Ca*. N. mikurensis sattes upp för analys av blodprov från friska människor som blivit bitna av fästingar, ingått i fästingstudien STING och därefter utvecklat symtom. Två patienter diagnostiserades med *Ca*. N. mikurensis och beskrivs. Delarbete III fokuserar på att med hjälp av nyuppsatt epidemiologisk typningsmetod jämföra olika europeiska kliniska stammar av *Ca.* N. mikurensis för att se om dessa är genetiskt lika varandra och få en bättre uppfattning om släktskapet till övriga medlemmar i bakteriefamiljen *Anaplasmataceae*. Vi fann att isolaten var väldigt lika varandra och skiljdes åt avseende ett fåtal nukleotider. *Ehrlichia ruminantium* som orsakar svår sjukdom hos boskap beskrivs här som *Ca.* N. mikurensis närmaste släkting. I delarbete IV använder vi oss av ny sekvenseringsteknik för att helgenomsekvensera denna bakterie direkt från blod taget från patienter diagnostiserade med neoehrlichios. Detta lyckas och vi beskriver storleken på genomet hos *Ca*. N. mikurensis som är ca 1.1 Mb och vi finner även att de tre sekvenserade isolaten från de svenska patienterna är väldigt lika. Våra studier har redan hjälpt flera patienter men det finns fortfarande mycket att lära sig om denna nya infektionssjukdom. Arbetet fortsätter och förhoppningsvis kommer våra resultat att ledatill ännu bättre diagnostiska möjligheter för dessa patienter och en ökad kunskap om *Ca.*N.mikurensis!

List of publications

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

I. **Grankvist A**, Andersson PO, Mattsson M, Sender M, Vaht K, Hoper L, Sakiniene E, Trysberg E, Stenson M, Fehr J, Pekova S,Bogdan C, Bloemberg G, Wenneras C
Infections with the tick-borne bacterium "*Candidatus Neoehrlichia mikurensis*" mimic non-infectious conditions in patients with B cell malignancies or autoimmune disease
Clin. Infect. Dis 2014; 58: 1716–1722.

II. **Grankvist A***, Sandelin LL*, Andersson J, Fryland L, Wilhelmsson P, Lindgren PE, Forsberg P, Wenneras C
Infections with *Candidatus Neoehrlichia mikurensis* and Cytokine Responses in 2 persons bitten by ticks, Sweden
Emerg. Infect. Dis 2015; 21: 1462-1465.
*Both authors contributed equally

III. **Grankvist A**, Moore ER, Svensson Stadler L, Pekova S, Bogdan C, Geissdorfer W, Grip-Linden J, Brandstrom K, Marsal J, Andreasson K, Lewerin C, Welinder-Olsson C, Wenneras C
Multilocus sequence analysis of clinical "*Candidatus* Neoehrlichia mikurensis*"* strains from Europe
J. Clin. Microbiol 2015; 53: 3126-3132.

IV. **Grankvist A**, Sikora P, Wennerås C
The sequence and *de novo* assembly of *Candidatus* Neoehrlichia mikurensis genome from a clinical isolate
In manuscript.

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Abbreviations

Ank	Ankyrin protein
Ca. N. mikurensis	Candidatus Neoehrlichia mikurensis
CDS	Coding sequence
CLL	Chronic lymphocytic leukemia
ClpB	Caseinolytic Peptidase B
CSF	Cerebrospinal fluid
CT	Cycle threshold
CTL	Cytotoxtic T-lymphocytes
CRP	C-reactive protein
DC	Denditric cell
DLCBL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema migrans
EMLA	*Ehrlichia muris*-like agent
FAM	Fluorescein
FtsZ	Filamenting temperature-sensitive mutant Z
GEM	Gel bead in Emulsion
HGA	Human granulocytic anaplasmosis
HME	Human monocytic ehrlichiosis
HMW	High molecular weight
IFA	Immunofluorescence assay
IL	Interleukin
INF	Interferon
IRE	Ixodes ricinus
ISE	Ixodes scapularis
LGL	Large granular lymphocyte leukemia
LPS	Lipopolysaccharides
LipA	Lipase A
Mb	Mega base
MIP	Major intrinsic protein
MLSA	Multilocus sequence assay
MLST	Multilocus sequence typing
NGS	Next generation sequencing
NO	Nitric oxide
NK	Natural killer

OMP	Outer membrane protein
PTLD	Post-transplant lymphoproliferative disorder
PAMPS	Pathogen-associated molecular pattern
PE	Phycoerythrin
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SNV	Single-nucleotide variants
TBE	Tick-borne encephalitis
TBD	Tick-Borne Diseases
T4SS	Type IV secretion system
TNF	Tumor necrosis factor
UPGMA	Unweighted pair group method with arithmetic mean
VEGF	Vascular endothelial growth factor

Introduction

Ticks

Ticks are external parasites that live by feeding on the blood of mammals, birds and reptiles. They are widely distributed over the world and they even existed at the age of the dinosaurs[1]. There are two major families of ticks, the *Ixodidae,* or hard ticks, and the *Argasidae,* or soft ticks. Hard ticks consist of a hard shield that covers the dorsal region and they have a head with mouth and feeding parts in the front whereas soft ticks have their mouthparts on the underside of the body. In Europe, *Ixodid* ticks, and *Ixodes ricinus*

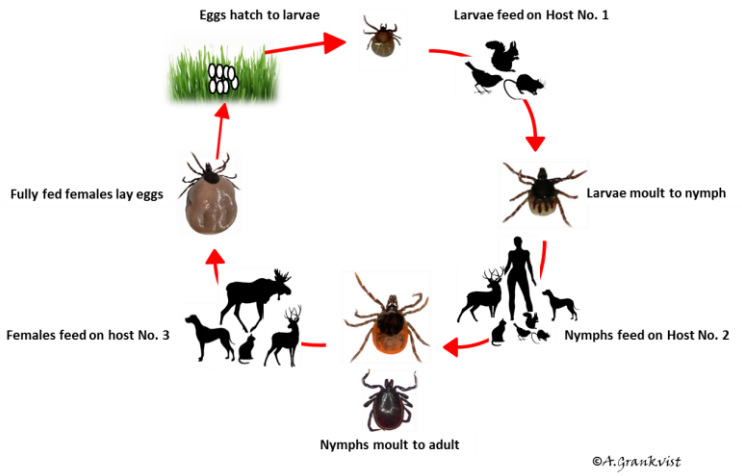

Figure 1 Tick life cycle
An adult female tick lays up to 3,000 eggs. When the larvae emerge, they feed and after feeding, they detach from their host and moult to nymphs in the vegetation. During moulting the tick sheds the previous skin or shell. The nymphs then feed on larger hosts and moult to adults. Female adults attach to larger hosts, feed and copulate with an adult male and after that, lay eggs. Males do not need to feed but occupy larger hosts primarily for mating. This life cycle can take between two to three years depending on climate.

in particular, are important vectors of human and animal parasitic, bacterial and viral pathogens[2]. Ticks of the genus *Ixodes* find their host using the sensory organs on their front legs (Haller´s organ). These organs can sense carbon dioxide, temperature, odors and movement, all of which signal the appearance of a blood-filled host[3].

In Sweden, the most common hard tick is *Ixodes ricinus.* This tick species has a three-host life cycle; it depends on three vertebrate hosts for blood meals (Figure 1). *I. ricinus* is able to take advantage of a multitude of host species, which differ with respect to the number of ticks and the different life stages they feed[4]. *Larvae* and nymphs mainly feed on small rodents and birds whereas adults feed on multiple host species, often roe deer, *Capreolus capreolus*[5].

In Europe, the *Ixodes* tick is found primarily in woodlands with a permanent layer of leaves and bushes that protect them from sunlight. This is the preferred microhabitat of the off-host phases, characterized by relative humidity, which should not go below 80% for the survival of the ticks. There, all developmental stages (unfed as well as engorged ticks) are able to survive winter[6].The ticks become active when the temperature exceeds 4-5˚C, i.e. they start to quest for hosts. The ticks can quest for several months if there is a suitable habitat, but they must replenish their reserves of water by frequent journeys from the surface vegetation down to the base layer of the vegetation[7]. In northern Europe, the peak of questing adult and nymphal ticks usually occurs in spring and early summer[7]. Low-level activity takes place during mid-summer although questing may persist throughout the summer in cool and humid conditions, especially in relatively protected habitats. In many areas, a second peak of activity occurs in autumn. Larval ticks typically begin questing slightly later than the adults and nymphs, appearing in large numbers in early summer and reaching their highest activity peaks in mid-summer. *I. ricinus* seasonal ac-

tivity is controlled by a biological strategy where ticks avoid questing at unfavorable times of the year. In that way they can increase their survival by responding to current conditions[7].

Tick-borne infections

During the last decades the emergence of tick-borne infections has increased remarkably in Europe[8]. This may be in part the result of advances in molecular biology since several new Rickettsial diseases, Ehrlichial diseases, and novel agents of *Borrelia* and *Babesia* genera have been recognized with the development of new diagnostic tools. The emergence of vector borne infectious diseases is a growing health concern for humans and livestock[8] since the most common zoonotic vector are the ticks, and in Europe the main vector is *Ixodes ricinus*.

The geographic distribution of ticks continues to expand and climate change is one reason for the spread of *Ixodes* ticks[9]. Some of the pathogens transmitted by the *Ixodes ricinus* tick include *Borrelia burgdoferi* sensu lato complex causing borreliosis, *Anaplasma phagocytophilum* causing human granulocytic anaplasmosis, *Fransicella tularensis* causing tularaemia, *Rickettsia helvetica* causing spotted fever rickettsiosis, *Babesia divergens* and *Babesia microti* responsible for babesiosis, *Candidatus* Neoehrlichia mikuensis causing neoehrlichiosis and tick-borne encephalitis virus (TBE)[9]. Althought the primary vector for ehrlichiosis is *Amblyomma americanum, Ixodes* ticks can also transmit Ehrlichial species [10] (Table 1).

Table 1 Tick-borne infections pose a threat to public health around the world, and they seem to be on the rise.

Disease in humans Pathogen	Tick vector	Geography
Borreliosis *Borrelia burgdorferi* sensu lato (bacteria)	*Ixodes species*	Global
Tick-borne encephalitis Japanese encephalitis virus	*Ixodes species*	Europe Asia
Spotted fevers *Rickettsia species* (bacteria)	*Ixodes species* *Dermacentor species* *Rhipicephalus sanguineus*	Global
Babesiosis *Babesia species* (protozoan)	*Ixodes species*	USA Europe Asia
Tick-borne relapsing fever *Borrelia species* (bacteria)	*Ixodes species* *Ornithodoros species*	Global
Ehrlichiosis *Ehrlichia species* (bacteria)	*Ixodes species* *Amblyomma species* *Dermacentor variabilis*	North America South America Africa Asia
Anaplasmosis *Anaplasma phagocytophilum* (bacteria)	*Ixodes species*	America Europe Asia
Neoehrlichiosis *Candidatus* Neoehrlichia mikurensis (bacteria)	***Ixodes ricinus*** *Ixodes species* *[Dermacentor species]*	Europe Asia
Tick-borne tularaemia *Francisella tularensis* (bacteria)	*Ixodes species* *Amblyomma species* *Dermacentor species*	North America Europe Russia

Borreliosis

In this thesis I have chosen to remove "Lyme" from Borreliosis since Lyme disease is caused by *Borrelia burgdorferi* sensu stricto in North America, whereas species belonging to the *Borrelia burgdorferi* sensu lato complex prevail in Europe. Consequently, the clinical manifestations described for Lyme disease differ from those characteristic of Borreliosis in Europe and in Asia.

Borreliosis can affect several parts of the human body and is the most common tick-borne disease in the northern hemisphere. The

earliest symptom is the red skin lesion called *erythema migrans* (EM). EM can be visualized days to weeks after a tick bite and can enlarge from the site of the tick bite. Patients can experience flu-like symptoms and the diagnosis is most often clinical since the patients are usually seronegative for *Borrelia* at this stage [11].

After EM, neuroborreliosis is the second most common manifestation of disease in Sweden[12]. It appears 4-6 weeks after the tick bite with neurological symptoms such as facial palsy, nerve root pain, headache and fever. The diagnosis is based on medical history, clinical symptoms and serological analysis of serum and cerebrospinal fluid (CSF)[11].

Later manifestations of *Borreliosis* include arthritis that is characterized by inflammation of large joints, and this condition causes a significant proportion of arthritis cases in children[13]. Acrodermatitis, a slow growing chronic skin condition that is most commonly seen on hands and feet in people older than 40 years. This condition almost only occurs in Europe[14]. Lymphocytoma, a rare skin manifestation of Borreliosis, which can appear in the same area as a previous EM. Finally, carditis, which is a treatable condition of the heart and the least common manifestation of borreliosis in Sweden[12].

Anaplasmosis

Anaplasma phagocytophilum causes human granulocytic anaplasmosis (HGA) and in Europe, the tick *Ixodes ricinus* is identified as the main vector transmitting the disease. The target cells of the bacteria are the neutrophils and the seroprevalence of *A. phagocytophilum* is between 5.7-8.1% in European populations [15] and 8-21% in Sweden[16,17,18,19]. Despite the high seroprevalence of *Anaplasma phagocytophilum* antibodies in Sweden there are few case reports of anaplasmosis in Scandinavia[20]. In northern Europe, *A.*

phagocytophilum is more commonly the cause of tick-borne fever in domestic ruminants, horses and dogs[21]. The bacterium infects the cytoplasm of circulating neutrophils, seen as intracellular inclusions known as *morulae* (microcolonies)[22]. *Morulae* can be detected in blood smears during acute febrile episodes. However, in Europe, *morulae* are a rare finding and symptomatic HGA infections are rarely diagnosed[23]. It is speculated that the European *A. phagocytophilum* strains are less virulent compared to the strains circulating in the USA[22,23]. The general clinical features of HGA are febrile illness with headache, myalgia and malaise accompanied by leukopenia, thrombocytopenia and elevated levels of hepatic enzymes[10]. The symptoms are generally mild to moderate, and subclinical infections occur. However, life-threatening complications have been reported in immune compromised individuals[19]. Today's diagnostic tool for HGA is serology using an indirect fluorescent antibody method (IFA) but PCR of peripheral blood is also possible[24].

Ehrlichiosis

Human monocytic ehrlichiosis (HME) is mainly caused *by Ehrlichia chaffeensis* and the primary tick-vector for HME is the Lone Star tick, *Amblyomma americanum*. Following transmission via a tick bite, *E. chaffeensis* enters and multiplies within monocytes or macrophages forming intracellular microcolonies (*morulae*). So far, there are no reported cases of HME outside of the USA[25]. The average incidence of HME in the USA is estimated to 0.7 cases/million population but can be much higher in endemic areas[10]. HME is a more severe disease than HGA and can be fatal even in immune competent patients, almost 50% of the cases require hospitalization[10]. Common symptoms are fever, headaches and myalgia. Gastrointestinal symptoms have been reported, mainly in children. Life-threatening manifestations include cardiovascular failure, haemorrhages, hepatic failure and respiratory distress syndrome.

HME can cause neurological manifestations such as meningitides but *morulae* are rarely identified in CSF monocytes[10,26]. A diagnosis of HME can be confirmed in several ways. Laboratory tests include serology, detection of *morulae* in blood smears, and detection of *Ehrlichial* DNA by PCR of blood or CSF samples.

Ehrlichia ewingii and *Ehrlichia canis* were believed to be exclusively canine pathogens until human cases of infection were described[27,28]. Most human cases of *E. ewingii* infection (HEE) have occurred among immune suppressed patients and the bacteria may be visualized as *morulae* in granulocytes and the clinical manifestations appear to be milder than HME [27]. *Ehrlichia canis* is transferred most commonly between dogs by *Rhipicephalus sanguineus*, the brown dog tick that rarely bites humans[29]. But, since the human cases of *E. canis* have been reported from Venezuela, which is a tropical country with high rates of *E. canis* infections among dogs, it is surmised that the human infections are also transmitted by a bite of *R.sanguineus*[28].

Recently, a new member of the genus *Ehrlichia* was reported to cause human infection, an *Ehrlichia muris*-like agent (EMLA). It is transmitted by *Ixodes scapularis* and the cases were from the northern part of the USA. All patients were immune suppressed and had fever, fatigue, and headache as the main symptoms[30].

Spotted fevers

The term Tick-borne Spotted Fevers is a catch-all term used to describe illnesses caused by ticks infected with *Rickettsia* bacteria. The most common forms of spotted fevers are African tick bite fever caused by *R. africae*, Mediterranean spotted fever caused by *R. conorii*, and the most severe form of them all is Rocky Mountain spotted fever[31], caused by *Rickettsia rickettsia*, primarily transmitted by the *Dermacentor variabilis* tick. Typically, symptoms of

Rocky Mountain spotted fever include a severe headache, chills, extreme exhaustion and muscle pains. Symptoms begin suddenly 3 to 12 days after a tick bite and on the first days of fever, a rash appears on the wrists, palms, ankles, soles, and forearms. It rapidly extends to the neck, face, armpits, buttocks, and trunk. These rashes are due to the bacterium infecting and multiplying within vascular endothelial cells, causing vasculitis of small to medium-sized blood vessels[32]. Spotted fever group rickettsiosis includes a large number of species, of which 24 are recognized human pathogens[33].

In Sweden and Denmark, where the most common tick species is *Ixodes ricinus, Rickettsia helvetica* is the dominant *Rickettsial species* with a variable seroprevalence of 1.7–17.3 % [34,35]. Despite this rather high prevalence, no significant difference were shown in the seroprevalence of antibodies against spotted fever Rickettsia between patients investigated for neuroborreliosis and healthy blood donors in a recent Danish study[35]. Case reports from Sweden suggests that infection with *R. helvetica* may be involved in both sarcoidosis and in chronic perimyocarditis with sudden cardiac death in two young men [36,37]. There are also several reports of severe cases with neurological manifestations and meningitides caused by *R. helvetica* and *R. felis* [38-40].

Tick-borne relapsing fever

Tick-borne relapsing fever is characterized by recurring febrile episodes that last up to 3 days and are separated by afebrile periods of several days. Along with fever, patients may experience a wide range of nonspecific symptoms (e.g., headache, myalgia, arthralgia, shaking chills, and abdominal complaints). Neurological symptoms are common during relapsing fever and the most common symptoms are meningitis and facial palsy. Patients who are not treated with antibiotics typically experience several episodes of fever before illness resolves[41]. The illness in Europe is caused by infection

with *Borrelia* species that mainly are transmitted by *Ornithodoros* soft ticks (e.g., *B. hispanica, B. persica* and *B. caucasica*)[42]. Recently, it has been discovered that human agents of relapsing fever may also be vectored by *Ixodid* ticks, chiefly the emerging pathogen *Borrelia miyamotoi*[43]. Human infection with *B. miyamotoi* has been reported from Russia, North America and Japan but in recent years cases from Europe have started to show up[44-46]: The first case was an elderly immune competent woman who sought medical care 3 weeks after a tick-bite because of fever, myalgia, headache, weight loss and an erythematous skin lesion[45]. The patient recovered fully without antibiotic treatment. Another two cases with severe manifestations, notably meningoencephalitis afflicting highly immune compromised patients from Germany and Netherlands have also been reported[44,46]. There are so far three reported case of meningoencephalitis caused by *B. miyamotoi* in the world[42].

Tick-borne tularaemia

Tularaemia is caused by infection with the facultative intracellular, gram-negative bacterium *Francisella tularensis* and has been recognized as a human pathogen since the beginning of the 20th century[47]. Humans can acquire this infection through several routes where tick bite is one of them. It can also be spread to humans by mosquitoes, contact with an infected animal, by drinking contaminated water, breathing contaminated dirt, or aerosol[48,49]. In Europe, the ticks *Dermacentor reticularis* and *Ixodes ricinus* are vectors for the bacterium, but in Sweden mosquitoes are considered to be the major transmitter of the bacteria[50].

The most common form of the disease in Sweden is ulceroglandular tularaemia, which is characterized by a skin ulcer at the site of infection and adjacent swollen regional lymph nodes and fever[51]. The mean incubation time is 3–5 days with a range of 1–21 days[48]. In some cases complications like soft tissue abscesses, pneumonia

and meningitis can occur[48]. The laboratory diagnosis is often based on the detection of specific serum antibodies and/or PCR for *F. tularensis* DNA in clinical samples. The bacterium can disseminate easily and is classified as a Category A critical biological agent and the risk of laboratory-associated infections is high[52].

The family *Anaplasmataceae*

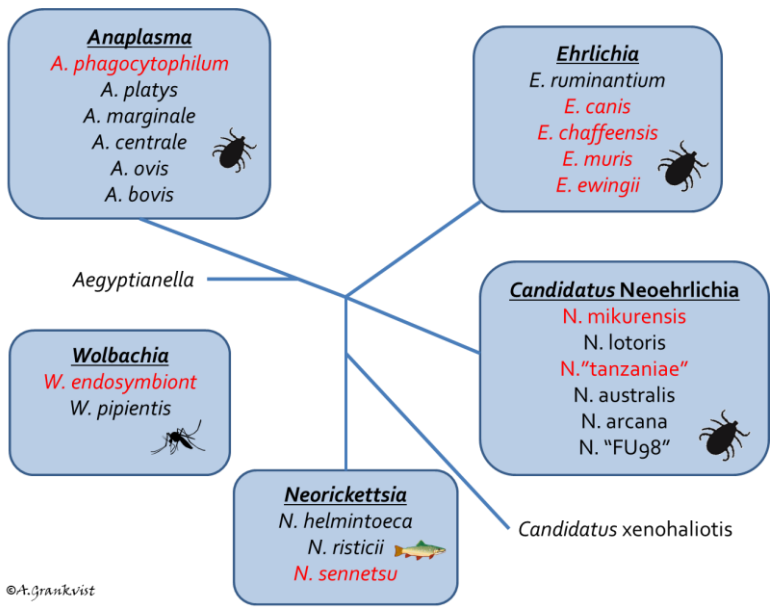

Figure 2 The family *Anaplasmataceae*
A map over the family *Anaplasmataceae*; the headings indicate genera and the species coloured in red are human pathogens. *Anaplasma, Ehrlichia* and *Candidatus* Neoehrlichia are mainly transmitted by different species of ticks. *Neorickettsia* are transmitted to mammals by eating infected fish or aquatic insects. *Wolbachia* infect arthropod species but have a role in the human infection *lymphatic filariasis.*

The family *Anaplasmataceae* (Figure 2) belongs to the order *Rickettsiales* together with the family *Rickettsiaceae*. All bacterial species in the *Anaplasmataceae* family are obligate intracellular

bacteria that infect invertebrates (pathogenic or non-pathogenic) but some species also infect mammals and birds. It has been proposed that one major difference between *Anaplasmataceae* and *Rickettsiaceae* are that the bacteria belonging to *Anaplasmataceae* are enclosed within membrane-bound compartments in the host cytoplasm in contrast to the *Rickettsiasceae* that are free in the cytoplasm [53,54]. Different stages of bacterial development have been suggested for *Anaplasmataceae* based on the reorganization of the bacterial DNA from an infectious form (dense-core) to a vegetative form (reticulate cell). The reticulate forms multiply by binary fission and form *morulae* and turn into dense-cored cells before being released from the host cell[54] (Figure 3).

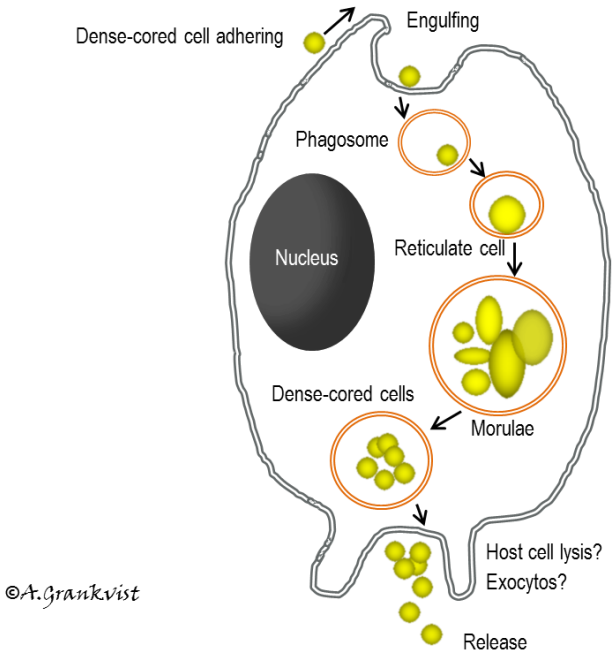

Figure 3 Intracellular life cycle of *Anaplasmataceae*
Dense-cored cells enter host cells and develop to replicating reticulate cells (vegetative form). A cluster of reticulate cells forms "*morulae*" that develop into mature dense-cored cells before being released by host cell rupture or exocytosis. Picture adapted from Pruneau et. al.[52]

Bacterial genera

Neorickettsia

Many species within the *Anaplasmataceae* family were known as veterinary pathogens before they were shown to infect humans. The first reported case of human infection by a bacterium belonging to the family *Anaplasmataceae* was in 1954[55]. *Neorickettsia sennetsu* caused infectious mononucleosis in a patient in Japan and the route of human infection was thought to be the consumption of raw fish[55]. In recent years, human cases of *N. sennetsu* infection have been identified in both Thailand and Laos by PCR and serology[55,56]. All *Neorickettsia* spp. are thought to be transmitted by parasitic flatworms, which transmit the organisms to an animal or human host. No other species in the genus have been documented to cause infection in humans but *N. helminthoeca* can cause salmon poisoning disease in dogs and *N. risticii* is the cause of Potomac horse fever[57,58].

Wolbachia

The genus *Wolbachia* was named by Hertig in 1936 and described as gram-negative, intracellular, *Rickettsiae*-like bacteria concentrated in the germline and somatic tissues of a broad range of insects and other arthropods[59,60]. When gene sequencing of bacteria increased dramatically in the early 1990´s, *Wolbachia* was included in the family *Anaplasmataceae* together with the genera *Ehrlichia*, *Neorickettsia*, and *Anaplasma*[61]. *Wolbachia* is vertically transmitted (also called mother-to-child transmission). In insects, it is common in both the male and female germlines but in the nematodes, it is present only in the female germline. *Wolbachia* is be-

lieved to have a neutral relationship with its host, existing as an endosymbiont[62].

Recently, *Wolbachia* got new attention when it was discovered that the bacteria have antiviral properties and are able to reduce the levels of dengue virus and other mosquito-borne human pathogens by upregulating immune effector genes when infecting the vector species[63]. So far, the only human infection with *Wolbachia* is the role the bacteria play in lymphatic filariasis (elephantiasis). This is a disease caused by parasitic worms that need the endosymbiont *Wolbachia* to infect and survive in the human lymphatic system[64]. When the worms located in the lymph system die they release the bacteria into their hosts thereby triggering an inflammatory response[64].

Anaplasma

In 1910, Theiler identified the first *Anaplasmataceae* species, *Anaplasma marginale*[65]. This pathogen was found in the red blood cells of cattle that suffered from severe anaemia. There are two more species in the genus that also infect ruminants: *A. centrale* (actually *A. marginale ss centrale*) and *A. ovis*. *A. marginale* is host-specific and is the major cause of anaplasmosis in cattle and has the widest geographic distribution among tick-borne pathogens, whereas *A. ovis* is a pathogen of sheep. *A. centrale* is less pathogenic for cattle and has been used as a live vaccine in Israel, Australia, Africa and South America[66]. *A. phagocytophilum* has been known to cause disease in domestic ruminants in Europe since 1951[67]. The bacteria were first classified as *Rickettsia phagocytophila* since they were assumed to belong to the class *Rickettsia*[67]. In 1962, they were renamed *Cytocetes phagocytophila* due to morphological similarities with *Cytoecetes microti*, an organism that is found in polymorphonuclear cells of voles[68]. In 1974, it changed name again to *Ehrlichia phagocytophila* and in 2001 after the reclassification of the genus *Ehrlichia*, the current name was

created[21,69]. In the beginning of the 1990s, the first human case of infection was described – a patient who died of severe febrile illness two weeks after a tick bite[70]. Small bacteria were discovered within the neutrophils in the blood of the patient and serological assays for the human monocytic ehrlichiosis (HME) agent, *Ehrlichia chaffensis* were negative. Even if serological assays indicated identical relationships to the veterinary pathogen, *E. phagocytophila*, the human agent was named human granulocytic ehrlichiosis (HGE) agent at that time[70]. *A. phagocytophilum* has, as its name implies, favouritism for phagocytic cells, and is one of very few bacteria that survive and replicate within neutrophilic granulocytes[21].

The bacterium counteracts neutrophil-mediated bacterial killing by triggering an anti-apoptotic cascade, which is important for its intracellular survival in the normally short-lived neutrophilic granulocytes[21,71]. In nature, the life cycle of *A. phagocytophilum* consists of mammalian and tick stages[53].Viable *A. phagocytophilum* organisms have been isolated from several mammalian hosts, e.g. cattle, sheep, dogs, horses and humans. In Europe, wild animals such as mice and bank voles are the primary reservoirs but domestic animals such as dogs can serve as secondary reservoirs for human infection[21].

Ehrlichia

The *Ehrlichia* genus contains five well-recognized species according to current taxonomy: E. *chaffeensis, E. ewingii, E. canis, E. muris* and *E. ruminantium*[69]. There are also a number of genetic variants of *Ehrlichia* that have not been clearly defined yet[72-74]. The main target cells for infection by *Ehrlichia* species of mammals are cells of the blood vascular system. *E. chaffeensis* (the agent of HME), the canine pathogen *E. canis* and the murine path-

ogen *E. muris* all multiply inside of monocytes and macrophages, whereas *E. ewingii* (the agent of HGE in humans) multiplies in granulocytes. *E. ruminantium* causes the disease heartwater in domestic ruminants. *E. ruminantium* replicates initially in macrophages and neutrophils near the site of infection. When these cells rupture, the organism disseminates via the bloodstream to invade endothelial cells of blood vessels in the body and in domestic ruminants there seems to be a predisposition for endothelial cells of the brain. Vasculitis leads to effusion in various sites, including the pericardial sac and thereof the name "heartwater"[75]. Heartwater is only seen in the southern hemisphere and has high mortality and decreases herd productivity, therefore the economic impact of the infection is very high[76].

Anaplasmataceae and the host cell

Bacteria belonging to the *Anaplasmataceae* must be very flexible to survive in different microenvironments. They need to escape the immune system during their extracellular and infective stages and once inside the host cell, they need to neutralize the innate immune response and sabotage cellular processes to be able to survive and replicate intracellularly. Among gram-negative bacteria, lipopolysaccharide (LPS) and peptidoglycan are important cell wall components. However, bacteria belonging to the *Anaplasmataceae* have only a thin outer membrane without LPS and no peptidoglycan; they have lost almost all genes for biosynthesis of these compounds[77]. It has been suggested that cholesterol is needed to compensate for the lack of LPS and peptidoglycan and to maintain the mechanical strength of the cell wall[77].

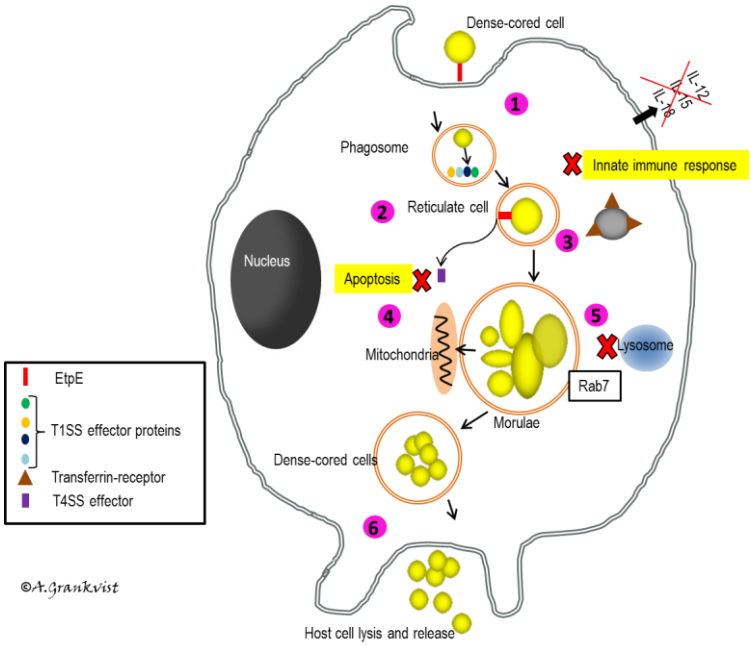

Figure 4. Ehrlichia´s strategies inside the host cell
(1) The dense-cored cell enter and attach by using Invasin EtpE. (2) The bacteria replicate in a phagosome (*Ehrlichia*-containing vacuole) that secrete T1SS effector proteins to escape host innate immune-response. (3) The dense-cored cell differentiate into reticulate cells and fuse with Transferrin-receptor endosome to acquire iron from the host cell and disrupt cell-signalling pathways to inhibit cytokine production and prevent innate immune responses. (4) Ehrlichia secretes T4SS effector protein to inhibit apoptosis and the reticulate cells divide to form microcolonies (*morulae*). The *morulae* secrete proteins to keep the mitochondria stabilized preventing triggering of apoptosis of the host cell. (5) To promote intracellular survival the bacterial protein Rab7 acidifies the late endosome to inhibit fusion with lysosome. (6) Dense-cored cells are released by exocytosis or rupture of the cells. Picture adapted from Mouméne et al. [77]

The lack of LPS facilitates the escape from macrophages and neutrophils since they cannot recognize pathogen-associated patterns (PAMP´s) such as LPS and peptidoglycan[78]. Other important mechanisms are the use of proteins, exemplified by the invasin EtpE that is required for the adhesion and invasion of the host cell (Fig 4). The *Ehrlichia* bacteria in the vacuoles (*Ehrlichia*-containing vacuole) also secrete effector proteins to escape the host immune response (Fig 4). Bacteria belonging to *Anaplasmataceae*

can also manipulate the host cell by inhibiting or delaying spontaneous cellular apoptosis to be able to replicate inside the host cell, e.g. *Ehrlichia* secrete T4SS effector proteins (Fig 4)[53,79]. *E. ewingii* can stabilize the mitochondrial membrane of neutrophils (host cell)[80] by unknown mechanisms but the end-result is mitochondrial time-out. An inactive mitochondrion will not trigger apoptosis of the infected cell and will not compete for nutrients with the *Ehrlichia* bacteria (Fig 4)[81]. Recent studies of the protein composition of *Ehrlichia chaffensis* vacuoles has shown that the bacterial protein Rab7 (characteristic late endosomal protein) acidifies the compartment at pH 5.2, which protects the vacuole for fusion with lysosomes[79,82] (Fig 4). *E. chaffeensis* is also able to inhibit the transcription of the cytokines IL-12, IL-15 and IL-18, thereby inhibiting the production of INF-ɣ from TH1 and NK cells, which reduces the activation of macrophages. In that way, *Ehrlichia* avoids intracellular killing by macrophages[79].Type IV secretion system (T4SS), is the general mechanism by which bacterial cells secrete or take up macromolecules. Bacteria belonging to the *Anaplasmataceae* have multiple copies of T4SS components and this system is up-regulated during infection[83]. The system secretes effector proteins, e.g. Ank 200 into the eukaryotic host cell and thereby facilitates intracellular bacterial survival, growth and virulence[53,79].

Genomes

The genomes of bacteria in this family are small (*A. phagocytophilum* 1.47 Mb, *A. marginale* 1.19 Mb, *E. ruminantium* 1.52 Mb, *E. chaffeensis* 1.18 Mb and *N. sennetsu* 0.859 Mb[53,84-86]), one quarter of the size of that of *Escherichia coli.* This is believed to result from reductive genome evolution, i.e., the intracellular bacteria have gotten rid of genes required for an extracellular lifestyle[87]. A remarkable finding in the *E. ruminantium* genome is the large

number of repetitive sequences, constituting 8.3 % of the chromosome, which results in the largest genome size in the family[84].

The genes that are absent from members in the family *Anaplasmataceae* are genes that encode proteins belonging to the category of central intermediary metabolism[53]. Obligate intracellular bacteria are unable to synthesize all the organic compounds required for growth (also known as an auxotroph) and acquire most amino acids and other metabolites from the host. A considerable part of the genome of these bacteria encodes outer-membrane protein 1 (OMP1) genes and some of them are unique to the family of *Anaplasmataceae* [86]. OMPs have many functions: ensuring the integrity and stability of the bacterial envelope, passive and active transport of substrates and nutrients by forming porine channels, cell-to-cell communication and adhesion to host cells. Since *Ehrlichia spp.* and *Anaplasma spp.* lack LPS these envelope OMP proteins are assumed to be a major part of the bacterial surface that is exposed to the host[53].

The expansion of OMP1 genes is shown by the repeats in the genomes of *Anaplasma spp*. and *Ehrlichia spp.* Moreover, other functionally important genes such as the type IV secretion genes, are duplicated and contribute to the repeats[86].One interesting thing, is that different OMPs are expressed in different host/vector cell types. In *A. phagocytophilum*, a higher proportion of up-regulated genes encoding OMPs are seen in human cell lines than in tick cell lines[88]. In contrast, the *E .ruminantium* MAP1 (OMP protein organized as a porin) gene is expressed only in tick cells (vector) and not in mammalian host cells[89]. Since OMP1 proteins are highly immunogenic they are good candidates as vaccine components[53,54,79]. Among nonliving vaccines, purified outer membranes have provided the best protection against infection and disease[90].

Candidatus Neoehrlichia mikurensis

Basic characteristics

Candidatus Neoehrlichia mikurensis is assumed to be an obligate intracellular gram-negative bacterium like the other members of the family *Anaplasmataceae*. This new bacterial species was named after its discovery in ticks and rodents on the Japanese island of Mikura in 2004[91]. The term *Candidatus* specifies that this bacterium is so far uncultivable[92]. However, we recently managed to cultivate this bacterium from six clinical isolates, so hopefully this prefix can be removed in the near future and the bacterium can be renamed *Neoehrlichia mikurensis*[93]. Before Kawahara's findings in 2004, the same bacterial species had been described and given other *Ehrlichial* names. Schouls et al. first described this agent in 1999, as an Ehrlichia-like species when it was found in *Ixodes ricinus* ticks collected in the Netherlands[94]. In the beginning of 2000, several reports followed from Norway, Russia and Italy about this new *Ehrlichia*-variant in *Ixodes* ticks[95-97]. Shpunov et al called this new species "*Ehrlichia*-like Schotti variant" when it was found in ticks in the Baltic region of Russia[98] and in 2003 the first identification of this *Ehrlichia*-like organism in small mammals were reported from China[99].

In 2004, Kawahara et al. showed an electron micrograph of an endothelial cell from the spleen of a rat with rounded, pleomorphic structures with a diameter of 0.5-1.2 µm[91]. These membrane-bound inclusions containing small cocci were the result of intraperitoneal infection of a laboratory rat with spleen homogenate from wild *Rattus norvegicus* that was PCR-positive for this new agent[91]. The structures inside the cytoplasm of the endothelial cell of the rat were not labelled with DNA probes or antibodies so it was not proven formally that this picture really displayed *Ca.* N. mikurensis.

Comparisons of the 16S rRNA and the *groEL* gene sequences of these Ehrlichia-like organisms revealed that this new bacterial species belonged to a new cluster in the family of *Anaplasmataceae*. More recently, new members have been added to the cluster of *Candidatus* Neoehrlichia: *Candidatus* Neoehrlichia lotoris (detected in raccoons from North America)[100]; *Candidatus* Neoehrlichia sp. FU98 (closely related to *Candidatus* Neoehrlichia lotoris and found in red foxes, a badger and one *Ixodes rugicollis* tick from Europe)[101,102]; *Candidatus* Neoehrlichia "Tanzaniae", detected in an immune competent young women from Austria believed to have contracted a febrile infectious disease in Tanzania[103]; *Candidatus* Neoehrlichia australis and *Candidatus* Neoehrlichia arcana, both described in *Ixodes holocyclus* ticks from Australia[104].

Epidemiology

The main vector of *Ca.* N. mikurensis is the *Ixodes ricinus* tick. This tick is widely distributed in Europe and many studies have shown that *Ca.* N. mikurensis is the third most common tick-borne pathogen in Europe after *Borrelia* and *Rickettsia species*[105,106]. *Ca.* N. mikurensis has been detected in ticks and rodents in several European countries, but also in China, Japan, Russia, Mongolia and South Korea[107,108]. So far, no *Ca.* N. mikurensis has been reported in ticks, humans or mammals from either the United Kingdom (UK) or America. The bacterium has been identified in *I. ricinus* collected both from the vegetation (prevalence of infection between 0.1-24%) and from wild and domestic vertebrates (0.3-31%)[108]. It has also been shown that *Ca.* N. mikurensis-infected ticks can be co-infected with other bacterial pathogens where *Borrelia burgdorferi* sensu lato seems to be the most common agent[108]. Besides detection in ticks belonging to the *Ixodes species,* it has been reported that this agent has been found in *Dermacentor reticulatus* in Germany[109,110] and in *Haemaphysalis concinna* in Mudanjiang,

China[111], but these few cases have probably no epidemiological relevance in the transmission of *Ca.* N. mikurensis.

The prevalence rates of *Ca.* N. mikurensis in ticks and wild animals seem to follow a seasonal pattern with low or no prevalence in May, increasing rates in June and July and the highest peaks in August (Germany)[112], September (Sweden)[113] or October (Netherlands)[114]. The prevalence of *Ca*. N. mikurensis in wild rodents varies between 1.1-27 % in China, 0-100% in Europe (4-19% in Sweden)[115].

Pathogenesis and transmission

Ca. N. mikurensis has been found in all stages of *Ixodes ricinus* ticks even if reports of detection of the bacterium in the larval stage are questionable since no vertical transmission seems to occur. [116,117]. The high prevalence of *Ca.* N. mikurensis in wild rodents (double that of prevalence in ticks) indicates that rodents are the reservoir of the infection that ensure the survival of *Ca*. N. mikurensis in the environment [112].

Several species of wild rodents has been described acting as reservoirs for the bacterium e.g voles, mice, rats and chipmunks[91,112,118,119]. The rodents do not seem to get affected by the bacteria and they are able to clear the infection by themselves[120]. The only known animal besides humans that gets symptomatic infection by *Ca.* N. mikurensis is the dog[121,122].

The transmission of *Ca.* N. mikurensis to humans is believed to occur via tick bites. The bacterium is inoculated through the skin via the bite of an infected tick. Many patients recall tick bites but this is not always the case, which is a known phenomenon with tick-borne infections[123]. Theoretically, it is possible that transmission of the infection can occur by blood transfusion, which has

been reported by other agents in the family of *Anaplasmataceae*[25,124].

The target cells of *Ca.* N. mikurensis in humans have been suggested to be both granulocytes and endothelial cells[91,125]. Recently, we published the successful cultivation of this agent in human endothelial cell lines, which has been our first choice of target cells since there is a high incidence of vascular events in patients diagnosed with neoehrlichiosis[93]. The endothelial cell lines were derived from skin microvasculature and the pulmonary artery, respectively, and the infection was transferred from infected tick cell lines.

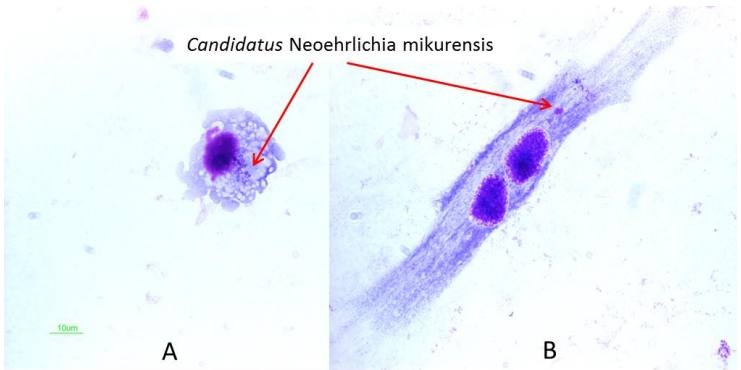

Figure 5 Visualization of *Candidatus* Neoehrlichia mikurensis infection in cell lines
A) Giemsa-stained cytocentrifuged smears of tick cells (IRE/CTVM20) infected *in vitro* with *Candidatus* Neoehrlichia mikurensis. B) Giemsa-stained preparation of a cutaneous microvasculature endothelial cell infected *in vitro* by *Candidatus* Neoehrlichia mikurensis. Arrows indicate bacterial inclusions. Photo by L.Wass ©

Figure 5A displays cells from the tick cell line IRE/CTV20 (tick cell line derived from *Ixodes ricinus*) infected with whole blood from a patient diagnosed with neoehrlichiosis. The photo is taken 8 weeks after inoculation. Small coccoid bacteria in cytoplasmic inclusions close to the cell nucleus can be visualized. This is the

same location that Yabsley et al. described for *Candidatus* Neoehrlichia lotoris, the closest relative of *Ca.* N. mikurensis[100].
The same phenomenon could be visualized in experimentally infected tick cells using Image flow cytometry (Fig. 6). The labelled bacteria appear in compact inclusions close to the host cell nuclei (Fig. 6). The close location to the host cell nucleus can be important for the bacteria for easy delivery of molecules (nucleomodulins) to hijack nuclear processes by targeting host DNA. Studies has indicate that nuclear delivery of factors by intracellular bacteria can sabotage host defences by directly interfering with transcription, chromatin-remodelling, RNA splicing or DNA replication and repair of the host cell[126].

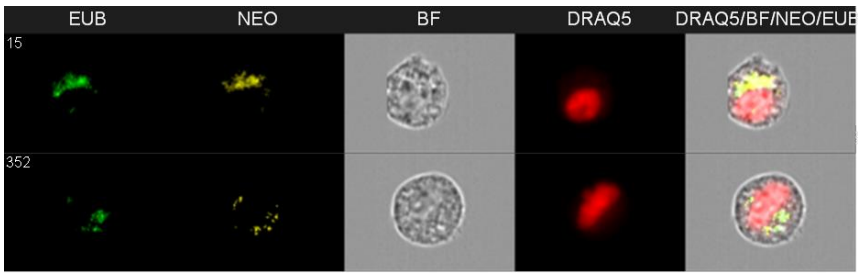

Figure 6 Image flow cytometer illustration of tick cells infected with *Candidatus* Neoehrlichia mikurensis. Tick cells from cell line IRE/CTVM shown after 25 weeks of inoculation with an infected blood sample from a human patient. The cells were labelled using panbacterial DNA probe (EUB) and *Candidatus* Neoehrlichia mikurensis-specific DNA probe (NEO). Red staining (DRAQ5) displays the host cell nucleus and the overlay image of all stains shows bacteria in the cytoplasm close to the host cell nucleus.

Our finding that endothelial cell lines are permissive for *Ca*. N. mikurensis infection together with our identification of the bacteria within so called "circulating endothelial cells" of infected patients strongly suggests that vascular endothelial cells are a target of the infection in humans[93]. Regarding the discovery by Pekova et al. demonstrating bacteria-like structures inside granulocytes of infected patients, it is possible that this finding reflected that the bacteria had been phagocytosed rather than infection of granulocyted by *Ca*. N. mikurensis[125]. If granulocytes were indeed the targets of this infection it should have been easier to localize the bacteria in

stained blood smears of infected patients. We have examined many blood smears from different patients and never detected any *morulae* or coccoid structures, nor have any other research groups. However, a close relative of *Ca*. N. mikurensis, *Ehrlichia ruminatium,* infects both vascular endothelial cells and circulating neutrophils so there is a possibility that *Ca.* N. mikurensis acts in the same way[127].

Clinical picture of neoehrlichiosis

The first case reports of human infection with *Ca.* N. mikurensis were published in 2010[128-130]. In 2014, we published an overview of the clinical picture of this new infectious disease, and together with our collaborators proposed to name this new infectious disease "neoehrlichiosis"[131]. Neoehrlichiosis is difficult to diagnose, especially among immune compromised patients, where the disease can mimic non-infectious conditions[131]. The most frequent symptoms in immune compromised patients are high remitting fever with chills and nightly sweats, migrating or localized pain (neck, temporal joint, knees, ankles or different muscles)[115,131].

Different types of skin rashes that can look like *erysipelas* or *erythema nodosum* can also occur[115]. There are patients that report less specific symptoms like diarrhoea, cough, and weight loss, most of which result from prolonged systemic inflammation[115]. However, the most severe findings among these patients are the high incidence of thromboembolic or vascular complications[115]. These events most often affect the venous part of the circulation, giving rise to deep vein thrombosis in the arms or legs but even pulmonary embolisms have occurred. Other frequent vascular complications are inflammation of superficial veins (thrombophlebitis) that can be extremely painful but there are also cases with severe arterial inflammation resulting in aneurysms or transitory ischemic attacks[128,131,132]. All these vascular events can result from infection of

the endothelial cells in the blood vessels and/or by the inflammatory response to the bacterial infection. In figure 7 the most common clinical signs in 27 diagnosed Swedish immune compromised patients are listed together with the most deviant laboratory parameters. The most typical laboratory findings among the patients are

Clinical sign	No. patients
Fever	27/27
Chills	11/27
Nightly sweats	4/27
Myalgia	11/27
Neck pain	7/27
Arthralgia	5/27
Headache	5/27
Painful subcutaneus veins	4/27
Abdominal pain	4/27
Deep vein thrombosis	11/27
Transitory ischemic attack	3/27
Pulmonary embolism	3/27
Other vascular/ thromboembolic events	3/27
Skin rash	8/27
Ankle edema	4/27

Laboratory parameter	Level
S-C-reactive protein	↑
White blood cell count	↑
B-Neutrophils	↑
B-Hemoglobin	↓
S-Sodium	↓
S-Lactate dehydrogenase	↑
B-platelet counts	↓
S-Aspartate aminotransferase	↑
S-Alanine aminotransferase	↑

Figure 7 Common clinical signs and laboratory parameters among immune suppressed patients with neoehrlichiosis. All patients have systemic inflammation demonstrated by high fever. Different types of pain, such as migrating muscle pain are also common. There are also a high rate of vascular and thromboembolic events. Increased C-reactive protein (serum), elevated blood cell count and anaemia are the most typical laboratory parameters. Abbrevations: B, blood; S, serum

increased acute phase reactant C-reactive protein (CRP) in serum, leukocytosis due to elevated levels of neutrophils, and anaemia. Moderately lowered platelet counts and slightly increased hepatic transaminases are less common[115].

Until recently, severe symptoms associated with neoehrlichiosis have mainly been seen in immune compromised patients. However, some of the last patients that we have diagnosed at our clinic, Clinical Microbiology Sahlgrenska University Hospital, have been

immune competent and some have been afflicted by severe vasculitis or thrombosis (data not published). All of the patients have been extensively investigated at their hospitals but none of them reported fever and they did not have abnormal laboratory parameters that pointed at infection. The patients also had lower loads of bacteria in their blood than what is seen among immune compromised patients. These are still unpublished cases but there is one published case from Germany with a fatal outcome of an immunocompetent patient[129]. This patient died due to an aneurysm of a cerebral artery that haemorrhaged. A post-mortem PCR analysis of a blood sample taken on the first day at the hospital showed infection with *Ca.* N. mikurensis.

The clinical picture of neoehrlichios in immune competent individuals can vary greatly. Asymptomatic or milder infection with fever and additional malaise, headache or stiff neck can be seen [111,133]. There are seven reported cases from Norway of immune competent patients who had EM after tick bite and where *Ca*. N. mikurenis was detected in blood, and no evidence of Borreliosis [134].

The incubation period between exposure to *Ca.* N. mikurensis and infection is still not clarified. It is hard to calculate since most of the patients have been investigated for their symptoms during a long time leading to considerable delay in diagnosis. A Chinese study suggests that the incubation period in immune competent persons has a range of 2-35 days[111]. Our two published immune competent patients had no measurable DNA levels in their blood when they attended the study at day 0. Both patients sought medical care because of rash after tick bites on day 77 respectively day 65, when *Candidatus* Neoehrlichia DNA could be detected. In immune suppressed patients, one would guess that the onset to infection would be shorter but many of our patients developed their first symptoms during the winter period[135]. This gives a rather long incubation time since no ticks are active in Sweden after November (ticks do not quest for host at temperatures below 5˚C).

Risk factors and immune response

Several factors make patients predisposed to develop severe neoehrlichiosis. Diseases like haematological malignancies (lymphoma and chronic lymphocytic leukaemia), autoimmune diseases (multiple sclerosis, or psoriasis) and rheumatic diseases (rheumatoid arthritis, systemic lupus erythematosus and granulomatosis with polyangiitis) have been described as underlying conditions[131,132,135]. Higher age, recent chemotherapy, corticosteroid treatment or treatment with rituximab is other risk factors. Rituximab is a monoclonal antibody directed against CD20 on B-cells and in Sweden this drug is used extensively among multiple sclerosis patients. Rituximab is also used to treat malignant B-cell lymphomas and a variety of systemic rheumatic diseases so the numbers of patients receiving this treatment is rather high. Splenectomy is also a predisposing factor for Neoehrlichial-infection; this organ is the largest lymphoid structure in the body with numerous immunological functions.

Many of the patients diagnosed with neoehrlichiosis have been splenectomised and have developed severe disease. The fact that many of the patients are splenectomised or have underlying diseases that affect the B-lymphocytes and are treated with rituximab (reducing the B-cells) indicates that B-cells are important immune cells to fight the infection. According to most of the literature, the adaptive humoral immune response which includes the antibody production from B-cells is not the first line defence against intracellular pathogens since the microbes are inaccessible for circulating antibodies[136]. However, Li et al. have shown that *Ehrlichia chaffeensis* can be found outside of host cells during an active infection, which makes them susceptible to antibody-mediated host defence explaining the importance of humoral immunity[137]. B-lymphocytes produce antibodies and convey immune memory. It is mainly neutrophils, macrophages and natural killer (NK) cells that mediate the innate immune response to intracellular pathogens. The

bacteria stimulate macrophages and dendritic cells to produce IL-12, which activates the NK-cells and they produce INF-ɣ that activate the macrophages, which promotes killing of the phagocytosed bacteria (Figure 8). However, innate immunity usually fails to eradicate the infection since intracellular bacteria are good at evolving strategies to resist elimination by phagocytes[138]. Patients with neoehrlichiosis have raised levels of leukocytes, in particular neutrophils[131]. This proves the involvement of these cells in infection with *Ca*. N. mikurensis probably both regarding their function as phagocytes but also as "cleaners" of dead cells and bacteria.

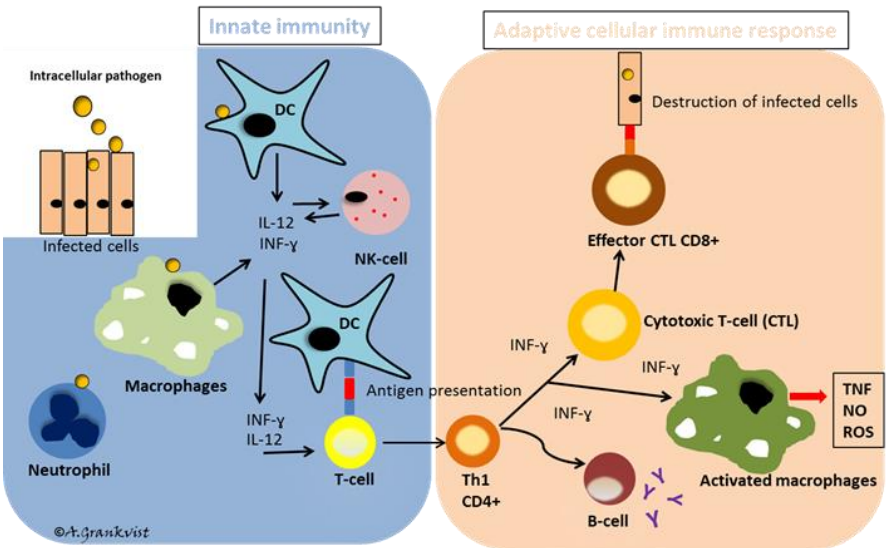

Figure 8 The immune response against intracellular pathogens
The innate immune response consists mainly of phagocytic cells, e.g. macrophages and neutrophilic granulocytes, natural killer (NK) cells and dendritic cells (DC). To eliminate the infection activation of adaptive cell-mediated response with T-cells is necessary. Abbreviations IL, interleukin; INF, interferon, TNF, tumour necrosis factor; ROS, reactive oxygen species; NO, nitric oxide.

Eradication of intracellular bacteria requires adaptive cell-mediated immunity[139]. This includes activation of macrophages by INF-ɣ produced by Th1 cells, which enhances the ability of the macro-

phage to produce substances like reactive oxygen species (ROS), nitric oxide (NO) and a large number of antimicrobial peptides, resulting in efficient killing of microbes that have managed to survive inside of phagocytes (Figure 8). INF-ɣ production by Th1 cells also stimulates the production of antibodies and the activation of cytotoxic T-cells (CTL) that eliminate infected cells mainly by cell-cell interaction but also by release of cytotoxic granules[140,141].

Diagnosis

Many of the patients diagnosed with neoehrlichiosis have been investigated for long periods of time to find the cause of their symptoms; the longest period among the patients that were diagnosed at the Department of Clinical Microbiology at Sahlgrenska Hospital was 1.5 years. Several patients have also been treated with various broad-spectrum antibiotics before they were diagnosed with neoehrlichiosis[131]. The delay of diagnosis probably has several reasons. (1) The bacterium does not grow in cell-free media such as blood culture bottles. (2) This is still a rather new infectious disease so the awareness among physicians is still very low. (3) The symptoms are often interpreted to be non-infectious and attributed to underlying diseases (recurrence of a hematologic malignancy or bout of systemic rheumatic or other autoimmune disease). (4) The diagnosis currently relies on molecular techniques such as pan-bacterial or specific PCR, which are only available at a few clinical laboratories.

Pan-bacterial PCR directed against the 16S rRNA gene is the most widely available diagnostic option for *Ca.* N. mikurensis at the moment. This assay is performed in clinical laboratories both in Sweden and in the wealthy parts of the world. Specific PCR done on clinical samples that is directed against the housekeeping genes *groEL* or 16S rRNA of *Ca*. N. mikurensis is in Sweden currently only analysed at the Department of Clinical Microbiology at

Sahlgrenska University Hospital. We have chosen to include a positive control plasmid in the PCR to enable calculation of the bacterial burden in analysed samples. Several studies have shown that infected immunosuppressed patients have a very high bacterial burden in blood[131,142]. The types of human samples that have yield positive PCR results in neoehrlichiosis patients so far are blood components (plasma, serum, whole blood and blood culture bottle contents), bone marrow and a skin biopsy [143]. Our experience is that the specific Neoehrlichia PCR has a better sensitivity in plasma than in whole blood. So far, no one has reported that *Candidatus* Neoehrlichia DNA has been detected in cerebrospinal fluid even if neurological symptoms have been observed in a few patients[131].

Treatment

The recommended treatment for neoehrlichiosis is oral doxycycline, 100 mg twice daily for three weeks. In case of hypersensitivity to doxycycline, Rifampin (300 mg twice a day for 3 weeks) also gives a complete response[115].We have also information regarding treatment failure when using a too low dose of doxycycline (100 mg once daily for 10 days preceded by a 200 mg start-up dose). The patient in question cleared his fever (which was deemed to be of uncertain origin and the treatment was empiric) and rapidly improved. However, 40 days after treatment the patient regained fever and stiff neck and follow-up PCR was positive for *Ca.* N. mikurensis. After adequate treatment with 200 mg doxycycline daily for 3 weeks, the patient achieved full recovery and the PCR was negative[24]. Most cases with neoerhlichiosis improve rapidly once given targeted antibiotics.

Aims

The aims of this thesis were:

- To map this new infectious disease with respect to what types of patients that are afflicted, the clinical picture displayed by the patient categories, and the pattern of laboratory findings seen in infected patients.
- Investigate if *Candidatus* Neoehrlichia mikurensis is only an opportunist that afflicts immune compromised patients or if immune competent persons can become sick.
- Establish the genetic relatedness of *Candidatus* Neoehrlichia mikurensis strains derived from patients in Europe.
- Perform *de novo* whole-genome sequencing of *Candidatus* Neoehrlichia mikurensis

Methods, Results and Discussion

Paper I

Data collection

Adult Swedish patients with possible ongoing *Ca.* N. mikurensis infection were recruited to the NEO-VÄST study. That is: a) patients that displayed at least one sign of systemic inflammation (fever, elevated inflammatory parameters such as C-reactive protein, erythrocyte sedimentation rate, white blood cell counts, etc.) b) among immunocompromised patients, e.g. haematology and rheumatology patients, and c) those with suspected recurrence of underlying disease but where something seemed amiss.

Study patients were examined by a physician according to a study protocol and various symptoms and clinical signs were noted as well as possible exposure to ticks. Host factors such as age, gender, underlying disease, immunosuppression including lack of spleen, and ongoing medication were registered. Blood samples were collected for clinical microbiological diagnosis of *Ca.* N. mikurensis, and blood chemical work-up. Serum samples were stored frozen for later immunological analyses. The Local Ethics Committee in Göteborg (394-12), Sweden approved the study in 2012. In paper I, all of the Swedish patients were from the NEO-VÄST study. Data, symptoms, follow-up and origin of the non-Swedish patients in this paper had been published before[125,129,142]. From the Swedish patients, the clinical and laboratory data were mainly derived from the patient's journals but also from attending physicians. Medical histories were reported by the patients themselves. GraphPad Prism 5.0 software was used for calculations.

Polymerase chain reaction

The diagnosis of neoehrlichiosis was based on PCR analysis of blood samples in all cases. EDTA-blood from the Swedish patients was processed at the Department of Clinical Microbiology Laboratory as follows: the plasma was concentrated (1600 x *g* for 5 minutes) and then DNA was extracted by a MagnaPure compact extraction robot (Roche, Basel, Switzerland) according to the manufacturer's protocol. This was not done for patient 1's sample, which was extracted manually with enzymatic treatment as previously published [144]. The DNA samples were then analysed with pan-bacterial PCR targeting region V1-V4 of the 16S rRNA gene (base pairs 23-806), followed by Sanger sequencing as described previously[144]. The sequences were analysed with the GeneBank-BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and showed 100% similarity to *Candidatus* Neoehrlichia mikurensis. For the patients diagnosed in Germany and Switzerland, almost the same procedures were performed; extraction from blood with extraction robot EZ1 (Qiagen, Hilden, Germany) in Switzerland or manually by QIAamp DNA mini kit (Qiagen, Hilden, Germany) in Germany, and pan-bacterial PCR against V1-V4 (base pairs 10-806 in Switzerland and base pairs 8-806 in Germany) followed by sequencing[129,142]. For the patients that were diagnosed in the Czech Republic, the extraction technique of the peripheral blood samples was not reported and the pan-bacterial PCR was against region V4-V8 (base pairs 783-1389) of the 16S rRNA gene[125].

Bacterial DNA loads were calculated in 5 patients by comparing cycle threshold (CT)-values from real-time PCR with standard curve generated from serial dilutions of a positive plasmid control. This was performed for two patients from Switzerland with the use of a *Ca.* N. mikurensis specific real-time PCR towards the 16S rRNA gene[142] and for three Swedish patients with a *Ca.* N. mikurensis specific real-time PCR against the heat shock protein *groEL*[145].

Results and discussion

This study was performed to make physicians aware of this new infectious disease that chiefly afflicts patients with certain autoimmune diseases or hematologic malignancies. The goal was to map this new disease to inform about neoehrlichiosis to avoid unnecessary suffering for affected persons. Many of these patients had been investigated by several different specialists during a long time before they were diagnosed with neoehrlichios. All but one of the patients had been hospitalized and two were admitted more than once. Several of the patients received immune suppressive therapy as a treatment against their symptoms since no infectious agent was discovered and the condition with systemic inflammation indicated recurrence of the underlying morbidity.

The 11 investigated patients in this study were all middle-aged or elderly. All patients had underlying diseases that influence the adaptive immune system (Table 2). Many of the patients (8/11) had no spleen, which in most cases had been removed due to their underlying disease. All patients had received some type of chemotherapy and/or immune suppressive treatment (7/11 systemic corticosteroids, 5/11 Rituximab, 4/11 Cyclophosphamide and 1/11 Methotrexate). Despite the high administration rate of immune suppressive treatment and the diagnostic delay, no patients died of the infection. The patients had high loads of bacterial DNA both in blood and in bone marrow (median value 2 x 10^6 bacterial gene copies/mL). This calculation is based on the assumption that *Ca*. N. mikurensis contains only 1 copy of the 16S rRNA gene and of the *groEL* gene, respectively. This theory seems to be correct since we have now managed to perform *de novo* whole-genome sequencing and annotation of these genes (paper IV). The heavy burden of bacteria in these patients probably reflects their impaired immune state since we haven't noticed these low CT-values among immune competent patients.

Table 2 Host factors of the studied patients diagnosed with neoehrlichiosis

Patient	Age	Sex	Hematological malignancy	Autoimmune disease	Asplenic
1	77	M	B-CLL		Yes
2	75	M	B-CLL		Yes
3	67	F	Later developed Follicular lymphoma	SLE	Yes (inborn)
4	67	F	T-LGL	Psoriasis artropathy	Yes
5	54	M	None	Psoriasis	No
6	59	M	DLCBL	Rheumatoid arthritis	Yes
7	68	M	CLL		Yes
8	58	M	Follicular lymphoma		No
9	55	F	Mantle cell lymphoma		Yes
10	58	M	PTLD	Sclerosing cholangitis	Yes
11	69	M	None	Chronic inflammatory demyelinating polyneuropathy	No

Abbreviations: M, male; F, female; B-CLL, B-cell chronic lymphocytic leukemia; SLE, Systemic lupus erythematosus; T-LGL, T-cell large granular lymphocyte leukemia; DLCBL, diffuse large B-cell lymphoma; CLL, chronic lymphocytic leukemia; PTLD, post-transplant lymphoproliferative disorder.

All patients had high fever with peaks of up to 40˚C and many experienced chills and nightly sweats. The majority (8/11) was bothered by different types of localized pain (migrating muscle pain, stiff neck, joint pain and tender subcutaneous veins). Less common symptoms were skin rashes of the lower extremities, diarrhoea, swollen ankles, cough and weight loss. The pain in subcutaneous veins and the swollen ankles was probably due to infection of endothelial cells since it has been established that endothelial cells are one target cell of infection for *Ca.* N. mikurensis[93]. The most severe findings among these patients and the clinical sign that seems to characterize neoehrlichios are the vascular and thromboembolic events that occur among many of the patients (6/11 in this study). Two patients had deep vein thrombosis above the knee, one patient developed deep vein thrombosis in both the upper arm and leg and one patient developed deep vein thrombosis, pulmonary embolism and transitory ischemic attacks. There was an additional patient with transitory ischemic attacks and both patients had re-

peated ischemic episodes that engaged both sides of the body. One patient also suffered from severe arterial inflammation with aneurysms.

The pathogenic mechanism behind these complications probably depends on infection of the endothelial cells. But there may be additional pathogenic mechanisms involved. Blood clot formation can be a process to limit an infectious process that cannot be controlled by the immune system[146]. The vascular complications may indicate that the depressed B-cell immune responses displayed by many of these patients might reflect that B-cells are important for elimination of this bacterium. It has been shown that B-cells and antibodies can play an important role in host defence against an intracellular infection, and not only T-cell immunity, which is considered as the main defence mechanism[147].

A well-functioning spleen in the fight of the infection is probably also important since many of the patients lacked the spleen. Since the spleen is one of the sites where natural IgM antibodies are produced, an important part of the innate immune system, we investigated whether patients with severe neoehrlichiosis might have low levels of such antibodies. However, half of the patients had depressed levels, and half had normal levels of natural IgM antibodies, and no relationship with splenectomy was seen[148]. We speculate that the main function of the spleen in the fight against neoehrlichiosis infection may be the production of specific IgM and IgG from memory B-cells[148].

The only consistent laboratory finding among the patients was the raise of the acute phase reactant C-protein in serum (19-370 mg/L; reference level <5 mg/L). Raised levels of white blood cells (9/11) and of serum lactate dehydrogenase (5/11) with anaemia (9/11) and hyponatremia (6/11) were also common parameters. Lowered platelet counts and elevated liver enzymes were rare findings.

These are common laboratory finding among patients with systemic inflammation and nothing that is specific for neoehrlichiosis, which also makes these patients more difficult to diagnose. Diagnosis currently relies on pan-bacterial or specific PCR, which are not used as first-line procedures in febrile patients, especially not on blood samples, where cultivation in cell-free blood culture bottles is the gold standard.

All patients in this study recovered within a week after doxycycline therapy was initiated. It was seen that 100 mg doxycycline twice daily for a 3-week period is sufficient to clear the infection and today when we have more experience, we know that the dosage used for empiric treatment of infections of unknown etiology (100 mg daily for 10-14 days) is not enough to clear the infection with *Ca.* N. mikurensis as documented for one neoehrlichiosis patient who relapsed[24].

Paper II

Patient population

The Tick-Borne Diseases (TBD) STING-study was initiated in 2007 by researchers at Linköping University[149]. The aim of the study was to investigate the prevalence of pathogens in ticks that have fed on humans and the risk of developing tick-borne diseases after a bite by an infected tick. Blood samples (EDTA blood and serum) from tick-bitten persons residing in Sweden or the Åland Islands, Finland were collected. The patient samples, two different health questionnaires, and the ticks that had bitten the persons were collected at the study's 34 primary health care centres, both at the time of inclusion in the study and at the end of the 3-month study period. If study participants developed symptoms during the study period they were asked to visit a health care centre where a physi-

cian examined them, filled in a questionnaire, and collected new blood samples. The patients were treated with antibiotics at the doctor's discretion. The study was approved by the Ethics Committee of the Medical Faculty, Linköping University (M132-06), and by the local Ethics Committee of Åland Health Care, 2008-05-23. All patient samples in paper II were frozen plasma or serum samples from patients recruited to the STING-study. We analysed the 102 out of 3248 participants who had sought medical care during the 3-month study period.

Polymerase chain reaction

Bacterial DNA was extracted from 400 µl of EDTA-plasma with a MagnaPure Compact extraction robot (Roche, Basel, Switzerland). A newly designed real-time PCR for clinical use, specific for *Ca.* N. mikurensis were used. The PCR was developed based on homology analysis of the *groEL* genes present in public databases. Detailed methodology is described in paper II. A 169-bp segment of the house keeping gene *groEL* was selected and a Taqman probe with fluorescent label fluorescein (FAM) was created. The specificity of the assay was difficult to test because of the lack of DNA from closely related species. Therefore we decided to confirm all positive samples with 16S-rRNA PCR followed by sequencing (described in paper I). The analytical sensitivity of the assay was determined using a synthetic plasmid that contained the 169-bp sequence of the *groEL* gene cloned into a pUC57 vector (Genescript, Piscataway, NJ, USA) and the limit of detection was 4 copies of the *groEL* gene. To evaluate the suitability of the specific assay in the routine diagnostic laboratory, patient specimens from earlier diagnosed neoehrlichiosis patients were tested and compared to the results of the pan-bacterial 16S rRNA PCR assay and complete concordance was shown. By generating a standard curve from 10-

fold serial dilutions of the positive control plasmid we were able to estimate the number of *groEL* gene copies in patient samples.

Anaplasma phagocytophilum serology

A commercial indirect immunofluorescence antibody (IFA) assay for analysis of IgG antibodies to a human isolate of *A. phagocytophilum* (Focus Diagnostics, Cypress, CA, USA) was used according to the manufacturer's recommendations. Diluted patient serum (1:64) was placed on a glass slide coated with *A. phagocytophilum*-infected HL-60 cells and incubated. After incubation the slide was washed and each well overlaid with fluorescein-labelled antibody to human IgG and incubated. The slide was then washed, mounted and examined by fluorescence microscopy. Positive reactions appear as green fluorescent dots within the cells. Serum samples were regarded as positive at a dilution of 1:64.

Borrelia serology

Patient serum samples were analysed for antibodies against *Borrelia burgdorferi* sensu lato using the RecomBead Borrelia IgM and IgG Kit (Mikrogen Diagnostic, Neuried, Germany) according to the manufacturer's protocol. This is a luminex-based immunoassay to detect antibodies against seven different *Borrelia* antigens and the technique is described in Figure 9.

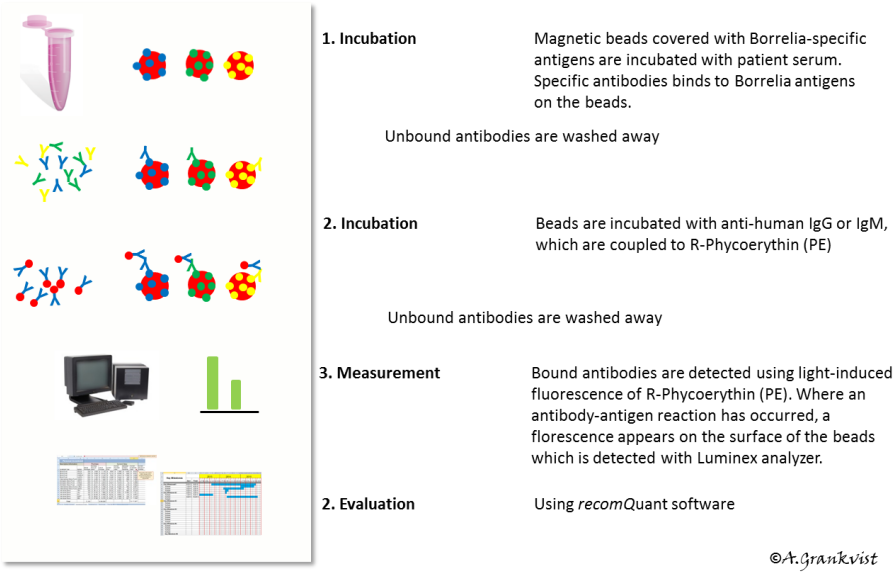

Figure 9 The luminex technique used for Borrelia serology. Patient serum is incubated together with magnetic beads covered with Borrelia specific antigens; OspC (outer surface protein C), OspA (outer surface protein A), VlsE (variable major protein-like sequence-expressed), p100, p58, p18 and p39. The antibodies in the patient serum bind to the antigen on the beads. After several washes the beads are incubated with anti-human IgG or IgM coupled to R-Phycoerythin (PE). After a new round of washes bound antibodies are detected using fluorescence of PE, if a reaction between antibody-antigen has occurred. The results are evaluated in accompanying software, *recom*Quant.

Cytokine measurements

Cytokine levels in patient serum samples were analysed by using Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA). A total of 20 cytokines levels were measured by a luminex-based multiplex system[150]. Antibody directed against the cytokine of interest is coupled to color-coded polystyrene beads. The antibody-coupled beads can react with a serum sample containing an unknown amount of cytokine, or with a standard solution containing a known amount of cytokine. After a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the cytokine is added to the beads. The result is formation of a cluster of antibodies around the cytokine. The reaction mixture is detected by the addition of streptavidin-phycoerythrin (PE) which binds to biotinylated detection antibodies. The cytokine contents in each test well are drawn up into the array system, which identifies and quantitates each specific reaction based on bead colour and fluorescence. Unknown cytokine concentrations are automatically calculated by Bio-Plex Manager software (Bio-Rad, Hercules, CA, USA) using a standard curve derived from a recombinant cytokine standard[150]. Calculation of cytokine levels were done with GraphPad prism 5.0.

Results and discussion

The goal of this study was to investigate if *Ca*. N. mikurensis in addition to being an opportunist that afflicts immune compromised patients might also give rise to disease in persons with normal immune defences. We had the opportunity to collaborate with Linköping University and were able to analyse blood samples collected from healthy tick-bitten individuals participating in the STING-

study. In this study we focused on study persons who had sought medical care during the 3-month study period.

Ca. N. mikurensis DNA was detected in 2 (2%) of the 102 investigated patients from the STING study. The first patient was a healthy 68-year old woman from southeast Sweden. She developed a red rash after a tick bite on her right breast 77 days after she entered the study. *Ca*. N. mikurensis DNA was detected on day 77 (2200 gene copies/mL) and on day 169 (200 gene copies/mL). She also developed IgM against *Borrelia* outer surface protein C (OspC) on day 77 and had pre-existing Borrelia-specific IgG titers that expanded from the inclusion to day 169. She also had unchanged IgG against *A. phagocytophilum* from day 0 to day 169. At the health care visit on day 77, the patient was given the diagnosis of *erythema migrans* and received phenoxymethylpenicillin (1gx3d for 10 days) and the rash disappeared. The rash at the breast may have been caused by co-infection with a *Borrelia* spp. since the patient increased in the antibody titers during the study period.

Patient 2 was a 57-year old woman who lived in Kalmar, Sweden. She had a medical history of allergy and had been treated for borreliosis 8 years earlier. She sought medical care on day 65 for a red rash on the left breast after a tick-bite 1.5 month before. She had lower levels of *Ca*. N. mikurensis DNA (260 gene copies/ mL on day 65 and 1300 gene copies /mL on day 98) than patient 1 and was seronegative for Borrelia during the entire study period. She had increased antibody levels of IgG against *A. phagocytophilum* throughout the study (-/+ day 0, + day 65 and ++/day 98). She was also treated with phenoxymethylpenicillin (1g, 3x/d for10 days) for her rash. Despite being seronegative for *Borrelia* the rash might have been caused by a *Borrelia* spp. It is well-known that patients with Borrelia infections do not always develop specific antibodies[151]. However, it has also been shown that 20% of patients with *erythema migrans* lack detectable *Borrelia* DNA as determined by PCR performed on skin biopsies collected from the

rash[152]. This indicates that other tick-borne agents than *Borrelia* may be able to cause *erythema migrans*. Therefore, immune suppressed patients with *erythema migrans* might be considered to be treated with doxycycline instead of penicillin to avoid development of severe neoehrlichiosis. Patient 2 had increasing levels of IgG against *A. phagocytophilum* during the study but this seroconversion might reflect serologic cross-reactivity with *Ca*. N. mikurensis. Wass et al showed that every fifth neoehrlichiosis patient had low titers of *A. phagocytophilum* antibodies in the blood at the time of diagnosis[24].

Table 3 Analysed cytokines and their role.

Cytokine	Full name	Source	Biological role	Classification in this study
IL-12p70	Interleukin-12	Dentritic cells, Macrophages	Differentation of T-cells	Th1 cytokine
IFN-γ	Interferon-γ	Th1-cells, cytotoxic T-cells and natural killer cells	Activator of macrophages Differentiation of cytotoxic T cells	Th1 cytokine
IP-10	Interferon gamma-induced protein-10	Neutrophils and monocytes	Promotes the migration of activated Th1 cells	Th1 cytokine
MCP-1	Monocyte chemoattractant protein-1	Monocytes, macrophages and dendritic cells	Recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation	Th1 cytokine
IL-1ß	Interleukin-1ß	Macrophages	Cell proliferation, differentiation, and apoptosis	Proinflammatory
IL-6	Interleukin-6	Several cells but especially Macrophages	Induces fever, production of cortisol and stimulates antibody production	Proinflammatory
TNF-α	Tumor necrosis factor-α	Macrophages	Induces fever and induction of cytokine secretion	Proinflammatory
MIP-1ß	Macrophage inflammatory protein-1β	Neutrophils, monocytes, B cells and T cells	Chemoattractant for natural killer cells, monocytes and other immune cells	Proinflammatory
IL-8	Interleukin-8	Macrophages, endothelial cells and others	Induce chemataxis and activation of neutrophils	Neutrophilia
IL-17A	Interleukin-17A	Activated T cells	Attracts neutrophils and acts as a mediator in several chronic inflmmatory diseases	Neutrophilia
G-CSF	Granulocyte-colony stimulating factor	Fibroblast in bone marrow	Stimulates the survival, proliferation, differentiation, and function of neutrophils	Neutrophilia
GM-CSF	Granulocyte-macrophage colony-stimulating factor	Fibroblast in bone marrow	Stimulates stem cells to produce granulocytes and monocytes	Neutrophilia

Reference; Inflammation, Mölne and Wold 2007 [153]

This was also shown for an immune competent neoehrlichiosis patient in Switzerland[130]. Since there are relative high rates of seropositivity to *A. phagocytophilum* in Sweden in the order of 11-17%[19,24] despite a rather low prevalence in ticks (1.3-15%), this seropositivity may depend on cross-reactivity to *Ca*. N. mikurensis, which is more frequently detected in Swedish ticks (6%)[120].

The cytokine levels in serum were raised in both patients for 12 cytokines (Table 3[153]). Out of the studied cytokines, 8 cytokines showed concentrations that were very low or under the detection limit and were therefore not further studied (IL-1ß, IL-2, IL-5, IL-10, VEGF, PDGEF-BB, IL-13 and IL-4). An interesting finding was that the cytokine levels seemed to mirror the numbers of *Ca*. N. mikurensis gene copies (Paper II). The cytokine response in patient 1 may have been caused by *Borrelia* spp. since this patient had a slight increase in antibodies against *Borrelia* spp during the study. However, this patient also had the highest concentration of *Ca*. N. mikurensis (2,200 gene copies/ mL) in blood which coincided with peak levels of Th1 cytokines (IL-12p70, IFN-ɣ, IP-10 and MCP-1). This indicated that the cytokine response substantially depended on the *Ca*. N. mikurensis infection since Th1-like immune responses are required to eliminate intracellular pathogens[140].

Both these patients were immune competent and as the most severe forms of neoehrlichiosis have so far mainly been detected in immune suppressed patients with diseases that affect the B-cells (paper I), our findings may be taken to support that B-cells are important for the defense against *Ca*. N. mikurensis since the patients in this study cleared the infection by themselves. Further studies on cytokines levels from these two different patient groups (immune suppressed vs immune competent) would be interesting to understand if there are dissimilar infectious defense mechanisms in these two patient groups. In this study we have besides the cytokines involved in Th1-like response also selected cytokines based on findings derived from neoehrlichiosis in immune suppressed

patients, who present with systemic inflammation (IL-1ß, IL-6, TNF-α and MIP-1ß), and with neutrophilia (IL-8, IL-17A, G-CSF and GM-CSF). This study has shown that immune competent persons may be asymptomatically infected by *Ca*. N. mikurensis during a very long period and that an erythematous rash in tick-bitten persons in Sweden might be caused by *Ca*. N. mikurensis. This has also been noticed in a Norwegian study were seven patients with EM had *Ca*. N. mikurensis DNA in their blood [134].

Paper III

Clinical samples

All Swedish patients in this study participated in the NEO-VÄST study described in Paper I. All patients were immune suppressed and had sought medical care due to prolonged fever and different symptoms consistent with neoehrlichiosis (Paper I). The patients were diagnosed with analysis of DNA (extracted from EDTA-plasma) by real-time PCR targeting the *groEL* gene (Paper II) followed by confirmation using pan-bacterial PCR with subsequent sequencing (Paper I). Details of the Czech and German patients have been described earlier [125,129] and extracted frozen DNA from these patients was kindly distributed by the scientists who authored the case studies in question.

Multilocus sequence analysis

A new bacterial strain is most often phylogenetically placed on the basis of 16S rRNA gene analysis to start with. However, there are disadvantages in using the 16S rRNA gene as a phylogenetic marker since it has insufficient resolution at species level[154]. A higher resolution can be obtained by performing phylogenetic analyses based on several protein-coding genes (housekeeping

genes) since they evolve at a slow but constant rate and have better resolution power, especially at the genus level but even below[154]. Internal fragments of these protein-coding genes are sequenced and subsequently used to calculate phylogenetic trees. Sequences that differ by even a single nucleotide for each gene are assigned as different alleles, making this kind of assay highly suitable for detecting genetic changes within and between species. In 2005, Gevers et al. introduced this type of genotyping method called multilocus sequence analysis (MLSA)[155]. MLSA is based on multilocus sequence typing (MLST) but in MLST the downstream analyses are based on allele numbers and sequence types to estimate relatedness among isolates and it ignores the number of nucleotide differences between alleles.

A critical point in MLSA studies is the selection of genes. It is clear that housekeeping genes coding for proteins with important functions should be considered because they are stable with respect to rapid genetic modifications, genes are often selected independently for each new taxon investigated[154,155].

Target loci and primer design

There were only a few limited genes that had been sequenced and deposited in gene banks for *Ca*. N. mikurensis (partial sequences of 16S rRNA, *gltA* and *groEL*)[91] at the start of this study. In order to establish a MLSA scheme we studied protocols developed for other species belonging to the *Anaplasmataceae* family, i.e., *Ehrlichia ruminantium*[156] and *Wolbachia pipientis*[157]. Alignments of 10 different whole-genome–sequenced bacterial strains belonging to the *Anaplasmataceae* (Table 4) were done for several genes and primers were designed manually. The primers were selected to cover regions where the different strains had corresponding nucleotide sequences. Selected genes and corresponding primer sequences are presented in paper III.

Table 4 *Anaplasmataceae* strains used for alignments and primer design

Bacterial strains	NCBI Reference Sequence
Ehrlichia canis strain Jake chromosome, complete genome	NC_007354.1
Ehrlichia ruminantium strain Welgevonden, complete genome	NC_006832.1
Ehrlichia ruminantium strain Gardel, complete genome	NC_006831.1
Ehrlichia chaffeensis strain Arkansas, complete genome	NC_007799.1
Anaplasma phagocytophilum strain HZ, complete genome	NC_007797.1
Anaplasma centrale strain Israel, complete genome	NC_013532.1
Anaplasma marginale strain St. Maries, complete genome	NC_004842.2
Anaplasma marginale strain Florida, complete genome	NC_012026.1
Wolbachia endosymbiont strain TRS of Brugia malayi, complete genome	NC_006833.1
Wolbachia species strain wRi, complete genome	NC_012416.1

Polymerase chain reaction and Sanger sequencing

In order to get optimal sequences, each PCR reaction were optimized and unique PCR protocols were established for each gene (paper III). All PCR products were purified (paper III) and subjected to cycle sequencing in both directions using BigDye Terminator v 3.1. All samples were analysed with Sanger sequencing using the ABI Prism 3130 genetic analyser (Applied Biosystems, CA, USA). Sanger sequencing is described in Figure 10. The sequences were edited and aligned as described in paper III.

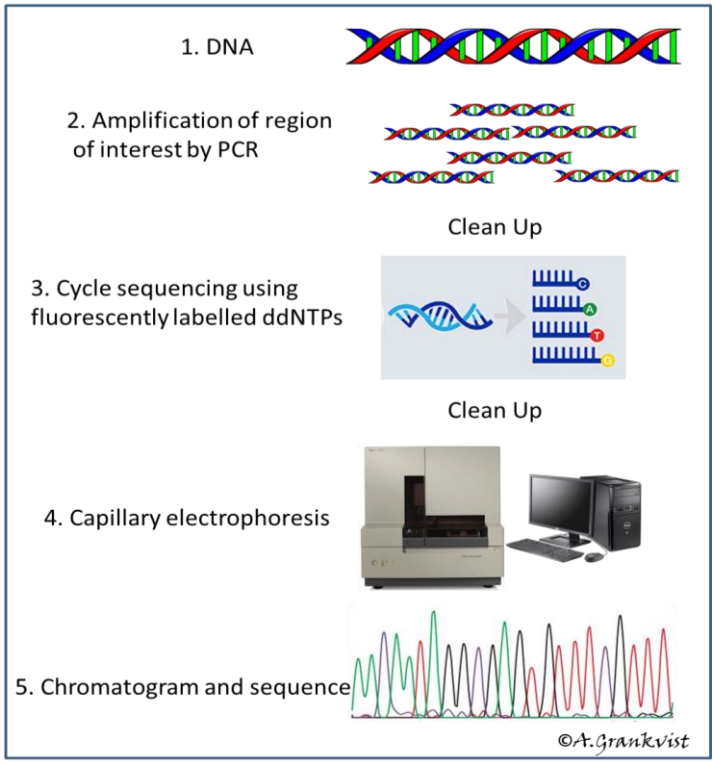

Figure 10 Sanger sequencing technology. 1) Extracted DNA, where the region of interest is amplified by PCR (2). The PCR-product is cleaned up and subjected to cycle sequencing (3) where incorporation of fluorescent dideoxynucleotides (ddNTP) occurs. This product is then cleaned up to remove excess primers and unincorporated nucleotides before it is loaded on an automated DNA-sequencing instrument for electrokinetic separation (4) where a laser reads the sequence and detects the fluorescent intensity that is translated into a "peak" and several peaks creates a sequence (5).

Results and discussion

In this paper we wanted to study the genetic relationship between strains of *Ca*. N. mikurensis that had caused infection in humans, as well as determine the phylogenetic relationship between other members of the family of *Anaplasmataceae* and this new agent. All 12 patients in this study were immune suppressed due to underlying diseases (hematologic, rheumatologic) and immune suppressive

therapy, i.e., rituximab or methotrexate. Three of nine Swedish patients were described in paper III (the rest were described in paper I) and the geographic location of all Swedish patients is shown in Figure 1, paper III.

Unweighted pair group method with arithmetic mean (UPGMA) was constructed individually for each gene. This showed overall low genetic diversity among the clinical isolates. There were only two deviations, one nucleotide exchange in the *lipA* gene at position 114 (T instead of C) seen in clinical isolates from three patients (SE01, SE02 and SE09) and one nucleotide substitution in the *clpB* locus (G instead of C) in a strain from one of the Czech patients, CZ01 (Figure 2 and 3, paper III). The nucleotide exchange in *clpB* locus was non-synonymous and resulted in an amino acid change of valine for leucine in position 421; of noteis that *E. ruminantium* also has a leucine in this position (paper III).

These results revealed three sequence types among the European clinical *Ca*. N. mikurensis isolates, one recovered in the western part of Sweden (with *lipA* exchange), one from central Europe (CZ1, with *clpB* exhange), and a pan-European genotype (with identical sequences in both genes). Loewenich et al. had previously reported sequence variations in the *groEl* and 16S rRNA genes among *Ca*. N mikurensis isolates from different kinds of hosts (ticks, rodents, dog and human) but this might reflect bacterial adaptation to survive in different host species[129]. However, Li et al. reported that different gene clusters were found in the same rodent species from different regions in China, which suggests that the genetic diversity might not be associated with different host species, at least not among Chinese strains[118]. The same authors also reported genetic differences between strains infecting humans in China and Europe, respectively, with regard to sequences of the *groEl* and the *rrs* genes[118]. The main vector of *Ca*. N. mikurenis in Europe is *I. ricinus*, whereas in China it is *I. persculcatus* that is the main tick species[158], which might explain the different se-

quence variants. The Chinese variant of neoehrlichiosis also seems to differ from the European one, since infected immune competent individuals in China appear to have more systemic inflammation with prominent fever than has been seen among European immune competent patients[111].

The MLSA scheme we developed for *Ca*. N. mikurensis was able to distinguish between *Ca*. N. mikurensis and other species in the family of *Anaplasmataceae*. We were at this point rather surprised that *E. ruminantium* was a closer relative to our agent than was *A. phagocytophilum* (Fig 4, paper III). *E. ruminantium* has so far not been found to cause infection in humans but is the agent of heartwater in domestic ruminants[75]. Today, when we know that *Ca*. N. mikurensis is capable of infecting endothelial cells, which is also the main target cell for *E. ruminantium* infection, this finding is not surprising[93].

This study has several limitations. The main challenge was to extract sufficiently high concentrations of *Ca*. N. mikurensis DNA from limited EDTA-blood volumes (collected before the patients were treated with doxycycline). The patients were selected because of their high loads of bacteria in their blood, which is needed for this type of DNA sequence-based analysis of multiple genes. The fact that all patients were immune suppressed and consequently had a more severe infection with higher amounts of bacteria, might have contributed to the low genetic diversity in this study. In this project it would have been interesting to compare the immune suppressed patients with the healthy individuals that carry the bacteria to see if the same strains infect both patients groups. But, we were not able to perform MLSA on strains isolated from immune competent persons because of insufficient amounts of bacterial DNA were recovered from them.

Another limitation is that this MLSA scheme was constructed based on multilocus sequence typing (MLST) protocols developed

for related species, which may not have been the best genotyping markers for *Ca*. N. mikurensis. MLST is usually applied for investigate relationship between strains that belong to a well-defined species and normally involves 7 housekeeping genes, which is more preferable for epidemiologic purposes[159].

Our suggestion in this publication was to perform this MLSA analyse once we had managed to cultivate this uncultivatable species, to obtain higher amounts of bacterial DNA and thereby be able to optimize and sequence additional genes. By now, when we have succeed to perform whole-genome sequencing directly from clinical samples (paper IV) we no longer believe this MLSA assay will contribute to any interesting findings. However, it was very interesting to note that the results from the whole-genome sequencing had a very good concordance with the MLSA results as the *Ca*. N. mikurensis whole-genome sequenced strains also showed very limited genetic variation.

Paper IV

Candidatus Neoehrlichia mikurensis isolates

EDTA-blood samples from three immune suppressed patients diagnosed with neoehrlichios were used for this study. Clinical data regarding these patients have been published before[93]. The embryo-derived tick-cell line IRE/CTVM20 (obtained from *I. ricinus*) was inoculated with whole-blood from a diagnosed patient (SE18) to establish a culture of this agent. The tick-cell line was incubated for 21 weeks and continuously monitored by PCR and image stream analysis to control the infection stage[93].

To be able to perform whole-genome sequencing, high-molecular weight DNA was extracted using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) described in paper IV. DNA yields and

purity were measured with Tape Station (Agilent Technologies, Santa Clara, California, USA) before proceeding to the 10X instrument.

10X Chromium technique

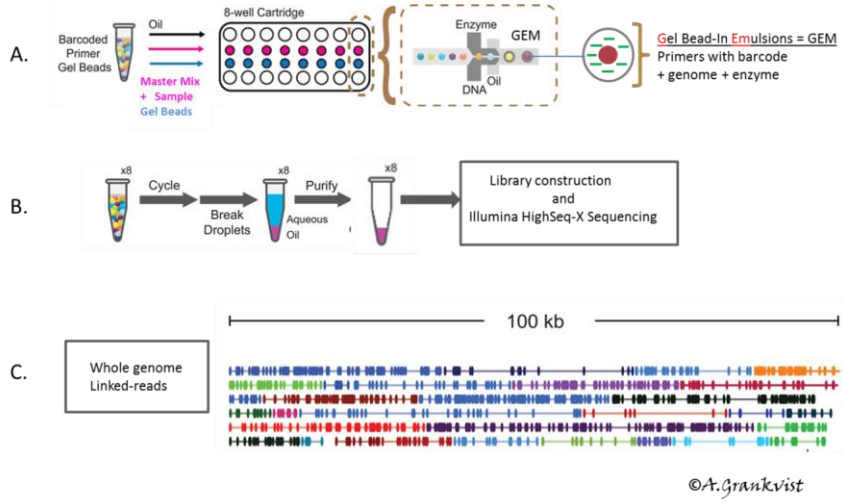

Figure 11 Overview of 10X Chromium technology. A) Gel beads loaded with primers labelled with unique barcodes are mixed inside an oil-droplet (GEM) with HMW DNA and enzymes. B) All GEMs are amplified and the droplets are dissolved and the products are purified and library is constructed and Illumina adapters are incorporated. The samples are then sequenced with Illumina technology (in this case Illumina HighSeq-X). C) Each line represents linked-reads with the same barcode. Picture is adapted from Zheng G. et.al. [161]

10X Chromium is a rather new microfluidic technology that has been developed to allow analysis of small amounts of input DNA, 1 ng of high molecular weight (HMW) genomic DNA is sufficient. Each input DNA fragment is incorporated into an individual gel-bead in emulsion (GEM), and subsequent biochemistry generates mini-libraries of NGS-ready molecules tagged with a barcode unique for each GEM (Fig 11)[160]. Barcode-tagged DNA molecules are released from each droplet and library preparation process is

performed. The resulting libraries then undergo standard Illumina short-read sequencing. A computational algorithm (Supernova assembler) uses the barcodes to link sequencing reads back to the original HMW DNA molecule[160]. The workflow for the technique is described in Figure 11[161].

Illumina sequencing technique

Illumina NGS workflows include four basic steps:

1. Library preparation: in this case, pooling of droplets, end-repair, and ligation of P7 sequencing adaptor. These adaptors have flow cell binding sites, which allow the library fragment to attach to the flow cell surface (the Illumina flow cell is the where the sequencing chemistry occurs)[162].

2. Cluster generation: the library is loaded into a flow cell and the fragments are hybridized to the flow cell surface. Each bound fragment is amplified in to a clonal cluster through bridge amplification. When cluster generation is complete, the templates are ready for sequencing[162].

3. Sequencing: the clusters in the flow cell are read one nucleotide at a time in repetitive cycles. During these cycles, fluorescently labelled dNTPs are incorporated into the growing DNA chain[162].

4. Data Analysis: in this study there were no reference sequences available for alignments. *De novo* sequencing refers to the sequencing of a novel genome where there is no reference sequence. The assembly of the genome and annotated genes is described in paper IV.

Results and discussion

The goal with this paper was to perform *de novo* whole-genome sequencing of *Ca*. N. mikurensis. These in order to gain new knowledge about the bacterium and maybe identify target molecules that can be used as diagnostic tools for this agent, and also acquire better understanding of its pathogenic mechanisms.

Surprisingly, no sequencing data were obtained from the tick-cell line cultures. The cultures we used were from one of the first attempts in culturing this agent and now, when we have gained more knowledge, we believe these cultures were harvested too late. The cell lines were heavily infected by the bacteria, which had probably started to undergo degradation. Our hypothesis had been that sequencing from cultures might be an advantage to achieve a higher amount of bacterial DNA, which had been the limitation in earlier whole-genome sequencing attempts of this agent. However, it is possible that the bacterium has host-specific attributes so that its virulence changes depending on the host[163]. Since, the culturing into tick-cell lines take more than 20 weeks for the infection to be established some genetic changes that enhance adaptation to a new host are likely to occur[93,164]. With these thoughts in mind it is better to sequence the bacteria directly from the infected human host if we want to study pathogenomics associated with severe complications in humans.

The sequencing of the clinical isolates from patient plasma was successful, despite their low contents of bacterial DNA after sequencing. The samples only contained 0.1 to 5.1% bacterial DNA sequences (the rest was human). The highest amount of bacterial DNA was enriched from the first sample obtained from patient SE24, which probably depended on the fact that the DNA was extracted from fresh plasma (the other plasma samples had been stored frozen before extraction). The successful sequencing outcome when using human plasma directly may depend on the fact

that contamination of the bacterial DNA with human DNA is advantageous when using 10X technology. The human DNA fragments are larger than the bacterial DNA fragment, allowing for the later to "hitchhike" on the larger DNA human fragment, which apparently protects and enhances the recovery and integrity of the bacterial DNA.

All sequenced patients in this study were immune suppressed. The immune suppressed patients have (as we know so far) the highest concentrations of bacteria in their blood and were therefore selected for this study. Patient SE24 (designated reference genome for *Ca*. N. mikurensis) had a high bacterial burden of 5.8×10^8 gene copies/mL blood (Paper IV, table 1). Now, when we became educated in how to sequence directly from clinical samples it would be interesting to perform whole-genome sequencing of strains isolated from immune competent individuals. Even if we are unlikely to obtain the entire whole-genome sequence from immune competent patients (depending on their lower bacterial burden), we now have a reference genome to compare sequence data with. It will now also be possible to study selected genes that could be involved in the pathogenesis of this agent.

The size of the genome of *Ca*. N. mikurensis was estimated to be 1.1 mbp and was found to contain 886 coding sequence (CDS) annotations, 924 gene annotations, including all six MLSA-genes, 36 tRNAs, as well as a single copy each of the genes encoding 5S rRNA, 16S rRNA and, 23S rRNA, respectively. Similar to previously sequenced members of the order *Rickettsiales Ca*. N. mikurensis has a single rRNA operon and the 16S rRNA gene is separated from the 23S-5S gene pair by 426 kb[84]. It is more common for bacteria to have several rRNA operons and it is has been proposed that the number of rRNA operons may contribute to the rapidity with which microbes can synthesize ribosomes and respond to changes in growth conditions[165,166]. It is also assumed that multiple copies of rRNA operons in prokaryotic organisms are re-

quired to achieve high growth rates and this can explain way it takes a long time to establish *Ca*. N. mikurensis infection in cell cultures, as well as the long incubation period of neoehrlichiosis in patients[166].

The locations of the previously identified MLSA genes are visualized in Figure 2 paper IV, and the characteristics of the *Ca*. N. mikurensis SE24 reference genome is summarized in Table 2, paper IV. *Ca*. N. mikurensis has one of the smallest genomes in the family *Anaplasmataceae* and similar to several members in the family it also has numerous repeats in the genome. So far, we have identified that *Ca*. N. mikurensis harbours three repetitive gene families (Table 3, paper IV). The expansion of the outer membrane protein (OMP) family we have identified, is also found by close relatives to *Ca*. N. mikurensis and may contribute to the cross-reactive antibodies to *Anaplasma phagocytophilum*[24]. Outer membrane proteins are a common theme among pathogenic bacteria, thought to be of importance for bacterial interactions with eukaryotic hosts. OMPs have a large repertoire of functions, including bacterial invasion, transportation of various molecules, adhesion of the bacteria to the host cell and signalling pathways[167].

Studies of the surface proteins of *Ca*. N. mikurensis will likely increase our understanding of the interaction of bacteria with their host cells. A very promising finding is that we have identified a surface antigen that seems to be specific for *Ca*. N. mikurensis (position 577689-578213) that could be a candidate antigen for the development of *Ca*. N. mikurensis-specific serological assays. If we are able to design an enzyme-linked immunosorbent assay (ELISA) with this surface antigen, we can easily study larger populations of humans and increase our knowledge regarding the prevalence of exposure of various groups of healthy individuals and patients to this emerging pathogen in different areas of Sweden. We might also be able to produce monoclonal antibodies to this surface antigen, which could prove to be a valuable tool for future

studies of the disease mechanisms and immune defences that are operative in neoehrlichiosis.

In this study we sequenced clinical isolates from three patients with neoehrlichiosis and the geographic origin of these patients are shown in Figure 1, paper IV. The genetic variation between the strains was very low, SE26 differed by 245 single-nucleotide variants (SNV) from SE24, and SE20 differed by 153 SNV from the SE24 reference. These findings support the results from the MLSA study that revealed an overall low genetic diversity among clinical isolates in Europe based on sequencing of six housekeeping genes (paper III). It would be interesting to whole-genome sequence all clinical strains that we have collected during the years (both Swedish and European) to see if the genetic variations are consistently low for this agent, and to determine the number of genotypes that exist. In addition, it would be of interest to sequence strains collected from diverse hosts to evaluate if there are genomic variations of *Ca*. N. mikurensis in different hosts.

This work will hopefully advance our understanding of the pathogenic mechanisms used by this emerging pathogen and also contribute to the development of new diagnostic assays to facilitate and improve the ability to diagnose patient suffering from neoehrlichiosis. A broader arsenal of diagnostic tools may contribute to identify patients with other clinical pictures and with distinct risk factors from those that are known at present.

Concluding remarks and future perspectives

The papers included in this thesis have contributed to a lot of new knowledge about this new human pathogen. In paper I we were able to describe this new infectious disease, termed neoehrlichiosis when we summarised new and earlier published cases regarding host factors, clinical picture, and laboratory findings. I think that this publication has been of great value to spread information of neoehrlichiosis and surely contributed to finding of new patients.

At the beginning we thought that neoehrlichiosis only caused infections in immune suppressed patients but the study described in paper II showed that immune competent patients can also become infected even if the symptoms are milder than those of immune suppressed patients.

Paper III was the firsts paper published where more than three genes of *Ca*. N. mikurensis were analysed regarding genetic diversity. Even if the method used in this paper has its limitations, especially now when we have succeed in sequencing the entire genome of *Ca*. N. mikurensis, the results in this publication have been supported by the whole-genome results.

The last work in this thesis, paper IV, will hopefully contribute to a lot of new knowledge about this agent and the genetics involved in causing severe infection in many patients. The results from this paper may also contribute to the development of new diagnostic tools that may prove to be of great value for diagnosing future patients.

The journey with discovering and characterizing a new human pathogen is fascinating and challenging. From the start, when we diagnosed one of the first human neoehrlichiosis patients in the world, to developing clinical assays that up to date have diagnosed

39 Swedish patients (paper II) makes me proud. Many of the patients that we have diagnosed during these studies have been in really bad condition as a result of the infection and most of them have recovered completely when they finally got their diagnosis and could receive the correct treatment. The main goal with this thesis has been to spread the word about this new infectious disease and we have managed to take a step in right direction. The work does not end here; we have a lot of more questions to answer and an inexhaustible hunger for knowledge regarding (*Candidatus*) Neoehrlichia mikurensis!

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