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Leishmaniasis in Sweden

Molecular, diagnostic and epidemiological studies of the parasite *Leishmania* in a nonendemic country

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Molecular, diagnostic and epidemiological studies of the parasite *Leishmania* in a non-endemic country

Sara Karlsson Söbirk

LUND UNIVERSITY

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Abstract:

Leishmaniasis is one of the Neglected Tropical Diseases that primarily affects the most vulnerable population in tropical and subtropical parts of the world. The incidence and characteristics of patients with leishmaniasis in Sweden was not previously known, and evaluations of current diagnostic methods had not been performed in a patient population corresponding to a non-endemic country with imported cases of leishmaniasis from different regions.

In a retrospective, epidemiological register study, we present the first estimation of annual incidence of leishmaniasis in Sweden 1993-2016 (0.023 to 0.35 per 100 000/year), and found, in concordance with reports from other non-endemic countries, an increase in incidence the last years of the study period. We describe patient characteristics that may be useful for identification and correct diagnosis of future patients with leishmaniasis presenting to Swedish health care units. In our evaluation of five serological tests for visceral and mucocutaneous leishmaniasis, the tests showed lower sensitivities and specificities in our selection of samples than in previous publications, performed in endemic countries.

In search for effective vaccine- and drug candidates, the interaction between *Leishmania* parasites and the human immune system is of great interest. We demonstrated with different methods, that several human chemokines have a direct antimicrobial activity on promastigotes of *Leishmania mexicana*, not mediated through their effects on leukocytes. This finding is the first proof of direct antiparasitic effect of chemokines on eukaryotic parasites, and encourages further studies of chemokines and chemokine receptors as possible drug targets. We further explored the group of enzymes called glycoside hydrolases in the published genomes of *Leishmania spp*. Including the conserved chitinase previously described in *Leishmania mexicana*, we found five putatively secreted and highly conserved glycoside hydrolases in need of further characterization.

In the light of previous studies and the results presented in this thesis, it is important to further investigate the interaction between *Leishmania* parasites and different components of the human immune response, for development of vaccines and better drugs to decrease morbidity and mortality of the leishmaniases worldwide. For non-endemic countries like Sweden, it is of value to follow and describe incidence of the disease and patient characteristics. Multi-center studies are needed to evaluate treatment outcome for each clinical presentation and to also in non-endemic countries define risk factors for developing disease, including new immunomodulatory drugs.

Key words: Leishmania, epidemiology, Sweden, leishmaniasis, immune response, chemokines, CAZymes

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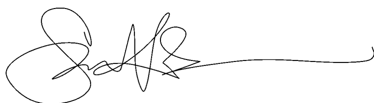
Signature  Date 2019-02-25

Leishmaniasis in Sweden

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LUND
UNIVERSITY

Cover image *Leishmaniasis pointed out*

by Sara Karlsson Söbirk, with the help of Daniel Karlsson

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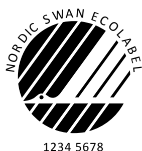

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To my family

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I Human chemokines as antimicrobial peptides with direct parasiticidal effect on *Leishmania mexicana in vitro.*
Sara Karlsson Söbirk, Matthias Mörgelin, Arne Egesten, Paul Bates, Oonagh Shannon and Mattias Collin. 2013 PLoS ONE. 8, 3, e58129

IIa Imported leishmaniasis in Sweden 1993-2016.
Sara Karlsson Söbirk, Malin Inghammar, Mattias Collin, Leigh Davidsson. 2018 Epidemiology and Infection. 146, 10, 1267-1274

IIb Comments on letter to the editor by Faniyan et al. in response to Imported leishmaniasis in Sweden 1993–2016.
Sara Karlsson Söbirk, Malin Inghammar, Mattias Collin, Leigh Davidsson. 2018 Epidemiology and Infection. Published online 6 November 2018. 1-1

III Comparative evaluation of five serological tests for the diagnosis of leishmaniasis in a non-endemic setting.
Sara Karlsson Söbirk, Georgina Isak, Leigh Davidsson. 2019
Submitted.

IV Exploring glycoside hydrolases in human pathogenic *Leishmania* species.
Sara Karlsson Söbirk, Eleni Bratanis, Mattias Collin. 2019
Manuscript in preparation.

Other publications

Cutaneous, mucocutaneous and visceral leishmaniasis in Sweden from 1996-2016: a retrospective study of clinical characteristics, treatments and outcomes. Hedvig Glans, Leif Dotevall, Sara Karlsson Söbirk, Anna Färnert, Maria Bradley. BMC Infectious Diseases 2018:18:632-642.

Clinical isolates of *Enterococcus faecalis* aggregate human platelets. Magnus Rasmussen, Daniel Johansson, Sara K Söbirk, Matthias Mörgelin, Oonagh Shannon. Microbes and Infection. 2010:12(4):295-301.

Primary *Klebsiella pneumoniae* liver abscess with metastatic spread to lung and eye, a North-European case report of an emerging syndrome. Sara K Söbirk, Carsten Struve, Sanda G Jacobsson. Open Microbiology Journal 2010:4:5-7.

Abbreviations

BCE	Before current era
CAZymes	Carbohydrate active enzymes
CL	Cutaneous leishmaniasis
DAT	Direct agglutination assay
DMARD	Disease modifying anti-rheumatic drug
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoassay
ICD	International classification of diseases and related health problems
LST	Leishmanin (Montenegro) skin test
MCL	Mucocutaneous leishmaniasis
ML	Mucosal leishmaniasis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NNN	Novy-MCNeal-Nicolle (medium)
NO	Nitric oxide
NTD	Neglected tropical diseases
PCR	Polymerase chain reaction (method)
PHAS	Public Health Agency of Sweden
PKDL	Post Kala-Azar dermal leishmaniasis
RESURS	Resor och Turism i Norden AB (konsultföretag)
RDT	Rapid diagnostic test
VL	Visceral leishmaniasis
WB	Western blotting (method)
WHO	World Health Organisation

Abstract

Leishmaniasis is one of the Neglected Tropical Diseases that primarily affects the most vulnerable population in tropical and subtropical parts of the world. However, with increased travel to and migration from endemic countries, a rise in incidence has been seen also in European non-endemic countries. The incidence and characteristics of patients with leishmaniasis in Sweden was not previously known, and evaluations of current diagnostic methods had not been performed in a patient population corresponding to a non-endemic country with imported cases of leishmaniasis from different regions. Once infected through the bite of an infected sandfly, an adequate cellular immune response of the host is essential for cure in leishmaniasis, and immunocompromised individuals are at increased risk of disease. In search for effective vaccine- and drug candidates, the interaction between *Leishmania* parasites and the human immune system is of great interest. Many human chemokines, which both have chemotactic effects on leucocytes and may act as antimicrobial peptides, are up- or down regulated in leishmaniasis lesions, possibly playing a role in control of infection. The Leishmania-produced enzyme LmexCht1 has previously been shown to be a virulence factor and comes from a group of enzymes, some of which are pathogen-produced with direct actions on human immunoglobulins.

We demonstrated with different methods, that several human chemokines have a direct antimicrobial activity on promastigotes of *Leishmania mexicana*, not mediated through their effects on leukocytes. This finding is the first proof of direct antiparasitic effect of chemokines on eukaryotic parasites, and encourages further studies of chemokines and chemokine receptors as possible drug targets. We further explored the group of enzymes called glycoside hydrolases in the published genomes of *Leishmania* spp. Including the conserved chitinase previously described in *Leishmania mexicana*, we found five putatively secreted and highly conserved glycoside hydrolases in need of further characterization. We also show that supernatant from cultured promastigotes had enzymatic activity on glycosidic bonds of human IgG, probably caused by carbohydrate active enzymes secreted by the parasites.

In a retrospective, epidemiological register study, we present the first estimation of annual incidence of leishmaniasis in Sweden 1993 through 2016 (0.023 to 0.35 per 100 000/year), and found, in concordance with reports from other non-endemic countries, an increase in incidence the last years of the study period. We describe patient characteristics that may be useful for identification and correct diagnosis of future patients with leishmaniasis presenting to Swedish health care units. We also evaluated five serological methods for the diagnosis of visceral leishmaniasis and mucocutaneous leishmaniasis in the mixed population of imported leishmaniasis. All tests showed lower sensitivities and specificities in our selected samples than

in previous publications, performed in endemic countries. Some immunosuppressed patients with confirmed leishmaniasis were negative in all tests.

In the light of previous studies and the results presented in this thesis, it is important to further investigate the interaction between *Leishmania* parasites and different components of the human immune response, for development of vaccines and better drugs to decrease morbidity and mortality of the leishmaniases worldwide. For non-endemic countries like Sweden, it is of value to follow and describe incidence of the disease and patient characteristics. Multi-center studies are needed to evaluate treatment outcome for each clinical presentation and to also in non-endemic countries define risk factors for developing disease, including new immunomodulatory drugs.

Populärvetenskaplig sammanfattning

Ungefär 350 miljoner människor lever i områden där de kan smittas av den encelliga parasiten *Leishmania* genom bett av sandmyggor. Parasiten tar sig in i makrofager, de celler i kroppen som normalt är programmerade för att ta död på inkräktande mikrober. Leishmaniaparasiterna undgår avdödning genom flera olika mekanismer, och kan istället föröka sig i makrofagerna och så småningom sprida sig i kroppen dolda för de funktioner som normalt känner igen och aktiverar immunförsvaret. Flertalet personer som blir infekterade med *Leishmania* blir inte sjuka, parasiterna kan finnas kvar i kroppen under många år utan att orsaka sjukdom. Men för minst 1 miljon människor årligen leder infektionen till sjukdom i hud (kutan leishmaniasis), slemhinnor (mukokutan leishmaniasis) eller djupare organ (visceral leishmaniasis).

Visceral leishmaniasis är den allvarligaste formen av sjukdomen, och leder oftast till döden om den inte upptäcks och behandlas i tid. Kutan leishmaniasis, som är vanligast, kan vara självläkande men ger ibland stora missprydande ärr i ansikte, på armar eller ben som kan leda till social stigmatisering. Mukokutan leishmanisis som förekommer i Syd- och Mellanamerika, kan bryta ned mjukdelar och brosk i näsa, mun och svalg, och ibland ge svårigheter att andas och äta. Människor med försvagat immunförsvar p.g.a. undernäring, hiv-infektion eller immunsänkande mediciner har stor risk att bli sjuka både vid nysmitta och genom reaktivering av parasiter vilande i kroppen efter tidigare infektion.

Det existerar inget effektivt och säkert vaccin mot leishmaniasis, och de flesta läkemedel som används mot allvarliga former av sjukdomen är dyra och har allvarliga biverkningar. Det finns alltså ett stort behov av nya angreppspunkter för vaccin och för bättre läkemedel. Eftersom ett välfungerande immunsvar från kroppens vita blodkroppar krävs för att kunna läka ut sjukdomen, skulle studier av mekanismer i samspelet mellan parasiterna och olika delar av människans immunsvar kunna identifiera sådana angreppspunkter.

Vi har i två av studierna i detta avhandlingsarbete undersökt möjliga mekanismer i samspelet mellan parasiten och människans immunförsvar. I det första arbetet har vi tittat på så kallade chemokiner, mycket små molekyler som tillverkas och utsöndras av många av kroppens celler, och som hittills är mest kända för de effekter de har på olika typer av vita blodkroppar. Några av chemokinerna har tidigare även visat sig ha en direkt avdödande funktion på många olika bakterietyper och på svampceller, de fungerar då som så kallade antimikrobiella peptider. Våra studier visade att flera chemokiner också har en direkt avdödande effekt på odlade leishmaniaceller, och att de alltså kan fungera som antimikrobiella peptider även på parasiter. I avhandlingens fjärde arbete undersöker vi istället enzymer utsöndrade av Leishmania-parasiterna, som vi tror

kan ha sjukdomsfrämjande funktion vid infektion. Vi kunde genom att studera gensekvensen i publicerade hela arvsmassor (genom) för flera olika Leishmania-arter visa att en grupp av enzymer (fem så kallade glykosidhydrolaser) har bevarats relativt oförändrade genom parasitens mångmiljonåriga evolution. Ett av dessa enzym, ett chitinas, kan ha vissa funktioner viktiga för parasitens överlevnad i sandflugan, men är enligt tidigare fynd troligen också viktig vid kutan leishmaniasis. Vi gjorde många försök att framställa enzymet i ren form i laboratoriet, vilket krävs för att fortsatt studera dess funktion. Även om vi ännu inte kunnat renframställa enzymet, har vi visat att parasiterna i odlingsbuljong verkar utsöndra kolhydrataktiva enzymer med aktivitet på sockerkedjor hos mänskliga antikroppar (IgG).

Båda dessa mekanismer skulle behöva undersökas i upprepade försök för fler leishmania-arter i olika utvecklingsstadier och studeras med många andra metoder för att komma underfund med om de är lovande som angreppspunkt för utveckling av vaccin eller läkemedel.

I Sverige är leishmaniasis en mycket ovanlig sjukdom, eftersom sandmyggan som sprider sjukdomen mellan djur och människor inte finns i norra Europa. De patienter som diagnosticerats har antingen invandrat från, eller varit på resa i, något av det hundratal länder där infekterade sandmyggor finns. Tidigare har ingen nationell sammanställning av sjukdomen gjorts, och vi har inte haft kunskap om hur många patienter som diagnosticeras i svensk sjukvård. Genom att analysera information från olika dataregister på socialstyrelsen och diagnostiska laboratorier kunde vi för första gången beskriva sjukdomen i Sverige, och göra en uppskattning av hur många fall vi haft per år under tiden 1993 till 2016, var patienter sökt sjukvård, var de smittats och andra fakta om dessa patienter. Eftersom varje enskild läkare mycket sällan stöter på patientgruppen, är det viktigt att sprida denna information, så att de fall som finns får rätt diagnos och behandling. Vi tog också fram information som visar att många som bor i Sverige är födda i eller har rest till länder där de kan ha smittats. Personer som en gång smittats kan ha en vilande infektion. Parasiterna kan långt senare börja dela sig och orsaka sjukdom hos patienter med nedsatt immunförsvar, t.ex. orsakad av hiv eller läkemedel.

För att en läkare ska kunna ställa diagnosen leishmaniasis hos en patient, bör patienten dels ha symptom som passar med sjukdomen, och dels mikrobiologiska prover som stöd. Antikroppspåvisning är ett komplement till andra, bättre mikrobiologiska metoder som påvisar hela parasiter (med mikroskopi eller odling) eller parasitens arvsmassa (med molekylära metoder). Vi utvärderade fem olika metoder för att i ett blandat patientmaterial kunna påvisa antikroppar mot *Leishmania* som en hjälp för att kunna ställa diagnosen visceral leishmaniasis och mucocutan leishmaniasis, de två allvarligaste formerna av sjukdomen. Vi såg att

flera personer som hade allvarliga former av sjukdomen inte hade positivt antikroppstest, och några av dessa var patienter med nedsatt immunförsvar. Flera av testerna blev positiva hos patienter med andra parasitsjukdomar än leishmanisis, vars prover var med som kontrollgrupp i studien.

Sammanfattningsvis har studierna som ingår i avhandlingen dels belyst några aspekter av leishmaniaparasitens interaktion med människans immunförsvar, och dels beskrivit sjukdomen i Sverige under 23 år samt jämfört olika metoder för att påvisa antikroppar i blod vid allvarliga former av leishmaniasis.

Vi fortsätter våra försök att framställa eller rena och vidare undersöka LmexCht1, ett enzym som påverkar storleken av hudförändringar vid leishmaniainfektion, och vars like från andra mikrober har effekt på antikroppar som är viktiga i immunförsvaret mot infektion.

Vi planerar också att följa utvecklingen av sjukdomen i Sverige de närmsta åren, och tillsammans med forskare i andra europeiska länder belysa riskfaktorer för sjukdom, typiska symptom och fynd vid infektion av en viss Leishmania-art, samt i ett större patientmaterial få möjlighet att jämföra hur effektiv olika typer av behandling är.

Introduction

Leishmaniasis in evolution and human history

The leishmaniases are diseases caused by intracellular protozoan parasites from the genus *Leishmania*, spread by phlebotomine sandflies mainly from the genera *Phlebotomous* and *Lutzomyia*. (1) In humans, infection not initially cleared by the immune system may lead to cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL) or latent infection, described below in the section for clinical disease.

The parasite was discovered in the late 1800s, by several independent researchers. Ronald Ross, a British medical doctor working for the Indian government to investigate kala-azar (VL) named the new genus and species *Leishmania donovani* after two of them, the Scottish pathologist William Boog Leishman (1865-1926) and the Irish doctor Charles Donovan (1863-1951). (2)

Although our knowledge of the parasite is only about 120 years, it has been a companion of humans and other mammals throughout our evolution. Phylogenetic analyses estimate that *Leishmania* evolved at least 90-100 million years ago, before the supercontinent Gondawa broke up into the present continents. (3) Studies on nuclear DNA sequence divergence between the different *Leishmania* species leads to similar results as for mammals that diverged in evolution 10-80 million years ago, comparable to the divergence of what is today mouse and man. (4)

Little is known about leishmaniasis in early human history, but from ancient times, leishmaniasis-like illnesses are described in Egyptian medical documents from 1500BCE (VL), on tablets in an Assyrian Library from the 7th century BCE (CL) and DNA from *Leishmania donovani* was recently found in human mummies from a tomb in West Thebes dating back to 2050-1650 BCE. In medieval time, Arabic scientists have described CL in the Baghdad region, and the Persian philosopher and physician Avicenna (980-1037) wrote about Balkh sores, probably caused by *Leishmania tropica* in northern Afghanistan. (2) (5,6) And from the 1600s to the discovery of the parasite in the 1880s, there are several descriptions of clinical course of oriental sores (CL) in the middle east and MCL-like conditions in the native population in South America. In India, a condition with acute anaemia,

hypersplenism and intermittent fever described in the 19th century was named kala-azar (black fever/disease, after dark/greyish appearance of skin during VL). Outbreaks of kala-azar were registered in India and West Bengal the decades before the causative microorganism was discovered. (2)

After break up of the continents, *Leishmania* parasites evolved further into the variants we have classified into more than 50 different species, of which at least 20 are known to cause human disease (Table 1). (7-11) Leishmaniasis in humans is most commonly caused by species within the subgenus *Leishmania Leishmania*, present both in the Old World (Eurasia and Africa) and the New World (the Americas). Species within the subgenus *Leishmania Viannia* are present only in the New World. Since description of the first morphologic differences between *Leishmania* species a century ago there has to date been a constant reclassification of species, and several changes of their names. (11)

Table 1. Human pathogenic species of *Leishmania* and their principal tropism
From WHO 2010, with modifications (7,8,10-12)
(Species status or taxonomic position within parantheses are under discussion.)

Subgenus	*L. (Leishmania)*	*L. (Leishmania)*	*L. (Viannia)*	*L. (Viannia)*
Old World =Eurasia, Africa	*L. donovani* *L. infantum*	*L. major* *L. tropica* *(L. killicki)* *L . aethiopica* *L. infantum* *(L. siamensis)*		
New World =The Americas	*L. infantum*	*L. infantum* *L. mexicana* *(L. pifanoi)* *L. venezuelensis* *(L. garnhami)* *L. amazonensis*	*L. braziliensis* *L. guyanensis* *L. panamensis* *L. shawi* *L. naïffi* *L. liainsoni* *L. lindenbergi* *L. peruviana* *(L. colombensis)*	*L. braziliensis* *L. panamensis*
Tropism	**Viscerotropic**	**Dermotropic**	**Dermotropic**	**Mucotropic**

Morphology, ecology and epidemiology

Life cycle of parasite and vector-borne transmission

The unicellular parasite exists in two major forms. In the phlebotomine sandfly (the intermediate host, or vector), it is a slender motile flagellated cell of 6-12 µm called promastigote. Within the parasitophorous vacuole of the mammalian macrophage, it transforms into the 1-5 µm ovoid amastigote form, without protruding flagellum, but with a nucleus and a kinetoplast visible in Giemsa-stained slides, traditionally used for diagnosis. (13)

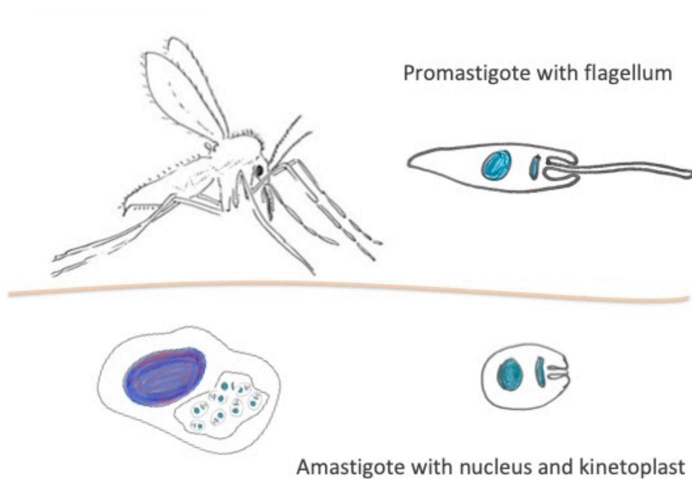


Figure 1
Within the phlebotomine sandfly, promastigotes exist in different developmental stages. In the parasitophorous vaculoe of mammalian macrophages, it divides as the amastigote form of *Leishmania*.

98 different species of phlebotomine sandflies are proven or suspected to be vectors of the leishmaniases. (14) They reside in humid, somewhat cooler microenvironments in rural or semi-urban areas in regions of Asia, Africa, America and the Mediterranean. (10) The adult sandflies, 3 mm in body length, are unable to fly at wind speeds exceeding their own maximal speed, 1m/s. The female sandfly makes a wound in the skin with its proboscus, and feeds after injecting the salivary gland contents into the haemorrhagic pool. (14)

The geographical distribution of phlebotomine sandflies is restricted to humid areas with temperatures above 15.6°C at least three months of the year. They are present in moist environments throughout tropics and subtropics globally. In Europe, they have previously been found around the Mediterranean region, but

recently also in Switzerland, France, Belgium, Germany, Austria and new areas in northern Italy. Endemic areas are predicted to expand further with global warming. (15)

If feeding on a *Leishmania*-infected animal, the amastigote-containing macrophages end up in the midgut of the sandfly, surrounded by a peritrophic matrix containing chitin and glycoproteins. Before the sandfly approximately 4 days later defecate the remainders of the blood meal, the amastigotes need to transform into motile promastigotes, escape the peritrophic matrix and start the migration to the stomodeal valve, from which it may infect a new host if the sandfly bites another animal. (13) (16)

Transmission of leishmaniasis is often zoonotic, with at least one animal reservoir and sandflies biting both animals and humans. Reservoir animals include rodents, dogs, foxes, cats and also domestic animals. (10) When no known animal reservoir exists, and the parasites are transmitted between humans with help of the sand fly vector, transmission is anthroponotic, as is believed to be the case for VL caused by *L. donovani* in the Indian subcontinent. (17)

Epidemiology

Geographical distribution

The distribution of leishmaniasis differs between CL and VL, and few areas in the more than 90 endemic countries have high burdens of both (Fig 2). (18) It is estimated that 1.7 billion people live in areas where they are at risk for infection. Globally between 0.7-1.2 million cases of CL and 0.2-0.4 million cases of VL occur each year. (19) Factors determining typical areas for transmission varies, probably due to presence of reservoir animals, which sandfly species that is the local vector, and preferences regarding climate and environment for both. Built-up areas are more likely to be at risk in Europe, Africa and Asia, while local climate factors affect risk for transmission more in the Americas. (18) Climate changes with increased temperatures and rainfall may alter the geographical distribution of vectors, reservoirs and the human leishmaniases. (20)

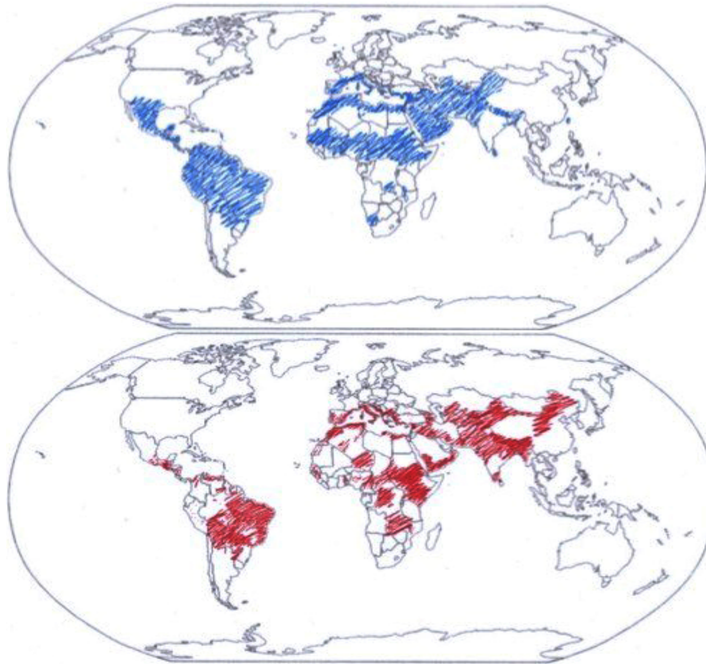


Figure 2 Geographical distribution of cutaneous (above in blue) and visceral leishmaniasis (below in red). From WHO (https://www.who.int/leishmaniasis/burden/en/) and Pigott, 2014.

The leishmaniases are diseases, which affect the more vulnerable population in the society. Within endemic areas one of the highest risk factors for leishmaniasis is poverty, which also affects morbidity and mortality. People with poor housing, or who sleep outdoors, are more exposed to bites of infected sandflies. A diagnosis of leishmaniasis is often an economic burden to the family, due to loss of income and costly treatment. (21-24) Armed conflicts following migration and increased exposure in refugee camps have caused epidemics of leishmaniasis in many regions. (25) (26) During the past years in Syria, the estimated incidence of CL in the northern parts of the country more than doubled during the first part of the war. (27) Refugees and migrants are over-represented among patients diagnosed with imported leishmaniasis, also in non-endemic countries. (28-32) Malnutrition and hiv-infection leads to immunosuppression, which is a risk factor for developing disease, leads to diagnostic challenges and affects treatment outcome. (33)

Other human activities, which may cause changes in the geographical distribution of transmission of leishmaniasis include deforestation and irrigation in farming. Vector control campaigns for malaria transmitting mosquitoes may also decrease numbers of phlebotomine sandflies. (9,34)

Human disease, diagnosis and treatment options

Historically, clinical forms of leishmaniasis have been grouped into Old and New World disease with the different endemic species typically presenting with visceral, cutaneous or mucocutaneous manifestations (Table 1). (10)

Visceral leishmaniasis

Visceral leishmaniasis (VL) is the most serious form of the disease, and is typically caused by the species *Leishmania donovani* and *Leishmania infantum*, both within the *Leishmania donovani* complex. In areas where these species are endemic, VL mainly affects children 1-4 years old, and immunosuppressed adults. (10) Infected macrophages travel from the skin to deep tissues in liver, spleen, bone marrow and lymph glands, where they gradually replace normal cells resulting in splenomegaly, hepatomegaly and a dysfunctional bone marrow. Symptoms of fever, malaise, weight loss and discomfort in the left hypochondrium develop gradually after an incubation period that may be as short as 10 days, but is usually months to years. Clinical findings include hepatosplenomegaly, wasting, anaemia and lymphadenopathy. Some symptoms show regional variation. Darkening of skin (giving name to kala-azar, which means black fever/disease) is more common in India. In Sudan and East Africa, patients with VL may also present with skin ulcers or nodules containing parasites. (10)

In regions where VL normally does not occur, changes in the environment resulting in different conditions for reservoirs or vectors or human migration may cause epidemics of leishmaniasis in people of all ages. In those cases, symptoms of VL are more acute and similar to those for non-immune persons infected when entering an endemic area. They may have a more acute onset with high, undulating fever, chills, profound sweating and rapid weight loss. (10)

HIV infection and medically induced immunosuppression are increasingly common in patients diagnosed with VL. Symptoms and typical presentation in these groups are often different than in previously immunocompetent individuals, described above. (10,33)

According to WHO recommended case definitions, a case of VL is ’a person showing clinical signs (mainly prolonged irregular fever, splenomegaly and weight loss) with serological and/or parasitological confirmation’. (10)

Post Kala-Azar Dermal Leishmaniasis (PKDL)

PKDL, with multiple macules, papules or nodules on the skin containing viable parasites, is a condition which occurs after apparent cure of VL caused by *L.*

donovani in some patients. Typically, it developes in 6-12 months after treatment for VL. In Africa (where it is more common), PKDL often resolves spontaneously, but the condition usually requires treatment in Asia. (10)

Figure 3 A child in Ethiopia with VL causing gross enlargement of liver and spleen (marked in green) and PKDL in a Sudanese child after treatment of VL (top).
WHO https://www.who.int/leishmaniasis/resources/photo_gallery/gallery/en/ (only modification: blurred eyes)

Cutaneous leishmaniasis

For cutaneous leishmaniasis (CL), there is a wide variety of clinical presentations, depending on parasite species and strain as well as genetic and immunological factors of the host. All human pathogenic *Leishmania* species can cause CL, but common dermotropic ones within the subgenus *Leishmania Leishmania* are *L. major, L. tropica, L. aethiopica* in the 'Old World', and *L. mexicana, L. venezuelensis, L. amazonensis* in the Americas. *Leishmania infantum* is both viscerotropic (causing VL), and a cause of CL around the Mediterranean. Endemic in the Americas, species within the subgenus *Leishmania Viannia* are all dermotropic, but some of them (*L. braziliensis* and *L. panamensis*) may also cause MCL.(Table 1) CL is usually a benign condition, healing without treatment over

time in the majority of patients, but leaves disfiguring scars, which may cause social stigma.

Around the site of the sandfly bite, amastigotes start to divide within dermal macrophages, and after an incubation period of up to three years (usually 2-8 weeks), the infection results in an area of erythema, leading to an inflammatory papule, usually just a few millimetres in diameter. The papule increases in size, and progresses to a nodule or a plaque, which often ulcerates, causing a wound, which is typically not painful, and with discoloration of surrounding skin. There may be multiple lesions and/or smaller satellite lesions surrounding the larger lesion. The progression is slow (taking weeks to months), as is healing after an adaptive cellular immune response has made it possible to fight the infection. (35)

WHO suggest a case definition of CL where clinical findings of lesions are always combined with parasitological confirmation of the diagnosis. Although only smears and culture are mentioned(10), proof of Leishmania-DNA from the lesion with molecular methods is also accepted as parasitological confirmation.

Figure 4 CL in the face of a child in Afghanistan, Kabul
WHO/C.Black (only modification: blurred eyes)

Mucocutaneous and mucosal leishmaniasis

Months to years after healing of CL lesions caused by *L. braziliensis*, *L. panamensis* or *L. guyanensis*, parasites disseminated to nasal or oropharyngeal mucosae, may cause muocoutaneous leishmaniasis, MCL. Most patients with CL

caused by these species do not develop MCL. Those with large, long-lasting lesions, infected in Bolivia, immunosuppressed or being male are at higher risk for later MCL. The lesions, which usually start on the border between skin and mucosa, may progress to very large destructive lesions causing defects in soft and cartilaginous tissues of mouth, nose and palate (Espundia) and eventually cause difficulties eating and breathing. (6,36)

In the literature, this condition is usually (but not always) distinguished from mucosal forms of leishmaniasis caused by other species, often without a history of CL. Mucosal leishmaniasis (ML) may be caused by *L. infantum*, *L. tropica* or *L. major*, and is more common in immunosuppressed patients, sometimes present at the same time as VL. (10,35)

A case of MCL, according to WHO recommended classification, is a person showing clinical signs (mucosal lesions) with parasitological and/or serological confirmation. (10)

Latent infection

Most humans bitten by *Leishmania*-infected sandflies do not develop disease. Both asymptomatic infection, and leishmaniasis which has healed with or without treatment, may lead to latent infection in which some amastigotes remain viable within the asymptomatic host for several years, controlled by the immune system. (37-39)

Diagnosis

As the typical symptoms of VL, CL or MCL are far from unique for these diseases, *Leishmania*-specific laboratory tests are required to confirm the diagnosis.

Microscopy

Examination in light microscopy of aspirates or biopsy punch smears from infected tissue has been the main confirmatory test for leishmaniasis since discovery of the parasite. If VL is suspected, aspirates from spleen or bone marrow are samples most likely to be positive. In tegumentary leishmaniasis (CL, MCL, ML), aspirates, curettage or biopsies from mucosa or skin can be used. Classically, slides are dyed with Giemsa, and round to ovoid amastigotes with both a nucleus and a kinetoplast, preferably seen within tissue macrophages, confirms the diagnosis (Fig 5). Although the specificity is close to 100%, sensitivity for microscopy is low for all tissues (53-86%) except for splenic aspirates (93-99%)

also in parasitology reference centres with experienced staff. (10,40) Immunofluorescence microscopy methods increase sensitivities somewhat. (41)

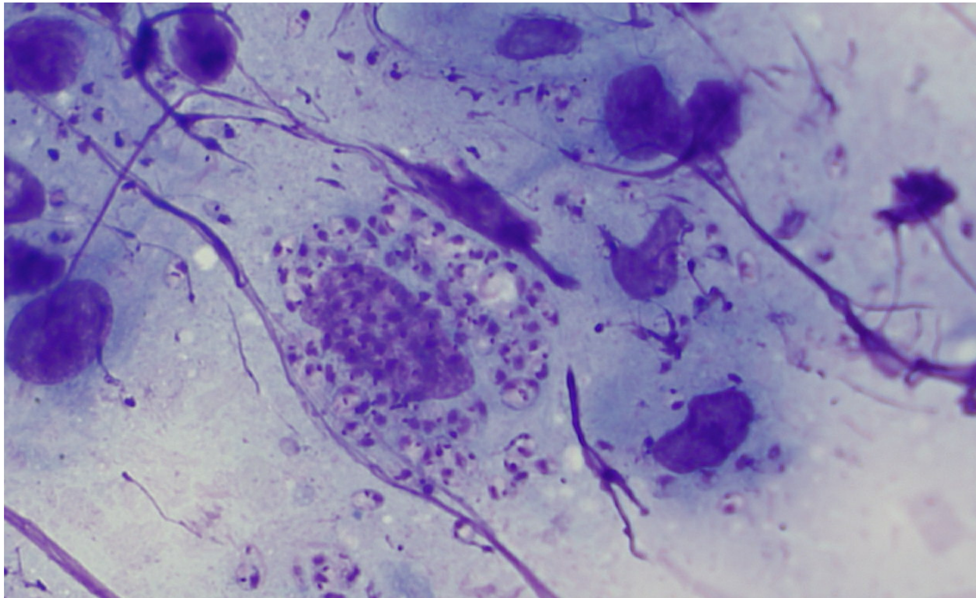


Figure 5 Giemsa coloured impression smear from skin biopsy with a *Leishmania* infected macrophage. Both intra- and extracellular amastigotes have a nucleus and the typical kinetoplast. 100x light microscopy.

Culture

Cultures of Leishmania parasites in the promastigote form can readily be performed in special medium (NNN-medium or substituted liquid cell culture medium). However, it is time consuming, and few diagnostic laboratories offer *Leishmania* culture. Culture thus increases the sensitivity compared to microscopy alone(42), but it often takes days to weeks before the intracellular amastigotes in the sample have transformed to motile promastigotes and started to divide so that they can be visualised in light microscopy (Fig 6).

Figure 6 Cultured promastigotes of *Leishmania mexicana*
100x light microscopy, wet preparation without fixation or dye.

Molecular methods

The most sensitive way, which is increasingly used to diagnose leishmaniasis in high-resource settings, are molecular methods, detecting also very small amounts of *Leishmania*-DNA within a sample. (43) (44) This has lead to concerns of PCR-methods detecting parasites in patients with latent infection, who suffer from diseases with symptoms similar to those of leishmaniasis. Many different molecular methods are used, with varying precision and sensitivity. (43,45-47) (48) (12) Most sensitive are quantitative PCR-methods, which can also be used on peripheral blood from patients with VL. (43,44) Nowadays, PCR is the first choice for species-identification and usually involves sequencing or gel analysis of amplified genes (such as HSP70 or ITS1). (12) This type of PCR is commonly used directly on skin biopsies, other tissues and cultured strains. Although somewhat less sensitive than the quantitative PCRs mentioned above, it is still more sensitive than microscopy and culture together. (40,42)

Serology

In some areas with high burdens of VL, health care does not have access to diagnostic laboratories. Serological methods (detecting anti-Leishmania antibodies in peripheral blood) are frequently used as the first, and sometimes the only test to confirm the diagnosis on patients with typical clinical findings. Many tests using

different techniques are available on the market. (49-60) Immunofluorescence assays (IFAT) require an immunofluorescence microscope and Enzyme-linked immunoassays (ELISAs) are usually read using specialized equipment and both are therefore more suitable for high resource diagnostic laboratories. Other tests e.g. the direct agglutination assay (DAT) and immunochromatographic rapid diagnostic tests (RDTs, often detecting antibodies against the leishmanial antigen K39), are developed for use in lower resource settings in endemic areas and are read optically without equipment. Western blotting (WB), which detects antibodies against pathogen-specific proteins separated by denaturing gel electrophoresis blotted to nitrocellulose filter paper, is often used as confirmatory test. However, it is not suitable for a field setting with many different users, as variation in interpretation of optically read bands is common. Some serological tests use *L. donovani* strains as the target antigen, and some antigens from *L. infantum*. The tests have shown different prestanda in studies from different regions, which is probably due to variation in which *Leishmania* species, and possibly strain, that is endemic in the area of the study population. (59,61) There is no single test which has proven to be the most sensitive or specific for all areas. (51,62)

Other methods

Assays to detect *Leishmania* antigen in urine or blood have been developed, but have so far showed low to moderate sensitivity, except in immunosuppressed patients. (63,64) As immunosuppressed patients may lack detectable anti-Leishmania antibodies, but still have a high burden of parasites, an antigen test could be a useful complement in this group.

An interferon-gamma release assay (Modified Quanitferon test) has been tried both in humans and dogs as a marker of latent infection. (65,66)

The leishmanin (or Montenegro) skin test (LST), based on a delayed type of hypersensitivity response, has been used e.g. to support the diagnosis of MCL where parasites have been difficult to demonstrate. After intradermal injection of cultured inactivated parasites in the forearm, the diameter of the induration is measured after 48-72hrs. (41,67) However, for most forms of leishmaniasis, LST results correlate poorly with disease. (68)

The choice of diagnostic methods

Visceral leishmaniasis is a disease with high mortality if left untreated. Delay of a correct diagnosis may cause increased morbidity or mortality. (69) Many of the differential diagnoses, such as haematological diseases, malaria and typhoid fever are also serious conditions important to diagnose and treat properly. Cutaneous leishmaniasis may cause disfiguring scars and, in some cases, later MCL, the risks

of both being reduced with proper treatment in time. (10) It is important to have both highly sensitive and specific diagnostic methods, as there may be serious consequences for the patient both in the case of a missed diagnosis, and if the leishmaniasis diagnosis is made erroneously when the patients symptoms are caused by another disease. Parasitological confirmation (with microscopy, culture and/or molecular methods) should be performed whenever possible. Detection of leishmania-DNA is usually the single method with highest sensitivity, but microscopy is usually faster, and a combination of test methods increases the sensitivity. (40,42) Serology is frequently used world-wide, and may, according to the case-classifications suggested by WHO, be the only confirmatory test to base a diagnosis of VL or MCL on (10). However, there are well-known cross-reactivities with other parasitoses(56), and patients in endemic areas without symptoms of disease or people having been exposed to infective sandflies when travelling to an endemic area, may also have detectable anti-Leishmania antibodies without disease. In a non-endemic high resource area like Sweden, diagnosis of leishmaniasis should not be made based on serology as the only confirmatory due to both false-positive and false-negative results with serological tests. Methods are available for all health care providers to confirm the parasites in infected tissue. (70)

Treatment

No effective vaccine exists against human leishmaniasis, and there are no current recommendations concerning prophylactic treatment. Once a diagnosis of clinical disease is made, there are several treatments options for both visceral and tegumentary leishmaniasis (VL+MCL). All recommended anti-leishmania drugs for systemic use may have potentially serious side effects, including anaphylactic-like infusion reactions, hypokalaemia, nephrotoxicity (amphotericin B and liposomal amhotericin B), cardiotoxicity, anaemia, leukopaenia, thrombocytopaenia (pentavalent antimonials), hepatotoxicity (previous drugs plus paromomycin and miltefosine). Gastrointestinal side effects as anorexia, nausea, vomiting, elevated pancreas enzymes are also common. In many areas, there is a limited access to drugs due to infrastructure or cost. (71) Recommended choice of treatment for CL depends on infecting species, size and location of lesions, presence of immunosuppression and possibility for long-time follow-up of the patient. Options include; local wound care, paromomycin ointments, thermotherapy, cryotherapy, intralesional pentavalent antimonials and also systemic treatment with the same agents as are used for VL. (72) (35)

Only recently have the molecular tools for high quality species identification direct from tissue samples been made available. Previous studies of treatment outcome for the many different regimens used for CL often lack information of infecting

species. Patients included in the studies are heterogenous when it comes to number and location of lesions, age, immune status and follow up. (73,74) Therefore, there is no consensus on which treatment is the best choice for CL of a certain type, caused by a certain species. International guidelines list several options (mentioned above). (10,35,72,75) There is a need for larger multicentre studies looking into patient, parasite, choice of treatment and with a systematic follow up of outcome to evaluate efficacy of currently used CL treatments, and for combinations of drugs for CL and VL. WHO has appointed leishmaniasis one of the Neglected Tropical Diseases (NTDs) and promotes more research for safe, effective and affordable medicines, as well as diagnostic tools and vaccines. (9)

Although a very important subject to study and one of my current research interests, different aspects of treatment of leishmaniasis are not included in this thesis.

Leishmania and the Human Immune Response

Immune evasion strategies of Leishmania

Throughout its life cycle, the *Leishmania* parasite has to withstand the immune system of the host. In reservoir animals or humans, it is dividing inside macrophages, the cell usually responsible for phagocytosis and clearance of intruding pathogens. In order to succeed, it has evolved several immune-evasion-strategies. In early infection, promastigotes are phagocytosed by neutrophils or dendritic cells recruited to the site of the sandfly bite. The neutrophils undergo apoptosis (programmed cell death), and scavenging macrophages clean up the endogenous apoptotic cells. Neutrophils and dendritic cells thus act as Trojan horses, letting the parasites enter macrophages without activating their parasite-killing mechanisms. (76-79) Both promastigote and amastigote forms of the parasite further have different ways to alter the environment and composition of the parasitophorous vacuole of the macrophage, preventing the normal events which lead to killing of microbes within the phagolysosome. (80,81) Complement opsonisation has been shown to facilitate promastigote uptake by macrophages. Gp63, a surface glycan previously shown to be a virulence factor in promastigotes of *L. major, L. mexicana* and *L. donovani*, cleaves C3b on the surface membrane of the parasite. This inhibits complement-mediated lysis and promotes parasite uptake by the macrophages via complement receptor 3 (CR3). (82-84)

These are some of the known strategies for *Leishmania* parasites to persist and thrive within the host, evading the immune system evolved to protect against invading parasites.

Immune response eventually fights back

An adequate adaptive T-cell response is needed to heal clinical infection with *Leishmania* parasites, regardless of if treatment is given or not. An effective immune response to infection is possible when Leishmania-specific CD4+ T-cells differentiate into T helper(Th)1-cells. Th1-cells activate macrophages and secrete IFN-γ and TNF-α, both important for parasite elimination. CD8+ T-cells have a role in differentiation of CD4+ T-cells into Th-cells. They may play a role in immunity after infection, but are also involved in inflammatory processes and tissue damage in lesions. (85) Even after an effective cellular immune response leading to clinical cure, parasites may persist in the host for many years. (39) There is some evidence that persisting live parasites may be important for sustained immunity against new infection in CL. (86)

Table 2. Chemokines used in screen, previous reports of antimicrobial activity.
Table from 'Human chemokines as antimicrobial peptides with direct parasiticidal effect on *Leishmania mexicana* in vitro', Söbirk et al. PLoS One 2013, for references, see Paper I.

Chemokine new/old name	Main leukocyte targets	Antimicrobial activity	Calculated pI
CXCL2 /GRO-β	Neutr, Mo, Mast	*E. coli, S. aureus*	10.27
CXCL6 /GCP-2	Neutr, Mo, Mast	*S. pyogenes, S. aureus, E. coli, P. aeruginosa, S. dysgalactiae*	9.06
CXCL8 /IL-8	Neutr, Mo, Mast	*S. aureus, S. typhimurium, C. albicans*	8.97
CXCL9 /MIG	Th1, NK, pDC, Mast	*E. coli, S. aureus, S. pyogenes, N. gonorrhoeae*	10.83
CXCL10 /IP-10	Th1, NK, pDC, Mast	*E. coli, S. aureus*	10.52
CCL2 /MCP-1	Mo, Bas, MemT	No activity on *E. coli*, *S. aureus*	9.58
CCL3 / MIP-1α	mDC, Mo, MemT, Th1, Treg, NK, pDC	No activity on *E. coli*, *S. aureus*	4.60
CCL20 /MIP-3α	BC, mDC, MemT	*E. coli, S. aureus, C. albicans*	10.08
CCL27 /CTACK	MemT	*C. albicans*. No activity on *E. coli, S. aureus*	9.11
CCL28 /MEC	MemT	*S.pyogenes, S.aureus, E.coli, C.albicans*	10.23

Human chemokines are important players in Leishmania infection

Chemokines are small extracellular signalling proteins with chemotactic properties, secreted by leukocytes and other nucleated cells. They are divided into four families, based on arrangements of conserved cysteine residues (XC-, CC-, CXC-, CX_3C-chemokines). Many of them have a role in recruiting and activating different types of leukocytes in leishmaniasis, and act through various chemokine-receptors on the target cells. (87-91) Chemokines and chemokine-receptors are subject to studies as possible drug targets for inflammatory conditions and tumours. (92-96) Certain chemokines have been shown to be up- or down regulated in CL-lesions, although different studies have shown varying chemokine-profiles for different types of lesions and *Leishmania* spp. (97-100) (101) Some chemokines have other effects than the chemotactic properties first

discovered. Several chemokines have been shown to have direct antimicrobial properties against bacteria and/or *Candida* species (Table 2). (102) They also share many properties with the increasing group of known antimicrobial peptides (AMPs). Both chemokines and AMPs are small (8-10 kDa), cationic molecules, and conserved as important components of the innate immunity in a wide range of species, and they are up-regulated in response to inflammatory or infectious stimuli. (102-105) Many antimicrobial peptides are described to have an effect on *Leishmania* spp. (81,106,107) Although the effect of chemokines on various protozoan infections have been studied in several murine knock-out-models(87), the direct interaction between parasites and chemokines (apart from their effect on leukocytes) has not been previously studied.

Carbohydrate Active Enzymes / LmexCht1

Many pathogen–derived enzymes are known to affect the immune system, resulting in increased chances of survival of the bacterium/fungus/parasite in the host. In parasitic protozoa, numerous proteases interact with different parts of the immune system in different stages of the parasites' life cycle. Among the proteases of the *Leishmania spp.*, cysteine peptidases have been shown to be important virulence factors. (108) Another group of enzymes are the carbohydrate active enzymes (CAZymes). They catalyze the synthesis and breakdown of glycosidic bonds, and account for 1–3% of the proteomes of most organisms. (109,110) Their targets, glycosylated molecules derived from both parasite and host have a role in infection and survival of the amastigote within the macrophage. (111) In *Leishmania*, CAZymes have been described, most of them from the groups of glycoside hydrolases (among them a chitinase) and glycosyltransferases. (112-114) With the publication of the whole genomes of three *Leishmania spp.,* the sequences of putative CAZymes count to 279. Most of them have yet to be characterized. CAZymes of other microbes have been shown to interact with and modulate the immune systems of their hosts. (115) Two glycoside hydrolases, the endoglycosidases EndoS and EndoE, derived from gram–positive bacteria, inactivate immunoglobulins by hydrolysing conserved glycans in their effector part. (116-118) Chitin and chitinases have also been shown to interact with the cellular immune system, modulating a type 2 inflammatory response with implications for allergic inflammation and tissue remodelling. (119) Pathogenic *Leishmania spp.* all have one conserved gene coding for a chitinase. For *L. mexicana*, this chitinase (LmexCht1) has been shown to be important for survival and multiplication in their sand fly vectors, and may play a role when the parasite has to penetrate the peritrophic matrix, containing chitin. However, the chitinase seems to be a virulence factor also in the mammalian host, increasing the size of lesions in a mouse infection model. (114,120-122)

Leishmaniasis in the immunosuppressed

As an adaptive cellular immune response is crucial to control infection, individuals with genetic, acquired or medically induced immunosuppression have increased morbidity and mortality in leishmaniasis. (33) This is due to failure to control a new infection, reactivation of a latent infection, and/or lack of the Th1-cellular immune response required for recovery from clinical disease. HIV-infected patients, especially with CD4+ T-cells below 200cells/mL, are now over-represented among VL-patients in southern Europe, East Africa and Asia. (33,123) Patients with medically induced immunosuppression are also a group in which leishmaniasis is increasingly reported. (124) Within the fields of rheumatology, haematology and gastroenterology, various immunosuppressive drugs have been associated with leishmaniasis. Many case reports are recently published concerning anti-tumour necrosis factor-alpha (TNF- α) antagonist drugs such as infliximab(125-128), but other immunosuppressive drugs (azathioprine, methotrexate, cyclosporine, cyclophosphamide and steroids) also increase the risk of clinical disease. (33)

Rationale for choice of studies

Although continuous surveillance of the incidence of leishmaniasis occurs in most endemic countries, few non-endemic countries collect annual national data on the disease. This information, together with characteristics about patients, infecting *Leishmania* species and probable countries of infection may be important in order for health care staff to suspect and correctly investigate and treat patients with leishmaniasis. The incidence and characteristics of patients with leishmaniasis in Sweden was not previously known, and evaluations of current diagnostic methods had not been performed in a patient population corresponding to ours.

An adequate cellular immune response of the host is essential for cure in leishmaniasis, and patients with immunosuppression are at increased risk of disease. Both in order to better understand the pathogenesis of leishmaniasis and in search for new vaccine- and drug candidates, the interaction between *Leishmania* parasites and the human immune system is of great interest. Many human chemokines are up- or down regulated in leishmaniasis lesions, possibly playing a role in control of infection. Previously, the direct effect of human chemokines on cultured *Leishmania* parasites had not been studied. Further, the *Leishmania* produced enzyme LmexCht1 is a virulence factor in a mouse model of leishmaniasis, and it comes from a group of enzymes, Glycoside hydrolases, some of which are pathogen-produced with direct actions on human immunoglobulins. Glycoside hydrolases from *Leishmania* spp. have previously not been studied for

their possible effects in the interaction between the parasite and the human immune response.

Aims

General aims with this thesis were to investigate epidemiological and clinical aspects of leishmaniasis in Sweden in order to increase awareness of the disease, to elucidate some aspects of the interaction between the parasite and the human immune system and to validate serological tools as a complement for diagnosis of VL and MCL.

Specific aims for each paper were;

Paper I

> To investigate possible direct parasiticidal properties of human chemokines by screening for antimicrobial effect of the most probable candidates on cultured *Leishmania mexicana* promastigotes.

Paper II

> To estimate incidence of imported leishmaniasis in Sweden and to describe the clinical presentation, patient characteristics, probable country where the infection was acquired and causative species.

Paper III

> To find a combination of two serological methods that can be used in a non-endemic setting to detect VL and MCL with the highest sensitivity and specificity.

Paper IV

> To investigate and describe putatively secreted carbohydrate active enzymes in the group Glycoside hydrolases encoded by *Leishmania spp*.

Present investigation

For detailed descriptions of methods, materials and results in the present studies, the reader is referred to each paper in the end of this thesis. Ethic approval for study II-IV was given by the Regional Ethics Committee in Lund (Dnr 2014/646, Dnr 2016/1101).

Paper I and additional experiments

An experimental study

In this study, all experiments were conducted in the research laboratory at the Division of Infection medicine, University of Lund. We cultured promastigotes of *Leishmania mexicana* parasites in liquid medium. After washing the promastigotes several times to minimize influence of peptides in the culture medium, the cells were exposed to 10 different chemokines proven to have antimicrobial properties on bacteria and/or fungi. Effects were compared to Amphotericin B (used to treat leishmaniasis), and three of the chemokines were tested in different concentrations, to see if there was a dose-response gradient. Cell viability of Leishmania-cells after exposure to chemokines was measured as a decrease in mitochondrial activity with MTT assays. To determine if the peptides acted by increasing permeability in Leishmania-cell membranes after exposure to chemokines, the promastigotes were incubated in propidium iodide, and the intracellular contents of propidium iodide in comparison to controls was measured with flow cytometer. Scanning electron microscopy was used to visualize changes in morphology of the promastigotes. We chose established methods well described in the literature to characterize the leishmanicidal activity of antimicrobial peptides. {LuqueOrtega:bf}

When screening ten human chemokines for antimicrobial effect on promastigotes of *Leishmania mexicana* we showed, for the first time, a direct antiparasitic activity of several of the chemokines on the parasite cells. The damage caused was demonstrated with different experiments (Table 3); With flow cytometry, we could show a breach in plasma membrane integrity, by entrance of propidium iodide (PI), a dye with molecular size of 668Da. We could also demonstrate an

aggregation of cells, both by the eye and in light microscopy. The morphology of cells were also altered by several of the chemokines, as has been shown before for antimicrobial peptides with direct action on *Leishmania* parasites. With MTT, the mitochondrial activity of the cells were measured after exposure of the chemokines, and viability of the cells decreased most after exposure for CXCL6, CXCL9 and CCL28. Our study indicates a direct interaction between the chemokines and parasite cells, which has not been described before.

Table 3. Observed activity of chemokines on promastigotes of *Leishmania mexicana.*
Cytotoxic activity in the MTT-assay (quantified – to +++), membrane damage measured by entry of PI in flow cytometry (quantified – to +++), aggregation of cells observed by the eye and in light microscopy (semiquantified – to +++), and morphologic changes of the cells visible in SEM (- or +) are summarized. N.D. = not done.

Chemokine	Cytotoxic activity	*Membrane damage*	*Aggregation of cells*	Morphologic changes in SEM
CXCL2	++	++	+	+
CXCL6	+++	+++	+	+
CXCL8	-	-	-	N.D.
CXCL9	+++	++	-	+
CXCL10	++	++	-	-
CCL2	-	-	-	+
CCL3	-	-	-	N.D.
CCL20	++	+	++	+
CCL27	-	-	+	+
CCL28	+++	++	+++	+
Amph.B	+++	++	-	+

Table from 'Human chemokines as antimicrobial peptides with direct parasiticidal effect on *Leishmania mexicana* in vitro', Söbirk et al. PLoS One 2013

Among the chosen chemokines, CXCL8, CCL2, CCL3 and CCL27 did not show antiparasitic activity in our study. As cationic properties of AMPs are believed to be important for their antimicrobial activity, the lower pI of these chemokines may partly (but not completely) explain their lesser antiparasitic effect. Human lesions of cutaneous leishmaniasis have nevertheless shown elevated levels of CCL2 and CCL3 (97) (101), and *L. major* incubated with human blood mononuclear cells increase their production of CCL2 and CXCL8. (129) These mechanisms could be beneficial for the parasite, as CCL2, CCL3 and CXCL8 do not seem to cause direct damage (in our study), but their increased expression at the site of the lesion could help to recruit new mononuclear cells (CCL2, CCL3 and CXCL8) or neutrophils (CXCL8) for the parasites to infect.

As our finding is limited to one *Leishmania* species, and the promastigote form of the parasite, further studies of the antimicrobial properties of chemokines in amastigote forms of different species of *Leishmania* would be interesting, as the amastigotes are the predominating form of the parasite in the host.

However, axenic amastigote cultures are difficult to perform, and in an intracellular macrophage infection model an effect by chemokines on the intracellular amastigotes could be an indirect effect through actions of the macrophage. We tried to transform promastigote cultures to amastigotes in a few clinical strains of *Leishmania* (130) (131), and preliminary data show a possible action also on these cells by CXCL6 and CXCL 10 in an MTT-assay (Fig 7) and through entrance of vital dyes demonstrated by flow cytometry (Fig 8), but questions arise whether these were true amastigotes, or just morphologically changed promastigotes with the ability to divide in conditions described for axenic culture of amastigotes (131).(Data not published.)

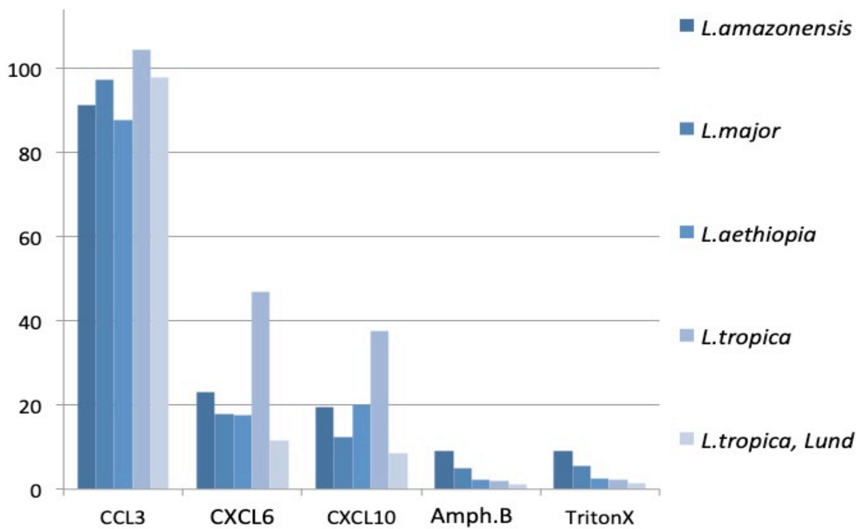


Figure 7
Decreased mitochondrial activity after exposure to CXCL6 and CXCL10 of axenically grown amastigotes of five clinical strains of *Leishmania.*
MTT assay. Y-axis show OD in percent of negative control. Data not published.

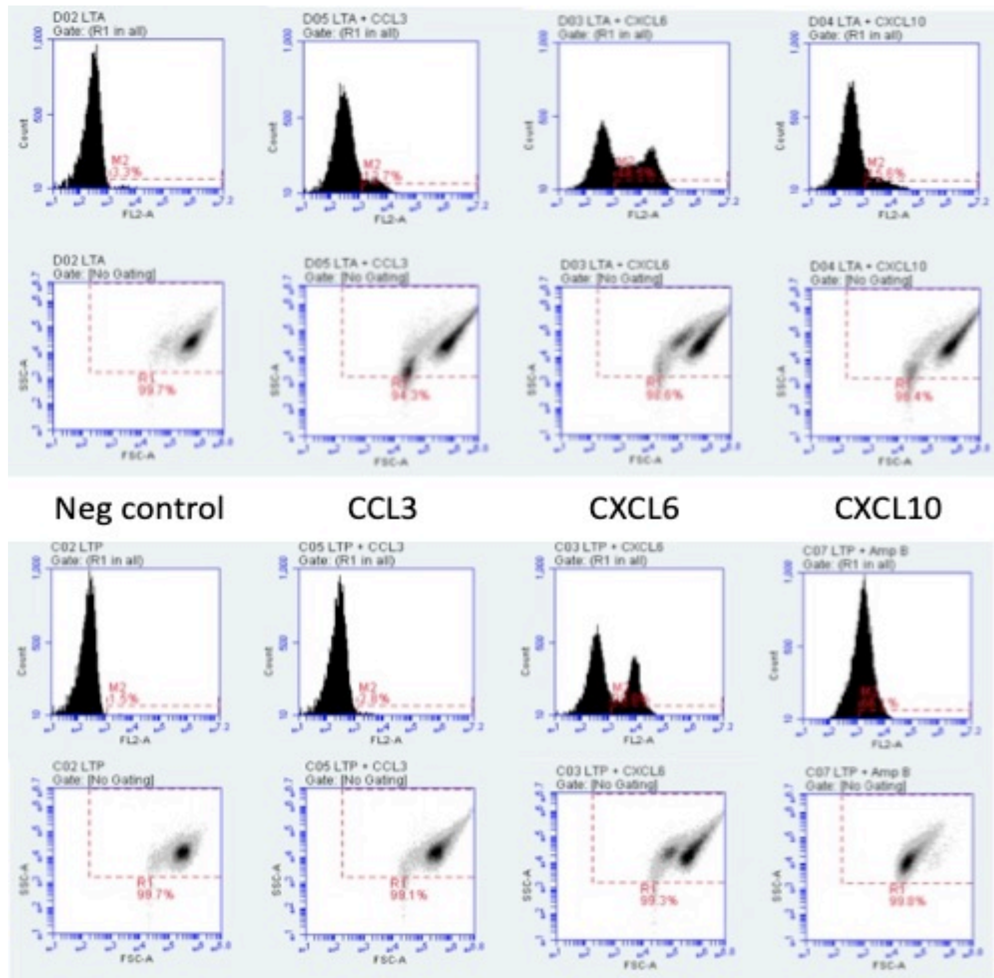


Figure 8 Plasma membrane permeability after exposure to CCL3, CXCL6 and CXCL10 of axenically grown amastigotes of a clinical *L. tropica* strain.
Shown with through entrance of propidium iodode, visualised with flow cytometry. The same assay performed with cultured promastigotes of the same *L. tropica* strain below, for comparison. Data not published

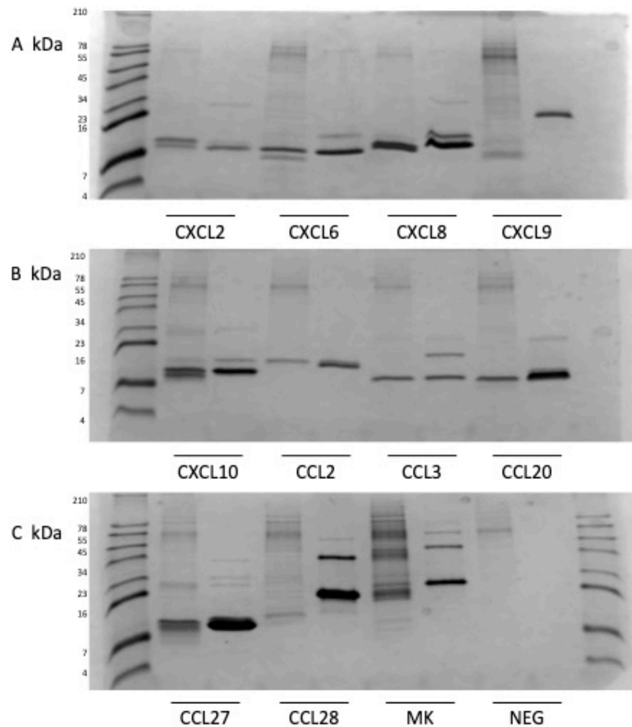
As mechanisms have been described in which Leishmania-produced metalloproteases protect against antimicrobial peptide-induced killing of *Leishmania major* (81), and many immune evasion strategies are known within the genus, we were interested to see whether promastigotes of *Leishmania mexicana* might also have an action on the chemokines? We could demonstrate that promastigotes incubated with chemokines interacted with the chemokines in different ways (Fig 9, data unpublished). Some chemokines seemed to be cleaved or degraded. CXCL9 disappeared from the supernatant of the incubation reaction, possibly bound to the promastigotes in the pellet. These are early findings, and

will have to be repeated and further studied, but could point towards a mechanism which has evolved as a result of the direct contact between parasite and host-derived chemokines.

Figure 9

Promastigotes interact with chemokines and change their bands in supernatants separated on SDS PAGE after incubation as previously described (Paper I).

Left lanes show supernatant from reactions with sorbitol buffer containing chemokines incubated with *L. mexicana* promastigotes, right lanes chemokines incubated under the same conditions without promastigotes. CCL3 was added as a negative peptide control, and the negative control in C was incubated in sorbitol buffer without chemokines. Unpublished data.



Chemokines and chemokine-receptors are involved in the pathogenesis of many inflammatory and infectious diseases, and have therefore been of interest as drug targets, either to stimulate or to block their functions. (96) One example is hiv-infection, where entry of the virus into human cells is facilitated when the chemokine receptors CCR5 and CXCR4 act as co-receptors to the CD4 membrane receptor. (132) Our findings suggest a direct interaction between leishmania parasites and some chemokines with direct cytotoxic effect (CXCL2, CXCL6, CXCL9, CXCL10, CCL20 and CCL28) which could not be seen for human cells (CCL28), and which is therefore probably not a general cytotoxic activity. Incubation with promastigotes and some chemokines also changed the appearance of the chemokines in the supernatant (CXCL9, CCL20, CCL27, CCL28). Although the latter is preliminary data (not published), it could be due to a direct interaction, perhaps through binding of the chemokine to the parasite surface membrane, or through actions of molecules secreted by the parasite.

However, our published findings are restricted to the promastigote (non-human) form of only one species (*L. mexicana*) in sorbitol buffer under *in vitro* study conditions with all its limitations. Comparison with real events in human disease should be made with caution.

As we know that chemokines and chemokine-receptors are involved in the pathogenesis of leishmaniasis (87) (133), elucidation (*in vitro* and *in vivo*) on the mechanisms of their direct and indirect actions during human disease is needed to evaluate if they should be further studied for use locally or systemically, alone or in combination with other drugs, to treat leishmaniasis.

Paper II (a,b)

A retrospective, epidemiological register study

This study was conducted on data from several registers, prospectively compiled in 1993 through 2016. In order to include data from as many patients as possible diagnosed with leishmaniasis in Sweden during these years, information on persons with ICD-diagnoses of leishmaniasis reported to the Swedish Patient Registry were included, as well as data from databases recorded by diagnostic laboratories at the Public Health Agency of Sweden (PHAS) and in Region Skåne. Separate incidence numbers were calculated for probable cases (reported ICD-diagnoses to the Swedish Patient Registry) and laboratory-confirmed cases. Patient characteristics were recorded and described (age, sex, probable country of infection, infecting *Leishmania* species and geographical distribution of diagnosing clinics in Sweden). Denominator data from Statistics Sweden and data from RESURS, a company mapping travel habits, were used to describe Swedish residents possibly at risk for leishmaniasis. In the earliest years of the study, the diagnosis leishmaniasis could have been made after microscopy of smears in the local clinic, without samples being sent to the PHAS or the laboratory in Skåne, and we may therefore have missed some cases. However, since molecular methods increased the sensitivity and made species identification easier in 2010, we believe that almost all patients with a parasitologically confirmed diagnosis are in the registers of PHAS. ICD-diagnoses are sometimes recorded and reported erroneously, and this could be a source of error in our group of probable disease.

Results and discussion

As human leishmaniasis is not a notifiable disease in Sweden, previously, we have not had any reliable information on how many cases were diagnosed within the Swedish health care. Reports from other non-endemic countries indicated an increasing incidence of imported leishmaniasis. (134-138) In our descriptive

retrospective study we provide the first estimation of the incidence of leishmaniasis in Sweden, and describe the characteristics of patients with a laboratory-confirmed disease. Annual incidence ranged from 0,023 to 0.35 per 100 000 Swedish residents, and we saw a rapid increase in cases diagnosed 2013-2016. Many patients (77 of 182) were infected with *L. tropica*, and most of them (50/77) were infected in Syria, and account for most of the increase of cases seen the last years. However, we could also show that there were many patients infected with *L.major* (31/182), *L.donovani* complex (22/182) and species within the *L. Viannia* subgenus. Most cases were CL, and only twelve of the 182 had other clinical presentations (6 MCL, 5 VL and 1 PKDL). We could also show that the diagnostic samples were not primarily sent by centres in the university hospitals. The geographical distribution of cases seemed to correspond well with the size of each region and city. We did not have complete data on clinical characteristics of patients, like information on comorbidity including immunosuppression. However, the information available revealed that some of the patients included in our study had acquired or medically induced immunosuppression. As this is an increasing group of patients internationally, it would have been interesting to study whether the proportion of immunosuppressed patients are increasing amongst those diagnosed with leishmaniasis in Sweden. To complete the discussion, we looked into data from Statistics Sweden, showing that 9.5% of the Swedish population in 2017 was born in Leishmania-endemic countries. In 2014, Swedish residents made 6.8 million trips with at least one overnight stay to Leishmania-endemic countries. Those having spent time in endemic areas may be at risk of developing disease some time during their lives, and the rise in use of immunosuppressive therapies may become a problem. Also in Sweden, we have seen a number of patients on biological DMARD treatment with different presentations of leishmaniasis after visiting endemic areas in Southern Europe. (128)

Our study presents the first estimation of the incidence of human leishmaniasis in Sweden, and registers kept over time at the Swedish Patient Registry and PHAS made it possible to collect information on a comparatively large number of patients with this rare disease for a non-endemic country (182 confirmed, 299 probable). (28,30,136,138,139) However, the true incidence of CL, MCL and VL respectively is probably higher than our estimations. In a case of CL probably caused by *L. major*, where the evolution of lesions suggest an imminent healing without treatment, or where the patient is diagnosed and treated outside Sweden, the physician in charge may have been satisfied with following the patient to see that the lesion cures, and no samples would have be sent to the laboratoryIn some cases, the treating physician may have been satisfied with a microscopy-result from the pathology-department together with a travel history to a country where only one *Leishmania* species is endemic. In both cases above, our study design would have included the patients in the probable group only, they would have

been missed in the group of confirmed cases. . Some cases of CL, MCL or VL may never have been suspected, as the disease is rare, and few medical specialities can be expected to know when to suspect leishmaniasis. Never suspected cases will be missed altogether in our study, and in most epidemiological studies of leishmaniasis.

Correspondence about the paper

A group of researchers from paediatric, university and emergency medicine departments in the USA did pay attention to our published paper, and communicated discussing the results in a letter to the editor in Epidemiology and Infection. (140) They had recently been looking into the subject due to a 12-year old boy investigated for skin lesions after a visit to the Middle East. In the letter, they commented on the diagnostic challenges of investigating a rare diagnosis in a non-endemic country. They were interested in the fact that we reported a high proportion of individuals aged below 18 in the last years of our study, and asked for a discussion of any underlying cause. Fanyan et. Al. suggested that we should discuss 'the variances in incidence to help readers understand whether there were specific natural or epidemiological factors or whether it was the complex interplay of socio-economic factors, environmental and climate change, zoonotic seroprevalence or unclear causative factors that contribute to the variations in incidence.' (140) For our reply, please see Paper IIb. (141) Although this correspondence does not present original research data, it has been included in this thesis as an example of communication concerning our findings, which is an important task both during PhD studies and for universities in general.

Paper III

A laboratory study comparing different diagnostic methods on a selection of serum samples

In this study, all assays were conducted at the National Reference Laboratory for Parasitology at the Public Health Agency (PHAS) in Stockholm. We chose four commercially available methods for detection of anti-leishmania antibodies in patients with VL or MCL, and added the serological method used for over 30 years at PHAS(142), resulting in five different assays for detecting anti-Leishmania antibodies. Serum samples from patients with verified VL and MCL were used as gold standard, and serum samples from several groups without VL or MCL were used to estimate sensitivity, specificity and accuracy of the different tests in a population of leishmania patients infected with different species as is the case in a non-endemic country like Sweden. In general, when evaluating a

diagnostic method, using fresh serum samples in a prospective study with complete information on patients, an accepted gold standard method to compare against and with a standardized protocol for diagnostic samples and data collection would be to prefer. However, for a disease as rare as VL in Sweden, this type of evaluation would take decades, leading to other problems, such as inter-reader-variability. We do not know if our chosen study group correctly represents the exact proportions in patients for whom the test will be used, but the positive patients (true VL/MCL) are taken from the biobanked serum samples from PHAS, most of them sent for *Leishmania* serology testing.

Results and discussion

Our hypothesis was that two different serological tests would perform better used in combination than one test alone. Each of the serological tests that were validated in our study had previously been tested in larger populations than ours for their sensitivities and specificities. (50,51) (52,58,59,61,143) However, no study had compared the five different tests in a mixed population, with VL and MCL-patients infected with different *Leishmania* species from different countries, in a non-endemic setting. In order to be able to properly interpret the results of a chosen test, it is important to also validate the test in the setting in which it will later be used. In our selection of samples, all tests had lower sensitivities and specificities compared to previous studies. The tests with highest accuracies (DAT and rK39 RDT) were negative in samples from patients infected with *L. Viannia* spp. Sensitivities of tests either used alone or in combination ranged from 82-94% and differences in sensitivity between the tests were not statistically significant in our population (88 samples in total). A combination of tests did not improve accuracies compared to the results from the best test alone. Specificities of the tests/test combinations varied widely between 61% (WB) and 98% (rK39 RDT). Although important for interpretation of test results, we did not calculate positive and negative predictive values for the tests to detect anti-Leishmania antibodies in patients with VL or MCL. This is due to the fact that we do not know whether our selection of samples correctly reflects the population in which the test will be used, nor can we be sure about the expected prevalence of the disease in the same group. However, as the incidence of VL/MCL is very low in Sweden(40), and the current serology method used at the Public Health Agency of Sweden had <7% positivity in the samples sent for detection of anti-Leishmania antibodies in 2017 (data not published), one could expect as many false positives as true positives in the population for which the test will be used if the specificity of the test is around 92%.

In concordance with previous findings, some patients having spent time in endemic regions without symptoms of VL/MCL were positive in serology (especially in WB) and three of the tests (IFAT, ELISA, WB) were positive in a

majority of patients with other parasitic diseases without symptoms or signs of leishmaniasis. Some patients with symptoms and parasitological confirmation of VL/MCL did not have any detectable antibodies, and the majority of them had a known immunosuppression. These results emphasize the importance, if serology is used in the investigation of VL and MCL, to also pursue the ambition to receive a parasitological confirmation through microscopy, culture and/or molecular methods. The fact that the DAT and rK39 RDT tests had the highest specificity in our study may be due to that our golden standard was patients with clinical VL or MCL. Any of the tests results detecting true anti-Leishmania antibodies due to previous, cutaneous or latent disease were considered as false positives in our study and would have impaired estimated specificities and accuracies.

Paper IV

An in silico study with comparison of genes in published genomes and incipient laboratory studies of possible activity

We aligned the sequences of the Glycoside Hydrolases known from other *Leishmania* species with the genome published by the *Leishmania mexicana* Genome Project. Most of them gave matches in 13 of the genes (10 good matches, 3 partial matches) probably coding for glycoside hydrolases also in this species. Using the software tool Signal P, we predicted 7 of them to be secreted proteins, and they were chosen for further investigation. The gene sequences were analysed for the presence of conserved domains and for homology, in order to reconstruct phylogenetic trees for the candidate proteins. Laboratory analyses performed at the Division of Infection medicine, University of Lund included PCR analyses to detect the 7 candidate genes in 14 clinical *Leishmania* strains and analyses of enzymatic activity on human IgG from supernatants of promastigote cultures of some strains.

Results and discussion

We searched the CAZy database for all Leishmania-derived putative glycoside hydrolases, aligned them with the published genome of Leishmania mexicana and used sequences of the probably secreted proteins for bioinformatic and molecular studies. We found six genes that seemed to be highly conserved in *Leishmania* spp, some of which were similar to glycoside hydrolases of other non-related pathogenic microbes as bacteria. With primers designed for the genes in *L. mexicana*, we amplified the genes in several clinical *Leishmania* strains of different species. Culture supernatants from the same strains were used to show enzymatic activity on glycosidic bonds on a protein with the size of the heavy

chain of IgG, probably caused by a carbohydrate enzyme secreted by the parasites. These findings encourage further studies in the field of CAZymes in *Leishmania* with possible action on components of the human immune system.

Concluding remarks

-The human chemokines CXCL2, CXCL6, CXCL9, CXCL10, CCL20 and CCL28 have direct antimicrobial effects on promastigotes of *Leishmania mexicana* in vitro. Apart from adding to the knowledge about the antimicrobial properties of chemokines and their possible role in leishmaniasis, this finding may encourage topical or systemically administered chemokines or chemokine-stimulators as targets for future studies.

-The first estimation of annual incidence of imported leishmaniasis in Sweden ranged from 0,023 to 0,35 per 100 000 with a rapid increase in 2013-2016. Many patients were infected in Syria and Afghanistan, but cases were diagnosed from all endemic continents, and there was a large variation in infecting species. Among laboratory-verified patients, molecular methods were most likely to be positive, and a combination of different methods increased the sensitivity. Our retrospective study may raise awareness of this rare imported disease amongst healthcare providers in Sweden so that patients with CL, VL or MCL will receive a correct diagnosis and the appropriate treatment.

-In the evaluation of five serological methods to detect anti-Leishmania antibodies in a selection of biobanked serum samples at the PHAS, no test or combination of tests had a higher sensitivity than 93%, or a higher specificity than 98% to detect VL. Surprisingly, no combination of two tests showed a higher accuracy than either the DAT or rK39 RDT alone (92% and 93%, respectively). Some patients with parasitologically confirmed VL were negative with all serological tests.

-*In silico* analysis identified seven candidate extracellular glycoside hydrolases in *Leishmania* spp. Detailed phylogeny indicated that most proteins are conserved among *Leishmania* species, and that all except the putative GH65 enzyme that resembles bacterial enzymes, are related to eukaryotic glycoside hydrolases. Enzymatic activity of the supernatants of cultured promastigotes reveals a possible action on the heavy chain of IgG, which could be of importance for infection and survival of the parasite within the host.

Future Perspectives

The complex interplay between *Leishmania* parasites and the immune system of the host has been studied for decades, but some questions remain to be answered, and there is still no effective and safe vaccine or drug for VL without serious side effects. In the light of the results of this thesis, two areas of further studies could reveal possible targets for drug or vaccine development. First, human chemokines' role in leishmaniasis, specifically their direct interaction with the parasites, could be studied also for amastigotes and with other methods to reveal the mechanisms behind our findings. Secondly, the conserved chitinase in *Leishmania,* which previously has been shown to be a virulence factor also in the host, and is expressed in larger amounts in amastigotes than in promastigotes, could be active in the parasites' defence against humoral or cellular immunity of the host. Recombinant expression or purification of this enzyme is needed for further characterization.

It is important to continue to follow the epidemiological situation of leishmaniasis in Sweden and other non-endemic countries to keep health care providers in affected specialities informed about the rare disease. With the large variety of clinical presentations, infecting species and choice of treatments, international multi-centre studies are needed to collect data in a systematic way from a sufficient number of patients in order to draw correct conclusions about treatment outcome. Medical immunosuppression is being used for an increasing number of diagnoses, and there is a concern for an increased incidence of leishmaniasis both in endemic areas and in non-endemic areas where people may reactivate a latent infection from previous stay in endemic areas, or be at risk for clinical disease visiting the same for business or vacation. With larger study populations and international databases, trends and risks for certain drugs may be discovered earlier. Although diagnostic tools to diagnose active disease are satisfactory, there is still a need for studies on how to best diagnose latent disease in order to assess risks with transfusion, transplantation and immunosuppressive medical treatment.

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Appendices, Paper I-IV

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