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# LIST OF PUBLICATIONS

This thesis is based on the following publications:

1. **Paape, D.**, Barrios-Llerena, M. E., Le Bihan, T., Mackay, L., Aebischer, T. (2010). Gel Gel free analysis of the proteome of intracellular Leishmania mexicana. Mol. Biochem. Parasitol. 169 (2) 108-114

2. Barrios-Llerena, **Paape, D.**, M. E., Le Bihan, T., Mackay, L., Aebischer, T. (2010) Proteome of the intracellular *Leishmania mexicana* harbouring parasitophourous vacuole (manuscript in preparation)

3. **Paape, D.**, Lippuner, C., Schmid, M., Ackermann, R., Barrios-Llerena, M. E., Zimny-Arndt, U., Brinkmann, V., Arndt, B., Pleissner, K. P., Jungblut, P. R., and Aebischer, T. (2008). Transgenic, fluorescent *Leishmania mexicana* allow direct analysis of the proteome of intracellular amastigotes. Mol. Cell Proteomics. 7 (9) 1688-1701

Further Publications:

4. Lippuner, C., **Paape, D.**, Paterou, A., Brand, J., Richardson, M., Smith, A. J., Hoffmann, K., Brinkmann, V., Blackburn, C., and Aebischer, T. (2009). Real-time imaging of *Leishmania mexicana*-infected early phagosomes: a study using primary macrophages generated from green fluorescent protein-Rab5 transgenic mice. FASEB J. 23 (2) 483-491

5. Mylonas, K. J., Nair, M. G., Prieto-Lafuente, L**., Paape, D.**, and Allen, J. E. (2009). Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. J.Immunol. 182 (5) 3084-3094

6. Paterou A., **Paape D.**, Lippuner C., Aebischer T. (2007) *Leishmania* infections: A cell biology centred view from an immunology and vaccinology perspective. Science and Culture May-June (73) No. 5-6, pp.150-158

# ABBREVIATIONS

| | |
|---|---|
| 1-DE | 1-dimensional SDS PAGE |
| 2D | two dimensional |
| 2-DE | two dimensional gel electrophoresis |
| Bmdm | bone marrow derived murine macrophages |
| CD40L | CD40 ligand |
| cDNA | complementary DNA |
| CE | cholesteryl ester |
| DC | dendritic cell |
| DCL | diffuse cutaneous leishmaniasis |
| DECR | 2,4-dienoyl CoA reductase |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylene diamine tetraacetic acid |
| FAD | flavin adenine dinucleotide |
| FC | free cholesterol |
| FCS | fetal calf serum |
| FFA | free fatty acids |
| FFA | free fatty acids |
| FMN | flavin mononucleotide |
| G | gauge |
| gDNA | genomic DNA |
| HCl | hydrogen chloride |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| IgG | immunoglobulin |
| IL | interleukin |
| *L.* | *Leishmania* |
| LCL | localized cutaneous leishmaniasis |
| LDL | low-density lipoprotein |
| LPDS | lipoprotein deficient serum |
| MEM | minimum essential medium |
| MES | 2-(N-Morpholino)ethanesulfonic acid |
| MOI | multiplicity of infection |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| MΦ | macrophage |
| NADPH | Nicotinamide adenine dinucleotide phosphate reduced form |
| NaOH | sodium hydroxide |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PFA | paraformaldehyde |
| PM | plasma membrane |
| PUFA | poly unsaturated fatty acids |
| PV | parasitophorous vacuole |

| | |
|---|---|
| qRT-PCR | quantitative Reverse Transcription PCR |
| RNA | ribonucleic acid |
| RPMI | Roswell Park Memorial Institute |
| RT | room temperature |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SDM | semi defined medium |
| SDS | sodium dodecyl sulphate |
| spp. | species |
| SSC | sodium chloride, sodium citrate buffer |
| TBE | Tris-borate-EDTA buffer |
| TBS | Tris buffered saline |
| TGFβ | Transforming growth factor β |
| TM | trans membrane |
| TRIS | 2-Amino-2-hydroxymethyl-propane-1,3-diol |
| Tris | tris(hydroxymethyl)aminomethane |
| UTR | untranslated region |
| VL | disseminated visceral Leishmaniasis |
| Vol. | volumes |

# ZUSAMMENFASSUNG

Bestandteil dieser Arbeit waren zwei Proteomanalysen des Parasiten *Leishmania mexicana*. In der ersten wurde das Proteom von aus ihrem intrazellulären Habitat aufgereinigten Amastigoten, mit dem Proteom von Promastigoten, der extrazellulären Lebensform, verglichen.

Der Fokus der zweiten Proteomanalyse war die Identifizierung von bei Amatigoten sezernierten Proteinen. Insgesamt wurden mehr als 1800 Proteine identifiziert, von denen mehr als 740 zuvor noch nicht detektiert worden waren. Diese Analysen tragen großen Anteil an den nun insgesamt 2500 ermittelten Proteinen, was ungefähr 30%, des auf dem Genom-basierenden vorhergesagten Proteomes entspricht. Außerdem konnten 143 potentiell sezernierte Proteine ermittelt werden.

Bioinformatische Analayse, der den identifizierten Proteine entsprechenden Gene, machte einen klaren translationalen Bias im Codongebrauch bei Leishmanien deutlich. Der Vergleich des Proteomes von Promastigoten und Amastigoten detektierte im letzteren Lebensstadium hochregulierte Proteine. Analyse der entsprechenden Genloci identifizierte charakteristische Sequenzmotive in den 3'-untranslatierten Regionen, diese stellen möglicherweise auf Translationsebene wirkende regulatorische Elemente dar.

Eine Untergruppe, der in Amastigoten hochregulierten Proteine, waren Enzyme des katabolen Fettsäurestoffwechsels. Daraus wurde die Hypothese abgeleitet, dass Proteine dieser Untergruppe Virulenzfaktoren sein könnten. Die 2,4 Dienoyl-CoA Reduktase (DECR), wurde für weitere Untersuchungen ausgewählt, da sie zwar homolog zu einen äquivalenten bakteriellen Protein ist, aber keine Homologie zu äquivalenten Proteinen höherer Eukaryoten aufweist.

Um zu analysieren, ob DECR zur Virulenz und Pathogenität beiträgt, wurden Parasiten hergestellt, die kein *decr* Gen mehr besitzen. Durch Infektion von BALB/c Mäusen, welche anfällig gegenüber *Leishmanien* sind, konnte gezeigt werden, dass Parasiten, denen *decr* fehlte, eine verminderte Virulenz gegenüber dem Wildtyp aufwiesen.

Eine weitere Hypothese war, dass Parasiten zur Deckung ihres eigenen Energie-Metabolismus der Wirtszelle Fettsäuren entziehen, und dies die Lipid-Homeostase der Wirtszelle beeinflusst. Zellen kontrollieren ihre Lipid-Homeostase mit Hilfe von Transkriptionsfaktoren. Die Quantifizierung der Genexpression der durch diese Transkriptionsfaktoren regulierten Gene, machte deutlich, dass Gene deren Proteine in der Biosynthese von Cholesterol beteiligt sind, in infizierten Makrophagen höher exprimiet waren. Durch biochemische Methoden konnte gezeigt werden, dass im Parasiten-enthaltendem Kompartiment der Wirtszelle, Cholesterol zurückbehalten wird.
Zusammenfassend wurde ein neuer Virulenzfaktor in Leishmanien identifiziert, welcher ein Kandidat für die Entwicklung eines neuen anti-parasitären Medikaments sein könnte. Außerdem wurde gezeigt, dass Leishmanien die Lipid-Homeostase von Makrophagen verändern, was Einfluss auf die gesamte Immunantwort nehmen könnte.

# SUMMARY

Two proteome studies of the parasite *Leishmania mexicana* were performed. The first study compared proteomes of amastigotes, purified from their intracellular habitat, to promastigotes, the extracellular life cycle form. Identification of proteins secreted by amastigotes was the focus of the second study. More than 1800 leishmanial proteins were identified, of which more than 740 were not previously reported, and contributed to the current 2500 detected proteins for that genus. That equals approximately 30% of the proteome predicted based on genome data. Moreover 143 potentially secreted proteins were identified.

Bioinformatic analyses of genes corresponding to protein datasets revealed clear evidence for translational bias in codon usage in *Leishmania*. Comparison of the promastigotes and amastigote proteomes led to the identification of upregulated proteins in amastigotes. Analysis of the respective loci identified characteristic sequence motifs in the 3'untranslated regions, which may represent, or be linked to, translational control elements.

A subset of proteins upregulated in amastigotes was involved in the catabolism of fatty acids. This led to the hypothesis that proteins of this subset may be virulence factors. A candidate, 2,4 dienoyl-CoA-reductase (DECR), was chosen for further investigation. The leishmanial DECR is related to an equivalent bacterial protein but has no homology to equivalent higher eukaryotic DECR enzymes. For the analysis of its contribution to virulence and pathogenicity, *decr* deficient parasites were generated. Infection of BALB/c mice, which are susceptible to *Leismania* spp., revealed that compared to wild type parasites, those devoid of *decr* were less virulent.

It was further hypothesised, that the parasites sequester fatty acids for their own energy metabolism and this alters the lipid homeostasis of the host cell. Cells control their lipid homeostasis by transcription factors. Quantitative reverse transcription-polymerase chain reaction of target genes of those transcription factors revealed that the genes involved in cholesterol biosynthesis were upregulated in infected macrophages. Further analysis revealed that cholesterol is retained in parasite harbouring compartments.

In summary, a novel virulence factor in *Leishmania* was identified, which could be an appropriate target for the development of novel anti-parasitic drugs. In addition *Leishmania* alters the lipid homeostasis of the macrophage which could be linked to immuno-modulation.

# 1 INTRODUCTION

## 1.1 *LEISHMANIA* AND LEISHMANIASES

*Leishmania* spp. are obligate intracellular protozoan parasites. They oscillate between the extracellular and intracellular life cycle stages, promastigotes and amastigotes respectively. Promastigotes are elongated, flagellated and motile and exist in the sandfly vector. Amastigotes are round, aflagellated and non motile and reside mainly inside macrophages (MΦ) and dendritic cells (DC). Promastigotes proliferate in the gut of the sandfly (hindgut for leishmanial organisms of the *Viannia* subgenus; midgut for organisms of the *Leishmania* subgenus see Table 1). They migrate to the proboscis where they become metacyclic, the non-dividing infective form. During a blood meal metacyclic promastigotes are transmitted to mammals, including humans, by the female sandfly of the genus *Lutzomyia* in the New World and of the genus *Phlebotomus* in the Old World (Alexander, J. *et. al.*, 1999; see Figure 1 for whole life cycle). New world and old world species of *Leishmania* exist with different occurrence, clinical pathology and transmission (TABLE 1). Once inside the host, promastigotes are phagocytosed by professional phagocytic cells such as neutrophils, MΦ and DC.

Until now it is unclear how the promastigotes enter the MΦs *in vivo*. It is believed that during a blood meal both dead and live promastigotes are transmitted into the host. Dead parasites present phosphatidyl serine, a surface apoptotic marker and possibly other apoptotic markers. Due to the bite neutrophils are attracted to the site of infection within minutes and phagocytose live and dead parasites (Peters, N. C. *et. al.*, 2008). It is assumed that due to the phagocytosis of dead parasites an anti-inflammatory cytokine milieu is generated by neutrophils, with relatively high levels of transforming growth factor-β (TGF-β) and low levels of tumour necrosis factor-α (TNF-α). This silences the surrounding neutrophils and prevents an oxidative burst that would kill the parasite (van Zandbergen, G. *et. al.*, 2006). Inside the neutrophils promastigotes do not transform into amastigotes. Instead they increase the life span of neutrophils. Eventually infected neutrophils undergo apotosis and are phagocytose by MΦs which were also attracted to the site of infection (Alexander, J. *et. al.*, 1999; van Zandbergen, G. *et. al.*, 2002; van

Zandbergen, G. *et. al.*, 2004). Thereby the MΦs are not activated, and clearance of parasites is prevented. This theory is called the Trojan horse or silent entry theory (Laskay, T. *et. al.*, 2008). Another study also reported that neutrophils are involved in the establishment of disease, as depletion of neutrophils significantly reduced the number of viable parasites detected at site of infection (Peters, N. C. *et. al.*, 2008). Balanco *et al.* reported that *L. amazonensis* amastigotes present phosphatidyl serine on their surface and infected macrophages produce TGF-ß as well as interleukin-10 (IL-10; (Balanco, J. M. F. *et. al.*, 2001). Also metacyclic promastigotes, see below, present phosphatidyl serine on the outer leaflet of membrane, (Barcinski, M. A. *et. al.*, 2003). This could promote their silent entry into the neutrophils.

In another hypothesis, neutrophils phagocytose only dead parasites, and live parasites escape phagocytosis (Peters, N. C. *et. al.*, 2008). Neutrophils undergo apoptosis, when attracted MΦs reach the site of infection and this effect is strengthened by the presence of live parasites (Allenbach, C. *et. al.*, 2008). The apoptotic neutrophils are phagocytosed by MΦs along with the promastigotes. Due to the presence of the apoptotic neutrophils the MΦ are silenced.

Inside the MΦ and DCs phagocytosed promastigotes reside initially in early endosomes. These early endosomes mature to late endosomes and eventually to the parasitophorous vacuole (PV) a late endosome early lysosome like compartment. Promastigotes transform to amastigotes inside the PV (Lippuner, C. *et. al.*, 2009). Depending on the cell lineage (MΦ or DC) as well as the parasite species, the size of the PV and the parasite proliferation rate varies. Parasites either share a communal PV or reside in single PVs (Antoine, J. C. *et. al.*, 1998).

During a blood meal parasites are transmitted back into another sandfly. Amastigotes retransform into promastigotes in the gut, start to proliferate and migrate to the proboscis and become metacyclic again, thus completing the cycle.

*Leishmania spp.* infections are a major cause of morbidity and mortality, with an estimated worldwide prevalence of 12 million people, with increasing tendencies in many areas of the world. There are an estimated 1.5 – 2 million new cases and 60,000 to 70,000 deaths each year, and 350 million people are at risk of infection

and disease (see http://www.who.int/vaccine_research/diseases/soa_parasitic/en/index3.html; (Reithinger, R. *et. al.*, 2007). An increasing problem is co-infections with human immune deficiency virus in south-western Europe (France, Italy, Portugal and Spain) as well as in Middle and South America, Africa and Asia (http://www.who.int/leishmaniasis/burden/hiv_coinfection/burden_hiv_coinfection/en/index.html).

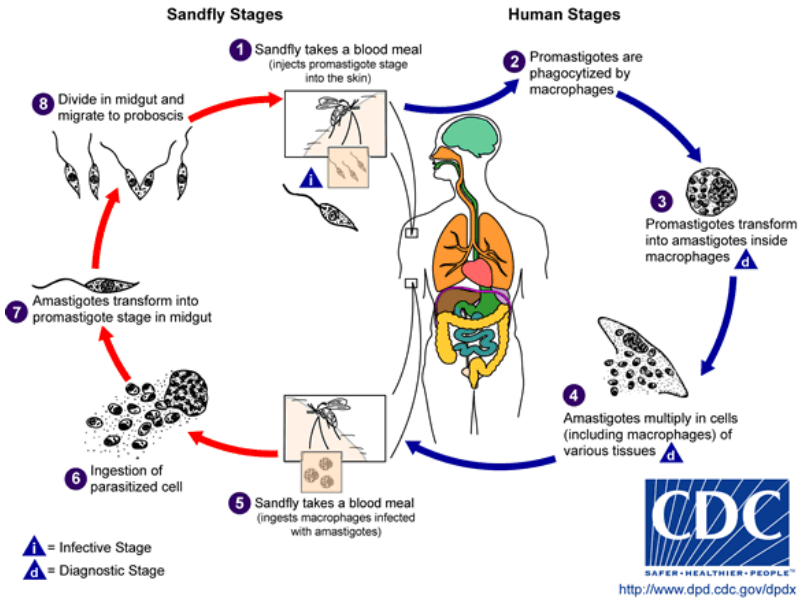


FIGURE 1. Life cycle of *Leishmania spp.*

Metacyclic promastigotes are transmitted by infected female sandfly vectors. They inject the parasites from their proboscis into the host during a blood meal (1). Inside the host promastigotes are phagocytosed by MΦ (see text for details) (2) and other professional phagocytotic cells. Within MΦ and dendritic cells promastigotes transform to aflagellated amastigotes (3) and start to proliferate (4). Depending on the Leishmania species and the immunological status of the host a certain type of disease will develop. During another blood meal of an infected sandfly, parasites are retransmitted as intracellular amastigotes (5, 6). In the midgut phagocytotic cells decompose and amastigotes are released. Subsequently they retransform to promastigotes (7), proliferate (8) and migrate to the proboscis where they become metacyclic, the non-dividing infective stage (1) and the cycle is complete.
Picture from: http://www.dpd.cdc.gov/DPDx/HTML/Leishmaniasis.htm

*Leishmania* spp. are the causal agents of leishmaniases, a spectrum of diseases ranging from a self healing cutaneous to mucocutaneous, to disseminated visceral forms.

The cutaneous form is characterized by ulcerative skin lesions at the site of infection, called localized cutaneous leishmaniasis (LCL) or multiple non-ulcerative nodules, called diffuse cutaneous leishmaniasis (DCL), whereas the mucocutaneous form leads to an inflammation of the mucosa and finally results in the destruction of the mucosa.

The disseminated visceral (VL) form is characterized by infection of the spleen, liver and bone marrow, and is fatal if untreated.

TABLE 1: Species of *Leishmania* that cause human disease

| | Main clinical pathology | Transmission cycle | Main geographical distribution |
|---|---|---|---|
| **New World Leishmania spp.** | | | |
| L (Viannia)* braziliensis | LCL, mucosal | Zoonotic | South America, parts of Central America, Mexico |
| L (Viannia) panamensis | LCL, mucosal | Zoonotic | Northern South America and southern Central America |
| L (Viannia) peruviana | LCL | Zoonotic | Peru |
| L (Viannia) guyanensis | LCL | Zoonotic | South America |
| L (Viannia) Lainsoni | LCL | Zoonotic | South America |
| L (Viannia) colombiensis | LCL | Zoonotic | Northern South America |
| L (Leishmania) amazonensis | LCL, DCL | Zoonotic | South America |
| L (Leishmania) mexicana | LCL, DCL | Zoonotic | Central America, Mexico, USA |
| L (Leishmania) pifanoi€ | LCL | Zoonotic | South America |
| L (Leishmania) venezuelensis | LCL | Zoonotic | Northern South America |
| L (Leishmania) garnhami | LCL | Zoonotic | South America |
| **Old World Leishmania spp.** | | | |
| L (Leishmania) aethiopica | LCL, DCL | Zoonotic | Ethiopia, Kenya |
| L (Leishmania) killicki | LCL | Zoonotic | North Africa |
| L (Leishmania) major | LCL | Zoonotic | Central Asia, north Africa, middle east, East Africa |
| L (Leishmania) tropica | LCL | Anthroponotic | Central Asia, middle east, parts of north Africa, southeast Asia |
| L (Leishmania) donovani | Visceral, LCL | Anthroponotic | Africa, central Asia, southeast Asia |
| **Old & New World Leishmania spp.** | | | |
| L (Leishmania) infantum | Visceral, LCL | Zoonotic | Europe, north Africa, Central America, South America |

LCL=localised cutaneous leishmaniasis. DCL=diffuse cutaneous leishmaniasis. *Subgenus is given in parentheses. Zoonotic transmitted from animals to human; anthroponotic transmitted from human to human.
Southeast Asia includes the Indian subcontinent and China.
Reprinted from Lancet Infect.Dis, Vol. 7, Reithinger, R., Dujardin, J. C., Louzir, H., Pirmez, C., Alexander, B., and Brooker, S., Cutaneous leishmaniasis, 581-596, Copyright 2007, with permission from Elsevier.

### 1.1.1 THERAPY OF LEISHMANIASES

The most commonly used drugs for first line treatment are pentavalent antimonials (sodium stibogluconate and meglumine antimonate). The main problem for this group of drugs is the intrinsic difference in species sensitivity which results in variable efficacy. *Leishmania donovani* and *L. braziliensis* are reported to be

three- to fivefold more sensitive to sodium stibogluconate than *L. major*, *L. mexicana* and *L. tropica* (Croft, S. L. *et. al.*, 2006). Furthermore, in the late 1970s the occurrence of resistance was reported in an endemic region of VL in North Bihar, India. Despite steady increase of dosage and duration of treatment, a decline in efficacy was evident (Croft, S. L. *et. al.*, 2006). This emergence of resistance is linked to widespread misuse of the drug due to poverty and the low standard of education.

Another widely used drug, used especially as a second line treatment, is amphotericin B. It interacts with ergosterol, the major sterol in the membranes of *Leishmania* and fungi. So far no definite resistance has been reported in the field but *in vitro* resistant *L. donovani* promastigotes have been raised (Mbongo, N. *et. al.*, 1998). Therefore the possibility of an emergence of resistance in the field cannot be excluded.

Miltefosine (hexadecylphosphocholine) has been recently introduced as an anti-leishmanial drug and others such as paromomycin are in clinical trials. Miltefosine is thought to inhibit phosphatidycholine, phosphatidylethanol-amine and sterol biosynthesis (Urbina, J. A. 1997). Cure rates of over 95% against visceral leishmaniasis were reported (Jha, T. K. *et. al.*, 1999). A study investigated the efficacy of miltefosine against cutaneous leishmaniasis. The drug was administered in regions in Colombia where *L.* (*vianna*) *panamensis,* and in regions in Guatemala where *L. vianna braziliensis* and *L. mexicana,* are endemic. Cure rates of over 90% were reported for the Colombian site but less than 55% for Guatemalan site (Soto, J. *et. al.*, 2004). Very promising results have been reported from the anti-fungal azoles (ketoconazole and itraconazole). These inhibit the biosynthesis of ergosterol in fungi as well as *Leishmania* spp. (Croft, S. L. *et. al.*, 2006).

The licensed drugs in use require a strict and long regimen of administration, some have severe side effects and the treatment is expensive. These reasons, as well as the variable efficacies of drugs to different *Leishmania* spp., necessitate the development of either new drugs or vaccines, which ideally will be inexpensive, easy to administer and produce fewer side effects.

### 1.1.2 TOWARDS NEW CANDIDATES FOR DRUG AND VACCINE DEVELOPMENT

As mentioned above there is a need to identify new vaccine candidates or candidates for drug development. This is possible via microarray or proteomic analysis. The latter is more representative in the case of *Leishmania* for reasons given below. Selection of suitable drug candidates, e.g. enzymes involved in parasite specific reactions, or enzymes catalysing reactions via a different mechanism than the host, leads to the next step: Compound screening. Proteomics of the amastigotes will also identify proteins which are of high abundance, which is an important parameter for the vaccine candidate selection (Aebischer, T. *et. al.*, 2000; Sabarth, N. *et. al.*, 2002).

Genomes of kinetoplastids share a common feature, genes are arranged in polycistronic units (El Sayed, N. M. *et. al.*, 2005). These units are transcribed constitutively into RNA precursors (Clayton, C. E. 2002; Leifso, K. *et. al.*, 2007). Processing of these transcribed polycistronic units by *trans* splicing and polyadenylation leads to individual RNA molecules (Liang, X. H. *et. al.*, 2003). Due to the constitutive transcription, the regulation of gene expression occurs at a post transcriptional level (Clayton, C. E. 2002). Hence the mRNA level generally does not correlate with the actual protein levels expressed, therefore using a microarray is not adequate, whereas proteomics reflects actual protein levels. The completion and annotation of the genome sequence of *L. major*, *L. infantum* and *L. braziliensis* (Ivens, A. C. *et. al.*, 2005; Peacock, C. S. *et. al.*, 2007) enabled proteomic analysis of *Leishmania* (Dea-Ayuela, M. A. *et. al.*, 2006; Foucher, A. L. *et. al.*, 2006; McNicoll, F. *et. al.*, 2006; Nugent, P. G. *et. al.*, 2004; Walker, J. *et. al.*, 2006). There is a high degree of sequence conservation and synteny of their genes throughout the genome among these species (Peacock, C. S. *et. al.*, 2007). This allows even proteomic analysis of unsequenced species of that genus (Nugent, P. G. *et. al.*, 2004). Proteomics analysis of the intracellular lifecycle stage is especially important in order to identify pathways which are activated within the host (Davis, A. J. *et. al.*, 2004).

Therefore proteome studies were performed in order to compare expressed proteins in the two different life cycle stages (see above for a selection of references). Proteomics of intracellular pathogens is hampered by the contamination of the sample with host cell material. Therefore, axenic amastigotes

(amastigote-like forms) have been used to substitute for the intracellular life cycle stage. Axenic amastigotes can be grown host-cell-free under conditions mimicking the intracellular habitat, i.e. low pH and higher temperature (Bates, P. A. 1994). However, they can only be grown from few species and genome wide profiling suggested that axenic amastigotes are more closely related to promastigotes than to *ex vivo* isolated amastigotes (Holzer, T. R. *et. al.*, 2006). Thus, current datasets on amastigote proteomes likely underestimate differences and could miss important changes.

In my diploma thesis[1] a purification method was developed to overcome these hurdles (FIGURE 2) and an initial list of proteins, present in pro- and amastigotes was obtained by mass spectrometric analyses. This list has been used as a basis for further analysis and complemented by additional proteomic investigations on the amastigote form of the parasite. These studies, as outlined later, indicated that enzymes involved in fatty acid metabolism were upregulated in amastigotes.

In the early 1980s it was reported that lesion derived amastigotes utilise more triglycerides and non-esterified fatty acids than promastigotes (Hart, D. T. and Coombs, G. H. 1982). Therefore, it was very likely that the data regarding higher abundance of fatty acid metabolism involved proteins would be confirmed. Thus, an overview of the lipid metabolism of the host cell as well as of *Leishmania* spp. is given below.

[1] Daniel Paape 'Ein Vergleich der Proteome von *Leishmania mexicana* Promastigoten versus Amastigoten' Diploma thesis, Freie Universität Berlin, Department Biology, Chemistry and Pharmacy in 2005

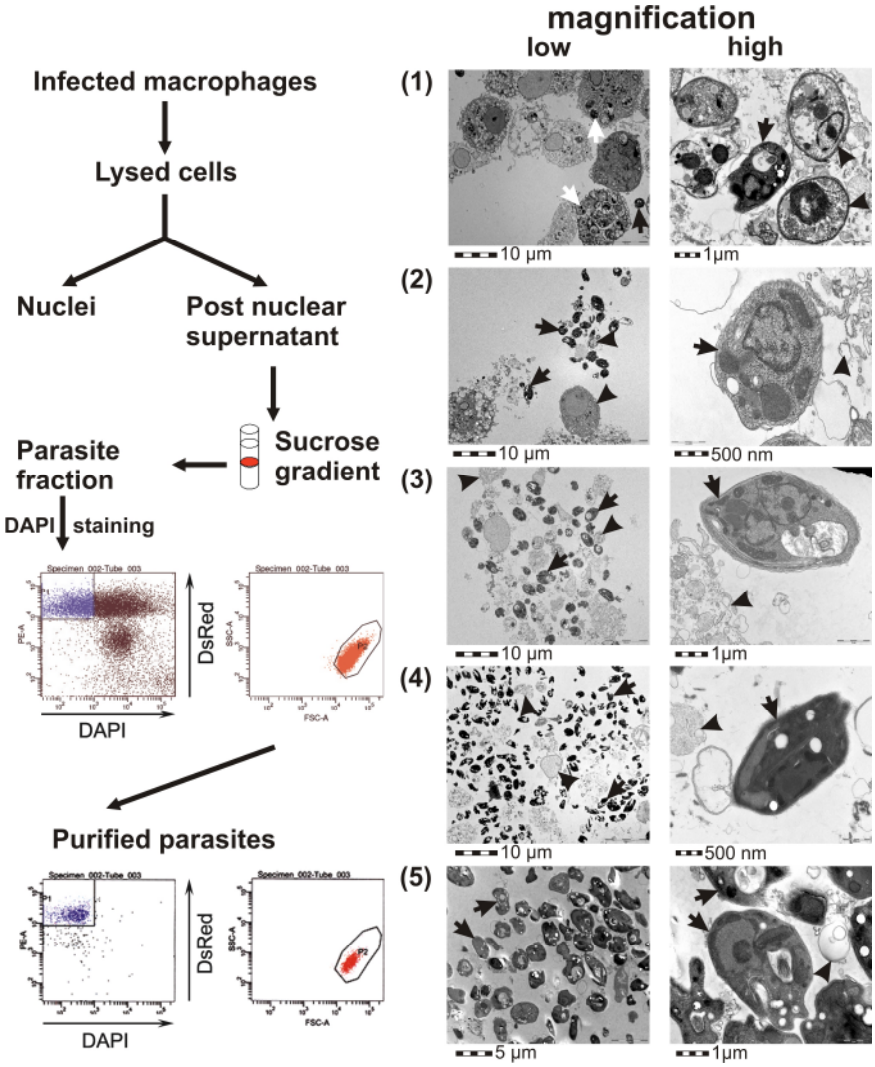


FIGURE 2. Purification scheme of amastigote isolation from infected macrophages. **Left)** *L. mexicana::DsRed* infected macrophages were lysed and nuclei pelleted at low centrifugal force. The post nuclear supernatant was overlaid onto a discontinuous sucrose gradient. The parasite containing fraction was harvested after centrifugation and stained with DAPI. Subsequently, this material was sorted by fluorescence activated cell sorting (FACS), focusing on $DsRed^{+}/DAPI^{-}$ (blue population) and forward scatter (FSC)/ side scatter (SSC; red population). Sorted parasites were re-analysed (bottom dot blot). **Right)** Analytical electron microscopy of parasite containing fractions. Infected macrophages (1) with intracellular amastigotes (white arrowheads) and after lysis (2), clearly visible free parasites (black arrows) and host cell material (black arrowheads). Post nuclear supernatant (3) and harvested sucrose gradient fraction (4) still contain host cell derived material which is almost completely absent in the sorted parasites (5), black arrows, free amastigotes; black arrowhead, loose membrane.

## 1.2 FATTY ACID METABOLISM / BETA-OXIDATION

### 1.2.1 LOW-DENSITY LIPOPROTEIN AND ITS PROCESSING

Human as well as murine cells in peripheral organs are supplied with cholesterol and fatty acids by low density lipoproteins (LDL). These consist of cholesteryl ester (CE), apolipoprotein B 100 (apoB100) and lipids. Cholesterol comprises 60% of the total lipid and approximately 80% is esterified with fatty acids. Among these fatty acids linoleic acid accounts for 50%, oleate and palmitate comprise 20% and 15%, respectively. Stearate, palmitoleate and arachidonate make up the remaining 15% (Goldstein, J. L. and Brown, M. S. 1977). Cholesterol and CE form the apolar core of LDL. This apolar core is surrounded by phospholipids which account for 30% of the total lipid mass of LDL and consists primarily of phosphatidylcholine (65%) and sphingomyelin (25%). It has been also reported, that five different types of glycosphingolipids are contained in LDL (Goldstein, J. L. and Brown, M. S. 1977).

LDL is endocytosed in a receptor mediated manner. The LDL-receptor (LDLR) binds apoB100 and internalises LDL via clathrin coated pits (Schmid, S. L. 1997; Maurer, M. E. and Cooper, J. A. 2006). After internalisation the clathrin coat is shed and the resulting vesicle is a mildly acidic (pH ~ 6.0) early endosome. The decreased pH leads to a dissociation of LDL from its receptor. The latter is conveyed back to the plasma membrane (PM) in a recycling endosome whereas the early endosome, containing the LDL, matures into the late endosome and lysosome. During this maturation the pH becomes gradually more acidic. In the lysosome LDL disintegrates, apolipoprotein is degraded, and lysosomal acid lipase (Lipa) hydrolyses CE to free fatty acids (FFA) and free cholesterol (FC; see Figure 4).

The parasites reside within the PV and depend on nutrients within this compartment to thrive. The constant supply of molecules is assured by fusion and fission events between endosomes and phagosomes where contents are exchanged (Desjardins, M. *et. al.*, 1994; Desjardins, M. *et. al.*, 1994; Vieira, O. V. *et. al.*, 2002).

Hence the PV where the parasites reside contains also LDL and FFA and FC released through hydrolysis from CE. Therefore the parasite can utilise FFA for its own energy metabolism.

### 1.2.2 FATTY ACID TRANSPORT

It has been reviewed that in eukaryotes FFA complexed with albumin rapidly adsorb to the outer membrane leaflet, pass through the bilayer and desorb from the inner leaflet in a quantitative manner (Hamilton, J. A. 1998).

Berman and colleagues, have shown that amastigotes also take up albumin-complexed FFAs very rapidly (Berman, J. D. *et. al.*, 1987). Furthermore uptake of albumin-complexed FFA is augmented at lower pH in mammalian cells (Spector, A. A. 1969). Besides the albumin-complexed FFA as they occur in the blood circulation, uncomplexed FFA adsorb measurably faster to membranes as studies with mammalian cells showed (Simard, J. R. *et. al.*, 2008; Simard, J. R. *et. al.*, 2008) and diffuse through the membrane, see below. The latter is most likely the case when FFA are released through hydrolysis from CE in the lysosome. Translocation of FFA can be either a passive diffusion or a protein mediated process. Hamilton proposed that the translocation of un-ionized FFA is a simple passive diffusion process called flip-flop (Hamilton, J. A. 1998). Due to the acidic environment of the PV it is highly probable that the FFAs are protonated. Flip-flop of the anionic form of FFA is very slow and apparently protein mediated. Garlid *et. al.* proposed a mechanism where un-ionized FFA flip-flop from the cytosolic leaflet of the inner mitochondrial membrane to the matrix side and either desorb or become un-protonated, thus delivering a proton to the matrix. Hence the net charge of the inner leaflet would become negative, because the flip-flop of ionized FFA is very slow. The mitochondrial uncoupling protein (UCP), an anion transporter, translocates the ionized FFA back to the cytosolic side where it gets protonated again, this is called protonophoretic cycle (Garlid, K. D. *et. al.*, 1996). So far it is still unclear if there is a FFA translocase in mammalian membranes. The main focus was on CD36, fatty acid transport protein (FATP) and caveolin-1, but there is no proof that these proteins transport FFA across the lipid bilayer or if they only facilitate the translocation. It has been shown that FATP activates FFA to form acyl-CoA, which cannot diffuse through the membrane. Caveolin-1 is a

peripheral membrane protein with a large intracellular domain. Diffusion of fatty acids through membranes is a rapid process as well as lateral diffusion. In the ordered phase of the membrane close to caveolin-1 regions also rich in cholesterol diffusion processes are probably slower and limited. Therefore it was proposed that caveolin-1 is involved in segregation or organisation of FFA for subsequent extraction from the PM by specialised proteins, see below. An enhanced FFA uptake has been detected for CD36 but the mechanism remains unclear (Hamilton, J. A. 2007).

At the inner membrane leaflet FFA desorb and diffuse through the cytosol either in an unbound form or attached to a group of proteins called fatty acid binding proteins (FABPs) (Furuhashi, M. and Hotamisligil, G. S. 2008). FABPs increase the dissociation from membranes and cytoplasmic diffusion (Chmurzynska, A. 2006). Clarke and colleagues overexpressed a membrane form of FABP (Stremmel, W. *et. al.*, 1985) and noted an increased rate of fatty acids transported across the sarcolemmal membrane (Clarke, D. C. *et. al.*, 2004). An interesting point is why there should be FFA translocases if the membrane is permeable for them.

### 1.2.3 CHOLESTEROL HOMEOSTASIS AND TRANSPORT

Cholesterol is a major component of the PM and of the membrane of other organelles. Cholesterol regulates the fluidity and permeability of membranes. Furthermore it forms cholesterol-enriched microdomains, so called lipid rafts, which are implicated in signalling and trafficking (Simons, K. and Ikonen, E. 1997; Simons, K. and Toomre, D. 2000).

Cholesterol synthesis in mammals occurs in all nucleated cells and the rate limiting step is carried out by hydroxymethylglutaryl CoA reductase (HMG-CoAR). This enzyme is embedded in the ER membrane and possesses a sterol sensing domain (SSD) which is crucial for the association with a group of ER retention proteins called insulin-inducible genes (Insigs). At high sterol levels cholesterol binds to the SSD of HMG-CoAR, and this results in a conformational change which favours an interaction with Insig-1 or -2. A fraction of Insig-1 is associated with proteins which lead to ubiquitination, extraction from the ER-membrane and degradation of HMG-CoAR. Hence at high sterol levels HMG-CoAR is degraded by the ER-associated protein degradation pathway (Raghow, R. *et. al.*, 2008; Figure 5).

Cholesterol also enters the cell via LDL, see section 1.2.1 for details.
Cholesterol export from late endosomes and lysosomes remains unclear but two proteins seem to be involved, which are known from Niemann-Pick Type C (NPC) disease. The disease is characterised by accumulation of unesterified cholesterol, sphingolipids and other lipids within the endosomal and lysosomal system. It is caused by mutations in either npc1 or 2. NPC1 is a transmembrane protein and contains a SSD domain. It is predominantly located within the late endosomal membrane but also transiently associated with lysosomes and the trans-Golgi network (Chang, T. Y. *et. al.*, 2005). It is thought that it functions as a lipid permease but an activity has not been determined yet. NPC2 is a soluble lysosomal protein with a high affinity for cholesterol and a lower affinity for fatty acids (Chang, T. Y. *et. al.*, 2005). Most of the newly synthesized cholesterol is rapidly delivered to the PM in an energy dependent manner which does not involve NPC1 (Liscum, L. *et. al.*, 1989). After it reaches the PM newly synthesized cholesterol may recycle between PM and recycling endosomes. It accumulates in late endosomes and lysosomes in NPC1 mutant but not in normal cells (Chang, T. Y. *et. al.*, 2005). NPC1 and 2 are assumed to target the cholesterol to the endoplasmic reticulum (ER) where it becomes esterified and is eventually stored as CE in lipid droplets, see section 1.4.

Cholesterol is removed from cells by the reverse cholesterol transport (RCT). Hereby cholesterol is transported by high density lipoprotein (HDL) from the peripheral tissue via the blood to the liver, and eventually excreted into the bile. HDL particles are synthesised in the liver and are mainly composed of apolipoprotein A1 (apoA1) and apoA2 as well as phospholipids. These cholesterol free pre-HDL particles circulate in the blood and become cholesterol laden by interacting with ATP-binding cassette transporters. This involves mainly ATP-binding cassette transporter A1 (ABCA1) as well as others, like ABCG1. ABCA1 proteins promote phospholipid and cholesterol export to lipid poor apoA1 or apoE. ABCG1 promotes the efflux of cholesterol and oxysterols to more lipid enriched HDL particles (Tall, A. R. 2008). FC becomes esterified by Lecithin:cholesterol

acyltransferase (LCAT). This may help the passive export of cholesterol by the ABCG1 pathway (Tall, A. R. 2008).

In the liver scavenger receptor B1 (SR-B1) recognizes HDL and a selective uptake of cholesterol is mediated. In humans an indirect pathway is likely to be more relevant. Hereby triglycerides of very low density lipoprotein (VLDL) are exchanged by cholesteryl esters of HDL. This is mediated by cholesteryl ester transfer protein (CETP). Therefore VLDL is processed to LDL, which is then phagocytosed by the LDL receptor pathway. The subsequent action of the extracellular located hepatic lipase on triglyceride-enriched HDL decreases the HDL particle size by hydrolysing the triglycerides (Zhong, S. *et. al.*, 1994), and released FFA and glycerol are taken up by the liver.

### 1.2.4 BETA-OXIDATION

β-oxidation is a process which produces acetyl-CoA from activated FFA (in general called acyl-CoA) in mitochondria or peroxisomes. FFAs become activated in the cytosol in a two step reaction. In the first step fatty acyl-CoA synthetase transfers Adenosin monophosphate (AMP) from Adenosin triphosphate (ATP) to the carboxyl group of the fatty acid. In the second step a thiol group of Coenzyme A (CoA) displaces the AMP and forms a thioester with the fatty acid resulting in a fatty acyl-CoA. The liberated inorganic pyrophosphate is immediately hydrolyzed to two phosphates by inorganic pyrophosphatase. The latter reaction drives the otherwise endergonic reaction forward.

After import into the mitochondria or peroxisomes the acyl-CoA enters the β-oxidation pathway which consists of 4 reoccurring steps: Oxidation, hydration, another oxidation and thiolysis.

The first step is catalyzed by acyl-CoA dehydrogenase. A double bond between C2 and C3 of acyl-CoA is formed and FAD functions as an electron acceptor. The resulting trans-$\Delta^2$-enoyl-CoA is hydrated at the C3 position in the second step by enoyl-CoA hydratase. This leads to the formation of L-β-hydroxy acyl-CoA. In the second oxidation catalysed by β-hydroxyacyl-CoA dehydrogenase, the newly formed hydroxyl group is converted into a keto group. In this reaction $NAD^+$ serves as an electron acceptor and β-ketoacyl-CoA is generated. At the final step of the sequence, catalyzed by β-ketoacyl-CoA thiolase, another thiol group is

inserted at the C3 position of β-ketoacyl-CoA, and acetyl-CoA, and acyl-CoA, shortened by two carbon atoms, are liberated. The acyl-CoA enters another cycle of β-oxidation. Acetyl-CoA enters the tri-carbon-cycle leading to ATP production, in mammals, it is also a precursor molecule for the synthesis of FA, cholesterol and ketone bodies (Eaton, S. *et. al.*, 1996; Poirier, Y. *et. al.*, 2006; Ikonen, E. 2008).

### 1.2.5 BETA-OXIDATION IN *LEISHMANIA* SPP.

The sequenced genome of *L. major* Friedlin (Ivens, A. C. *et. al.*, 2005) indicates that all four core enzymes of the β-oxidation are present; the genome data can be accessed via the GeneDB database at http://www.genedb.org/genedb/leish/index.jsp. Based on homology the following genes are candidates to form the core enzymes; acyl-CoA dehydrogenases LmjF07.0460, LmjF28.2510 and LmjF35.2730; enoyl-CoA hydratase LmjF18.0580, LmjF29.2310, LmjF32.3680, LmjF35.0360 and LmjF33.2600. The final one is also assigned as a β-hydroxyacyl-CoA dehydrogenase. Another putative β-hydroxyacyl-CoA dehydrogenase is LmjF36.1140. Also there are three putative β-ketoacyl-CoA thiolases encoded in the *L. major* genome, LmjF23.0690, LmjF31.1630 and LmjF31.1640.

So far it is still not clear whether the β-oxidation in *Leishmania*, is exclusively glycosomal (a peroxisome like compartment in trypanosomatids), exclusively mitochondrial, or if parts of it occur in both organelles. Furthermore, it has not been shown that all four core enzymes are localised at the glycosome. Hart and Opperdoes (Hart, D. T. and Opperdoes, F. R. 1984) could identify β-hydroxyacyl-CoA dehydrogenase in the glycosome. And recently in a genome wide *in silico* prediction for peroxisomal targeting sequences (PTS), Opperdoes could identify a PTS for the putative multifunctional enzyme enoyl-CoA hydratase/enoyl-CoA isomerase/3-hydroxyacyl-CoA dehydrogenase (LmjF23.2600) and β-ketoacyl-CoA thiolase (LmjF23.0690) (Opperdoes, F. R. and Szikora, J. P. 2006; Michels, P. A. *et. al.*, 2006). Acyl-CoA dehydrogenase has not been shown to be a glycosomal enzyme, neither could a PTS be predicted.

## 1.3 OXIDATION OF POLYUNSATURATED FATTY ACIDS

The oxidation of polyunsaturated fatty acids needs auxiliary enzymes in addition to those of the β-oxidation pathway. In eukaryotes for example, degradation of linoleic acid will form an ester with a cis-$\Delta^3$, cis-$\Delta^6$ configuration after three cycles through the β-oxidation pathway (Figure 3). This cannot be processed by the enoyl-CoA-hydratase, which only acts on trans double bonds. The first auxiliary enzyme $\Delta^3$-*cis*-$\Delta^2$-*trans*-enoyl-CoA-isomerase isomerises the *cis*-$\Delta^3$- to the *trans*-$\Delta^2$-enoyl-CoA. The resulting product undergoes another cycle of the β-oxidation and the first step of the next cycle, catalysed by acyl-CoA-dehydrogenase, forming trans-$\Delta^2$, cis-$\Delta^4$-enoyl-CoA. This cannot be used by the enzymes of the β-oxidation. At first the cis-$\Delta^4$ doublebond has to be reduced. This reduction reaction is catalysed by 2,4-dienoyl-CoA-reductase (DECR). DECR uses NADPH to reduce the doublebond and the product is trans-$\Delta^3$-enoyl-CoA. Finally this is converted again by enoyl-CoA-isomerse to a trans-$\Delta^2$-enoyl-CoA. The remaining reactions of the β-oxidation can now take place.

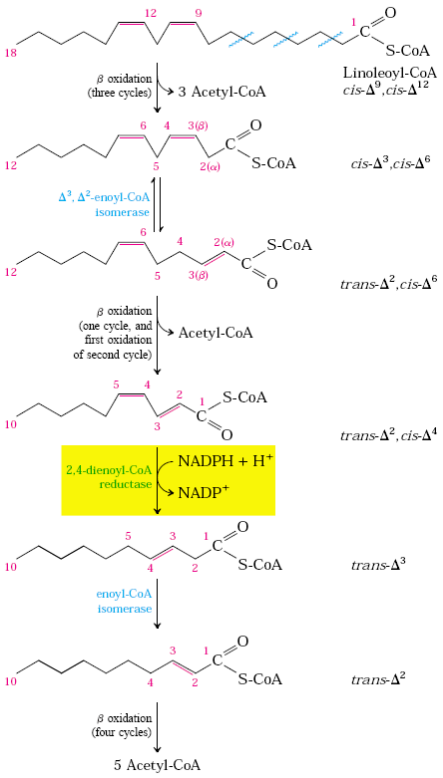


**FIGURE 3: Oxidation of polyunsaturated fatty acids.**

As an example activated linoleate is shown. Oxidation requires two other auxiliary enzymes enoyl-CoA-isomerase and 2,4 dienoyl CoA-reductase (DECR).

Reaction catalysed by DECR is highlighted by the yellow box.

## 1.4 NETWORK OF FATTY ACID METABOLISM AND TRANSCRIPTION

Peripheral cells, such as MΦ, are supplied with fatty acids and cholesterol by LDL as mentioned previously. Macrophages also take up oxidised LDL (oxLDL) as well as acetylated LDL (acLDL). All three types of LDL are taken up by receptor mediated endocytosis, but it has been reported that in human monocyte derived macrophages LDL can be taken up in a receptor independent way (Kruth, H. S. *et. al.*, 2002) after stimulation of protein kinase C (Kruth, H. S. *et. al.*, 2005). Modified LDL is endocytosed by macrophages by scavenger receptors, i.e. oxLDL is taken up by CD36, SR-B1 and SR-A. The latter two also recognize acLDL. Furthermore oxLDL can be endocytosed by oxidised LDL-receptor.

The phagocytosed LDL gets transported to the lysosme where it disintegrates and lysosomal acid lipase hydrolyses CE to FFA and FC. As mentioned above it still remains unclear how the FC is exported from the lysosome. Two proteins are probably involved in this process, NPC1 and 2 which help to target cholesterol to the ER where it becomes esterified by acyl-CoA-cholesteryl-acyl-transferase (Soat). After esterification it is stored in the cytosol as CE in lipid droplets. Cellular cholesterol undergoes a constant cycle of esterification and ester hydrolysis, and net breakdown of CE in lipid droplets only occurs when cellular cholesterol levels fall. Two enzymes, a Lipase (Lipe) and Soat, maintain this equilibrium between FFA/FC and CE, see Figure 4. Soat esterifies FFA and FC to CE and Lipe hydrolyses it.

The cell also has mechanisms to sense its lipid status. Several transcription factors are involved and become activated either if the cell is deprived of cholesterol or by the presence of lipids, such as unsaturated FA or oxysterols. These transcription factors regulate the expression of genes in order to restore lipid homeostasis in the cell. For example, as a result of cholesterol deprivation genes involved in cholesterol biosynthesis are transcribed. If the level of oxidised cholesterol (oxysterols) is elevated genes involved in the export of cholesterol are expressed. See below for a more detailed view in the regulation of these transcription factors.

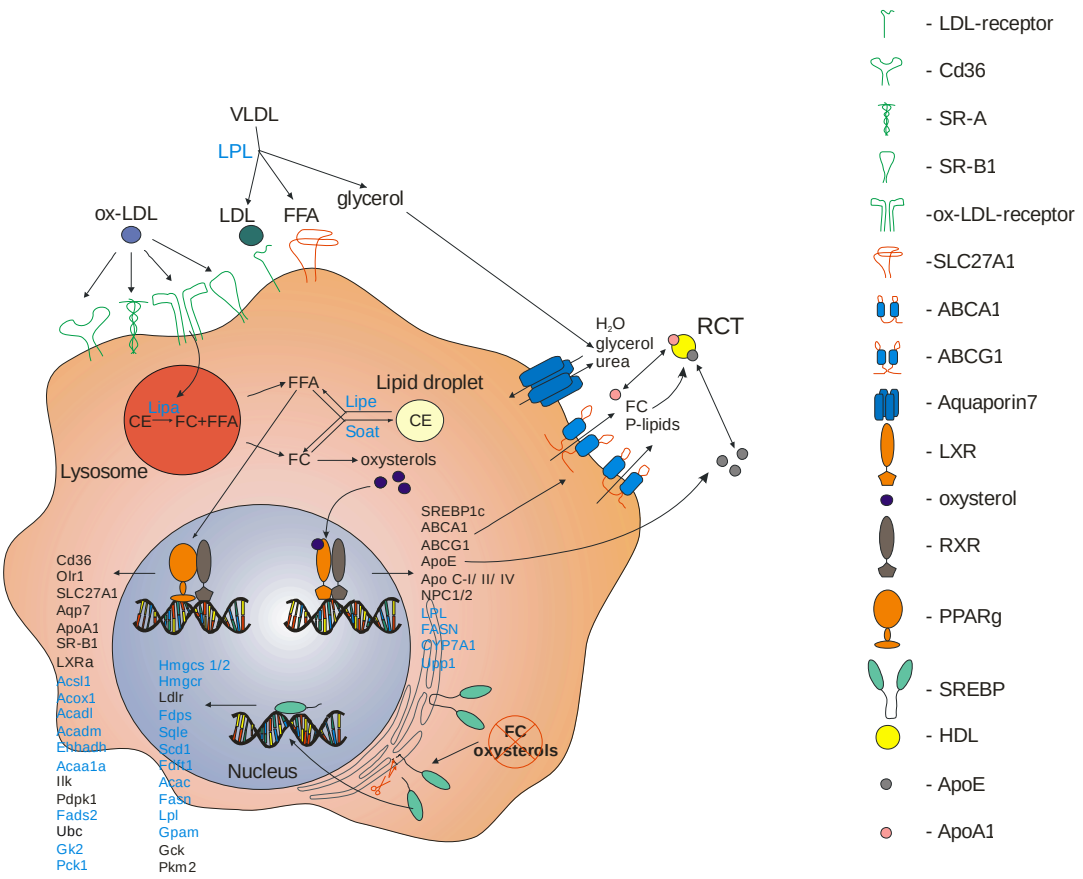


**FIGURE 4: Correlation between the status of free fatty acids and cholesterol derivatives and transcription in the example of a macrophage.**

LDL and LDL derivatives are endocytosed. These disintegrate in the lysosome where the cholesteryl ester (CE) is hydrolysed to free fatty acids (FFA) and free cholesterol (FC). FFA and FC are exported into the cytosol where an equilibrium is maintained by esterification, carried out by an acyl-CoA-cholesteryl-acyl-transferase (Soat), and hydrolysis, carried out by a lipase (Lipe). CE is stored in the cytosol as lipid droplets. The macrophage senses an unbalanced equilibrium by changes in transcription factor activation, such as PPARγ, LXR and SREBP. FFA translocate into the nucleus and bind to PPARγ. PPARγ becomes activated and induces transcription of a subset of genes. LXR targets a different subset of genes and depends on oxysterols as an activator. In cholesterol and oxysterols depleted cells, SREBP is transported to the Golgi-apparatus and cleaved off the membrane. It translocates into the nucleus resulting in another subset of genes being transcribed.

LDL – low density lipoprotein; oxLDL – oxidised LDL; RCT – reverse cholesterol transport; P-lipids –phospholipids.

In light blue are genes encoding for enzymes, in black are other genes, see text for explanation of the specific genes.

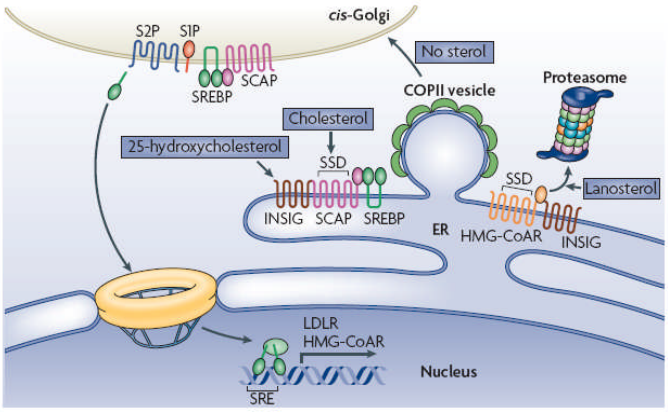


**FIGURE 5: Sterol regulatory binding protein (SREBP) and cholesterol regulation of gene transcription.**

In presence of cholesterol or oxysterols the SREBP-cleavage-activating protein (SCAP)-SREBP complex is retained in the ER by insulin-inducible genes (INSIGs). If cholesterol or oxysterol is depleted, SCAP-SREBP complexes dissociate from INSIG and translocate from the ER to the Golgi apparatus in COPII vesicles. In the Golgi apparatus SREBP becomes liberated by the action of two proteases, site 1 proteases (SP1) and SP2. SREBP forms homodimers and translocates to the nucleus where it binds to sterol regulatory elements (SRE) in promoters and activates gene transcription (such as hydroxymethylglutaryl CoA reductase (*HMG-CoAR*)) and the low density lipoprotein receptor (*LDLR*). HMG-CoAR also possesses a sterol sensing domain (SSD) similar to SCAP. At high cholesterol levels HMG-CoAR is associated with INSIG which leads to the extraction of HMG-CoAR from the ER and its degradation. On the other hand in the absence of cholesterol, HMG-CoAR is not associated with INSIG and cholesterol can be synthesized.

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Sterol Regulatory Element Binding Proteins (SREBP) are a family of transcription factors consisting of three members, SREBP-1a, SREBP-1c and SREBP-2 (Brown, M. S. and Goldstein, J. L. 1997). These proteins survey intracellular and membrane levels of FC and FFA and connect it with *de novo* lipid biosynthesis. SREBP-1a and SREBP-2 are ubiquitously expressed and in contrast SREBP-1c is only expressed in the liver after food intake (Raghow, R. *et. al.*, 2008). They are expressed as large precursors that are inserted into the ER membrane through two membrane spanning domains. SREBP is retained in the ER by a C terminal

interaction of SREBP with SREBP-cleavage-activating protein (SCAP; Figure 5). SCAP also possesses a SSD and serves as a membrane sterol sensor. It binds cholesterol and undergoes a conformational change inhibiting interaction with COPII-coat proteins Sar1 and Sec23/24 and the complex SCAP/SREBP is not transported to the Golgi apparatus via COPII vesicle. The inhibition of SCAP binding to COPII proteins is enhanced by INSIGs. Apart from HMG-CoA reductase Insigs also interact with SCAP in a sterol dependent manner (Bengoechea-Alonso, M. T. and Ericsson, J. 2007). In cholesterol depleted cells, SCAP dissociates from INSIG and translocates together with SREBP to the Golgi-apparatus. Here SREBP is cleaved by two proteases, site 1 (SP1) protease and thereafter by site 2 (SP2) protease, lumenal and intra-membrane proteolysis, respectively.

Oxysterols interact with INSIG and cause a conformational change upon binding which in turn changes the conformation of SCAP and the SCAP-SREBP complex is retained in the ER.

The cholesterol level in the ER is low and it fluctuates much more than that of the plasma membrane (Tabas, I. 2002). Therefore small changes can be sensed rapidly. After cleavage in the Golgi-apparatus SREBP forms homodimers and translocates to the nucleus where it binds to sterol regulatory elements (SRE) and transcription of target genes is activated (Figure 4). SREBPs predominantly activate genes involved in lipid synthetic pathways like cholesterol synthesis, FA synthesis, lipogenesis, and triglyceride and phospholipid synthesis. The three SREBP transcription factors do not activate the same set of genes. Overexpression of just one form of SREBP in mice revealed that SREBP-1c activates mainly genes involved in FA synthesis, whereas target genes of SREBP-2 are involved in cholesterol synthesis (Ahmed, M. H. and Byrne, C. D. 2007). Among these genes involved in cholesterol synthesis are 3-hydroxy-3-methylglutaryl-CoA-synthase (*hmgcs*), 3-hydroxy-3-methylglutaryl-CoA-reductase (*hmgcr*), farnesyl diphosphate synthase (*fdps*) squalene synthase (*Fdft1*), LDL-receptor (Horton, J. D. *et. al.*, 1998; Goldstein, J. L. and Brown, M. S. 1990) and squalene epoxidase (*sqle*) (Murphy, C. *et. al.*, 2006).

SREBP also induces expression of enzymes involved in FA synthesis and uptake, including acetyl-CoA-carboxylase (*acac*), fatty acid synthase (*fasn*), stearoyl-CoA

desaturase 1 (*scd1*), and lipoprotein lipase (*lpl*) (Kim, J. B. and Spiegelman, B. M. 1996; Lopez, J. M. *et. al.*, 1996; Magana, M. M. and Osborne, T. F. 1996; Shimano, H. *et. al.*, 1996; Tontonoz, P. *et. al.*, 1993).

Peroxisome proliferator-activated receptors (PPAR) and Liver-X receptors (LXRα and LXRβ) belong to the nuclear receptor superfamily which functions as ligand dependent TFs when complexed with the obligate 9-cis retinoid acid receptor α (RXRα).

Three isotypes of PPARs have been identified, PPARα, γ and β/δ. All exhibit a level of sequence and structural homology, but display a different expression pattern and ligand specificity. PPARα is mainly expressed in the liver, heart, kidney, intestine and skeletal muscles i.e. mainly in tissues which utilize fatty acids as main energy source (Desvergne, B. and Wahli, W. 1999). It is also the predominant isoform in murine lymphocytes (T- and B-cells) (Jones, D. C. *et. al.*, 2002). PPAR β/δ is ubiquitously expressed in human and rat and often at higher levels than the other two isoforms. Despite its ubiquitous expression, expression levels vary from tissue to tissue. PPARγ is mainly expressed in adipose tissue in human and rat. In rat, it is also expressed in the spleen, Peyer's patches and the intestine. In contrast, PPARγ is found in the liver and heart of humans, but neither in the intestines nor in the spleen (Desvergne, B. and Wahli, W. 1999). PPARγ is furthermore expressed in MΦ and upregulated in resident MΦ upon stimulation with interleukin 4 (IL-4) and highly upregulated in thioglycollate-elicited peritoneal MΦ (inflammatory MΦs) (Huang, J. T. *et. al.*, 1999).

Ligands for all three PPARs are unsaturated fatty acids, for example, ω-3 polyunsaturated fatty acids (PUFAs) (α- and γ-linolenic acid, eicosapentaenoic and docohexaenoic acid), ω-6 PUFAs (linoleic and arachidonic acid) and ω-9 PUFAs (palmitoleic and oleic acid). In contrast to PUFAs, saturated fatty acids are poor ligands for PPARs and a potent binding to PPARα and β/δ has only been detected for palmitic and stearic acid. Eicosanoids also function as ligands. Hydroxyeicosapentaenoic acid is primarily a ligand for PPARα whereas linoleic acid metabolites 9 and 13-hydroxy-10,12-octadecadienoic acid (9-HODE and 13-HODE, respectively) function as ligands of PPARγ (for review see (Desvergne, B.

and Wahli, W. 1999; Tontonoz, P. and Spiegelman, B. M. 2008; Nagy, L. *et. al.*, 1998).

PPARs function in a similar way as LXRs; they are pre-bound to their respective DNA recognition site, the peroxisome proliferators response elements (PPRE), in the promoter region of target genes. In the absence of ligands PPAR/RXR is associated with co-repressor proteins. A conformational change upon ligand binding leads to the dissociation of co-repressor molecules, recruitment of co-activator molecules and target genes are transcribed (Ricote, M. and Glass, C. K. 2007). Furthermore PPARs can also act as negative regulators of transcription in a ligand –dependent manner. The mechanism of this so called transrepression remains unclear.

In addition, PPARγ regulates lipid metabolism and has an anti-inflammatory function in macrophages. Activation of PPARγ in macrophages promotes lipid clearance through regulation of CD36, LXR, and ABC transporter expression (Tontonoz, P. and Spiegelman, B. M. 2008).

Target genes of PPAR are scavenger receptor CD36 (scavenging of oxLDL), oxidized-LDL-receptor (Orl1), solute carrier family 27 (fatty acid transporter) (SLC27A1), glycerol transporter aquaporin 7 (Aqp7), apoA1, LXRα, fatty acid binding protein (Fabp), acyl-CoA synthase (Acsl1) , acyl-CoA-oxidase (Acox1), long and medium chain acyl-CoA-dehydrogenase (Acadl and Acadm, respectively), enoyl-CoA hydratase/3-Hydroxyacyl-CoA dehydro-genase (Ehhadh), integrin linked kinase (Ilk), adipose differentiation related protein (Adfp), glycerol kinase (GK2), and phosphoenolpyruvate-carboxykinase (Pck) (Chawla, A. *et. al.*, 2001; Dalen, K. T. *et. al.*, 2006; Di Poi, N. *et. al.*, 2002; Lehrke, M. and Lazar, M. A. 2005; Odegaard, J. I. *et. al.*, 2007; Tontonoz, P. *et. al.*, 1998).

LXR/RXR heterodimers function as sensors of cellular oxysterols. The complex is already bound to its respective recognition site, the LXR response element (LXRE) of a target gene. In the presence of oxysterols the LXR/RXR becomes activated and expression of genes, that control sterol and fatty acid metabolism and homeostasis, increases (Edwards, P. A. *et. al.*, 2002). It is assumed that in the absence of oxysterols, repressor proteins are bound to the LXR/RXR complex and

transcription is inhibited. Basal transcription occurs if the LXR or RXR ligand is present at low levels due to the dissociation of parts of the repressor machinery and recruiting of co-activators. In the presence of both or high levels of either LXR/RXR ligand the complete repressor machinery dissociates and additional co-activators are recruited and transcription is enhanced. LXRα is activated only by a specific subset of oxysterols and the strongest is 22(R)-hydoxycholesterol (22(R)-HC), while its enantiomer is completely inactive (Janowski, B. A. *et. al.*, 1996) thus location and stereochemistry are important for activity. The other most potent activators are in the following order from highest to lowest: 20(S)-HC, 22,20-HC and 24-HC (Janowski, B. A. *et. al.*, 1996). Another potent ligand albeit to a lesser extent than the aforementioned, is 27-HC. It is generated from cholesterol by mitochondrial sterol-27-hydroxylase (Cyp27A1). Two groups found that in human MΦ RXR and PPARɣ ligands synergistically stimulate Cyp27A1 expression. The produced 27-HC induces LXRα hence LXRα target genes (Quinn, C. M. *et. al.*, 2005; Szanto, A. *et. al.*, 2004).

LXRα target genes are SREBP1c, ABCA1, ABCG1, apoE, Fasn as well as NPC1 and 2 (Costet, P. *et. al.*, 2000; Joseph, S. B. *et. al.*, 2002; Kennedy, M. A. *et. al.*, 2001; Laffitte, B. A. *et. al.*, 2001; Repa, J. J. *et. al.*, 2000; Rigamonti, E. *et. al.*, 2005).

As lesion derived amastigotes utilise more triglycerides and non-esterified fatty acids than promastigotes (Hart, D. T. and Coombs, G. H. 1982), the effects on the MΦ lipid metabolism upon amastigote infection is going to be investigated.

## 1.5 OBJECTIVES

The focus of this thesis has three main objectives:

The first objective was proteomic analysis of amastigotes and to determine differences in comparison to promastigotes.

The second objective was to assess the relevance of increased fatty acid metabolism in amastigotes for virulence and pathogenicity.

And finally, the third objective was to analyse the effects of *Leishmania* infection on host cell lipid homeostasis.