University of Granada

Faculty of Medicine

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Epidemiological Studies on Zoonotic Leishmaniasis and New Trials for Studying the Effect of Melatonin on the Parasite

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Epidemiological Studies on Zoonotic Leishmaniasis and New Trials for Studying the Effect of Melatonin on the Parasite

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Dedication

This work is dedicated to Soul of my father, my mother and my sisters and all family, and to the people who dedicate their lives to seek for the truth…

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Acknowledgement

First of all, I wish to express my sincere gratitude and all thanks to our God for giving me everything I need in my life, easiness the difficulties and supporting me to finish this work. Praise be to our God, the Cherisher and Sustainer of the worlds, who with his grace this work has been done...

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Funding and publications during candidature

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II. Scientific publications:

- The present work has been developed in the laboratories of the following departments, department of pharmacology, department of Microbiology of school of medicicne, university of Granada, Granada, Spain, as well as part has been carried out in service of microbiology and parasitology. Another part from the present work has been done in the laboratory of parasitology, department of veterinary sciences, University of Turin, Italy. Parts from the results of the present works have been published in the following scientific journals:
- El presente trabajo se ha desarrollado en los laboratorios del Departmento de Faramcologia, departmento de de Microbiologia de la facultad de medicina, del Universidad de Granada y en el servicio de Microbiología y Parasitología del Hospital Universitario Virgen de las Nieves - Universidad de Granada, España. Parte del presente trabajo se se ha desarrollado en el Laboratorio del parasitología, departamento de ciencias veterinarias de la Universidad de Turín, Italia. Partes de los resultados de este trabajo han sido publicados en las siguentes revistas:

- **Authors**: Ehab Kotb Elmahallawy, Aroa Jiménez-Aranda, Antonio Sampedro Martínez, Javier Rodriguez-Granger, Miguel Navarro-Alarcón, José Gutiérrez-Fernández and Ahmad Agil
  Title: Activity of melatonin against *Leishmania infantum* promastigotes by mitochondrial dependent pathway.
  Reference: Chemico-Biological Interactions Journal. 2014; 220C:84-93.

- **Authors**: Ehab Kotb Elmahallawy, Antonio Sampedro Martinez, Javier Rodriguez Granger, Yannick Hoyos-Mallecot, Ahamd Agil, Jose Mari Navarro Mari and Jose Gutierrez Fernandez
  Title: Diagnosis of Leishmaniasis.
  Reference: The Journal of Infection in Developing Countries. 2014 8(8):961-72.

- **Authors**: Ehab Kotb Elmahallawy and Ahmad Agil.
  Title: Treatment of Leishmaniasis: A Review and Assessment of recent research.
  Reference: Current Pharmaceutical Design. 2015; 21(18):2259-75.

- **Authors**: Ehab Kotb Elmahallawy; Elena Cuadros-Moronta; Mª del Carmen Liébana Martos; Antonio Sampedro-Martínez, Javier Rodriguez-Granger, Ahmad Agil, José M Navarro-Marí and José Gutierrez-Fernández
  Title: Seroprevalence of *Leishmania* infection among asymptomatic renal transplant recipients from southern Spain.
  Reference: Transplant Infectious Disease. 2015 Aug 19. doi: 10.1111/tid.12444.

- **Authors**: Ehab Kotb Elmahallawy, Javier Ortega Luque, Abdelkarim saleh Aloweidi, José Gutiérrez Fernández, Antonio Sampedro Martínez, Javier Rodriguez-Granger, Abdullah Kaki, and Ahmad Agil.
  Title: Potential relevance of melatonin against some infectious agents: A Review and Assessment of Recent Research.
  Reference: current medicinal chemistry. 2015 Aug 26 Accepted.

- **Authors**: Ehab Kotb Elmahallawy, Marco Poggi, Gabriele Cieri, Ahmad Agil, Stefania Zanet, Anna Trisciuoglio and Ezio Ferroglio.
  Title: Cross-sectional Epidemiological survey of *Leishmania infantum* in cats from an endemic region in Northwestern Italy.
  Reference: Acta Tropica-S-15-00778. 2015, under review.

III. Communications and Congresses

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1. **Elmahallawy EK** ,Jiménez-*Aranda* A, Velasco-Pérez, L, Ruiz, OM, Rodriguez-Granger J, Gutiérrez Fernández J, Agil A. *In vitro* antileishmanial activity of melatonin. V Reunión de Jóvenes Farmacólogos de Andalucía. 9 July 2013, Departamento de Farmacología Universidad de Malaga, Spain.
2. **Ehab Kotb Elmahallawy**, Javier Rodriguez-Granger, Jose Gutiérrez Fernández, Ahmad Agil. Possible involvement of mitochondrial complex II in melatonin-induced apoptosis in *Lesihmania.* V Programa Jornadas Neurociencias. 12 March 2014, Instituto de Neurociencias, Universidad de Granada, Spain.

3. **Elmahallawy EK**, Martínez A, Rodriguez-Granger J, Fernández JG, and Agil A. Melatonin-induced apoptosis and mitochondrial alterations in *Leishmania infantum* promastigotes. VI Reunión de Jóvenes Farmacólogos de Andalucía. 12 June 2014, Departamento de Farmacología Universidad de Sevilla, Spain (Best oral presentation award by the Spanish Society of Pharmacology).

IV. Collaboration

Por otro lado, el doctorando estaba Colaborando con nuestra grupo de investigación. Asi que partes de los resultados de este trabajo han sido publicados en las siguientes revistas, reuniones y conferencias:

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Title: Melatonin increases intracellular calcium in liver, muscle, white adipose tissues and pancreas of diabetic obese rats.
Reference: Food & Function 2015 Aug 5;6(8):2671-8.

1. Velasco-Pérez L., Jiménez-Aranda A., **Elmahallawy EK**. , Ortiz-Ruiz M., Kaki A., Agil A. Chronic melatonin administration has beneficial effects in neuropathic pain in Zucker diabetic (ZDF) fatty rats. V Reunión de Jóvenes Farmacólogos de Andalucía. 9 July 2013, Departamento de Farmacología Universidad de Malaga, Spain.
2. Velasco-Pérez L., Jiménez-Aranda A., **Elmahallawy EK**. , Ortiz-Ruiz M., Kaki A., Agil A. Chronic melatonin administration has beneficial effects in neuropathic pain in Zucker diabetic (ZDF) fatty rats. V Reunión de Jóvenes Farmacólogos de Andalucía. 9 July 2013, Departamento de Farmacología Universidad de Malaga, Spain.

3. **Elmahallawy EK**, Ortiz-Ruiz M and Agil A. Involvement of mitochondrial membrane permeability transition pore opening in melatonin hepato-protective effects in Zucker diabetic fatty (ZDF) rats. VI Reunión de Jóvenes Farmacólogos de Andalucía. 12 June 2014, Departamento de Farmacología Universidad de Sevilla, Spain.

4. Ortiz-Ruiz M, **Elmahallawy EK**, Agil A. Chronic melatonin administration improves the mitocondrial stress in liver of Zucker diabetic fatty (ZDF) rats. VI Reunión de Jóvenes Farmacólogos de Andalucía. 12 June 2014, Departamento de Farmacología Universidad de Sevilla, Spain.

V. Awards

4. Best oral presentation award on VI Reunión de Jóvenes Farmacólogos de Andalucía. 12 June 2014, Departamento de Farmacología Universidad de Sevilla, Spain on his presentation entitled ‘’Melatonin-induced apoptosis and mitochondrial alterations in *Leishmania infantum* promastigotes’’.

List of abbreviations

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Abbreviation	Mean
2-ME	2- mercaptoethanol
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
AIDS	Acquired immune deficiency syndrome
ALT	alanine transaminase
ALP	alkaline phosphatase
ACL	anthroponotic cutaneous leishmaniasis
AVL	anthroponotic visceral leishmaniasis
Ab	Antibody
Ag	Antigen
APC	antigen presenting cells
AST	aspartate aminotransferase
BSA	Bovine serum albumin
Ca^{2+}	calcium
CDC	Center of Disease control
CF	Central nervous system
CHOL	cholesterol
CFT	Complement fixation test
IC_{50}	concentration of inhibition of parasite growth by 50%
CREA	creatinine
CSA	Crude soluble antigen
CL	Cutaneous leishmaniasis
CSA	Cyclosporin A
DPI	Days Post Infection
◦C	degree Celsius
Dnase I	Deoxyribonuclease I
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
DAT	Direct agglutination test

Na_2HPO_4	Di-sodium hydrogen Phosphate
D.W	Distilled water
ETC	electron transport chain
ER	Endoplasmic reticulum
E.	Entamoeba
ELISA	Enzyme Linked Immuno-sorbant Assay
EOS	eosinophils
EDTA	Ethylene diamine tetraacetic acid
FAST	fast agglutination screening test
FIV	Feline immunodeficiency virus
FeLV	Feline leukemia virus
CFC	final concentration of control culture
FDA	Food and Drug Administration
GLU	glucose
GST	glutathione-S transferase
HCT	hematocrit
Hb	hemoglobin
HAART	Highly Active Anti-Retroviral Therapy
H	histones
h	hour
HIV	Human Immunodeficiency Virus
H_2O_2	Hydrogen peroxide
IgG	Immune globulin G
ICT	Immunochromatographic test
IFAT	immunofluorescence assay
IL	interleukin
IU	international unit
I.P	Intra- peritoneal
kDa	kilodalton
rK	kinesin-related protein recombinant antigen
kDNA	kinetoplast DNA

LAT	latex agglutination test
L.	*Leishmania*
L	Liter
LYM	lymphocytes
Mφ	Macrophage
MCL	Magnesium chloride
MHC	major histocompatibility complex
µM	micro molar
MCM	micro-culture method
µg	microgram
mM	mille Mole
mL	milliliter
mm	millimeter
medRNA min	miniexon-derived RNA
MEM	Minimum Essential culture Medium
min	minutes
MON	monocytes
M	Mole
MCL	Mucocutaneous leishmaniasis
nm	nanometers
nM	nanomolar
NK	Natural Killer
NEU	neutrophils
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NOS/ iNOS	nitric oxide synthase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OPD	Ortho-Phenylene-diamine
PP	Patent Period
Sbv	Pentavalent Antimonial
PPI%	percentage of parasite inhibition
P.	*Phlebotomus*

PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline-Tween-20
PHOS	phosphorus
P.	*Plasmodium*
PAGE	Polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
p.i.	Post Infection
PKDL	Post Kala Azar Dermal Leishmaniasis
K	potassium
KCL	Potassium Chloride
K H2PO4	Potassium di-hydrogen phosphate
PP	Pre-Patent Period
P- Value	Probability value
PLT	platelets count
RDT	Rapid Diagnostic Test
RBC	red-blood cell count
ROT	rhoptry protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
SSC	saline sodium citrate buffer
SAG	Sodium antimony gluconate
$NaHCO_3$	Sodium bicarbonate
Na_2CO_3	Sodium carbonate
Na CL	Sodium chloride
STE	Sodium Chloride-Tris-EDTA buffer
SDS	Sodium dodecyl sulfate
Na OH	Sodium hydroxide
SSG	Sodium stibogluconate
Sol	Solution
SPF	specific pathogen free
SD	Standard deviation

S. c.	Subcutaneous
H_2SO_4	Sulfuric acid
Th	T helper cell
3' UTR	three prime untranslated region
TP	total protein
T.gondi	*Toxoplasma gondii*
T.	*Trypanosoma*
TNF-α	Tumor Necrosis Factor-α
VL	Visceral Leishmaniasis
VL/HIV	Visceral Leishmaniasis HIV co infection
WB	Western blotting
WBC	white-blood cell count
WHO	World Health Organization
ZCL	zoonotic cutaneous leishmaniasis
ZVL	zoonotic visceral leishmaniasis

Index

Index

No.	Title	Page N.
(1)	**Summary/Resumen**	1
(2)	**Introduction**	
	1. Background	8
	2. Diagnosis of Leishmaniasis	21
	3. Treatment of Leishmaniasis	30
	4. Potential relevance of melatonin against some infectious agents	51
(3)	**Hypothesis and objectives**	64
	1. Hypothesis	
	2. Objectives	
(4)	**Material and Methods**	
	❖ Experimental Design	
	1. Diagnostic part:	68
	1.1. Determination of *Leishmania* seroprevalence among renal transplant recipients	
	1.1.1. Study area and population	
	1.1.2. Sample collection and screening using IFAT.	
	1.2. Epidemiological survey of *Leishmania infantum* in cats	
	1.2.1. Study population and preparation of serum samples	
	1.2.2. Clinical and laboratory diagnosis	
	1.2.3. Western blotting and SDS- polyacrylamide gel electrophoresis	
	1.2.4. Preparation of blood samples and extraction of DNA	
	1.2.5. Qualitative PCR for detection of *Leishmania* and *Mycoplasma* DNA	
	1.2.6. Detection of FELV antigen and FIV antibody	
	2. Melatonin treatment part:	71
	2.1. Materials	
	2.2. *Leishmania* cells and culture conditions	
	2.3. Drug assessment on promastigote growth inhibition assay	

	2.4. Mitochondrial studies **2.4.1.** Mitochondrial Isolation **2.4.2.** Protein concentration measurement **2.4.3.** Calcium Retention Capacity **2.4.4.** Determination of Mitochondrial Nitrites **2.4.5.** Determination of Mitochondrial Superoxide Dismutase Activity **2.4.6.** Spectrophotometric Assays of Individual Respiratory Chain Complexes **3. Statistical analysis**	75
(5)	**Results** **1.** Seroprevalence of *Leishmania* infection among asymptomatic renal transplant recipients from southern Spain **2.** Cross-sectional Epidemiological survey of *Leishmania infantum* in cats from an endemic region in Northwestern Italy **3.** Activity of melatonin against *Leishmania infantum* promastigotes by mitochondrial dependent pathway	77 79 82
(6)	**Discussion** **1**. General aspects 2. Leishmaniasis among renal transplant patients 3. Role of cats in *L.infantum* epidimiology 4. Melatonin and parasites	87
(7)	**Conclusion/ Conclusión**	101
(8)	**Bibliography**	104

SUMMARY

Summary

Leishmaniasis remains an important public health problem caused by protozoan of genus *Leishmania* and transmitted by the bite of a female phlebotomine sand fly. Humans, rodents, and some animal species are considered reservoir for the disease. Among other animal species, the dogs are the most important reservoirs in a domestic environment, maintaining the endemic focus of the parasite. The disease has been also linked to tropical and subtropical regions besides being an endemic disease in the Mediterranean basin and South America. Depending on the infecting parasite species and host immune response, three forms of the disease are known: cutaneous, mucocutaneous, and disseminated visceral leishmaniasis of fatal prognosis.

The recent years have witnessed extraordinary potential progress and ever growing in organ transplantation worldwide as a consequence of sustained economic growth and the higher investments in tertiary healthcare policies in many developing countries. Spain is widely known as one of the countries with the highest transplant rates. As result of lack of routine serology for blood or organ donors in areas of high endemicity, transplanted recipients are susceptible to a broad spectrum infectious agents resulting in different symptoms. *Leishmania* is considered one of opportunistic infections but it is not common disease among transplanted patients, however, the growing pool of transplant survivors and high migration dynamics steadily increases the numbers of infected cases among transplant recipients especially among renal transplanted recipients mainly in southern Europe, particularly with visceral leishmanisis (VL).

Despite several leishmanial researches, many questions are still unanswered. Early case detection followed by adequate treatment represents the key to the control of the disease that may improve the prognosis and can reduce transmission. Diagnosis of *Leishmania* infection is still somewhat controversial due to absence of gold standard technique that appears mandatory to establish effective strategic programmes. Current tools of diagnosis are several and they rely mainly on parasite detection by microscopic examination or by molecular biology-based assays for detecting parasite DNA (PCR) but none of these methods have become popular in field diagnosis. The conventional parasitological techniques are also risky, time consuming, invasive for the patient and require skilled personnel. The molecular methods also require the availability of software, probes and primers, which cannot easily applied especially in field settings and still not affordable for many clinical and scientific laboratories in developing countries.

Interestingly, serological diagnosis using several tests is an alternative tool for the parasitological diagnosis; can be used for a large-scale and decentralized diagnosis, however, all the serological techniques share many drawbacks like that related to sensitivity or specificity have been reported.

On the other hand, the presence of different *Leishmania* species and various manifestations also complicates the therapeutic approach, especially in immunocompromised patients. A limited number of effective antileishmanial agents are available for chemotherapy, and many of them are expensive with severe side effects or have a markedly reduced effectiveness due to the development of drug resistance. Based upon several published works, plants have different biologically active compounds in their organs, which can be pharmacologically studied. Melatonin is an indoleamine synthesized and released by several organs. Several studies have included the relationship between melatonin and many parasitic or viral diseases.

Given above information, there is a genuine need to develop a novel effective and less toxic antileishmanial drug for amelioration of patient's life quality besides the necessity for surveying strategy using a rapid and reliable diagnostic test in one of animal reservoirs in certain endemic area (cats).

The scheduling of the thesis has been divided into several phases: the first part includes reviews of the literature about current status of epidemiology, development in diagnosis and treatment of the disease for better understanding the gaps in disease management. The second part of the thesis, the experimental part, includes two phases; the first phase of the present work included a serological study about *Leishmania* infection among transplanted organ recipients from southern Spain followed by assessment of the occurrence of *Leishmania infantum* in domestic cats from an endemic region in North-western Italy, by the association of both serological and molecular tests. In the second phase of our work, we have studied the effect of melatonin against the parasite *in vitro.*

Our results provide an evidence that a relatively high prevalence of *L.infantum* was recorded among kidney transplanted recipients, where 30 (4.08%) samples were positive for *L. infantum* out of 625 examined serum samples. Regarding the cross sectional survey in cats, we have found

that 33 samples (13.12%) were positive for *L. infantum* out of 250 examined serum samples from cats, whereas of the 282 blood samples, 80 (28.37%) were positive.

In accordance with therapeutic trials, melatonin not only demonstrated a significant antileishmanial activity *in vitro* but was also accompanied by an alteration of the several mitochondrial parameters, including calcium homeostasis and by changes in some mitochondrial parameters critical to parasite survival.

These multiple results suggest that 1) the routine serological testing for VL should be initially considered before undergoing transplantation for both donor and recipient transplant patients living or traveling in endemic areas to prevent such serious post-transplantation infection, 2) High prevalence of *L.infantum* among cats in the studied area which show the importance of cats not only as reservoir for the disease, but also the need for further future research for accurate diagnosis of this zoonosis, 3) Western blot and PCR would be a novel potential tools in diagnosis of *Leishmania* infection in cat, and 4) melatonin may be a potent antileishmanial agent, and therefore further research is warranted to elucidate the effects of melatonin *in vivo* and in association with other antileishmanial drugs combined with examination the role of melatonin receptors in these effects and their underlying mechanisms. These observations together could be of special attention for the people to identify the risk factors of transmission of such protozoan and would be helpful in earlier detection, treatment and as a consequence in eradication of this neglected disease.

Resumen

La leishmaniasis es a día de hoy un conjunto de enfermedades de interés internacional causadas por protozoos del género *Leishmania* y transmitido por la picadura de un *flebotomo* femenino. Los seres humanos, roedores, y algunas otras especies animales se consideran reservorio de dicha enfermedad. Entre otras especies animales, los perros son los reservorios más importantes en el entorno doméstico, manteniendo el foco endémico del parásito. La enfermedad se ha vinculado también a regiones tropicales y subtropicales, además de ser una enfermedad endémica en la cuenca del Mediterráneo y América del Sur. Dependiendo de la especie causante de la enfermedad, así como de la respuesta inmunitaria del huésped, se conocen tres formas de la enfermedad: cutánea, mucocutánea, y visceral, ésta última diseminada y en ausencia de tratamiento suele resultar fatal. Los últimos años han sido testigos de avances de extraordinario potencial y de crecientes casos de transplantes de órganos en todo el mundo como consecuencia de un crecimiento económico continuado y una mayor inversión en políticas de salud terciarias en muchos países en desarrollo. España es ampliamente conocida como uno de los países con las tasas más altas de trasplante. Como resultado de la falta de serología rutinaria de sangre o de órganos donantes en áreas de alta endemicidad, los receptores trasplantados son susceptibles a un amplio espectro de agentes infecciosos que resultan en síntomas diferentes. La *Leishmania* es considerada un agente infeccioso oportunista, pero no es una enfermedad común entre los pacientes trasplantados. Sin embargo, el creciente número de supervivientes de trasplante y la alta tasa de migración aumenta constantemente el número de casos de infección entre los receptores de trasplante, especialmente entre los receptores de transplantes renales y en el sur de Europa, y en particular a la leishmaniasis visceral (LV).

A pesar de las varias investigaciones en *Leishmania*, muchas preguntas siguen sin respuesta. La detección temprana de los casos, seguida de un tratamiento adecuado, representan la clave para el control de la enfermedad con lo que se podría mejorar el pronóstico y reducir la transmisión. En los últimos años se ha producido un extraordinario avance en el diagnóstico de la infección por *Leishmania*, pero el principal reto sigue siendo la falta de técnica de referencia, que parece obligatoria para establecer programas estratégicos eficaces para controlar y erradicar la enfermedad. Las herramientas actuales de diagnóstico son variadas y se basan principalmente en la detección de parásitos mediante examen microscópico o mediante ensayos basados en la

biología molecular para la detección de ADN del parásito (PCR), pero ninguno de estos métodos se han hecho populares en el diagnóstico de campo. Las técnicas parasitológicas convencionales también son arriesgadas, lentas, invasivas para el paciente y requieren personal cualificado. Los métodos moleculares también requieren de la disponibilidad de software, sondas y cebadores, que no son fácilmente aplicables, sobre todo en el campo, y todavía no son asequibles para muchos laboratorios clínicos y científicos en los países en desarrollo. El diagnóstico serológico es una herramienta alternativa para el diagnóstico parasitológico; puede ser utilizado para una gran escala y el diagnóstico descentralizado, sin embargo, casi todas las técnicas serológicas comparten muchos inconvenientes como los relacionados con la sensibilidad o especificidad entre diferentes ubicaciones. De hecho, la presencia de diferentes especies de *Leishmania* y diversas manifestaciones también complican el enfoque terapéutico, especialmente en pacientes inmunodeprimidos, ya que los rendimientos de quimioterapia convencionales ofrecen pobres resultados e inconsistentes. Solo un número limitado de agentes eficaces anti-*Leishmania* están disponibles para la quimioterapia, y muchos de ellos son caros y o bien tienen efectos secundarios graves, o presentan una eficacia marcadamente reducida debido al desarrollo de resistencia a los medicamentos. Sobre la base de varios trabajos publicados, las plantas tienen diferentes compuestos biológicamente activos en sus órganos, que pueden ser estudiados farmacológicamente. La melatonina es un indolamina sintetizada y liberada principalmente por la glándula pineal durante la oscuridad. Varios estudios han incluido la relación entre la melatonina y muchas enfermedades parasitarias o virales.

Basándonos en esta información, existe una auténtica necesidad de desarrollar un nuevo fármaco anti-*Leishmania*, eficaz y menos tóxico, para la mejora de la calidad de vida del paciente junto a la necesidad de conseguir una prueba de diagnóstico rápida y fiable para el diagnóstico de la infección por *Leishmania*, ya sea en humanos o en los principales animales reservorios. La programación de la tesis se ha dividido en varias fases: la primera parte incluye una revisión de la literatura sobre el estado actual epidemiologíco, desarrollo en el diagnostic, y el tratamiento de la enfermedad para evaluar las deficiencias en la gestión de la leishmaniasis. La segunda parte de la tesis, que es la parte experimental, incluye dos fases; la primera fase de este trabajo incluyó un estudio serológico sobre la infección causada por *Leishmania* entre los receptores de órganos trasplantados del sur de España, siguió por un estudio epidemiológico sobre la presencia de

Leishmania infantum en los gatos (domésticos) de una región endémica en el norte-oeste de Italia (Liguria), por la asociación de ambas pruebas serológicas (western blotting) y moleculares (PCR). En la segunda fase de nuestro trabajo, hemos estudiado el efecto de la melatonina contra el parásito *in vitro*.

Nuestros resultados evidencian que una prevalencia relativamente alta de *L. infantum* se registró entre los receptores de trasplante de riñón, donde 30 (4.08%) muestras fueron positivas para *L. infantum* de 626 muestras de suero examinadas. En cuanto a la encuesta epdimiological en los gatos, también hemos encontrado que 33 muestras (13.12%) fueron positivos de 250 muestras de suero examinadas para *L. infantum* con el western blotting, mientras que de las 282 muestras de sangre, 80 (28.37%) fueron positivas. Por otra parte con respecto a los ensayos terapéuticos, la melatonina no sólo demostró una actividad significativa contra *Leishmania in vitro*, sino también fue acompañada por una alteración de varios parámetros mitocondriales, incluyendo la homeostasis del calcio y por cambios en algunos parámetros mitocondriales críticos para la supervivencia del parásito.

Estos múltiples resultados sugieren que 1) las pruebas serológicas de rutina para LV deben ser tenidas en cuenta inicialmente a la hora de realizarse un trasplante, habiendo de llevarse a cabo en el donante y en el receptor si éstos viven o viajan a áreas endémicas para evitar la grave infección post-trasplante, 2) la melatonina puede ser un agente anti-leishmania potente, y por lo tanto es necesaria más investigación para dilucidar los efectos de la melatonina *in vivo* y en asociación con otros fármacos contra *leishmania*, combinados con el examen del papel de los receptores de melatonina en estos efectos y sus mecanismos subyacentes, 3) alta prevalencia de *L. infantum* entre los gatos en el área del estudio que muestran la importancia de los gatos no sólo como reservorio de la enfermedad, sino también la necesidad de una mayor investigación en el futuro para el diagnosis esta zoonosis, y 4) Western blot y PCR podrían ser unas potencial herramientas en el diagnóstico de la infección por *Leishmania* en muestras en los gatos. En conclusion, estas observaciones en conjunto podrían ser de especial interés para identificar los factores de riesgo de transmisión a las personas de dichos protozoos y serían útiles para la detección temprana, el tratamiento y, como consecuencia, para la erradicación de esta enfermedad olvidada.

INTRODUCTION

1. Background

General remarks

The term leishmaniasis refers to a heterogeneous group of diseases with various clinical syndromes, caused by obligate intracellular protozoa of the genus *Leishmania*. This protozoan parasite causes a widespread disease in tropical and subtropical regions [1-4]. The disease is mainly transmitted to humans by the bite of infected female phlebotomine sand fly [4], resulting in one or more of a spectrum of manifestations known as leishmaniasis. Three main forms of the disease are known: cutaneous, mucocutaneous, or visceral leishmaniasis [4]. This group of diseases is found in 98 countries around the world and affects a total of 12 million people [1, 3, 5], whereas approximately 350 million people are at risk for infection, in addition to an estimated 500 000- 2 000 000 million new cases and 20 000 to 50 000 deaths occur annually [1, 3, 5-7].

Among other forms, cutaneous leishmaniasis (CL) is the most common form of the disease, which characterized by skin lesions like ulcers can heal spontaneously but may result in disfiguring scars [5]. About 95% of the total cases of CL occur in the Mediterranean basin, Americas, and the Middle East and Central Asia while over two-third of CL new cases occur in six countries: Afghanistan, Algeria, Brazil, Colombia, Iran and the Syrian Arab Republic [4]. It is also estimated that 0.7 - 1.3 million new cases occur worldwide annually [4]. Mucocutaneous leishmaniasis (MCL) is another form of the disease, which results in partial or complete destruction of mucous membranes of the nose, mouth and throat. Several studies have been reported that 90% of MCL cases occur in the Plurinational State of Bolivia, Brazil and Peru [4].

Visceral Leishmaniasis (VL) is the most fatal form, which is associated with 200 000-400 000 new annual cases worldwide. The disease (VL) starts with skin ulcers and later accompanied with irregular bouts of fever, low red blood cells, anaemia, weight loss, enlarged spleen and liver and often fatal in absence of proper intervention [5, 8, 9]. High endemicity of this form was reported in the Indian subcontinent and East Africa; particularly, more 90% of new cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan [4, 10].

It should be stressed that the clinical features of VL can be mistaken with some diseases such as malaria, tuberculosis, typhoid fever and leprosy [11]. Hence, the reliable laboratory methods become mandatory for accurate diagnosis and early case detection followed by adequate treatment, together is the key to the control of the disease.

Regarding the causative protozoan, there are more than 20 species of *Leishmania* infect humans, and they are classified mainly by geographical distribution (Table 1) and the clinical characteristics of the disease [4, 6, 10]. Importantly, the aggressiveness of the individual species, their organ preference and the host immune status are the principal determinant of disease course. In fact, HIV/*Leishmania* co-infection represents a distinct challenge to leishmaniasis approach with particular epidemiological features, diagnosis and treatment in immunocompromised patients [10, 12, 13]. Risk factors include: poverty, malnutrition, deforestation, and urbanization increase the threating [4].

CL is caused by *L. major*, *L. tropica*, *L. aethiopica* (Old World CL), *L. infantum*, *L. chagasi* (Mediterranean and Caspian Sea region CL), *L. amazonensis*, *L. mexicana*, *L. braziliensis*, *L. panamesis*, *L. peruviana*, and *L. guayanensis* (New World CL) [6, 14-16]. MCL or espundia, is caused by *L. brazilensis*, *L. panamensis*, *L. guyanensis* in the New World (Western Hemisphere, specifically the Americas), whereas in the Old World (Estern Hemisphere, mainly Africa, Asia, and Europe), it is mainly caused by *L. infantum* and *L. donovani* [6, 9, 10, 14].

Clinical forms of the Disease	**Causative agents**	**Region**
CL	*L. tropica*	Mediterranean and countries, Afghanistan
	L. major	Middle East, Western and Northern Africa, Kenya
	L. aethiopica	Ethiopia
	L. Mexicana	Central America, Amazon regions
MCL	*L. braziliensis*	Brazil, Peru, Ecuador, Columbia, Venezuela
VL	*L. donovani*	China, India, Iran, Sudan, Kenya, Ethiopia
	L. infantum	Mediterranean countries (Spain)
	L. chagasi	Brazil, Columbia, Venezuela, Argentina

Table 1: Different forms of *leishmaniasis* with their distribution, adapted from: [17].

On the other hand, VL, which also known as kala-azar black fever or Dumdum fever, is caused by *L. donovani* species complex that consist mainly of *L.* infantum, *L. donovani*, and *L. chagasi* [5, 6, 10, 14, 18]. Taken into account, these species occasionally may cause other forms of disease [5].

Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL and characterized by skin lesions such as erythematous, maculo-papular, or nodular rash on the face, trunk, or other part of the body; those lesions is usually 6 months or several years after initial treatment of VL. This form is commonly noticed in *L. donovani* infection in Sudan and Indian subcontinent where it follows 50-60% and 10% of treated VL cases, respectively [19, 20]. Importantly, those infected people with chronic PKDL are considered important reservoir host. The following sections will review some general remarks about the epidemiological aspects of the disease, aiming for getting more information about the disease [21, 22].

Systematic and Morphology of the parasite

The first report about the disease and its etiological agents (*Leishmania)* was in 1903 by the Scottish pathologist William Boog Leishman and Charles Donovan, independently of each other [23]. *Leishmania* are protozoan hemoflagellates (2-5µm large) belonging to the order of *Kinetoplastida* (Figure 1). They are unicellular eukaryotes having a well-defined nucleus and other cell organelles including flagella and a deeply staining structure at the end of a flagellum called kinetoplasts [24]. A single locomotory flagellum, free or attached to a pellicle as an undulating membrane, originates from the *kinetoplast* [24].

It is widely known that all parasites species undergo obligate changes, altering their morphology and propagations in their respective invertebrate host. *Leishmania* exists in two stages on its lifecycle; the amastigote form which is an intracellular and non-motile form, devoid of external flagella, however, a short flagellum is embedded at the anterior end without projecting out. This form of the parasite is oval in shape, and measures 3–6 µm in length and 1–3 µm in breadth. It is found in the mononuclear phagocytes and circulatory systems of mammalian hosts including humans. On the other hand, the promastigote form is an extracellular motile form, found in the alimentary tract of sandflies; it is larger and highly elongated than amastigote form, measuring 15-30 µm in length and 5 µm in width. Promastigote has spindle shape, with long flagellum projected externally at the anterior end and the nucleus lies at the center.

Among 35 species of genus *Leishmania*, more than 20 of them can be transmitted to humans [25, 26], and therefore the taxonomy of *Leishmania* organisms is complex. On the basis of the location of the parasite within the insect gut, species of the genus *Leishmania* are classified into two subgenera; *Leishmania* and *Viannia*. The subgenus *Leishmania* includes pathogenic medically important vectors of the Old World species: *L. tropica*, *L. aethiopica*, *L. major*, *L. infantum* and *L. donovani*, while the medical important species of the *L. mexicana* group comprise *L. mexicana*, *L. amazonensis*, *L. venezuelensis* and *L. pifanoi* belong the New World.

In subgenus *Viannia*, the parasite develops in the hind gut of the insect and is found only in Central and South America. The most important species have public health importance for this subgenus include: *L. braziliensis*, L. *guyanensis*, *L. panamensis*, *L. infantum* and *L. peruviana* [27, 28]. It should be emphasized that the hybrids might be involved as reported in Brazil between *Leishmania (V.) guyanensis and Leishmania (V.) shawi shawi* [29].

Vectors, Transmission and Reservoirs

The parasite is naturally transmitted by the bite of 30 species of infected female sandflies belong the genus *Phlebotomous* in the Old World and *Lutzomyia* in the New World [30, 31]. Taken into account, the sand fly are very active from dusk to dawn while they are less active during the hottest time of the day. The majority of people might not realize the presence of sand flies as they are very small (2–3 mm), and therefore, they can pass through the holes netting.

Three mechanisms for transmission of the disease by the bite of an infected sand fly are known: (1) regurgitation of metacyclic promastigotes from the thoracic midgut into the skin during the blood meal (common) [32]; (2) precipitation of the infective stage from the proboscis into the skin [33]; (3) inoculation of metacyclic promastigotes from the salivary glands into the skin with the saliva [34]. However, it should be borne into mind that in some countries, such as Spain, besides the traditional epidemiological pattern involved in a zoonotic infection, namely infected dog –sandfly vector- human infection [35, 36].

Among others, *Leishmania* can also be transmitted to human by contaminated sharing among intravenous drug abusers [37], by blood transfusion [38], by venereal infections [39-41], congenitally from mother to infant [42], and by direct contact from animal to animal as reported in infected dogs that have co-habited with *Leishmania*-positive dogs [43].

In fact, these later routes of transmission are very rare *versus* the vector-borne transmission. Humans and rodents are considered reservoir for the disease, however, the dogs are the most important reservoirs in a domestic environment [4, 44]. Therefore, it is not surprising to state that dogs are the main peridomestic reservoirs for zoonotic leishmaniasis in the Mediterranean area [45-48], where the disease is referred as canine leishmaniosis (CanL) and occurs as cutaneous or cutaneous-visceral form with several clinical manifestations. The most common clinical signs appear in the form of generalized lymphadenomegaly, splenomegaly, a pale mucous membrane and weight loss and the disease is often associated with skin abnormalities with dry exfoliative dermatitis, ulcers, periorbital or diffuse alopecia and onychogryphosis. Likewise, the most important laboratory findings represent by an increase in gammaglobulins, hypoalbuminaemia, hyperproteinemia and anaemia. Some dogs show no systemic clinical signs but can have severe renal failure [49-51].

As previously mentioned, rodents, in particular, *Rattus rattus* and *Rattus norvegicus*, and *Mus musculus*, also play an important role as a possible reservoir of *leishmania* in some regions like Italy, Spain, Yugoslavia , and Saudi Arabi [22, 44, 45, 52-54]. Wild canids including wolves, foxes, jackals, hoary zorros, bush dogs, sloths and armadillos are also involved in the epidemiological cycle as sylvatic reservoirs in some countries [55-58]. Bats and wild mammals are also reported to play a role in transmission of the infection [59-61].

The role of cats as reservoirs of *Leishmania* is still controversial, however, cats remain secondary reservoirs, rather than accidental ones [57, 62]. Few reports about leishmaniosis in cats (FL) are available, however, in some immunosuppressed conditions like feline virus infection and tumor may accelerate the contraction of infection [63-65]. *L. infantum* and *L donovani* are the most common *Leishmania* species in domesticated cats in the Mediterranean area and Middle East [66], while *L. chagasi*, *L. braziliensis*, *L. amazonensis* and *L. mexicana* are the major species in Central and South America [67-70].

Other domestic animal species such as pigs, goats, cattle and horses may act as incidental reservoirs but they do not play a major significant role in transmission and only rare cases of CL have been reported in Africa and South America [71-79]. Few sporadic cases of equine leishmaniosis (EquL) have been also reported in Europe, in the form of self-healing, skin-dwelling disease [57, 80, 81]. The affected domestic animals showed body weight loss, skin

lesions and swollen lymph nodes and sometimes lameness and recurrent fever [82], but VL has not been reported in equines. The role of the domestic chicken (Gallus gallus) in the epidemiology of VL in Brazil was reported in 2002 [83]. Actually, chickens represent the link between the sylvatic and domestic transmission cycles as they are very attractive to *phlebotomine* sandflies and also to reservoir hosts of *L. infantum* infection, such as the crab-eating fox Cerdocyon thous [83]. Hence, chickens must be considered as a risk factor for the presence of vectors and reservoir hosts of *Leishmania* next to human habitations in some regions [83]. Some studies have also concluded that raising pigs, chickens, or other livestock in the adjacency of urban dwellings significantly increases the risk of CanL as chicken blood provides a nutritive meal for sand fly [83-86].

Pathogen Life Cycle

As previously mentioned, *Leishmania* is a protozoan parasite transmitted by the bite of female *phlebotomine* sand flies (*Phlebotomus* and *Lutzomyia* species) to the mammalian host [4]. The female's sandflies inject their saliva that contains metacyclic promastigotes (infective stage) into the skin during blood meals, causing some allergic reactions (Fig. 1). In the mammalian host, promastigotes are phagocytized by macrophages and occupy an intracellular niche then transform into amastigotes, mainly within macrophages of the host where they multiply in phagolysosomes of phagocytes as amastigotes which exists within a parasitophorous vacuole and unaffected by cellular digestive enzymes. Amastigotes multiply by simple division and proceed to infect new mononuclear phagocytic cells and affect different tissues, depending in part on which *Leishmania* species is involved. Parasite, host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral forms.

When a female sand fly bites an infected vertebrate host, they ingest macrophages infected with amastigotes with the blood meal, and then a chitin-based peritrophic membrane is synthesized around the blood-meal. In the sand fly's midgut (in the hindgut for *Viannia* subgenus) and over 4-25 days, the amastigote differentiates by binary fission into elongated, motile procyclic promastigotes, which multiply, differentiate into metacyclic promastigotes (flagellated forms), which are resistant to the digestive enzymes, migrate to the proboscis and are finally transmitted to the next host during the next blood meal [27, 87, 88].

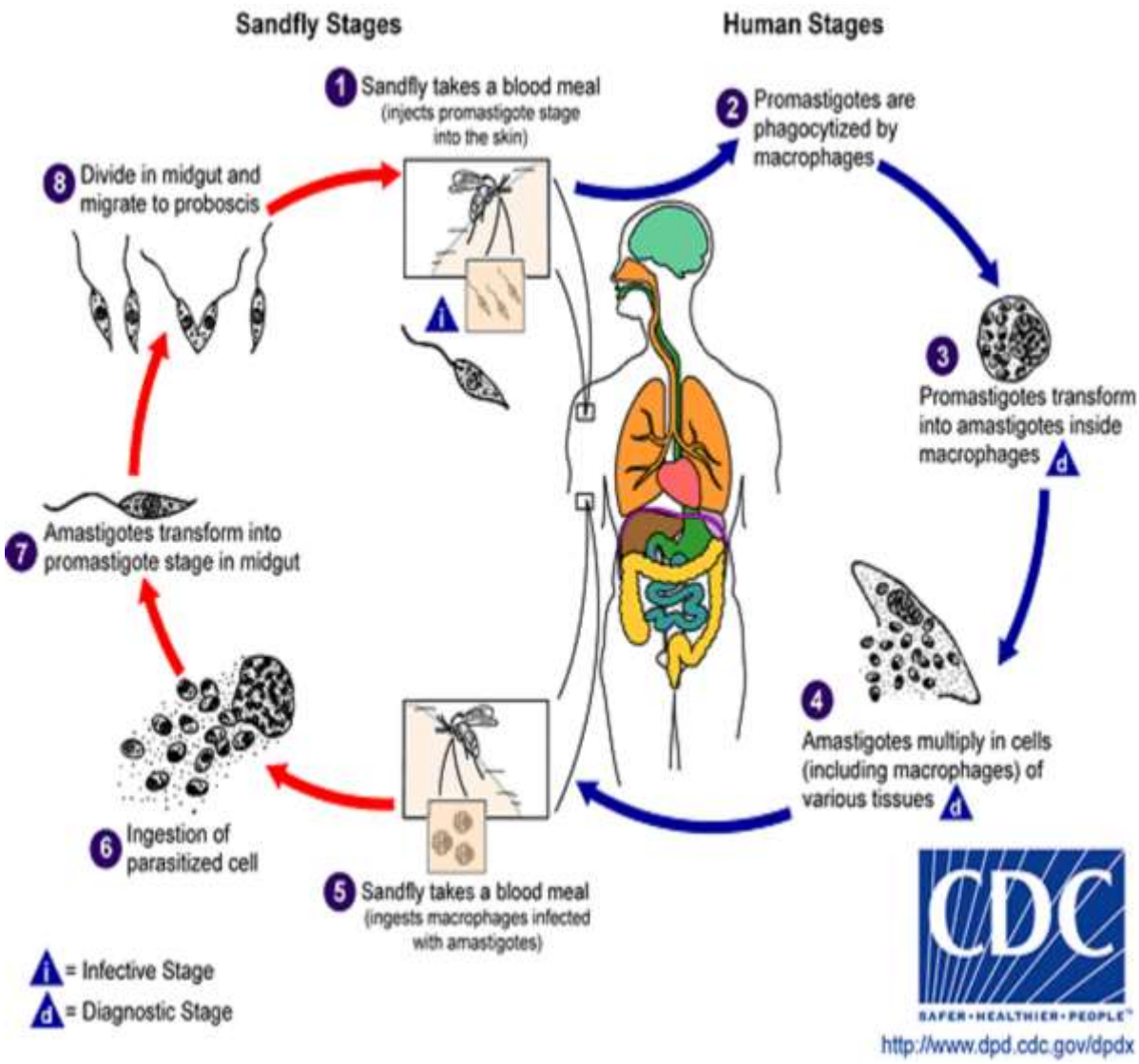

Figure 1: Life cycle and Morphological Stages of *leishmania, adopted from CDC website (http://www.cdc.gov/parasites/leishmaniasis/biology.html).*

Manifestations of Disease

Depending on the causative species and the form involved, the disease is manifested in humans in several clinical forms: CL, MCL, VL, and PKDL [4, 89]. These forms are usually developed after vector bite but the latency may occur from a week to years [90, 91]. Therefore, the characterization of the disease mainly depends on the infecting species and the immunological responses of the host [90, 92].

The cutaneous form of the disease (CL) is very common and characterized by skin lesions which develop within weeks or months after being bitten and these lesions may be either localized or diffused [4, 89, 93]. However, it should be borne in mind that some people don't develop any clincal signs [94]. The localized cutaneous form manifested as crusted papules or ulcers on exposed skin where lesions may be associated with sporotrichotic spread, while the disseminated form appears in the form of multiple, widespread non-tender, non-ulcerating cutaneous papules and nodules; similar to lepromatous leprosy lesions. These lesions are common on uncovered areas of the skin that are such as hands, the face, or lower legs in the form of nodular plaques, and eventually lead to open sores with a raised border and central crater [90, 93]. They are usually painless but can be painful, particularly after become secondary infection with bacteria. Some people may develop satellite lesions and swollen glands near the sores (regional lymphadenopathy), and nodular lymphangitis. These previously mentioned lesions may resolve quickly after two to three months without treatment or become chronic, lasting months or years [90, 92, 95]. This form of the disease (CL) is of great potential concern, especially with some species of the *Leishmania* in South and Central America, where the infection may progress from the skin to the mucosal surfaces of the nose or mouth and cause sores [90, 93].

Mucocutaneous form (MCL) is manifested as ulcerative or granulomatous lesions of the nasal, oral and pharyngeal mucosa and may result in disfiguration and ulcerative tissue destruction that may results in perforation of the nasal septum [92, 96, 97]. Presence of granulation, erosion, and ulceration of the palate, lips, pharynx, and larynx, with sparing of the bony structures; may be occur. Gingivitis, periodontitis, localized lymphadenopathy, optical and genital mucosal involvement was also reported in severe cases [91, 98-101]. In fact, this form is less common but was reported in travelers and expatriates whose cases of CL were inadequately treated or absence of treatment at all, and mostly in Latin America [90, 102-106].

As previously mentioned, VL is the most life-threatening form of leishmaniasis, with very high rate (95 %) of mortality in absence of treatment [107, 108]. In this form, the parasite spreads into the following organs: lymph nodes, kidneys, spleen, bone marrow, liver, pancreas, testicles, lungs, eyes, joints and bones; and therefore, all these organs may develop a granulomatous reaction with variable numbers of amastigotes. VL may also behave the chronic way and characterized by fever, lymphadenopathy, hepatosplenomegaly, anaemia, leukopenia,

thrombocytopenia, weight loss, besides progressive emaciation and weakness; which is mainly resulted from proliferation of the parasite in macrophages and organs associated with the reticuloendothelial system [4, 57, 82, 109-113]. Also, silent or latent infection has been reported [94, 108].

Advances in the epidemiology and the geographical distribution of Leishmaniasis

To our knowledge, the disease is widely known as the second-largest parasitic killer in the world affecting a variety of settings including rain forests, deserts, rural and peri-urban areas [4, 93]. Regarding the zoonotic aspect of the disease, leishmaniasis has two zoonotic forms in Europe: zoonotic cutaneous leishmaniasis (ZCL) and zoonotic visceral leishmaniasis (ZVL). Zoonotic visceral Leishmaniasis (ZVL), caused by *L. infantum*, occurs in most countries of the Mediterranean region, while zoonotic cutaneous leishmaniasis (ZCL), is caused by *L. major* and occurs in Afghanistan, Egypt, Iran, Iraq, Jordan, Libya, Morocco, Palestine, Pakistan, Saudi Arabia, Sudan, Syria, Tunisia and Yemen [114].

ZCL may also occurs in the same areas endemic for VL but it seems that there are many subclinical cases than those registered [115, 116]. These two forms are usually associated with *L. infantum*, being the most common etiological agent of leishmaniasis in Europe [117]. This parasite species might result in a latent public health threat but some immunosuppressive cases [117], such as human immunodeficiency virus (HIV) co- infection, organ transplanted patients [118] or immunosuppressive therapies, reactivate the latent infection [119]. However, isolation of parasites from patients with CL in France, Spain, Italy and Malta revealed only one species of *L. infantum* [120-122], among other species.

Leishmaniasis may also occur as an anthroponotic cutaneous Leishmaniasis (ACL) which is usually caused by *L. tropica* and sporadically occurs in of the western Mediterranean, Spain and Greece [2, 121, 123, 124], however, this form is mainly found in Afghanistan, Iran, Iraq, Morocco, Pakistan, Saudi Arabia, Syria and Yemen [114], whereas Anthroponotic visceral leishmaniasis (AVL), is another form caused by *L. donovani* and mainly occurs in Sudan and Somalia [114]. In fact, the epidemiology of leishmaniasis depends on several factors such as the parasite species, local ecologic settings of the transmission sites, current and past exposure of the human population to the parasite and human behavior. It seems that the disease is found with

higher tendency in the tropics, subtropics, where conditions are favorable for its vector. The ecological characteristics range from rainy forests in Central and South America to deserts in western Asia and the Middle East, and therefore, climate and other environmental changes have the potential to expand the geographic range of the sand fly vectors and the distribution of leishmaniasis in the world. Taken into account, leishmaniasis usually is more common in rural than in urban areas, however, its presence in the outskirts of some cities explains the hypothesis state that the disease is a climate-sensitive disease and is strongly affected by changes in rainfall, atmospheric temperature and humidity. Such epidemiological aspects gave the patchy distribution of the disease with discrete transmission foci all over the world, depending on microecological conditions [125-127]. This geographic expansion of the disease has led to the necessity of establishing more effective control measures which is mainly based on accurate diagnostic tools.

As previously mentioned, leishmaniasis is a climate-sensitive disease. Therefore, global warming, changes and land degradation together strongly influence the incidence and epidemiology of leishmaniasis via several mechanisms [52, 128-130]. The changes in the environment a part from urbanization, domestication of the transmission cycle and the incursion of agricultural farms and settlements into forested areas, markedly involve the epidemiological pattern [131-134]. Additional occupational activities such as deforestation and hunting may also influence the risk of infection by increasing. Therefore, the infection rate is often high among people living at the edge of natural foci, close to the sylvatic cycle [135-142]. In this regard, geographical information systems (GIS) may provide a valuable tool for basic and operational research on the epidemiology of disease, including epidemiological surveys [143-145]. The history of previous exposure seems an important aspect as large proportion of the adult population will gain acquired immunity to the parasite. Nevertheless, the immunosuppressed individuals who enter an endemic area are highly at risk for the disease. Hence, leishmaniasis is also often associated with migration and movement of individuals into endemic areas or enzootic transmission cycles as reported in military activities and conflict areas [131, 132, 146, 147].

Regarding the age, it is mainly depend on the parasite species, the history of population exposure and the immune status of the individual. In endemic foci, where the causative parasite is *L. infantum*, the median age of clinical VL patients tends to be younger (usually younger than 5

years) than that of *L. donovani* [148, 149]. Moreover, young age with malnutrition seems to predispose humans to VL in South American [150, 151]. Indeed, it seems that the young adults are more susceptible to the infection than the other ages, however, some studies on VL in Brazil for dogs have not evidenced sexual, racial or age range related to the infection [136, 138, 139].

In concern with the sex as epidemiological aspect, several studies have reported more cases in male than female, however, the sex ratio can be accurately ascertained only in community-based studies [136, 138]. The breeds may also involve the epidemiological pattern since it has been reported that miniature breeds are less affected because they usually live inside the houses [152-154]. Another important epidemiological aspect is the poverty which increases the risk for leishmaniasis though different mechanisms [136]. Poor housing and peridomestic sanitary conditions may increase sandfly breeding and resting sites, and therefore, their access to humans [136]. Overcrowding into a small space may also attract sandflies by providing a large biomass for blood-meals, and therefore, large outbreaks in highly populated cities also occur, especially during war and population migration. The host genetic background may play a role in disease manifestations [155-157].

In concern with the geographic distribution, leishmaniasis is generally found on all inhibited continents except Australia and Antarctica; hence, the disease afflicts approximately 350 million people in 98 countries or territories around the world [4, 158, 159]. As previously mentioned, at least 20 morphologically indistinguishable species of *Leishmania* can infect humans and mainly classified by geographical distribution and the clinical characteristics of the disease they afflict [6]. The HIV/*Leishmania* co-infection represents additional challenge to the approach to leishmaniasis with diagnosis and treatment as the clinical course of the disease is even less specific and can be masked by other associated opportunistic infection [12, 159, 160].

Among 20 well-recognized *Leishmania* species, 13 species have zoonotic importance, which results in different forms among the Old and the New Worlds [57]. In the Old World, throughout the Mediterranean Basin of North Africa, the Middle East and Southern Europe, the CL form occur predominantly and is also partly endemic in sub-Sahara Africa, Southern Asia, the western parts of India and China [114]. The most severe form, VL, affects poor populations in remote areas across South Central Asia and China, East Africa, South America, Middle East and the

Mediterranean region. In this concern, more than 90% of the world's cases of VL occur in rural and suburban areas of India, Sudan, Bangladesh, Nepal, and Brazil [4, 21, 57, 161, 162].

The African continent, in particular the East and North, is considered the origin of leishmaniasis, from where the disease is spreading to Southern Europe [163]. In East Africa, there are frequent outbreaks of VL in the northern Acacia–Balanite savanna, southern savanna and forest areas where sandflies live. On the other hand, CL is found in Ethiopia and other places in East Africa, where increased human–fly contact occurs in villages built on rock hills or river banks. In the Mediterranean Basin, VL is the main form of the disease which is more common in rural areas in villages and mountainous regions. Leishmaniasis is also found across much of Asia, and Middle East. In South-East Asia, VL is the main form of the disease and the transmission of the disease is generally found in rural areas with a heavy annual rainfall and abundant vegetation. Among other Asian countries, leishmaniasis occurs commonly in Afghanistan[164], where Kabul is estimated as the largest focus of CL in the world, with around 67,500 cases in 2004, which is partly presumed to bad sanitation.

The epidemiology of CL in the Americas is somewhat complex, with variations in transmission cycles, *Leishmania* species, reservoir hosts, sandfly vectors, clinical manifestations and response to therapy even in the same geographical area. The disease can be found from Texas (USA) to Argentina in South America. On the other hand, MCL mostly occurs in the New World (Brazil and Central America), while the cases of leishmaniasis evaluated in the United States reflect travel and immigration patterns [103]. For example, many cases of VL and CL have been reported by U.S. troops stationed in Saudi Arabia and Iraq since the Gulf War of 1990, and also, travelers have been acquired the disease in tourist destinations in Latin America [103, 165].

Current situation of the disease in Europe

As previously mentioned, leishmaniasis is found endemic in Europe, particularly in Mediterranean basin. Several autochthonous cases have been reported but the first case of *L. donovani* in Europe have been detected in Cyprus [116, 166], and later it has been estimated that around 700 new cases of leishmaniasis occur in southern European countries [2, 21, 104]. The World Health Organization (WHO) has estimated approximately 410–620 VL cases each year

during 2003 to 2008 in only nine countries of the European Union (EU), in particular Bulgaria, Macedonia, Greece, Croatia, Italy, Southern parts of France and the Iberian Peninsula [167].

WHO data also revealed that co-infection cases with *Leishmania*/HIV are higher in Spain, compared with other Mediterranean countries [160], more specifically, one third of patients hospitalized with leishmaniasis have been described to be co-infected with HIV, which has alarming effect about this zoonosis [22, 35, 168-170]. For example, Madrid was recently reported to be the largest community with outbreak of leishmaniasis in Europe with 446 cases in the period from 2009 to 2012 [35]. This high prevalence of human infections could be attributed to two main reasons, including humans are frequently bitten by sandflies and infection is widespread in a highly susceptible host (dogs) [124, 171]. As a result of absence of systematic analyses of leishmaniasis in travellers visiting endemic areas in Europe [105, 172], a large series at the north European countries with natural transmission of VL and CL have reported that mainly acquired the infection during holidays in southern Europe [104, 169, 173-177].

In accordance with the vector, the disease is mainly transmitted in Europe by sand flies of the genus *Phlebotomus* but rodents and canines are important common reservoirs [2, 124]. The seroprevalence of canine Leishmaniasis (CanaL) ranged from 5–30% in some countries of southern EU, which means that infection rates may reach values of 40–80% [2, 178, 179]. Wildlife and hares were recently shown to play an important role in transmission in some countries like Spain [180, 181].

Five major species of the subgenus *Larroussius* (*P. ariasi*, *P. neglectus*, *P. perfiliewi*, *P. perniciosus* and *P. tobbi*) are considered the competent vectors in the Mediterranean area of Europe [124]. They are not equally distributed and more than one species of this subgenus may transmit the parasite at the same place [120]. In Spain, P. *ariasi*, *P. perniciosus* and *P. langeroni* are the main vectors but *P. papatasi*, *P. sergenti* and *P. longicuspis* may play a role in transmission of the disease [182-184], while in Greece, *P. sergenti* is the main vector of *L. tropica* [120, 185-187]. The clinical course of the disease in Europe, like other countries, is slow chronic course and may results in deaths due to inappropriate management of VL cases, however, these cases are rare and more frequent among VL/ HIV co-infection cases or, in case of young children, malnutrition associated with late diagnosis.

2. Diagnosis of Leishmaniasis

Adapted from a previous review article published by the same author as a part from the thesis*: Ehab Kotb Elmahallawy, Antonio Sampedro Martínez, Javier Rodriguez-Granger, et al. Diagnosis of Leishmaniasis***.** *Review Article. J Infect Dev Ctries 2014; 8(8):961-972.*

Diagnosis of visceral leishmaniasis

In developing countries where the disease is not prevalent, the existence of laboratory facilities enables an adequate and efficient follow-up of the disease. However, in developing countries with large numbers of patients in rural areas, simple diagnostic tools are necessary for field use [188]. Laboratory diagnosis of VL includes microscopic observation and culture from adequate samples, antigen detection, serological tests, and detection of parasite DNA.

Culture and microscopic observation

Definitive VL diagnosis is supported by direct demonstration of parasites in clinical specimens and specific molecular methods [189-191]. The commonly used samples are splenic or bone marrow aspirates. The presence of amastigotes can also be determined in other samples such as liver biopsies, lymph nodes, and buffy coats of peripheral blood. The sensitivity of the bone marrow stained with Giemsa is about 60% to 85%. In splenic aspirates, the sensitivity is higher (93%)[192], but sampling is associated with a risk of fatal hemorrhage in inexperienced hands. To increase the sensitivity, fluorescent dye-conjugated antibodies can be used [193].

The sensitivity in peripheral blood smears is low, especially in individuals with low parasitemia. In addition, results are dependent on technical expertise and the quality of prepared slides. Culture of the parasite can improve diagnostic sensitivity, but is tedious, time-consuming, and expensive, and thus seldom used for clinical diagnosis. There are new culture methods that improve sensitivity, such as the micro-culture method (MCM); recent modifications of this method involve using the buffy coat and peripheral blood mononuclear cells [194, 195].

The culture media used may be biphasic and may include Novy-MacNeal-Nicolle medium and Tobie's medium (conversion of amastigotes to promastigotes and monophasic medium), Schneider's insect medium, M199, and Grace's insect medium (amplifying parasite number) [193].

Antigen detection in urine

Several studies have demonstrated leishmanial antigen in the urine of VL patients. In 1995, De Colmenares *et al*. reported two polypeptide fractions of 72–75 kDa and 123 kDa in the urine of kala-azar patients [196]. In 2002, Sarkari *et al*. described a urinary 5–20 kDa carbohydrate-based, heat stable antigen of VL patients[197]. A latex agglutination test (KAtex, Kalon Biological, UK) for detection of this antigen in urine samples was evaluated using samples from confirmed cases and controls from endemic and non-endemic regions. This test showed good specificity (82% to 100%), but had low to moderate sensitivity that ranged from 47% to 95% [198-202]. Nowadays, this method is useful for the diagnosis of disease in cases with deficient antibody production.

In this respect, this method has reported 100% sensitivity and 96% specificity in immunocompromised patients [203]. In another study, 87% specificity and 85% sensitivity were obtained for primary VL in HIV-co-infected patients, and the method had predictive capabilities in the follow-up of treatment and detection of subclinical infection in *Leishmania*/HIV co-infected cases[204]. Another urinary leishmanial antigen, a low-molecular weight, heat-stable carbohydrate has been detected in the urine of VL patients by an agglutination test with 60% to 71% sensitivity and 79% to 94% specificity [205]. In summary, the latex agglutination test is simple, easy to perform, inexpensive, rapid, and can be used as a screening test. Efforts are being made to improve the performance of this technique, because it promises to be a test of cure in populations of developing areas [192].

Serological diagnosis

Specific serological diagnosis is based on the presence of a specific humoral response. Current serological tests are based on four formats: indirect fluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA), western blot, and direct agglutination test (DAT). The sensitivity depends upon the assay and its methodology, but the specificity depends on the antigen rather than the serological format used. It has been noted that all antibody detection tests share the same drawbacks; the antibodies remain positive for many months after the patient has been cured and do not differentiate between current and past infection. In endemic regions, asymptomatically infected persons can also be positive in these tests. Table 2 presents a summary of all the serological and antigen detection techniques commonly used for leishmaniasis diagnosis.

Indirect fluorescent antibody (IFA) test

The IFA test shows acceptable sensitivity (87%–100%) and specificity (77%–100%) [205, 206]. Promastigote forms should be the antigens of choice for diagnosis of visceral leishmaniasis by the IFA test because they minimize cross-reactivity with trypanosomal sera [207]. The antibody response is detectable very early in infection and becomes undetectable six to nine months after cure; hence, if the antibodies persist in low titers, it is a good indication of a probable relapse [208]. The need for a sophisticated laboratory with a fluorescence microscope restricts use of the IFA test to reference laboratories.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is the preferred laboratory test for serodiagnosis of VL. The technique is highly sensitive, but its specificity depends upon the antigen used. Moreover, this assay can be performed easily and is adaptable for use with several antigenic molecules. One of the antigens used in the ELISA test is a crude soluble promastigote antigen (CSA) that is obtained by freezing and thawing live promastigotes. The sensitivity of ELISA using CSA ranges from 80% to 100%, and specificity ranges from 84% to 95% [209, 210]. Cross-reactivity with sera from patients with tuberculosis, trypanosomiasis, and toxoplasmosis has been reported [211-215]. When selective antigenic molecules were used (with molecular weights of 116 kDa, 72 kDa, and 66 kDa), the specificity approached 100%, while the sensitivity was very low (37%) [195, 216]. Other purified antigens used were a 36-kDa glycoprotein [217], metabolic antigens released by *L. donovani* [218], and A2 proteins implicated in the development of visceral disease[219-221].

A conserved portion of a kinesin-related protein recombinant antigen from a cloned protein of *L. chagasi*, called rK39, has been reported to be highly reactive to sera from human and canine VL cases when run in an ELISA format[222, 223]. Using this recombinant antigen, 99% specificity and sensitivity were reported in immunocompetent patients with clinical VL. In India, this antigen was reported with a sensitivity of 98% and a specificity of 89%[224]; however, a report from Sudan and other countries revealed that this antigen showed low sensitivity (75%) and specificity (70%)[225]. In HIV-positive patients, rK39-ELISA showed higher sensitivity (82%) than the IFA test (54%), with higher predictive value for detecting VL [226]. With successful

therapy, antibody titers declined steeply at the end of treatment and during follow-up; in contrast, patients who relapsed showed increased titers of antibodies to rK39. This suggests the possible application of rK39-ELISA in monitoring drug therapy and detecting relapse of VL [216]. In another study, two hydrophilic antigens of *Leishmania chagasi* were used (rk9 and rk26), leading to an increase in the list of available antigens for serodiagnosis of VL [227]. Another kinesin recombinant related protein used in ELISA assay is rKE16. The use of this antigen for VL diagnosis has been very sensitive and specific as rK39 when tested in patients from China, Pakistan, and Turkey [228].

Another new assay, based on the detection of the K28 fusion protein in studies performed in Sudan and Bangladesh with 96% sensitivity in Sudan and 98% in Bangladesh, has been developed [229]. Moreover, heat shock proteins HPS70 or histones proteins H2A, H2B, H3, and H4 may have potential use for serodiagnosis of VL [230, 231]; furthermore, lipid-binding proteins (LBPs) as antigens have shown high levels of sensitivity and an absence of cross-reactions with the sera of patients with other diseases [232]. The ELISA test, due to the requirement of skilled personnel, sophisticated equipment, and electricity, is not used in endemic regions for the diagnosis of VL.

Immunoblotting (western blot)

For this type of testing, promastigotes are cultured to log phase, lysed, and the proteins are separated on SDS-PAGE. The separated proteins are electrotransferred onto a nitrocellulose membrane and probed with serum from the patient. The western blot technique provides detailed antibody responses to various leishmanial antigens[233, 234], and has been found to be more sensitive than the IFA test and ELISA, especially in co-infected HIV patients with VL [235-237], but the drawbacks of the technique (equipment and time requirements, cumbersomeness, and cost) limit its use to research laboratories.

Direct agglutination test (DAT)

The DAT is based on direct agglutination of *Leishmania* promastigotes that react specifically with anti-*Leishmania* antibodies in the serum specimen, resulting in agglutination of the promastigotes. Whole, trypsinized, coomassie-stained promastigotes can be used either as a suspension or in freeze-dried form that can be stored at room temperature for at least two years, facilitating its use in the field [238, 239]. Chappuis *et al*. [240], in a meta-analysis that included

thirty studies evaluating DAT, found that the DAT had sensitivity and specificity estimates of 94.8% (95% confidence intervals [CI], 92.7–96.4) and 97.1% (95% CI, 93.9–98.7), respectively. Moreover, in settings where parasitological confirmation is not feasible, the freeze-dried DAT together with classical clinical features of VL can be used for diagnosis at a cut-off for positive DAT, which is 1:12,800 as in endemic areas. The DAT is simpler than many other tests but presents severe problems in terms of reproducibility of results, which depends on antigen elaboration[241]. A new antigen elaboration method, the EasyDAT described in 2003, shows the same sensitivity, specificity, and durability as the traditional DAT antigen method but offers the additional advantages of cost reduction and standardization [242].

Although the DAT for the serodiagnosis of visceral leishmaniasis has high sensitivity and specificity, it still has some limitations; among these are the relative long incubation time (18 hours) and the serial dilutions of the samples that must be made. In order to circumvent these problems, Schoone *et al*. in 2001 [243] developed a fast agglutination screening test (FAST). The FAST utilizes only one serum dilution (qualitative result) and requires three hours of incubation. This makes the test very suitable for the screening of large populations. The sensitivity and specificity of FAST were found to be 91.1%–95.4% and 70.5%–88.5%, respectively [244, 245]. Anti-*Leishmania* antibodies may persist for years as a result of previous VL infection, so titers measured by DAT may remain positive for up to five years after recovery in > 50% of VL patients, which may limit the DAT's widespread applicability in regions of endemicity. Although the DAT is the first real field test, it remains the serological test of choice as well as the first antibody detection test for VL used in field settings, particularly in many developing countries and in *Leishmania*/HIV co-infections [237, 246, 247].

Immunochromatographic assay (IC)

The immunochromatographic test using rK39 antigen (39 amino-acid-repeat recombinant leishmanial antigen from *L. chagasi*) has become popular in recent years. It is a qualitative membrane-based immunoassay with nitrocellulose strips impregnated with recombinant K39 *Leishmania* antigen. A drop of blood or serum is smeared over the pad of the strips and dipped in a small amount of buffer; the results are ready within a few minutes. In clinical cases of VL, the rK39 IC showed variation in the sensitivity and specificity among different populations.

The rK39 IC showed 100% sensitivity and 93%–98% specificity in India [248, 249], 90% sensitivity and 100% specificity in Brazil [250], and 100% sensitivity and specificity in the Mediterranean area [251]. In other reports in southern Europe, the rK39 IC test was positive in only 71.4% of the cases of VL [252]; in Sudan, rK39 IC showed a sensitivity of 67% [225]. These differences in sensitivity may be due to differences in the antibody responses observed in different ethnic groups. The rK39 IC assay has proven to be versatile in predicting acute infection, and it is the only available format for diagnosis of VL with acceptable sensitivity and specificity levels. It is also easy to use in the field, rapid (15–20 minutes), cheap, and gives reproducible results. Like the DAT assay, IC is positive in a significant proportion of healthy individuals in endemic regions and for long periods after cure; hence, this limits its usefulness in persons with a previous history of VL who present with recurrence of fever and splenomegaly, as these tests cannot discriminate between a case of VL relapse and other pathologies.

Latex agglutination test (LAT)

The LAT is one of the recently developed rapid diagnostic tests for the rapid detection of anti-*Leishmania* antibodies against the A2 antigen derived from the amastigote form as well as those against crude antigens derived from the promastigote form of an Iranian strain of *L. infantum*. In a comparative study with the DAT, the sensitivity of tested human sera from DAT-confirmed patients yielded 88.4% sensitivity, while the specificity was 93.5% on A2-LAT amastigote, with a higher degree of similarity in accuracy to the DAT [253, 254].

Molecular methods: polymerase chain reaction (PCR)

Although different molecular methods have successively been evaluated for leishmaniasis diagnosis, PCR-based assays are the main molecular diagnostic tools, especially in immunosupressed patients [255-258]. PCR protocols to detect *Leishmania* DNA in VL diagnosis have used a variety of samples, including spleen, lymph node, and bone marrow aspirates, whole blood, and buffy coat [256, 259-265]. There are different target sequences used, which include ribosomal RNA genes, kinetoplast DNA (kDNA), miniexon-derived RNA (medRNA), and the β-tubulina gene region[266].Many PCR-based methods for diagnosis of VL have been described with different specificities and sensitivities; PCR assay sensitivity depends on the sample used. Sensitivity is highest (near 100%) in spleen or bone marrow samples [257, 260, 267]. The ideal sample is peripheral blood due to its non-invasive character. Using peripheral blood, the sensitivity ranges described vary from 70% to 100% [257, 266, 267].

A comparative clinical study between conventional microbiologic techniques and a leishmania species-specific PCR assay in HIV-co-infected and HIV-uninfected patients has shown the sensitivity of the Leishmania species-specific PCR to be 95.7% for bone marrow and 98.5% for peripheral blood samples; the sensitivity in HIV-co-infected and non-HIV-co-infected adults was 100% [258]. A PCR-ELISA was used to diagnose VL in HIV-negative patients; peripheral blood was used and yielded a high sensitivity [268]. A similar PCR-based technique was applied in the diagnosis of VL in HIV patients, with good results [189]. Recently, an evaluation of an oligochromatography-PCR for diagnosis of VL, cutaneous leishmaniasis (CL), and post kala-azar dermal leishmaniasis (PKDL), showed a high sensitivity (> 95%) on lymph, blood, and bone marrow samples from confirmed VL patients [269].

Another interesting approach is a rapid fluorogenic PCR technique. Wortmann *et al*. used a fluorescent DNA probe for a conserved rRNA gene that is amplified using flanking primers; this technique using clinical samples showed great sensitivity and specificity [262]. The real-time PCR has the advantage of being quantitative, which could be useful in the follow-up of treatment, allowing for the assessment of the parasite burden [270, 271]. However, PCR techniques remain complex and expensive, and in most VL-endemic countries, they are restricted to a few teaching hospitals and research centers.

Diagnosis of VL-HIV co-infection

According to the World Health Organization (WHO)[272], an estimated 35 million people worldwide are living with HIV. Immunosuppression may reactivate latent *Leishmania* infection in asymptomatic patients and among HIV/AIDS patients. It is known that *Leishmania* has emerged as an opportunistic disease among HIV patients in endemic areas [22, 273-275]. Moreover, it has been noted that the risks of clinical VL in HIV patients increased by 100 to 1,000 times [274]. Although cases of co-infection have so far been reported in 33 countries worldwide, most of the cases have been found in sub-Saharan African countries, especially in East Africa. In Humera (in northwest Ethiopia), the proportion of VL patients co-infected with HIV increased from 18.5% in 1998–99 to 40% in 2006 [276].

VL in HIV patients has atypical clinical presentation; only 75% of HIV-infected patients, as opposed to 95% of non-HIV-infected patients, exhibit the characteristic clinical pattern – namely, fever, splenomegaly, hepatomegaly, and gastrointestinal involvement [277-280]. The

diagnostic principles remain the same as those for non-HIV-infected patients. The presence of *Leishmania* amastigotes in the bone marrow can often be demonstrated in buffy coat preparation and in unusual locations (stomach, colon, or lungs) [281], but it has lower sensitivity in VL-HIV patients. For HIV patients, the sensitivity of antibody-based immunological tests such as the IFA test and ELISA is low. Serological tests have limited diagnostic value because over 40% of co-infected individuals have no detectable specific antibody levels against *Leishmania* [12]. In their meta-analysis, Cota *et al*. [237] summarized the accuracy of different serological techniques used for diagnosing HIV-co-infected persons. The estimated sensitivities using random effect models and their respective 95% confidence intervals for the other tests were: IFA test, 51%; ELISA, 66% (40% to 88%); DAT, 81% (61% to 95%); and immunoblotting, 84% (75% to 91%). The estimated specificity using random effect models and their respective confidence intervals for the following tests were: immunoblotting, 82% (65% to 94%); ELISA, 90% (77% to 98%); IFA test, 93% (81% to 99%); and DAT, 90% (66% to 100%).

Thus, due to the low sensitivity of the serological tests for VL diagnosis in HIV-infected patients, at least two different serological tests should be used for each patient to increase the sensitivity of antibody detection [160]. The detection of polypeptide fractions of 72–75 kDa and 123 kDa of *Leishmania* antigen in the urine of patients with VL was 96% sensitive and 100% specific; furthermore, these antigens were not detectable after three weeks of treatment, suggesting a good prognostic value [196]. In conclusion, serology should not be used to rule out a diagnosis of VL among HIV-infected patients; an additional specially recommended serological test and/or molecular or parasitological methods may be necessary if the results of serological tests are negative.

Serological Test	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages	Ref.
(A) Antibody detection **1) IFA test**	77–100	Positive in the early stages of infection and undetectable six to nine months after cure.	Requires sophisticated laboratory Not applicable in the field.	The possible a possibility of cross reaction with *Trypanosoma* sera. Lower recorded sensitivity which ranged from 28 to 98%.	[205-207]
2) ELISA with: **CSA** **Fucose-mannose** **rK39 antigen**	96 84-95 96 98-99	86-100 84 – 95 95-100 99-100	Can be used to screen a large scale of epidemiologic studies	Sensitivity and specificity is greatly influenced by the antigen used. Requires skilled personnel, sophisticated equipment, and electricity.	[209, 210, 217, 222, 223]
3) Western blot	90–98	88 – 100	Provides detailed antibody responses to various leishmanial antigens.	Time consuming, technically cumbersome, and expensive	[233-237]
4) DAT	85 – 100	91 - 100	Rapid test, applicable in the field.	Limited to use in regions of endemicity. Long incubation time is needed. Unavailability of commercial source of the positive antigen and fragility of is aqueous form.	[238, 240, 241, 243]
5) IC test with rK39	90-100	93–100	Inexpensive, rapid, simple, and can be performed by untrained person	Low sensitivity and specificity reported in Sudan (67%) and southern Europe (71%).	[248-250, 252]
(B) Antigen detection **Latex agglutination test in urine (Katex)**	79–100	60–100	Simple, easy performing, Useful in diagnosis of disease immunocompromised patients.	Difficult to distinguish weakly positive from negative results and the urine must be boiled to avoid false-positive reactions	[197, 198, 200]

Table 2: Serological and antigen detection techniques commonly used for leishmaniasis diagnosis [15].

3. Treatment of Leishmaniasis: A Review and Assessment of recent research

Adapted from a previous review article published by the same author as a part from his thesis: *Ehab Kotb Elmahallawy and Ahmad Agil. Current Pharmaceutical Design. 2015; 21(18):2259-75.*

General remarks

Over the past couple of decades, research on *Leishmania* has made a significant progress in various directions but many questions have not been answered so far. Treatment remains the mind-boggling question, presumably due to the existence of various species of *Leishmania* and different clinical manifestations which complicate the treatment strategy.

Treatment of CL is fraught with controversy, the lesions could resolve spontaneously, but sometimes may evolve into MCL [282]. The aim of the treatment in CL cases is to prevent the development of mucosal forms of the disease and to limit tissue damage, especially on the face, and therefore the patients should be given systemic treatment. *Leishmania* species are temperature sensitive that makes local treatment with heat, infrared heat therapy or even carbon dioxide laser (CO_2 laser) may hasten the resolution of Old and New World CL lesions [283, 284]. Cryotherapy, using Liquid nitrogen application, has also been successful in uncomplicated lesions of *L. tropica* and *L. major* infections with 69% cure rate, but this strategy can be painful [285]. Topical applications, using intralesional injection of some antileishmanial agents, have been known to be useful in the treatment of some cases of CL, depending on the infecting species and the risk of serious complications [286]. Trials with 15% paromomycin and 12% methylbenzethonium application have been used successfully with CL infected cases in the Middle East and in Latin America [287].

Immunotherapy represents an additional method of treatment [288, 289]. In this regard, a combination of heat-killed *Leishmania* and live bacille Calmette–Guérin (BCG), defined that recombinant interferon-gamma (IFN-γ) or granulocyte-macrophage colony-stimulating factor together with pentavalent antimony; has been used with higher efficacy for the treatment of CL, MCL and even VL in some cases, but this method consumes a longer period time [290-295]. MCL is successfully treated with systemic drugs with the combination of pentavalent antimony and pentoxifylline [293, 294].

To date, the reliable human vaccine together with the ideal strategy to control parasite vectors and reservoirs is still out of reach. Treatment of VL or PKDL is mainly based on leishmanicidal drugs in order to cure the patient and to prevent the development of resistance and relapse.

Around 25 compounds and formulations are available for the treatment of leishmaniasis in humans, but not all are in use. These agents are associated with toxicity and/or drug resistance problems, making the development of an effective drug against all forms of Leishmaniasis indispensable [296, 297]. The treatment strategy principally consists of pentavalent antimonials that have been in use since the early 20th century, especially for VL [152, 298, 299]. In the recent years, however, there has been an alarming increase in reports of primary resistance, irregular effectiveness, serious side effects, and relapse [300, 301]. The second line of treatment includes liposomal amphotericin B and miltefosine [299, 302]. Liposomal amphotericin B is highly effective antiparasitic agent, but it is costly and associated with serious adverse effects [303]. Miltefosine was the first oral drug approved against VL, but it is associated with teratogenicity and the efficacy is highly dependent on the *Leishmania* species infecting the patient [14, 282, 304]. Confronted with resistant leishmaniasis, new antileishmanial agents have been developed such as tamoxifen and paramomycin [18, 305].

As previously mentioned, many of available antileishmanial drugs share the same adverse effects of high toxicity, high cost issues, treatment for long duration, and poor patient compliance [297, 306]. Moreover, the efficacy of these agents is impaired by the development of drug resistance and frequent cases of relapse [6, 306]. Drug combinations have been introduced in the treatment strategy and demonstrated positive results with a short-term solution and high efficacy which seems promising [13, 307, 308]. Importantly, treatment of VL in co-infected persons with HIV or AIDS represents an additional challenge. The intention of this review is not to summarize in detail all of the knowledge in this field, but rather to highlight several facts about the current status of available antileishmanial agents and their adverse effects (Table 3).

Pentavalent Antimony Compounds (SbV)

Despite SbV compounds being used since the mid of 1930s for the treatment all forms of leishmaniasis [309], they still remain to be recommended candidates for the treatment of VL and CL in many areas, especially in developing countries [310, 311].

SbV is the inactive form of the drug, which converts in macrophages to the active form (Sb III)[312]. The bioavailability and efficacy of SbV against VL and CL differ according to the available compounds. Two commercial SbV compounds are available: branded meglumine antimoniate (Glucantime, Sanofi-Aventis) contains approximately 8.5% Sbv and mainly used in English-speaking countries, whereas in Latin America and French-speaking parts of Africa and Europe; generic sodium stibogluconate (SSG, Pentostam, GlaxoSmithKline) is largely used and contains approximately 10% Sbv [313, 314]. The generic form was produced in India and found to be safer, cheaper and more effective than the branded version for the treatment of VL [315, 316].

The exact mechanism of action of SbV compounds remains unknown. It was reported that SbV compounds inhibit amastigote glycolytic activity and fatty acid oxidation with influencing macrophage parasiticidal activity [317]. This inhibition is accompanied by a dose-dependent inhibition in net formation of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) [318, 319].

As mentioned above, SbV compunds enter the host cells, cross the phagolysosomal membrane and convert into trivalent antimony (Sb III). Then, Sb III act against amastigotes by compromising the cells thiol redox potential through induction of intracellular thiols efflux, in consequence, inhibit trypanothione reductase (TR) that protects the parasite from oxidant damage and toxic heavy metals, which in turn leads to initiation of apoptosis [319]. Also, this reduction can be non-enzymatic under acidic conditions, such as those found in the phagolysosome (e.g. glutathione (GSH), glycylcysteine and trypanothione), or enzymatic by thiol-dependent reductase (TDR1) or antimonite reductase (ACR2) which increase the sensitivity of *Leishmania* to SbV [320]. SbV can also kill the parasite by indirect mechanisms, such as increasing cytokine levels [321]. Additionally, antimonials may directly affect the molecular processes of the parasite at the DNA level, which in turn induce DNA damage *in vivo* [322] and inhibit DNA topoisomerase I [323].

VL patients seem to tolerate SbV better than CL or MCL patients. SSG is considered the drug of choice for persistent PKDL in Sudan, but requires prolonged hospitalization with daily injections [324, 325]. Likewise, SbV drugs remain the standard for the treatment of CL in America, but this is counteracted by some adverse effects such as toxicity, poor tolerability, high potential recurrence rate (20–45%), and development of occasional resistance [311, 313, 326]. The cure

rate was 60–70% using Sbv compounds with *L. braziliensis* in patients from Brazil and Colombia, while it was higher (95%) in US troops infected with *L. panamensis* in Panama, but the later was associated with some drawbacks [313]. SbV drugs are also safe drugs for the treatment of ML caused by *L. braziliensis* with 75% cure rate for mild cases, however, it was only 10-63% in more advanced cases in Brazil with 30% relapse rate [311, 313, 327, 328].

The potential adverse effects of Sbv compounds were nausea, vomiting, abdominal pain, myalgia, arthralgia, malaise, neutropenia, and thrombocytopenia associated with hypoplasia of the bone marrow [282, 329-331]. Clinically apparent pancreatitis has been recorded in many cases [332, 333]. Reversible elevations of hepatocellular and pancreatic enzymes can also develop [332, 334]. Among others, renal tubular acidosis, nephrotoxicity and cardiotoxicity are considered the most serious issues [335, 336]. SbV compounds also cause dose-dependent electrocardiographic changes, including prolonged Q-T intervals, nonspecific ST-T-wave and T-wave flattening or inversion effects on the electrocardiogram [297, 307, 337]. Serious toxicity and Sudden death have been observed with higher doses, especially with generic SbV preparations in patients who concomitantly possess cardiac disease, renal failure, liver disease, advanced HIV infection or advanced age and pregnant women [282]. The death in such cases is presumably due to cardiac arrhythmias and acute renal failure; therefore, special attention should be given to those patients prior to therapy. The emergence of resistance of resistant strains of *Leishmania* to SbV compounds, in both CL and VL, has been reported in some endemic areas, such as India with efficacy less than 40% [326, 338-341].

Despite these drawbacks, SbV remain efficacious and widely considered to be one of the safest parental drugs when given at appropriate doses in some areas [311]. Several recent attempts have been reported in order to reduce the toxicity and improve delivery of antimonies [301], including liposome- based formulations for VL treatment [342] and cyclodextrin-based formulation for oral delivery [343].

Amphotericin B

It is antifungal macrolide originally extracted from Streptomyces nodosus, a filamentous bacterium, to treat systemic Mycoses [344]. Presently, a high cure rate (90-95%) can be achieved against leishmaniasis using the drug [296, 345, 346]. Two forms of amphotericin are available: conventional amphotericin B deoxycholate and Liposomal amphotericin B [346].

The action of amphotericin against leishmaniasis resulted from formation of complexes with 24-substituted sterols of the biological membrane such as ergosterol, causing alteration of ion balance and cell death [347, 348]. A previous study has revealed that increased calcium permeability is not responsible for the rapid lethal effects of amphotericin on *Leishmania* [349], however, a more recent study concluded that amphotericin produces osmotic cell lysis by the formation of aqueous pores in the membrane of promastigotes, establishing the role of Ca^{2+} in amphotericin-induced apoptosis through osmotic alteration [303, 350].

Since there was a steady increase of resistance to antimonials, amphotericin B deoxycholate was successfully introduced in the 1990s as a second-line drug for MCL and resistant cases of VL, particularly in India with 90-100% cure rate [302, 351, 352]. Although amphotericin deoxycholate is effective and less expensive than liposomal amphotericin B, the former drug is associated with the following side effects: frequent and severe fever, chills and thrombophlebitis, muscle and joint pain, gastro-intestinal cramps, cardiac arrhythmias, liver disorders, peripheral neuropathy and convulsions, anemia and hypokalemia, and occasionally developed resistance or toxicity in many cases, and hence administration of amphotericin deoxycholate requires careful and slow i.v. injection, and should be avoided in elderly patients or pregnant women [18, 353-355]. Additionally, renal impairment represents one of the major side effects of amphotericin B. Accordingly, careful monitoring of renal function and hospitalization during the administration (4–5 weeks) seems mandatory, but increases the cost of therapy. The previous adverse effects of amphotericin deoxycholate discourage the use of the drug in the treatment of VL or CL [356].

Lipid formulations of amphotericin B include: liposomal amphotericin B (AmBisome), amphotericin B lipid complex, and amphotericin B cholesteryl sulphate, have been developed to improve the pharmacokinetic characters of the drug, the tolerability in the patient, and to minimize the side effects [344, 356-358]. Liposomes are artificial lipid particles which are rapidly assimilated then target the mononuclear phagocyte system where *L.donovani* amastigotes reside and develop [311, 359]. Importantly, the smaller liposomes stay in the blood stream longer than the free drug, which improve the drug bioavailability within shorter courses of therapy [358]. AmBisome has been approved as therapeutic agent for VL with low toxicity and high cure rate (90-95%) [360-362]. Accordingly, LAmB (Ambisome, Gilead Sciences, USA) is considered the only antileishmanial drug approved in 1997 by the Food and Drug Administration (FDA) for treatment of MCL, VL and PKDL in all regions [359, 363-365].

It is also considered the first-line treatment against MCL, VL and PKDL in most non-endemic countries in all ages, but the drug response was mixed with scares studies in CL [359, 363-365]. Interestingly, liposomal Amphotericin was 100% effective in Leishmania / HIV co-infection cases [366]. The lack of stability at high temperature and high cost, represent the main limitations of amphotericin forms which limit their practical value in less developed countries [367, 368]. In fact, the high cost of liposomal amphotericin B has been reduced for use in endemic countries, but more drastic reduction is still needed [369]. According to a recent study, a much cheaper and alternative therapy has been developed using conventional amphotericin B diluted in a commercially available lipid emulsion and has achieved high efficacy against VL in both immunocompetent and immunocompromised patients [370]. The other lipid-associated amphotericin B preparations also appear to be effective, but have not been sufficiently studied [356, 371].

Paromomycin (Monomycin or Aminosidine)

Paromomycin is a parenteral antibacterial aminoglycoside, designed against cryptosporidiosis and amoebiasis [372]. Among other properties, paromomycin showed good efficacy against many infectious diseases; particularly protozoa like *Leishmania* [373, 374]. It is also considered the second choice for the treatment of leishmanisis alone or in association with SbV with variable results, presumably due to the species of *Leishmania* involved, the geographical location, and the drug combination [375-377]. Paromomycin was introduced in the 1960s as an ointment (15% paromomycin) for the treatment of CL and showed 87% cure rate [378]. It was also very effective ointment for the treatment of CL in Peru, especially the uncomplicated cases with 90-95% cure rate, however, in Brazil and by parenteral administration of paromomycin sulfate, the cure rate was low (48 - 67 %) [142, 379, 380]. In fact, topical paromomycin was effective against CL caused by *L. major* and *L. mexicana* because these species do not tend to cause visceral or mucocutaneous disease [355]. On the other hand, the efficacy of the drug against *L. tropica* infection cases has been disappointing; skin rashes and local pruritus have also been encountered; therefore, topical therapy is not recommended for the treatment of New World species [355, 381].

Paromomycin was introduced in 1990s for the treatment of VL with high efficacy to treat Sbv-resistant VL in India through an IV administration [382, 383] and it is poorly absorbed into

systemic circulation after oral administration than intramuscular injection [384]. The patients treated with paromomycin in phase II in India achieved response rates of 77–97% *versus* 66 % for antimonials [375]. In a phase III trial in Bihar (India), the final cure rate was 94.6% against VL, simulating that of amphotericin B deoxycholate, while it was 94.2 % in phase IV in the same region [373, 385]. The therapeutic efficacy of paromomycin in Africa varied substantially between geographical areas, where the cure rate was 14.3–46.7 %, 80 %, and 75–96.6 % in Sudan, Kenya, and Ethiopia, respectively [386]. There are no reliable data on the efficacy of paromomycin for the Mediterranean and Latin America [387].

The mechanism of action of paromomycin in *Leishmania* is not yet precisely understood, however, it has been reported to bind to the 30S ribosomal subunit, and therefore interfers with initiation of protein synthesis by fixing the 30S-50S ribosomal complex at the start codon of mRNA, in turn leads to accumulation of abnormal initiation complex [377, 388, 389]. Other mechanisms were suggested such as alterations in membrane fluidity and lipid metabolism following low Mg^{2+} concentration, and impairment the mitochondrial membrane potential, which in turn leads to dysfunction in the respiratory systems [390, 391].

High efficacy (94.6%), excellent tolerability, and the very cheap price (US$ 10–20) are the major advantages of paromomycin [373, 392]. On the other hand, it showed a lower incidence of side effects, including reversible high-tone ototoxicity and nephrotoxicity, vestibular instability, increase in hepatic transaminases and long duration of injection [373, 393]. Paromomycin resistant isolates would probably be developed clinically [394], following a 60 day parenteral course for treatment of two *L. aethiopica* cases, isolates taken from relapsing patients were three to five fold less sensitive to paromomycin after treatment than the isolates taken before treatment in an amastigote-macrophage assay [394, 395].

Pentamidine

Pentamidine, an antimicrobial drug, has good clinical activity in treating leishmaniasis since 1980s and mostly used as second-line therapeutic option in antimony-resistant VL treatment in India and Sudan with a high cure rate (93%) [48, 396, 397]. By 1990, the cure rate has been drastically reduced to 70-80% in certain areas in India [318, 351, 398]. With respect to CL and MCL, pentamidine is the drug of choice for treatment *L. viannia guyanensis* in French Guiana, *L. panamensis* in Colombia, and *L. braziliensis* in Brazil, and achieved response rates of 90-93%,

therefore, the Pan-American Health Organization (PAHO) recommended pentamidine as a second-line drug against *leishmania* [399-401]. However, a low cure rate was reported (35%) in patients infected with *L. braziliensis* in Peru [402, 403].

The mechanism of action of pentamidine is unknown, however, there are some evidences about the involvement of the drug on mitochondrial function or through selective modification of ubiquitin [404, 405]. Pentamidine acts on parasite genome by hampering replication and transcription at the mitochondrial level. Polyamines are substituted at nuclei acid binding sites, which preferentially bind to kinetoplast DNA [296]. Likewise, pentamidine can be taken up by purine receptors as in *Trypansoma brucei*, hence, the parasite is unable to synthesize their own adenine, in turn triggers parasite death by inhibiting the enzymes and interacting with DNA [406].

The drug can be given as an intramuscular injection or intravenous with a 5-15 and 54 minutes half-life, respectively [355, 384]. However, pentamidine is widely known in combination therapy [326], some serious adverse effects have been reported in the treatment of VL patients, including fall in blood pressure with intravenous injection, low efficacy resulted from development of drug resistance, toxicity hazards due to vascular collapse, pancreatitis, and reversible renal dysfunction [387, 407]. The most serious drawback is life-threatening hypoglycemia that may be followed by persistent diabetes mellitus or even the diabetic cases getting worse [297, 318, 407]. Such adverse effects combined with drug resistance and few data about pentamidine efficacy in the Mediterranean area and Latin America might potentiate the complete desertion of the drug in some countries [387, 408].

Miltefosine (Hexadecylphosphocholine)

Miltefosine is an alkylphosphocholin, was originally developed as antineoplastic drug then implicated as the first orally effective antileishmanial agent [302, 409-412]. Miltefosine yielded cure rate of 94–97% for the treatment of Kala-azar in India [413]. It is well-tolerated for the treatment of all forms of leishmaniasis [298, 413, 414], therfore, it is considered ‘’the first line’’ treatment of VL in India, Nepal and Bangladesh among all ages, especially children’s [415, 416].

Phase 2 and 3 drug studies showed high efficacy (93-100%) in patients infected with VL in India [417-419]. On the other hand, the final cure rate was only 82-85% in phase 4 trial, however, it is still imperative to prevent the emergence of resistance [420, 421].

Although the exact mechanism of drug activity against the parasite is not fully understood, some studies have reported the direct involvement of a putative miltefosine transporter (LdMT), B-subunit LdRos3 and P-type ATPase in miltefosine and phospholipids translocation in *Leishmania* [422]. On the basis of the other published scientific researches, impairment of lipid metabolism and alteration of the plasma membrane permeability could be involved, resulting in fast drug metabolism and efflux of the drug, in turn trigger parasite apoptosis [422, 423]. Miltefosine also stimulates IFN-gamma-dominated antileishmanial immune response via production of inducible nitric oxide synthetase 2 (iNOS2) of the host cell, and therefore catalyzes the generation of nitric oxide (NO) which kills the parasite within the macrophage [424]. It has been hypothesized that miltefosine causes programmed cell death in *Leishmania* via mitochondrial dependent pathway through its interaction with membrane constituents, affecting cell signaling pathways that cause modulation of cell surface receptors, inositol metabolism, inhibition of protein kinase C and other mitogenic pathways, and consequently trigger the apoptotic pathway [423, 425, 426].

Miltefosine is effective against CL and seems promising for the treatment of ML in Bolivian patients [409, 427]. High doses of miltefosine reported 91-94% cure rate in CL caused by *L. panamensis* in Colombian patients and 82% for *L. braziliensis* in Bolivian patients, respectively, while the efficacy was only 53% in curing of infections caused by *L. viannia braziliensis* in Guatemala [409, 411, 428]. The initial cure rate was 96% with PKDL [413, 429].

Miltefosine toxicity is not very common, however, it is associated with teratogenicity and gastrointestinal side-effects [297, 392]. Skin allergy, elevated hepatic transaminase concentrations, high blood urea nitrogen and creatinine have been also observed but these effects decrease with continuation of the drug. The relatively high cost of miltefosine and a long half-life (approximately 152 hours), represent additional challenges [416, 419, 430-432].

Miltefosine administered biweekly did not prevent visceral relapse in patients co-infected with HIV, however, it remains effective when retreatment is needed but with lower initial response (64%) [393]. For this reason, miltefosine should not be used alone either in the treatment of patients co-infected with VL and HIV or in pregnant women or who could become pregnant within 3 months after treatment [282, 420, 433]. The geographic location, causative species, immune status, and age, seem to be mandatory factors in the response to miltefosine treatments [428, 431, 432].

Despite these limitations, miltefosine is now recommended in combination with AmBisome to prevent the drug resistance [410]. Further studies are mandatory to elucidate the cure rate for miltefosine, especially in Latin America and Mediterranean region due to lack of reliable data [434].

Azole Drugs

Metronidazole and other imidazole-derived antifungal medications, including Ketoconazole, miconazole, cotrimazole, posaconazole, itraconazole, fluconazole and terbinafine, have been studied as antileishmanial agents [282, 435-438]. They have been reported to be effective in the treatment of some VL cases in India, either alone or in association with another antileishmanial agent [302, 439-441]. A combination of allopurinol and ketoconazole reported good results in renal transplant patients with rare cases of nephrotoxicity [442]. Both ketoconazole and itraconazole have also shown some effectiveness with CL and MCL, but yielded variable cure rate (23-80%), depending on the species and the region [443-447].

In this regard, oral ketoconazole and Fluconazole have been used against *L. major* and *L. mexicana* in Old World to hasten CL healing and acheived curing rate of 79-89% in uncomplicated cases [287, 435]. Itraconazole has been used in Old and New World, but the studies consistently demonstrated a gradient of reduced efficacy [448]. Although, ketoconazole was more effective than antimony in treatment of localized CL caused by *L. mexicana* (89% cure rate), it was less effective in *L. viannia braziliensis* (30% cure rate) infections [446, 449, 450]. Simulating to other azole antifungals, azole drugs, exert their effects through inhibition of some enzymes like cytochrome P450 14-alpha-demethylase (P45014DM) and 14 alpha-demethylase, a key enzymes in the synthesis of ergosterol [451-454].

The major drawbacks of azole derivatives are low or failure of efficacy against some parasite species such as *L. tropica*, *L. aethiopica*, and *L. braziliensis*, and reports of toxicity represent, hence, the routine blood tests for liver function in the patients on long-term azole therapy seem to be mandatory [296, 311, 324, 439, 455-457]. Less common side effects were observed with ketoconazole such as endocrine dysfunction, reduction in cortisol levels, hyponatremia, and adrenal insufficiency [311, 439, 458]. Itraconazole is better tolerated than ketoconazole, however, it elevates alanine aminotransferase levels, few cases develop congestive heart failure, and of low efficacy in India (66%) [459].

Fluconazole has also been associated with QT interval prolongation, which may lead to serious cardiac arrhythmias or lethal hepatotoxicity, and therefore contraindicated in hypersensitive patients to azole derivatives, pregnant and lactating mothers [460, 461]. Furthermore, the efficacy of treatment with azole derivatives was based on uncontrolled studies with conflicting results [439, 462].

Sitamaquine

Sitamaquine is a potential orally active 8-aminoquinoline analog discovered by the Walter Reed Army Institute, in collaboration with GlaxoSmithKline, for the potential treatment of VL [463, 464]. It was first synthesized as an antimalarial agent in the US [465]. Later on, it is considered the second oral medication against VL, even for compassionate use in HIV/ VL co- infections [307, 408]. On the other hand, topical sitamaquine was not active for the treatment of experimental CL caused by *L. major* in BALB/c mice [466]. This 8-aminoquinolone was first introduced in March 2002 [467] and was shown to be a promising candidate for the treatment of VL with 27-87% cure rate in preliminary clinical studies (Phase II trials) in Africa, Brazil, and India [463, 468-470].

The antileishmanial action of sitmaquine metabolites is not fully understood, however, the intracellular targets of the drug are mitochondria and acidocalcisomes [471-473]. It has been suggested that the alteration of *Leishmania* morphology [474, 475], or the greater affinity of the drug for negative phospholipids are the possible mechanisms through which sitmaquine act [475]. Sitamaquine also interacts with anionic phospholipids, then with phospholipid acyl chains to insert within biological membranes and accumulates in *Leishmania* cytosol via sterol-independent process [476]. Indeed, sitamaquine efflux is related to energy-dependent mechanism [475]. It was noticed that high amount of protein in the medium could potentiate the activity of sitamaquine on *L. donovani* promastigotes, possibly suggesting that interaction occurs between proteins and the antileishmanial action of sitamaquine [475-477]. A more recent study showed that sitamaquine targets succinate dehydrogenase, resulting in oxidative stress and apoptosis in *L. donovani* treatment of CL and visceral experimental models of leishmaniasis [305, 478, 479]. Tamoxifen exert its effects against the parasite through induction of a rapid colonization of parasitophorous vacuoles harboring *Leishmania* [480].

On the other hand, the use of tamoxifen in the treatment of leishmaniasis has encountered the following risks: impairment of bone development in children or childbearing age women, and associated with endometrial cancer in women [481, 482].

Imiquimod, a topical imidazole quinolone, is a novel immune response modifier that results in an induction of macrophage activation with the production of pro-inflammatory cytokines, and therefore potentiate the beneficial effect of the drug in combination with pentavalent antimony in relapsed CL caused by *L. chagasi and L. tropica* [483, 484]. Imiquimod may be effective in antimony-refractory cases but sever irritation at the site of application could be resulted [484].

promastigotes [485].

Sitamaquine administration route is generally well tolerated by VL patients. Besides its pharmacokinetics data in humans, sitamaquine showed short elimination half-life (26 hour) than miltefosine (150-200 hour) [486], that weigh in favor of improving the bioavailability and a low probability of resistance emergence.

Some adverse effects have been pointed out during the selection of a sitamaquine-resistant clone of *L. donovani* in laboratory, with low efficacy after treatment in phase II clinical [463]. The resistance to sitamaquine was also reported *in vitro* [487]. Other effects such as abdominal pains, cyanosis, a recognized glucose-6- phosphate deshydrogenase (G6PD) deficiency, and renal adverse effects, were reported [463, 466, 473]. Therfore, sitamaquine alone remains insufficient for the treatment.

Allopurinol

Allopurinol is a purine analog; It was first synthesized as antineoplastic drug [488]. It is widely used today for the treatment of CL, MCL, and PKDL [489-491]. The drug also showed activity against canine Leishmaniasis (CanaL) with high efficacy for maintaining clinical remission with long-term intermittent administration [492]. It was shown to be effective against VL, especially among transplanted organ patients either alone or in combination with other drugs such as fluconazole [493-495].

The suggested mechanism of action of allopurinol is through purine salvage pathway as alternative substrate for hypoxanthine guanine phosphoribosyl transferase (HGPRTase), well-known xanthine oxidase inhibitor, which results in incorporation of allopurinol riboside into RNA, then inhibit protein synthesis in the parasite [451].

Though, allopurinol was reported to be inexpensive and orally effective against CL, the drug did not show activity in placebo-controlled double-blinded trial in colombian patients with CL caused by *L. panamensis* or *L. braziliensis*, even when combined with probenecid [311, 496-498]. Allopurinol was reported to be associated with a higher incidence of relapse (56%) and lower efficacy *versus* pentavalent antimony in treatment of VL in HIV-infected patients [297, 499]. The experience with allopurinol in treatment of VL is out of date due to dissimilar results and the majority of the studies based upon combination therapy of allopurinol with other drugs [489, 500].

Other Alternative Drugs:

A number of other drugs, include some secondary metabolites from plants, are under clinical evaluation or reported to have antileishmanial activity based upon relatively small studies either *in vitro* and/or *in vivo* [302].

Tamoxifen is a nonsteroidal triphenylethylene derivative and exhibits *in vitro* antileishmanial activity against several *Leishmania* species [478, 501]. It was effective in the treatment of CL and visceral experimental models of leishmaniasis [305, 478, 479]. Tamoxifen exert its effects against the parasite through induction of a rapid colonization of parasitophorous vacuoles harboring *Leishmania* [480]. On the other hand, the use of tamoxifen in the treatment of leishmaniasis has encountered the following risks: impairment of bone development in children or childbearing age women, and associated with endometrial cancer in women [481, 482].

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Azithromycin has shown *in vitro* and *in vivo* activities against CL and MCL [502, 503], but failure of treatment in Brazil and a very low cure rate (45.5 %) in treatment of CL caused by *L. braziliensis* in Argentina was also recorded [498, 504].

Melatonin, in a recent work, we have shown for the first time that exogenous melatonin, neurohormone, could lower the number of viable promastigotes of *L. infantum*, accompanied by impairment of some mitochondrial parameters which target the parasite viability [505]. Further

research is warranted to elucidate the mechanisms and adverse effects of these alternative drugs, either alone or in association with other antileishmanial drugs, in an attempt to develop a novel chemotherapeutic strategy.

Combination Therapy

As previously mentioned, the available drugs being alone (monotherapy) for the treatment of leishmaniasis share some drawbacks such as field administration difficulties, toxicity and resistance issues. Hence, combination therapy appears to be more effective and promising alternative strategy which has recently been implemented in many endemic areas [308, 506]. In this form of treatment, two or more drugs are combined together to give a better efficacy and decrease the emergence of resistance. Several studies have included the major advantages of antileishmanial drug combination *versus* monotherapy, especially lower cost, shorter treatment period, the lower overall dose of drugs which reduce the toxicities and improve compliance [308, 408, 507]. The combination strategy should be applied with care to avoid development of resistance and consequently loss of efficacy [282, 308].

Several drug combinations were examined with encouraging results. Currently, the most successful combination is sodium stibogluconate (SSG) plus paromomycin for 17 days which achieved higher cure rate of VL than antimonials alone [508]. The efficacy of this combination was 93% in India and East Africa, while there were no differences in the efficacy of the combination treatment *versus* monotherapy in Ethiopia, Kenya, and Sudan, presumably due to regional variation [509-511]. A combination of antimonials plus allopurinol, ketoconazole, fluconazole, or itrakonazole has been used, but with insufficient evidences to make a recommendation [512]. The cure rate was 98–99% in India using three separate combinations: liposomal amphotericin B (LAmB) plus miltefosine (oral) or liposomal amphotericin B plus paromomycin (intramuscular) or co-administration of miltefosine plus paromomycin (intramuscular); this strategy was cheap, safe, and effective within a short duration (7- 10 days) to treat antimony-resistant VL infection [307, 369, 410, 513-515].

Interestingly, several studies have emphasized the benefits of a combination of low-dose or short course of effective antileishmanial drugs with immunomodulator products as a successful approach for effective treatment of leishmaniasis [516-518]. The main purpose of the immunomodulator is to enhance the immune response through activation of macrophages and

enhancement of NO production to eliminate the infection [326, 519-521]. In this regard, several clinical trials using rIFN-γ with Sbv were useful in treating severe or Sb refractory VL in Brazil [522, 523]. Likewise, low-dose or short course of amphotericin B with Tuftsin can induce the effector immune response by stimulating white blood cells, in addition to a wide spectrum of biological activities like enhancement of phagocytosis [524, 525]. A combination of miltefosine with tuftsin or picroliv was also found to augment the efficacy of miltefosine against experimental VL than miltefosine alone [516, 526].

A combination of allopurinol, Imiquimod, itraconazole or oral pentoxifylline plus meglumine antimoniate may be a highly effective regime (86% cure rate) against CL, especially in its chronic form [294, 447, 527, 528]. Though, a combination of intravenous stibogluconate and allopurinol showed higher efficacy against CL caused by *L. viannia panamensis* compared to stibogluconate alone, this effect was not recorded in the treatment of MCL [446]. Taken into consideration, combination strategy is limited only to the approved drugs for short-term which complicates understanding the mechanisms underlying the effects and may couple with risk; nevertheless, this regime offers major advantages over monotherapy and remains a priority.

Synergistic role of immune system in the therapeutic efficacy of some antileishmanial drugs

Understanding the immune pathways of leishmaniasis is critical to identify novel drug targets and therapeutic strategies that may exhibit their effects through the immunological cross-wires of pathogenic cross-talk [299]. The cell-mediated immunity is essential for parasite clearance in many cases of leishmanial infections, which represented by effective mechanisms of macrophage to decimate intracellular pathogens (amastigotes) via generation of toxic nitrogen and oxygen metabolites like NO and reactive oxygen species (ROS) [529]. It seems that immune system plays an important synergistic role in the therapeutic efficacy of many of antileishmanial drugs [517, 530, 531]. This synergetic interaction is classically associated with a depression of Th1 cells and preferential expansion of Th2 cells, by skewing T cells toward Th1 responses [94, 299, 532-534].

The process of parasite clearance is suppressed by the infection itself, which down-regulates the requisite of pro-inflammatory signaling pathways between macrophage and T cell such as the interleukin (IL) 12, major histocompatibility complex (MHC) presentation, rIFN-γ, and persistent tumor necrosis factor-alpha (TNF-a), in whole or in part, explain the beneficial role of

Th1 inflammation to the host [535-538]. On the other hand, Th2 response has controversial involvement in disease susceptibility, mainly conducted by the major cytokines such as IL-4, IL-17, and IL-22 [532, 539, 540]. These cytokines have an important regulatory function during *Leishmania* infection though their action on neutrophils [532, 539, 540]. It is therefore not surprising that T cell compartment is necessary for the action of some antileishmanial agents [424, 517, 541].

In fact, the most commonly used drugs are known to have direct or indirect immunomodulatory effects [517, 541, 542]. In this regard, the efficacy of antimonials is contingent on host T-cell function and underlying immune bias [543, 544]. Sodium antimony gluconate (SAG) can activate both innate and adaptive immune compartments through its role in reticuloendothelial system [545]. This action was confirmed by the loss of effectiveness of antimonial therapy in mice without a functional T-cell response or without key Th1 effector cytokines such as rIFN-γ [546]. Stibogluconate also inhibits host cell tyrosine phosphatases, which in turn leads to an increase in secretion of cytokines [321]. Moreover, SAG has been reported to induce both ROS and NO production in murine macrophages and promote two waves of killing of *L. donovani* amastigotes [547].

Several previous reports have shown *in vitro* and *in vivo* immunomodulation by miltefosine in *Leishmania*-infected animals [424, 541, 548]. Indeed, miltefosine did not induce a humoral response, natural killer (NK) cell activation or cytotoxic spleen cells in host cells, but induced immunological and inflammatory effects on isolated mononuclear cells and macrophages [548-550]. Miltefosine also activates intracellular signaling pathways through the production of cytokines, NO, TNF-a, and promotes IFN-gamma-dominated anti-leishmanial immune response, which could mediate the immunomodulation of the host cell and kill the parasite within the macrophage [548, 551]. The combination of paromomycin and miltefosine also targets Toll-like receptors (TLR4) in the host defense, which in turn induce Th-1-biased immunomodulation for parasite killing in VL [552, 553]. Recently, Ghosh and co-authors have studied the host cell-dependent mechanism of four antileishmanial drugs (SAG, miltefosine, amphotericin B and paromomycin) for killing of intracellular parasites and their cytotoxic effect on macrophages [554]. Indeed, any alteration on macrophage membrane fluidity has a bearing effect on T cell-stimulation ability [555].

Ghosh and co-authors have observed that *in vitro* and *in vivo* treatment of macrophages with certain doses of miltefosine, SAG or paromomycin enhanced T cell-stimulating ability, which was evidenced by a significantly high IL-2 level and generation of ROS, nitrite, interleukin-12 and TNF-α *versus* the untreated macrophages [554]. In contrast, amphotericinB-macrophages failed to show any appreciable increase in IL-2 production from a T cell hybridoma [554]. It is therefore hypothesized that most antileishmanial drugs target the immune system directly or indirectly.

Leishmania–HIV Co-Infection

The evidence for treatment of CL and MCL/ HIV co-infection cases is still very limited, however, some cases of ML have successfully been treated with local treatment, while other cases may argue in favor of systemic treatment due to the risk of subsequent visceralization [556].

The treatment of VL/ HIV co-infection cases is similar to that of the VL patients without HIV infection with some precautions due to low CD4 counts (90-100 cells/mm^3), combined with high frequency of leukopenia, lymphocytopenia, and thrombocytopenia [557]. These co-infection cases are usually associated with low cure rate, high relapse rate, and treatment-associated mortality, particularly with antimonials [142, 315, 558-560].

WHO recommended liposomal amphotericin B as a preferred therapy [282, 359, 365]. In fact, the validity and dose of the amphotericin may differ, according to the geographical zone of infection. The majority of studies were undertaken to date in Europe where amphotericin B is considered a valid resource for the treatment of VL-HIV co-infection, unlike the developing countries due to high cost of the drug [282, 561].

The use of antimonials compounds in the treatment of co-infection cases yielded unsteady results, depending on the region. In this concern, meglumine antimoniate reported lower cure rate (58%) in VL/ HIV patients from Ethiopia and having many side effects, including pancreatitis and drug resistance, then the efficacy lowered to 35% in Bihar (India) [562, 563]. Relatively high rates of fatal toxicity were also recorded with antimonials and long duration of treatment (30–60 days) which requires close monitoring, hence these compounds are not tolerated and not always successful [558, 564, 565]. The response to antimonial compounds in Europe ranged from 33-82%, with frequent cases of relapse [560, 566, 567].

Most studies recommended liposomal amphotericin B, because of its safety profile and well-tolerated by the patients [359, 568]. The relapse rate (1-year) was reduced to 50% in the patients who received prophylactic amphotericin B lipid complex, while the relapse rate was 78% in patients without prophylaxis [569].

The second-line treatment options for VL/ HIV-coinfected patients include miltefosine and paromomycin. Miltefosine had an acceptable safety profile, and the drug-related toxicities were manageable with moderate efficacy, however, this efficacy was lower than SSG with percentage of mortalities in east Africa [564, 570-572]. Moreover, miltefosine-treated patients were proposed to relapse the infection with stoppage of treatment [573].

It should be emphasized that effective highly active antiretroviral treatment (HAART) may be beneficial to VL/ HIV-coinfected patients, as it was proved to reduce the development of the disease and increase the survival of the patients, but of partial effect on relapse [142, 381, 574]. The development of PKDL was also reported in HIV/AIDs infected cases undergoing HAART as a manifestation of immune reconstitution syndrome [566, 572].

The previously mentioned shortcomings support the necessity of multidrug combinational therapy over the prolonged period of treatment in immunosuppressed patients infected with *Leishmania* [575]. There is no data about drug combination in VL/HIV Co-infected patients and the available data originated from a reports of Ethiopian patients treated with drug combination such as miltefosine-SSG combinations [308, 558].

Another combination of Sbv compounds with allopurinol or azole derivatives or rIFN-γ have used, but with insufficient evidences to make a recommendation [512]. Novel combination regimens of intravenous pentamidine and oral fluconazole were also reported to be effective against VL/HIV co-infected patients, but with several cases of relapses after failure of treatment using the other antileishmanial agents [576].

Drug	Formulation and route of administration	Regimen of administration	Cure rate (%)	Potential adverse effects	Comments	References
Pentavalent antimony (SbV)	Sodium stibogluconate (i.v. or i.m.) Meglumine antimonite (i.v. or i.m.)	For CL: 20 mg Sb/kg daily for 20 days. For MCL and VL: 20 mg Sb/kg daily for 28–30 days. For east African PKDL: 20 mg Sb/kg daily for 60 days.	10–95%, according to the region, species infected, and form of the infection.	Nausea, abdominal pain, myalgia, arthralgia, pancreatitis, renal tubular acidosis, nephrotoxicity and cardiotoxicity.	Emergence of Clinical resistance of *Leishmania* could be noted. Shouldn't be used in patients with cardiac disease, renal failure, liver disease, advanced HIV infection, and pregnant women.	[149, 311, 313, 326-333, 336, 338-341]
Amphotericin B	Amphotericin B deoxycholate (i.v.) Liposomal amphotericin (i.v.)	For VL: 0.5–1 mg/kg/day daily or every other day for up to 8 weeks. For PKDL: the same dose of VL daily or on alternate days for up to 4 months. For CL and MCL: the same VL regime but continues for 20–30 days in CL and for MCL is up to 45 days. For VL in immunocopetent patients: 3.0 mg/kg body weight on days 1 through 5, 14, and 21. For VL in immunocompromised patients: 4.0 mg/kg body weight on days 1 through 5, 10, 17, 24, 31, and 38. CL and MCL: 3 mg/kg/day up to ten doses, given within a 21-day period.	90-100% for CL, VL, and MCL. 90-100% for VL while 84% in CL.	Renal impairment, thrombophlebitis, gastrointestinal cramps, cardiac arrhythmias, liver disorders, peripheral neuropathy, and convulsions. Highly toxic, development of resistance, anaphylactic reactions. Very little experience in Eastern Africa. High cost.	Not indicated in elder patients or pregnant women. Contraindicated in patients who develop hypersensitivity reactions. Further studies concerning its effect against CL and MCL are mandatory.	[18, 302, 351-359] [350, 358-362, 364-371]
Paromomycin	Paromomycin sulfate (i.v. /i.m. or oral)	For VL: 15–20mg/kg/day for 21 days. For CL: topically in a 15% ointment together with 12% methyl benzothonium chloride daily for 20 days. For CL (Parenterally): IM at a dose of 12–18 mg/kg/day for 14 day. For MCL: 16 mg/kg/day for 20 days.	In VL: 77-94% In CL: 90-95% cure rate for topical application and 47-90% for parenteral administration. MCL: 0-67%.	Ototoxicity, vestibular instability and nephrotoxicity. Mild pain at the injection site, and long duration of injection.	Drug resistance could develop especially with CL and Inadequate data regarding its use in pregnancy. Therapy is not recommended for treatment for New World species to avoid the progress to mucocutaneous disease. No reliable data concerning its use in the Mediterranean area	[350, 355, 370, 373, 375-378, 385, 387, 394, 395, 577]

					and Latin America.	
Drug	**Formulation and route of administration**	**Regimen of administration**	**Cure rate (%)**	**Potential adverse effects**	**Comments**	**References**
Pentamidine	Pentamidine isethionate Pentam – 300 (i.m. or i.v.)	For VL/MCL: 4 mg/kg three times weekly for 15–30 week. For CL: 3–4 mg/kg/day every other day, for a total of 4–10 injections.	In VL: 93 % but in recent studies in India, it was 70-80%. In CL and MCL: 90-93%.	Life-threatening hypoglycemia, persistent diabetes mellitus. Development of drug resistance, lower efficacy, toxicity hazards, pancreatitis, and reversible renal dysfunction. Liver enzyme abnormalities, bone marrow effects, nephrotoxicity and cardiotoxicity.	Little literature about use of pentamidine in the Mediterranean area and Latin America against VL. First-line CL option in Pregnancy. A low cure rate was reported (35%) in patients infected with *L. braziliensis*. Patients on this drug require careful observation.	[48, 297, 318, 387, 396-398, 403, 407]
Miltefosine	Hexadecylphosphocholine (Impavido, Zentaris) per oral	For VL: up to 150 mg for adult for 28 days. For CL: 2.5 mg/kg/day 28 days. For MCL: doses of 2.5–3.3 mg/kg/day for 28 days. For PKDL: 2.5 mg/kg/day for 12 weeks.	VL: 93-100% while in phase IV and Ethiopia was 85% and 64%, respectively. CL: 53-100%. MCL: 58-83%. PKDL: 96%.	Many cases of relapse, drug resistance development, and teratogenic effect.	Should not be used in pregnant women or who could become pregnant within 3 months after treatment. Should not be used alone in treatment of patients co-infected with VL and HIV. Recommended in childhood against VL and considered the first line of treatment of VL in India.	[297, 392, 393, 411-413, 428-434, 534]

Drug	Formulation and route of administration	Regimen of administration	Cure rate (%)	Potential adverse effects	Comments	References
Azole Derivatives	Ketoconazole, fluconazole, and itraconazole (per oral) Miconazole or clotrimazole (topical application)	For CL: Ketoconazole: 600 mg/day for 4-6 weeks for the adult while for children's, the dose is 200mg/day for 28-30 day. Fluconazole: 200- 400 mg/day for 6 weeks. Itraconazole 200 mg bid for 28 days. 2 % miconazole or 1 % clotrimazole cream applied on the lesion twice a day for 30 days. For MCL: doses of 4 mg/Kg/day from oral drugs for 6-24 weeks. For VL: itraconazole 600 mg/day orally for up to 24 months.	Ketoconazole and Fluconazole yielded 79 - 89% cure rate against *L. major* and *L. mexicana* in Old World. CL and MCL: 23-80%. Higher efficacy against VL without any relapses.	Failures of response, ineffective against some species of the parasite such as *L. tropica*, *L. aethiopica*, and *L. braziliensis* or toxic. Reduction in cortisol levels, adrenal insufficiency, and hypoadrenalism. Itraconazole elevates alanine aminotransferase levels; some cases develop congestive heart failure and hepatotoxicity.	A variable or low efficacy of Itrakonazole and ketoconazole Against *L. viannia braziliensis* (30%). Require routine blood tests for liver function. Fluconazole is contra-indicated in pregnant and lactating mothers. Few data about the efficacy of azole derivatives against VL.	[287, 302, 311, 324, 439-450, 455, 456, 458, 461]

Table 3: The available antileishmanial agents combined with regimen of administration, cure rate, and potential adverse effects [578].

4. Potential relevance of melatonin against some infectious agents: A Review and Assessment of Recent Research

Adapted from a previous review article published by the same author as a part from his thesis: *Ehab Kotb Elmahallawy, Javier Ortega Luque, Abdelkarim saleh Aloweidi, et al. Curr Med Chem. 2015 Aug 26.*

Contents

1. Melatonin synthesis, mammalian subtypes, precursors and their role in cell biology.
2. Physiological and therapeutic effects of melatonin
2.1 Effects of melatonin on immune system
2.2 Antioxidant action of melatonin
2.3 Melatonin in bacterial and viral infection
2.4 Melatonin and parasites
2.4.1 Melatonin and Apicomplexa
2.4.1.1 Melatonin and malaria
2.4.1.2 Melatonin and toxoplasmosis
2.4.2 Melatonin and trypanosomasis
2.4.3 Melatonin and schistosomiasis
2.4.4 Melatonin and amoebiasis
2.4.5 Melatonin and Leishmaniasis

1. Melatonin synthesis, mammalian subtypes, precursors and their role in cell biology

Melatonin, N-acetyl-5-methoxytryptamine, is an indoleamine released by the pineal gland with peak concentrations at night and is thought to participate in regulation of circadian rhythms in many eukaryotes, including vertebrates, invertebrates, higher plants and dinoflagellates [579, 580]. Taken into account, the secretion of this natural hormone is not confined exclusively to the pineal gland, but other peripheral organs and tissues including retina, gastrointestinal tract, Harderian gland, skin, leukocytes, thymus and bone marrow cells also produce melatonin but not extrapineal melatonin retains the chronobiotic properties [581, 582].

Melatonin is synthesized from tryptophan and converted into serotonin in the circulatory system [583]. Serotonin is transformed into N-acetylserotonin via arylalkylamine-N-acetyl transferase enzyme which is then metabolized into melatonin by hydroxyindole-O-methyltransferase

enzyme [583]. Melatonin is released immediately into the blood capillaries and rapidly distributed throughout the body tissues with high affinity in the cerebrospinal fluid [584], due to its amphiphilic nature which enables it to cross all biologic barriers and gets free access to all cellular compartments, especially nucleus and mitochondria [585].

To our knowledge, there are two mammalian subtypes of G protein-coupled receptor (GPCR) binds to melatonin receptors; MT1 (Mel1a) and MT2 (Mel1b), which mediate most of the regulatory functions of melatonin [586-588]. Though these receptors mainly expressed in central nervous system (CNS), they also present in peripheral organs [589]. Taken into account, both MT1 (Mel1a) and MT2 receptors are of similar binding properties, however, human MT2 receptor has shown a lower affinity to melatonin *versus* human MT1 receptor [590]. The previously mentioned receptors (MT1 and MT2) seem to be extremely important in regulation of cell cycle in some infectious agents like parasites [591, 592]. Also, melatonin binds to other cellular targets such as calmodulin (CaM), calreticulin, quinone reductase 2 (MT3 binding site), and tubulin, explaining that some effects of melatonin are independent of the activation of membrane-bound receptors [593, 594].

Besides its great role in circadian rhythm, melatonin been implicated in a wide array on the plethora of processes of cell biology and physiological functions in many infectious agents [595-602]. Importantly, the underlying mechanisms of these effects are various and may involve intracellular antioxidant enzymes, receptor-mediated and receptor-independent actions [603]. The following explanation will discuss some physiological and therapeutic implications of melatonin and their potential relevance against some infectious agents (Figure: 2).

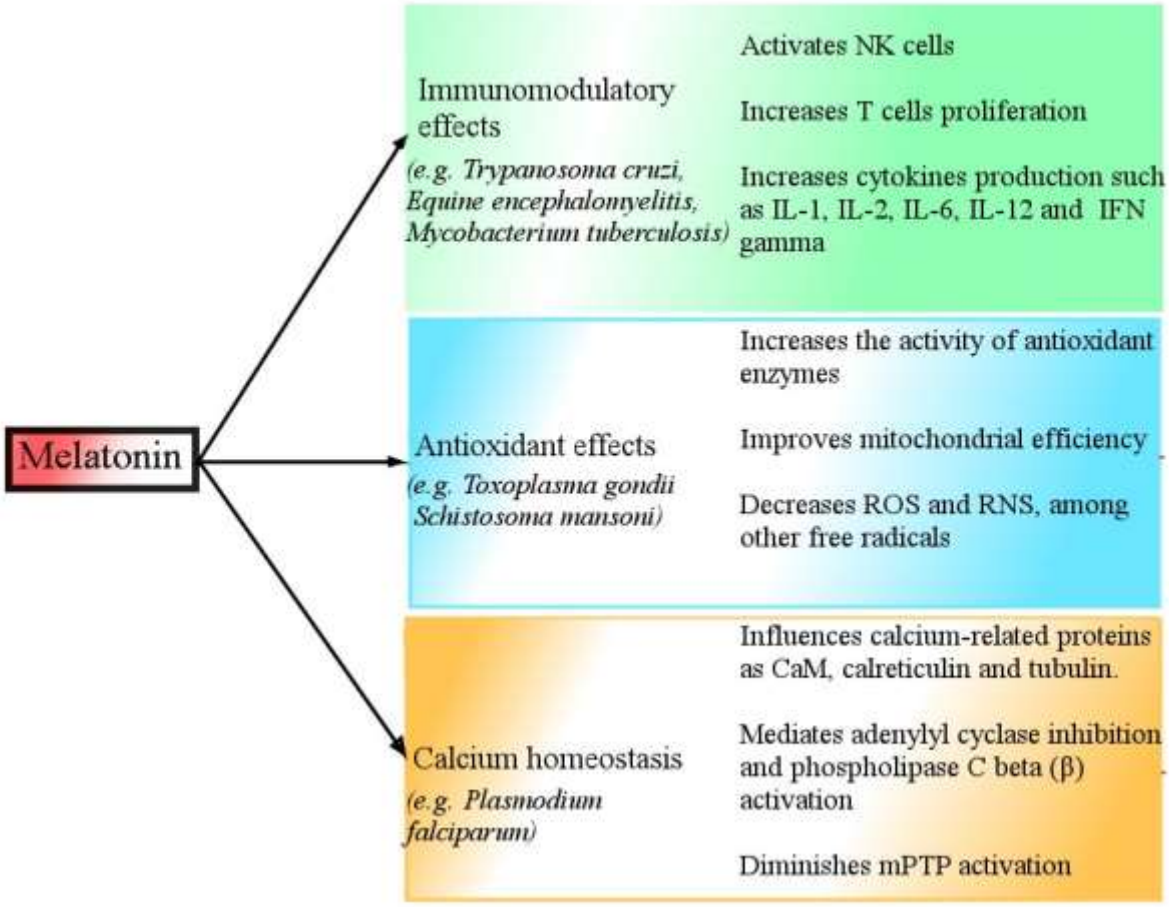

Figure 2: Summarize the physiological and therapeutic implications of melatonin and their potential relevance against some infectious agents [592, 597, 604-621].

2. Physiological and therapeutic effects of melatonin

2.1 Effects of melatonin on immune system

Melatonin has been recognized as neuroendocrine–immunological network modulator due to its affinity to T-lymphocytes (CD4+) and innate immunity [622-625]. Several previous studies have reported the immunomodulatory effect in both animals and humans as it enhances innate and acquired immunity through activation of natural killer (NK) cells and antibody-dependent cell-mediated cytotoxicity and subsequently increases T cells proliferation and production of cytokines [605, 606, 622, 626, 627]. This may justify the immunotherapeutic potential of melatonin which counteracts the induced-immunosuppression by acute stress, ageing, bacterial and viral infections [607, 608, 623].

As previously mentioned, leukocytes, bone marrow cells, thymocytes and epithelial cells have been reported to produce melatonin [581, 609, 628]. Even more, cultured human lymphocytes were able to release large amount of melatonin which has autocrine, endocrine, intracrine, and/or paracrine effects, and therefore coordinates immune response [626]. The presence of melatonin's receptors (especially MT1) in different immune cells of thymus and spleen also implicates the modulatory and anti-inflammatory effects of melatonin [585, 629]. These effects are mainly mediated through its effect on certain receptors in immune organs and immunocompetent cells of many mammals, as well as human [630-632]. The involvement of receptors MT2 in melatonin's modulatory effects have been explored in mice through enhanced splenocyte and lymphocytes proliferation, while this effect was blocked by the MT2 antagonist luzindole [609, 622, 633-635]. Melatonin also regulates hematopoiesis indirectly through its action on certain receptors located on bone marrow cells and via the induction of T-helper-cell-derived opioid cytokines [636], or directly through its action on some progenitor cells such as NK cells, pre-B cells, and monocytes [610, 637]. Hence, the anticancer action of melatonin may be attributed to activation of lymphocytes, monocytes and macrophages which also prevents tumor development [638, 639]. Likewise, activation of melatonin receptors has been reported to enhance the secretion of cytokines by T-helper cell Type 1 (Th1), like interleukin-2 (IL-2) and gamma-interferon (IFNc) [605, 609, 636]. Interestingly, activation of T-helper cells type 1, monocytes, and/or monocyte-derived cells by melatonin was found to enhance the production IL-1, IL-6, IL-12, IFN-γ, and macrophagecolony stimulating factor (M-CSF), which together act through binding to nuclear RZR/ROR receptors subfamily belongs retinoic acid receptor and membrane MT1 and MT2 receptors [587, 610-612, 640]. These previously mentioned cytokines may counteract stress-induced immunosuppression in several infectious cases [610], besides their role in immunomodulation process [624].

Melatonin also promotes the expression of major histocompatibility complex (MHC) class II and transforming growth factor (TGF)-β in antigen-presenting cells (APC) [605, 641]. In addition to promotion or suppression of Th-2 responses in some cases, melatonin involved in down-regulation of cyclooxygenase expression in macrophages and 5-lipoxygenase which antagonizes prostaglandin synthesis [581, 607, 608, 623].

On the other hand, the role of melatonin in other autoimmune diseases is still controversial and the mechanism of action is poorly understood, however, some studies related such effects to the

balance between Th1/Th2 and others suggest its contribution to the immune system homeostasis [610, 642]. In this regard, this compound has pro-inflammatory action in rheumatoid arthritis patients where a high plasma level of melatonin was found and the synovial macrophages of the patients respond to exogenous melatonin with an increased production of IL-12 and nitric oxide (NO)[641, 643]. Hence, melatonin antagonists may achieve therapeutic effects in such cases and further studies are necessary to understanding the underlying mechanisms of action [642].

2.2 Antioxidant action of melatonin

Oxidative stress is a common term refers to the disturbance in the balance between the reactive oxygen species (ROS) and the antioxidant defense [644] which accompanied several pathological conditions such as parasitic infection and aging [645, 646]. Based upon several published works, melatonin has shown a potential antioxidant effect resulted from both hydrophilic and hydrophobic features of this indolamine that allow it to cross several body barriers [613]. Also, melatonin indirectly regularizes the activity of several antioxidant enzymes, increases the efficiency of mitochondrial bioenergetics, and reduces the electron leakage from the mitochondria, which in turns lowers the free radical generation and augments the efficiency of other antioxidants [614, 615, 647]. Additionally, melatonin has a great scavenging activity for the free radicals, including hydroxyl radicals like hydroperoxyl radical, NO, singlet oxygen or peroxynitrite anion ($ONOO^{-}$), which explains the role of melatonin as a potent scavenger of mutagenic and carcinogenic hydroxyl radical ($OH^{-\bullet}$) [585, 614]. Interestingly, this anti-inflammatory action of melatonin results from its inhibitory effect on inducible nitric oxide synthase (NO synthase), which consequently reduces the oxidative damage and protects from NO-mediated mitochondrial blockade under acute or chronic conditions [609, 628, 648-650]. Hence, some clinical trials suggested that melatonin can contribute efficiently to several metabolic functions [651].

2.3 Role of melatonin in bacterial and viral infection

Several published works have reported the beneficial effect of melatonin in bacterial and viral infections [607, 625, 652, 653]. Indeed, administration of melatonin was found to be effective in controlling chlamydial infection and bacterial infection caused by *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Dichelobacter nodosus*, in addition to many viral infections such as *Equine encephalomyelitis* virus and *Ebola* virus disease [607, 653-659]. The activity of melatonin in these cases is mainly attributed to its free radical scavenger activity, regulation of

bacterial growth, depletion of some intracellular substrates like iron, and/or immunomodulatory-adjuvant activities [657, 660-662].

2.4 Melatonin and parasites

General remarks about role of calcium homeostasis in biology of some parasites and its relation to anti- parasitic effects of melatonin

In fact, the cell function in parasites is coordinated using a second messenger signaling cascades involving cyclic adenosine monophosphate (cAMP) and calcium (Ca^{2+}) [663-673], which control many critical events including host cell invasion, gliding motility, parasite differentiation and egress [674-682]. Even more, calcium binding proteins such as CaM and calcium-dependent protein kinase (CDPK) genes play critical roles in protein secretion, host cell invasion and parasite differentiation [679, 683]. Calmodulin (CaM), the ubiquitous intracellular calcium binding natural regulator, has been identified in American and African trypanosomes, *Leishmania braziliensis*, *Leishmania tropica* and *Leishmania donovani* [684-686]. It shared 99% amino acid sequence identity between trypanosomatids [684-686] and related to various functions in trypanosomatids like cAMP-dependent phosphodiestherase stimulation in *Trypanosoma cruzi (T. cruzi)* [687-689], $Ca^{2+/}$calmodulin ($Ca^{2+/}$CaM)-dependent protein kinase (TcCaM K) [690, 691], and transduction mechanisms of the cGMP-nitric oxide pathway in *T. cruzi* [692-694]. These events allowed CaM to act as: a mediator of Ca^{2+} functions, calcium sensor, and signal transducer to many proteins which are able to bind to CaM and unable to bind calcium [99].

Moreover, some scientists have reported other important functions of Ca^{2+} in regulation of cellular differentiation, cAMP levels in *T. brucei* [695-697], and cAMP phosphodiesterase in *T. cruzi* [688, 698]. It was also proposed that an inositol 1, 4, 5-trisphosphate ($InsP_3$)- dependent calcium response in *Plasmodium* species (spp.) and *T. gondii* [699]. Calcium is also considered the main controller of protein secretion, invasion, motility, and egress of *Toxoplasma* [700, 701], while it is very critical for developmental regulation and cyclic nucleotide signaling in *Plasmodium* with involvement of many stages of invasion and motility of the parasite including erythrocyte invasion stage by merozoites [702], besides its important role in the sexual multiplication in the mosquito vector [703-708].

Similar to eukaryotic cells, intracellular Ca^{2+} is finely regulated in trypanosomatids by various organelles [675, 694, 709], including mitochondria, endoplasmic reticulum, Golgi and acidocalcisomes which are known as major calcium storage sites [694, 710]. Moreover, trypanosomatids possess acidocalcisomes which involved in bioenergetics besides a single mitochondrion which represents 12% of the parasite volume and capable for accumulation large amounts of polyphosphates together with Ca^{2+} ions [711-713]. Accordingly, any fluctuations in cytosolic Ca^{2+} level ($[Ca^{2+}]i$) could control many cellular functions in such organisms [676, 679]. In this regard, many of available antiprotozoal agents exert their effects through alteration of Ca^{2+} homeostasis in the parasite and/or through impairment of the activity some mitochondrial parameters [714, 715].

According to the latest publications, melatonin has shown a wide range of activity against various parasites [653, 716]. To our knowledge, the signal transduction mechanisms of melatonin for its receptors are different among the various tissues and cell types [601, 717, 718]. It has been reported that melatonin exerted its effects in such pathological conditions through its influence on some intracellular proteins like CaM [616, 719], calreticulin [720], or tubulin [721], antagonizing the binding of Ca^{2+} to CaM [722]. As mentioned above, there are two mammalian subtypes of G protein coupled heterodimers participate in signaling pathways, leading to downstream effects on Ca^{2+} channels, Ca^{2+} signaling and changes in extracellular-signal-regulated kinases which give melatonin and its derivatives a pleiotropic nature [581, 723, 724]. MT1 melatonin receptor could also mediate adenylyl cyclase inhibition and phospholipase C beta (β) activation through its coupling to different G proteins. Therefore, activation of MT1 receptor may activate a large variety of G proteins which inhibit the cyclic adenosine monophosphate (cAMP) signal transduction cascade and their accumulation decrease the activity of protein kinase A and cAMP response element binding CREB) [617, 618, 725]. Melatonin (MT1) receptors also regulate ion fluxes besides its influence on calcium-activated potassium channels [726-728]. Likewise, several studies have reported a numerous safeguarding mitochondrial effects of melatonin, which is mainly attributed to its role on respiratory electron flux [585, 626] or through its unique effect in alteration of Ca^{2+}-induced mitochondrial permeability transition pore (mPTP), which is found to be a gatekeeper of apoptotic and necrotic cell death [581, 592].

Taken together, these previous events have a strong influence on the control of some infectious agents, especially the parasitic type, since disruption of Ca^{2+} homeostasis may result in cell death [137], however, it should be borne in mind that the suggested mechanisms underlying this activity seem to be different among these parasites (Figure 3). The following section will highlight several facts about the potential activity of melatonin against several global infectious diseases caused by a group of parasites.

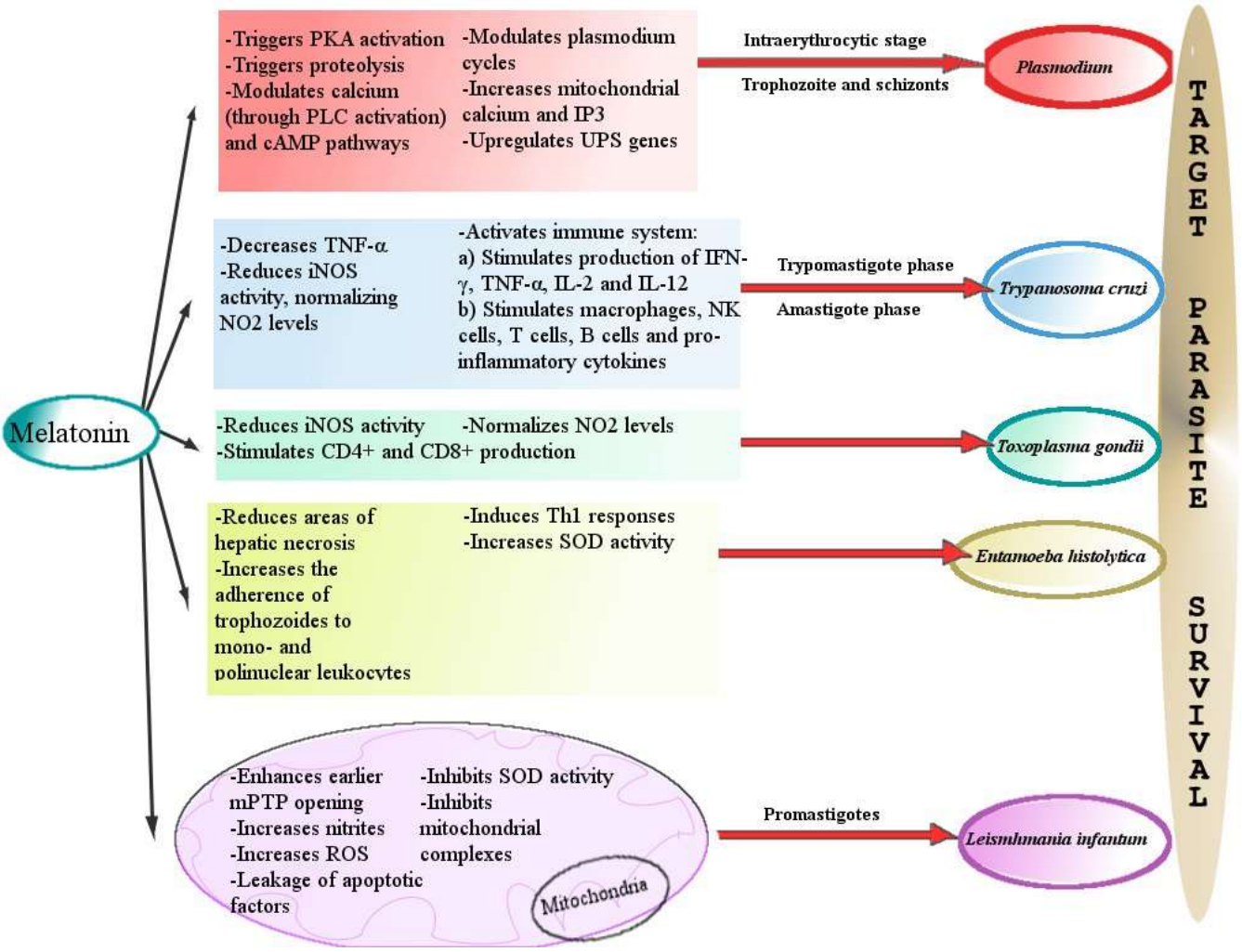

Figure 3: Summarize the action of melatonin with *Plasmodium*, *Toxoplasma gondii*, *Trypansoma cruzi*, *Entamoeba histolytica*, and *Leishmania infantum* promastigotes [597, 600, 602, 604, 653, 729-738]

2.4.1 Melatonin and Apicomplexa

2.4.1.1 Melatonin and malaria

Malaria is mosquito-borne infectious disease of humans and other animals caused by protozoan of genus *Plasmodium* and mainly transmitted via the bites of infected mosquitoes [739]. More than 220 million cases of malarial infections are reported every year, and the disease kills between 473,000 and 789,000 people worldwide, mainly in Africa [611, 739, 740]. *Plasmodium falciparum* (*P. falciparum*), *Plasmodium malariae* (*P. malariae*), *Plasmodium vivax* (*P. vivax*), *Plasmodium knowlesi* (*P. knowlesi*) and *Plasmodium ovale* (*P. ovale*) are the main causative species [739, 740]. The parasite multiplies in the liver of human, and then infects red blood cells (RBCs); this stage (erythrocytic) occurs after 48 hours in *P. falciparum* and consists of ring, trophozoite, schizonts and ultimately give rise to merozoites that release into the blood stream at a specific time of the day-night cycle [640].

Melatonin and its precursors are widely known as a nocturnal signal can regulate the cell physiology of the parasite, besides their critical role in the synchronization of maturation of the parasite and its survival in the host [598, 612, 628, 729, 730]. In this regard, several studies have reported that melatonin drives a temporal regulation in some species of *Plasmodium* either *in vivo* or *in vitro* [592, 741].

Importantly, melatonin drives as a second messenger through modulation of Ca^{2+} and cyclic-AMP pathways, besides its role in activation of Protein kinase A (PKA), a class of cAMP-dependent enzymes which modulates the cell cycle [15, 730]. The level of extracellular calcium is a critical event for the invasion of the parasite into RBCs, exflagellation process as a step of sexual stage of the life cycle, and *Plasmodium* kinases [742-744].

Indeed, melatonin and its derivatives promote $[Ca^{2+}]I$ increase by mobilizing it from internal stores either by direct uncaging (Photolytically) of $InsP_3$ within the intraerythrocytic stage of the parasite or by increasing parasite inositol phosphate formation, which subsequently modulate the *P. falciparum* cell cycle [602, 732, 745]. Therefore, they could regulate and modulate the life cycle of human malaria parasite, *P. chabaudi* and *P. falciparum*, *in vivo* and *in vitro* [602, 730], by mobilization of Ca^{2+} from internal Ca^{2+} pools of parasite trophozoite, augmenting the proportion of schizonts and cytosolic free Ca^{2} [729].

Furthermore, recent studies have revealed that melatonin up regulates the genes related to ubiquitin-proteosome-protein system (UPS) which involved in specific functions related to pathogenesis and virulence of *P. falciparum* [600, 746]. It should be also pointed out that exogenous melatonin remarkably prevents development of mitochondrial pathology and mitochondrial oxidative stress in hepatocytes, which in turns prevents hepatic cell damage resulting from malaria infection [730, 747, 748].

On the other hand, melatonin is known as a potent antioxidant agent protects malarial parasites from ROS attacks in the oxygen rich environment at erythrocytic stage [14, 748]. Arguably, the blockade of melatonin's nocturnal action on malaria parasite growth or the circadian changes in the melatonin levels of the host using common melatonin antagonists or some derivatives seems extremely important in combating this disease [713, 745]. Taken these facts together, melatonin and its derivatives exhibit potent antimalarial effects.

2.4.1.2 Melatonin and toxoplasmosis

Toxoplasmosis is a worldwide parasitic zoonotic disease caused by protozoan of genus *Toxoplasma gondii,* which is considered a causative agent of death in the United States [749]. Most warm-blooded animals can be infected, including humans, but the primary host is family Felidae [749].

Several studies have investigated the effect of artificial supplementation of melatonin and/or zinc on the response of immune system to *T. gondii*. Melatonin has shown an important role in activation of cellular immunity by stimulating $CD4^+$ and $CD8^+$ production [619, 653, 750, 751].

Furthermore, NO levels increase in *Toxoplasma* infection, particularly in the chronic phase of the infection in Sprague-Dawley rats, which increases in melatonin deficiency.

Hence, melatonin reduces the activity inducible nitric oxide synthase (iNOS) activity which enhance the immune system by the activation of Astrocytes and HUVEC cells, resulting in NO release in the presence of the parasite and the later might be beneficial to the host, as it normalizes nitrites (NO_2^-) levels [731, 752]. Taken together, melatonin could be an adjunctive therapy for treatment of *Toxoplasma* retinochoroiditis, especially in immunosuppressed individuals.

2.4.2 Melatonin and trypanosomasis

Trypanosomiasis is a group of parasitic diseases of vertebrates, mainly caused by protozoan parasite of genus *Trypanosoma*. The parasite has three different stages: trypomastigote,

amastigote, and epimastigote [753]; the transformation of epimastigote form into the metacyclic trypomastigotes is mainly occurred during darkness period [754].

There are two main forms of Trypanosomiasis; Human African Trypanosomiasis which is common disease in 36 countries of sub-Saharan Africa with more than 60 million people at risk [755] and is caused by *Trypanosoma bruceigambiense* or *Trypanosoma bruceirhodesien*se while tsetse flies are responsible for transmission of the disease to human [756].

The other form, American trypanosomiasis (Chagas disease), is caused by *Trypanosoma cruzi* (*T. cruzi*) and transmitted mostly by insects known as *Triatominae* [757], resulting in in 21,000 cases of deaths annually, mainly in Latin America [757]. Sudden death in acute patients may be resulted from congestive heart failure associated with myocarditis or meningoencephalitis [758], while most of the patients develop the chronic form of the disease [759].

Several studies have reported the significant contribution of melatonin in controlling *T. cruzi* multiplication *in vivo* and *in vitro* [760-762]. In this regard, melatonin treatment (5 mg/kg orally), prior to experimental infection or during the infection, resulted in reduction of the levels of IL-10, IL- 4, tumor growth factor-β and NO, while it increased the number of macrophages and enhance the release of IL-12, IL-2, TNF-α and IFN-γ [597, 620, 733, 763]. In such cases, melatonin up-regulates Th-1 immune response and suppressed Th-2 response [597, 734, 764], which promotes a reduction in blood and tissue parasites, and therefore reduce the parasitemia combined with the blockade of prostaglandin E2 synthesis [599, 765].

Administration of melatonin during the acute phase of infection with the parasite may possess a dual effect (promoting and inhibitory) on *T. Cruzi* life cycle, based upon the period of exposure and the concentration used [736, 765]. In this regard, melatonin administered during the acute phase of *T. cruzi* infection resulted in reduction of the parasitemia [762], inhibition of parasite propagation or killing the parasite through its action on the immune system, as it activated the macrophages as a result of enhanced NO production. This later product is considered the major effectors' molecule of *T. cruzi* intracellular amastigote killing [735, 760, 766].

It has also been proposed that ROS and oxidative stress play an important role in expansion of the systemic complications of Chagas, especially cardiomyopathy [767-770]. As consequences, mitochondrial functional decline, combined with loss of the scavenger activity for ROS, resulting in sustained oxidative stress during infection [768, 769]. Furthermore, NO accumulation was found to slow down the electron transport chain, which inhibits the production

of Adenosine triphosphate (ATP), higher ROS production, and in turn increases the susceptibility of cell death [771]. Indeed, melatonin could protect mitochondria by counteracting the oxidative damage and prevent the development of heart damage [772].

During the chronic phase of the disease, melatonin could be beneficial for combating the disease progression [768, 773]. It could reduce the oxidative stress accompanying the myocardial damage, which represented by reduction in the number of trypomastigotes, fewer amastigote carriage, lower tissue disorganization in the heart, and higher number of leucocytes resulted from activation of Th-1 inflammatory response [597, 736, 762].

Therefore, administration of melatonin agonist like the MT1/MT2 agonist (ramelteon) in Chagas´ disease during the acute phase may enhance the immune response without impairment in NO production, while high doses of melatonin during the chronic course of the disease lowers the oxidative stress, preserves the mitochondria and prevents the development of cardiomyopathy [773]. These findings prove that melatonin either alone or in association with other drugs such as meloxicam could be helpful therapy in American trypanosomiasis [597, 762].

2.4.3 Melatonin and schistosomiasis

Schistosomiasis (Bilharzia) is neglected disease caused by parasitic worms of genus *Schistosoma* [774]. The disease affects almost 210 million people worldwide [774] and is considered the second devastating parasitic disease after malaria, especially in poor societies with unclean water and inadequate sanitation [775].

Melatonin enhanced the protective immune response against *Schistosoma mansoni* in hamster infected with *Schistosoma mansoni* using cercarial and soluble worm antigens [596]. Indeed, melatonin has been postulated to be protective against the pathological changes in *Schistosoma mansoni*-infected mice, which may be resulted from its antioxidant and free radical scavenging activity that reduces the oxidative damage and increases the survival rate [621].

2.4.4 Melatonin and amoebiasis

Amebiasis is a parasitic infection of the large intestine, sometimes involving the liver caused by *Entamoeba histolytica,* and estimated to cause 70,000-100,000 deaths per year worldwide [776, 777].

França-Botelho and co-authors have studied the effect of melatonin administration (15 mg/kg body weight subcutaneously) in experimental amoebiasis (*in vivo* and *in vitro*) and on the

relationship between trophozoites of the virulent strain HM1-IMSS of *E. histolytica* and human blood cells [737]. They have noticed a marked decrease in the amoebic necrotic areas in liver infiltrated with large quantities of mononuclear inflammatory cells, which explains the enhanced adherence of the parasite trophozoites to mononuclear and polymorph nuclear leukocytes (PMN) [653, 737]. Therefore, it is suggested that melatonin administration resulted in induction of Th1 responses and could establish its role as an adjuvant therapeutic agent in amebiasis [653, 737].

2.4.5 Melatonin and leishmaniasis

Leishmaniasis is a group of neglected diseases, caused by infection by flagellate protozoa of the genus *Leishmania* and present in all inhabited continents with a clear endemicity in tropic and subtropics areas [15, 778].

Despite several leishmanial researches, a limited number of effective and less toxic antileishmanial agents are available which is mainly encountered by the development of drug resistance [779, 780]. As previously mentioned, *Leishmania* is a member of the trypanosomatidae family possess a large mitochondrion which represents 12% of the parasite volume and can accumulate large amounts of Ca^{2+} [713], besides its role as in important target for many of the available antileishmanial agents [714, 781, 782]. Recently, melatonin has shown antileishmanial activity against the promastigotes phase of *Leishmania infantum*, together with marked alteration in parasite mitochondrial calcium level and significant alteration in some mitochondrial parameters, and therefore target parasite survival [738].

Hypothesis and Objectives

1. Hypothesis

The last decade has witnessed an extraordinary progress in the spread of the disease to urban areas which led to the necessity for more surveying and search for novel drug targets due to absence the drug of choice, aiming to construct effective control measures adopted to eliminate transmission and prevent new epidemics. The working hypothesis may switch on the light for the surveillance of such diseases in human and feline using some diagnostic methods such as IFAT, WB and PCR. The working hypothesis also proposed in this study is that exogenous melatonin, as a potent natural antioxidant would target the parasite survival and thereby, may be play a future role as antileishmanial agent.

2. Objectives

General Objective

The main objective was to get more information about epidemiology of Leishmaniasis through a serological study about *Leishmania* infection among kidney transplanted patients from southern Spain combined with assessment the prevalence of *L. infantum* infection among domestic cats from highly endemic area, named Liguria which is a coastal region of north-western Italy. The other part was mainly undertaken to assess the effects of melatonin against the parasite *in vitro*. The expected outcomes of our study is better understanding the incidence of the disease among asymptomatic renal recipients populations and in cats from endemic area in Italy, besides the involvement of new diagnostic technique both WB and PCR in diagnosis of the infection to identify the risk factors of transmission of such protozoan and the potential reservoir for the infection in this area. The study also tested the association of *Leishmania* infection in cats to some infectious agents like haemotropic *Mycoplasma* species and reoviruse including Feline immunodeficiency virus (FIV) and Feline leukemia virus (FeLV) and the possible association between these agents and the anemia status of the possible examined animal. On the other hand, the other present work outcome was the activity of melatonin against the parasite. We further hope to provide new diagnostic and /or therapeutic perspectives for combating this neglected disease.

2.1. Specific aims:

2.1.1. To assess the Seroprevalence of *Leishmania* infection among asymptomatic renal transplant recipients from southern Spain.

2.1.2. To evaluate the occurrence of *Leishmania infantum* in cats from an endemic region in Northwestern Italy.

2.1.3. To investigate the effects of melatonin against *L. infantum* and, if so, to study the possible underlying mechanisms of this activity.

Material and Methods

Experimental Design

1. Diagnostic part:

1.1. Determination of *Leishmania* seroprevalence among renal transplant recipients

1.1.1. Study area and population: Serum samples from 625 individual renal transplant patients were collected for one year (April 2013-April 2014). Age, gender, the cause of the transplant, the survival time after transplantation and the type of immunosuppressive therapy were controlled. Any individuals with fever or infectious disease were excluded from the study. All patients were living in neighbouring Granada provinces for at least the last ten years, for which our hospital is a referral centre for renal and liver transplantation. The study was approved by the Ethical Committee of Research of the University Hospital Virgen de las Nieves (Granada, Spain), before initiation of data collection.

1.1.2. Sample collection and screening using IFAT: To investigate *Leishmania* infection, peripheral blood was collected into sterile Vacutainer™ tubes. Samples were centrifuged at 4ºC and stored at -80°C until serological tests were performed. All patients had a low level of immunosuppression at the time of blood sampling.

Serum samples were screened for leishmaniasis with indirect fluorescent antibody test (IFAT). The IFAT for determination of antibodies against *L. infantum* (*Leishmania* IFI IgG Kit, Vircell, Santa Fe, Granada, Spain) was performed according to the manufacturer's instructions.

When samples were positive with a titer of 1:80, a twofold serum dilution was done until reached 1:1280 dilution. As shown in the kit prescription IFAT is considered positive with promastigotes fluorescence with a cutoff dilution of 1:40 due to the endemicity of the area of study. This result is considered as indeterminate in this assay.

1.2. Epidemiological survey of *Leishmania infantum* in cats

1.2.1. Study population and preparation of serum samples

A total number of 346 domestic cats (n=250 serum and n=282 blood samples) from Liguria, a coastal region located in Northwestern Italy, were routinely sampled between 2005 and 2013

during veterinary clinic visit and underwent screening. To investigate Leishmania infection, 2mL of blood was collected with Ethylene diamine tetraacetic acid (EDTA) and without anticoagulant (through jugular vein puncture), from the animal in clean sterile tube. Serum samples were centrifuged and stored at 80°C until testing. The tests were performed in the Department of Veterinary Sciences, division of Parasitology.

2.1.2.2. Clinical and laboratory diagnosis

Cats were examined clinically for any lesion together with routine hematochemical examination. For the complete blood count (CBC), Pentra60 Horiba ABXTM used to count the blood cells, whereas for a biochemical MIRA Plus Horiba ABXTM (chemical liquid) and for a serum protein electrophoresis, SAE-NT of Chemetron was used. The haematochemical parameters included the following: white-blood cell count(WBC), monocytes (MON), neutrophils (NEU), eosinophils (EOS), lymphocytes (LYM), red-blood cell count (RBC), hemoglobin (Hb), hematocrit (HCT), platelets count (PLT); albumin (ALB), total protein (TP), creatinine (CREA), blood urea nitrogen (BUN), alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), cholesterol (CHOL), triglycerides (TG), glucose (GLU), calcium (Ca), potassium (K) and phosphorus (PHOS). Serum samples were also serologically screened using *L. infantum* promastigotes as antigen with western blotting. The molecular detection was carried out using PCR using blood samples.

2.1.2.3. Western blotting and SDS- polyacrylamide gel electrophoresis

Serum samples were screened by WB using *L. infantum* promastigotes as antigen according to the method described elsewhere [783, 784] with slight modifications using horseradish peroxidase labelled Goat Anti-Cat IgG (H+L) Antibody, diluted 1:8000 (Novex by Life Tecnologies, USA). The resulting bands were compared using as marker Prestained Protein Molecular Weight Marker (Fermentas International Inc, Ontario, Canada) and Biotinylated SDS PAGE Standards broad range (BioRad Laboratories, Hercules, California, USA). Samples were considered positive by WB when at least two bands of 169, 115, 66, or 33 kDa could be detected [784].

2.1.2.4. Preparation of blood samples and extraction of DNA

Total genomic DNA from was extracted from 200 µL of whole blood using the commercial kit GenomeElute under conditions suggested by the manufacturer (Sigma–Aldrich). The DNA was then stored in sterile DNAse- and RNAse-free microtubes and kept at –20°C.

2.1.2.5. Qualitative polymerase chain reaction (PCR) for detection of Leishmania and Mycoplasma DNA

We adapted a previously described PCR protocol [785] that uses primers mRV1 5' CTTTTCTGGTCCCGCGGGTAGG-3' and mRV2 (5'-CCACCTGGCCTATTTTACACCA-3') to amplify a 145 bp fragment present on the highly reiterated kDNA minicircle of *L. infantum*. The PCR reaction mixture (25 µl) contained ≈ 100 ng of DNA template, 2.5 µl 10X PCR buffer, 5 µl of Q Buffer, 2.5 UI of HotStarTaq DNA Polymerase (Qiagen, Milan, Italy), 0.5 µl of dNTPs mix (10 mM of each dNTP, Sigma-Aldrich, St. Louis,MO, USA), and 12.5 pmol of each primer. An initial denaturation step of 15 min at 95°C was followed by 35 repeats of 15 min at 95°C, 1 min at 60°C, and 1 min at 72°C, and a final elongation step of 10 min at 72°C.

To detect the presence of haemotropic *mycoplasmas*, we developed a multiplex PCR protocol targeting the 16s rRNA gene. Species specific forward primers (MhfF 5'-TCTTTGGTTTCGGCCAAAGAT-3', MhmF 5'-GCTTGATAGGAAATGATTAAGC-3', and MtcF 5'-TCCTCCATCAGACAGAAGGGGGA-3' for *Mycoplasma haemofelis, Candidatus Mycoplasma haemominutum, and Candidatus Mycoplasma turicensis*, respectively) were used together with a common reverse primer MycR 5'-GGGTATCTAATCCCATTGC-3'.

The multiplex PCR was optimized in a final volume of 25 µL, using Promega PCR Master Mix (Promega Corporation, WI, USA), together with 1µM of primers MhfF and MtcF, 0.5µM of MhmF, 2 µM of the reverse primer MycR and ≈100 ng of DNA template.

The amplification included a 5 min denaturation step at 95°C followed by 40 repeats of 1 min at 95°C, 30 s at 60°C, and 3 min at 72°C and a final extension at 72°C for 10 min. PCR fragment size was estimated by comparison with two molecular weight standards: PCR 100 pb Low Ladder and pBR 322 HaeIII Digest (Sigma–Aldrich) after electrophoresis on a 2% agarose gel.

Gels were stained with MegaFluor kit (Euroclone, Milano, Italy) performed under the conditions suggested by the manufacturer and photographed on a Gel-Doc System (Bio-Rad).

Fifteen randomly selected positive PCR products were sequenced (Macrogen Inc., The Netherlands) and the resulting sequences were compared to those available in GenBank, to confirm PCR specificity.

2.1.2.6. Detection of FELV antigen and FIV antibody

To test the correlation between Retroviruses and *Leishmania* infection, 87 samples were tested for the presence of FeLV antigen and 89 samples for FIV antibody. Detection of FeLV antigen (p27) and FIV antibody was performed using immunochromatographic BVTTM until 2006. From 2007 onwards, ELISA Test Snap IDEXXTM commercial assay kit (SNAP® FIV Antibody/FeLV Antigen Combo Test; IDEXX Laboratories, Westbrook, ME) was used.

2. Melatonin treatment part

2.1. Materials: Melatonin, amphotericin B, and Dimethyl Sulfoxide (DMSO) were obtained from Sigma Chemicals (Madrid, Spain), while Alamar blue was obtained from Invitrogen™ of Life Technologies (Catalog N. DAL1025). Minimum Essential culture Medium (MEM; Sigma-Aldrich Corporation, St Louis, MO, USA), supplemented with 10% fetal calf serum (GIBCO, Invitrogen, NY, USA), 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich Corporation) was used in a growth inhibition assay to test the effect of melatonin on parasites. The required materials for following subcellular fractionation techniques such as Oregon green were obtained from Panreac (Madrid, Spain) and Invitrogen (Madrid, Spain). All the other remaining chemicals and reagents were purchased from Sigma-Aldrich (Madrid, Spain). All materials were of analytical grade.

2.2. *Leishmania* cells and culture conditions

L. infantum MHOM/Fr/LEM75 (PB75) strain was used in a preliminary screening test of the effects of melatonin. *L. infantum* Promastigotes were grown in 25-mL culture flasks using MEM at 26°C supplemented with 10% fetal calf serum,100 IU/mL penicillin, and 100 µg/mL streptomycin [786, 787]. The parasites were maintained by weekly transfer from previous culture into new medium.

2.3. Drug assessment on promastigote growth inhibition assay

The susceptibility of promastigote to melatonin was assessed using the method described by Carrio and co-authors in three independent assays [788]. Briefly, a 5-day-old culture (log phase) was centrifuged at 1500-2000×g for 10 min at 4°C. The pellet was then resuspended in fresh MEM supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 µg/mL streptomycin at 26°C, and the promastigote count was adjusted to 1×10^6 cells/mL.

Tested agents were: melatonin at different concentrations (1, 10, 25, and 50 nanomolar [nM]), and 0.1 µM amphotericin B dissolved in 0.1% DMSO. DMSO at this concentration is known to have no inhibitory effects on parasite growth [789, 790].

100 µL of log-phase promastigotes of *L. infantum* (1×10^6 cells /mL) were seeded in 96-well culture plate (Nunc, Denmark) containing 100 µL of MEM medium per well treated with melatonin (1, 10, 25, or 50 nM) or 0.1 µM amphotericin B (reference drug) for 72 h at 26°C.

The Parasite survival and viability were monitored by direct counting of parasites using Neubauer hemocytometer. The results were expressed as the percentage of parasite inhibition (PPI) for each concentration used while the number of parasites counted in wells without drug was set as 100% parasite survival (control negative) [791].

$$\text{PPI} = \frac{\text{CFC}-\text{CFP}}{\text{CFC}} \times 100$$

Where CFC is the final concentration of control culture (cells /mL), and CFP is the final concentration of treated culture (cells /mL).

The leishmanicidal effect was expressed as the concentration inhibiting parasite growth by 50% (IC_{50}) which was calculated from the sigmoidal dose response curve. Results were verified by using the Alamar blue colorimetric assay, which has proven to be more accurate for estimation of cell growth and viability [792, 793].

The assay was carried out on microtiter plates by adding 20 µL Alamar blue 8 h before the end of the incubation period and then measuring absorbance of the treated samples and medium blank with a spectrophotometer at 570 nm and 600 nm.

The inhibition assays were repeated three times in triplicate wells. For mitochondrial and subcellular fractionation, a parallel technique for incubation of promastigote cultures was carried out in 25 mL culture flasks under the same treatment and incubation conditions. The experiment was repeated at least three times in three independent assays.

2.4. Mitochondrial studies

2.4.1. Mitochondrial Isolation

The mitochondrion of treated and untreated promastigote cultures was isolated according to a previously reported protocol [794, 795] with slight modifications. Cells were harvested by centrifugation at 1000 x g for 10 min, washed three times in saline-sodium citrate buffer (SSC), and then resuspended in Sodium Chloride-Tris-EDTA (STE) buffer, homogenized by passage through a 26 gauge needle, and centrifuged at 700 x g for 10 min. This centrifugation step was repeated until all cells were removed from the supernatant, which was then centrifuged at 8000 x g for 10 min. The pellet obtained was resuspended in STE buffer and incubated with 200 mg/mL of DNase I at 37 °C for 30 min in the presence of 7-mM $MgCl_2$. DNase I was then removed by washing thrice with 40-mM EDTA. The resulting fractions were then resuspended in 0.15- M NaCl, 0.1- M EDTA, and 0.05-M Na_2HPO_4.

2.4.2. Protein concentration measurement

Protein concentration of the isolated mitochondrion was measured according to Bradford method [796] using bovine serum albumin (BSA) as reference. Protein quantification was performed in mitochondrial suspension volumes of equal weight and volume (0.2 mL) to standardize the procedure.

2.4.3. Calcium Retention Capacity

The sensitivity of the mitochondrial Permeability Transition Pore (mPTP) opening to calcium ions and the calcium retention capacity of isolated mitochondria from treated and untreated cultures were assessed in the presence of 0.25 μM Oregon green using a fluorimetric assay. Pulses of 250 μM Ca^{2+} were injected until pore opening. One μM Cyclosporine A (CsA), the standard inhibitor of PTP, was added to evaluate the specificity [797]. mPTP opening was calculated from the area under the curve (AUC) of fluorescence signals over time.

2.4.4. Determination of Mitochondrial Nitrites

Determination of mitochondrial nitrites was performed using the Griess diazotization reaction, in which nitric oxide (NO) is spontaneously oxidized under physiological conditions and the resulting nitrate is measured spectrophotometrically [798, 799]. Measurements were performed at 548 nm on isolated mitochondria using a Griess Reagent Kit [Molecular Probes, G-7921], according to the manufacturer's instructions.

2.4.5. Determination of Mitochondrial Superoxide Dismutase Activity

Manganese (Mn) and Copper/Zinc (Cu/Zn) superoxide Dismutase (SOD) activities of isolated mitochondria were determined using a SOD Assay Kit-WST [Sigma-Aldrich, 19160, Switzerland], according to the manufacturer's instructions. Results were expressed as a percentage of inhibition of SOD activity.

2.4.6. Spectrophotometric Assays of Individual Respiratory Chain Complexes

Subsequent assays of respiratory enzymes activity were performed as described below, expressing the specific activity as nmol·min/mg protein.

Complex I. Submitochondrial fractions were incubated for 5 min in a medium containing 250 mM sucrose, 50 mM potassium-phosphate, 1 mM potassium cyanide (KCN), and 0.5 mM decylubiquinone, pH 7.4. The reaction was initiated by the addition of NADH. The specific activity was determined by the decrease in absorbance as a result of the oxidation of NADH at 340 nm [800].

Complex II. Submitochondrial fractions were added to the reaction buffer containing 1 M potassium phosphate, 1 M succinate, 0.1 % DCIP, 1 mM EDTA and Triton x-100, pH 7.4. The reaction was initiated by adding 0.5 %decylubiquinone (in ethanol) was added. The specific activity represented by the reduction in 2, 6-dichlorophenolindophenol was determined at 600 nm [801].

Complex III. Specific activity of isolated submitochondrial fractions was measured by monitoring the reduction in cytochrome *c* at 550 nm, through mixing of a submitochondrial fractions [0.03 mg/mL], with a reaction medium of 0.1 M potassium phosphate, 15 mM decylubiquinone, 0.1 M Sodium azide (NaN3) and 10% BSA [pH 7.5]. The reaction was initiated by adding 1% oxidized Cytochrome *c* [802].

3. Statistical analysis

Regarding the cross sectional survey in cats, we used generalized linear mixed models in which the result of the PCR and WB was the dichotomous response variable to identify possible associations between *L.infantum* prevalence and seroprevalence. The potential explanatory variables (covariates) considered were: individual factors (breed, age, sex), and hemato-biochemical parameters (WBC, MON, NEU, EOS, LYM, RBC, Hb, HCT, PLT, ALB, TP, CREA, BUN, ALT, AST, ALP, CHOL, TG, GLU, Ca, K and PHOS). Concurrent infection with FeLV, FIV and Mycoplasma were also considered as potential risk factors. The Variance inflation Factor (VIF) was used to test and avoid multicollinearity among predictors [803]. All the putative covariates were screened in univariate regression models. Variables identified as significant factors ($p \leq 0.05$) in the first univariate analysis were selected further tested by multivariate linear regression. Best model selection was performed using AIC (Akaike information Criterion), while the goodness-of-fit of the final model was assessed by computing the area under the curve (AUC) of the receiver operating characteristic plots. All statistical analysis were performed using R [804]. In case of testing the efficacy of melatonin against promastigotes culture of *Leishmani infantum*, values were expressed as means ± SEM. The data were analyzed using the Statistical Package for the Social Sciences (SPSS), Version 17.0 (IBM Inc, Michigan, IL, USA). Mean values were compared between treated and untreated promastigote cultures by using one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. The significance level was * $P < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Results

1. Seroprevalence of Leishmania infection among asymptomatic renal transplant recipients from southern Spain

Ehab Kotb Elmahallawy; Elena Cuadros-Moronta; Mª del Carmen Liébana Martos; et al.. Seroprevalence of Leishmania infection among asymptomatic renal transplant recipients from southern Spain. Transplant Infectious Disease. 2015 Aug 19. doi: 10.1111/tid.12444.

The aim of this study was undertaken to assess the seroprevalence of *Leishmania* infection among asymptomatic renal transplant recipient patients in Granada (Southern Spain) while they are tracking transplant.

During the study period, 625 patients with a mean time of ten years from transplantation to the last review were recruited at their transplant consultation. In terms of age distribution median age was 49 years, (range: 11-81 years). According to gender, 225 (36%) were female and 400 (64%) were male. Regarding the distribution by race, the caucasian race is predominant 621 (99.4%) within which 17 were romani ethnicity, and 4 (0.6%) were black. The etiology of renal failure that caused the transplant was: glomerular disease 174, (27. 8%), unknown 116 (18.6%), cystic disease 98 (15.7%), interstitial disease 84 (13, 4%), vascular disease 81 (13%), diabetes 27 (4.3%) and 45 other causes (6.1%). IFA test was performed on 625 serum samples. 30 samples were positive, 13 showed titers of 1:80, 15 showed titers of 1:160; 2 showed titers of 1:320. 14 additional samples showed titers of 1:40, considered as indeterminate. The characteristics of patients with *Leishmania* antibodies in relation to the variable studied are shown in the following table (table 4) and the prevalence was 4.8%. The immunosuppressive treatments after transplants for patients with positive serology included following regimens: thymoglobulin (administered only during the first three months after transplantation) plus mycophenolate mofetil plus prednisone in 10 patients (30,3%); tacrolimus plus mycophenolate mofetil plus prednisone in 8 patients (2.6%); prednisone plus mycophenolate mofetil plus basiliximab in 5 patients (16.6%); advagraf (tacrolimus once daily) plus basiliximab plus mycophenolate mofetil plus prednisone sodium in 4 patients (13.3%) and basiliximab plus cyclosporine plus mycophenolate mofetil plus prednisone in 3 patients (10%). All patients had a low level of immunosuppression at the time of blood sampling. There were no differences between the immunosuppressive treatment among patients with negative serology and positive serology. No patient developed leishmaniasis disease during the study period and to date.

Antibody titer (n)	**1:80 (n= 13)**	**1:160 (n= 15)**	**1:320 (n= 2)**
Age in years (range)	44.15 (29-66)	49.33 (29-67)	45.50 (43-48)
Sex			
Man	6 (46.2)	9 (60)	2 (100)
Woman	7 (53.8)	6 (40)	0 (0)
Race			
Mediterranean white	13 (100)	14 (93.3)	2 (100)
Nordic white	-	1 (6.7)	-
Renal failure type			
Unknown	4 (30.8)	3 (20)	1 (50)
Glomerular	5 (38.5)	3 (20)	-
Vascular	2 (15.4)	1 (6.7)	-
Systemic	2 (15.4)	-	-
Interstitial	-	2 (13.3)	1 (50)
Cystic	-	3 (20)	-
Diabetes	-	2 (13.3)	-
Survival time in years (range)	2.64 (1.58-3.72)	2.20 (0.3-3.67)	1.52 (0.44-2.61)

Table 4: Characteristics of patiens (n=30) grouped by antibody titer against *Leishmania infantum.*

* All patients had a low level of immunosuppression.

2. Cross-sectional Epidemiological survey of *Leishmania infantum* in cats from an endemic region in Northwestern Italy

Ehab Kotb Elmahallawy, Marco Poggi, Gabriele Cieri, et al.,.Cross-sectional Epidemiological survey of Leishmania infantum in cats from an endemic region in Northwestern Italy. Submitted. Acta Tropica-S-15-00778. 2015.

The present study was undertaken to assess the prevalence and current situation of *L. infantum* infection among domestic cats from a highly endemic area in northwestern Italy. We also aimed to identify the risk factors related to infection with *Leishmania infantum* protozoa. We tested the association between *Leishmania* infection and infectious agents like haemotropic *Mycoplasma* species and Retrovirus including *Feline immunodeficiency virus* (FIV) and *Feline leukemia virus* (FeLV). Hematobiochemical alterations were also considered as variables associated to infection.

Of the 250 sera tested with WB, 33 samples tested positive (P=13.20%; CI95% 9.56%-17.96%) for *L. infantum*, while of 282 blood samples tested with PCR, 80 samples were positive for *L. infantum* with a prevalence of 28.37% (CI95% 23.43%-33.89%). It was possible to test by both PCR and WB a total of 186 cats. Of these, 10 tested positive by both PCR and WB (Table 5).

	WB		
PCR	Negative	positive	**Total**
Negative	118	17	135
Positive	41	10	51
Total	159	27	186

Table 5: A total of 186 samples were tested for *L. infantum* by both polymerase chain reaction (PCR) and Western Blotting (WB), the results of both tests are summarized in the table. Sequencing confirmed the specificity of the protocol used as all the sequenced amplicons were identified as *L. infantum* (identity ≥98% to GenBank accession number: AB678348).

On the other hand, 17 out of the 167 samples tested with PCR for various species of *Mycoplasma* (P=10.18%; CI95% 6.45%-15.70%) were positive, including 8 which were also positive for *Leishmania* using PCR and other 3 were positive in WB and PCR.The prevalence of

Mycoplasma in PCR and WB positive samples was 7.2% compared to 22% in PCR and WB negative samples (P 0.002).

Out of the 89 also tested samples for FIV, 31 (P=34.83%; CI95% 25.75%-45.17%) were positive, whereas of 87 test FeLV performed, 22 (P=25.29%; IC95% 17.33%-35.33%) sample tested positive. PCR positivity for *L. infantum* resulted positively associated with higher values of BUN, NEU and with concomitant infection with FIV ($p<0.05$), while there was a significant negative correlation with low values of HCT, RBC and Hb ($p<0.05$). The best fitting generalized linear model included FIV, BUN and RBC (AUC= 0.82, AIC= 181.75).

For WB our data evidenced only a direct significant correlation between *Mycoplasma* infection and the presence of anti-*L. infantum* antibodies. The characteristics of those variables which were significantly associated to PCR or WB results are summarized in table 6.

WB associate covariates						
Qualitative variables		**Number of WB positive cats**	**Number of WB negative cats**	**P**	**OR**	**CI 95%**
Mycoplasma spp.	Pos	4	5	0.076	4.39	0.78 - 23.16
	Neg	15	84			
PCR associated covariates						
Qualitative variables		**Number of PCR positive cats**	**Number of PCR negative cats**	**P**	**OR**	**CI 95%**
FIV	Pos	23	18	0.006	3.77	1.43 - 10.43
	Neg	12	36			
Quantitative variables		**Mean value (min-max)**		**P**	**OR**	**CI 95%**
BUN	PCR pos	119 (18-600)		0.047	1.78	0.85-6.27
	PCR neg	93 (5.59-583)				
NEUTR	PCR pos	10.84 (0.21-37.5)		0.050	2.01	1.03-9.58
	PCR neg	8.30 (0.33-22.12)				
HCT	PCR pos	18.37 (4.6-43.4)		0.032	0.86	0.05-1.46
	PCR neg	24.94 (4.2-73.4)				
RBC	PCR pos	4.04 (0.95-10.12)		0.0480	0.78	0.01-10.45
	PCR neg	5.34 (1.04-11.65)				
Hb	PCR pos	6.15 (1.6-14.3)		0.050	0.81	0.44-7.43
	PCR neg	8.18 (1.7-27.1)				

Table 6: *L. infantum* PCR results were found to be significantly associated to Feline Immunodeficiency Virus (FIV) infection, to higher values of Blood Urea Nitrogen (BUN) and Neutrophils (NEUTR), and to low values of Hematocrit (HCT), Red blood cells (RBC), and Hemoglobin (Hb). Western blot (WB) positivity was instead positively associated to concurrent *Mycoplasma* spp. infection. The data of each covariate are summarized in the table.

3. Activity of melatonin against *Leishmania infantum* promastigotes by mitochondrial dependent pathway

Ehab Kotb Elmahallawy, Aroa Jiménez-Aranda, et al. Activity of melatonin against Leishmania infantum promastigotes by mitochondrial dependent pathway. Chem Biol Interact Journal. 2014; 220C:84-93.

The objective of the present investigation was to determine the effects of exogenous melatonin on *L.infantum* promastigote *in vitro*, analyzing the changes in parasite viability and some mitochondrial parameters in treated and untreated promastigote cultures.

Inhibitory effect of melatonin on promastigotes and determination of IC $_{50}$

Figure 4 depicts the inhibitory effects of melatonin against *L. infantum* promastigotes as a function of the concentration used. The percentage of parasite inhibition (PPI) with respect to controls values was 13.9%, 18.9%, 32.9%, and 58.3% at concentrations of 1, 10, 25, and 50 nM melatonin, respectively (IC $_{50}$ / 72 h = 42.8± 0.45 nM). Amphotericin B achieved a PPI value of 70.9%.

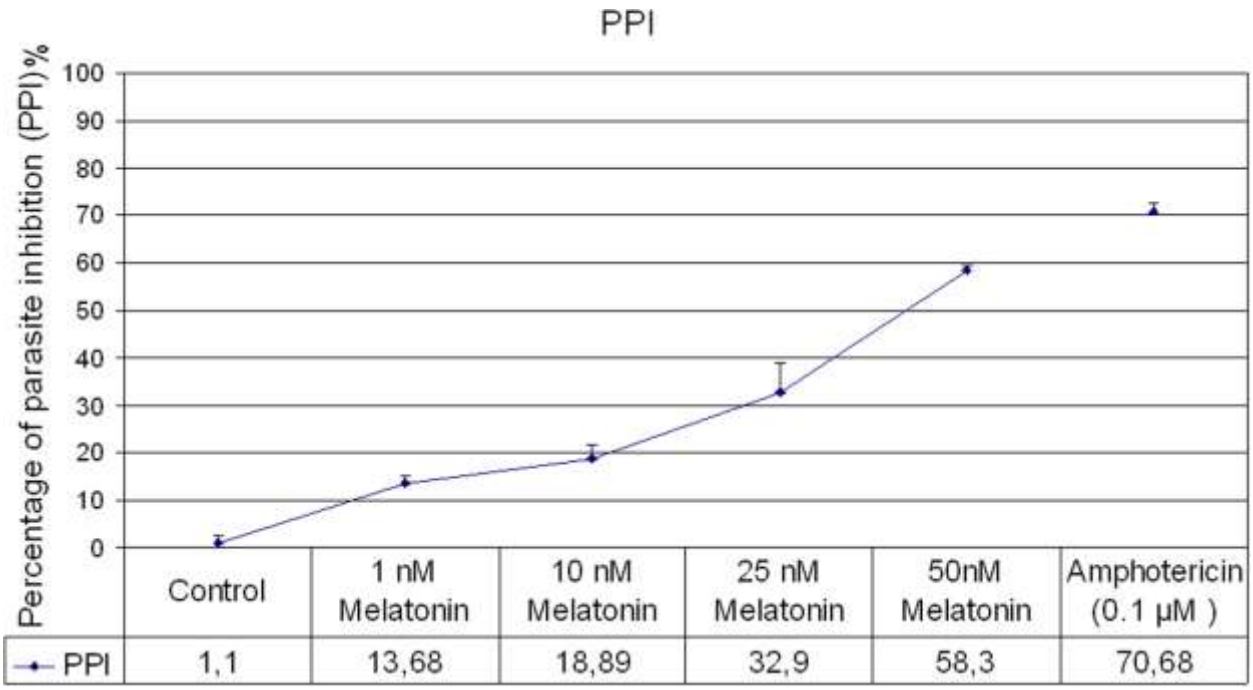

Figure 4: Percentage of parasite growth inhibition against tested agents after 72 hours of co-incubation. Values are means ± S.E.M.

Alteration of Calcium Retention Capacity and sensitivity of mPTP opening

Figure 5 shows the effects of treatment with melatonin (at 1, 10, 25, or 50 nM) or with amphotericin on calcium-induced mPTP opening of the isolated mitochondrial fractions in comparison to negative and positive (CsA-treated) controls. Addition of Ca^{2+} or melatonin stimulated the release of accumulated Ca^{2+} in all isolated mitochondrial fractions, and this release was hindered by CsA. The AUC value was significantly higher for treatments with 25 or 50 nM melatonin or amphotericin than for treatments with lower melatonin concentrations or for controls, indicating a greater mPTP opening, given that the mPTP opens earlier with lower Ca^{2+} retention capacity and vice versa.

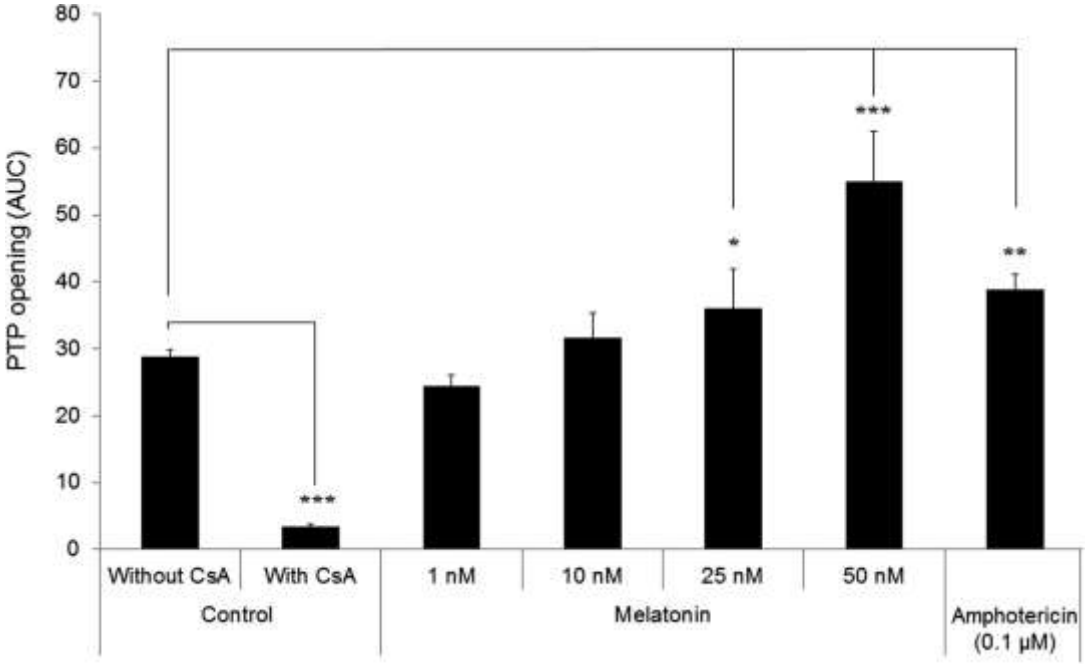

Figure 5: Calcium-induced permeability transition pore (PTP) opening of control (untreated) and amphotericin- and melatonin-treated promastigote cultures (1-50nM). Pulses of 250 µM Ca^{2+} were injected in the presence of 0.25 µM Oregon green until pore opening. 1 µM Cyclosporine A (CsA) was added *in vitro* to the isolated mitochondria. PTP opening was calculated from the Area under the Curve (AUC) of fluorescence signals versus time. Values are means ± S.E.M. Superscript letters refer to significant differences measured using one-way ANOVA followed by the Tukey post-hoc test. (* $P < 0.05$ in 25 nM melatonin-treated cultures; ** $P < 0.01$ in amphotericin-treated cultures; and *** $P < 0.001$ in CSA-treated mitochondria and 50 nM melatonin-treated cultures, all versus control cultures.

Melatonin increased Mitochondrial Nitrites Levels

As shown in Figure 6 A, the level of mitochondrial nitrites was significantly higher in the cultures treated with the highest melatonin concentrations (25 or 50 nM) than in the untreated cultures, reaching a similar level to that in the amphotericin-treated culture.

Melatonin non-significantly reduced SOD Activity

Figure 6 B depicts the mitochondrial SOD activity in treated and untreated cultures. Although SOD activity was lower in cultures treated with melatonin (especially 50 nM) than in controls, no significant difference was found with any melatonin concentration, whereas a significant reduction was observed with amphotericin.

Melatonin impaired activities of respiratory complexes I, II and III

Clearly, the promastigote cultures treated with 25 or 50 nM melatonin or with amphotericin showed significantly impaired complex I, II, and III activities in comparison to controls (Figures 7 A, B and C).

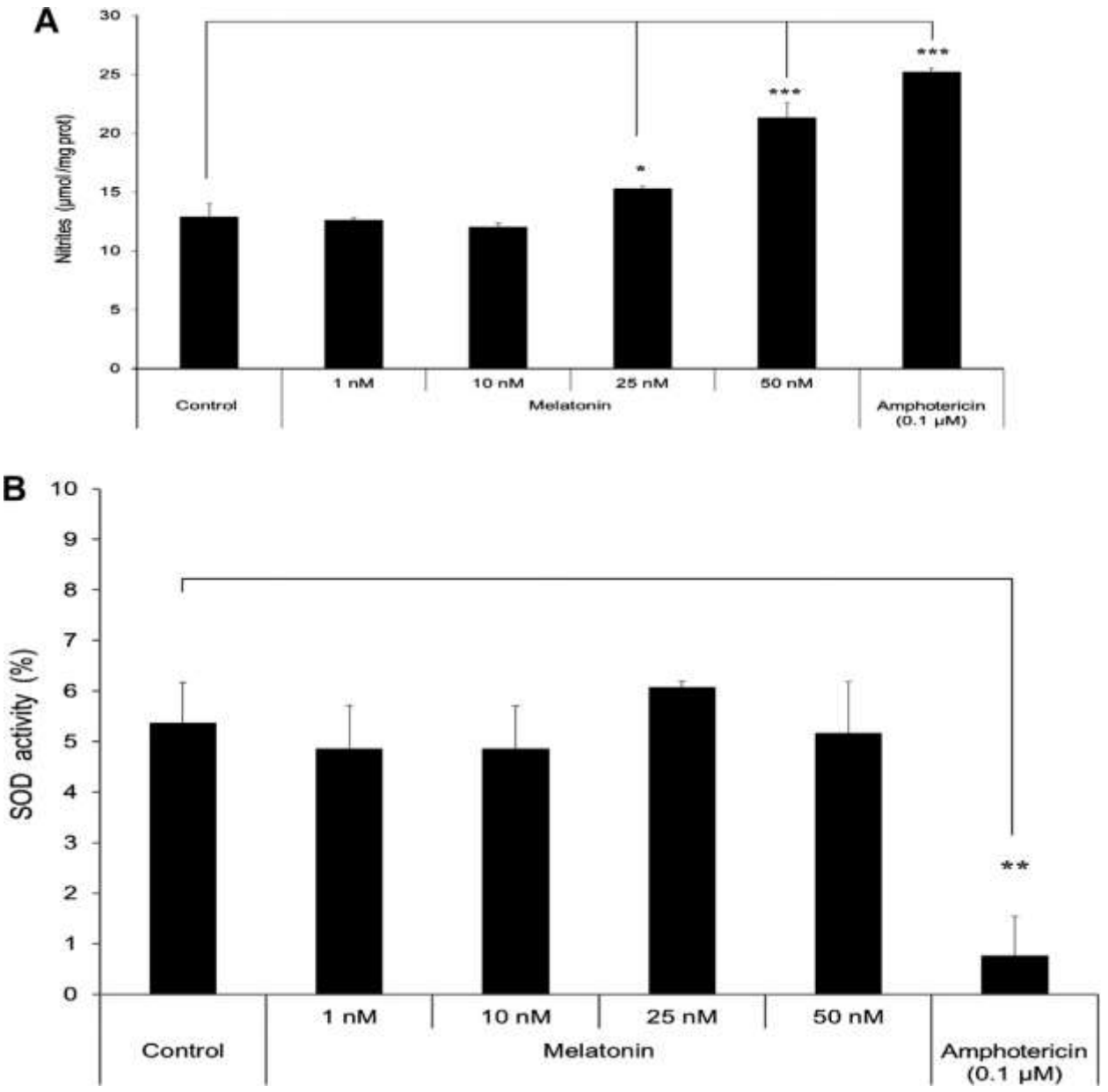

Figure 6 A. Nitrites concentration in mitochondria isolated from control (untreated), amphotericin, and melatonin-treated cultures.

Figure 6 B. Superoxide dismutase (SOD) activity in mitochondria isolated from control (untreated), amphotericin-, and melatonin-treated cultures. Values are means ± S.E.M. Superscript letters refer to significant differences measured using one-way ANOVA followed by the Tukey post-hoc test (*P <0.05 in 25 nM melatonin-treated cultures compared with control; *** P < 0.001 amphotericin and 50 nM melatonin-treated cultures versus control cultures).

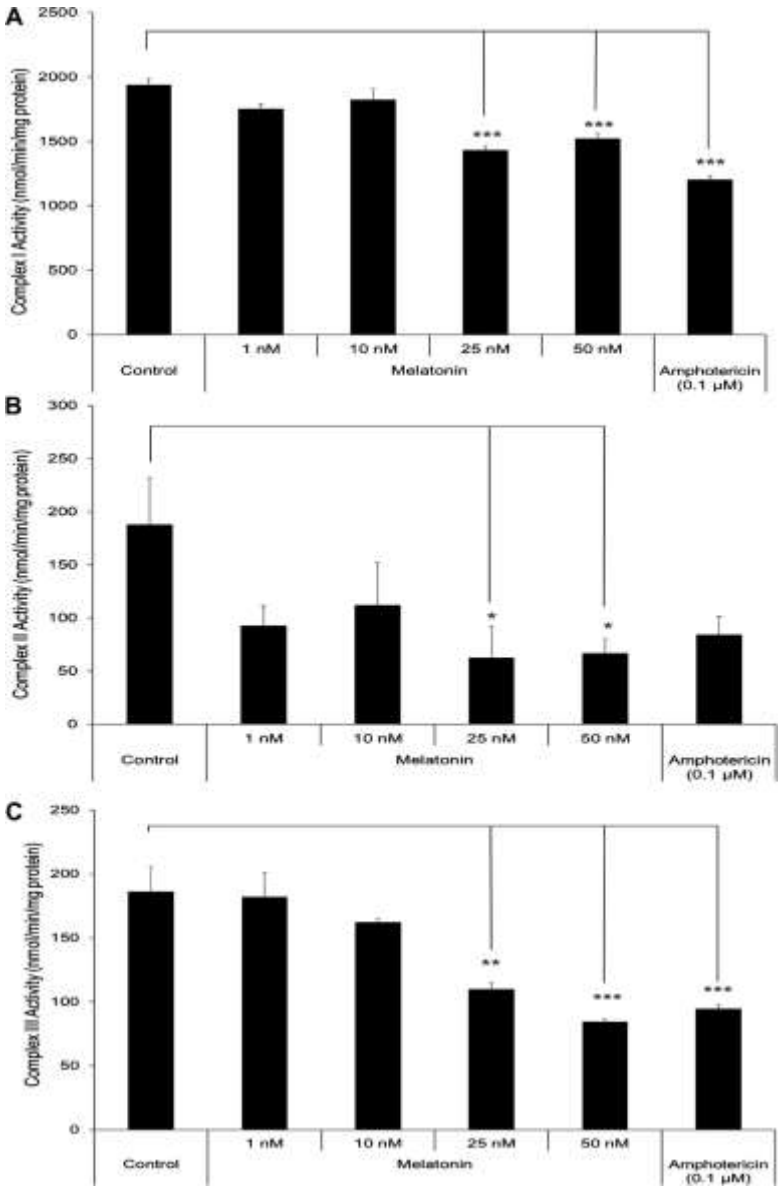

Figure 7 A, B, C. Activities of the electron transport chain complexes I, II, and III respectively, in mitochondria isolated from untreated and treated promastigote cultures. Values are the means ± S.E.M. Superscript letters refer to significant differences measured using one-way ANOVA followed by the Tukey post-hoc test (Complex I : *** P < 0.001 for amphotericin- and 25 and 50 nM melatonin-treated cultures versus control cultures; complex II: * P <0.05 melatonin treated cultures at concentrations of 25 and 50 nM compared with control cultures; and complex III: ** *P* < 0.01 for 25 nM melatonin-treated cultures; *** P < 0.001 for amphotericin- and 50 nM melatonin-treated cultures versus control cultures).

Discussion

1. General aspects

Leishmaniasis refers to a group of vector-borne diseases caused by an opportunistic intracellular protozoan parasite of the genus *Leishmania,* belonging to the family *Trypanasomatidae*, order *Kinetoplastida* [1, 282, 805]. The epidemiological profile of the disease include humans, rodents, domestic and wild animals but the dogs are the most important reservoirs in a domestic environment [44]. This clinically heterogeneous syndrome encompasses subclinical, localized (skin lesion), and disseminated (cutaneous, mucocutaneous, and visceral) infection and the spectrum of manifestations depends on the immune status of the host, on the parasite, and on immunoinflammatory responses. The impact of the disease on public health has been increased due to its zoonotic importance. Among 15 well-recognized species of subgenus *Leishmania*, 13 species are zoonotic [57, 282]. Furthermore, the distribution of disease has traditionally been linked to tropical and subtropical regions besides being endemic in many areas worldwide such as Mediterranean basin, East Africa and South America [1, 282].

The last decade has witnessed an extraordinary progress in the spread of the infection to new areas, in particular due to global change and/or increases in global movements of hosts and vectors [1, 167, 806-809]. This resulted in the appearance of new endemic disease foci together with increasing reports of new reservoirs and the new transmission routes. On the other hand, over the past decade, new formulations of standard drugs have become available and registered for use in many countries. However, although around 25 compounds and formulations are available to treat leishmaniasis in humans [373, 791], all are associated with toxicity and/or drug resistance problems, and there is an urgent need to develop an effective drug against all forms of leishmaniasis [299, 810]. In fact, many available antileishmanial agents can exert their effects through the disruption of Ca^{2+} homeostasis in the parasite and /or through changes in different mitochondrial parameters [714, 782].

With this background, the disease has spurred the interest of the scientific research towards the role of several animal species as potential reservoirs for the infection and as consequence led to the necessity for more surveying using a rapid and reliable diagnostic test and search for novel less toxic antileishmanial drug, aiming to construct effective control measures adopted to eliminate transmission and prevent new epidemics [1, 15, 780].

In this regard, our intial goal was to assess the included a seroloprevalence of *Leishmania* infection among transplanted organ recipients from southern Spain followed by investigation the role of domestic cats from an endemic region in North-western Italy in *Leishmania infantum* epidemiology, by the association of both serological and molecular tests. In the second phase of our work, we have studied the effect of melatonin against the promastigote phase of the parasite *in vitro.* The first results showed a relatively high prevalence of *L.infantum* was recorded among kidney transplanted recipients. These novel findings in this area suggest that routine serological testing for VL should be done before undergoing transplantation for both donor and recipient transplant patients living or traveling in endemic areas.

Furthermore, we have found high prevalence of *L.infantum* among cats in the studied, confirming the importance of cats not only as reservoir for the disease, but also the need for further future research for accurate diagnosis of this zoonosis. In accordance with therapeutic trials, melatonin not only demonstrated a significant antileishmanial activity against the promastigote phase of the parasite *in vitro* but was also accompanied by an alteration of the several mitochondrial parameters, including calcium homeostasis and by changes in some mitochondrial parameters critical to parasite survival. In conclusion, the findings presented in these papers represent the begining of novel strategy for surveying and combating leishmanisis.

2. Leishmaniasis among renal transplanted patients

As previously mentioned, VL is considered to be a well-established zoonotic disease in the area of Mediterranean basin, especially in southwestern Europe, where approximately 700 cases per year appear and the clinical form is frequently associated to immunosuppression[2, 277]. The actual prevalence of leishmaniasis in Spain is not completely known [168], however, in our geographical area, southern Spain, there are studies in Alpujarra Region (Granada province) and Axarquia region (Malaga province) of rural population, using the leishmanin skin test (LST) (test cell immunity), published in 1996 with average positivity rates exceeding 40% in both studies [811, 812]. Nationwide, more recent studies have been made in areas close to ours, such as the study performed on blood donors from Ibiza (Balearic Islands, Spain) in 2004 which indicated a high rate of asymptomatic infection on the island and another study in 2008 from Balearic Islands that confirms this high prevalence [813, 814]. Apart from these studies mentioned previously, the most studied groups of population have been HIV infected individuals and

intravenous drug users in our environment (southern Spain) [261, 815, 816]. There are not many publications about the known prevalence of *Leishmania* in special groups such as transplant recipients, oncological or other immunodeficient non HIV groups in Spain and the Mediterranean region.

Leishmaniasis is not a common disease among transplant recipient patients but with this growing pool of transplant survivors and increasing migration dynamics, the numbers of infected cases among transplant recipients are in a steady increase especially among renal transplant recipients mainly in southern Europe [817], which is considered to be an additional challenge [818, 819]. Transmission of VL to this specific group of population occurs through different routes: *Leishmania* transmission by sandflies after transplantation when the specific group of population is living in an endemic area or makes a short stay in an endemic area represents a route of transmission [820, 821], also it may have originated from a donor with an undiagnosed infection, or a recrudescence or to a latent infection in previously infected recipient during a period of immunosuppression. Infection can also be acquired through blood transfusion [822].

Although VL is a rare disease among transplant patients, the current situation has been changed and the number of published cases has increased in recent years[823]. More than 100 VL cases following transplantation have been reported worldwide, predominantly described in renal transplantation [819, 823, 824]. The prevalence of asymptomatic infections of *Leishmania* spp varied from 0.6% to 71% in endemic areas [117]. To detect asymptomatic carriers, direct method (PCR, culture, microscopic examination) or indirect methods (IFA test, direct agglutination test, ELISA with single or crude *Leishmania* antigens, Western blot, immunochromatographic techniques) can be used but there is not a gold standard test able to identify asymptomatic infection with high sensitivity and specificity [117, 825].

The prevalence of *Leishmania* infection in our study is in agreement with previous studies in different geographical areas of our country, Balearic Islands and Castilla León, with results of prevalence of 3.1 % and 4.9% in blood donors and general population respectively [814, 826]. More recent studies, like the one published in the province of Seville on a small population that included intravenous drug users and non-drug injectors, where the frequency of Leishmania–seropositive was high, showed prevalence of infection by *Leishmania* of 24% using PCR-ELISA. This study showed that a remarkable proportion of asymptomatic *Leishmania*

seropositive individuals at risk for parenterally transmited infections carry *Leishmania* kDNA in blood [261]. Some studies have been made in southern Spain in asymptomatic HIV population diagnosing by amplification of Kinetoplast DNA (kDNA) from peripheral blood showing a prevalence of 30.8% (28/92) infection by *Leishmania.* No patients showed positive results by other techniques like ELISA, WB or leishmanin skin test. Conversely, patients with a negative PCR result showed prevalence values respectively of 3.5%, 2.4% and 4.3% when tested by ELISA , WB and LST [816] . In other endemic zones for *L. infantum* like Brazil, studies have recently been published, which raise interest in the detection of asymptomatic infection in transplant population living in the endemic areas as well as the need to use more sensitive techniques for screening this population group, such as the research of Clemente et al. (2014) on asymptomatic *Leishmania* infection among liver donors and recipients. The results of this study show a prevalence of *Leishmania* infection of 1.5% using serological techniques which increased to 7.5 %, 8.9 % and 5.9 % respectively in blood samples, liver samples and splenic samples using molecular methods [824].

The results obtained in our study (4.8%) using a unique serological technique may be underestimated, because IFAT has less sensibility than other indirect assays (ELISA)[117]. The use of two serological methods could slightly increase the proportion of asymptomatic carriers detected [827, 828]. On the other hand, antibody detection may not reflect a chronic infection but more a recent contact with parasite followed by cure; PCR assay would have greater sensitivity [829] than the indirect method and reflects actual parasitism rather than a previous infection by *Leishmania*. Different studies highlight the added value of using a combination of tests (molecular and serological) to increase the capacity to detect asymptomatic *Leishmania* infections [117, 825]. It is at present not possible to predict exactly who and when among the asymptomatically infected people will develop VL disease [830]. Reasons causing an infection to remain asymptomatic or progress to VL are likely the result of a complex interaction between environment, parasite and host related factors [831].

Studies of the risk of progression from infection to disease have yielded contradictory results based on serological status, the data revealed strong associations between the magnitude of positive serology and risk of progressing to symptomatic VL [832].

International guidelines for the management of transplant recipients recommend specific serology in donors and recipients from endemic areas, regardless of laboratory test limitations and availability [833]. Given that patients such as transplant recipients are susceptible to develop leishmaniasis, and the clinical manifestations in these patients can be serious, it seems advisable to perform a screening of serological status in this population group and a more intense monitoring of patients with positive serology with high titers in order to prevent possible development of disease.

3. Role of cat in *Leishmania infantum* epidemiology

Our data shows that cats play an important role as *L.infantum* host, represented by higher prevalence of *Leishmania infantum* among the examined cats either serologically using WB or molecularly using PCR. This survey is the first epidemiological investigation performed about leishmaniasis in cats in this endemic area in North-western Italy (Liguria). Likewise, it gives us more evidences about the correlation between infection of cats with *L. infantum* as widely known opportunistic pathogen and concomitant infection with some pathogens like FIV, FeLV and three feline hemoplasma species that include: *Mycoplasma haemofelis*, *Mycoplasma haemominutum*, and *Mycoplasma turicensis*.

As previously mentioned, cats rarely show clinical signs of VL and usually limited to skin ulcers. A scant clinical cases appear with typical signs of cutaneous forms, including ulcer crusted dermatitis and nodular lesions on the nose, lips, ears, and eyelids [834]. Other groups may develop chronic ulceration, located particularly on the head and limbs [834]. In rare reported cases of VL, the infected cases showed visceral involvement of liver, spleen, lymph nodes and kidneys. Also, they are accompanied, almost always, with cutaneous manifestations [57, 63, 835, 836]; and there may also be local or generalized lymphadenopathy, ocular lesions, acute renal failure, epistaxis, lameness, anemia, hepatosplenomegaly, icterus, lymphoplasmocytic gastroenteritis with abundant *Leishmania* parasites, and membranous glomerulonephritis [834-837]. Despite this previous fact stats that leishmaniasis is not common in cats, some previous studies have examined the occurrence of feline leishmaniasis worldwide, especially in several countries where zoonotic leishmaniasis is present like America, Brazil and Iran [66-70, 838, 839].

In Europe, several clinical cases of feline leishmaniasis have been described since 1911 to date in France, Greece, Switzerland, Spain, Italy, and Portugal, with very controversial results among serological and molecular methods [63, 66, 67, 840-844]. In southern Europe where the disease is endemic, in particular Italy, several sero-epidemiological studies have shown a prevalence of *L.infantum* in cats ranged from 0.6% to 68%, which is differing among countries [62, 66, 839-842, 845-850], while the positivity to PCR ranging from 3% to 61% [841, 851]. This may explain the influence of geographical location on the epidemiological pattern of the disease and as consequences variation in the efficiency of serological test used in each area.

Among other serological tests, western blotting is used to detect and quantify proteins that react with a specific antibody. As reported in some previous studies with *Leishmania*, this test has shown a good sensitivity, greater than that IFAT and ELISA, which make it recommended mainly in doubtful cases besides its high specificity and sensitivity [852, 853]. Regarding our results, we have reported positivity of 28.37% using PCR which is nearly similar with a previous study using the same method [840]. Nevertheless, the reported prevalence rate, these findings highlights the role played by cats in the transmission of disease, which has been confirmed through sandflies infection contraction from cats naturally infected with *Leishmania* [854], suggesting that cats could play an important role as secondary reservoir host for *L. infantum*.

In the present study, the prevalence of infection using WB yielded 13.20% which is either lower or higher than previous reports in the same country or even worldwide; this may be attributed to the difference of serological technique used as the majority of studies have used either ELISA [62], IFAT [69, 840, 841, 845, 850] or even DAT [848], and may explain the role of WB as an accurate test to diagnose the clinical disease than to detect the infection. Furthermore, a previous study in Liguria and Tuscany has recorded much lower seroprevalence (0.9%) than our study using IFAT [845], which could be attributed to lower sensitivity of IFAT compared to WB and explains the possibility of other wild reservoirs increase [180, 855, 856] or even habit changes and expansion range of sandfly vectors which resulted in appearance of new endemic foci for the disease [806-809, 857]. As well, nearly similar results have been reported in some studies in the same country using different serological technique [847]. Beside the difference in the serological tests used, the difference in the prevalence rate between our study and the previous studies could be attributed to the geographic location which affects the degree of endemicity of the disease.

Taken into account, several serological studies have revealed that *Leishmania*-infected cats often develop a low level of humoral response or remain seronegative [841, 845].

To our knowledge, the polymerase chain reaction (PCR) is widely accepted as a molecular tool in epidemiological studies for identification and quantification of *Leishmania* spp. in various tissues and body fluids of reservoir or hosts [69, 785, 858]. Therefore, several studies have been recommended PCR as a highly efficient non-invasive tool for diagnosis and follow-up of the disease [258, 859, 860]. Our results reported either lower, higher or even nearly similar prevalence rate of *L. infantum* using PCR than in several previous studies [840, 841], which may be also attributed to difference of the geographic location which affect the degree of endemicity of the disease [1, 21, 57].

The role of some other pathogens like viral infection like FeLV or FIV or even *Mycoplasma* and its association to *Leishmania* infection is still unclear [845]. Based upon our going results, there was a statistical correlation between and positivity of sample to *L. infantum* using PCR and both FIV, anemic status of animal, blood urea nitrogen level and some other hematological parameters, whereas using WB, there was just association between positivity and *Mycoplasma* infection; which is similar to associations previously reported between these parameters and *Leishmania* infection [836, 845, 850, 861, 862]. This may suggest the role played some viruses like FIV as a retroviral infection in development of some opportunistic pathogens like *Leishmania* which is widely accepted [15]. However, it should be borne into mind that the only presence of FIV is not sufficient to demonstrate an immunodeficiency status and this needs additional immunological tests. On the other side, there was no a clear relationship between infected cats with FeLV to contract the infection by *Leishmania*, which is consistent with several previous results [62, 841, 842, 847, 849].

As shown, the use of direct methods (PCR) and indirect (WB) yielded discrepant results, which has allowed to show that the prevalence of infection in cats with double direct method than the serology. The explanation for this is not in the different sensitivity of the two methods, but rather in the fact that in species resistant to infection as the cat, the immune response activated is particularly of cell mediated than humoral immunity [861]. The data collected in our present work confirms the hypothesis suggesting that the cat predominantly a Th1 response, as evidenced by the high number infections (positivity to PCR) than the number of subjects with

circulating antibodies (positive to WB) and the limited non-specific clinical signs occurrence in animals in question [861, 863]. Also, our present data give more information about the association between *Leishmania* infection and both retrooviruses and hemoplasmosis, which seem to play a role in pathogenesis of disease or even contraction of the infection.

In conclusion, our study reports higher prevalence of *L.infantum* in cats which could be an important parameter when further epidemiological studies show xenodiagnosis. This means the infected cats are able to transmit the infection to the other reservoirs or vectors of the disease. Based upon these findings, our study might suggest including a routine serological testing for leishmaniasis in cats especially in endemic area. This contributes to the hypothesis that cats may be a *Leishmania* reservoir. Further studies are also needed to evaluate the role of cats in the epidemiology of *L. infantum* in the Mediterranean area. Other alternative prophylactic strategies may be also essential to reduce the risk of infection and identification for new models of leishmanial transmission.

4. Activity of melatonin against *L. infantum* promastigote

Our present work shows that exogenous melatonin reduces the number of viable *L. infantum* promastigote accompanied by alteration of some mitochondrial parameters that are target for parasite viability. This antileishmanial effect was associated with enhanced mPTP opening, suggesting alterations in intracellular Ca^{2+} distribution, and also an increase in mitochondrial nitrite levels and respiratory chain complex impairment. All of these effects may indirectly compromise parasite survival, leading to cell death.

To our knowledge, no published data are available on the potential biocidal activity of melatonin against *Leishmania*. Melatonin is known to regulate important physiological processes, including pubertal development, sleep wake cycle and seasonal adaptation [730]. Various studies have shown that melatonin has an antioxidant effect, a good pharmacological safety profile, and represents a potentially useful tool for stand- alone or adjuvant therapy for many degenerative and infectious diseases, including Chagas's disease and several immune disorders [610, 627, 716, 772, 864-871].

Among other properties, melatonin has also shown biocidal activity against a wide range of pathogenic agents including parasites [602, 660, 737]. As mentioned above, melatonin exert its

effects against some pathogenic agents through interaction through its interaction with intracellular proteins such as calreticulin [720], tubulin [721], or Calmodulin (CaM), antagonizing the binding of Ca^{2+} to CaM [722, 736]. Thus, MT1 and MT2 are transmembrane G-protein-coupled heterodimers whose signaling pathways lead to downstream effects on Ca^{2+} channels, Ca^{2+} signaling, and changes in mitogen-activated protein kinases and extracellular-signal-regulated kinases [724], explaining the role of melatonin in the regulation of Ca^{2+} ion fluxes in cells. However, it should be borne in mind that melatonin is also a very powerful free radical scavenger and antioxidant, and these actions do not require a receptor [716, 800].

As noted above, trypanosomatids possess a single mitochondrion that occupies 12% of the total volume of the parasite and has been recognized as drug target in many of these parasites [713]. In such cases, the antiparasitic agents target mitochondria through one or more of the following programmed cell death mechanisms: intracellular Ca^{2+} levels alteration [782, 872]; loss of membrane potential and ATP levels [771]; and increase of hydrogen peroxide (H_2O_2) and superoxide radical generation (O_2^-) [873]. The mitochondrion is known to be an essential organelle involved to be operative in the apoptotic pathway *in Leishmania*, but the main mechanisms by which the protozoal mitochondrion is involved in parasite apoptosis are not yet precisely understood [872]. Evidences have been published linking many leishmanicidal drugs with mitochondrial dysfunction, and the mitochondrion has become established as a target for antileishmanial drugs as in miltefosine and pentamidine [781, 874], which exert their effects via multiple pathways.

Ca^{2+} homeostasis is a crucial matter in all organisms [875]. In common with most eukaryotes, cell function in parasites is coordinated using second messenger signaling cascades involving cyclic adenosine monophosphate (cAMP) and Ca^{2+} [676, 876]. Moreover, in the family trypanosomatidae, Ca^{2+} is also involved in microtubule assembly, and differentiation processes, among numerous other functions [675]. Calcium antagonists, such as miltefosine, have shown antileishmanial activity by inducing a large increase in intracellular Ca^{2+}, thereby disrupting the parasite's intracellular Ca^{2+} homeostasis [781]. Other effective antileishmanial drugs against promastigote and amastigote phase, such as tafenoquine and amiodarone, were found to be partly localized in acidocalcisomes, important Ca^{2+} reservoirs in *Leishmania*; increasing the free cytosolic Ca^{2+} level mobilized from intracellular stores; disrupting Ca^{2+} homeostasis, resulting in mitochondrial damage [714, 782].

Thus, fluctuations of cytosolic free Ca^{2+} concentrations may regulate a various cellular functions in all eukaryotes. The lytic effect of trypanocidal drugs against *Leishmania* and African trypanosomiasis was attributed to the alteration of calcium homeostasis produced by Ca^{2+} mobilization from intracellular stores during the progression of apoptosis [676, 877]. It has also been proposed that cell damage results from the formation of reactive oxygen species (ROS) induced by Ca^{2+} alterations [878]. The oxidative phosphorylation process produces an accumulation of ROS such as H_2O_2 and O_2^- which are considered toxic products of mitochondrial respiration and have been implicated in altered Ca^{2+} homeostasis in parasites [879, 880]. Accumulation of these products in in the mitochondrion mediates the oxidation of critical thiol groups of adenine nucleotide translocase. This results in opening of the mPTP, which causes collapse of mitochondrial membrane potential ($\Delta\Psi$ m), loss of matrix solute, mitochondrial membrane swelling, and indirectly enhance the release of death factors into the cytosol, leading to cell death [881-883].

As shown in our results, a significantly higher mPTP opening were observed in melatonin-treated cultures of *L.infantum* promastigotes, especially at a dose of 50 nM, than in control or amphotericin-treated cultures, signifying a reduced Ca^{2+} retention capacity with an earlier PTP opening. As expected; CsA had a blocking effect on mPTP opening. Interestingly, these findings confirms the phenomenon stats that mPTP opening involved in respiratory chain inhibitor-induced dissipation of $\Delta\Psi$ m and corroborates with previous observations that PTP opening induces $\Delta\Psi$ m collapse through a proton dissipation pathway [884].

Clearly, our data also support the hypothesis that *Leishmania* parasite utilizes intracellular Ca^{2+} based signaling during maturation and suggests that melatonin may modulate the cell cycle of *Leishmania* through its action on mPTP opening, calcium retention capacity, and the release of intracellular Ca^{2+}, disrupting the Ca^{2+} homeostasis of the parasite [675]. This is considered the most striking effect of melatonin treatment in our work as stimulation of the Ca^{2+} -induced mPTP in mitochondria isolated from melatonin-treated cultures, which either alone or in association with the other mitochondrial parameters, indirectly acts as an initiator of induced cell death [883, 885-888]. Since mPTP is widely accepted be the most notorious of all the inner membrane megachannels and is considered the gatekeeper of apoptotic and necrotic cell death [883, 884, 888-892]. A previous study reported that increased calcium permeability is not responsible for the rapid lethal effects of amphotericin on *Leishmania* [349]; however, a more

recent study concluded that amphotericin produces osmotic cell lysis by formation of aqueous pores in in the membrane of promastigotes [350], establishing the role of Ca^{2+} in amphotericin-induced apoptosis through osmotic alteration, consistent with our results [303].

In accordance with mitochondrial nitrites (NO_2^-) level, results for the level of NO_2^- suggest a potential role for the mitochondrion in regulating nitrite concentrations, which are considered an indicator for the stable breakdown product of NO in mitochondria. Several studies have revealed a major role of NO in controlling *L. major* and *T. cruzi* multiplication *in vivo* and *in vitro* [760, 761]. Melatonin administered to *T.cruzi*-infected animals was found to increase NO production, inhibiting parasite propagation or killing the parasite [736]. NO accumulation was also reported to inhibit the mitochondrial respiratory chain, resulting in inhibition of ATP production, greater ROS production, and increased the susceptibility to cell death. This cytotoxicity was preceded by a decrease in $\Delta\Psi$ m followed by an increase in O_2^- and peroxynitrite levels [893].

Our results are in the same line, with a significant increase in nitrate level in cultures treated with 25 or 50 nM melatonin or amphotericin *versus* the control cultures. At 1 or 10 nM melatonin, however, the nitrite levels were non-significantly higher than those of control of control cultures. These non-significant variations may be attributable to the antioxidant effect of melatonin at these lower concentrations, hindering free radical production, given reports that melatonin and its metabolites are effective direct scavengers of nitric oxide free radical ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^-$) [894]. It should also be taken also into account that the induced nitrite levels in the previous studies were *in vivo* and therefore mainly based on the immune system, in which the activated macrophages were reported to synthesize NO, considered the major effector molecule of *T. cruzi* intracellular amastigote killing [735, 760].

Several studies have reported that *Leishmania* species are susceptible to ROS and reactive nitrogen species [895]. Three types of SOD have been identified: Cu/Zn-SOD, Mn-SOD, and Fe-SOD [896]. They are considered one of defense enzymes, protecting the parasites from mitochondrial-derived ROS damage and programmed cell death by detoxifying superoxide into hydrogen peroxide and oxygen, thereby maintaining the integrity of the mitochondrion [897].

Mitochondrial Fe-SOD has been shown to protect the *Leishmania* parasite from oxidative stress, and inhibit programmed cell death, as observed in miltefosine-induced cytotoxicity in *L. donovani* [897, 898]. However, we found non-significantly lower Cu/Zn-SOD activities in melatonin-treated cultures (all concentrations) than in control cultures. This lack of a significant

difference may be attributable to the modest effect of Cu/Zn-SOD in *Leishmania*, the antioxidant or protective effect of melatonin at lower concentrations, the parasite strain, among other possibilities. Consistent with these findings, a recent study reported the negligible inhibition of human Cu/Zn-SOD with pyrazole-based benzo[g]phthalazine derivatives on *L. infantum* and *L. braziliensis* [899].

It is noteworthy that many available antileishmanial agents act by impairing of mitochondrial complexes. Complexes I, III and IV are known to function as H^+ ions (protons) pumps, generating a proton electrochemical gradient that drives ATP synthesis, explaining why mitochondrial complexes are the primary source of endogenous ROS [900]. Thus, inhibition of these complexes by antileishmanial agents interferes with electron transport processes, resulting in increased ROS from diverted electron flow, as in the case of antimycin A and tafenoquine against *L. donovani* [782, 901].

In the present study, melatonin produced a significant inhibition of mitochondrial complexes I, II and III, which was marked at the highest concentrations of melatonin (25 and 50 mM). However, the activity of complex I is very low in *Leishmania*, and only the enzyme corresponding to rotenone-insensitive NADH: quinone oxidoreductase has been observed in *Trypanosoma* and *Leishmania* [902]. Therefore, these parasites possess only one NADH dehydrogenase gene in their total DNA that may be essential for parasite survival; explaining its appeal as a drug target [902]. Inhibition of complex I would result in generation of ROS and therefore leads to mitochondrial dysfunction, which in turn triggers the apoptotic mitochondrial pathway [782, 902, 903]. The present results are in agreement with the previous findings on the antileishmanial effect of luteolin resulted from reduction in the activities of electron transport chain complexes I, II, III, and IV [904].

According to results, a drastically lowered activity of complex II on melatonin-treated cultures versus control cultures was observed. This result correlates with report of induced death in *Leishmania* using new Benzophenone-Derived Bisphosphonium Salts, which inhibit complex II [905].

Several studies have described complex III as an attractive target for antiprotozoal drugs [904]. Tafenoquine was found to cause a mitochondrial dysfunction in *Leishmania* by complex III (cytochrome *c* reductase) inhibition [782]. In these cases, the stoppage of electron transfer may reduce the oxygen consumption rate, causing an imbalance of Ca^{2+} homeostasis and dissipating

the ΔΨ m that is accompanied by production of O_2^-, triggering death of the parasite [782, 895, 901, 906]. It should be stressed that inhibition of Complexes I , II and III results in an intracellular increase of Ca^{2+} that intimately associated with ROS generation, while the inhibition of complex II markedly alters Ca^{2+}[895, 905].

These are highly promising findings, given that complex I inhibition may result on mitochondrial hyperpolarization that is usually preceded by increased O_2^- production, while inhibition of complexes II and III might cause dissipation of ΔΨ m. Both hyperpolarization and loss of ΔΨ m might target the viability of the promastigotes [907, 908]. The inhibitory effect of melatonin on these mitochondrial complexes support the proposition that *Leishmania* mitochondria respond strongly to the inhibition of respiratory chain complexes [909].

The role of mitochondria in our findings may be initiated by the effects of melatonin on mPTP, a key implication, through disruption of Ca^{2+} homeostasis which indirectly drives to lethal effects resulting in cell death by apoptosis or necrosis [888]. As shown in the results, melatonin-treated cultures depleted some respiratory components, probably due to the opening of mPTP. Remarkably, impairment of activity of mitochondrial respiratory enzymes in treated cultures may push the mitochondria towards mitochondrial oxidative stress expressed by inhibition of electron transfer, together with inhibition of SOD and the resulted nitrosative stress which may act collectively as a major inducer of mPTP opening, thereby indirectly drives to lethal effects by apoptosis or necrosis [878, 910]. All of the above impressive changes in melatonin-treated cultures are in part attributable to alterations in mPTP opening, accompanied by higher mitochondrial nitrite levels and inhibition of the activity of mitochondrial complexes especially II and III, and these events may indirectly trigger death of the parasite by apoptosis or necrosis.

The present results indicate that melatonin exerts *in vitro* activity on promastigote phase of *L.infantum*, producing mitochondrial alterations in Ca^{2+} distribution, nitrite concentrations, and complexes I, II and III, thereby targeting the parasite survival. Further research is warranted to elucidate the *in vivo* effects of melatonin alone and in association with other antileishmanial drugs in order to develop a novel chemotherapeutic strategy against the disease. There is also a need to examine the mechanisms underlying these effects and the role of melatonin receptors.

Conclusions

Conclusions

1. According to the recorded prevalence in this study, transplant renal recipients are susceptible population to develop Leishmaniasis which emphasizes the impact of the disease in our area. Clearly, it is suggested to perform a dequete and intense monitoring in this type of patients with high titers in order to prevent the development of the serious and fatal forms of leishmaniasis.
2. Given the limitations of serological techniques, a combination of serological tests together with molecular techniques could of higher efficacy in such type of patients.
3. High prevalence of *L.infantum* in cats could be an important parameter when further epidemiological studies show xenodiagnosis. This means the infected cats are able to transmit the infection to the other reservoirs or vector of the disease.
4. Further efforts should be done to control feline leishmaniasis and the human disease in endemic areas and other alternative prophylactic strategies may be also essential to reduce the risk of infection and identification for new models of leishmanial transmission.
5. Melatonin exerts an interesting antileishmanial activity against the parasite *in vitro*. This observation together with its high pharmacological safety profile make melatonin a potentially useful tool for stand-alone or adjunct therapy for leishmaniasis. Hence, further studies are suggested to investigate the effects of melatonin *in vivo* in association with other available antileishmanial drugs, followed by investigation the role of receptores underlying these effects.

Conclusiones

1. En función de la prevalencia encontrada en este estudio, los receptores de trasplante renal son una población susceptible de desarrollar leishmaniasis, siendo por tanto importante en nuestra área llevar a cabo esta determinación en este tipo de pacientes y realizar una vigilancia más adecuada en aquellos pacientes con títulos mas altos, para prevenir la posible aparición de formas graves de *leishmnia*.
2. Dadas las limitaciones de las técnicas serológicas, el uso combinado de estas técnicas, junto con técnicas de biología molecular podría mejorar el diagnóstico de leishmaniasis en este tipo de pacientes.
3. La alta prevalencia de *L. infantum* en gatos podría ser un importante parámetro cuando estudios epidemiológicos muestran xenodiagnosis. Esto significa que los gatos infectados pueden transmitir la infección hacia otros reservorios o vectore de la enfermedad.
4. Deben realizarse más esfuerzos para controlar la leishmaniosis felina y la enfermedad humana en las zonas endémicas y otras estrategias profilácticas alternativas puede ser también necesarias para reducir el riesgo de infección y la identificación de nuevos modelos de transmisión de *Leishmania*.
5. La melatonina ejerce una actividad anti-leishmaniosis interesante contra el parásito in vitro. Esta observación, junto con su alto perfil de seguridad farmacológica hace a la melatonina una herramienta potencialmente útil para la administración por sí sola o terapia adjunta para la leishmaniasis. Por lo tanto es necesario más investigación en los efectos de la *melatonina in vivo* y en asociación con otros fármacos contra *Leishmania*, seguidos del papel de los receptores de melatonina en estos efectos y sus mecanismos subyacentes.

Bibliography

Bibliography

1. Clem A: A current perspective on leishmaniasis. *J Glob Infect Dis* 2010, 2(2):124-126.
2. Ready PD: Leishmaniasis emergence in Europe. *Euro Surveill* 2010, 15(10):19505.
3. Control of the leishmaniases. *World Health Organization technical report series* 2010(949):xii-xiii, 1-186, back cover.
4. WHO: Leishmaniasis Fact sheet N°375". World Health Organization. January 2014. Retrieved 17 February 2014.
5. Barrett MP, Croft SL: Management of trypanosomiasis and leishmaniasis. *British medical bulletin* 2012, 104:175-196.
6. Herwaldt BL: Leishmaniasis. *Lancet* 1999, 354(9185):1191-1199.
7. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY *et al*: Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012, 380(9859):2095-2128.
8. Rabkin SW, Klassen GA: Session on sodium and calcium in the management of hypertension summary of remarks of discussant and chairman. *Can J Physiol Pharmacol* 1986, 64(6):784-785.
9. Chang and Bray R: Human Parasitic Diseases: Leishmaniasis, Elsevier Science Publishers, Oxford, 1985.
10. Desjeux P: Human leishmaniases: epidemiology and public health aspects. *World health statistics quarterly Rapport trimestriel de statistiques sanitaires mondiales* 1992, 45(2-3):267-275.
11. Machado CM, Martins TC, Colturato I, Leite MS, Simione AJ, Souza MP, Mauad MA, Colturato VR: Epidemiology of neglected tropical diseases in transplant recipients. Review of the literature and experience of a Brazilian HSCT center. *Revista do Instituto de Medicina Tropical de Sao Paulo* 2009, 51(6):309-324.
12. Alvar J, Canavate C, Gutierrez-Solar B, Jimenez M, Laguna F, Lopez-Velez R, Molina R, Moreno J: *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. *Clin Microbiol Rev* 1997, 10(2):298-319.

13. Alvar J, Yactayo S, Bern C: Leishmaniasis and poverty. *Trends in parasitology* 2006, 22(12):552-557.
14. Desjeux P: Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 2004, 27(5):305-318.
15. Elmahallawy EK, Sampedro Martinez A, Rodriguez-Granger J, Hoyos-Mallecot Y, Agil A, Navarro Mari JM, Gutierrez Fernandez J: Diagnosis of leishmaniasis. *Journal of infection in developing countries* 2014, 8(8):961-972.
16. Chang K.P. B, R.S. : Human Parasitic Diseases: Leishmaniasis, Elsevier Science Publishers, Oxford, 1985.
17. Cheng CY, Mruk DD: The blood-testis barrier and its implications for male contraception. *Pharmacological reviews* 2012, 64(1):16-64.
18. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M: Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* 2007, 5(11):873-882.
19. Thakur CP, Kumar K: Post kala-azar dermal leishmaniasis: a neglected aspect of kala-azar control programmes. *Ann Trop Med Parasitol* 1992, 86(4):355-359.
20. Das VN, Pandey K, Verma N, Lal CS, Bimal S, Topno RK, Singh D, Siddiqui NA, Verma RB, Das P: Short report: Development of post-kala-azar dermal leishmaniasis (PKDL) in miltefosine-treated visceral leishmaniasis. *The American journal of tropical medicine and hygiene* 2009, 80(3):336-338.
21. Dujardin JC, Campino L, Canavate C, Dedet JP, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M: Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. *Emerg Infect Dis* 2008, 14(7):1013-1018.
22. Gil-Prieto R, Walter S, Alvar J, de Miguel AG: Epidemiology of leishmaniasis in Spain based on hospitalization records (1997-2008). *Am J Trop Med Hyg* 2011, 85(5):820-825.
23. Leishman WB: On the possibility of the occurrence of trypanomiasis in India". The British Medical Journal; Donovan, C. (1903). "Memoranda: On the possibility of the occurrence of trypanomiasis in India". . *The British Medical Journal* 1903.
24. Pulvertaft RJ, Hoyle GF: Stages in the life-cycle of *Leishmania donovani*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1960, 54:191-196.

25. Franco AM, Grimaldi G, Jr.: Characterization of Endotrypanum (Kinetoplastida: Trypanosomatidae), a unique parasite infecting the neotropical tree sloths (Edentata). *Memorias do Instituto Oswaldo Cruz* 1999, 94(2):261-268.
26. Momen H, Cupolillo E: Speculations on the origin and evolution of the genus Leishmania. *Memorias do Instituto Oswaldo Cruz* 2000, 95(4):583-588.
27. Killick-Kendrick R: The life cycles of Leishmania in the sand fly and transmission of leishmaniosis by bite. In: Canine leishmaniosis: moving towards a solution. . *Proceedings of the Second International Canine Leishmaniosis Forum Sevilla, Spain* 2002, pp. 57-68.
28. Schuster FL, Sullivan JJ: Cultivating of clinically significant hemoflagellates. . *Clinical Microbiology Review*, 2002, 15 (3):374-389.
29. Jennings YL, de Souza AA, Ishikawa EA, Shaw J, Lainson R, Silveira F: Phenotypic characterization of Leishmania spp. causing cutaneous leishmaniasis in the lower Amazon region, western Para state, Brazil, reveals a putative hybrid parasite, *Leishmania (Viannia) guyanensis x Leishmania (Viannia) shawi shawi*. *Parasite* 2014, 21:39.
30. Myler P, f FN: Leishmania: After The Genome. Caister Academic Press. p. [page needed]. ISBN = 978-1-904455-28-8. 2008.
31. Harms G, U. B: Leishmaniosen-importierte Krankheiten. . *Dtsch Arztebl* 2000, 31/32:1589–1592.
32. Schlein Y, Jacobson RL, Messer G: Leishmania infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. *Proceedings of the National Academy of Sciences of the United States of America* 1992, 89(20):9944-9948.
33. Killick-Kendrick R: Biology of Leishmania in phlebotomine sandflies. In: Biology of the Kinetoplastida. W.H. R. Lumsden, D.A. Evans (eds), . *New York, Academic Press* 1979, 2:395-460.
34. Killick-Kendrick R: Leishmaniosis – an English disease of the future? . *Bull Trop Med Int Health 4, 5* 1996.
35. Arce A, Estirado A, Ordobas M, Sevilla S, Garcia N, Moratilla L, de la Fuente S, Martinez AM, Perez AM, Aranguez E *et al*: Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(30):20546.

36. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L: Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. *Trends in parasitology* 2008, 24(7):324-330.

37. Cruz I, Morales MA, Noguer I, Rodriguez A, Alvar J: Leishmania in discarded syringes from intravenous drug users. *Lancet* 2002, 359(9312):1124-1125.

38. Owens SD, Oakley DA, Marryott K, Hatchett W, Walton R, Nolan TJ, Newton A, Steurer F, Schantz P, Giger U: Transmission of visceral leishmaniasis through blood transfusions from infected English foxhounds to anemic dogs. *J Am Vet Med Assoc* 2001, 219(8):1076-1083.

39. Symmers WS: Leishmaniasis acquired by contagion: a case of marital infection in Britain. *Lancet* 1960, 1(7116):127-132.

40. Chatterjee K: Dermal leishmanoid. In: Chatterjee KD Editor Parasitology, 12th edition. . *Calcutta: Chatterjee Medical Publishers* 1980, 64-65.

41. Silva FL, Oliveira RG, Silva TM, Xavier MN, Nascimento EF, Santos RL: Venereal transmission of canine visceral leishmaniasis. *Veterinary parasitology* 2009, 160(1-2):55-59.

42. Rosypal AC, Troy GC, Zajac AM, Frank G, Lindsay DS: Transplacental transmission of a North American isolate of *Leishmania infantum* in an experimentally infected beagle. *J Parasitol* 2005, 91(4):970-972.

43. Slappendel RJ, Teske E: 1999. A review of canine leishmaniosis presenting outside endemic areas. In: Canine Leishmaniosis: an update. Proceeding of the International Canine Leishmaniosis Forum, Killick-Kendrick (ed.), . *Wiesbaden* 1999: pp,54-59.

44. Gradoni L, Pozio E, Gramiccia M, Maroli M, Bettini S: Leishmaniasis in Tuscany (Italy): VII. Studies on the role of the black rat, Rattus rattus, in the epidemiology of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1983, 77(4):427-431.

45. WHO: The leishmaniases. Report of a WHO Expert Committee. *World Health Organization technical report series* 1984, 701:1-140.

46. Solano-Gallego L, Rodriguez-Cortes A, Trotta M, Zampieron C, Razia L, Furlanello T, Caldin M, Roura X, Alberola J: Detection of *Leishmania infantum* DNA by fret-based real-time PCR in urine from dogs with natural clinical leishmaniosis. *Veterinary parasitology* 2007, 147(3-4):315-319.

47. Miro G, Montoya A, Mateo M, Alonso A, Garcia S, Garcia A, Caballero MJ, Molina R: A leishmaniosis surveillance system among stray dogs in the region of Madrid: ten years of serodiagnosis (1996-2006). *Parasitology research* 2007, 101(2):253-257.
48. Zijlstra EE, Musa AM, Khalil EA, el-Hassan IM, el-Hassan AM: Post-kala-azar dermal leishmaniasis. *The Lancet Infectious diseases* 2003, 3(2):87-98.
49. Oliva G, Foglia Manzillo V, Pagano A: [Canine leishmaniasis: evolution of the chemotherapeutic protocols]. *Parassitologia* 2004, 46(1-2):231-234.
50. Ciaramella P, Oliva G, Luna RD, Gradoni L, Ambrosio R, Cortese L, Scalone A, Persechino A: A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. *The Veterinary record* 1997, 141(21):539-543.
51. Moritz A, Steuber S, Greiner M: Clinical follow-up examination after treatment of canine leishmaniasis. *The Tokai journal of experimental and clinical medicine* 1998, 23(6):279-283.
52. Ready PD: Leishmaniasis emergence and climate change. *Rev Sci Tech* 2008, 27(2):399-412.
53. Petrovic Z, Borjoski A, Savin Z: Les resultats de recherches sur le réservoir de *Leishmania donovani* dans une région endémique du Kala-azar. . *Proc Europ Multicoll Parasti Trogir* 1975. , 2, :97-98.
54. Di Bella C, Vitale F, Russo G, Greco A, Milazzo C, Aloise G, Cagnin M: Are rodents a potential reservoir for *Leishmania infantum* in Italy? *J Med Ecol* 2003. , 7 (Suppl.) 125-129.
55. Beck A, Beck R, Kusak J, Gudan A, Martinkovic F, Artukovic B, Hohsteter M, Huber D, Marinculic A, Grabarevic Z: A case of visceral leishmaniosis in a gray wolf (Canis lupus) from Croatia. *Journal of wildlife diseases* 2008, 44(2):451-456.
56. Gibson-Corley KN, Hostetter JM, Hostetter SJ, Mullin K, Ramer-Tait AE, Boggiatto PM, Petersen CA: Disseminated *Leishmania infantum* infection in two sibling foxhounds due to possible vertical transmission. *The Canadian veterinary journal La revue veterinaire canadienne* 2008, 49(10):1005-1008.
57. Gramiccia M, Gradoni L: The current status of zoonotic leishmaniases and approaches to disease control. *International journal for parasitology* 2005, 35(11-12):1169-1180.
58. Ashford RW, Bettini S: Ecology and epidemiology: Old World. In: The biology of Leishmaniases in Biology and Medicine. W. Peters, R. Killick-Kendrick (eds.), . 1987. , 1, :365-424.

59. De Lima H, Rodriguez N, Barrios MA, Avila A, Canizales I, Gutierrez S: Isolation and molecular identification of Leishmania chagasi from a bat (Carollia perspicillata) in northeastern Venezuela. *Memorias do Instituto Oswaldo Cruz* 2008, 103(4):412-414.

60. Dedet JP, Gay F, Chatenay G: Isolation of *Leishmania* species from wild mammals in French Guiana. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1989, 83(5):613-615.

61. Sobrino R, Ferroglio E, Oleaga A, Romano A, Millan J, Revilla M, Arnal MC, Trisciuoglio A, Gortazar C: Characterization of widespread canine leishmaniasis among wild carnivores from Spain. *Veterinary parasitology* 2008, 155(3-4):198-203.

62. Solano-Gallego L, Rodriguez-Cortes A, Iniesta L, Quintana J, Pastor J, Espada Y, Portus M, Alberola J: Cross-sectional serosurvey of feline leishmaniasis in ecoregions around the Northwestern Mediterranean. *The American journal of tropical medicine and hygiene* 2007, 76(4):676-680.

63. Mancianti F: [Feline leishmaniasis: what's the epidemiological role of the cat?]. *Parassitologia* 2004, 46(1-2):203-206.

64. Petersen CA: Leishmaniasis, an emerging disease found in companion animals in the United States. *Topics in companion animal medicine* 2009, 24(4):182-188.

65. Grevot A, Jaussaud Hugues P, Marty P, Pratlong F, Ozon C, Haas P, Breton C, Bourdoiseau G: Leishmaniosis due to *Leishmania infantum* in a FIV and FelV positive cat with a squamous cell carcinoma diagnosed with histological, serological and isoenzymatic methods. *Parasite* 2005, 12(3):271-275.

66. Diakou A, Papadopoulos E, Lazarides K: Specific anti-*Leishmania* spp. antibodies in stray cats in Greece. *J Feline Med Surg* 2009, 11(8):728-730.

67. Pennisi MG, Venza M, Reale S, Vitale F, Lo Giudice S: Case report of leishmaniasis in four cats. *Vet Res Commun* 2004, 28 Suppl 1:363-366.

68. Figueiredo FB, Bonna IC, Nascimento LD, Costa T, Baptista C, Pacheco TM, Amendoeira MR, Madeira Mde F: [Serological evaluation for detection of anti-Leishmania antibodies in dogs and cats in the district of Santa Rita de Cassia, municipality of Barra Mansa, State of Rio de Janeiro]. *Revista da Sociedade Brasileira de Medicina Tropical* 2009, 42(2):141-145.

69. Braga AR, Langoni H, Lucheis SB: Evaluation of canine and feline leishmaniasis by the association of blood culture, immunofluorescent antibody test and polymerase chain reaction. *J Venom Anim Toxins Incl Trop Dis* 2014, 20(1):5.
70. Trainor KE, Porter BF, Logan KS, Hoffman RJ, Snowden KF: Eight cases of feline cutaneous leishmaniasis in Texas. *Veterinary pathology* 2010, 47(6):1076-1081.
71. Muller N, Welle M, Lobsiger L, Stoffel MH, Boghenbor KK, Hilbe M, Gottstein B, Frey CF, Geyer C, von Bomhard W: Occurrence of *Leishmania* sp. in cutaneous lesions of horses in Central Europe. *Veterinary parasitology* 2009, 166(3-4):346-351.
72. Williams AO, Mutinga J, Rodgers M: Leishmaniasis in a domestic goat in Kenya. *Molecular and cellular probes* 1991, 5(5):319-325.
73. Mutinga MJ, Kihara SM, Lohding A, Mutero CM, Ngatia TA, Karanu F: Leishmaniasis in Kenya: description of leishmaniasis of a domestic goat from Transmara, Narok District, Kenya. *Tropical medicine and parasitology : official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit* 1989, 40(2):91-96.
74. van der Lugt JJ, Carlyon JF, de Waal DT: Cutaneous leishmaniasis in a sheep. *Journal of the South African Veterinary Association* 1992, 63(2):74-77.
75. Moraes-Silva E, Antunes FR, Rodrigues MS, da Silva Julião F, Dias-Lima AG, Lemos-de-Sousa V, de Alcantara AC, Reis EA, Nakatani M, Badaró R *et al*: Domestic swine in a visceral leishmaniasis endemic area produce antibodies against multiple *Leishmania infantum* antigens but apparently resist to *L. infantum* infection. . *Acta Trop* 2006, 98(2)::176-182.
76. Anjili CO, Ngichabe CK, Mbati PA, Lugalia RM, Wamwayi HM, Githure JI: Experimental infection of domestic sheep with culture-derived *Leishmania donovani* promastigotes. *Veterinary parasitology* 1998, 74(2-4):315-318.
77. Dubey JP, Bwangamoi O, Courtney SP, Fritz DL: Leishmania-like protozoan associated with dermatitis in cattle. *The Journal of parasitology* 1998, 84(4):865-867.
78. Brazil RP, Desterro MD, Nascimento SB, Macau RP: [Natural infection of a pig (Sus scrofa) by Leishmania in a recent focus of cutaneous leishmaniasis on the Island of Sao Luis, Maranhao]. *Memorias do Instituto Oswaldo Cruz* 1987, 82(1):145.
79. Mukhtar MM, Sharief AH, el Saffi SH, Harith AE, Higazzi TB, Adam AM, Abdalla HS: Detection of antibodies to *Leishmania donovani* in animals in a kala-azar endemic region in

eastern Sudan: a preliminary report. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2000, 94(1):33-36.

80. Vedovello Filho D, Jorge FA, Lonardoni MV, Teodoro U, Silveira TG: American cutaneous leishmaniasis in horses from endemic areas in the north-central mesoregion of Parana state, Brazil. *Zoonoses and public health* 2008, 55(3):149-155.
81. Lopes AP, Sousa S, Dubey JP, Ribeiro AJ, Silvestre R, Cotovio M, Schallig HD, Cardoso L, Cordeiro-da-Silva A: Prevalence of antibodies to *Leishmania infantum* and *Toxoplasma gondii* in horses from the north of Portugal. *Parasites & vectors* 2013, 6:178.
82. Quinnell RJ, Courtenay O: Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. *Parasitology* 2009, 136(14):1915-1934.
83. Alexander B, de Carvalho RL, McCallum H, Pereira MH: Role of the domestic chicken (Gallus gallus) in the epidemiology of urban visceral leishmaniasis in Brazil. *Emerging infectious diseases* 2002, 8(12):1480-1485.
84. Sant'anna MR, Nascimento A, Alexander B, Dilger E, Cavalcante RR, Diaz-Albiter HM, Bates PA, Dillon RJ: Chicken blood provides a suitable meal for the sand fly Lutzomyia longipalpis and does not inhibit *Leishmania* development in the gut. *Parasites & vectors* 2010, 3(1):3.
85. Moreira ED, Jr., de Souza VM, Sreenivasan M, Lopes NL, Barreto RB, de Carvalho LP: Peridomestic risk factors for canine leishmaniasis in urban dwellings: new findings from a prospective study in Brazil. *The American journal of tropical medicine and hygiene* 2003, 69(4):393-397.
86. Noguera P, Rondon M, Nieves E: Effect of blood source on the survival and fecundity of the sandfly Lutzomyia ovallesi Ortiz (Diptera: Psychodidae), vector of Leishmania. *Biomedica : revista del Instituto Nacional de Salud* 2006, 26 Suppl 1:57-63.
87. Sacks D, Kamhawi S: Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annual review of microbiology* 2001, 55:453-483.
88. Schuster FL, Sullivan JJ: Cultivation of clinically significant hemoflagellates. *Clinical microbiology reviews* 2002, 15(3):374-389.
89. Prevention) CCfDCa: Parasites home: leishmaniasis. Epidemiology & risk factors. Available at http://www.cdc.gov/parasites/leishmaniasis/epi.html. Accessed April 11, 2014.

90. Murray HW, Berman JD, Davies CR, Saravia NG: Advances in leishmaniasis. *Lancet* 2005, 366(9496):1561-1577.

91. Cardo LJ, Rentas FJ, Ketchum L, Salata J, Harman R, Melvin W, Weina PJ, Mendez J, Reddy H, Goodrich R: Pathogen inactivation of *Leishmania donovani infantum* in plasma and platelet concentrates using riboflavin and ultraviolet light. *Vox sanguinis* 2006, 90(2):85-91.

92. Lawyer P.G., P.V. P: Leishmaniasis and trypanosomiasis. In: B.F. Eldridge and J.D. Edman (eds). Medical Entomology. pp 231-298. Kluwer, The Netherlands. 2004.

93. World Health O: Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis. *World Health Organization technical report series* 2012(975):v-xii, 1-100.

94. Bogdan C: Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. *Cellular microbiology* 2008, 10(6):1221-1234.

95. Neuber H: Leishmaniasis. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* 2008, 6(9):754-765.

96. World Health O: Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, World Health Organization (WHO) (22–26 March 2010) Contract No.: no. 9492010.

97. Organization WH: Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis. *World Health Organization technical report series* 2012(975):v-xii, 1-100.

98. McHugh CP, Melby PC, LaFon SG: Leishmaniasis in Texas: epidemiology and clinical aspects of human cases. *The American journal of tropical medicine and hygiene* 1996, 55(5):547-555.

99. Lian LY, Pandalaneni SR, Todd PA, Martin VM, Burgoyne RD, Haynes LP: Demonstration of binding of neuronal calcium sensor-1 to the cav2.1 p/q-type calcium channel. *Biochemistry* 2014, 53(38):6052-6062.

100. Organization. WH: Leishmaniasis: the disease and its epidemiology. Available at http://www.who.int/leishmaniasis/disease_epidemiology/en/. Accessed April 10, 2014.

101. Prevention. CfDCa: Parasites home: leishmaniasis. Epidemiology & risk factors. Available at http://www.cdc.gov/parasites/leishmaniasis/epi.html. Accessed April 11, 2014).

102. Ahluwalia S, Lawn SD, Kanagalingam J, Grant H, Lockwood DN: Mucocutaneous leishmaniasis: an imported infection among travellers to central and South America. *BMJ* 2004, 329(7470):842-844.

103. Herwaldt BL, Stokes SL, Juranek DD: American cutaneous leishmaniasis in U.S. travelers. *Annals of internal medicine* 1993, 118(10):779-784.

104. Ehehalt U, Schunk M, Jensenius M, van Genderen PJ, Gkrania-Klotsas E, Chappuis F, Schlagenhauf P, Castelli F, Lopez-Velez R, Parola P *et al*: Leishmaniasis acquired by travellers to endemic regions in Europe: a EuroTravNet multi-centre study. *Travel medicine and infectious disease* 2014, 12(2):167-172.

105. Emeis M, Liesenfeld O, Henze G, Meredith S, Schoone G, Stuck B: [Imported visceral leishmaniasis (kala azar) in a German infant]. *Klinische Padiatrie* 1995, 207(6):347-349.

106. Gautret P, Cramer JP, Field V, Caumes E, Jensenius M, Gkrania-Klotsas E, de Vries PJ, Grobusch MP, Lopez-Velez R, Castelli F *et al*: Infectious diseases among travellers and migrants in Europe, EuroTravNet 2010. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2012, 17(26).

107. Choi CM, Lerner EA: Leishmaniasis as an emerging infection. *The journal of investigative dermatology Symposium proceedings / the Society for Investigative Dermatology, Inc [and] European Society for Dermatological Research* 2001, 6(3):175-182.

108. van Griensven J, Diro E: Visceral leishmaniasis. *Infectious disease clinics of North America* 2012, 26(2):309-322.

109. Lubbert C, Opitz BM, Harms-Zwingenberger G, Nietsch HH: [Fever, pancytopenia, and splenomegaly 8 months after a trip to Majorca Island (Spain)]. *Medizinische Klinik* 2008, 103(1):29-35.

110. Schmid MB, Leichsenring M, Keller C, Hegasy G: [Pancytopenia, fever, and splenomegaly in a 2-year-old boy]. *Deutsche medizinische Wochenschrift* 2009, 134(24):1274-1277.

111. Lainson R, Shaw JJ, Silveira FT, Fraiha H: Leishmaniasis in Brazil. XIX: visceral leishmaniasis in the Amazon Region, and the presence of Lutzomyia longipalpis on the Island of Marajo, Para State. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1983, 77(3):323-330.

112. Lainson R: The American leishmaniases: some observations on their ecology and epidemiology. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1983, 77(5):569-596.

113. Aoun K, Amri F, Chouihi E, Haouas N, Bedoui K, Benikhlef R, Ghrab J, Babba H, Chahed MK, Harrat Z *et al*: [Epidemiology of *Leishmania (L.) infantum, L. major and L. killicki* in

Tunisia: results and analysis of the identification of 226 human and canine isolates]. *Bulletin de la Societe de pathologie exotique* 2008, 101(4):323-328.

114. Postigo JA: Leishmaniasis in the World Health Organization Eastern Mediterranean Region. *International journal of antimicrobial agents* 2010, 36 Suppl 1:S62-65.
115. Lachaud L, Dedet JP, Marty P, Faraut F, Buffet P, Gangneux JP, Ravel C, Bastien P, Working Group for the Notification of Human Leishmanioses in F: Surveillance of leishmaniases in France, 1999 to 2012. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(29):20534.
116. Schantz PM, Steurer FJ, Duprey ZH, Kurpel KP, Barr SC, Jackson JE, Breitschwerdt EB, Levy MG, Fox JC: Autochthonous visceral leishmaniasis in dogs in North America. *Journal of the American Veterinary Medical Association* 2005, 226(8):1316-1322.
117. Michel G, Pomares C, Ferrua B, Marty P: Importance of worldwide asymptomatic carriers of *Leishmania infantum (L. chagasi)* in human. *Acta tropica* 2011, 119(2-3):69-75.
118. Elmahallawy E.K, Cuadros-Moronta E, María Carmen Liébana-Martos MC, Rodriguez-Granger JM, Sampedro-Martínez A, Agil A, Navarro-Marí JM, Gutiérrez-Fernández J, J. B-S: Seroprevalence of *Leishmania* infection among asymptomatic renal transplant recipients from southern Spain. *JMM* 2015.
119. Zanger P, Gabrysch S: Leishmaniasis in the era of tumor necrosis factor alpha antagonist therapy--a research agenda for Europe. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(30):20542.
120. Killick-Kendrick R: Phlebotomine vectors of the leishmaniases: a review. *Medical and veterinary entomology* 1990, 4(1):1-24.
121. Gradoni L: Epidemiological surveillance of leishmaniasis in the European Union: operational and research challenges. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(30):20539.
122. Pace D: Leishmaniasis. *The Journal of infection* 2014, 69 Suppl 1:S10-18.
123. Aguado M, Espinosa P, Romero-Mate A, Tardio JC, Cordoba S, Borbujo J: Outbreak of cutaneous leishmaniasis in Fuenlabrada, Madrid. *Actas dermo-sifiliograficas* 2013, 104(4):334-342.
124. Antoniou M, Gramiccia M, Molina R, Dvorak V, Volf P: The role of indigenous phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the

Mediterranean region. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(30):20540.

125. Mauricio IL, Stothard JR, Miles MA: The strange case of Leishmania chagasi. *Parasitology today* 2000, 16(5):188-189.
126. Singh S: Changing trends in the epidemiology, clinical presentation, and diagnosis of Leishmania-HIV co-infection in India. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* 2014, 29:103-112.
127. Mollalo A, Alimohammadi A, Shirzadi MR, Malek MR: Geographic information system-based analysis of the spatial and spatio-temporal distribution of zoonotic cutaneous leishmaniasis in Golestan Province, north-east of Iran. *Zoonoses and public health* 2015, 62(1):18-28.
128. Confalonieri UE, Margonari C, Quintao AF: Environmental change and the dynamics of parasitic diseases in the Amazon. *Acta tropica* 2014, 129:33-41.
129. Bounoua L, Kahime K, Houti L, Blakey T, Ebi KL, Zhang P, Imhoff ML, Thome KJ, Dudek C, Sahabi SA *et al*: Linking climate to incidence of zoonotic cutaneous leishmaniasis (*L. major*) in pre-Saharan North Africa. *International journal of environmental research and public health* 2013, 10(8):3172-3191.
130. Fischer D, Thomas SM, Beierkuhnlein C: Temperature-derived potential for the establishment of phlebotomine sandflies and visceral leishmaniasis in Germany. *Geospatial health* 2010, 5(1):59-69.
131. Desjeux P: The increase in risk factors for leishmaniasis worldwide. *Trans R Soc Trop Med Hyg* 2001, 95(3):239-243.
132. Desjeux P: Worldwide increasing risk factors for leishmaniasis. *Medical microbiology and immunology* 2001, 190(1-2):77-79.
133. Gonzalez C, Paz A, Ferro C: Predicted altitudinal shifts and reduced spatial distribution of *Leishmania infantum* vector species under climate change scenarios in Colombia. *Acta tropica* 2014, 129:83-90.
134. Gonzalez C, Wang O, Strutz SE, Gonzalez-Salazar C, Sanchez-Cordero V, Sarkar S: Climate change and risk of leishmaniasis in north america: predictions from ecological niche models of vector and reservoir species. *PLoS neglected tropical diseases* 2010, 4(1):e585.

135. Trotz-Williams L, Gradoni L: Disease risks for the travelling pet Leishmaniasis.. *In Practice* 2003, 25,: 190–197.

136. al. BRe: Social epidemiology of leishmaniasis in the central jungle of Peru. In: Walton BC, Wijeyaratne PM, Moddaber F, eds. Research on control strategies for the leishmaniases. *Proceedings of an international workshop held in Ottawa, Canada, 1-4 June, 1987 Ottawa, Canada, International Development Research Centre (IDRC), 1988* 1987.

137. Netto EM, Cuba CC, Costa JL, Barreto AC, Rosa C, Marsden PD: Recurrence of South American tegumentary leishmaniasis. *Lancet* 1986, 1(8479):501.

138. Gomes Ade C, Yamamoto YI, Capinzaiki AN, Amaral NM, Guimaraes AJ: [Ecological aspects of American cutaneous leishmaniasis. 9. Prevalence/incidence of the human infection in the municipality of Pedro de Toledo and Miracatu, Sao Paulo, Brazil]. *Revista do Instituto de Medicina Tropical de Sao Paulo* 1992, 34(2):149-158.

139. Tolezano JE: Ecoepidemiological aspects of American cutaneous leishmaniasis in the state of Sao Paulo, Brazil. *Memorias do Instituto Oswaldo Cruz* 1994, 89(3):427-434.

140. Pupo Nogueira Neto J, Basso G, Cipoli AP, El Kadre L: American cutaneous leishmaniasis in the State of Sao Paulo, Brazil--epidemiology in transformation. *Annals of agricultural and environmental medicine : AAEM* 1998, 5(1):1-5.

141. da Costa SM, Cechinel M, Bandeira V, Zannuncio JC, Lainson R, Rangel EF: Lutzomyia (Nyssomyia) whitmani s.l . (Antunes & Coutinho, 1939)(Diptera: Psychodidae: Phlebotominae): geographical distribution and the epidemiology of American cutaneous leishmaniasis in Brazil--mini-review. *Memorias do Instituto Oswaldo Cruz* 2007, 102(2):149-153.

142. Davies CR, Kaye P, Croft SL, Sundar S: Leishmaniasis: new approaches to disease control. *BMJ* 2003, 326(7385):377-382.

143. Tsegaw T, Gadisa E, Seid A, Abera A, Teshome A, Mulugeta A, Herrero M, Argaw D, Jorge A, Aseffa A: Identification of environmental parameters and risk mapping of visceral leishmaniasis in Ethiopia by using geographical information systems and a statistical approach. *Geospatial health* 2013, 7(2):299-308.

144. Bhunia GS, Kesari S, Jeyaram A, Kumar V, Das P: Influence of topography on the endemicity of Kala-azar: a study based on remote sensing and geographical information system. *Geospatial health* 2010, 4(2):155-165.

145. Shimabukuro PH, da Silva TR, Ribeiro FO, Baton LA, Galati EA: Geographical distribution of American cutaneous leishmaniasis and its *phlebotomine* vectors (Diptera: Psychodidae) in the state of Sao Paulo, Brazil. *Parasites & vectors* 2010, 3:121.

146. Antinori S, Gianelli E, Calattini S, Longhi E, Gramiccia M, Corbellino M: Cutaneous leishmaniasis: an increasing threat for travellers. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2005, 11(5):343-346.

147. Pavli A, Maltezou HC: Leishmaniasis, an emerging infection in travelers. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* 2010, 14(12):e1032-1039.

148. Control of the leishmaniases. Report of a WHO Expert Committee. *World Health Organization technical report series* 1990, 793:1-158.

149. World Health O: Control of the leishmaniases. *World Health Organization technical report series* 2010(949):xii-xiii, 1-186, back cover.

150. Harrison LH, Naidu TG, Drew JS, de Alencar JE, Pearson RD: Reciprocal relationships between undernutrition and the parasitic disease visceral leishmaniasis. *Rev Infect Dis* 1986, 8(3):447-453.

151. Schaefer KU, Kurtzhals JA, Sherwood JA, Githure JI, Kager PA, Muller AS: Epidemiology and clinical manifestations of visceral and cutaneous leishmaniasis in Baringo District, Rift Valley, Kenya. A literature review. *Tropical and geographical medicine* 1994, 46(3):129-133.

152. Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, Desjeux P, Wasunna MK, Bryceson AD: Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *The Lancet Infectious diseases* 2002, 2(8):494-501.

153. Killick-Kendrick R: Breeding places of Phlebotomus ariasi in the cevennes focus of leishmaniasis in the south of France. *Parassitologia* 1987, 29(2-3):181-191.

154. Feliciangeli MD: Natural breeding places of phlebotomine sandflies. *Medical and veterinary entomology* 2004, 18(1):71-80.

155. Jeronimo SM, Duggal P, Ettinger NA, Nascimento ET, Monteiro GR, Cabral AP, Pontes NN, Lacerda HG, Queiroz PV, Gomes CE *et al*: Genetic predisposition to self-curing infection with the protozoan *Leishmania chagasi*: a genomewide scan. *The Journal of infectious diseases* 2007, 196(8):1261-1269.

156. Jeronimo SM, Holst AK, Jamieson SE, Francis R, Martins DR, Bezerra FL, Ettinger NA, Nascimento ET, Monteiro GR, Lacerda HG *et al*: Genes at human chromosome 5q31.1 regulate delayed-type hypersensitivity responses associated with Leishmania chagasi infection. *Genes and immunity* 2007, 8(7):539-551.

157. Blackwell JM, Fakiola M, Ibrahim ME, Jamieson SE, Jeronimo SB, Miller EN, Mishra A, Mohamed HS, Peacock CS, Raju M *et al*: Genetics and visceral leishmaniasis: of mice and man. *Parasite immunology* 2009, 31(5):254-266.

158. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S: Cutaneous leishmaniasis. *The Lancet Infectious diseases* 2007, 7(9):581-596.

159. Petri WA Jr, R. H: Entamoeba species, including Amebiasis. In: Mandell GL, JE Bennett, R Dolin, eds. Mandell, Douglas, and Bennett's principles and practice of infectious diseases, 7th ed. Philadelphia: Churchill Livingstone; 2010. p. 3411–25. 2010.

160. Desjeux P, Alvar J: Leishmania/HIV co-infections: epidemiology in Europe. *Ann Trop Med Parasitol* 2003, 97 Suppl 1:3-15.

161. (2011). WHO: Elimination visceral leishmaniasis: a multiple pronged approach. Available: http://www.who.int/tdr/news/2011/vl-elimination/en/. Accessed May 2014.

162. Lawyer PG, Perkins PV: Leishmaniosis and trypanosomiasis. In: Medical Entomology, Revised Edition, by B. F. Eldridge and J. D. Edman (eds.), Kluver Academic Publishers, Great Britain, pp. 231-298. *Kluver Academic Publishers, Great Britain, pp 231-298* 2004.

163. Aoun K, Bouratbine A: Cutaneous leishmaniasis in North Africa: a review. *Parasite* 2014, 21:14.

164. Kennedy K: "VCS Advocacy in the News: VA May Designate 9 Infectious Diseases as Related to Gulf War". Veterans for Common Sense. Retrieved 10 February 2011, (2010). 2010.

165. Kennedy K: "VCS Advocacy in the News: VA May Designate 9 Infectious Diseases as Related to Gulf War". Veterans for Common Sense. Retrieved 10 February 2011. 2010.

166. Antoniou M, Haralambous C, Mazeris A, Pratlong F, Dedet JP, Soteriadou K: *Leishmania donovani* leishmaniasis in Cyprus. *The Lancet Infectious diseases* 2008, 8(1):6-7.

167. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, Team WHOLC: Leishmaniasis worldwide and global estimates of its incidence. *PloS one* 2012, 7(5):e35671.

168. Botet Fregola J, Portus Vinyeta M: [Leishmaniasis in peninsular Spain. A historical-bibliographic review (1912-1985)]. *Rev Sanid Hig Publica (Madr)* 1993, 67(4):255-266.

169. Monge-Maillo B, Norman FF, Cruz I, Alvar J, Lopez-Velez R: Visceral leishmaniasis and HIV coinfection in the Mediterranean region. *PLoS neglected tropical diseases* 2014, 8(8):e3021.

170. Suarez Rodriguez B, Isidoro Fernandez B, Santos Sanz S, Sierra Moros MJ, Molina Moreno R, Astray Mochales J, Amela Heras C: [Review of the current situation and the risk factors of *Leishmania infantum* in Spain]. *Revista espanola de salud publica* 2012, 86(6):555-564.

171. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L: Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. *Medical and veterinary entomology* 2013, 27(2):123-147.

172. Berens-Riha N, Fleischmann E, Pratlong F, Bretzel G, von Sonnenburg F, Loscher T: Cutaneous leishmaniasis (*Leishmania tropica*) in a German tourist after travel to Greece. *Journal of travel medicine* 2009, 16(3):220-222.

173. Wall EC, Watson J, Armstrong M, Chiodini PL, Lockwood DN: Epidemiology of imported cutaneous leishmaniasis at the Hospital for Tropical Diseases, London, United Kingdom: use of polymerase chain reaction to identify the species. *The American journal of tropical medicine and hygiene* 2012, 86(1):115-118.

174. Malik AN, John L, Bruceson AD, Lockwood DN: Changing pattern of visceral leishmaniasis, United Kingdom, 1985-2004. *Emerging infectious diseases* 2006, 12(8):1257-1259.

175. Weitzel T, Muhlberger N, Jelinek T, Schunk M, Ehrhardt S, Bogdan C, Arasteh K, Schneider T, Kern WV, Fatkenheuer G *et al*: Imported leishmaniasis in Germany 2001-2004: data of the SIMPID surveillance network. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 2005, 24(7):471-476.

176. Bart A, van Thiel PP, de Vries HJ, Hodiamont CJ, Van Gool T: Imported leishmaniasis in the Netherlands from 2005 to 2012: epidemiology, diagnostic techniques and sequence-based species typing from 195 patients. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(30):20544.

177. Mansueto P, Seidita A, Vitale G, Cascio A: Leishmaniasis in travelers: a literature review. *Travel medicine and infectious disease* 2014, 12(6 Pt A):563-581.

178. Franco AO, Davies CR, Mylne A, Dedet JP, Gallego M, Ballart C, Gramiccia M, Gradoni L, Molina R, Galvez R *et al*: Predicting the distribution of canine leishmaniasis in western Europe based on environmental variables. *Parasitology* 2011, 138(14):1878-1891.

179. Cortes S, Vaz Y, Neves R, Maia C, Cardoso L, Campino L: Risk factors for canine leishmaniasis in an endemic Mediterranean region. *Veterinary parasitology* 2012, 189(2-4):189-196.

180. Ruiz-Fons F, Ferroglio E, Gortazar C: *Leishmania infantum* in free-ranging hares, Spain, 2004-2010. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(30):20541.

181. Millan J, Ferroglio E, Solano-Gallego L: Role of wildlife in the epidemiology of *Leishmania infantum i*nfection in Europe. *Parasitology research* 2014, 113(6):2005-2014.

182. Killick-Kendrick R: The biology and control of phlebotomine sand flies. *Clinics in dermatology* 1999, 17(3):279-289.

183. Aransay AM, Testa JM, Morillas-Marquez F, Lucientes J, Ready PD: Distribution of sandfly species in relation to canine leishmaniasis from the Ebro Valley to Valencia, northeastern Spain. *Parasitology research* 2004, 94(6):416-420.

184. Martin-Sanchez J, Gallego M, Baron S, Castillejo S, Morillas-Marquez F: Pool screen PCR for estimating the prevalence of *Leishmania infantum* infection in sandflies (Diptera: Nematocera, Phlebotomidae). *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2006, 100(6):527-532.

185. Ntais P, Sifaki-Pistola D, Christodoulou V, Messaritakis I, Pratlong F, Poupalos G, Antoniou M: Leishmaniases in Greece. *The American journal of tropical medicine and hygiene* 2013, 89(5):906-915.

186. Gkolfinopoulou K, Bitsolas N, Patrinos S, Veneti L, Marka A, Dougas G, Pervanidou D, Detsis M, Triantafillou E, Georgakopoulou T *et al*: Epidemiology of human leishmaniasis in Greece, 1981-2011. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(29):20532.

187. Frank C, Hadziandoniou M, Pratlong F, Garifallou A, Rioux JA: Leishmania tropica and *Leishmania infantum* responsible for cutaneous leishmaniasis in Greece: sixteen autochthonous cases. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1993, 87(2):184-185.

188. Santarém N, Silva Cd: Diagnosis of visceral leishmaniasis. In: Mendez-Vilas A, editor. Communicating current research and educational topics and trends in applied microbiology. . *Badajoz: Formatex* 2007:839-846.

189. Del Olmo Martinez L, Aller de la Fuente R, Velayos Jimenez B, Fernandez Salazar L, Gonzalez Hernandez JM: [Visceral leishmaniasis diagnosed by duodenal biopsy]. *Rev Esp Enferm Dig* 2009, 101(6):439-440.

190. Artan R, Yilmaz A, Akcam M, Aksoy NH: Liver biopsy in the diagnosis of visceral leishmaniasis. *J Gastroenterol Hepatol* 2006, 21(1 Pt 2):299-302.

191. Reus M, Garcia B, Vazquez V, Morales D, Fuster M, Sola J: Visceral leishmaniasis: diagnosis by ultrasound-guided fine needle aspiration of an axillary node. *Br J Radiol* 2005, 78(926):158-160.

192. Srivastava P, Dayama A, Mehrotra S, Sundar S: Diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2011, 105(1):1-6.

193. Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW, Kager PA: Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. *Trans R Soc Trop Med Hyg* 1992, 86(5):505-507.

194. Allahverdiyev AM, Bagirova M, Uzun S, Alabaz D, Aksaray N, Kocabas E, Koksal F: The value of a new microculture method for diagnosis of visceral leishmaniasis by using bone marrow and peripheral blood. *Am J Trop Med Hyg* 2005, 73(2):276-280.

195. Maurya R, Mehrotra S, Prajapati VK, Nylen S, Sacks D, Sundar S: Evaluation of blood agar microtiter plates for culturing *leishmania* parasites to titrate parasite burden in spleen and peripheral blood of patients with visceral leishmaniasis. *J Clin Microbiol* 2010, 48(5):1932-1934.

196. De Colmenares M, Portus M, Riera C, Gallego M, Aisa MJ, Torras S, Munoz C: Short report: detection of 72-75-kD and 123-kD fractions of *Leishmania* antigen in urine of patients with visceral leishmaniasis. *Am J Trop Med Hyg* 1995, 52(5):427-428.

197. Sarkari B, Chance M, Hommel M: Antigenuria in visceral leishmaniasis: detection and partial characterisation of a carbohydrate antigen. *Acta tropica* 2002, 82(3):339-348.

198. Attar ZJ, Chance ML, el-Safi S, Carney J, Azazy A, El-Hadi M, Dourado C, Hommel M: Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta tropica* 2001, 78(1):11-16.

199. El-Safi SH, Abdel-Haleem A, Hammad A, El-Basha I, Omer A, Kareem HG, Boelaert M, Chance M, Hommel M: Field evaluation of latex agglutination test for detecting urinary antigens in visceral leishmaniasis in Sudan. *East Mediterr Health J* 2003, 9(4):844-855.

200. Rijal S, Boelaert M, Regmi S, Karki BM, Jacquet D, Singh R, Chance ML, Chappuis F, Hommel M, Desjeux P *et al*: Evaluation of a urinary antigen-based latex agglutination test in the diagnosis of kala-azar in eastern Nepal. *Tropical medicine & international health : TM & IH* 2004, 9(6):724-729.

201. Diro E, Techane Y, Tefera T, Assefa Y, Kebede T, Genetu A, Kebede Y, Tesfaye A, Ergicho B, Gebre-Yohannes A *et al*: Field evaluation of FD-DAT, rK39 dipstick and KATEX (urine latex agglutination) for diagnosis of visceral leishmaniasis in northwest Ethiopia. *Trans R Soc Trop Med Hyg* 2007, 101(9):908-914.

202. Malaei S, Mohebali M, Akhoundi B, Z Z: Evaluation of latex agglutination test (Katex) for the detection of urinary antigens in human visceral leishmaniasis. . *J Tehran Un Med Sci* 2006, 4:: 101-108.

203. Vilaplana C, Blanco S, Dominguez J, Gimenez M, Ausina V, C TU, Munoz C: Noninvasive method for diagnosis of visceral leishmaniasis by a latex agglutination test for detection of antigens in urine samples. *J Clin Microbiol* 2004, 42(4):1853-1854.

204. Boelaert M, El-Safi S, Hailu A, Mukhtar M, Rijal S, Sundar S, Wasunna M, Aseffa A, Mbui J, Menten J *et al*: Diagnostic tests for kala-azar: a multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. *Trans R Soc Trop Med Hyg* 2008, 102(1):32-40.

205. Boelaert M, Rijal S, Regmi S, Singh R, Karki B, Jacquet D, Chappuis F, Campino L, Desjeux P, Le Ray D *et al*: A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. *The American journal of tropical medicine and hygiene* 2004, 70(1):72-77.

206. Iqbal J, Hira PR, Saroj G, Philip R, Al-Ali F, Madda PJ, Sher A: Imported visceral leishmaniasis: diagnostic dilemmas and comparative analysis of three assays. *Journal of clinical microbiology* 2002, 40(2):475-479.

207. Badaro R, Reed SG, Carvalho EM: Immunofluorescent antibody test in American visceral leishmaniasis: sensitivity and specificity of different morphological forms of two Leishmania species. *The American journal of tropical medicine and hygiene* 1983, 32(3):480-484.

208. Singh S, Sivakumar R: Recent advances in the diagnosis of leishmaniasis. *J Postgrad Med* 2003, 49(1):55-60.

209. Ryan JR, Smithyman AM, Rajasekariah GH, Hochberg L, Stiteler JM, Martin SK: Enzyme-linked immunosorbent assay based on soluble promastigote antigen detects immunoglobulin M (IgM) and IgG antibodies in sera from cases of visceral and cutaneous leishmaniasis. *Journal of clinical microbiology* 2002, 40(3):1037-1043.

210. Rajasekariah GH, Ryan JR, Hillier SR, Yi LP, Stiteler JM, Cui L, Smithyman AM, Martin SK: Optimisation of an ELISA for the serodiagnosis of visceral leishmaniasis using in vitro derived promastigote antigens. *Journal of immunological methods* 2001, 252(1-2):105-119.

211. Smrkovski LL, Larson CL: Antigenic cross-reactivity between Mycobacterium bovis (BCG) and *Leishmania donovani*. *Infect Immun* 1977, 18(2):561-562.

212. Choudhry A, Guru PY, Saxena RP, Tandon A, Saxena KC: Enzyme-linked immunosorbent assay in the diagnosis of kala-azar in Bhadohi (Varanasi), India. *Trans R Soc Trop Med Hyg* 1990, 84(3):363-366.

213. Kumar R, Pai K, Pathak K, Sundar S: Enzyme-linked immunosorbent assay for recombinant K39 antigen in diagnosis and prognosis of Indian visceral leishmaniasis. *Clin Diagn Lab Immunol* 2001, 8(6):1220-1224.

214. Bray R: Immunodiagnosis of leishmaniasis. In Chang KP, Bray RS, editors. Leishmaniasis. *Amsterdam: Elsevier* 1985:177-181.

215. Bray R: Immunodiagnosis of leishmaniasis. In Cohen S, Sadun EH, editors. Immunology of parasitic infections. Oxford: Blackwell. . 1976:65-76.

216. Vinayak VK, Mahajan D, Sobti RC, Singla N, Sundar S: Anti-66 kDa antileishmanial antibodies as specific immunodiagnostic probe for visceral leishmaniasis. *Indian J Med Res* 1994, 99:109-114.

217. Palatnik-de-Sousa CB, Gomes EM, Paraguai-de-Souza E, Palatnik M, Luz K, Borojevic R: *Leishmania donovani*: titration of antibodies to the fucose-mannose ligand as an aid in diagnosis and prognosis of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1995, 89(4):390-393.

218. Martin SK, Thuita-Harun L, Adoyo-Adoyo M, Wasunna KM: A diagnostic ELISA for visceral leishmaniasis, based on antigen from media conditioned by *Leishmania donovani* promastigotes. *Ann Trop Med Parasitol* 1998, 92(5):571-577.

219. Carvalho FA, Charest H, Tavares CA, Matlashewski G, Valente EP, Rabello A, Gazzinelli RT, Fernandes AP: Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant *Leishmania donovani* A2 antigen. *Diagn Microbiol Infect Dis* 2002, 43(4):289-295.

220. McCall LI, Zhang WW, Matlashewski G: Determinants for the development of visceral leishmaniasis disease. *PLoS Pathog* 2013, 9(1):e1003053.

221. Rafati S, Hassani N, Taslimi Y, Movassagh H, Rochette A, Papadopoulou B: Amastin peptide-binding antibodies as biomarkers of active human visceral leishmaniasis. *Clin Vaccine Immunol* 2006, 13(10):1104-1110.

222. Burns JM, Jr., Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG: Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. *Proceedings of the National Academy of Sciences of the United States of America* 1993, 90(2):775-779.

223. Badaro R, Benson D, Eulalio MC, Freire M, Cunha S, Netto EM, Pedral-Sampaio D, Madureira C, Burns JM, Houghton RL *et al*: rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. *The Journal of infectious diseases* 1996, 173(3):758-761.

224. Singh DP, Goyal RK, Singh RK, Sundar S, Mohapatra TM: In search of an ideal test for diagnosis and prognosis of kala-azar. *J Health Popul Nutr* 2010, 28(3):281-285.

225. Zijlstra EE, Nur Y, Desjeux P, Khalil EA, El-Hassan AM, Groen J: Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. *Trop Med Int Health* 2001, 6(2):108-113.

226. Houghton RL, Petrescu M, Benson DR, Skeiky YA, Scalone A, Badaro R, Reed SG, Gradoni L: A cloned antigen (recombinant K39) of Leishmania chagasi diagnostic for visceral leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy. *J Infect Dis* 1998, 177(5):1339-1344.

227. Mohapatra TM, Singh DP, Sen MR, Bharti K, Sundar S: Compararative evaluation of rK9, rK26 and rK39 antigens in the serodiagnosis of Indian visceral leishmaniasis. *J Infect Dev Ctries* 2010, 4(2):114-117.

228. Sivakumar R, Sharma P, Chang KP, Singh S: Cloning, expression, and purification of a novel recombinant antigen from *Leishmania donovani*. *Protein Expr Purif* 2006, 46(1):156-165.

229. Pattabhi S, Whittle J, Mohamath R, El-Safi S, Moulton GG, Guderian JA, Colombara D, Abdoon AO, Mukhtar MM, Mondal D *et al*: Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. *PLoS Negl Trop Dis* 2010, 4(9).

230. Amorim AG, Carrington M, Miles MA, Barker DC, de Almeida ML: Identification of the C-terminal region of 70 kDa heat shock protein from *Leishmania (Viannia) braziliensis* as a target for the humoral immune response. *Cell Stress Chaperones* 1996, 1(3):177-187.

231. Perez-Alvarez MJ, Larreta R, Alonso C, Requena JM: Characterisation of a monoclonal antibody recognising specifically the HSP70 from *Leishmania*. *Parasitol Res* 2001, 87(11):907-910.

232. Maache M, Azzouz S, Diaz de la Guardia R, Alvarez P, Gil R, de Pablos LM, Osuna A: Host humoral immune response to Leishmania lipid-binding protein. *Parasite Immunol* 2005, 27(6):227-234.

233. Brito ME, Mendonca MG, Gomes YM, Jardim ML, Abath FG: Identification of potentially diagnostic *Leishmania braziliensis* antigens in human cutaneous leishmaniasis by immunoblot analysis. *Clinical and diagnostic laboratory immunology* 2000, 7(2):318-321.

234. Ravindran R, Anam K, Bairagi BC, Saha B, Pramanik N, Guha SK, Goswami RP, Banerjee D, Ali N: Characterization of immunoglobulin G and its subclass response to Indian kala-azar infection before and after chemotherapy. *Infection and immunity* 2004, 72(2):863-870.

235. Mary C, Lamouroux D, Dunan S, Quilici M: Western blot analysis of antibodies to *Leishmania infantum* antigens: potential of the 14-kD and 16-kD antigens for diagnosis and epidemiologic purposes. *The American journal of tropical medicine and hygiene* 1992, 47(6):764-771.

236. Santos-Gomes G, Gomes-Pereira S, Campino L, Araujo MD, Abranches P: Performance of immunoblotting in diagnosis of visceral Leishmaniasis in human immunodeficiency virus-Leishmania sp.-coinfected patients. *Journal of clinical microbiology* 2000, 38(1):175-178.

237. Cota GF, de Sousa MR, Demarqui FN, Rabello A: The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. *PLoS neglected tropical diseases* 2012, 6(5):e1665.

238. Oskam L, Nieuwenhuijs JL, Hailu A: Evaluation of the direct agglutination test (DAT) using freeze-dried antigen for the detection of anti-*Leishmania* antibodies in stored sera from

various patient groups in Ethiopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1999, 93(3):275-277.

239. Fakhar M, Motazedian MH, Hatam GR, Asgari Q, Monabati FA, . KM: Comparative performance of direct agglutination test indirect immunofluorescent antibody test polymerase chain reaction, bone marrow aspiration method for diagnosis of Mediterranean visceral leishmaniasis. . *African J Microbiol* 2012, 6:: 5777-5781.
240. Chappuis F, Rijal S, Soto A, Menten J, Boelaert M: A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *Bmj* 2006, 333(7571):723.
241. Boelaert M, El Safi S, Mousa H, Githure J, Mbati P, Gurubacharya V, Shrestha J, Jacquet D, De Muynck A, Le Ray D *et al*: Multi-centre evaluation of repeatability and reproducibility of the direct agglutination test for visceral leishmaniasis. *Tropical medicine & international health : TM & IH* 1999, 4(1):31-37.
242. Gomez-Ochoa P, Castillo JA, Lucientes J, Gascon M, Zarate JJ, Arbea JI, Larraga V, Rodriguez C: Modified direct agglutination test for simplified serologic diagnosis of leishmaniasis. *Clin Diagn Lab Immunol* 2003, 10(5):967-968.
243. Schoone GJ, Hailu A, Kroon CC, Nieuwenhuys JL, Schallig HD, Oskam L: A fast agglutination screening test (FAST) for the detection of anti-Leishmania antibodies. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2001, 95(4):400-401.
244. Hailu A, Schoone GJ, Diro E, Tesfaye A, Techane Y, Tefera T, Assefa Y, Genetu A, Kebede Y, Kebede T *et al*: Field evaluation of a fast anti-*Leishmania* antibody detection assay in Ethiopia. *Trans R Soc Trop Med Hyg* 2006, 100(1):48-52.
245. Akhoundi B, Mohebali M, Babakhan L, Edrissian GH, Eslami MB, Keshavarz H, Malekafzali H: Rapid detection of human *Leishmania infantum* infection: a comparative field study using the fast agglutination screening test and the direct agglutination test. *Travel Med Infect Dis* 2010, 8(5):305-310.
246. Pedras MJ, de Gouvea Viana L, de Oliveira EJ, Rabello A: Comparative evaluation of direct agglutination test, rK39 and soluble antigen ELISA and IFAT for the diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2008, 102(2):172-178.

247. Schallig HD, Schoone GJ, Kroon CC, Hailu A, Chappuis F, Veeken H: Development and application of 'simple' diagnostic tools for visceral leishmaniasis. *Med Microbiol Immunol* 2001, 190(1-2):69-71.

248. Sundar S, Reed SG, Singh VP, Kumar PC, Murray HW: Rapid accurate field diagnosis of Indian visceral leishmaniasis. *Lancet* 1998, 351(9102):563-565.

249. Sundar S, Pai K, Sahu M, Kumar V, Murray HW: Immunochromatographic strip-test detection of anti-K39 antibody in Indian visceral leishmaniasis. *Annals of tropical medicine and parasitology* 2002, 96(1):19-23.

250. Carvalho SF, Lemos EM, Corey R, Dietze R: Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *The American journal of tropical medicine and hygiene* 2003, 68(3):321-324.

251. Brandonisio O, Fumarola L, Maggi P, Cavaliere R, Spinelli R, Pastore G: Evaluation of a rapid immunochromatographic test for serodiagnosis of visceral leishmaniasis. *Eur J Clin Microbiol Infect Dis* 2002, 21(6):461-464.

252. Jelinek T, Eichenlaub S, Loscher T: Sensitivity and specificity of a rapid immunochromatographic test for diagnosis of visceral leishmaniasis. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 1999, 18(9):669-670.

253. Ghatei MA, Hatam GR, Hossini MH, Sarkari B: Performance of latex agglutination test (KAtex) in diagnosis of visceral leishmaniasis in Iran. *Iran J Immunol* 2009, 6(4):202-207.

254. Akhoundi B, Mohebali M, Shojaee S, Jalali M, Kazemi B, Bandehpour M, Keshavarz H, Edrissian GH, Eslami MB, Malekafzali H *et al*: Rapid detection of human and canine visceral leishmaniasis: assessment of a latex agglutination test based on the A2 antigen from amastigote forms of *Leishmania infantum*. *Exp Parasitol* 2013, 133(3):307-313.

255. Piarroux R, Gambarelli F, Dumon H, Fontes M, Dunan S, Mary C, Toga B, Quilici M: Comparison of PCR with direct examination of bone marrow aspiration, myeloculture, and serology for diagnosis of visceral Leishmaniasis in immunocompromised patients. *J Clin Microbiol* 1994, 32(3):746-749.

256. Pizzuto M, Piazza M, Senese D, Scalamogna C, Calattini S, Corsico L, Persico T, Adriani B, Magni C, Guaraldi G *et al*: Role of PCR in diagnosis and prognosis of visceral leishmaniasis

in patients coinfected with human immunodeficiency virus type 1. *J Clin Microbiol* 2001, 39(1):357-361.

257. Lachaud L, Dereure J, Chabbert E, Reynes J, Mauboussin JM, Oziol E, Dedet JP, Bastien P: Optimized PCR using patient blood samples for diagnosis and follow-up of visceral Leishmaniasis, with special reference to AIDS patients. *J Clin Microbiol* 2000, 38(1):236-240.

258. Antinori S, Calattini S, Longhi E, Bestetti G, Piolini R, Magni C, Orlando G, Gramiccia M, Acquaviva V, Foschi A *et al*: Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. *Clin Infect Dis* 2007, 44(12):1602-1610.

259. Liarte DB, Mendonca IL, Luz FC, Abreu EA, Mello GW, Farias TJ, Ferreira AF, Millington MA, Costa CH: QBC for the diagnosis of human and canine american visceral leishmaniasis: preliminary data. *Rev Soc Bras Med Trop* 2001, 34(6):577-581.

260. da Silva ES, Gontijo CM, Pacheco Rda S, Brazil RP: Diagnosis of human visceral leishmaniasis by PCR using blood samples spotted on filter paper. *Genet Mol Res* 2004, 3(2):251-257.

261. Martin-Sanchez J, Pineda JA, Morillas-Marquez F, Garcia-Garcia JA, Acedo C, Macias J: Detection of *Leishmania infantum* kinetoplast DNA in peripheral blood from asymptomatic individuals at risk for parenterally transmitted infections: relationship between polymerase chain reaction results and other *Leishmania* infection markers. *Am J Trop Med Hyg* 2004, 70(5):545-548.

262. Wortmann G, Sweeney C, Houng HS, Aronson N, Stiteler J, Jackson J, Ockenhouse C: Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. *Am J Trop Med Hyg* 2001, 65(5):583-587.

263. Gangneux JP, Menotti J, Lorenzo F, Sarfati C, Blanche H, Bui H, Pratlong F, Garin YJ, Derouin F: Prospective value of PCR amplification and sequencing for diagnosis and typing of old world *Leishmania* infections in an area of nonendemicity. *J Clin Microbiol* 2003, 41(4):1419-1422.

264. Otero AC, da Silva VO, Luz KG, Palatnik M, Pirmez C, Fernandes O, Palatnik de Sousa CB: Short report: occurrence of *Leishmania donovani* DNA in donated blood from seroreactive Brazilian blood donors. *Am J Trop Med Hyg* 2000, 62(1):128-131.

265. Sreenivas G, Ansari NA, Kataria J, Salotra P: Nested PCR assay for detection of *Leishmania donovani* in slit aspirates from post-kala-azar dermal Leishmaniasis Lesions. *J Clin Microbiol* 2004, 42(4):1777-1778.

266. Singh N, Curran MD, Rastogil AK, Middleton D, Sundar S: Diagnostic PCR with *Leishmania donovani* specificity using sequences from the variable region of kinetoplast minicircle DNA. *Trop Med Int Health* 1999, 4(6):448-453.

267. Osman OF, Oskam L, Zijlstra EE, Kroon NC, Schoone GJ, Khalil ET, El-Hassan AM, Kager PA: Evaluation of PCR for diagnosis of visceral leishmaniasis. *J Clin Microbiol* 1997, 35(10):2454-2457.

268. De Doncker S, Hutse V, Abdellati S, Rijal S, Singh Karki BM, Decuypere S, Jacquet D, Le Ray D, Boelaert M, Koirala S *et al*: A new PCR-ELISA for diagnosis of visceral leishmaniasis in blood of HIV-negative subjects. *Trans R Soc Trop Med Hyg* 2005, 99(1):25-31.

269. Saad AA, Ahmed NG, Osman OS, Al-Basheer AA, Hamad A, Deborggraeve S, Buscher P, Schoone GJ, Schallig HD, Laurent T *et al*: Diagnostic accuracy of the Leishmania OligoC-TesT and NASBA-Oligochromatography for diagnosis of leishmaniasis in Sudan. *PLoS Negl Trop Dis* 2010, 4(8):e776.

270. Bossolasco S, Gaiera G, Olchini D, Gulletta M, Martello L, Bestetti A, Bossi L, Germagnoli L, Lazzarin A, Uberti-Foppa C *et al*: Real-time PCR assay for clinical management of human immunodeficiency virus-infected patients with visceral leishmaniasis. *J Clin Microbiol* 2003, 41(11):5080-5084.

271. Mary C, Faraut F, Lascombe L, Dumon H: Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. *J Clin Microbiol* 2004, 42(11):5249-5255.

272. WHO: World Health Organization: HIV/AIDS fact sheet nº 360. Available: http://www.who.int/mediacentre/factsheets/fs360/en/. Accessed 5 May 2014. 2013.

273. Shafiei R, Mohebali M, Akhoundi B, Galian MS, Kalantar F, Ashkan S, Fata A, Farash BR, Ghasemian M: Emergence of co-infection of visceral leishmaniasis in HIV-positive patients in northeast Iran: a preliminary study. *Travel Med Infect Dis* 2014, 12(2):173-178.

274. Sinha PK, van Griensven J, Pandey K, Kumar N, Verma N, Mahajan R, Kumar P, Kumar R, Das P, Mitra G *et al*: Liposomal amphotericin B for visceral leishmaniasis in human immunodeficiency virus-coinfected patients: 2-year treatment outcomes in Bihar, India. *Clin Infect Dis* 2011, 53(7):e91-98.

275. ter Horst R, Tefera T, Assefa G, Ebrahim AZ, Davidson RN, Ritmeijer K: Field evaluation of rK39 test and direct agglutination test for diagnosis of visceral leishmaniasis in a population with high prevalence of human immunodeficiency virus in Ethiopia. *Am J Trop Med Hyg* 2009, 80(6):929-934.

276. WHO: World Health Organization (2007) Report of the Fifth Consultative Meeting on Leishmania /HIV Co-infection Addis Ababa, Ethiopia, 20–22 March 2007. Available: http://www.who.int/leishmaniasis/resources/Leishmaniasis_hiv_coinfection5.pdf2. Accessed May 2014. 2014.

277. Fernandez-Guerrero ML, Aguado JM, Buzon L, Barros C, Montalban C, Martin T, Bouza E: Visceral leishmaniasis in immunocompromised hosts. *Am J Med* 1987, 83(6):1098-1102.

278. Mondain-Miton V, Toussaint-Gari M, Hofman P, Marty P, Carles M, De Salvador F, Miton F, Le Fichoux Y, Dellamonica P: Atypical leishmaniasis in a patient infected with human immunodeficiency virus. *Clin Infect Dis* 1995, 21(3):663-665.

279. Albrecht H: Leishmaniosis--new perspectives on an underappreciated opportunistic infection. *AIDS* 1998, 12(16):2225-2226.

280. Rosenthal E, Marty P, le Fichoux Y, Cassuto JP: Clinical manifestations of visceral leishmaniasis associated with HIV infection: a retrospective study of 91 French cases. *Ann Trop Med Parasitol* 2000, 94(1):37-42.

281. Carnauba D, Jr., Konishi CT, Petri V, Martinez IC, Shimizu L, Pereira-Chioccola VL: Atypical disseminated leishmaniasis similar to post-kala-azar dermal leishmaniasis in a Brazilian AIDS patient infected with *Leishmania (Leishmania) infantum chagasi*: a case report. *Int J Infect Dis* 2009, 13(6):e504-507.

282. WHO: Control of the leishmaniases. *World Health Organ Tech Rep Ser* 2010(949):xii-xiii, 1-186, back cover.

283. Bumb RA, Prasad N, Khandelwal K, Aara N, Mehta RD, Ghiya BC, Salotra P, Wei L, Peters S, Satoskar AR: Long-term efficacy of single-dose radiofrequency-induced heat therapy vs.

intralesional antimonials for cutaneous leishmaniasis in India. *Br J Dermatol* 2013, 168(5):1114-1119.

284. Shamsi Meymandi S, Zandi S, Aghaie H, Heshmatkhah A: Efficacy of CO(2) laser for treatment of anthroponotic cutaneous leishmaniasis, compared with combination of cryotherapy and intralesional meglumine antimoniate. *J Eur Acad Dermatol Venereol* 2011, 25(5):587-591.
285. Negera E, Gadisa E, Hussein J, Engers H, Kuru T, Gedamu L, Aseffa A: Treatment response of cutaneous leishmaniasis due to *Leishmania aethiopica* to cryotherapy and generic sodium stibogluconate from patients in Silti, Ethiopia. *Trans R Soc Trop Med Hyg* 2012, 106(8):496-503.
286. Asilian A, Sadeghinia A, Faghihi G, Momeni A: Comparative study of the efficacy of combined cryotherapy and intralesional meglumine antimoniate (Glucantime) vs. cryotherapy and intralesional meglumine antimoniate (Glucantime) alone for the treatment of cutaneous leishmaniasis. *Int J Dermatol* 2004, 43(4):281-283.
287. Gonzalez U, Pinart M, Reveiz L, Alvar J: Interventions for Old World cutaneous leishmaniasis. *The Cochrane database of systematic reviews* 2008(4):CD005067.
288. Garcia Bustos MF, Barrio AB, Parodi Ramoneda CM, Ramos F, Mora MC, Convit J, Basombrio MA: Immunological correlates of cure in the first American cutaneous leishmaniasis patient treated by immunotherapy in Argentina. A case report. *Invest Clin* 2011, 52(4):365-375.
289. Badaro R, Lobo I, Munos A, Netto EM, Modabber F, Campos-Neto A, Coler RN, Reed SG: Immunotherapy for drug-refractory mucosal leishmaniasis. *J Infect Dis* 2006, 194(8):1151-1159.
290. Almeida RP, Brito J, Machado PL, AR DEJ, Schriefer A, Guimaraes LH, Carvalho EM: Successful treatment of refractory cutaneous leishmaniasis with GM-CSF and antimonials. *Am J Trop Med Hyg* 2005, 73(1):79-81.
291. Badaro R, Lobo I, Nakatani M, Muinos A, Netto EM, Coler RN, Reed SG: Successful use of a defined antigen/GM-CSF adjuvant vaccine to treat mucosal Leishmaniasis refractory to antimony: A case report. *Braz J Infect Dis* 2001, 5(4):223-232.
292. Convit J, Ulrich M, Polegre MA, Avila A, Rodriguez N, Mazzedo MI, Blanco B: Therapy of Venezuelan patients with severe mucocutaneous or early lesions of diffuse cutaneous

leishmaniasis with a vaccine containing pasteurized *Leishmania* promastigotes and bacillus Calmette-Guerin: preliminary report. *Mem Inst Oswaldo Cruz* 2004, 99(1):57-62.

293. Da-Cruz AM, de Oliveira MP, De Luca PM, Mendonca SC, Coutinho SG: Tumor necrosis factor-alpha in human american tegumentary leishmaniasis. *Mem Inst Oswaldo Cruz* 1996, 91(2):225-229.
294. Machado PR, Lessa H, Lessa M, Guimaraes LH, Bang H, Ho JL, Carvalho EM: Oral pentoxifylline combined with pentavalent antimony: a randomized trial for mucosal leishmaniasis. *Clin Infect Dis* 2007, 44(6):788-793.
295. Sundar S, Singh VP, Sharma S, Makharia MK, Murray HW: Response to interferon-gamma plus pentavalent antimony in Indian visceral leishmaniasis. *J Infect Dis* 1997, 176(4):1117-1119.
296. Mishra J, Saxena A, Singh S: Chemotherapy of leishmaniasis: past, present and future. *Current medicinal chemistry* 2007, 14(10):1153-1169.
297. Sundar S, Chatterjee M: Visceral leishmaniasis - current therapeutic modalities. *Indian J Med Res* 2006, 123(3):345-352.
298. Alvar J, Croft S, Olliaro P: Chemotherapy in the treatment and control of leishmaniasis. *Adv Parasitol* 2006, 61:223-274.
299. Murray HW: Treatment of visceral leishmaniasis (kala-azar): a decade of progress and future approaches. *Int J Infect Dis* 2000, 4(3):158-177.
300. Faraut-Gambarelli F, Piarroux R, Deniau M, Giusiano B, Marty P, Michel G, Faugere B, Dumon H: *In vitro* and *in vivo* resistance of *Leishmania infantum* to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. *Antimicrob Agents Chemother* 1997, 41(4):827-830.
301. Frezard F, Demicheli C, Ribeiro RR: Pentavalent antimonials: new perspectives for old drugs. *Molecules* 2009, 14(7):2317-2336.
302. Sundar S, Chakravarty J: Leishmaniasis: an update of current pharmacotherapy. *Expert opinion on pharmacotherapy* 2013, 14(1):53-63.
303. Mbongo N, Loiseau PM, Billion MA, Robert-Gero M: Mechanism of amphotericin B resistance in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* 1998, 42(2):352-357.

304. Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, Boelaert M, Dujardin JC, Chakravarty J: Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin Infect Dis* 2012, 55(4):543-550.

305. Miguel DC, Zauli-Nascimento RC, Yokoyama-Yasunaka JK, Pereira LI, Jeronimo SM, Ribeiro-Dias F, Dorta ML, Uliana SR: Clinical isolates of New World *Leishmania* from cutaneous and visceral leishmaniasis patients are uniformly sensitive to tamoxifen. *Int J Antimicrob Agents* 2011, 38(1):93-94.

306. Garcia-Hernandez R, Manzano JI, Castanys S, Gamarro F: *Leishmania donovani* develops resistance to drug combinations. *PLoS Negl Trop Dis* 2012, 6(12):e1974.

307. Sundar S, Sinha PK, Rai M, Verma DK, Nawin K, Alam S, Chakravarty J, Vaillant M, Verma N, Pandey K *et al*: Comparison of short-course multidrug treatment with standard therapy for visceral leishmaniasis in India: an open-label, non-inferiority, randomised controlled trial. *Lancet* 2011, 377(9764):477-486.

308. van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, Boelaert M: Combination therapy for visceral leishmaniasis. *Lancet Infect Dis* 2010, 10(3):184-194.

309. Drugs for parasitic infections. *Med Lett Drugs Ther* 1974, 16(2):5-12.

310. Singh RK, Pandey HP, Sundar S: Visceral leishmaniasis (kala-azar): challenges ahead. *Indian J Med Res* 2006, 123(3):331-344.

311. Berman JD: Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 1997, 24(4):684-703.

312. Ephros M, Bitnun A, Shaked P, Waldman E, Zilberstein D: Stage-specific activity of pentavalent antimony against *Leishmania donovani* axenic amastigotes. *Antimicrob Agents Chemother* 1999, 43(2):278-282.

313. Herwaldt BL, Berman JD: Recommendations for treating leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. *The American journal of tropical medicine and hygiene* 1992, 46(3):296-306.

314. Wortmann G, Miller RS, Oster C, Jackson J, Aronson N: A randomized, double-blind study of the efficacy of a 10- or 20-day course of sodium stibogluconate for treatment of cutaneous leishmaniasis in United States military personnel. *Clin Infect Dis* 2002, 35(3):261-267.

315. Ritmeijer K, Veeken H, Melaku Y, Leal G, Amsalu R, Seaman J, Davidson RN: Ethiopian visceral leishmaniasis: generic and proprietary sodium stibogluconate are equivalent; HIV co-infected patients have a poor outcome. *Trans R Soc Trop Med Hyg* 2001, 95(6):668-672.

316. Moore E, O'Flaherty D, Heuvelmans H, Seaman J, Veeken H, de Wit S, Davidson RN: Comparison of generic and proprietary sodium stibogluconate for the treatment of visceral leishmaniasis in Kenya. *Bull World Health Organ* 2001, 79(5):388-393.

317. Baiocco P, Colotti G, Franceschini S, Ilari A: Molecular basis of antimony treatment in leishmaniasis. *J Med Chem* 2009, 52(8):2603-2612.

318. Thakur CP, Kumar M, Pandey AK: Comparison of regimes of treatment of antimony-resistant kala-azar patients: a randomized study. *Am J Trop Med Hyg* 1991, 45(4):435-441.

319. Wyllie S, Cunningham ML, Fairlamb AH: Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. *J Biol Chem* 2004, 279(38):39925-39932.

320. Ashutosh, Sundar S, Goyal N: Molecular mechanisms of antimony resistance in Leishmania. *J Med Microbiol* 2007, 56(Pt 2):143-153.

321. Pathak MK, Yi T: Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines. *J Immunol* 2001, 167(6):3391-3397.

322. Lima MI, Arruda VO, Alves EV, de Azevedo AP, Monteiro SG, Pereira SR: Genotoxic effects of the antileishmanial drug Glucantime. *Arch Toxicol* 2010, 84(3):227-232.

323. Torres DC, Adaui V, Ribeiro-Alves M, Romero GA, Arevalo J, Cupolillo E, Dujardin JC: Targeted gene expression profiling in *Leishmania braziliensis* and *Leishmania guyanensis* parasites isolated from Brazilian patients with different antimonial treatment outcomes. *Infect Genet Evol* 2010, 10(6):727-733.

324. el Hassan AM, Ghalib HW, Zijlstra EE, Eltoum IA, Satti M, Ali MS, Ali HM: Post kala-azar dermal leishmaniasis in the Sudan: clinical features, pathology and treatment. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1992, 86(3):245-248.

325. Musa AM, Khalil EA, Younis BM, Elfaki ME, Elamin MY, Adam AO, Mohamed HA, Dafalla MM, Abuzaid AA, El-Hassan AM: Treatment-based strategy for the management of post-kala-azar dermal leishmaniasis patients in the Sudan. *J Trop Med* 2013, 2013:708391.

326. Croft SL, Coombs GH: Leishmaniasis--current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol* 2003, 19(11):502-508.

327. Lessa HA, Carvalho EM, Marsden PD: Eustachian tube blockage with consequent middle ear infection in mucosal leishmaniasis. *Revista da Sociedade Brasileira de Medicina Tropical* 1994, 27(2):103.

328. Franke ED, Wignall FS, Cruz ME, Rosales E, Tovar AA, Lucas CM, Llanos-Cuentas A, Berman JD: Efficacy and toxicity of sodium stibogluconate for mucosal leishmaniasis. *Annals of internal medicine* 1990, 113(12):934-940.

329. Khalil EA, Ahmed AE, Musa AM, Hussein MH: Antimony-induced cerebellar ataxia. *Saudi Med J* 2006, 27(1):90-92.

330. Subba Raju BV, Gurumurthy S, Kuhls K, Bhandari V, Schnonian G, Salotra P: Genetic typing reveals monomorphism between antimony sensitive and resistant *Leishmania donovani* isolates from visceral leishmaniasis or post kala-azar dermal leishmaniasis cases in India. *Parasitology research* 2012, 111(4):1559-1568.

331. Thakur CP, Kumar K: Efficacy of prolonged therapy with stibogluconate in post kala-azar dermal leishmaniasis. *The Indian journal of medical research* 1990, 91:144-148.

332. Gasser RA, Jr., Magill AJ, Oster CN, Franke ED, Grogl M, Berman JD: Pancreatitis induced by pentavalent antimonial agents during treatment of leishmaniasis. *Clin Infect Dis* 1994, 18(1):83-90.

333. Shahian M, Alborzi A: Effect of meglumine antimoniate on the pancreas during treatment of visceral leishmaniasis in children. *Medical science monitor : international medical journal of experimental and clinical research* 2009, 15(6):CR290-293.

334. Collin S, Davidson R, Ritmeijer K, Keus K, Melaku Y, Kipngetich S, Davies C: Conflict and kala-azar: determinants of adverse outcomes of kala-azar among patients in southern Sudan. *Clin Infect Dis* 2004, 38(5):612-619.

335. Zaghloul IY, Al-Jasser M: Effect of renal impairment on the pharmacokinetics of antimony in hamsters. *Ann Trop Med Parasitol* 2004, 98(8):793-800.

336. Matoussi N, Ameur HB, Amor SB, Fitouri Z, Becher SB: [Cardiotoxicity of n-methyl-glucamine antimoniate (Glucantime). A case report]. *Medecine et maladies infectieuses* 2007, 37 Suppl 3:S257-259.

337. Antezana G, Zeballos R, Mendoza C, Lyevre P, Valda L, Cardenas F, Noriega I, Ugarte H, Dedet JP: Electrocardiographic alterations during treatment of mucocutaneous leishmaniasis with meglumine antimoniate and allopurinol. *Trans R Soc Trop Med Hyg* 1992, 86(1):31-33.

338. Ouellette M, Papadopoulou B: Mechanisms of drug resistance in *Leishmania*. *Parasitol Today* 1993, 9(5):150-153.

339. Neves LO, Talhari AC, Gadelha EP, Silva Junior RM, Guerra JA, Ferreira LC, Talhari S: A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin B for the treatment of cutaneous leishmaniasis by *Leishmania guyanensis*. *Anais brasileiros de dermatologia* 2011, 86(6):1092-1101.

340. Inocencio da Luz R, Romero GA, Dorval ME, Cruz I, Canavate C, Dujardin JC, Van Assche T, Cos P, Maes L: Drug susceptibility of *Leishmania infantum* (syn. Leishmania chagasi) isolates from Brazilian HIV-positive and HIV-negative patients. *The Journal of antimicrobial chemotherapy* 2011, 66(3):677-679.

341. Rojas R, Valderrama L, Valderrama M, Varona MX, Ouellette M, Saravia NG: Resistance to antimony and treatment failure in human *Leishmania (Viannia)* infection. *The Journal of infectious diseases* 2006, 193(10):1375-1383.

342. Schettini DA, Ribeiro RR, Demicheli C, Rocha OG, Melo MN, Michalick MS, Frezard F: Improved targeting of antimony to the bone marrow of dogs using liposomes of reduced size. *Int J Pharm* 2006, 315(1-2):140-147.

343. Demicheli C, Ochoa R, da Silva JB, Falcao CA, Rossi-Bergmann B, de Melo AL, Sinisterra RD, Frezard F: Oral delivery of meglumine antimoniate-beta-cyclodextrin complex for treatment of leishmaniasis. *Antimicrob Agents Chemother* 2004, 48(1):100-103.

344. Lemke A, Kiderlen AF, Kayser O: Amphotericin B. *Appl Microbiol Biotechnol* 2005, 68(2):151-162.

345. Singh S, Sivakumar R: Challenges and new discoveries in the treatment of leishmaniasis. *J Infect Chemother* 2004, 10(6):307-315.

346. Barratt G, Legrand P: Comparison of the efficacy and pharmacology of formulations of amphotericin B used in treatment of leishmaniasis. *Curr Opin Infect Dis* 2005, 18(6):527-530.

347. Goad LJ, Holz GG, Jr., Beach DH: Sterols of Leishmania species. Implications for biosynthesis. *Mol Biochem Parasitol* 1984, 10(2):161-170.

348. Roberts CW, McLeod R, Rice DW, Ginger M, Chance ML, Goad LJ: Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. *Mol Biochem Parasitol* 2003, 126(2):129-142.

349. Cohen BE, Benaim G, Ruiz MC, Michelangeli F: Increased calcium permeability is not responsible for the rapid lethal effects of amphotericin B on Leishmania sp. *FEBS Lett* 1990, 259(2):286-288.

350. Ramos H, Valdivieso E, Gamargo M, Dagger F, Cohen BE: Amphotericin B kills unicellular leishmanias by forming aqueous pores permeable to small cations and anions. *The Journal of membrane biology* 1996, 152(1):65-75.

351. Mishra M, Biswas UK, Jha DN, Khan AB: Amphotericin versus pentamidine in antimony-unresponsive kala-azar. *Lancet* 1992, 340(8830):1256-1257.

352. Thakur CP, Singh RK, Hassan SM, Kumar R, Narain S, Kumar A: Amphotericin B deoxycholate treatment of visceral leishmaniasis with newer modes of administration and precautions: a study of 938 cases. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1999, 93(3):319-323.

353. Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, Marzochi MC, Andrade CA: Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta tropica* 2011, 118(2):87-96.

354. Laniado-Laborin R, Cabrales-Vargas MN: Amphotericin B: side effects and toxicity. *Rev Iberoam Micol* 2009, 26(4):223-227.

355. Balana-Fouce R, Reguera RM, Cubria JC, Ordonez D: The pharmacology of leishmaniasis. *General pharmacology* 1998, 30(4):435-443.

356. Sundar S, Mehta H, Suresh AV, Singh SP, Rai M, Murray HW: Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2004, 38(3):377-383.

357. Torchilin VP: Lipid-core micelles for targeted drug delivery. *Curr Drug Deliv* 2005, 2(4):319-327.

358. Yardley V, Croft SL: Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis. *Antimicrobial agents and chemotherapy* 1997, 41(4):752-756.

359. Meyerhoff A: U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 1999, 28(1):42-48; discussion 49-51.

360. Berman JD, Badaro R, Thakur CP, Wasunna KM, Behbehani K, Davidson R, Kuzoe F, Pang L, Weerasuriya K, Bryceson AD: Efficacy and safety of liposomal amphotericin B (AmBisome) for visceral leishmaniasis in endemic developing countries. *Bull World Health Organ* 1998, 76(1):25-32.

361. Davidson RN, Di Martino L, Gradoni L, Giacchino R, Russo R, Gaeta GB, Pempinello R, Scott S, Raimondi F, Cascio A *et al*: Liposomal amphotericin B (AmBisome) in Mediterranean visceral leishmaniasis: a multi-centre trial. *Q J Med* 1994, 87(2):75-81.

362. Sundar S, Agrawal G, Rai M, Makharia MK, Murray HW: Treatment of Indian visceral leishmaniasis with single or daily infusions of low dose liposomal amphotericin B: randomised trial. *Bmj* 2001, 323(7310):419-422.

363. Wortmann G, Zapor M, Ressner R, Fraser S, Hartzell J, Pierson J, Weintrob A, Magill A: Lipsosomal amphotericin B for treatment of cutaneous leishmaniasis. *Am J Trop Med Hyg* 2010, 83(5):1028-1033.

364. Musa AM, Khalil EA, Mahgoub FA, Hamad S, Elkadaru AM, El Hassan AM: Efficacy of liposomal amphotericin B (AmBisome) in the treatment of persistent post-kala-azar dermal leishmaniasis (PKDL). *Ann Trop Med Parasitol* 2005, 99(6):563-569.

365. Bern C, Adler-Moore J, Berenguer J, Boelaert M, den Boer M, Davidson RN, Figueras C, Gradoni L, Kafetzis DA, Ritmeijer K *et al*: Liposomal amphotericin B for the treatment of visceral leishmaniasis. *Clin Infect Dis* 2006, 43(7):917-924.

366. Murdaca G, Setti M, Campelli A, Ghio M, Puppo F, Indiveri F: [Liposomal Amphotericin B for treatment of acute phase and secondary prophylaxis of visceral leishmaniasis in a HIV positive patient]. *Infez Med* 2000, 8(4):241-244.

367. Berman J, Dietze R: Treatment of visceral leishmaniasis with amphotericin B colloidal dispersion. *Chemotherapy* 1999, 45 Suppl 1:54-66.

368. Sundar S, Goyal AK, Mandal AK, Makharia MK, Singh VP, Murray HW: Amphotericin B lipid complex in the management of antimony unresponsive Indian visceral leishmaniasis. *J Assoc Physicians India* 1999, 47(2):186-188.

369. Sundar S, Goyal AK, More DK, Singh MK, Murray HW: Treatment of antimony-unresponsive Indian visceral leishmaniasis with ultra-short courses of amphotericin-B-lipid complex. *Ann Trop Med Parasitol* 1998, 92(7):755-764.

370. Olliaro P, Sundar S: Anthropometrically derived dosing and drug costing calculations for treating visceral leishmaniasis in Bihar, India. *Tropical medicine & international health : TM & IH* 2009, 14(1):88-92.

371. Pearson RD, Jeronimo SM, Lareau SM: US Food and Drug Administration Approval of Liposomal Amphotericin B for the Treatment of Visceral Leishmaniasis: A Model for Orphan Drug Development. *Current infectious disease reports* 1999, 1(5):415-416.

372. Courtney KO, Thompson PE, Hodgkinson R, Fitzsimmons JR: Paromomycin as a therapeutic substance for intestinal amebiasis and bacterial enteritis. *Antibiot Annu* 1959, 7:304-309.

373. Sundar S, Jha TK, Thakur CP, Sinha PK, Bhattacharya SK: Injectable paromomycin for Visceral leishmaniasis in India. *N Engl J Med* 2007, 356(25):2571-2581.

374. Ben Salah A, Ben Messaoud N, Guedri E, Zaatour A, Ben Alaya N, Bettaieb J, Gharbi A, Belhadj Hamida N, Boukthir A, Chlif S *et al*: Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. *N Engl J Med* 2013, 368(6):524-532.

375. Jha TK, Olliaro P, Thakur CP, Kanyok TP, Singhania BL, Singh IJ, Singh NK, Akhoury S, Jha S: Randomised controlled trial of aminosidine (paromomycin) v sodium stibogluconate for treating visceral leishmaniasis in North Bihar, India. *Bmj* 1998, 316(7139):1200-1205.

376. Barrett MP, Mottram JC, Coombs GH: Recent advances in identifying and validating drug targets in trypanosomes and leishmanias. *Trends Microbiol* 1999, 7(2):82-88.

377. Sundar S, Chakravarty J: Paromomycin in the treatment of leishmaniasis. *Expert Opin Investig Drugs* 2008, 17(5):787-794.

378. El-On J, Livshin R, Even-Paz Z, Hamburger D, Weinrauch L: Topical treatment of cutaneous leishmaniasis. *J Invest Dermatol* 1986, 87(2):284-288.

379. Asilian A, Jalayer T, Nilforooshzadeh M, Ghassemi RL, Peto R, Wayling S, Olliaro P, Modabber F: Treatment of cutaneous leishmaniasis with aminosidine (paromomycin) ointment: double-blind, randomized trial in the Islamic Republic of Iran. *Bull World Health Organ* 2003, 81(5):353-359.

380. Romero GA, Lessa HA, Orge MG, Macedo VO, Marsden PD: [Treatment of mucosal leishmaniasis with aminosidine sulfate: results of two year follow-up]. *Rev Soc Bras Med Trop* 1998, 31(6):511-516.

381. WHO, Organization. WH: Leishmaniasis Fact sheet N°375". *January 2014 Retrieved 17 February 2014* 2014.

382. Neal RA, Allen S, McCoy N, Olliaro P, Croft SL: The sensitivity of Leishmania species to aminosidine. *J Antimicrob Chemother* 1995, 35(5):577-584.

383. Scott JA, Davidson RN, Moody AH, Grant HR, Felmingham D, Scott GM, Olliaro P, Bryceson AD: Aminosidine (paromomycin) in the treatment of leishmaniasis imported into the United Kingdom. *Trans R Soc Trop Med Hyg* 1992, 86(6):617-619.

384. Tracy JW, LT. W: In: Hardman JG, Limbird LE, Eds. The pharmacological basis of therapeutics. New York: McGraw Hill 2001; pp. 1097. 2001.

385. Sinha PK, Jha TK, Thakur CP, Nath D, Mukherjee S, Aditya AK, Sundar S: Phase 4 pharmacovigilance trial of paromomycin injection for the treatment of visceral leishmaniasis in India. *Journal of tropical medicine* 2011, 2011:645203.

386. Hailu A, Musa A, Wasunna M, Balasegaram M, Yifru S, Mengistu G, Hurissa Z, Hailu W, Weldegebreal T, Tesfaye S *et al*: Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicentre, open-label, randomized trial. *PLoS Negl Trop Dis* 2010, 4(10):e709.

387. Monge-Maillo B, Lopez-Velez R: Therapeutic options for visceral leishmaniasis. *Drugs* 2013, 73(17):1863-1888.

388. Maarouf M, Lawrence F, Croft SL, Robert-Gero M: Ribosomes of *Leishmania* are a target for the aminoglycosides. *Parasitol Res* 1995, 81(5):421-425.

389. Hirokawa G, Kaji H, Kaji A: Inhibition of antiassociation activity of translation initiation factor 3 by paromomycin. *Antimicrob Agents Chemother* 2007, 51(1):175-180.

390. Jhingran A, Chawla B, Saxena S, Barrett MP, Madhubala R: Paromomycin: uptake and resistance in *Leishmania donovani*. *Mol Biochem Parasitol* 2009, 164(2):111-117.

391. Maarouf M, de Kouchkovsky Y, Brown S, Petit PX, Robert-Gero M: In vivo interference of paromomycin with mitochondrial activity of *Leishmania*. *Exp Cell Res* 1997, 232(2):339-348.

392. Sundar S, Chakravarty J: Recent advances in the diagnosis and treatment of kala-azar. *Natl Med J India* 2012, 25(2):85-89.

393. Sundar S, Rai M: Treatment of visceral leishmaniasis. *Expert Opin Pharmacother* 2005, 6(16):2821-2829.

394. Llanos-Cuentas A, Echevarria J, Seas C, Chang E, Cruz M, Alvarez E, Rosales E, Campos P, Bryceson A: Parenteral aminosidine is not effective for Peruvian mucocutaneous leishmaniasis. *The American journal of tropical medicine and hygiene* 2007, 76(6):1128-1131.

395. Teklemariam S, Hiwot AG, Frommel D, Miko TL, Ganlov G, Bryceson A: Aminosidine and its combination with sodium stibogluconate in the treatment of diffuse cutaneous leishmaniasis caused by Leishmania aethiopica. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1994, 88(3):334-339.

396. Nok AJ: Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human African trypanosomiasis. *Parasitol Res* 2003, 90(1):71-79.

397. Thakur CP: Epidemiological, clinical and therapeutic features of Bihar kala-azar (including post kala-azar dermal leishmaniasis). *Trans R Soc Trop Med Hyg* 1984, 78(3):391-398.

398. Das VN, Siddiqui NA, Pandey K, Singh VP, Topno RK, Singh D, Verma RB, Ranjan A, Sinha PK, Das P: A controlled, randomized nonblinded clinical trial to assess the efficacy of amphotericin B deoxycholate as compared to pentamidine for the treatment of antimony unresponsive visceral leishmaniasis cases in Bihar, India. *Ther Clin Risk Manag* 2009, 5(1):117-124.

399. PAHO, salud. Opdl: Tratamiento de las enfermedades parasitarias. In Gui´a para el Tratamiento de las enfermedades infecciosas 2013–2014. *Washington, DC: OPS; p 212–3.*

400. de Paula CD, Sampaio JH, Cardoso DR, Sampaio RN: [A comparative study between the efficacy of pentamidine isothionate given in three doses for one week and N-methil-glucamine in a dose of 20mgSbV/day for 20 days to treat cutaneous leishmaniasis]. *Rev Soc Bras Med Trop* 2003, 36(3):365-371.

401. Amato V, Amato J, Nicodemo A, Uip D, Amato-Neto V, Duarte M: [Treatment of mucocutaneous leishmaniasis with pentamidine isothionate]. *Ann Dermatol Venereol* 1998, 125(8):492-495.

402. Nacher M, Carme B, Sainte Marie D, Couppie P, Clyti E, Guibert P, Pradinaud R: Influence of clinical presentation on the efficacy of a short course of pentamidine in the treatment of cutaneous leishmaniasis in French Guiana. *Ann Trop Med Parasitol* 2001, 95(4):331-336.

403. Andersen EM, Cruz-Saldarriaga M, Llanos-Cuentas A, Luz-Cjuno M, Echevarria J, Miranda-Verastegui C, Colina O, Berman JD: Comparison of meglumine antimoniate and pentamidine for peruvian cutaneous leishmaniasis. *The American journal of tropical medicine and hygiene* 2005, 72(2):133-137.

404. Nguewa PA, Fuertes MA, Cepeda V, Iborra S, Carrion J, Valladares B, Alonso C, Perez JM: Pentamidine is an antiparasitic and apoptotic drug that selectively modifies ubiquitin. *Chem Biodivers* 2005, 2(10):1387-1400.

405. Sun T, Zhang Y: Pentamidine binds to tRNA through non-specific hydrophobic interactions and inhibits aminoacylation and translation. *Nucleic Acids Res* 2008, 36(5):1654-1664.

406. "WHO, Drugs". Who.int. 2011-12-28. Retrieved 2013-03-26.]. . 2013.

407. Berman J: Recent Developments in Leishmaniasis: Epidemiology, Diagnosis, and Treatment. *Curr Infect Dis Rep* 2005, 7(1):33-38.

408. Ouellette M, Drummelsmith J, Papadopoulou B: Leishmaniasis: drugs in the clinic, resistance and new developments. *Drug Resist Updat* 2004, 7(4-5):257-266.

409. Soto J, Toledo J, Valda L, Balderrama M, Rea I, Parra R, Ardiles J, Soto P, Gomez A, Molleda F *et al*: Treatment of Bolivian mucosal leishmaniasis with miltefosine. *Clin Infect Dis* 2007, 44(3):350-356.

410. Sundar S, Rai M, Chakravarty J, Agarwal D, Agrawal N, Vaillant M, Olliaro P, Murray HW: New treatment approach in Indian visceral leishmaniasis: single-dose liposomal amphotericin B followed by short-course oral miltefosine. *Clin Infect Dis* 2008, 47(8):1000-1006.

411. Soto J, Arana BA, Toledo J, Rizzo N, Vega JC, Diaz A, Luz M, Gutierrez P, Arboleda M, Berman JD *et al*: Miltefosine for new world cutaneous leishmaniasis. *Clin Infect Dis* 2004, 38(9):1266-1272.

412. Sindermann H, Engel J: Development of miltefosine as an oral treatment for leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2006, 100 Suppl 1:S17-20.

413. Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, Junge K, Bryceson A, Berman J: Oral miltefosine for Indian visceral leishmaniasis. *The New England journal of medicine* 2002, 347(22):1739-1746.

414. Berman J, Bryceson AD, Croft S, Engel J, Gutteridge W, Karbwang J, Sindermann H, Soto J, Sundar S, Urbina JA: Miltefosine: issues to be addressed in the future. *Trans R Soc Trop Med Hyg* 2006, 100 Suppl 1:S41-44.

415. Singh UK, Prasad R, Mishra OP, Jayswal BP: Miltefosine in children with visceral leishmaniasis: a prospective, multicentric, cross-sectional study. *Indian J Pediatr* 2006, 73(12):1077-1080.

416. Bhattacharya SK, Jha TK, Sundar S, Thakur CP, Engel J, Sindermann H, Junge K, Karbwang J, Bryceson AD, Berman JD: Efficacy and tolerability of miltefosine for childhood visceral leishmaniasis in India. *Clin Infect Dis* 2004, 38(2):217-221.

417. Sundar S, Jha TK, Thakur CP, Bhattacharya SK, Rai M: Oral miltefosine for the treatment of Indian visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2006, 100 Suppl 1:S26-33.

418. Sundar S, Rosenkaimer F, Makharia MK, Goyal AK, Mandal AK, Voss A, Hilgard P, Murray HW: Trial of oral miltefosine for visceral leishmaniasis. *Lancet* 1998, 352(9143):1821-1823.

419. Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C, Voss A, Berman J: Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *N Engl J Med* 1999, 341(24):1795-1800.

420. Bhattacharya SK, Sinha PK, Sundar S, Thakur CP, Jha TK, Pandey K, Das VR, Kumar N, Lal C, Verma N *et al*: Phase 4 trial of miltefosine for the treatment of Indian visceral leishmaniasis. *J Infect Dis* 2007, 196(4):591-598.

421. Rahman M, Ahmed BN, Faiz MA, Chowdhury MZ, Islam QT, Sayeedur R, Rahman MR, Hossain M, Bangali AM, Ahmad Z *et al*: Phase IV trial of miltefosine in adults and children for treatment of visceral leishmaniasis (kala-azar) in Bangladesh. *Am J Trop Med Hyg* 2011, 85(1):66-69.

422. Perez-Victoria FJ, Gamarro F, Ouellette M, Castanys S: Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *J Biol Chem* 2003, 278(50):49965-49971.

423. Paris C, Loiseau PM, Bories C, Breard J: Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* 2004, 48(3):852-859.

424. Wadhone P, Maiti M, Agarwal R, Kamat V, Martin S, Saha B: Miltefosine promotes IFN-gamma-dominated anti-leishmanial immune response. *J Immunol* 2009, 182(11):7146-7154.

425. Ruiter GA, Zerp SF, Bartelink H, van Blitterswijk WJ, Verheij M: Anti-cancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase-Akt/PKB survival pathway. *Anticancer Drugs* 2003, 14(2):167-173.

426. Verma NK, Singh G, Dey CS: Miltefosine induces apoptosis in arsenite-resistant *Leishmania donovani* promastigotes through mitochondrial dysfunction. *Exp Parasitol* 2007, 116(1):1-13.

427. Soto J, Toledo J, Gutierrez P, Nicholls RS, Padilla J, Engel J, Fischer C, Voss A, Berman J: Treatment of American cutaneous leishmaniasis with miltefosine, an oral agent. *Clin Infect Dis* 2001, 33(7):E57-61.

428. Soto J, Rea J, Balderrama M, Toledo J, Soto P, Valda L, Berman JD: Efficacy of miltefosine for Bolivian cutaneous leishmaniasis. *The American journal of tropical medicine and hygiene* 2008, 78(2):210-211.

429. Ramesh V, Katara GK, Verma S, Salotra P: Miltefosine as an effective choice in the treatment of post-kala-azar dermal leishmaniasis. *Br J Dermatol* 2011, 165(2):411-414.

430. Sundar S, Murray HW: Availability of miltefosine for the treatment of kala-azar in India. *Bulletin of the World Health Organization* 2005, 83(5):394-395.

431. Perez-Victoria FJ, Sanchez-Canete MP, Seifert K, Croft SL, Sundar S, Castanys S, Gamarro F: Mechanisms of experimental resistance of *Leishmania* to miltefosine: Implications for clinical use. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* 2006, 9(1-2):26-39.

432. Soto J, Berman J: Treatment of New World cutaneous leishmaniasis with miltefosine. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2006, 100 Suppl 1:S34-40.

433. Sundar S, Olliaro PL: Miltefosine in the treatment of leishmaniasis: Clinical evidence for informed clinical risk management. *Therapeutics and clinical risk management* 2007, 3(5):733-740.

434. Rijal S, Ostyn B, Uranw S, Rai K, Bhattarai NR, Dorlo TP, Beijnen JH, Vanaerschot M, Decuypere S, Dhakal SS *et al*: Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2013, 56(11):1530-1538.

435. Alrajhi AA, Ibrahim EA, De Vol EB, Khairat M, Faris RM, Maguire JH: Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major*. *N Engl J Med* 2002, 346(12):891-895.

436. Mishra M, Thakur BD, Choudhary M: Metronidazole and Indian kala-azar: results of a clinical trial. *Br Med J (Clin Res Ed)* 1985, 291(6509):1611.

437. Al-Abdely HM, Graybill JR, Loebenberg D, Melby PC: Efficacy of the triazole SCH 56592 against Leishmania amazonensis and *Leishmania donovani* in experimental murine cutaneous and visceral leishmaniases. *Antimicrob Agents Chemother* 1999, 43(12):2910-2914.

438. Thakur CP, Sinha PK: Inefficacy of ethambutol, ethambutol plus isoniazid, INH plus rifampicin, co-trimoxazole and metronidazole in the treatment of kala-azar. *J Trop Med Hyg* 1989, 92(6):383-385.

439. Wali JP, Aggarwal P, Gupta U, Saluja S, Singh S: Ketoconazole in the treatment of antimony- and pentamidine-resistant kala-azar. *The Journal of infectious diseases* 1992, 166(1):215-216.

440. Jha TK, Singh NK, Jha S: Ketoconazole in antimonial unresponsive cases of Kala-azar. *The Journal of the Association of Physicians of India* 1995, 43(5):347.

441. Angarano G, Maggi P, Coppola SL, Cavaliere RL: Itraconazole as maintenance therapy for visceral leishmaniasis in HIV-infected patients. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 1998, 17(5):365-367.

442. Hueso M, Bover J, Seron D, Gil-Vernet S, Rufi G, Alsina J, Grinyo JM: The renal transplant patient with visceral leishmaniasis who could not tolerate meglumine antimoniate-cure with ketoconazole and allopurinol. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 1999, 14(12):2941-2943.

443. Boecken G, Sunderkotter C, Bogdan C, Weitzel T, Fischer M, Muller A, Lobermann M, Anders G, von Stebut E, Schunk M *et al*: [Diagnosis and therapy of cutaneous and mucocutaneous Leishmaniasis in Germany]. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* 2011, 9 Suppl 8:1-51.

444. Calvopina M, Guevara AG, Armijos RX, Hashiguchi Y, Davidson RN, Cooper PJ: Itraconazole in the treatment of New World mucocutaneous leishmaniasis. *International journal of dermatology* 2004, 43(9):659-663.

445. Iniguez E, Sanchez A, Vasquez MA, Martinez A, Olivas J, Sattler A, Sanchez-Delgado RA, Maldonado RA: Metal-drug synergy: new ruthenium(II) complexes of ketoconazole are highly active against *Leishmania major* and *Trypanosoma cruzi* and nontoxic to human or murine normal cells. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2013, 18(7):779-790.

446. Navin TR, Arana BA, Arana FE, Berman JD, Chajon JF: Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. *The Journal of infectious diseases* 1992, 165(3):528-534.

447. Rodriguez LV, Dedet JP, Paredes V, Mendoza C, Cardenas F: A randomized trial of amphotericin B alone or in combination with itraconazole in the treatment of mucocutaneous leishmaniasis. *Memorias do Instituto Oswaldo Cruz* 1995, 90(4):525-528.

448. Nassiri-Kashani M, Firooz A, Khamesipour A, Mojtahed F, Nilforoushzadeh M, Hejazi H, Bouzari N, Dowlati Y: A randomized, double-blind, placebo-controlled clinical trial of itraconazole in the treatment of cutaneous leishmaniasis. *Journal of the European Academy of Dermatology and Venereology : JEADV* 2005, 19(1):80-83.

449. Saenz RE, Paz H, Berman JD: Efficacy of ketoconazole against *Leishmania braziliensis* panamensis cutaneous leishmaniasis. *The American journal of medicine* 1990, 89(2):147-155.

450. Ozgoztasi O, Baydar I: A randomized clinical trial of topical paromomycin versus oral ketoconazole for treating cutaneous leishmaniasis in Turkey. *International journal of dermatology* 1997, 36(1):61-63.

451. Monzote L: Antileishmanial patents antileishmanial current drugs and relevant patents. *Recent Pat Antiinfect Drug Discov* 2011, 6(1):1-26.

452. Vannier-Santos MA, Urbina JA, Martiny A, Neves A, de Souza W: Alterations induced by the antifungal compounds ketoconazole and terbinafine in *Leishmania*. *J Eukaryot Microbiol* 1995, 42(4):337-346.

453. Andrade-Neto VV, Cicco NN, Cunha-Junior EF, Canto-Cavalheiro MM, Atella GC, Torres-Santos EC: The pharmacological inhibition of sterol biosynthesis in Leishmania is counteracted by enhancement of LDL endocytosis. *Acta Trop* 2011, 119(2-3):194-198.

454. Loose DS, Kan PB, Hirst MA, Marcus RA, Feldman D: Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. *J Clin Invest* 1983, 71(5):1495-1499.

455. Dan M, Verner E, el-On J, Zuckerman F, Michaeli D: Failure of oral ketoconazole to cure cutaneous ulcers caused by Leishmania braziliensis. *Cutis* 1986, 38(3):198-199.

456. Khalil EA, Nur NM, Zijlstra EE, El-Hassan AM, Davidson RN: Failure of a combination of two antifungal drugs, terbinafine plus itraconazole, in Sudanese post kala-azar dermal leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1996, 90(2):187-188.

457. McGwire BS, Satoskar AR: Leishmaniasis: clinical syndromes and treatment. *QJM* 2014, 107(1):7-14.

458. Weinrauch L, Livshin R, el-On J: Ketoconazole in cutaneous leishmaniasis. *The British journal of dermatology* 1987, 117(5):666-668.

459. Albanese G, Giorgetti P, Santagostino L, Crippa D, Sala G: Cutaneous leishmaniasis. Treatment with itraconazole. *Arch Dermatol* 1989, 125(11):1540-1542.

460. Rossi S: Australian Medicines Handbook; 2006. ISBN 0-9757919-2-3 Rossi S, editor. Australian Medicines Handbook 2006. Adelaide: Australian Medicines Handbook; 2006. ISBN 0-9757919-2-3

461. Kragie L, Turner SD, Patten CJ, Crespi CL, Stresser DM: Assessing pregnancy risks of azole antifungals using a high throughput aromatase inhibition assay. *Endocrine research* 2002, 28(3):129-140.

462. Sundar S, Kumar K, Singh VP: Ketoconazole in visceral leishmaniasis. *Lancet* 1990, 336(8730):1582-1583.

463. Dietze R, Carvalho SF, Valli LC, Berman J, Brewer T, Milhous W, Sanchez J, Schuster B, Grogl M: Phase 2 trial of WR6026, an orally administered 8-aminoquinoline, in the treatment of visceral leishmaniasis caused by *Leishmania chagasi*. *Am J Trop Med Hyg* 2001, 65(6):685-689.

464. Kinnamon KE, Steck EA, Loizeaux PS, Hanson WL, Chapman WL, Jr., Waits VB: The antileishmanial activity of lepidines. *Am J Trop Med Hyg* 1978, 27(4):751-757.

465. Elderfield R.C., Mertel H.E., Mitch R.T., I.M. W, E.J. W: Synthesis of primaquine and certain of its analogs. . *Journal of the American Chemical Society,* 1955, 77, :4816-4819.

466. Garnier T, Brown MB, Lawrence MJ, Croft SL: In-vitro and in-vivo studies on a topical formulation of sitamaquine dihydrochloride for cutaneous leishmaniasis. *J Pharm Pharmacol* 2006, 58(8):1043-1054.

467. Yeates C: Sitamaquine (GlaxoSmithKline/Walter Reed Army Institute). *Curr Opin Investig Drugs* 2002, 3(10):1446-1452.

468. Wasunna MK, Rashid JR, Mbui J, Kirigi G, Kinoti D, Lodenyo H, Felton JM, Sabin AJ, Albert MJ, Horton J: A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya. *Am J Trop Med Hyg* 2005, 73(5):871-876.

469. Jha TK, Sundar S, Thakur CP, Felton JM, Sabin AJ, Horton J: A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. *Am J Trop Med Hyg* 2005, 73(6):1005-1011.

470. Sherwood JA, Gachihi GS, Muigai RK, Skillman DR, Mugo M, Rashid JR, Wasunna KM, Were JB, Kasili SK, Mbugua JM *et al*: Phase 2 efficacy trial of an oral 8-aminoquinoline (WR6026) for treatment of visceral leishmaniasis. *Clin Infect Dis* 1994, 19(6):1034-1039.

471. Vercesi AE, Docampo R: Ca2+ transport by digitonin-permeabilized *Leishmania donovani*. Effects of Ca2+, pentamidine and WR-6026 on mitochondrial membrane potential in situ. *Biochem J* 1992, 284 (Pt 2):463-467.

472. Vercesi AE, Rodrigues CO, Catisti R, Docampo R: Presence of a Na(+)/H(+) exchanger in acidocalcisomes of *Leishmania donovani* and their alkalization by anti-leishmanial drugs. *FEBS Lett* 2000, 473(2):203-206.

473. Loiseau PM, Cojean S, Schrevel J: Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. *Parasite* 2011, 18(2):115-119.

474. Langreth SG, Berman JD, Riordan GP, Lee LS: Fine-structural alterations in Leishmania tropica within human macrophages exposed to antileishmanial drugs *in vitro*. *J Protozool* 1983, 30(3):555-561.

475. Duenas-Romero AM, Loiseau PM, Saint-Pierre-Chazalet M: Interaction of sitamaquine with membrane lipids of *Leishmania donovani* promastigotes. *Biochim Biophys Acta* 2007, 1768(2):246-252.

476. Coimbra ES, Libong D, Cojean S, Saint-Pierre-Chazalet M, Solgadi A, Le Moyec L, Duenas-Romero AM, Chaminade P, Loiseau PM: Mechanism of interaction of sitamaquine with *Leishmania donovani*. *J Antimicrob Chemother* 2010, 65(12):2548-2555.

477. Lopez-Martin C, Perez-Victoria JM, Carvalho L, Castanys S, Gamarro F: Sitamaquine sensitivity in *Leishmania* species is not mediated by drug accumulation in acidocalcisomes. *Antimicrob Agents Chemother* 2008, 52(11):4030-4036.

478. Miguel DC, Zauli-Nascimento RC, Yokoyama-Yasunaka JK, Katz S, Barbieri CL, Uliana SR: Tamoxifen as a potential antileishmanial agent: efficacy in the treatment of *Leishmania braziliensis* and *Leishmania chagasi* infections. *J Antimicrob Chemother* 2009, 63(2):365-368.

479. Miguel DC, Yokoyama-Yasunaka JK, Uliana SR: Tamoxifen is effective in the treatment of Leishmania amazonensis infections in mice. *PLoS Negl Trop Dis* 2008, 2(6):e249.

480. Miguel DC, Yokoyama-Yasunaka JK, Andreoli WK, Mortara RA, Uliana SR: Tamoxifen is effective against Leishmania and induces a rapid alkalinization of parasitophorous vacuoles harbouring *Leishmania (Leishmania) amazonensis* amastigotes. *J Antimicrob Chemother* 2007, 60(3):526-534.

481. Iqbal J, Ginsburg OM, Wijeratne TD, Howell A, Evans G, Sestak I, Narod SA: Endometrial cancer and venous thromboembolism in women under age 50 who take tamoxifen for prevention of breast cancer: a systematic review. *Cancer Treat Rev* 2012, 38(4):318-328.

482. Karimian E, Chagin AS, Gjerde J, Heino T, Lien EA, Ohlsson C, Savendahl L: Tamoxifen impairs both longitudinal and cortical bone growth in young male rats. *J Bone Miner Res* 2008, 23(8):1267-1277.

483. Arevalo I, Tulliano G, Quispe A, Spaeth G, Matlashewski G, Llanos-Cuentas A, Pollack H: Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. *Clin Infect Dis* 2007, 44(12):1549-1554.

484. Arevalo I, Ward B, Miller R, Meng TC, Najar E, Alvarez E, Matlashewski G, Llanos-Cuentas A: Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod, an immunomodulator. *Clin Infect Dis* 2001, 33(11):1847-1851.

485. Carvalho L, Luque-Ortega JR, Lopez-Martin C, Castanys S, Rivas L, Gamarro F: The 8-aminoquinoline analogue sitamaquine causes oxidative stress in *Leishmania donovani* promastigotes by targeting succinate dehydrogenase. *Antimicrob Agents Chemother* 2011, 55(9):4204-4210.

486. Theoharides AD, Chung H, Velazquez H: Metabolism of a potential 8-aminoquinoline antileishmanial drug in rat liver microsomes. *Biochem Pharmacol* 1985, 34(2):181-188.

487. Bories C, Cojean S, Huteau F, Loiseau PM: Selection and phenotype characterisation of sitamaquine-resistant promastigotes of *Leishmania donovani*. *Biomed Pharmacother* 2008, 62(3):164-167.

488. Robins RK: Potential Purine Antagonists. I. Synthesis of Some 4,6-Substituted Pyrazolo \3,4-d] pyrimidines1. *Amer Chem Soc* 1956, 78 (4):784

489. Martinez S, Gonzalez M, Vernaza ME: Treatment of cutaneous leishmaniasis with allopurinol and stibogluconate. *Clin Infect Dis* 1997, 24(2):165-169.

490. Ramesh V, Kumar J, Kumar D, Salotra P: A retrospective study of intravenous sodium stibogluconate alone and in combinations with allopurinol, rifampicin, and an immunomodulator in the treatment of Indian post-kala-azar dermal leishmaniasis. *Indian J Dermatol Venereol Leprol* 2010, 76(2):138-144.

491. Reveiz L, Maia-Elkhoury AN, Nicholls RS, Romero GA, Yadon ZE: Interventions for American cutaneous and mucocutaneous leishmaniasis: a systematic review update. *PLoS One* 2013, 8(4):e61843.

492. Ginel PJ, Lucena R, Lopez R, Molleda JM: Use of allopurinol for maintenance of remission in dogs with leishmaniasis. *J Small Anim Pract* 1998, 39(6):271-274.

493. Jha TK: Evaluation of allopurinol in the treatment of kala-azar occurring in North Bihar, India. *Trans R Soc Trop Med Hyg* 1983, 77(2):204-207.

494. Harzallah K, Belhadj R, Jemli B, Haloues M, Berraies N, Gargouri S, Hmida J, Battikh R, Manaa J: Visceral leishmaniasis in a renal transplant recipient treated with allopurinol. *Saudi J Kidney Dis Transpl* 2010, 21(1):105-108.

495. Colakoglu M, Yaylali GF, Colakoglu NY, Yilmaz M: Successful treatment of visceral leishmaniasis with fluconazole and allopurinol in a patient with renal failure. *Scand J Infect Dis* 2006, 38(2):152-154.

496. Guderian RH, Chico ME, Rogers MD, Pattishall KM, Grogl M, Berman JD: Placebo controlled treatment of Ecuadorian cutaneous leishmaniasis. *Am J Trop Med Hyg* 1991, 45(1):92-97.

497. Esfandiarpour I, Alavi A: Evaluating the efficacy of allopurinol and meglumine antimoniate (Glucantime) in the treatment of cutaneous leishmaniasis. *Int J Dermatol* 2002, 41(8):521-524.

498. Velez I, Agudelo S, Hendrickx E, Puerta J, Grogl M, Modabber F, Berman J: Inefficacy of allopurinol as monotherapy for Colombian cutaneous leishmaniasis. A randomized, controlled trial. *Ann Intern Med* 1997, 126(3):232-236.

499. Ribera E, Ocana I, de Otero J, Cortes E, Gasser I, Pahissa A: Prophylaxis of visceral leishmaniasis in human immunodeficiency virus-infected patients. *Am J Med* 1996, 100(5):496-501.

500. Torrus D, Boix V, Massa B, Portilla J, Perez-Mateo M: Fluconazole plus allopurinol in treatment of visceral leishmaniasis. *J Antimicrob Chemother* 1996, 37(5):1042-1043.

501. Bonano VI, Yokoyama-Yasunaka JK, Miguel DC, Jones SA, Dodge JA, Uliana SR: Discovery of synthetic leishmania inhibitors by screening of a 2-arylbenzothiophene library. *Chem Biol Drug Des* 2014, 83(3):289-296.

502. Sampaio RN, Lucas IC, Costa Filho AV: The use of azythromycin and N-methyl glucamine for the treatment of cutaneous Leishmaniasis caused by *Leishmania (Leishmania) amazonensis* in C57BL6 mice. *An Bras Dermatol* 2009, 84(2):125-128.

503. Silva-Vergara ML, Silva Lde A, Maneira FR, da Silva AG, Prata A: Azithromycin in the treatment of mucosal leishmaniasis. *Rev Inst Med Trop Sao Paulo* 2004, 46(3):175-177.

504. Teixeira AC, Paes MG, Guerra Jde O, Prata A, Silva-Vergara ML: Failure of both azithromycin and antimony to treat cutaneous leishmaniasis in Manaus, AM, Brazil. *Rev Inst Med Trop Sao Paulo* 2008, 50(3):157-160.

505. Elmahallawy EK, Jiménez-Aranda A, AS. M, Rodriguez-Granger J, Navarro –Alarcón M, J. G-F, Agil A: Activity of melatonin against *Leishmania infantum* promastigotes by mitochondrial dependent pathway. *Chem Biol Interact* 2014, 220(C):84-93.

506. Musa A, Khalil E, Hailu A, Olobo J, Balasegaram M, Omollo R, Edwards T, Rashid J, Mbui J, Musa B *et al*: Sodium stibogluconate (SSG) & paromomycin combination compared to SSG for visceral leishmaniasis in East Africa: a randomised controlled trial. *PLoS Negl Trop Dis* 2012, 6(6):e1674.

507. Nwaka S, Hudson A: Innovative lead discovery strategies for tropical diseases. *Nat Rev Drug Discov* 2006, 5(11):941-955.

508. Thakur CP, Kanyok TP, Pandey AK, Sinha GP, Zaniewski AE, Houlihan HH, Olliaro P: A prospective randomized, comparative, open-label trial of the safety and efficacy of paromomycin (aminosidine) plus sodium stibogluconate versus sodium stibogluconate alone for the treatment of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2000, 94(4):429-431.

509. Chunge CN, Owate J, Pamba HO, Donno L: Treatment of visceral leishmaniasis in Kenya by aminosidine alone or combined with sodium stibogluconate. *Trans R Soc Trop Med Hyg* 1990, 84(2):221-225.

510. Seaman J, Pryce D, Sondorp HE, Moody A, Bryceson AD, Davidson RN: Epidemic visceral leishmaniasis in Sudan: a randomized trial of aminosidine plus sodium stibogluconate versus sodium stibogluconate alone. *J Infect Dis* 1993, 168(3):715-720.

511. Melaku Y, Collin SM, Keus K, Gatluak F, Ritmeijer K, Davidson RN: Treatment of kala-azar in southern Sudan using a 17-day regimen of sodium stibogluconate combined with paromomycin: a retrospective comparison with 30-day sodium stibogluconate monotherapy. *Am J Trop Med Hyg* 2007, 77(1):89-94.

512. Laguna F, Lopez-Velez R, Soriano V, Montilla P, Alvar J, Gonzalez-Lahoz JM: Assessment of allopurinol plus meglumine antimoniate in the treatment of visceral leishmaniasis in patients infected with HIV. *J Infect* 1994, 28(3):255-259.

513. Omollo R, Alexander N, Edwards T, Khalil EA, Younis BM, Abuzaid AA, Wasunna M, Njoroge N, Kinoti D, Kirigi G *et al*: Safety and efficacy of miltefosine alone and in combination with sodium stibogluconate and liposomal amphotericin B for the treatment of primary visceral leishmaniasis in East Africa: study protocol for a randomized controlled trial. *Trials* 2011, 12:166.

514. Seifert K, Croft SL: *In vitro* and *in vivo* interactions between miltefosine and other antileishmanial drugs. *Antimicrob Agents Chemother* 2006, 50(1):73-79.

515. Berman JD: U.S Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clin Infect Dis* 1999, 28(1):49-51.

516. Shakya N, Sane SA, Gupta S: Antileishmanial efficacy of fluconazole and miltefosine in combination with an immunomodulator--picroliv. *Parasitol Res* 2011, 108(4):793-800.

517. Saha P, Mukhopadhyay D, Chatterjee M: Immunomodulation by chemotherapeutic agents against Leishmaniasis. *Int Immunopharmacol* 2011, 11(11):1668-1679.

518. Goto H, Lindoso JA: Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Rev Anti Infect Ther* 2010, 8(4):419-433.

519. Murray HW, Brooks EB, DeVecchio JL, Heinzel FP: Immunoenhancement combined with amphotericin B as treatment for experimental visceral leishmaniasis. *Antimicrob Agents Chemother* 2003, 47(8):2513-2517.

520. Chattopadhyay A, Jafurulla M: A novel mechanism for an old drug: amphotericin B in the treatment of visceral leishmaniasis. *Biochem Biophys Res Commun* 2011, 416(1-2):7-12.

521. Banerjee A, De M, Ali N: Complete cure of experimental visceral leishmaniasis with amphotericin B in stearylamine-bearing cationic liposomes involves down-regulation of IL-10 and favorable T cell responses. *J Immunol* 2008, 181(2):1386-1398.

522. Squires KE, Rosenkaimer F, Sherwood JA, Forni AL, Were JB, Murray HW: Immunochemotherapy for visceral leishmaniasis: a controlled pilot trial of antimony versus antimony plus interferon-gamma. *Am J Trop Med Hyg* 1993, 48(5):666-669.

523. Badaro R, Johnson WD, Jr.: The role of interferon-gamma in the treatment of visceral and diffuse cutaneous leishmaniasis. *J Infect Dis* 1993, 167 Suppl 1:S13-17.

524. Wardowska A, Dzierzbicka K, Mysliwski A: [Tuftsin--new analogues and properties]. *Postepy Biochem* 2007, 53(1):60-65.

525. Wardowska A, Dzierzbicka K, Trzonkowski P, Mysliwski A: The biological activity of new tuftsin derivatives--induction of phagocytosis. *Acta Pol Pharm* 2006, 63(5):378-381.

526. Shakya N, Sane SA, Haq W, Gupta S: Augmentation of antileishmanial efficacy of miltefosine in combination with tuftsin against experimental visceral leishmaniasis. *Parasitol Res* 2012, 111(2):563-570.

527. Llanos-Cuentas A, Echevarria J, Cruz M, La Rosa A, Campos P, Campos M, Franke E, Berman J, Modabber F, Marr J: Efficacy of sodium stibogluconate alone and in combination with allopurinol for treatment of mucocutaneous leishmaniasis. *Clin Infect Dis* 1997, 25(3):677-684.

528. Esfandiarpour I, Dabiri SH: Treatment of cutaneous leishmaniasis recidivans with a combination of allopurinol and meglumine antimoniate: a clinical and histologic study. *Int J Dermatol* 2007, 46(8):848-852.

529. Seifert K, Escobar P, Croft SL: *In vitro* activity of anti-leishmanial drugs against *Leishmania donovani* is host cell dependent. *J Antimicrob Chemother* 2010, 65(3):508-511.

530. Doenhoff MJ, Modha J, Lambertucci JR, McLaren DJ: The immune dependence of chemotherapy. *Parasitol Today* 1991, 7(1):16-18.

531. Berger BJ, Fairlamb AH: Interactions between immunity and chemotherapy in the treatment of the trypanosomiases and leishmaniases. *Parasitology* 1992, 105 Suppl:S71-78.

532. Alexander J, Brombacher F: T helper1/t helper2 cells and resistance/susceptibility to leishmania infection: is this paradigm still relevant? *Front Immunol* 2012, 3:80.

533. Awasthi A, Mathur RK, Saha B: Immune response to *Leishmania* infection. *Indian J Med Res* 2004, 119(6):238-258.

534. Bimal S, Sinha S, Singh SK, Narayan S, Kumar V, Verma N, Ranjan A, Sinha PK, Das VN, Pandey K *et al*: *Leishmania donovani*: CD2 biased immune response skews the SAG mediated therapy for a predominant Th1 response in experimental infection. *Experimental parasitology* 2012, 131(3):274-282.

535. Gombos I, Kiss E, Detre C, Laszlo G, Matko J: Cholesterol and sphingolipids as lipid organizers of the immune cells' plasma membrane: their impact on the functions of MHC molecules, effector T-lymphocytes and T-cell death. *Immunol Lett* 2006, 104(1-2):59-69.

536. Carvalho EM, Johnson WD, Barreto E, Marsden PD, Costa JL, Reed S, Rocha H: Cell mediated immunity in American cutaneous and mucosal leishmaniasis. *J Immunol* 1985, 135(6):4144-4148.

537. Silveira FT, Lainson R, De Castro Gomes CM, Laurenti MD, Corbett CE: Immunopathogenic competences of *Leishmania* (V.) braziliensis and L. (L.) amazonensis in American cutaneous leishmaniasis. *Parasite Immunol* 2009, 31(8):423-431.

538. Sacks D, Noben-Trauth N: The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol* 2002, 2(11):845-858.

539. Aseffa A, Gumy A, Launois P, MacDonald HR, Louis JA, Tacchini-Cottier F: The early IL-4 response to *Leishmania major* and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory CD4+CD25+ T cells. *J Immunol* 2002, 169(6):3232-3241.

540. Pitta MG, Romano A, Cabantous S, Henri S, Hammad A, Kouriba B, Argiro L, el Kheir M, Bucheton B, Mary C *et al*: IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*. *J Clin Invest* 2009, 119(8):2379-2387.

541. Murray HW, Delph-Etienne S: Visceral leishmanicidal activity of hexadecylphosphocholine (miltefosine) in mice deficient in T cells and activated macrophage microbicidal mechanisms. *J Infect Dis* 2000, 181(2):795-799.

542. Hartley MA, Kohl K, Ronet C, Fasel N: The therapeutic potential of immune cross-talk in leishmaniasis. *Clin Microbiol Infect* 2013, 19(2):119-130.

543. Li J, Sutterwala S, Farrell JP: Successful therapy of chronic, nonhealing murine cutaneous leishmaniasis with sodium stibogluconate and gamma interferon depends on continued interleukin-12 production. *Infect Immun* 1997, 65(8):3225-3230.

544. Nabors GS, Farrell JP: Successful chemotherapy in experimental leishmaniasis is influenced by the polarity of the T cell response before treatment. *J Infect Dis* 1996, 173(4):979-986.

545. Croft SL, Yardley V: Chemotherapy of leishmaniasis. *Curr Pharm Des* 2002, 8(4):319-342.

546. Haldar AK, Sen P, Roy S: Use of antimony in the treatment of leishmaniasis: current status and future directions. *Mol Biol Int* 2011, 2011:571242.

547. Mookerjee Basu J, Mookerjee A, Sen P, Bhaumik S, Banerjee S, Naskar K, Choudhuri SK, Saha B, Raha S, Roy S: Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages. *Antimicrob Agents Chemother* 2006, 50(5):1788-1797.

548. Ponte CB, Alves EA, Sampaio RN, Urdapilleta AA, Kuckelhaus Cdos S, Muniz-Junqueira MI, Kuckelhaus SA: Miltefosine enhances phagocytosis but decreases nitric oxide production by peritoneal macrophages of C57BL/6 mice. *Int Immunopharmacol* 2012, 13(1):114-119.

549. Hilgard P, Kampherm E, Nolan L, Pohl J, Reissmann T: Investigation into the immunological effects of miltefosine, a new anticancer agent under development. *J Cancer Res Clin Oncol* 1991, 117(5):403-408.

550. Zeisig R, Rudolf M, Eue I, Arndt D: Influence of hexadecylphosphocholine on the release of tumor necrosis factor and nitroxide from peritoneal macrophages *in vitro*. *J Cancer Res Clin Oncol* 1995, 121(2):69-75.

551. Sau K, Mambula SS, Latz E, Henneke P, Golenbock DT, Levitz SM: The antifungal drug amphotericin B promotes inflammatory cytokine release by a Toll-like receptor- and CD14-dependent mechanism. *J Biol Chem* 2003, 278(39):37561-37568.

552. Aderem A, Ulevitch RJ: Toll-like receptors in the induction of the innate immune response. *Nature* 2000, 406(6797):782-787.

553. Das S, Rani M, Pandey K, Sahoo GC, Rabidas VN, Singh D, Das P: Combination of paromomycin and miltefosine promotes TLR4-dependent induction of antileishmanial immune response *in vitro*. *J Antimicrob Chemother* 2012, 67(10):2373-2378.

554. Ghosh M, Roy K, Roy S: Immunomodulatory effects of antileishmanial drugs. *J Antimicrob Chemother* 2013, 68(12):2834-2838.

555. Chakraborty D, Banerjee S, Sen A, Banerjee KK, Das P, Roy S: *Leishmania donovani* affects antigen presentation of macrophage by disrupting lipid rafts. *J Immunol* 2005, 175(5):3214-3224.

556. Faucher B, Pomares C, Fourcade S, Benyamine A, Marty P, Pratlong L, Faraut F, Mary C, Piarroux R, Dedet JP *et al*: Mucosal *Leishmania infantum* leishmaniasis: specific pattern in a multicentre survey and historical cases. *J Infect* 2011, 63(1):76-82.

557. Laguna F, Adrados M, Alvar J, Soriano V, Valencia ME, Moreno V, Polo R, Verdejo J, Jimenez MI, Martinez P *et al*: Visceral leishmaniasis in patients infected with the human immunodeficiency virus. *Eur J Clin Microbiol Infect Dis* 1997, 16(12):898-903.

558. Alvar J, Aparicio P, Aseffa A, Den Boer M, Canavate C, Dedet JP, Gradoni L, Ter Horst R, Lopez-Velez R, Moreno J: The relationship between leishmaniasis and AIDS: the second 10 years. *Clin Microbiol Rev* 2008, 21(2):334-359, table of contents.

559. van Griensven J, Carrillo E, Lopez-Velez R, Lynen L, Moreno J: Leishmaniasis in immunosuppressed individuals. *Clin Microbiol Infect* 2014, 20(4):286-299.

560. Laguna F, Lopez-Velez R, Pulido F, Salas A, Torre-Cisneros J, Torres E, Medrano FJ, Sanz J, Pico G, Gomez-Rodrigo J *et al*: Treatment of visceral leishmaniasis in HIV-infected patients: a randomized trial comparing meglumine antimoniate with amphotericin B. Spanish HIV-Leishmania Study Group. *AIDS* 1999, 13(9):1063-1069.

561. Foti G: [Treatment of visceral leishmaniasis]. *Minerva Med* 2001, 92(4):245-249.

562. Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, Kumar PC, Murray HW: Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis* 2000, 31(4):1104-1107.

563. Hailu W, Weldegebreal T, Hurissa Z, Tafes H, Omollo R, Yifru S, Balasegaram M, Hailu A: Safety and effectiveness of meglumine antimoniate in the treatment of Ethiopian visceral leishmaniasis patients with and without HIV co-infection. *Trans R Soc Trop Med Hyg* 2010, 104(11):706-712.

564. Ritmeijer K, Dejenie A, Assefa Y, Hundie TB, Mesure J, Boots G, den Boer M, Davidson RN: A comparison of miltefosine and sodium stibogluconate for treatment of visceral

leishmaniasis in an Ethiopian population with high prevalence of HIV infection. *Clin Infect Dis* 2006, 43(3):357-364.

565. Hurissa Z, Gebre-Silassie S, Hailu W, Tefera T, Lalloo DG, Cuevas LE, Hailu A: Clinical characteristics and treatment outcome of patients with visceral leishmaniasis and HIV co-infection in northwest Ethiopia. *Trop Med Int Health* 2010, 15(7):848-855.
566. Pintado V, Lopez-Velez R: HIV-associated visceral leishmaniasis. *Clin Microbiol Infect* 2001, 7(6):291-300.
567. Laguna F, Videla S, Jimenez-Mejias ME, Sirera G, Torre-Cisneros J, Ribera E, Prados D, Clotet B, Sust M, Lopez-Velez R *et al*: Amphotericin B lipid complex versus meglumine antimoniate in the treatment of visceral leishmaniasis in patients infected with HIV: a randomized pilot study. *J Antimicrob Chemother* 2003, 52(3):464-468.
568. Murray HW: Leishmaniasis in the United States: treatment in 2012. *Am J Trop Med Hyg* 2012, 86(3):434-440.
569. Lopez-Velez R, Videla S, Marquez M, Boix V, Jimenez-Mejias ME, Gorgolas M, Arribas JR, Salas A, Laguna F, Sust M *et al*: Amphotericin B lipid complex versus no treatment in the secondary prophylaxis of visceral leishmaniasis in HIV-infected patients. *J Antimicrob Chemother* 2004, 53(3):540-543.
570. Sindermann H, Engel KR, Fischer C, Bommer W: Oral miltefosine for leishmaniasis in immunocompromised patients: compassionate use in 39 patients with HIV infection. *Clin Infect Dis* 2004, 39(10):1520-1523.
571. Marques N, Sa R, Coelho F, Oliveira J, Saraiva Da Cunha J, Melico-Silvestre A: Miltefosine for visceral leishmaniasis relapse treatment and secondary prophylaxis in HIV-infected patients. *Scand J Infect Dis* 2008, 40(6-7):523-526.
572. Antinori S, Longhi E, Bestetti G, Piolini R, Acquaviva V, Foschi A, Trovati S, Parravicini C, Corbellino M, Meroni L: Post-kala-azar dermal leishmaniasis as an immune reconstitution inflammatory syndrome in a patient with acquired immune deficiency syndrome. *Br J Dermatol* 2007, 157(5):1032-1036.
573. Troya J, Casquero A, Refoyo E, Fernandez-Guerrero ML, Gorgolas M: Long term failure of miltefosine in the treatment of refractory visceral leishmaniasis in AIDS patients. *Scand J Infect Dis* 2008, 40(1):78-80.

574. Paredes R, Munoz J, Diaz I, Domingo P, Gurgui M, Clotet B: Leishmaniasis in HIV infection. *J Postgrad Med* 2003, 49(1):39-49.

575. Grabmeier-Pfistershammer K, Poeppl W, Brunner PM, Rappersberger K, Rieger A: Clinical Challenges in the Management of Leishmania/HIV Coinfection in a Nonendemic Area: A Case Report. *Case Rep Infect Dis* 2012, 2012:787305.

576. Rybniker J, Goede V, Mertens J, Ortmann M, Kulas W, Kochanek M, Benzing T, Arribas JR, Fatkenheuer G: Treatment of visceral leishmaniasis with intravenous pentamidine and oral fluconazole in an HIV-positive patient with chronic renal failure--a case report and brief review of the literature. *Int J Infect Dis* 2010, 14(6):e522-525.

577. Hailu A, Musa A, Wasunna M, Balasegaram M, Yifru S, Mengistu G, Hurissa Z, Hailu W, Weldegebreal T, Tesfaye S *et al*: Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicentre, open-label, randomized trial. *PLoS neglected tropical diseases* 2010, 4(10):e709.

578. Elmahallawy EK, Agil A: Treatment of leishmaniasis: a review and assessment of recent research. *Current pharmaceutical design* 2015, 21(17):2259-2275.

579. Lerner AB, Case JD, Takahashi Y, Lee TH, Mori N: Isolation of melatonin, a pineal factor that lightens melanocytes. . *J Am Chem Soc* 1958., 80, 2587.

580. Cassone VM, Natesan AK: Time and time again: the phylogeny of melatonin as a transducer of biological time. *J Biol Rhythms* 1997, 12(6):489-497.

581. Hardeland R, Cardinali DP, Srinivasan V, Spence DW, Brown GM, Pandi-Perumal SR: Melatonin--a pleiotropic, orchestrating regulator molecule. *Prog Neurobiol* 2011, 93(3):350-384.

582. Agil A, Elmahallawy EK, Rodriguez-Ferrer JM, Adem A, Bastaki SM, Al-Abbadi I, Fino Solano YA, Navarro-Alarcon M: Melatonin increases intracellular calcium in the liver, muscle, white adipose tissues and pancreas of diabetic obese rats. *Food Funct* 2015.

583. Axelrod J, Wurtman RJ: Photic and neural control of indoleamine metabolism in the rat pineal gland. *Adv Pharmacol* 1968, 6(Pt A):157-166.

584. Cardinali DP, Pevet P: Basic aspects of melatonin action. *Sleep Med Rev* 1998, 2(3):175-190.

585. Acuna Castroviejo D, Lopez LC, Escames G, Lopez A, Garcia JA, Reiter RJ: Melatonin-mitochondria interplay in health and disease. *Curr Top Med Chem* 2011, 11(2):221-240.

586. Reppert S, Weaver D, Godson C: Melatonin receptors step into the light: cloning and classification of subtypes. *Trends Pharmacol Sci* 1996, 17((3)):100-102.

587. Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J: International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. *Pharmacol Rev* 2010, 62(3):343-380.

588. Legros C, Devavry S, Caignard S, Tessier C, Delagrange P, Ouvry C, Boutin JA, Nosjean O: Melatonin MT(1) and MT(2) receptors display different molecular pharmacologies only in the G-protein coupled state. *Br J Pharmacol* 2014, 171(1):186-201.

589. Zlotos DP, Jockers R, Cecon E, Rivara S, Witt-Enderby PA: MT1 and MT2 melatonin receptors: ligands, models, oligomers, and therapeutic potential. *J Med Chem* 2014, 57(8):3161-3185.

590. Dubocovich ML, Markowska M: Functional MT1 and MT2 melatonin receptors in mammals. *Endocrine* 2005, 27(2):101-110.

591. Srinivasan V, Ahmad AH, Mohamed M, Zakaria R: Melatonin effects on *Plasmodium* life cycle: new avenues for therapeutic approach. *Recent Pat Endocr Metab Immune Drug Discov* 2012, 6(2):139-147.

592. Srinivasan V, Mohamed M, Zakaria R, Ahmad AH: Malaria, anti malarial drugs and the role of melatonin. *Infect Disord Drug Targets* 2012, 12(5):371-379.

593. Pandi-Perumal SR, Trakht I, Srinivasan V, Spence DW, Maestroni GJ, Zisapel N, Cardinali DP: Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. *Prog Neurobiol* 2008, 85(3):335-353.

594. Boutin JA: [Melatonin binding site MT3 is QR2: state of the art]. *J Soc Biol* 2007, 201(1):97-103.

595. Srinivasan V, Zakaria R, Mohamed M, Saleh RM: Effects of melatonin derivatives on human malaria parasite *Plasmodium falciparum*. *Recent Pat Endocr Metab Immune Drug Discov* 2014, 8(2):102-108.

596. Soliman MF, El Shenawy NS, El Arabi SE: Schistosoma mansoni: melatonin enhances efficacy of cercarial and soluble worm antigens in the induction of protective immunity against infection in the hamster. *Exp Parasitol* 2008, 119(2):291-295.

597. Santello FH, Frare EO, Caetano LC, AlonsoToldo MP, do Prado JC, Jr.: Melatonin enhances pro-inflammatory cytokine levels and protects against Chagas disease. *Journal of pineal research* 2008, 45(1):79-85.

598. Reiter RJ, Tan DX, Terron MP, Flores LJ, Czarnocki Z: Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. *Acta Biochim Pol* 2007, 54(1):1-9.

599. Oliveira LG, Kuehn CC, Santos CD, Toldo MP, do Prado JC, Jr.: Enhanced protection by melatonin and meloxicam combination in experimental infection by *Trypanosoma cruzi*. *Parasite Immunol* 2010, 32(4):245-251.

600. Koyama FC, Ribeiro RY, Garcia JL, Azevedo MF, Chakrabarti D, Garcia CR: Ubiquitin proteasome system and the atypical kinase PfPK7 are involved in melatonin signaling in *Plasmodium falciparum*. *J Pineal Res* 2012, 53(2):147-153.

601. Furuyama W, Enomoto M, Mossaad E, Kawai S, Mikoshiba K, Kawazu S: An interplay between 2 signaling pathways: melatonin-cAMP and IP3-Ca2+ signaling pathways control intraerythrocytic development of the malaria parasite *Plasmodium falciparum*. *Biochem Biophys Res Commun* 2014, 446(1):125-131.

602. Alves E, Bartlett PJ, Garcia CR, Thomas AP: Melatonin and IP3-induced Ca2+ release from intracellular stores in the malaria parasite *Plasmodium falciparum* within infected red blood cells. *J Biol Chem* 2011, 286(7):5905-5912.

603. Reiter RJ, Tan DX, Manchester LC, Pilar Terron M, Flores LJ, Koppisepi S: Medical implications of melatonin: receptor-mediated and receptor-independent actions. *Adv Med Sci* 2007, 52:11-28.

604. Elmahallawy EK, Luque JO, Aloweidi AS, Fernandez JG, Martinez AS, Rodriguez-Granger J, Kaki A, Agil A: Potential relevance of melatonin against some infectious agents: A Review and Assessment of Recent Research. *Current medicinal chemistry* 2015.

605. Carrillo-Vico A, Reiter RJ, Lardone PJ, Herrera JL, Fernandez-Montesinos R, Guerrero JM, Pozo D: The modulatory role of melatonin on immune responsiveness. *Curr Opin Investig Drugs* 2006, 7(5):423-431.

606. Pioli C, Caroleo MC, Nistico G, Doria G: Melatonin increases antigen presentation and amplifies specific and non specific signals for T-cell proliferation. *International journal of immunopharmacology* 1993, 15(4):463-468.

607. Srinivasan V, Mohamed M, Kato H: Melatonin in bacterial and viral infections with focus on sepsis: a review. *Recent Pat Endocr Metab Immune Drug Discov* 2012, 6(1):30-39.

608. Ben-Nathan D, Maestroni GJ, Lustig S, Conti A: Protective effects of melatonin in mice infected with encephalitis viruses. *Arch Virol* 1995, 140(2):223-230.

609. Carrillo-Vico A, Lardone PJ, Fernandez-Santos JM, Martin-Lacave I, Calvo JR, Karasek M, Guerrero JM: Human lymphocyte-synthesized melatonin is involved in the regulation of the interleukin-2/interleukin-2 receptor system. *J Clin Endocrinol Metab* 2005, 90(2):992-1000.

610. Maestroni GJ: The immunotherapeutic potential of melatonin. *Expert Opin Investig Drugs* 2001, 10(3):467-476.

611. Guerrero JM, Pozo D, Garcia-Maurino S, Osuna C, Molinero P, Calvo JR: Involvement of nuclear receptors in the enhanced IL-2 production by melatonin in Jurkat cells. *Ann N Y Acad Sci* 2000, 917:397-403.

612. Garcia-Maurino S, Pozo D, Calvo JR, Guerrero JM: Correlation between nuclear melatonin receptor expression and enhanced cytokine production in human lymphocytic and monocytic cell lines. *J Pineal Res* 2000, 29(3):129-137.

613. Johns JR, Platts JA: Theoretical insight into the antioxidant properties of melatonin and derivatives. *Org Biomol Chem* 2014, 12(39):7820-7827.

614. Zhang HM, Zhang Y: Melatonin: a well-documented antioxidant with conditional pro-oxidant actions. *J Pineal Res* 2014, 57(2):131-146.

615. Reiter RJ, Tan DX, Mayo JC, Sainz RM, Leon J, Czarnocki Z: Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochim Pol* 2003, 50(4):1129-1146.

616. Hardeland R: Melatonin: signaling mechanisms of a pleiotropic agent. *Biofactors* 2009, 35(2):183-192.

617. McNulty S, Ross AW, Barrett P, Hastings MH, Morgan PJ: Melatonin regulates the phosphorylation of CREB in ovine pars tuberalis. *J Neuroendocrinol* 1994, 6(5):523-532.

618. Gazarini ML, Beraldo FH, Almeida FM, Bootman M, Da Silva AM, Garcia CR: Melatonin triggers PKA activation in the rodent malaria parasite Plasmodium chabaudi. *J Pineal Res* 2011, 50(1):64-70.

619. Avunduk AM, Avunduk MC, Baltaci AK, Mogulkoc R: Effect of melatonin and zinc on the immune response in experimental Toxoplasma retinochoroiditis. *Ophthalmologica Journal*

international d'ophtalmologie International journal of ophthalmology Zeitschrift fur Augenheilkunde 2007, 221(6):421-425.

620. Brazao V, Del Vecchio Filipin M, Santello FH, Caetano LC, Abrahao AA, Toldo MP, do Prado JC, Jr.: Melatonin and zinc treatment: distinctive modulation of cytokine production in chronic experimental *Trypanosoma cruzi* infection. *Cytokine* 2011, 56(3):627-632.
621. El-Sokkary GH, Omar HM, Hassanein AF, Cuzzocrea S, Reiter RJ: Melatonin reduces oxidative damage and increases survival of mice infected with Schistosoma mansoni. *Free Radic Biol Med* 2002, 32(4):319-332.
622. Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ: A review of the multiple actions of melatonin on the immune system. *Endocrine* 2005, 27(2):189-200.
623. Carrillo-Vico A, Lardone PJ, Alvarez-Sanchez N, Rodriguez-Rodriguez A, Guerrero JM: Melatonin: buffering the immune system. *Int J Mol Sci* 2013, 14(4):8638-8683.
624. Reiter RJ, Paredes SD, Manchester LC, Tan DX: Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. *Crit Rev Biochem Mol Biol* 2009, 44(4):175-200.
625. Calvo JR, Gonzalez-Yanes C, Maldonado MD: The role of melatonin in the cells of the innate immunity: a review. *J Pineal Res* 2013, 55(2):103-120.
626. Carrillo-Vico A, Calvo JR, Abreu P, Lardone PJ, Garcia-Maurino S, Reiter RJ, Guerrero JM: Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance. *FASEB J* 2004, 18(3):537-539.
627. Agil A, Reiter RJ, Jimenez-Aranda A, Iban-Arias R, Navarro-Alarcon M, Marchal JA, Adem A, Fernandez-Vazquez G: Melatonin ameliorates low-grade inflammation and oxidative stress in young Zucker diabetic fatty rats. *J Pineal Res* 2013, 54(4):381-388.
628. Lardone PJ, Carrillo-Vico A, Molinero P, Rubio A, Guerrero JM: A novel interplay between membrane and nuclear melatonin receptors in human lymphocytes: significance in IL-2 production. *Cell Mol Life Sci* 2009, 66(3):516-525.
629. Ishii H, Tanaka N, Kobayashi M, Kato M, Sakuma Y: Gene structures, biochemical characterization and distribution of rat melatonin receptors. *J Physiol Sci* 2009, 59(1):37-47.
630. Guerrero JM, Reiter RJ: Melatonin-immune system relationships. *Curr Top Med Chem* 2002, 2(2):167-179.

631. Calvo JR, Rafii-el-Idrissi M, Pozo D, Guerrero JM: Immunomodulatory role of melatonin: specific binding sites in human and rodent lymphoid cells. *J Pineal Res* 1995, 18(3):119-126.

632. Maestroni GJ, Sulli A, Pizzorni C, Villaggio B, Cutolo M: Melatonin in rheumatoid arthritis: synovial macrophages show melatonin receptors. *Ann N Y Acad Sci* 2002, 966:271-275.

633. Drazen DL, Bilu D, Bilbo SD, Nelson RJ: Melatonin enhancement of splenocyte proliferation is attenuated by luzindole, a melatonin receptor antagonist. *Am J Physiol Regul Integr Comp Physiol* 2001, 280(5):R1476-1482.

634. Drazen DL, Nelson RJ: Melatonin receptor subtype MT2 (Mel 1b) and not mt1 (Mel 1a) is associated with melatonin-induced enhancement of cell-mediated and humoral immunity. *Neuroendocrinology* 2001, 74(3):178-184.

635. Wei W, Shen YX, Dai M, Chen Q: Effects and mechanisms of melatonin on immune responses in mice of different months. *Acta Pharmacol Sin* 2003, 24(7):719-723.

636. Sze SF, Liu WK, Ng TB: Stimulation of murine splenocytes by melatonin and methoxytryptamine. *J Neural Transm Gen Sect* 1993, 94(2):115-126.

637. Maestroni GJ, Hertens E, Galli P, Conti A, Pedrinis E: Melatonin-induced T-helper cell hematopoietic cytokines resembling both interleukin-4 and dynorphin. *J Pineal Res* 1996, 21(3):131-139.

638. Miller SC, Pandi-Perumal SR, Esquifino AI, Cardinali DP, Maestroni GJ: The role of melatonin in immuno-enhancement: potential application in cancer. *Int J Exp Pathol* 2006, 87(2):81-87.

639. Lissoni P: The pineal gland as a central regulator of cytokine network. *Neuro Endocrinol Lett* 1999, 20(6):343-349.

640. von Gall C, Stehle JH, Weaver DR: Mammalian melatonin receptors: molecular biology and signal transduction. *Cell Tissue Res* 2002, 309(1):151-162.

641. Lin GJ, Huang SH, Chen SJ, Wang CH, Chang DM, Sytwu HK: Modulation by melatonin of the pathogenesis of inflammatory autoimmune diseases. *Int J Mol Sci* 2013, 14(6):11742-11766.

642. Kalpakcioglu B, Senel K: The role of melatonin in rheumatic diseases. *Infect Disord Drug Targets* 2009, 9(4):453-456.

643. Sanchez-Barcelo EJ, Mediavilla MD, Tan DX, Reiter RJ: Clinical uses of melatonin: evaluation of human trials. *Curr Med Chem* 2010, 17(19):2070-2095.

644. Valko M, Morris H, Cronin MT: Metals, toxicity and oxidative stress. *Curr Med Chem* 2005, 12(10):1161-1208.

645. Pratap Singh K, Zaidi A, Anwar S, Bimal S, Das P, Ali V: Reactive oxygen species regulates expression of iron-sulfur cluster assembly protein IscS of *Leishmania donovani*. *Free Radic Biol Med* 2014, 75:195-209.

646. Lowes DA, Webster NR, Murphy MP, Galley HF: Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. *Br J Anaesth* 2013, 110(3):472-480.

647. Mogulkoc R, Baltaci AK, Oztekin E, Aydin L, Sivrikaya A: Melatonin prevents oxidant damage in various tissues of rats with hyperthyroidism. *Life Sci* 2006, 79(3):311-315.

648. Ortiz F, Garcia JA, Acuna-Castroviejo D, Doerrier C, Lopez A, Venegas C, Volt H, Luna-Sanchez M, Lopez LC, Escames G: The beneficial effects of melatonin against heart mitochondrial impairment during sepsis: inhibition of iNOS and preservation of nNOS. *J Pineal Res* 2014, 56(1):71-81.

649. Galley HF, Lowes DA, Allen L, Cameron G, Aucott LS, Webster NR: Melatonin as a potential therapy for sepsis: a phase I dose escalation study and an ex vivo whole blood model under conditions of sepsis. *J Pineal Res* 2014, 56(4):427-438.

650. Joshi N, Biswas J, Nath C, Singh S: Promising Role of Melatonin as Neuroprotectant in Neurodegenerative Pathology. *Mol Neurobiol* 2014.

651. Cipolla-Neto J, Amaral FG, Afeche SC, Tan DX, Reiter RJ: Melatonin, energy metabolism, and obesity: a review. *J Pineal Res* 2014, 56(4):371-381.

652. Tunon MJ, San-Miguel B, Crespo I, Laliena A, Vallejo D, Alvarez M, Prieto J, Gonzalez-Gallego J: Melatonin treatment reduces endoplasmic reticulum stress and modulates the unfolded protein response in rabbits with lethal fulminant hepatitis of viral origin. *J Pineal Res* 2013, 55(3):221-228.

653. Vielma JR, Bonilla E, Chacin-Bonilla L, Mora M, Medina-Leendertz S, Bravo Y: Effects of melatonin on oxidative stress, and resistance to bacterial, parasitic, and viral infections: a review. *Acta Trop* 2014, 137:31-38.

654. Regodon S, Ramos A, Morgado S, Tarazona R, Martin-Palomino P, Rosado JA, Miguez Mdel P: Melatonin enhances the immune response to vaccination against A1 and C strains of Dichelobacter nodosus. *Vaccine* 2009, 27(10):1566-1570.

655. Valero N, Nery A, Bonilla E, Espina LM, Chacin-Bonilla L, Anez F, Maldonado M, Melean E: Antagonistic effect of luzindole in mice treated with melatonin during the infection with the venezuelan equine encephalomyelitis virus. *Neurochem Res* 2009, 34(2):268-273.

656. Rahman MA, Azuma Y, Fukunaga H, Murakami T, Sugi K, Fukushi H, Miura K, Suzuki H, Shirai M: Serotonin and melatonin, neurohormones for homeostasis, as novel inhibitors of infections by the intracellular parasite chlamydia. *J Antimicrob Chemother* 2005, 56(5):861-868.

657. Ozkan E, Yaman H, Cakir E, Deniz O, Oztosun M, Gumus S, Akgul EO, Agilli M, Cayci T, Kurt YG *et al*: Plasma melatonin and urinary 6-hydroxymelatonin levels in patients with pulmonary tuberculosis. *Inflammation* 2012, 35(4):1429-1434.

658. Tekbas OF, Ogur R, Korkmaz A, Kilic A, Reiter RJ: Melatonin as an antibiotic: new insights into the actions of this ubiquitous molecule. *J Pineal Res* 2008, 44(2):222-226.

659. Tan DX, Korkmaz A, Reiter RJ, Manchester LC: Ebola virus disease: potential use of melatonin as a treatment. *J Pineal Res* 2014, 57(4):381-384.

660. Bonilla E, Valero N, Chacin-Bonilla L, Medina-Leendertz S: Melatonin and viral infections. *J Pineal Res* 2004, 36(2):73-79.

661. Silvestri M, Rossi GA: Melatonin: its possible role in the management of viral infections--a brief review. *Ital J Pediatr* 2013, 39:61.

662. Regodon S, Ramos A, Miguez MP, Carrillo-Vico A, Rosado JA, Jardin I: Vaccination prepartum enhances the beneficial effects of melatonin on the immune response and reduces platelet responsiveness in sheep. *BMC Vet Res* 2012, 8:84.

663. McDonough KA, Rodriguez A: The myriad roles of cyclic AMP in microbial pathogens: from signal to sword. *Nat Rev Microbiol* 2012, 10(1):27-38.

664. Botsford JL, Harman JG: Cyclic AMP in prokaryotes. *Microbiol Rev* 1992, 56(1):100-122.

665. Rodriguez A, Martinez I, Chung A, Berlot CH, Andrews NW: cAMP regulates Ca2+-dependent exocytosis of lysosomes and lysosome-mediated cell invasion by trypanosomes. *J Biol Chem* 1999, 274(24):16754-16759.

666. Claustrat B, Brun J, Chazot G: The basic physiology and pathophysiology of melatonin. *Sleep Med Rev* 2005, 9(1):11-24.

667. Merckx A, Nivez MP, Bouyer G, Alano P, Langsley G, Deitsch K, Thomas S, Doerig C, Egee S: *Plasmodium falciparum* regulatory subunit of cAMP-dependent PKA and anion channel conductance. *PLoS Pathog* 2008, 4(2):e19.

668. Read LK, Mikkelsen RB: Comparison of adenylate cyclase and cAMP-dependent protein kinase in gametocytogenic and nongametocytogenic clones of *Plasmodium falciparum*. *J Parasitol* 1991, 77(3):346-352.

669. Eaton MS, Weiss LM, Kim K: Cyclic nucleotide kinases and tachyzoite-bradyzoite transition in *Toxoplasma gondii*. *Int J Parasitol* 2006, 36(1):107-114.

670. Mol M, Patole MS, Singh S: Signaling networks in Leishmania macrophages deciphered through integrated systems biology: a mathematical modeling approach. *Syst Synth Biol* 2013, 7(4):185-195.

671. Swierczewski BE, Davies SJ: A schistosome cAMP-dependent protein kinase catalytic subunit is essential for parasite viability. *PLoS Negl Trop Dis* 2009, 3(8):e505.

672. Lopes AH, Gomes MT, Dutra FL, Vermelho AB, Meyer-Fernandes JR, Silva-Neto MAC, Souto-Padrón T, Vieira DP: Intracellular Signaling Pathways Involved in Cell Differentiation in Trypanosomatids *The Open Parasitology Journal* 2010, 4:102-110

673. Beraldo FH, Almeida FM, da Silva AM, Garcia CR: Cyclic AMP and calcium interplay as second messengers in melatonin-dependent regulation of *Plasmodium falciparum* cell cycle. *J Cell Biol* 2005, 170(4):551-557.

674. Besteiro S, Tonn D, Tetley L, Coombs GH, Mottram JC: The AP3 adaptor is involved in the transport of membrane proteins to acidocalcisomes of *Leishmania*. *J Cell Sci* 2008, 121(Pt 5):561-570.

675. Moreno SN, Docampo R: Calcium regulation in protozoan parasites. *Curr Opin Microbiol* 2003, 6(4):359-364.

676. Moreno SN, Ayong L, Pace DA: Calcium storage and function in apicomplexan parasites. *Essays Biochem* 2011, 51:97-110.

677. Raabe AC, Wengelnik K, Billker O, Vial HJ: Multiple roles for *Plasmodium berghei* phosphoinositide-specific phospholipase C in regulating gametocyte activation and differentiation. *Cell Microbiol* 2011, 13(7):955-966.

678. Alano P, Billker O: Gametocytes and Gametes. In Molecular Approaches to Malaria, I.W. Sherman, ed. (Washington, D.C.: ASM Press), pp. 191–219. 2005. .

679. Docampo R, Vercesi AE, Huang G: Mitochondrial calcium transport in trypanosomes. *Mol Biochem Parasitol* 2014, 196(2):108-116.

680. Docampo R, Moreno SN, Plattner H: Intracellular calcium channels in protozoa. *Eur J Pharmacol* 2014, 739:4-18.

681. Vassella E, Reuner B, Yutzy B, Boshart M: Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. *J Cell Sci* 1997, 110 (Pt 21):2661-2671.

682. Santos DO, Oliveira MM: Effect of cAMP on macromolecule synthesis in the pathogenic protozoa *Trypanosoma cruzi*. *Mem Inst Oswaldo Cruz* 1988, 83(3):287-292.

683. Racioppi L, Means AR: Calcium/calmodulin-dependent protein kinase kinase 2: roles in signaling and pathophysiology. *J Biol Chem* 2012, 287(38):31658-31665.

684. Chin D, Means AR: Calmodulin: a prototypical calcium sensor. *Trends Cell Biol* 2000, 10(8):322-328.

685. Miranda A, Samudio F, Saldana A, Castillo J, Brandao A, Calzada JE: The calmodulin intergenic spacer as molecular target for characterization of *Leishmania* species. *Parasit Vectors* 2014, 7:35.

686. Benaim G, Cervino V, Villalobo A: Comparative phosphorylation of calmodulin from trypanosomatids and bovine brain by calmodulin-binding protein kinases. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1998, 120(1):57-65.

687. Seebeck T, Schaub R, Johner A: cAMP signalling in the kinetoplastid protozoa. *Curr Mol Med* 2004, 4(6):585-599.

688. Tellez-Inon MT, Ulloa RM, Torruella M, Torres HN: Calmodulin and Ca2+-dependent cyclic AMP phosphodiesterase activity in *Trypanosoma cruzi*. *Mol Biochem Parasitol* 1985, 17(2):143-153.

689. Ruben L, Haghighat N, Campbell A: Cyclical differentiation of Trypanosoma brucei involves changes in the cellular complement of calmodulin-binding proteins. *Exp Parasitol* 1990, 70(2):144-153.

690. Ogueta SB, Macintosh GC, Tellez-Inon MT: Stage-specific substrate phosphorylation by a Ca2+/calmodulin-dependent protein kinase in *Trypanosoma cruzi*. *J Eukaryot Microbiol* 1998, 45(4):392-396.

691. Fujisawa H: Regulation of the activities of multifunctional Ca2+/calmodulin-dependent protein kinases. *J Biochem* 2001, 129(2):193-199.

692. Paveto C, Pereira C, Espinosa J, Montagna AE, Farber M, Esteva M, Flawia MM, Torres HN: The nitric oxide transduction pathway in *Trypanosoma cruzi*. *J Biol Chem* 1995, 270(28):16576-16579.

693. Mayer B: Nitric oxide/cyclic GMP-mediated signal transduction. *Ann N Y Acad Sci* 1994, 733:357-364.

694. Benaim B, Garcia CR: Targeting calcium homeostasis as the therapy of Chagas' disease and leishmaniasis - a review. *Trop Biomed* 2011, 28(3):471-481.

695. Voorheis HP, Martin BR: Characteristics of the calcium-mediated mechanism activating adenylate cyclase in Trypanosoma brucei. *Eur J Biochem* 1981, 116(3):471-477.

696. Bowles DJ, Voorheis HP: Release of the surface coat from the plasma membrane of intact bloodstream forms of *Trypanosoma brucei* requires Ca2+. *FEBS Lett* 1982, 139(1):17-21.

697. Stojdl DF, Clarke MW: Trypanosoma brucei: analysis of cytoplasmic Ca2+ during differentiation of bloodstream stages *in vitro*. *Exp Parasitol* 1996, 83(1):134-146.

698. Moreno SN, Silva J, Vercesi AE, Docampo R: Cytosolic-free calcium elevation in *Trypanosoma cruzi* is required for cell invasion. *J Exp Med* 1994, 180(4):1535-1540.

699. Passos AP, Garcia CR: Inositol 1,4,5-trisphosphate induced Ca2+ release from chloroquine-sensitive and -insensitive intracellular stores in the intraerythrocytic stage of the malaria parasite P. chabaudi. *Biochem Biophys Res Commun* 1998, 245(1):155-160.

700. Billker O, Lourido S, Sibley LD: Calcium-dependent signaling and kinases in apicomplexan parasites. *Cell Host Microbe* 2009, 5(6):612-622.

701. Morlon-Guyot J, Berry L, Chen CT, Gubbels MJ, Lebrun M, Daher W: The *Toxoplasma gondii* calcium-dependent protein kinase 7 is involved in early steps of parasite division and is crucial for parasite survival. *Cell Microbiol* 2014, 16(1):95-114.

702. Gao X, Gunalan K, Yap SS, Preiser PR: Triggers of key calcium signals during erythrocyte invasion by *Plasmodium falciparum*. *Nat Commun* 2013, 4:2862.

703. Ishino T, Orito Y, Chinzei Y, Yuda M: A calcium-dependent protein kinase regulates Plasmodium ookinete access to the midgut epithelial cell. *Mol Microbiol* 2006, 59(4):1175-1184.

704. Siden-Kiamos I, Ecker A, Nyback S, Louis C, Sinden RE, Billker O: *Plasmodium berghei* calcium-dependent protein kinase 3 is required for ookinete gliding motility and mosquito midgut invasion. *Mol Microbiol* 2006, 60(6):1355-1363.

705. Baton LA, Ranford-Cartwright LC: How do malaria ookinetes cross the mosquito midgut wall? *Trends Parasitol* 2005, 21(1):22-28.

706. Ono T, Cabrita-Santos L, Leitao R, Bettiol E, Purcell LA, Diaz-Pulido O, Andrews LB, Tadakuma T, Bhanot P, Mota MM *et al*: Adenylyl cyclase alpha and cAMP signaling mediate *Plasmodium* sporozoite apical regulated exocytosis and hepatocyte infection. *PLoS Pathog* 2008, 4(2):e1000008.

707. Gaur D, Chitnis CE: Molecular interactions and signaling mechanisms during erythrocyte invasion by malaria parasites. *Curr Opin Microbiol* 2011, 14(4):422-428.

708. Vaid A, Thomas DC, Sharma P: Role of Ca2+/calmodulin-PfPKB signaling pathway in erythrocyte invasion by *Plasmodium falciparum*. *J Biol Chem* 2008, 283(9):5589-5597.

709. Benaim G, Romero PJ: A calcium pump in plasma membrane vesicles from *Leishmania braziliensis*. *Biochim Biophys Acta* 1990, 1027(1):79-84.

710. Michelangeli F, Ogunbayo OA, Wootton LL: A plethora of interacting organellar Ca2+ stores. *Curr Opin Cell Biol* 2005, 17(2):135-140.

711. Docampo R, de Souza W, Miranda K, Rohloff P, Moreno SN: Acidocalcisomes - conserved from bacteria to man. *Nat Rev Microbiol* 2005, 3(3):251-261.

712. Vercesi AE: Ca2+ homeostasis and mitochondrial bioenergetics in *Trypanosoma cruzi*. *Braz J Med Biol Res* 1994, 27(2):501-503.

713. Fidalgo LM, Gille L: Mitochondria and trypanosomatids: targets and drugs. *Pharm Res* 2011, 28(11):2758-2770.

714. Serrano-Martin X, Garcia-Marchan Y, Fernandez A, Rodriguez N, Rojas H, Visbal G, Benaim G: Amiodarone destabilizes intracellular Ca2+ homeostasis and biosynthesis of sterols in *Leishmania mexicana*. *Antimicrob Agents Chemother* 2009, 53(4):1403-1410.

715. Vercesi AE, Hoffmann ME, Bernardes CF, Docampo R: ATP and Ca2+ homeostasis in *Trypanosoma cruzi*. *Braz J Med Biol Res* 1993, 26(4):355-363.

716. Bagnaresi P, Nakabashi M, Thomas AP, Reiter RJ, Garcia CR: The role of melatonin in parasite biology. *Mol Biochem Parasitol* 2012, 181(1):1-6.

717. Kilic U, Yilmaz B, Reiter RJ, Yuksel A, Kilic E: Effects of memantine and melatonin on signal transduction pathways vascular leakage and brain injury after focal cerebral ischemia in mice. *Neuroscience* 2013, 237:268-276.

718. Reppert SM, Weaver DR, Godson C: Melatonin receptors step into the light: cloning and classification of subtypes. *Trends Pharmacol Sci* 1996, 17(3):100-102.

719. Benitez-King G, Anton-Tay F: Calmodulin mediates melatonin cytoskeletal effects. *Experientia* 1993, 49(8):635-641.

720. Macias M, Escames G, Leon J, Coto A, Sbihi Y, Osuna A, Acuna-Castroviejo D: Calreticulin-melatonin. An unexpected relationship. *Eur J Biochem* 2003, 270(5):832-840.

721. Melendez J, Maldonado V, Ortega A: Effect of melatonin on beta-tubulin and MAP2 expression in NIE-115 cells. *Neurochem Res* 1996, 21(6):653-658.

722. Benitez-King G: Melatonin as a cytoskeletal modulator: implications for cell physiology and disease. *J Pineal Res* 2006, 40(1):1-9.

723. Chiang RP, Huang CT, Tsai YJ: Melatonin reduces median nerve injury-induced mechanical hypersensitivity via inhibition of microglial p38 mitogen-activated protein kinase activation in rat cuneate nucleus. *J Pineal Res* 2013, 54(2):232-244.

724. Dubocovich ML, Yun K, Al-Ghoul WM, Benloucif S, Masana MI: Selective MT2 melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. *FASEB J* 1998, 12(12):1211-1220.

725. Brydon L, Roka F, Petit L, de Coppet P, Tissot M, Barrett P, Morgan PJ, Nanoff C, Strosberg AD, Jockers R: Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. *Mol Endocrinol* 1999, 13(12):2025-2038.

726. Calamini B, Santarsiero BD, Boutin JA, Mesecar AD: Kinetic, thermodynamic and X-ray structural insights into the interaction of melatonin and analogues with quinone reductase 2. *Biochem J* 2008, 413(1):81-91.

727. Geary GG, Duckles SP, Krause DN: Effect of melatonin in the rat tail artery: role of K+ channels and endothelial factors. *Br J Pharmacol* 1998, 123(8):1533-1540.

728. Tjong YW, Li MF, Hung MW, Fung ML: Melatonin ameliorates hippocampal nitric oxide production and large conductance calcium-activated potassium channel activity in chronic intermittent hypoxia. *J Pineal Res* 2008, 44(3):234-243.

729. Beraldo FH, Garcia CR: Products of tryptophan catabolism induce Ca2+ release and modulate the cell cycle of *Plasmodium falciparum* malaria parasites. *J Pineal Res* 2005, 39(3):224-230.

730. Srinivasan V, Spence DW, Moscovitch A, Pandi-Perumal SR, Trakht I, Brown GM, Cardinali DP: Malaria: therapeutic implications of melatonin. *Journal of pineal research* 2010, 48(1):1-8.

731. Baltaci AK, Mogulkoc R, Turkoz Y, Bediz CS, Ozugurlu F: The effect of pinealectomy and zinc deficiency on nitric oxide levels in rats with induced *Toxoplasma gondii* infection. *Swiss Med Wkly* 2004, 134(23-24):359-363.

732. Beraldo FH, Mikoshiba K, Garcia CR: Human malarial parasite, *Plasmodium falciparum*, displays capacitative calcium entry: 2-aminoethyl diphenylborinate blocks the signal transduction pathway of melatonin action on the P. falciparum cell cycle. *J Pineal Res* 2007, 43(4):360-364.

733. Santello FH, Caetano LC, Filipin Mdel V, Brazao V, Caetano LN, Toldo MP, do Prado JC, Jr.: Does orchiectomy enhance the immune-stimulatory effects of melatonin during experimental Chagas' disease? *Res Vet Sci* 2012, 93(2):819-825.

734. Santello FH, Del Vecchio Filipin M, Caetano LC, Brazao V, Caetano LN, Dos Santos CD, Alonso Toldo MP, do Prado JC, Jr.: Influence of melatonin therapy and orchiectomy on T cell subsets in male Wistar rats infected with *Trypanosoma cruzi*. *J Pineal Res* 2009, 47(3):271-276.

735. Brunet LR: Nitric oxide in parasitic infections. *Int Immunopharmacol* 2001, 1(8):1457-1467.

736. Macias M, Rodriguez-Cabezas MN, Reiter RJ, Osuna A, Acuna-Castroviejo D: Presence and effects of melatonin in *Trypanosoma cruzi*. *Journal of pineal research* 1999, 27(2):86-94.

737. Franca-Botelho AC, Franca JL, Oliveira FM, Franca EL, Honorio-Franca AC, Caliari MV, Gomes MA: Melatonin reduces the severity of experimental amoebiasis. *Parasit Vectors* 2011, 4:62.

738. Elmahallawy EK, Jimenez-Aranda A, Martinez AS, Rodriguez-Granger J, Navarro-Alarcon M, Gutierrez-Fernandez J, Agil A: Activity of melatonin against *Leishmania infantum*

promastigotes by mitochondrial dependent pathway. *Chemico-biological interactions* 2014, 220:84-93.

739. WHO: Malaria Fact sheet N°94". . *Retrieved 28 August 2014* 2014.
740. Beare NA, Lewallen S, Taylor TE, Molyneux ME: Redefining cerebral malaria by including malaria retinopathy. *Future Microbiol* 2011, 6(3):349-355.
741. Bagnaresi P, Alves E, da Silva HB, Epiphanio S, Mota MM, Garcia CR: Unlike the synchronous *Plasmodium falciparum* and *P. chabaudi* infection, the *P. berghei* and *P. yoelii* asynchronous infections are not affected by melatonin. *Int J Gen Med* 2009, 2:47-55.
742. Wasserman M: The role of calcium ions in the invasion of *Plasmodium falciparum*. *Blood Cells* 1990, 16(2-3):450-451.
743. Deligianni E, Morgan RN, Bertuccini L, Kooij TW, Laforge A, Nahar C, Poulakakis N, Schuler H, Louis C, Matuschewski K *et al*: Critical role for a stage-specific actin in male exflagellation of the malaria parasite. *Cell Microbiol* 2011, 13(11):1714-1730.
744. Glushakova S, Lizunov V, Blank PS, Melikov K, Humphrey G, Zimmerberg J: Cytoplasmic free Ca2+ is essential for multiple steps in malaria parasite egress from infected erythrocytes. *Malar J* 2013, 12:41.
745. Schuck DC, Jordao AK, Nakabashi M, Cunha AC, Ferreira VF, Garcia CR: Synthetic indole and melatonin derivatives exhibit antimalarial activity on the cell cycle of the human malaria parasite *Plasmodium falciparum*. *Eur J Med Chem* 2014, 78:375-382.
746. Rivo YB, Alkarimah A, Ramadhani NN, Cahyono AW, Laksmi DA, Winarsih S, Fitri LE: Metabolite extract of Streptomyces hygroscopicus Hygroscopicus inhibit the growth of *Plasmodium berghei* through inhibition of ubiquitin - proteasome system. *Trop Biomed* 2013, 30(2):291-300.
747. Dey S, Guha M, Alam A, Goyal M, Bindu S, Pal C, Maity P, Mitra K, Bandyopadhyay U: Malarial infection develops mitochondrial pathology and mitochondrial oxidative stress to promote hepatocyte apoptosis. *Free Radic Biol Med* 2009, 46(2):271-281.
748. Guha M, Maity P, Choubey V, Mitra K, Reiter RJ, Bandyopadhyay U: Melatonin inhibits free radical-mediated mitochondrial-dependent hepatocyte apoptosis and liver damage induced during malarial infection. *J Pineal Res* 2007, 43(4):372-381.
749. KJ; R, (editors) RC: Sherris Medical Microbiology (4th ed.). . *McGraw Hill* 2004:pp. 723–727.

750. Baltaci AK, Mogulkoc R, Bediz CS, Pekel A: Effects of zinc deficiency and pinealectomy on cellular immunity in rats infected with *Toxoplasma gondii*. *Biol Trace Elem Res* 2005, 104(1):47-56.

751. Baltaci AK, Bediz CS, Mogulkoc R, Kurtoglu E, Pekel A: Effect of zinc and melatonin supplementation on cellular immunity in rats with toxoplasmosis. *Biol Trace Elem Res* 2003, 96(1-3):237-245.

752. Rozenfeld C, Martinez R, Figueiredo RT, Bozza MT, Lima FR, Pires AL, Silva PM, Bonomo A, Lannes-Vieira J, De Souza W *et al*: Soluble factors released by *Toxoplasma gondii*-infected astrocytes down-modulate nitric oxide production by gamma interferon-activated microglia and prevent neuronal degeneration. *Infect Immun* 2003, 71(4):2047-2057.

753. Hoare CA: [Rationalization of the terminology for the developmental stages of trypanosomatid flagellates]. *Med Parazitol (Mosk)* 1971, 40(3):307-309.

754. Noireau F, Diosque P, Jansen AM: *Trypanosoma cruzi*: adaptation to its vectors and its hosts. *Vet Res* 2009, 40(2):26.

755. WHO: "Trypanosomiasis, Human African (sleeping sickness)". Fact sheet N°259. World Health Organization. Retrieved 25 February 2014. 2014.

756. Henze K, Martin W: Evolutionary biology: essence of mitochondria. *Nature* 2003, 426(6963):127-128.

757. Rassi A, Jr., Rassi A, Marcondes de Rezende J: American trypanosomiasis (Chagas disease). *Infect Dis Clin North Am* 2012, 26(2):275-291.

758. Tanowitz HB, Machado FS, Jelicks LA, Shirani J, de Carvalho AC, Spray DC, Factor SM, Kirchhoff LV, Weiss LM: Perspectives on *Trypanosoma cruzi*-induced heart disease (Chagas disease). *Prog Cardiovasc Dis* 2009, 51(6):524-539.

759. Cunha Neto E: [Rethinking the pathogenesis of chronic chagasic cardiopathy at the closing of the millennium]. *Medicina (B Aires)* 1999, 59(5 Pt 1):496-500.

760. Reyes JL, Terrazas LI, Espinoza B, Cruz-Robles D, Soto V, Rivera-Montoya I, Gomez-Garcia L, Snider H, Satoskar AR, Rodriguez-Sosa M: Macrophage migration inhibitory factor contributes to host defense against acute *Trypanosoma cruzi* infection. *Infect Immun* 2006, 74(6):3170-3179.

761. Assreuy J, Cunha FQ, Epperlein M, Noronha-Dutra A, O'Donnell CA, Liew FY, Moncada S: Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major*. *Eur J Immunol* 1994, 24(3):672-676.

762. Santello FH, Frare EO, dos Santos CD, Toldo MP, Kawasse LM, Zucoloto S, do Prado JC, Jr.: Melatonin treatment reduces the severity of experimental *Trypanosoma cruzi* infection. *J Pineal Res* 2007, 42(4):359-363.

763. Oztekin E, Mogulkoc R, Baltaci AK, Tiftik AM: The influence of estradiol and progesterone and melatonin supplementation on TNF-alpha levels in ovariectomized and pinealectomized rats. *Acta Biol Hung* 2006, 57(3):275-281.

764. Santello FH, Frare EO, dos Santos CD, Caetano LC, Alonso Toldo MP, do Prado JC, Jr.: Suppressive action of melatonin on the TH-2 immune response in rats infected with *Trypanosoma cruzi*. *J Pineal Res* 2008, 45(3):291-296.

765. Zhang S, Li W, Gao Q, Wei T: Effect of melatonin on the generation of nitric oxide in murine macrophages. *Eur J Pharmacol* 2004, 501(1-3):25-30.

766. Rodrigues WF, Miguel CB, Chica JE, Napimoga MH: 15d-PGJ(2) modulates acute immune responses to *Trypanosoma cruzi* infection. *Mem Inst Oswaldo Cruz* 2010, 105(2):137-143.

767. Paes MC, Cosentino-Gomes D, de Souza CF, Nogueira NP, Meyer-Fernandes JR: The Role of Heme and Reactive Oxygen Species in Proliferation and Survival of *Trypanosoma cruzi*. *J Parasitol Res* 2011, 2011:174614.

768. Gupta S, Wen JJ, Garg NJ: [In Process Citation]. *Interdiscip Perspect Infect Dis* 2009, 2009:190354.

769. de Oliveira TB, Pedrosa RC, Filho DW: Oxidative stress in chronic cardiopathy associated with Chagas disease. *Int J Cardiol* 2007, 116(3):357-363.

770. Wen JJ, Vyatkina G, Garg N: Oxidative damage during chagasic cardiomyopathy development: role of mitochondrial oxidant release and inefficient antioxidant defense. *Free Radic Biol Med* 2004, 37(11):1821-1833.

771. Menna-Barreto RF, Salomao K, Dantas AP, Santa-Rita RM, Soares MJ, Barbosa HS, de Castro SL: Different cell death pathways induced by drugs in *Trypanosoma cruzi*: an ultrastructural study. *Micron* 2009, 40(2):157-168.

772. Cardinali DP, Alvarez CB: Melatonin in Chagas' disease. Possible therapeutic value. *Medicina (B Aires)* 2011, 71(5):477-483.

773. Oliveira LG, Kuehn CC, dos Santos CD, Miranda MA, da Costa CM, Mendonca VJ, do Prado Junior JC: Protective actions of melatonin against heart damage during chronic Chagas disease. *Acta Trop* 2013, 128(3):652-658.

774. Fenwick A: The global burden of neglected tropical diseases. *Public Health* 2012, 126(3):233-236.

775. Thetiot-Laurent SA, Boissier J, Robert A, Meunier B: Schistosomiasis chemotherapy. *Angew Chem Int Ed Engl* 2013, 52(31):7936-7956.

776. WHO: Life in the 21st Century: a vision for all. The World Health Report 1998. World Health Organization, Geneva, Switzerland. 1998.

777. Chacin-Bonilla L: [An update on amebiasis]. *Rev Med Chil* 2013, 141(5):609-615.

778. WHO: Technical Report Series., 2012. Research Priorities for Chagas Disease, Human African Trypanosomiasis and Leishmaniasis. Technical Report of the TDR Disease Reference Group on Chagas Disease, Human African Trypanosomiasis and Leishmaniasis. No. 975. 2012.

779. Sereno D, Maia C, Ait-Oudhia K: Antimony resistance and environment: Elusive links to explore during *Leishmania* life cycle. *Int J Parasitol Drugs Drug Resist* 2012, 2:200-203.

780. Elmahallawy EK, Agil A: Treatment of leishmaniasis: a review and assessment of recent research. *Curr Pharm Des* 2015, 21(18):2259-2275.

781. Luque-Ortega JR, Rivas L: Miltefosine (hexadecylphosphocholine) inhibits cytochrome c oxidase in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* 2007, 51(4):1327-1332.

782. Carvalho L, Luque-Ortega JR, Manzano JI, Castanys S, Rivas L, Gamarro F: Tafenoquine, an antiplasmodial 8-aminoquinoline, targets *leishmania* respiratory complex III and induces apoptosis. *Antimicrob Agents Chemother* 2010, 54(12):5344-5351.

783. Ferroglio E, Centaro E, Mignone W, Trisciuoglio A: Evaluation of an ELISA rapid device for the serological diagnosis of *Leishmania infantum* infection in dog as compared with immunofluorescence assay and Western blot. *Vet Parasitol* 2007, 144(1-2):162-166.

784. Ferroglio E, Trisciuoglio A, Gastaldo S, Mignone W, M. DP: Comparison of ELISA, IFAT and Western blot for the serological diagnosis of *Leishmania infantum* infection in dog. . *Parassitologia* 2002, 44(Suppl):64.

785. Lachaud L, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP, Bastien P: Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *J Clin Microbiol* 2002, 40(1):210-215.

786. Evans D: Handbook on isolation, characterization and cryopreservation of *Leishmania*. TDR.UNDP/World Bank/WHO. Special Programme for Research and Training in Tropical Diseases, 45. 1989.

787. Debrabant A, Joshi MB, Pimenta PF, Dwyer DM: Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. *Int J Parasitol* 2004, 34(2):205-217.

788. Carrio J, de Colmenares M, Riera C, Gallego M, Arboix M, Portus M: *Leishmania infantum*: stage-specific activity of pentavalent antimony related with the assay conditions. *Exp Parasitol* 2000, 95(3):209-214.

789. Jesus Corral-Caridad M, Moreno I, Torano A, Dominguez M, Alunda JM: Effect of allicin on promastigotes and intracellular amastigotes of *Leishmania donovani* and *L. infantum*. *Exp Parasitol* 2012, 132(4):475-482.

790. Bodley AL, McGarry MW, Shapiro TA: Drug cytotoxicity assay for African trypanosomes and Leishmania species. *J Infect Dis* 1995, 172(4):1157-1159.

791. Mesa-Valle CM, Castilla-Calvente J, Sanchez-Moreno M, Moraleda-Lindez V, Barbe J, Osuna A: Activity and mode of action of acridine compounds against *Leishmania donovani*. *Antimicrob Agents Chemother* 1996, 40(3):684-690.

792. Mikus J, Steverding D: A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue. *Parasitol Int* 2000, 48(3):265-269.

793. Rampersad SN: Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors (Basel)* 2012, 12(9):12347-12360.

794. Pallotti F, Lenaz G: Isolation and subfractionation of mitochondria from animal cells and tissue culture lines. *Methods Cell Biol* 2007, 80:3-44.

795. Renger HC, Wolstenholme DR: The form and structure of kinetoplast DNA of Crithidia. *J Cell Biol* 1972, 54(2):346-364.

796. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, 72:248-254.

797. Morales AI, Detaille D, Prieto M, Puente A, Briones E, Arevalo M, Leverve X, Lopez-Novoa JM, El-Mir MY: Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway. *Kidney Int* 2010, 77(10):861-869.

798. Stuehr DJ, Nathan CF: Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* 1989, 169(5):1543-1555.

799. Githinji EK, Irungu LW, Tonui WK, Rukunga GM, Mutai C, Muthaura CN, Lugalia R, Gikandi G, Wainaina CW, Ingonga JM *et al*: *In vitro* effects of Warburgia ugandensis, Psiadia punctulata and Chasmanthera dependens on *Leishmania major* promastigotes. *Afr J Tradit Complement Altern Med* 2010, 7(3):264-275.

800. Lopez A, Garcia JA, Escames G, Venegas C, Ortiz F, Lopez LC, Acuna-Castroviejo D: Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. *J Pineal Res* 2009, 46(2):188-198.

801. Ziegler DM, Doeg KA: Studies on the electron transport system. XLIII. The isolation of a succinic-coenzyme Q reductase from beef heart mitochondria. *Archives of Biochemistry and Biophysics* 1962, 97(1):41–50.

802. Rieske JS: Preparation and properties of reduced coenzyme Q-cytochrome c reductase (complex III of the respiratory chain). *Methods in Enzymology* 1967, 10:239-245.

803. Zuur A, Ieno E, Walker N, Saveliev A, Smith G: Mixed effects models and extensions in ecology with R, 1st edition. Springer, New York. 2009.

804. R Core Team: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/. 2015.

805. Romero HD, Silva Lde A, Silva-Vergara ML, Rodrigues V, Costa RT, Guimaraes SF, Alecrim W, Moraes-Souza H, Prata A: Comparative study of serologic tests for the diagnosis of asymptomatic visceral leishmaniasis in an endemic area. *Am J Trop Med Hyg* 2009, 81(1):27-33.

806. Ferroglio E, Romano A, Trisciuoglio A, Poggi M, Ghiggi E, Sacchi P, Biglino A: Characterization of *Leishmania infantum* strains in blood samples from infected dogs and humans by PCR-RFLP. *Trans R Soc Trop Med Hyg* 2006, 100(7):636-641.

807. Biglino A, Bolla C, Concialdi E, Trisciuoglio A, Romano A, Ferroglio E: Asymptomatic *Leishmania infantum* infection in an area of northwestern Italy (Piedmont region) where such infections are traditionally nonendemic. *J Clin Microbiol* 2010, 48(1):131-136.

808. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, Gramiccia M, Mortarino M, Pietrobelli M, Gradoni L: The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. *Trop Med Int Health* 2008, 13(2):256-264.

809. Ferroglio E, Maroli M, Gastaldo S, Mignone W, Rossi L: Canine leishmaniasis, Italy. *Emerg Infect Dis* 2005, 11(10):1618-1620.

810. Sereno D, Maia C, Aït-Oudhia K: Antimony resistance and environment: Elusive links to explore during *Leishmania* life cycle. *Inter J for Parasito: Drugs and Drug Resistance* 2012, 2:200–203.

811. Acedo Sanchez C, Martin Sanchez J, Velez Bernal ID, Sanchis Marin MC, Louassini M, Maldonado JA, Morillas Marquez F: Leishmaniasis eco-epidemiology in the Alpujarra region (Granada Province, southern Spain). *Int J Parasitol* 1996, 26(3):303-310.

812. Morillas F, Sanchez Rabasco F, Ocana J, Martin-Sanchez J, Ocana-Wihelmi J, Acedo C, Sanchiz-Marin MC: Leishmaniosis in the focus of the Axarquia region, Malaga province, southern Spain: a survey of the human, dog, and vector. *Parasitol Res* 1996, 82(6):569-570.

813. Riera C, Fisa R, Udina M, Gallego M, Portus M: Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. *Trans R Soc Trop Med Hyg* 2004, 98(2):102-110.

814. Riera C, Fisa R, Lopez-Chejade P, Serra T, Girona E, Jimenez M, Muncunill J, Sedeno M, Mascaro M, Udina M *et al*: Asymptomatic infection by *Leishmania infantum* in blood donors from the Balearic Islands (Spain). *Transfusion* 2008, 48(7):1383-1389.

815. Pineda JA, Gallardo JA, Macias J, Delgado J, Regordan C, Morillas F, Relimpio F, Martin-Sanchez J, Sanchez-Quijano A, Leal M *et al*: Prevalence of and factors associated with visceral leishmaniasis in human immunodeficiency virus type 1-infected patients in southern Spain. *J Clin Microbiol* 1998, 36(9):2419-2422.

816. Garcia-Garcia JA, Martin-Sanchez J, Gallego M, Rivero-Roman A, Camacho A, Riera C, Morillas-Marquez F, Vergara S, Macias J, Pineda JA: Use of noninvasive markers to detect

Leishmania infection in asymptomatic human immunodeficiency virus-infected patients. *J Clin Microbiol* 2006, 44(12):4455-4458.

817. Ma DD, Concannon AJ, Hayes J: Fatal leishmaniasis in renal-transport patient. *Lancet* 1979, 2(8137):311-312.
818. Antinori S, Schifanella L, Corbellino M: Leishmaniasis: new insights from an old and neglected disease. *Eur J Clin Microbiol Infect Dis* 2012, 31(2):109-118.
819. Simon I, Wissing KM, Del Marmol V, Antinori S, Remmelink M, Nilufer Broeders E, Nortier JL, Corbellino M, Abramowicz D, Cascio A: Recurrent leishmaniasis in kidney transplant recipients: report of 2 cases and systematic review of the literature. *Transpl Infect Dis* 2011, 13(4):397-406.
820. Vinhal FA, Afonso-Cardoso SR, Silva AG, Pereira CG, Sousa W, Botelho SM, Souza JB, Silva WD, Pena JD, Souza MA: A typical presentation of cutaneous leishmaniasis after renal transplantation. *Nephrol Dial Transplant* 2007, 22(12):3674.
821. Clemente WT, Couto CA, Ribeiro DD, de Medeiros Chaves Franca M, Sanches MD: An atypical course of visceral leishmaniasis (Kala-azar) in a liver transplant recipient. *Transplantation* 2007, 83(3):368-369.
822. le Fichoux Y, Quaranta JF, Aufeuvre JP, Lelievre A, Marty P, Suffia I, Rousseau D, Kubar J: Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. *J Clin Microbiol* 1999, 37(6):1953-1957.
823. Bouchekoua M, Trabelsi S, Ben Abdallah T, Khaled S: Visceral leishmaniasis after kidney transplantation: report of a new case and a review of the literature. *Transplantation reviews* 2014, 28(1):32-35.
824. Clemente WT, Rabello A, Faria LC, Peruhype-Magalhaes V, Gomes LI, da Silva TA, Nunes RV, Iodith JB, Protil KZ, Fernandes HR *et al*: High prevalence of asymptomatic *Leishmania* spp. infection among liver transplant recipients and donors from an endemic area of Brazil. *Am J Transplant* 2014, 14(1):96-101.
825. Srivastava P, Gidwani K, Picado A, Van der Auwera G, Tiwary P, Ostyn B, Dujardin JC, Boelaert M, Sundar S: Molecular and serological markers of *Leishmania donovani* infection in healthy individuals from endemic areas of Bihar, India. *Trop Med Int Health* 2013, 18(5):548-554.

826. Garrote JI, Gutierrez MP, Izquierdo RL, Duenas MA, Zarzosa P, Canavate C, El Bali M, Almaraz A, Bratos MA, Berbel C *et al*: Seroepidemiologic study *of Leishmania infantum* infection in Castilla-Leon, Spain. *Am J Trop Med Hyg* 2004, 71(4):403-406.

827. de Gouvea Viana L, de Assis TS, Orsini M, da Silva AR, de Souza GF, Caligiorne R, da Silva AC, Peruhype-Magalhaes V, Marciano AP, Martins-Filho OA *et al*: Combined diagnostic methods identify a remarkable proportion of asymptomatic *Leishmania (Leishmania) chagasi* carriers who present modulated cytokine profiles. *Trans R Soc Trop Med Hyg* 2008, 102(6):548-555.

828. Alborzi A, Pourabbas B, Shahian F, Mardaneh J, Pouladfar GR, Ziyaeyan M: Detection of *Leishmania infantum* kinetoplast DNA in the whole blood of asymptomatic individuals by PCR-ELISA and comparison with other infection markers in endemic areas, southern Iran. *Am J Trop Med Hyg* 2008, 79(6):839-842.

829. Mary C, Faraut F, Drogoul MP, Xeridat B, Schleinitz N, Cuisenier B, Dumon H: Reference values for *Leishmania infantum* parasitemia in different clinical presentations: quantitative polymerase chain reaction for therapeutic monitoring and patient follow-up. *Am J Trop Med Hyg* 2006, 75(5):858-863.

830. Gidwani K, Kumar R, Rai M, Sundar S: Longitudinal seroepidemiologic study of visceral leishmaniasis in hyperendemic regions of Bihar, India. *Am J Trop Med Hyg* 2009, 80(3):345-346.

831. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S: Asymptomatic *Leishmania* infection: a new challenge for *Leishmania* control. *Clin Infect Dis* 2014, 58(10):1424-1429.

832. Hasker E, Malaviya P, Gidwani K, Picado A, Ostyn B, Kansal S, Singh RP, Singh OP, Chourasia A, Kumar Singh A *et al*: Strong association between serological status and probability of progression to clinical visceral leishmaniasis in prospective cohort studies in India and Nepal. *PLoS Negl Trop Dis* 2014, 8(1):e2657.

833. Schwartz BS, Mawhorter SD: Parasitic infections in solid organ transplantation. *Am J Transplant* 2013, 13 Suppl 4:280-303.

834. Chatzis MK, Leontides L, Athanasiou LV, Papadopoulos E, Kasabalis D, Mylonakis M, Rallis T, Koutinas AF, Andreadou M, Ikonomopoulos J *et al*: Evaluation of indirect immunofluorescence antibody test and enzyme-linked immunosorbent assay for the diagnosis

of infection by *Leishmania infantum* in clinically normal and sick cats. *Experimental parasitology* 2014, 147:54-59.

835. Hervas J, Chacon-Manrique de Lara F, Lopez J, Gomez-Villamandos JC, Guerrero MJ, Moreno A: Granulomatous (pseudotumoral) iridociclitis associated with leishmaniasis in a cat. *Vet Rec* 2001, 149(20):624-625.
836. Hervas J, Chacon MDLF, Sanchez-Isarria MA, Pellicer S, Carrasco L, Castillo JA, Gomez-Villamandos JC: Two cases of feline visceral and cutaneous leishmaniosis in Spain. *J Feline Med Surg* 1999, 1(2):101-105.
837. Marcos R, Santos M, Malhao F, Pereira R, Fernandes AC, Montenegro L, Roccabianca P: Pancytopenia in a cat with visceral leishmaniasis. *Veterinary clinical pathology / American Society for Veterinary Clinical Pathology* 2009, 38(2):201-205.
838. Sarkari B, Hatam GR, Adnani SJ, Asgari Q: Seroprevalence of feline leishmaniasis in areas of Iran where *Leishmania infantum* is endemic. *Annals of tropical medicine and parasitology* 2009, 103(3):275-277.
839. Ayllon T, Tesouro MA, Amusategui I, Villaescusa A, Rodriguez-Franco F, Sainz A: Serologic and molecular evaluation of *Leishmania infantum* in cats from Central Spain. *Ann N Y Acad Sci* 2008, 1149:361-364.
840. Maia C, Gomes J, Cristovao J, Nunes M, Martins A, Rebelo E, Campino L: Feline *Leishmania* infection in a canine leishmaniasis endemic region, Portugal. *Vet Parasitol* 2010, 174(3-4):336-340.
841. Martin-Sanchez J, Acedo C, Munoz-Perez M, Pesson B, Marchal O, Morillas-Marquez F: Infection by *Leishmania infantum* in cats: epidemiological study in Spain. *Vet Parasitol* 2007, 145(3-4):267-273.
842. Spada E, Proverbio D, Migliazzo A, Della Pepa A, Roberta Perego R, G BDG: Serological and Molecular Evaluation of *Leishmania infantum* Infection in Stray Cats in a Nonendemic Area in Northern Italy. *ISRN Parasitology* 2013., 2013(Article ID 916376, 6 pages).
843. Miro G, Ruperez C, Checa R, Galvez R, Hernandez L, Garcia M, Canorea I, Marino V, Montoya A: Current status of *L. infantum* infection in stray cats in the Madrid region (Spain): implications for the recent outbreak of human leishmaniosis? *Parasit Vectors* 2014, 7:112.
844. Rufenacht S, Sager H, Muller N, Schaerer V, Heier A, Welle MM, Roosje PJ: Two cases of feline leishmaniosis in Switzerland. *Vet Rec* 2005, 156(17):542-545.

845. Poli A, Abramo F, Barsotti P, Leva S, Gramiccia M, Ludovisi A, Mancianti F: Feline leishmaniosis due to *Leishmania infantum* in Italy. *Vet Parasitol* 2002, 106(3):181-191.

846. Pennisi MG: Leishmaniosis of companion animals in Europe: an update. *Vet Parasitol* 2015, 208(1-2):35-47.

847. Vita S, Santori D, Aguzzi I, Petrotta E, Luciani A: Feline leishmaniasis and ehrlichiosis: serological investigation in Abruzzo region. *Vet Res Commun* 2005, 29 Suppl 2:319-321.

848. Cardoso L, Lopes AP, Sherry K, Schallig H, Solano-Gallego L: Low seroprevalence of *Leishmania infantum* infection in cats from northern Portugal based on DAT and ELISA. *Vet Parasitol* 2010, 174(1-2):37-42.

849. Duarte A, Castro I, Pereira da Fonseca IM, Almeida V, Madeira de Carvalho LM, Meireles J, Fazendeiro MI, Tavares L, Vaz Y: Survey of infectious and parasitic diseases in stray cats at the Lisbon Metropolitan Area, Portugal. *J Feline Med Surg* 2010, 12(6):441-446.

850. Ayllon T, Diniz PP, Breitschwerdt EB, Villaescusa A, Rodriguez-Franco F, Sainz A: Vector-borne diseases in client-owned and stray cats from Madrid, Spain. *Vector Borne Zoonotic Dis* 2012, 12(2):143-150.

851. Tabar MD, Altet L, Francino O, Sanchez A, Ferrer L, Roura X: Vector-borne infections in cats: molecular study in Barcelona area (Spain). *Vet Parasitol* 2008, 151(2-4):332-336.

852. Fernandez-Perez FJ, Gomez-Munoz MT, Mendez S, Alunda JM: *Leishmania*-specific lymphoproliferative responses and IgG1/IgG2 immunodetection patterns by Western blot in asymptomatic, symptomatic and treated dogs. *Acta Trop* 2003, 86(1):83-91.

853. Ozon C, Marty P, Pratlong F, Breton C, Blein M, Lelievre A, Haas P: Disseminated feline leishmaniosis due to *Leishmania infantum* in Southern France. *Vet Parasitol* 1998, 75(2-3):273-277.

854. Maroli M, Pennisi MG, Di Muccio T, Khoury C, Gradoni L, Gramiccia M: Infection of sandflies by a cat naturally infected with *Leishmania infantum*. *Vet Parasitol* 2007, 145(3-4):357-360.

855. Millan J, Zanet S, Gomis M, Trisciuoglio A, Negre N, Ferroglio E: An investigation into alternative reservoirs of canine leishmaniasis on the endemic island of Mallorca (Spain). *Transbound Emerg Dis* 2011, 58(4):352-357.

856. Zanet S, Sposimo P, Trisciuoglio A, Giannini F, Strumia F, Ferroglio E: Epidemiology of *Leishmania infantum*, *Toxoplasma gondii*, and *Neospora caninum* in *Rattus rattus* in absence of domestic reservoir and definitive hosts. *Vet Parasitol* 2014, 199(3-4):247-249.

857. Ferroglio E, Romano A, Dettoni F, Trisciuoglio A: Distribution of Phlebotomus perniciosus in North-Italy: a study on 18S rDNA of phlebotomine sand flies. *Vet Parasitol* 2010, 170(1-2):127-130.

858. keda-Garcia FA, M. M: Métodos de diagnóstico da leishmaniose visceral canina. *Rev Clín Vet* 2007, 71:34-42.

859. Mortarino M, Franceschi A, Mancianti F, Bazzocchi C, Genchi C, Bandi C: [Quantitative PCR in the diagnosis of Leishmania]. *Parassitologia* 2004, 46(1-2):163-167.

860. Gramiccia M: Recent advances in leishmaniosis in pet animals: epidemiology, diagnostics and anti-vectorial prophylaxis. *Vet Parasitol* 2011, 181(1):23-30.

861. Slappendel R, Ferrer L: Leishmaniasis. In: Saunders, W.B. (Ed.), Infectious Diseases of the Dog and Cat. . *Philadelphia,* 1998, pp. 450–458.

862. Roura X, Peters IR, Altet L, Tabar MD, Barker EN, Planellas M, Helps CR, Francino O, Shaw SE, Tasker S: Prevalence of hemotropic mycoplasmas in healthy and unhealthy cats and dogs in Spain. *J Vet Diagn Invest* 2010, 22(2):270-274.

863. Baneth G: Leishmaniasis. In Infectious Diseases of the Dog and Cat. C.E. *Greene, Ed: 685–698 WB Saunders Philadelphia , PA* 2006.

864. Buscemi N, Vandermeer B, Hooton N, Pandya R, Tjosvold L, Hartling L, Vohra S, Klassen TP, Baker G: Efficacy and safety of exogenous melatonin for secondary sleep disorders and sleep disorders accompanying sleep restriction: meta-analysis. *BMJ* 2006, 332(7538):385-393.

865. Zhdanova IV, Wurtman RJ, Regan MM, Taylor JA, Shi JP, Leclair OU: Melatonin treatment for age-related insomnia. *J Clin Endocrinol Metab* 2001, 86(10):4727-4730.

866. Terry PD, Villinger F, Bubenik GA, Sitaraman SV: Melatonin and ulcerative colitis: evidence, biological mechanisms, and future research. *Inflamm Bowel Dis* 2009, 15(1):134-140.

867. Siddiqi NJ, Pandey VC: Studies on hepatic oxidative stress and antioxidant defence systems during arteether treatment of *Plasmodium yoelii nigeriensis* infected mice. *Mol Cell Biochem* 1999, 196(1-2):169-173.

868. Seabra ML, Bignotto M, Pinto LR, Jr., Tufik S: Randomized, double-blind clinical trial, controlled with placebo, of the toxicology of chronic melatonin treatment. *J Pineal Res* 2000, 29(4):193-200.

869. Molina-Carballo A, Munoz-Hoyos A, Reiter RJ, Sanchez-Forte M, Moreno-Madrid F, Rufo-Campos M, Molina-Font JA, Acuna-Castroviejo D: Utility of high doses of melatonin as adjunctive anticonvulsant therapy in a child with severe myoclonic epilepsy: two years' experience. *J Pineal Res* 1997, 23(2):97-105.

870. Bonnefont-Rousselot D, Collin F: Melatonin: action as antioxidant and potential applications in human disease and aging. *Toxicology* 2010, 278(1):55-67.

871. Silman RE: Melatonin: a contraceptive for the nineties. *Eur J Obstet Gynecol Reprod Biol* 1993, 49(1-2):3-9.

872. Arnoult D, Akarid K, Grodet A, Petit PX, Estaquier J, Ameisen JC: On the evolution of programmed cell death: apoptosis of the unicellular eukaryote *Leishmania major* involves cysteine proteinase activation and mitochondrion permeabilization. *Cell Death Differ* 2002, 9(1):65-81.

873. Getachew F, Gedamu L: *Leishmania donovani* iron superoxide dismutase A is targeted to the mitochondria by its N-terminal positively charged amino acids. *Mol Biochem Parasitol* 2007, 154(1):62-69.

874. Mukherjee A, Padmanabhan PK, Sahani MH, Barrett MP, Madhubala R: Roles for mitochondria in pentamidine susceptibility and resistance in *Leishmania donovani*. *Mol Biochem Parasitol* 2006, 145(1):1-10.

875. Brini MC, Tito; Ottolini, Denis; Carafoli, Ernesto . : "Chapter 5 Intracellular Calcium Homeostasis and Signaling". In Banci, Lucia (Ed.). *Metallomics and the Cell Metal Ions in Life Sciences 12 Springer* 2013.

876. Doerig C, Baker D, Billker O, Blackman MJ, Chitnis C, Dhar Kumar S, Heussler V, Holder AA, Kocken C, Krishna S *et al*: Signalling in malaria parasites. The MALSIG consortium. *Parasite* 2009, 16(3):169-182.

877. Clarkson AB, Jr., Amole BO: Role of calcium in trypanocidal drug action. *Science* 1982, 216(4552):1321-1323.

878. Batandier C, Leverve X, Fontaine E: Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I. *J Biol Chem* 2004, 279(17):17197-17204.

879. Kroemer G, Reed JC: Mitochondrial control of cell death. *Nat Med* 2000, 6(5):513-519.

880. Turrens JF: Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 1997, 17(1):3-8.

881. Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F: The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings in situ. *J Biol Chem* 2001, 276(15):12030-12034.

882. Kanno T, Sato EE, Muranaka S, Fujita H, Fujiwara T, Utsumi T, Inoue M, Utsumi K: Oxidative stress underlies the mechanism for Ca(2+)-induced permeability transition of mitochondria. *Free Radic Res* 2004, 38(1):27-35.

883. Green DR, Kroemer G: The pathophysiology of mitochondrial cell death. *Science* 2004, 305(5684):626-629.

884. Ly JD, Grubb DR, Lawen A: The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. *Apoptosis* 2003, 8(2):115-128.

885. Green DR, Reed JC: Mitochondria and apoptosis. *Science* 1998, 281(5381):1309-1312.

886. Ichas F, Mazat JP: From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochim Biophys Acta* 1998, 1366(1-2):33-50.

887. Kushnareva YE, Sokolove PM: Prooxidants open both the mitochondrial permeability transition pore and a low-conductance channel in the inner mitochondrial membrane. *Arch Biochem Biophys* 2000, 376(2):377-388.

888. Zhivotovsky B, Orrenius S: Calcium and cell death mechanisms: a perspective from the cell death community. *Cell Calcium* 2011, 50(3):211-221.

889. Kroemer G, Galluzzi L, Brenner C: Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007, 87(1):99-163.

890. Di Lisa F, Bernardi P: Mitochondrial function as a determinant of recovery or death in cell response to injury. *Mol Cell Biochem* 1998, 184(1-2):379-391.

891. Kinnally KW, Peixoto PM, Ryu SY, Dejean LM: Is mPTP the gatekeeper for necrosis, apoptosis, or both? *Biochim Biophys Acta* 2011, 1813(4):616-622.

892. Dumas JF, Argaud L, Cottet-Rousselle C, Vial G, Gonzalez C, Detaille D, Leverve X, Fontaine E: Effect of transient and permanent permeability transition pore opening on NAD(P)H localization in intact cells. *J Biol Chem* 2009, 284(22):15117-15125.

893. Shiva S: Mitochondria as metabolizers and targets of nitrite. *Nitric Oxide* 2010, 22(2):64-74.

894. Laothong U, Pinlaor P, Hiraku Y, Boonsiri P, Prakobwong S, Khoontawad J, Pinlaor S: Protective effect of melatonin against Opisthorchis viverrini-induced oxidative and nitrosative DNA damage and liver injury in hamsters. *J Pineal Res* 2010, 49(3):271-282.

895. Das R, Roy A, Dutta N, Majumder HK: Reactive oxygen species and imbalance of calcium homeostasis contributes to curcumin induced programmed cell death in *Leishmania donovani*. *Apoptosis* 2008, 13(7):867-882.

896. Bannister JV, Bannister WH, Rotilio G: Aspects of the structure, function, and applications of superoxide dismutase. *CRC Crit Rev Biochem* 1987, 22(2):111-180.

897. Plewes KA, Barr SD, Gedamu L: Iron superoxide dismutases targeted to the glycosomes of Leishmania chagasi are important for survival. *Infect Immun* 2003, 71(10):5910-5920.

898. Getachew F, Gedamu L: *Leishmania donovani* mitochondrial iron superoxide dismutase A is released into the cytosol during miltefosine induced programmed cell death. *Mol Biochem Parasitol* 2012, 183(1):42-51.

899. Sanchez-Moreno M, Gomez-Contreras F, Navarro P, Marin C, Ramirez-Macias I, Olmo F, Sanz AM, Campayo L, Cano C, Yunta MJ: *In vitro* leishmanicidal activity of imidazole- or pyrazole-based benzo[g]phthalazine derivatives against *Leishmania infantum* and *Leishmania braziliensis* species. *J Antimicrob Chemother* 2012, 67(2):387-397.

900. Schagger H: Respiratory chain supercomplexes. *IUBMB Life* 2001, 52(3-5):119-128.

901. de Graaf AO, Meijerink JP, van den Heuvel LP, DeAbreu RA, de Witte T, Jansen JH, Smeitink JA: Bcl-2 protects against apoptosis induced by antimycin A and bongkrekic acid without restoring cellular ATP levels. *Biochim Biophys Acta* 2002, 1554(1-2):57-65.

902. Kitamura Y, Inden M, Miyamura A, Kakimura J, Taniguchi T, Shimohama S: Possible involvement of both mitochondria- and endoplasmic reticulum-dependent caspase pathways in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Neurosci Lett* 2002, 333(1):25-28.

903. Vaidya AB: Mitochondrial and plastid functions as antimalarial drug targets. *Curr Drug Targets Infect Disord* 2004, 4(1):11-23.

904. Sen N, Das BB, Ganguly A, Banerjee B, Sen T, Majumder HK: *Leishmania donovani*: intracellular ATP level regulates apoptosis-like death in luteolin induced dyskinetoplastid cells. *Exp Parasitol* 2006, 114(3):204-214.

905. Luque-Ortega JR, Reuther P, Rivas L, Dardonville C: New benzophenone-derived bisphosphonium salts as leishmanicidal leads targeting mitochondria through inhibition of respiratory complex II. *J Med Chem* 2010, 53(4):1788-1798.

906. Sudhandiran G, Shaha C: Antimonial-induced increase in intracellular Ca2+ through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. *J Biol Chem* 2003, 278(27):25120-25132.

907. Mehta A, Shaha C: Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. *J Biol Chem* 2004, 279(12):11798-11813.

908. Chinopoulos C, Tretter L, Adam-Vizi V: Reversible depolarization of in situ mitochondria by oxidative stress parallels a decrease in NAD(P)H level in nerve terminals. *Neurochem Int* 2000, 36(6):483-488.

909. Sen N, Majumder HK: Mitochondrion of protozoan parasite emerges as potent therapeutic target: exciting drugs are on the horizon. *Curr Pharm Des* 2008, 14(9):839-846.

910. Bernardi P, Krauskopf A, Basso E, Petronilli V, Blachly-Dyson E, Di Lisa F, Forte MA: The mitochondrial permeability transition from *in vitro* artifact to disease target. *FEBS J* 2006, 273(10):2077-2099.