Metadados:

Título: GENES ASSOCIADOS A RESISTÊNCIA A ANTIPARASITÀRIOS NA LEISHMANIOSE CUTÂNEA HUMANA

Resumo:

Introdução: A leishmaniose é uma zoonose causada pelo protozoário do gênero Leishmania spp., e apresenta diferentes manifestações clinicas, podendo ser leishmaniose cutânea (LC), mucocutânea (LMC) e visceral (LV). O desenvolvimento de cepas resistentes aos medicamentos utilizados no tratamento como o glucantime, está se tornando uma realidade cada vez mais presente. Fatores genéticos do parasita são ditos como importantes mecanismos de evasão a drogas, dentre eles umas das principais são as mutações no genoma e/ou a alteração da expressão gênica. Objetivo: Com isso, o objetivo deste trabalho foi identificar genes da Leishmania spp. causador da LC humana que possam estar associados a resistência a drogas antiparasitárias. Material e métodos: Para isso, foi realizada uma revisão sistemática utilizando o protocolo do PRISMA a fim de identificar os principais genes do parasita descritos na literatura associados ao desenvolvimento de resistência terapêutica. A partir dos resultados da revisão sistemática, foram identificados e selecionados para avaliação, em amostras de biópsia de pacientes infectados os genes AQP1, TRYR, MRPA, GAMA-GCS para avaliar a expressão gênica, AQP1 e HSP70 para avaliar mutações. Para a obtenção do material genético, foi realizada a extração de RNA e DNA de biopsias de pacientes diagnosticados clinicamente com leishmaniose cutânea separando em dois grupos, pacientes com um ciclo de tratamento com glucantime e pacientes com 2 ou mais ciclos. Para a avaliação da expressão gênica foi realizado RT-qPCR e para avaliação de mutações no genoma foi realizado o sequenciamento Sanger. Resultados: Foram obtidos 22 artigos, tendo como os principais genes associado a resistência: AQP1, MRPA, TRYR, GAMA-GCS, HSP70, ODC, TDR-1 e ARM58. Na avaliação experimental da expressão gênica, não obtivemos diferença estatística entre os grupos e não identificamos mutações descritas na revisão nas amostras analisadas. Conclusão: Na revisão realizada demonstrou que há alteração da expressão AQP1, MRPA, TRY, GAMA-GCS, ODC, TDR-1 e ARM58 em cepas resistentes, e que algumas mutações nos genes HSP70 e AQP1 podem levar ao desenvolvimento de cepas resistentes. Porém, os genes avaliados experimentalmente por este estudo não identificaram diferença entre os grupos. Mais estudos são necessários para a identificação de possíveis marcadores genéticos de resistência a medicamentos.

Abstract:

Introduction: Leishmaniasis is a zoonosis caused by the protozoan of the genus Leishmania spp., and it has different clinical manifestations, which can be cutaneous (LC), mucocutaneous (CML) and visceral (VL) leishmaniasis. The development of strains resistant to drugs used in treatment, such as glucantime, is becoming an increasingly present reality. Genetic factors of the parasite are said to be important mechanisms of drug evasion, among them one of the main ones are mutations in the genome and/or alteration of gene expression. Objective: With that, the objective of this work was to identify genes of Leishmania spp. cause of human CL that may be associated with resistance to antiparasitic drugs. Material and methods: For this, a systematic review was carried out using the PRISMA protocol in order to identify the main genes of the parasite described in the literature associated with the development of therapeutic resistance. Based on the results of the systematic review, AQP1, TRYR, MRPA, GAMA-GCS genes were identified and selected for evaluation in biopsy samples from infected patients to assess gene

expression, and AQP1 and HSP70 to assess mutations. To obtain the genetic material, RNA and DNA were extracted from biopsies of patients clinically diagnosed with cutaneous leishmaniasis, separating into two groups, patients with one cycle of treatment with glucantime and patients with 2 or more cycles. For the evaluation of gene expression, RT-qPCR was performed and for the evaluation of mutations in the genome, Sanger sequencing was performed. Results: 22 articles were obtained, with the main genes associated with resistance: AQP1, MRPA, TRYR, GAMA-GCS, HSP70, ODC, TDR-1 and ARM58. In the experimental evaluation of gene expression, we did not obtain statistical difference between the groups and we did not identify mutations described in the review in the analyzed samples. Conclusion: The review carried out showed that there is an alteration in the expression of AQP1, MRPA, TRY, GAMA-GCS, ODC, TDR-1 and ARM58 in resistant strains, and that some mutations in the HSP70 and AQP1 genes can lead to the development of resistant strains. However, the genes evaluated experimentally by this study did not identify differences between the groups. More studies are needed to identify possible genetic markers of drug resistance.

Palavras chave:

Leishmaniose Cutânea; resistência á drogas; Leishmania braziliensis; marcadores de resistência

Cutaneous Leishmaniasis; drug resistance; Leishmania braziliensis; resistance markers

Principais resultados:

Tabela 4: Descrição dos grupos de pacientes inclusos no estudo

Comentado [ls1]: TROQUE A ORDEM DAS TABELAS. PRIMEIRO A5 DEPOIS A 4

Ciclos de tratamento	Total de participantes	Média de idade	*p*	Tamanho da lesão	*p*	Quantidade de lesões	*p*	Duração	*p*	IDRM	*p*
1	7	22,2	0,3	2,5	0,9	2	0,4	2,7	0,8	13,6	0,4
2 ≥	9	36,2		2,8		1,7		5,8		16,6	
Total	**16**	**29,2**		**2,6**		**1,8**		**4,3**		**15,1**	

Tabela 5: Estratificação dos pacientes inclusos no estudo

Código de identificação	Sexo	Idade	Nº de ciclos	Status da lesão	Quantidade de lesões	Tamanho médio da lesão	Duração da lesão	Localização da lesão
2164	F	24	1	Cura Clínica	1	4,5	1	Joelho
2234	M	6	1	Cura Clínica	1	1	3	Braço
2166	M	20	1	Cura Clínica	1	2,5	5	Canela
2201	F	89	2	Cura Clínica	2	5	4	Cabeça
2005	M	15	2	Cura Clínica	1	1	Un	Cotovelo
2032	M	23	2	Cura Clínica	1	5	1,5	Canela
2168	F	42	3	Cura Clínica	2	3,2	5	Braço/antebraço
2139	F	39	3	Cura Clínica	2	2	3	Cabeça/pé
2009	F	24	4	Cura Clínica	1	1	4	Coxa/canela/antebraço
2189	M	60	2	Cura Clínica	1	0,5	20	Canela
2218	M	35	2	Cicatrização total	2	1,5	2	Antebraço/cotovelo
2069	M	26	1	Cicatrização total	1	3	4	Coxa

2072	M	42	1	Cicatrização parcial	1	2,5	2	Tronco
2078	F	6	1	Cicatrização parcial	1	2,5	3	Cabeça
2089	M	32	2	Cura Clínica	1	5	3	Canela
2135	F	35	1	Cicatrização parcial	7	2	2	Canela

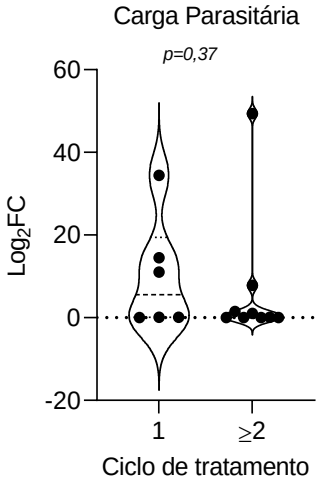


Figura 7: **Avaliação da carga parasitária dentre os participantes**. Nível de expressão gênica (Log_2FC) da proteína *rS8* para a determinação da carga parasitária entre os paciente com um ciclo de tratamento (1) e dois ou mais ciclos (≥) de tratamento utilizando o método de RT-qPCR. Foi aplicado teste T para análise da expressão gênica entre os grupos, tendo como *p* valor de 0,37.

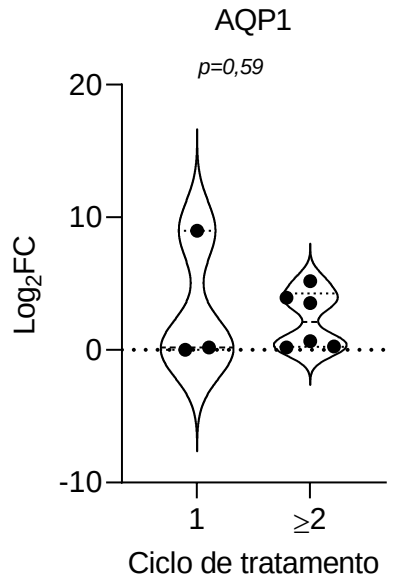


Figura 8: **Avaliação da expressão gênica de *aqp1* dentre os participantes**. Nível de expressão gênica (Log_2FC) da proteína AQP1 entre os paciente com um ciclo de tratamento (1) e dois ou mais ciclos (≥) de tratamento utilizando o método de RT-qPCR. Foi aplicado teste T para análise da expressão gênica entre os grupos, tendo como p valor de 0,59.

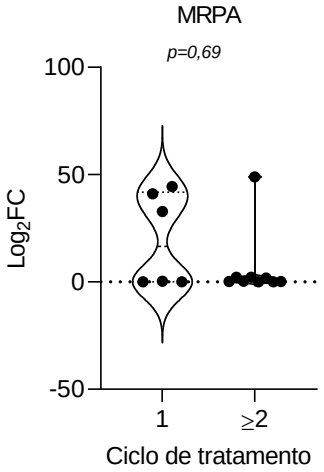
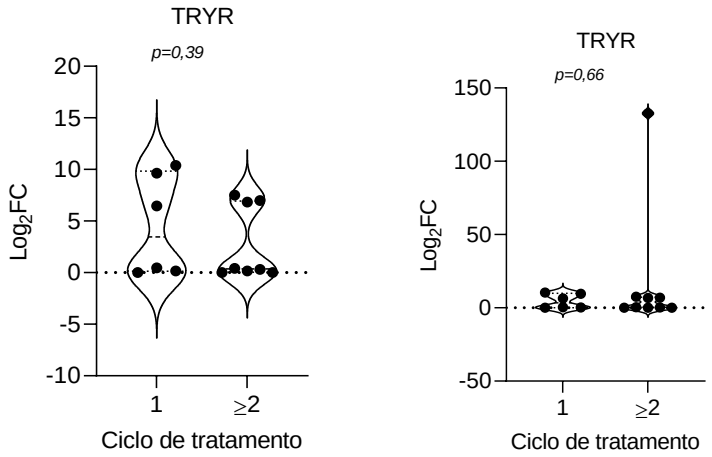


Figura 9: **Avaliação da expressão gênica de *mrpa* dentre os participantes**. Nível de expressão gênica (Log_2FC) da proteína MRPA entre os paciente com um ciclo de tratamento (1) e dois ou mais ciclos (≥) de tratamento utilizando o método de RT-qPCR. Foi aplicado teste T para análise da expressão gênica entre os grupos, tendo como p valor de 0,69



a **b**

Figura 10: **Avaliação da expressão gênica de *tryr* dentre os participantes**. Nível de expressão gênica (Log_2FC) da proteína TRYR entre os paciente com um ciclo de tratamento (1) e dois ou mais ciclos (≥) de tratamento utilizando o método de RT-qPCR. Análise realizada com amostra dita como *outlier* (a) e sem o *outlier* (b). Foi aplicado teste T para análise da expressão gênica entre os grupos, tendo como p valor de 0,66 (a) e 0,39 (b).

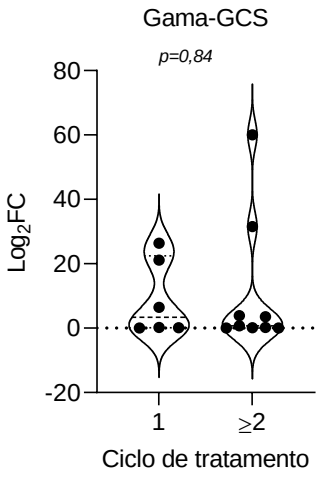


Figura 11: **Avaliação da expressão gênica de *gama-gcs* dentre os participantes**. Nível de expressão gênica (Log_2FC) da proteína Gama-GCS entre os paciente com um ciclo de tratamento (1) e dois ou mais ciclos (≥) de tratamento utilizando o método de RT-qPCR. Foi aplicado teste T para análise da expressão gênica entre os grupos, tendo como p valor de 0,84.

Tabela 4: Descrição dos pacientes inclusos na avaliação de mutações

Comentado [ls2]: TABELA 6?

Ciclos de tratamento	Total de participantes		Média de idade	Tamanho da lesão	Quantidade de lesões	Duração	IDRM
1	2		20	2,2	2,5	2,5	27
2 ≥	5		41,2	2,64	1,6	3,2	19,3
Total	7	**Média**	30,6	2,42	2,05	2,85	23,15

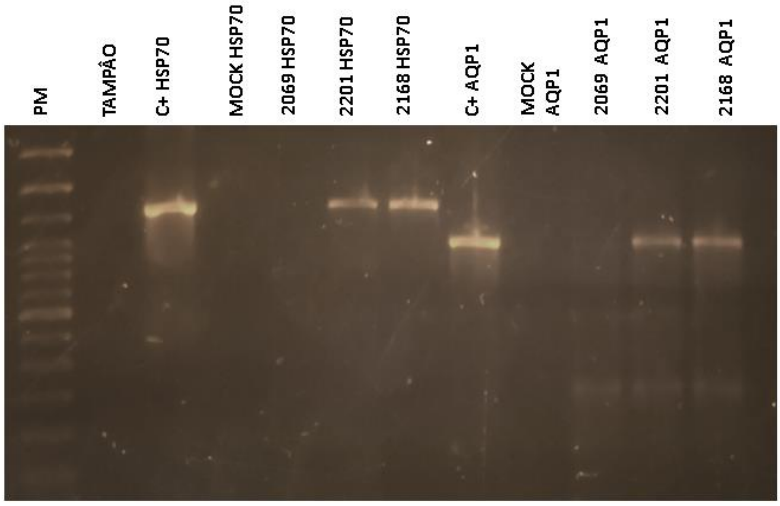


Figura 12: Gel de agarose a 1,5% apara a verificação da amplificação de DNA das amostras inclusas no estudo

Comentado [ls3]: LEGENDA DA FIGURA VAI EM BAIXO DA FIGURA

Artigo de revisão em professo de submissão novamente:

Genetic Factors of *Liehsmania spp.* Associated with Cutaneous Leishmaniasis Drug Resistance: A Systematic Review

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Abstract

Leishmaniasis is a zoonotic neglected disease caused by parasites of the genus *Leishmania (L) spp*. Some drugs can be used to treat cutaneous leishmaniasis (CL), such as the antimoniate of Meglumine, amphotericin B, miltefosine, and pentamidine. These drugs are chemotherapeutic, with strong adverse and toxic effects, and is usually intravenously administered, which makes patient treatment follow-up very difficult. Factors as gene expression and genome mutation of *Leishmania spp* seems to presents and important role in drug resistance development. This systematic review was carried out to summarize the literature findings that address the evaluation of parasite genetic markers that may be associated with LC drug resistance. The systematic review followed the PRISMA protocol collecting articles from PubMed, SciELO and LILACS databases. A total of 22 articles were selected, more of them from Iran (13). Twenty studies used antimony pentavalent as treatment and 17 evaluated gene expression, and 11 evaluated mutation. When evaluating gene expression, ABC transporters proteins family, AQP1, MRPA, TDR1 e TRYR were the most identified genes associated with drug resistance. Among the articles that evaluated mutation, only one identified mutation in HSP70 (T579A), and two identified mutations in AQP1 (A516C and G562A) associated with resistance. The development of further studies evaluating the genetic resistance factors of *Leishmania spp*. is necessary, in order to elucidate the parasite mechanisms and help the patient treatment and the infection control.

Key words: *Leishmania spp*., drug resistance, cutaneous leishmaniasis, genetic factors, systematic review

Key Findings

- AQP1, MRPA, TDR1, TRYR, HSP70, and genes related to ABC transport are possible candidates for drug resistance genetic marker, but cannot be evaluated alone.
- Antimony pentavalent is the most commonly described drug for Leishmanioses treatment.
- More than half of the selected articles evaluated gene expression of possible genes related to drug resistance, ratifying the role of parasite adaptation by gene expression modulation in the circumstances of the environment in which *Leishmania spp*. is located.
- There is a lack of standardization among the articles on the definition of resistance and sensitive of the parasite to drugs, as well as the best sample type to use, which makes difficult to evaluate and compare the information obtained from each study.

Introduction

Leishmaniasis is a zoonotic neglected disease caused by parasites of the genus *Leishmania (L) spp* (World Health Organization, 2010). More than 431 million people live in endemic areas, being susceptible to the infection, and at risk of developing associated diseases (Croft and Engel, 2006; Barkati, Ndao and Libman, 2019). With an incidence of 900,000 to 1.8 million cases worldwide every year (Nühs *et al.*, 2014), it is one of the leading causes of deaths resulting from infectious diseases (Ponte-Sucre *et al.*, 2017). Leishmaniasis affects many areas in the world, mainly developing countries from Central and South America, Southern Europe, Middle East, Indian subcontinent, and North and East Africa (Croft, Sundar and Fairlamb, 2006).

The most common clinical forms are cutaneous leishmaniasis (CL), followed by mucocutaneous (MCL), visceral (VL) and diffuse cutaneous (DCL). CL is acquired from the bite of the female phlebotomine sand flies infected by *Leishmania spp*. The skin lesion will be characterized by erythematous papules, which can become round-shaped ulcers with high edges (Reithinger *et al.*, 2007; World Health Organization, 2010). Some species are associated with the development of CL in the new world, such as *L. braziliensis, L. guyanensis,, L. amazonensis* and *L. paramensis,* and in the old world Leishmanias are *L. tropica* and *L. major* (Barkati, Ndao and Libman, 2019).

Many drugs can be used in CL treatment, such as the antimoniate of Meglumine, amphotericin B, miltefosine, and pentamidine (Croft, Sundar and Fairlamb, 2006). The therapeutic choice depends on age, gestation, presence of comorbidities, and the cost-benefit of the used drug toxicity, due to the side effects that may occur (Brasil. Ministério da Saúde., 2017). They are chemotherapeutic, with strong adverse reaction and toxic effects. The administration is intravenous, making the patient that live in rural areas have long distance journey for the drug

application, which makes the patient treatment follow-up very difficult (Antoniou and Gough, 2005).

The first indication of therapeutic failure was described in India, north of Bihar, in 1997 (Sereno, Harrat and Eddaikra, 2019). Few information is available about drug resistance mechanisms in CL, but factors of the host, parasite, or vector are important for this outcome (Ponte-Sucre *et al.*, 1997). Studies have shown that parasite genetic mutations and gene expression variations can be a very important factors for the therapeutic failure (Barkati, Ndao and Libman, 2019).

Several of genetic mechanisms that could lead to drugs resistance of cutaneous leishmaniasis is the gene copies number variation; aneuploidy; small and specific intrachromosomal regions; the presence of extrachromosomal DNA that could be exchanged between strains, and genome mutations (single nucleotide variation, insertions or deletions) (Papadopoulou *et al.*, 2016; Ponte-Sucre *et al.*, 2017). These adaptations play a key role in drug resistance and the transcriptional control is one of the most important mechanisms for therapeutic failure (Barkati, Ndao and Libman, 2019)**.** The resistance of *Leishmania* to drugs is complex and a few studies have investigated those adaptive mechanisms (Barrera *et al.*, 2017).

Some genes are shown to be potential drug resistance markers, such as Multi-drug resistance protein gene A and all protein in ABC Transporter (MRPA) (Rugani *et al.*, 2019), as well as Gamma Glutamylcysteine synthetase (GSH1) (Grondin *et al.*, 1997), Aquaglyceroporin 1 (AQP1) and Trypanothione reductase (TRYR), among others.

These genes have been described as associated with drug resistance, but there is no marker that defines and predicts the leishmaniases treatment outcome with clinical application. Therefore, it is necessary to identify the possible *Leishmania's.* genes that may be associated with drug resistance, such as genome mutations or gene expression changes. Thus, the aim of this systematic review is to summarize the literature findings regarding the genetic factors that may be associated with resistance to amphotericin B, antimonials, and miltefosine drugs in cutaneous leishmaniasis.

Materials and methods

Search strategy

This article is a systematic review study following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol (Kamioka, 2019), addressing the genetic factors associated with resistance to drugs amphotericin B, antimonial, and miltefosine in cutaneous leishmaniasis.

The database searches were performed on September 28th, 2021, using PubMed (National Center for Biotechnology Information NCBI), SciELO (Scientific Electronic Library Online) and LILACS (Literatura Latino-Americana e do Caribe em Ciências da Saúde) platforms. Keywords were identified using the Medical Subject Headings (MeSH) to construct the following search algorithm: ("cutaneous leishmaniasis" OR "*leishmania braziliensis*" OR "*leishmania amazonensis*" OR "*leishmania Guyanensis*" OR "*Leishmania major*" OR "*leishmania Viannia*") AND ("drug resistance" OR "treatment failure" OR resistance) AND (mutation* OR gene* OR polymorphism OR SNP OR expression OR RNA-seq).

Eligibility criteria

To selected the eligible articles to include in this study, the following inclusion criteria were applied: (i) articles that investigated mutations in the genome and/or changes in the gene expression of *Leishmania* associated with therapeutic resistance; (ii) articles that uses amphotericin B or antimonial and/or miltefosine drugs in the therapy of cutaneous leishmaniasis; (iii) studies that uses naturally resistant strains isolated from patients; (iv) articles in English, Portuguese or Spanish; (v) articles published from 2000 to September 2021.

The following exclusion criteria were also applied in the article selection: (i) review or case report articles; (ii) articles that do not measure, quantify, or identifies the presence of genetic factors of *Leishmania* in cutaneous Leishmaniasis; (iii) that used animals; (iv) that studied visceral or diffuse leishmaniasis; (v) studies that worked with laboratory induced mutation or expression.

Data extraction

Initially, two independent (RLA and TMSS) reviewers read the titles and abstracts, applying the selection criteria, for the first selection. For the second selection, the full articles were read and checked for eligibility. Divergent articles between the two reviewers were analyzed by a third reviewer (LAS).

The following data were then extracted from the selected articles: type of study; title; authors; year of publication; study location; type of sample; *Leishmania* species; used therapy; classification of resistance; the methodology for mutations identification; genome mutation analyzed genes; the method used for gene expression analysis; gene expression analyzed genes; genes associated with resistance; genes associated with susceptibility. The data obtained were summarized and synthesized on a Microsoft Excel 2019 spreadsheet.

Joanna Briggs Institute Critical Appraisal Checklist for Analytical Cross Sectional (Aromataris *et al.*, 2020) and Cohort Studies was used to evaluate the quality of the articles and risk of bias. To evaluate the risk of bias the percentage of answers "yes" on the checklist was calculate for each article. The articles were classified as low risk of bias with 70% or more, medium risk of bias with 50 - 69% and high risk of bias with 50% or less.

This systematic review protocol was approved by the PROSPERO (International prospective register of systematic reviews) with the registration number: CRD42021225134.

Results

A total of 1179 articles were identified by the platforms PubMed (1149), SciELO (7), and LILACS (23) search. Inclusion and exclusion criteria were applied and a total of 22 articles were eligible and selected for this study (Figure 1).

Of the 22 selected articles, eleven (50%) were cross-sectional and eleven (50%) were cohort studies. More than half of the studies obtained samples from Iran (59.1%), while the other studies were from Central and South America (36.4%) or did not inform the place of samples origin (4.5%). The Central and South America countries included are, Brazil, (22.7%) from Peru and Suriname, Colombia (13,6%) and Panama (4,5%) (Table 1). There is no consensus among articles on the classification of cure and treatment failure. Three (13,6%) articles defined treatment resistance as three or more cycles of the drug without cure, three months without cure was defined in six studies (27,3%), EC50 (half maximal effective concentration) in six studies (27,3%), no cure after 1 cycle as resistance in three articles (13,6%), active injury after two cycles

in one study (4,5%), relapse after six months in one study (4,5%) and two studies (9,2%) did not inform.

Regarding the methodology that was used to obtaining the genetic material of *Leishmania spp.,* fifteen (68,2%) articles isolated the parasite from lesions culture (biopsy or aspirated fluid). When the parasite culture growth count enters in logarithmic phase, the genetic material, DNA and/or RNA, were extracted. Two of those articles obtain the genetic material from the lesions aspirated fluid, and six, which use parasite obtain from biopsy, also used drug in the culture to confirm the sensitive and resistance of parasites. Others six (27,3%) articles obtained the genetic material straight from biopsies sample, and one (4,5%) did not report how the samples were obtained (Espada *et al.*, 2019) (Table 1).

The species evaluated were *Leishmania (L.) braziliensis*, *L. tropica* and *L. major* in seven article each, *L. panamensis* in three, and *L. guyanensis* in two studies. Of all the articles, most evaluated *Leishmania* gene expression (77,3%), and five evaluated only genome mutations. Six articles evaluated both, mutation and gene expression. Most authors (90,9%) evaluated the resistance of the antimony pentavalent (Table 1).

Of the eleven studies that evaluated mutations associated with drug resistance in tegumentary leishmaniasis, only one screened the complete genome. The other ten studies evaluated eight different gene regions. Of these, only AQP1 mutation A516C (present in *L. braziliensis*) (Rugani *et al.*, 2019), G562A (present in *L. panamensis*) (Alijani *et al.*, 2019), G700A (present in *L. major*) (Somee, Eslami and Vakili, 2021), and HSP70 T579A (present in *L. braziliensis*) were related to drug resistance to pentavalent antimony (Torres *et al.*, 2013) (Table 2).

Included studies evaluated gene expression related to drug resistance in 27 different genes. It was reported an increase in gene expression associated with resistance in ARM58 (1), γ-GCS (2), TRYR (1), MRPA (3), ABCC3 (3), Ubiquitin (1) and AAP3 (1), and ODC (1) (ornithine decarboxylase), and AQP1 (only 1 article). A reduction in gene expression associated with resistance was observed in AQP1 (8), MAPK (2), and ROS3 (2). Increased expression in ABCA3 (1) and a reduction in gene expression of ABCI4 (1) and gamma GCS (1) genes was observed associated with drug susceptibility (Table 3).

Discussion

The drug resistance and treatment failure in leishmaniasis is a challenge since the number of drugs available is limited and there is a lack of knowledge on the therapeutic resistance mechanisms, including genetic mechanisms (Croft, Sundar and Fairlamb, 2006). In this systematic review, we synthetized some genes that demonstrated to be a promising drugs resistance marker of cutaneous leishmaniasis, or could help predict the disease treatment outcome. Some of them are AQP1, MRPA, TRYR, γ-GCS, GSH1, and HSP70.

Drug resistance can be developed when the parasite are exposed to low concentrations of drugs, or can be associated with the immune evasion strategy used by *Leishmania* (change in enzyme activity or change in influx or efflux of the drug and thiol) (Lima *et al.*, 2007). *Leishmania* genome adaptation is also an important factor for the parasite's resistance to nitric oxide from phagolysosomes and drugs. Some of the adaptations mechanisms are: transcriptional control and regulation of drug target genes and genes involved in oxidative stress response (Barkati, Ndao and Libman, 2019). The presence of mutations may also confer *Leishmania* drug resistance (Barrera *et al.*, 2017) as well as copies number variation, aneuploidy, extrachromosomal DNA

and indels (Papadopoulou *et al.*, 2016). In present study, more than a half of the studies evaluated the expression of possible genes related to resistance, showing that transcriptional control is fundamental for the Kinetoplastids adaptation of the parasite (Fairlamb and Cerami, 1992; Barkati, Ndao and Libman, 2019)

Of all the genes that were identify in this work, aquaglyceroporin (AQP1) was shown to be one of the most studied in Leishmaniasis drug resistance assessment. AQP1 plays a very important role in *Leishmania's* osmotic regulation, since this proteins conducts water, unpaired polar solutes between extra and intracellular media (Plourde *et al.*, 2015), mediating the entry of various metabolites, including the pentavalent antimonial (Bhattacharjee, Rosen and Mukhopadhyay, 2009). With this, the overexpression would lead to hypersensitivity of the parasite (Croft, Sundar and Fairlamb, 2006) as well as mutations in this gene could alter the efflux and influx of drugs (Rugani *et al.*, 2019) (Figure 2).

Furthermore, AQPs can also be found in several organisms such as *Escherichia coli*, *Plasmodium falciparum, Toxoplasma Gondi*, *Trypanosoma spp* (Soveral and Casini, 2017). Munday and collaborators, in 2013, that carried out the introduction of AQP2 gene in resistant strains of *Trypanosoma brucei*, and this led to susceptibility to the drug pentamidine (Munday *et al.*, 2014).

The articles that evaluated mutation in the AQP1 gene demonstrated that a single change can modify the expression or the function of the protein, (Alijani *et al.*, 2019; Rugani *et al.*, 2019; Somee, Eslami and Vakili, 2021), and may interfere in the traffic of solutes (To *et al.*, 2015). Among the genetic variations found were one in *L. braziliensis*, and two in *L. major*, all used

pentavalent antimony as medicine for treatment (Table 2). This shows that *Leishmania spp.* strains are mutating and creating resistance to one of the most used drugs for the treatment of cutaneous leishmaniasis leading to a positive selective pressure for those strains. Other study have also shown a mutation in *L. guyanensis* that configures resistance (Monte-Neto *et al.*, 2015). So, the epidemiological and genomic surveillance is important to identify mutations and resistance strains.

Another important gene is MRPA. Nearly a half of the studies that evaluated MRPA gene expression showed an increase expression associated with drug resistance. This can also be a mechanism of influx or efflux of pentavalent antimony, since antimony can be stored in intracellular vesicles of antimony-thiol complexes, as a kidnapping inactivation (Figure 2) (Restrepo *et al.*, 2019; Sereno, Harrat and Eddaikra, 2019). Many studies also demonstrated that increased MRPA expression may be associated with drug resistance in injuries caused by *Leishmania tropica* (Kazemi-Rad *et al.*, 2013; Oliaee *et al.*, 2018; Mohebali *et al.*, 2019; Rugani *et al.*, 2019). This was also seen in other species such as *L. guyanensis*, *L. amazonensis*, and *L. braziliensis* (Moreira *et al.*, 2013), and in another Kinetoplastids like *Trypanosoma brucei* (Shahi, Krauth-Siegel and Clayton, 2002; Alibu *et al.*, 2006).

Other important gene reported by the literature is TDR1, an enzyme that is found in parasites of the genus *Leishmania* and in *Trypanosoma*. This protein is important for control in redox regulation (Fyfe *et al.*, 2012), and is most expressed in the amastigote phase, which could justify that the mammalian phase is the most sensitive to antimonials (Denton, McGregor and Coombs, 2004). The species may be a key factor for this gene evaluation, since the only study that had association with drug resistance evaluated in *L. tropica* (Oliaee *et al.*, 2018). In *Trypanosoma cruzi*, it has been demonstrated that the application of TDR1 protein in mice can modulate the

Comentado [ls4]: Eu to confusa aqui. Toda a sua discussão e os estudos usados nela fala de aumento de expressão de MRPA. Os que tiveram redução da expressão foi em relação a cepa não resistente? Qual o dado sobre isso?
Pq aqui ta meio repetitivo. Vc ta falando de outros estudos que não foram incluídos? Vc ta falando de outras epecies? So p deixar a msg mais clara

Comentado [RL5R4]: Não entendi. Não tem redução do MRPA associada a resistência. 6 estudos avaliaram a expressão genica, 3 encontraram aumento da expressão associada a resistência, os outros não acharam nenhuma alteração. Uma possível redução foi vista no gráfico do meu mestrado. Não há estudos que falem sobre redução com análise direto da amostra

Comentado [ls6]: Teve um estudo em L. braziliensis não? Foi so um estudo? Sua tabela fala diferente

Comentado [RL7R6]: O único estudo que descreveu associação foi só um e em tropica, o resto não viu associação.

immune response against the parasite, but those results was not demonstrated in *L. infantum* (Silva *et al.*, 2012). Considering this, more studies are needed to investigate this gene to ascertain the association of this gene with drug resistance.

The TRYR is another strong candidate as a maker for predicting therapeutic failure. This gene is describe as a low variability and is conserved among species (Torres *et al.*, 2013) and is associated with thiol biosynthesis/redox metabolism (Torres *et al.*, 2010) (Figure 2). In tripanossomatids, the trypanothione has been reported as the target for trivalent arsenic drugs (Romão *et al.*, 2006). This gene is more studied in drug development, since it is parasite specific and would probably not cause damage to the patient (Fairlamb *et al.*, 1992; Matadamas-Martínez *et al.*, 2019; Battista *et al.*, 2020; Tunes *et al.*, 2020). The development of new leishmanicidal drugs and the increasing number of studies in this area is important to highlight the relevance of TRYR as a marker of resistant strains. Because it is a gene exclusively found in the parasite, this may facilitate the application of this gene for drug resistance evaluation, allowing it to use genetic material directly from the lesion, making the appropriate intervention faster.

Among the genes identified in the literature, HSP70 can be the first line of defense against antimony, until others mechanisms are initiated (Brochu, Halmeur and Ouellette, 2004). This may favor the *Leishmania spp*. shape change, allowing the infection of macrophages. Mutations in this gene can affect those mechanisms, making the strain more resistant or more sensitive to the drug (Figure 2). In adults *Schistosoma mansoni* (Miller et al., 2000) and in *Plasmodium falciparum* (Shonhai, 2010), HSP70 is one of the most important proteins for drug action.

A set of proteins that make up the ABC transporter are important for development of glucantime-resistant *Leishmania* strains, like Y-GCS, ODC and GSH1. Y-GCS is an important enzyme for glutathione biosynthesis, and has a major role in cell defense in combating oxidative stress (Grondin *et al.*, 1997; Torres *et al.*, 2010). In the present review, some authors found thet the increasein gene expression would be associated with resistance (MOHEBALI et al., 2019; TORRES et al., 2010), and others found the opposite (Oliaee *et al.*, 2018) even used the same methodology, the same species of *Leishmania* (*L. tropica*), the same region (Iran) and the same drug to treatment (Oliaee *et al.*, 2018; Mohebali *et al.*, 2019). However, in a study using recombinant strains was observed an increase in the expression of Y-GCS (GHOBAKHLOO; MOTAZEDIAN; FARDAEI, 2016).

In *L. guyanensis* transfected with GSH1 and ODC overexpressed, confer resistance to Glucantime (Fonseca *et al.*, 2017). Parasites of the genus *Plasmodium* demonstrated an increase in the activity of enzymes related to GSH1, in resistance strains to chloroquine (Pérez-Rosado *et al.*, 2002). In this review, none of the articles showed association of the GSH1 gene to drug resistance in *Leishmania spp.* and within the studies that evaluated ODC, only one found that increase gene expression may be associated with drug resistance. Coons et al, in 1990, demonstrated that in a culture of *L. donovani* resistances strains of DL-alpha-difluoromethylornithine (DFMO), show an overexpression of ODC compared to susceptible wild strains (Coons *et al.*, 1990).

Comentado [ls8]: Foi em 1 ou 2?

Comentado [RL9R8]: Apenas 1

Therefore, the analysis alone of those genes that makes up the ABC transporter is not indicated as a good marker of drug resistance. Therefore, an analysis that considers all the genes, or a part of them together can contribute to a better prediction of drug resistance as described in Figure 2.

In this work, the most used drug is Antimonial pentavalent (Frézard, Demicheli and Ribeiro, 2009), which is one of the main drugs recommended by the WHO, and one of the most used in Brazil (Brasil. Ministério da Saúde., 2017) and Iran (Reithinger *et al.*, 2007). Little is known about the mechanisms of the action of antimony (Comandolli-Wyrepkowski *et al.*, 2020). Some hypotheses ae described in figure 3. This drug has several adverse effects such as anorexia, fever, vomiting, among others, may become toxic. Moreover, little is known about the their mechanism and action (Frézard, Demicheli and Ribeiro, 2009). There are few possibilities of pharmacological treatment of cutaneous Leishmaniasis (Ameen, 2007), there are Anfotericin B, Pentamidine and miltefosine, but all these options also have adverse effects or high cost (ANTONIOU; GOUGH, 2005).

There are no guidelines on the definition of what is cure and what is drug resistance. Also, there is no standardization for the adequate method of sample evaluation, which can be direct from biopsy or after the *Leishmania spp.* isolation from the biopsy. This can cause a difference in the results and interpretations, since some genes are present in other organisms, making it difficult to compare the data. Considering these, it is important to carry out studies with drug resistance classification guidelines, as well as standardized evaluation methodologies, to obtain a more reliably and applicable information.

Here, the exclusion of studies that used animals, genetic modified strains or induced the overexpression of possible genes related to resistance, made it possible to evaluate the genetic markers in the parasite natural infection and in the lesion. Although the aim of this study was to evaluate a more clinical applicable data, many drug resistance experimental studies were excluded been a possible study limitation. Despite this, evaluation of the genetic conditions at

the lesion site would demonstrate more truthfully what happens in the lesion of cutaneous leishmaniasis, the real conditions of parasite adaptation to drugs and to the mechanisms of the immune response, such as cytotoxic action. In addition, one limitation of the included studies is that most evaluated specific genes, missing a broader picture of transcriptome, as well as the complete genome alterations of the parasite.

Leishmaniasis is a neglected disease, and their prevention is not only based on vector control, but also in understanding the parasite molecular mechanisms and disease. The drug resistance molecular analyses are a growing area with the advance technological achievements. Investment in genome sequencing technology, gene expression evaluation, identification of mutations, aneuploidies, among others, has proven to be important tools to assist in public health. This make it possible to predict clinical outcomes, as well as drug resistant strains evaluation and disease control. Investments in this sector, as well as its application in Leishmaniasis became a fundamental and a necessary tool.

This study is the first work that brought all genes that may be related to natural drug resistance. Cutaneous Leishmaniasis is a public health problem and is becoming more common. Some genes like AQP1, MRPA, TDR1, TRYR, HSP70, and genes that are related to ABC transport, are possible markers to predict drug resistance. The joint evaluation of genome mutation or/and gene expression of multiple genes can contribute to drug resistance prevention. Here, it was presented that the studies developed on this field are very importance, and the technological progress can help to find the solution of this problem.

Author Contribution

R.L.A. performed the search and selection of the articles, data analysis, as well as manuscript writing. T.M.S.S. participated in the articles search and selection and data analysis. R.K. contribute in study design, writing revision and editing. C.A.F. and L.A.S. study design, formal analysis, writing, revision, editing and supervision. All authors have read and approved the final manuscript.

Financial Support

The FAPESB (Fundação de Amparo á Pesquisa do Estado da Bahia) contributed to the R.L.A. scholarship (BOL0603/2019) and CNPq (Conselho Nascional de Desenvolvimento Científico e Tecnológico) T.M.S.S. scholarship (130231/2020-7). This research is not for profit.

Conflicts of Interest

The authors declare there are no conflicts of interest.

Ethical Standards

Not applicable

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Table 1: Characteristics of the included articles (n=22)

Author and year	Type of study	Sample location	Sample type	*Leishmania* Specie	Therapy	Assessment of genetic factors
Torres, 2010	Cross-sectional	Brazil	Parasite obtained from the lesion	*L. braziliensis L. guyanensis*	antimony pentavalent	Expression
Adaui V, 2011	cohort	Peru	Parasite obtained from the lesion	*L. braziliensis*	antimony pentavalent	Expression
Adaui v, Maes, 2011	cohort	Peru/ Brazil/ Surinam	Parasite obtained from the lesion	*L. braziliensis*	antimony pentavalent	Mutation
Alizadeh R, 2011	Cross-sectional	Iran	Parasite obtained from the lesion	*L. tropica L. major*	antimony pentavalent	Mutation
Kazemi-Rad E, 2013 a	cohort	Iran	Parasite obtained from the lesion	*L. tropica*	antimony pentavalent	Expression
Kazemi-Rad E, 2013 b*	cohort	Iran	Parasite obtained from the lesion	*L. tropica*	antimony pentavalent	Expression
Torres DC, 2013	cohort	Brazil	Parasite obtained from the lesion	*L. braziliensis L. guyanensis*	antimony pentavalent	Mutation
Obonaga R, 2014	cohort	Colombia	Parasite obtained from the lesion	*L.panamensis L. braziliensis*	Miltefosine	Expression and mutation
Eslami G, 2016	cohort	iran	Biopsy of injury	*L. major*	antimony pentavalent	Expression
Hajjaran H, 2016	Cross-sectional	Iran	Parasite obtained from the lesion	*L.Tropica*	antimony pentavalent	Expression
Ghobakhloo N, 2016	cohort	Iran	Parasite obtained from the lesion	*L. major*	antimony pentavalent	Expression
Barrera MC, 2017	Cross-sectional	UN	Parasite obtained from the lesion	*L. panamensis*	antimony pentavalent	Expression
Oliaee RT, 2018	cohort	iran	Parasite obtained from the lesion	*L. tropica*	antimony pentavalent	Expression and mutation
Rugani JN, 2019	Cross-sectional	Brazil	Biopsy of injury	*L. braziliensis*	antimony pentavalent	Expression and mutation
Mohebali M, 2019	Cross-sectional	Iran	Parasite obtained from the lesion	*L.tropica*	antimony pentavalent	Expression
Alijani Y, 2019	cohort	Iran	Biopsy of injury	*L. major*	antimony pentavalent	Expression and mutation
Espada CR, 2019	Cross-sectional	Brazil	UM	*L. braziliensis*	miltefosine and amphotericin	Expression and mutation

Ahmadian S, 2019	cohort	Iran	Parasite obtained from the lesion	*L. major*	antimony pentavalent	Expression
Restrepo CM, 2019	Cross-sectional	Panama	Parasite obtained from the lesion	*L. panamensis*	antimony pentavalent	Mutation
Fozongari F, 2020	Cross-sectional	Iran	Biopsy of injury	*L. tropica*	antimony pentavalent	Mutation
Eslami G, 2021	Cross-sectional	Iran	Biopsy of injury	*L. major*	antimony pentavalent	Expression
Somee R, 2021	Cross-sectional	Iran	Biopsy of injury	*L. major*	antimony pentavalent	Expression and mutation

UN: uninformed. *RNAseq.

Table 2: Characteristics of selected studies that evaluated Leishmania genome mutations of genes associated with drug resistance (n=11)

Author and year	*Leishmania* Specie	Therapy	Definition of resistance		Method	Evaluated Genes	Drug-Resistance Associated Mutation
			Sensitive	Resistant			
Alizadeh R, 2011	*L. tropica L. major*	antimony pentavalent	UN	UN	Mutation screening/ RFLP	MDR region	UN
Adaui V, 2011 b	*L. braziliensis*	antimony pentavalent	EC50	EC50	MLMT	Variations**MLMT	No association
Torres DC, 2013	*L. braziliensis* *L. guyanensis*	antimony pentavalent	no cure > 3 cycles	3 cycles without cure	Sanger sequencing	AQP1, hsp70, MRPA and TRYR	hsp70: T579A
Obonaga R, 2014	*L.panamensis* *L. braziliensis*	Miltefosine	EC50 and cure	EC50 and incomplete healing	Sanger sequencing	LBMT	No association
Oliaee RT, 2018	*L. tropica*	antimony pentavalent	1 cycle with cure and no recurrence for 6 months	Active injuries after 2 cycles	Sanger sequencing	AQP1, γ-GCS, MRPA, TDR1 and TR	No association
Rugani JN, 2019	*L. braziliensis*	antimony pentavalent	UN	UN	Sanger sequencing	AQP1	AQP1: A516C
Restrepo CM, 2019	*L. panamensis*	antimony pentavalent	EC50	EC50	illumine sequencing (genoma)	Variations*	No association
Alijani Y, 2019	*L. major*	antimony pentavalent	cure in 3 months	No cure in 3 months	PCR-RFLP	AQP1	AQP1: G562A
Espada CR, 2019	*L. braziliensis*	miltefosine e amphotericin	EC50	EC50	Sanger sequencing	MT e ROS3	No association
Fozongari F, 2020	*L. tropica*	antimony pentavalent	Cure with 1 cycle	No cure with 1 cycle	Sanger sequencing	Variations*	4 mutations could generate changes in the TRYR protein
Somee R, 2021	*L. major*	antimony pentavalent	Cure with 1 cycle	No cure with 1 cycle	Sanger sequencing	AQP1	G700A

PCR-RFLP: Restriction fragment length polymorphism. MDR: Multidrug drug region. MLMT: Multilocus microsatellite typing. AQP1: Aquaglyceroporin 1. MT: Miltefosine Transporter. γ-GCS : γ-glutamylcysteine synthetase. MRPA: Multidrug resistance protein A. TR: Trypanothione reductase. TDR1: Thiol dependentreduxtase. Hsp70: shock proteins 70. UN: uninformed.

NA: No association. *estimation of chromosome somy, detection of genetic amplifications, copy number variations, single nucleotide variations and indels over 3 par of bases. **single nucleotide vatiations and phylogeny. EC50: median 50% effective concentration

Table 3: Characteristics of selected studies that evaluated Leishmania expression of genes associated with drug resistance (n=17)

Author and year	*Leishmania* Species	Therapy	Resistance rating		Rated gene	Resistance associated gene
			Sensitive	Resistant		
Kazemi-Rad, 2013 *	*L. tropica*	antimonial pentavalente	Cure with 3 or less cycles	No cure with 3 or more cycles	Ubiquitina and AAP3	↑Ubiquitina and AAP3
Kazemi-Rad,2013	*L. tropica*	antimony pentavalent	Cure with 3 or less cycles	No cure with 3 or more cycles	AQP1, MAPK, (ABC) MRPA, PGK, PTP	↑MRPA, PTP and PGK. ↓AQP1 e MAPK
Obonaga R,2014	*L.panamensis* *L. braziliensis*	Miltefosina	EC50 and complete healing	EC50 and incomplete healing	MT, ABCC2, ABCC3, ABCA2, ABCA3, ABCG4 e ABCG6	NA
Eslami,2016	*L. major*	antimony pentavalent	Cure in 3 months	No cure in 3 months	AQP1	↓AQP1
Oliaee,2018	*L. tropica*	antimony pentavalent	1 cycle with cure and no recurrence for 6 months	Active injuries after 2 cycles	AQP1, γ-GCS, MRPA, TDR1 and TR	↓ AQP1, γ-GCS and TDR1 ↑ MRPA AQP1 ** γ-GCS **
Mohebali,2019	*L.Tropica*	antimony pentavalent	Cure without recurrence in 6 months	Relapse in 6 months	γ-GCS, ODC, TRYR, AQP1 and MRPA	↑γ-GCS, TRYR and MRPA ↓AQP1
Torres, 2010	*L. braziliensis* *L. guyanensis*	antimony pentavalent	cure in 3 months	No cure in 3 months	AQP1, GSH1, TRYR, MRPA TDR1 E GSH2	↑ γ-GCS *L. guyanensis* ↓*AQP1 L braziliensis*

Adaui, 2011	*L. braziliensis*	antimony pentavalent	EC50	EC50	ACR2, GSH2, MRPA, PABP, PAP14, S8, TDR1, META 1, TRYR, ODC, GSH1, AQP1	↑ODC and TRYR
Ghobakhloo, 2016	*L. major*	antimony pentavalent	cure in 3 months	No cure in 3 months	AQP1, TDR-1, γ-GCS	NA
Hajjaran, 2016	*L.Tropica*	antimony pentavalent	Cure with < 3 cycles	3 or more cycles without cure.	LACK1	↓LACK1
Barrera, 2017	*L. panamensis*	antimony pentavalent	EC50	EC50	abca2, abca3, abcc2, abcc3, abcg4, abcg6, AQP1, sams, sahh, B-tubulin	↑ABCC3 ↓AQP1
Ahmadian S, 2019	*L. major*	antimonial pentavalente	cure in 3 months	No cure in 3 months	J-binding protein 1 and 2	NA
Rugani, 2019	*L. braziliensis*	antimony pentavalent	UN	UN	MRPA, AQP1, GSH1, ABCG2, ABCI4, AMR56, ARM58	↓AQP1 ↑ARM58
Alijani Y, 2019	*L. major*	antimony pentavalent	cure in 3 months	No cure in 3 months	AQP1	↑AQP1
Espada, 2019	*L. braziliensis*	miltefosine e anfotericina B	EC50	EC50	MT and ROS3	↓ROS3
Eslami G	*L. major*	antimony pentavalent	Cure with 1 cycle	No cure with 1 cycle	AQP1	↓AQP1
Somee R	*L. major*	antimony pentavalent	Cure with 1 cycle	No cure with 1 cycle	AQP1 and MAPK1	↓AQP1 and ↓ MAPK1

* RNAseq, ** Negative correlation between the level of expression of AQP1 and γ-GCS and the duration of the lesion in responsive patients (r=-1). MDR: Multidrug drug region. AQP1: Aquaglyceroporin 1. MT: Miltefosine Transporter. γ-GCS: γ-glutamylcysteine synthetase. MRPA: Multidrug resistance protein A. TR: Trypanothione reductase. TDR1: Thiol dependent reduxtase. UN: uninformed. LACK1: Leishmania-activated C kinase gene PGK: phosphoglycerate kinase PTP: tyrosine phosphatase protein AAP3: amino acid permease ACR2: As/Sb Reductase PABP: RNA-binding protein, ODC: ornithine decarboxylase, ROS3: complex Miltefosine Transporter, META1: putative infective insect stage-specific Protein, S8: 40S ribosomal protein S8, PAP14: poly(A) polymerase, putative ABC: ABC-transporter AMR56/58: antimony resistance marker NA: No association, MAPK1: Mitogen-Activated Protein Kinase.

Figure 1: Flowchart of this systematic review study selection

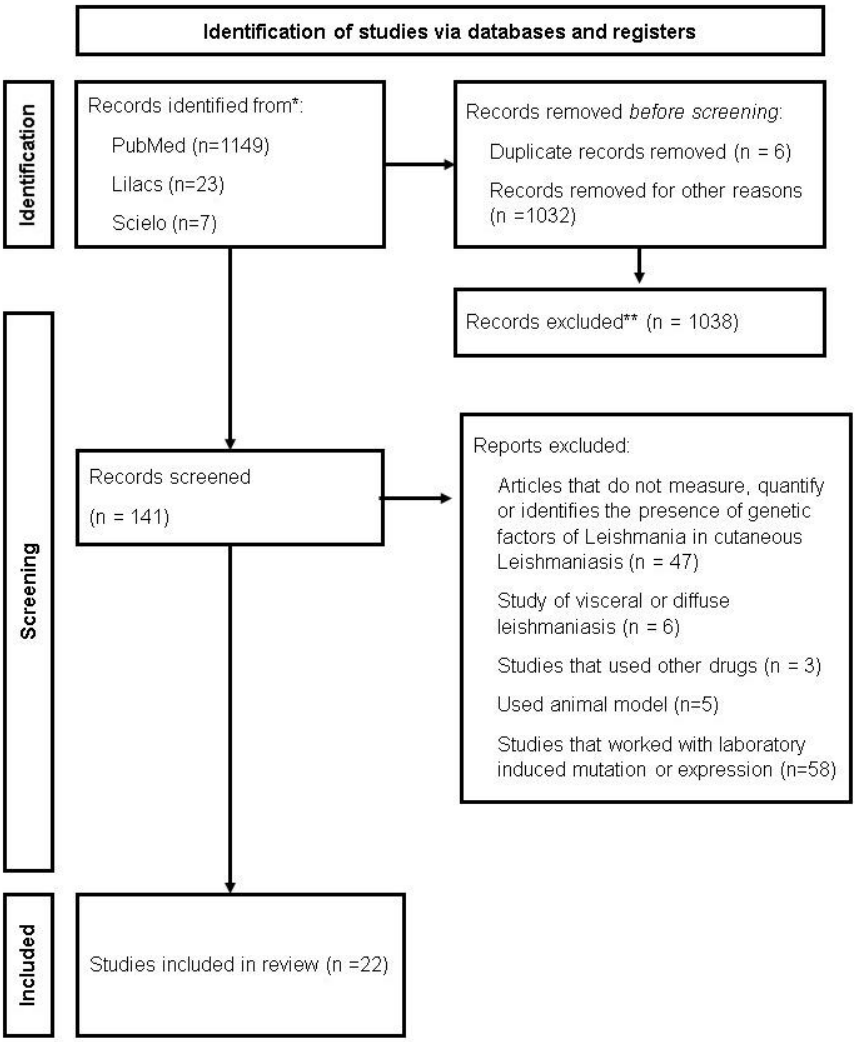
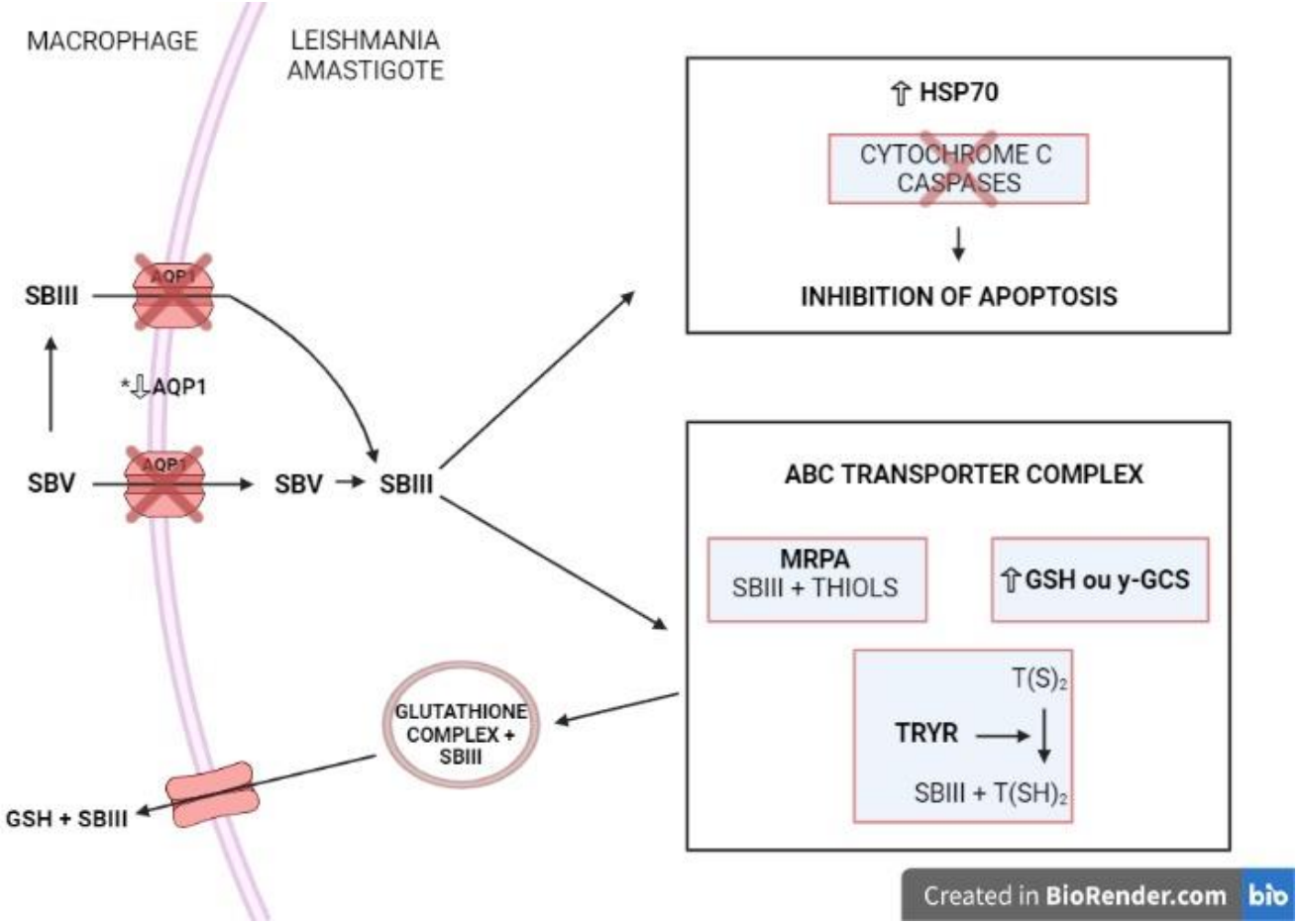
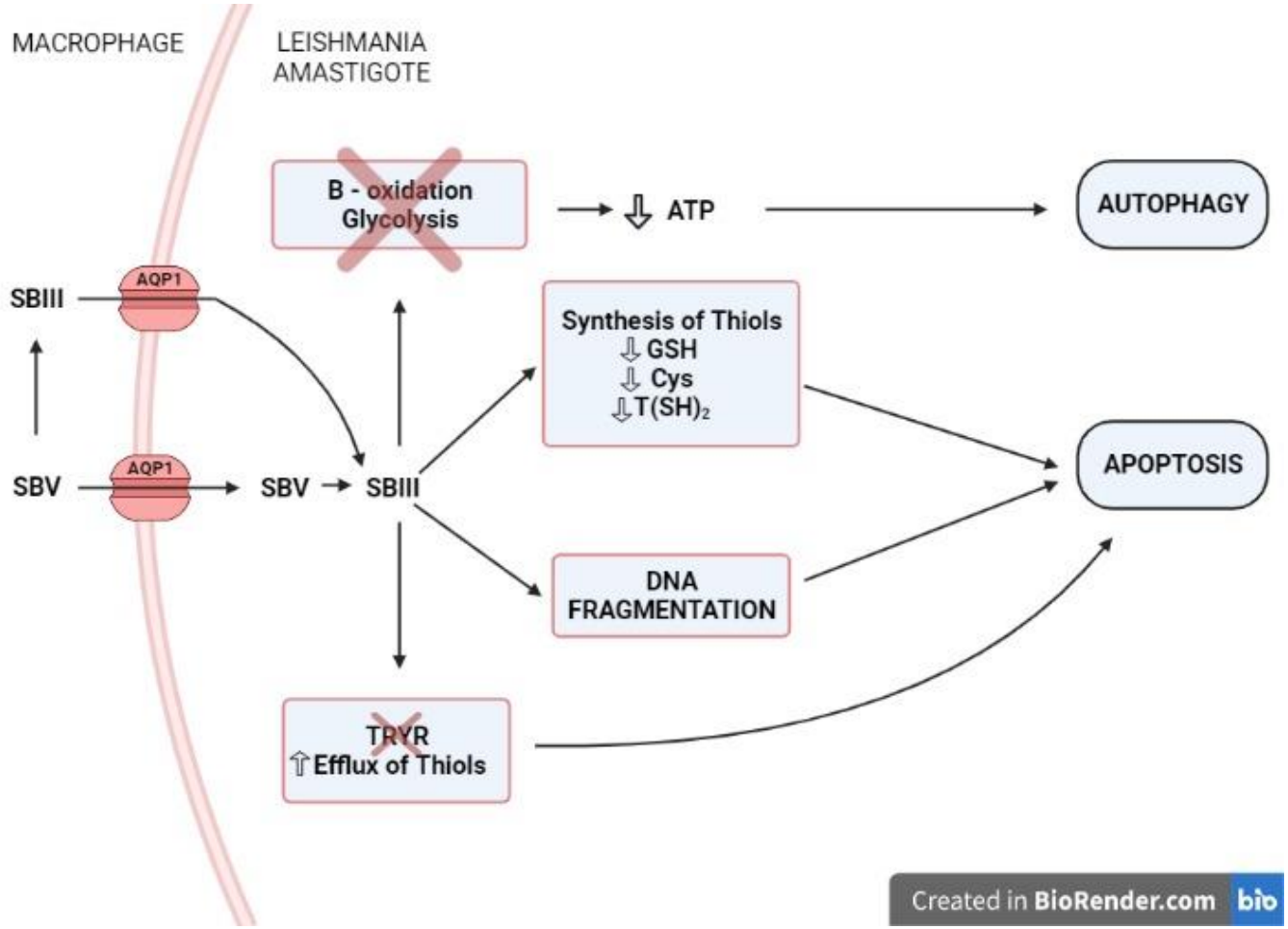


Figure 2: Gene regulatory mechanisms for the development of antimony resistance



SBIII: trivalent antimony; SBV= pentavalent antimonial HSP70= shock proteins 70; MRPA= Multidrug resistance protein A; GSH1= Glutamate cysteine; γ-GCS: γ-glutamylcysteine synthetase; TRYR= Trypanothione reductase. .

Figure 3: Possibles mechanisms of action of the antimony



SBIII: trivalent antimony; SBV= pentavalent antimonial HSP70= shock proteins 70; MRPA= Multidrug resistance protein A; GSH1= Glutamate cysteine; γ-GCS: γ-glutamylcysteine synthetase; TRYR= Trypanothione reductase.