Recently, there has been a renewed interest in the development of new drugs for the treatment of leishmaniasis. This has spurred the need for pharmacodynamic markers to monitor and compare therapies specifically for visceral leishmaniasis, in which the primary recrudescence of parasites is a particularly long-term event that remains difficult to predict. We performed a systematic review of studies evaluating biomarkers in human patients with visceral, cutaneous, and post-kala-azar dermal leishmaniasis, which yielded a total of 170 studies in which 53 potential pharmacodynamic biomarkers were identified. In conclusion, the large majority of these biomarkers constituted universal indirect markers of activation and subsequent waning of cellular immunity and therefore lacked specificity. Macrophage-related markers demonstrate favorable sensitivity and times to normalcy, but more evidence is required to establish a link between these markers and clinical outcome. Most promising are the markers directly related to the parasite burden, but future effort should be focused on optimization of molecular or antigenic targets to increase the sensitivity of these markers. In general, future research should focus on the longitudinal evaluation of the pharmacodynamic biomarkers during treatment, with an emphasis on the correlation of studied biomarkers and clinical parameters.

Significant progress has been made in the past few decades in our understanding of the pathophysiology and immunological mechanisms involved in the fatal parasitic infection visceral leishmaniasis (VL) and its dermal counterpart, cutaneous leishmaniasis (CL). Despite this progress, these scientific efforts have not directly led to new and better treatment options for patients suffering from these neglected tropical diseases. Fortunately, public interest and momentum in drug discovery and development for the leishmaniasis have been renewed, which is substantiated, for instance, by the Drugs for Neglected Diseases initiative (DNDi) in the last decade (1, 2). This renewed interest stipulates the need for more modalities to compare and monitor therapeutic interventions.

Classical clinical features used to evaluate individual treatment responses of patients with VL include the normalization of spleen/liver size, defervescence, and the normalization of blood cell counts (as an indicator of recovering bone marrow). Likewise, for CL, the sizes of the inner and outer borders of cutaneous lesions are used as proxy determinants of parasite biomass, although reepithelialization, crusting, and a multiplicity of skin lesions complicate interpretation. These individual clinical features are, however, rarely used in the quantitative comparison of antileishmanial therapies in the context of a clinical trial. Within such trials, the current standard confirmation of initial cure for VL is a Leishmania-negative spleen or bone marrow aspirate confirmed by microscopy, a very invasive semiquantitative technique which cannot be regularly repeated (3–7). For CL, the confirmation of initial cure is much less clear: most clinical trials have defined “cure” as the absence of all inflammatory signs (skin edema and/or hardening) and complete scarring or reepithelialization of ulcerative lesions at the 3-month follow-up (8–10).

For both VL and CL, confirmation of a final cure as a primary endpoint is even more complicated by the long time periods between initial cure and recrudescence of parasites, requiring long follow-up periods (up to 6 or 12 months) to establish final cure (11). Parasite recrudescence is a rare and slow-developing event which is difficult to predict, mainly because little is known about the causes or risk factors. To compare the efficacies of treatment regimens, sensitive and specific markers that correlate with treatment effect and can predict long-term clinical outcome, by noninvasive sampling methods, are urgently needed.

The general definition of biomarkers, a neologism for “biological markers,” was previously established by the working group on biomarkers of the U.S. National Institutes of Health (NIH) as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention” (12). The use of biomarkers as surrogate endpoints in trials for leishmaniasis may have several possible advantages. First, they can be used for additional (earlier) analyses because primary clinical endpoints are both sparse and available only after a very long period of follow-up. Second, biomarker measurements are faster and less invasive than conventional clinical evaluations. Third, the use of biomarkers may allow the design of smaller, more efficient clinical studies, thereby speeding up the regulatory evaluation and approval of drugs (13). This systematic review focuses on the identification and evaluation of biomarkers to monitor treatment response in cases of VL, CL, and post-kala-azar dermal leishmaniasis (PKDL), with a fo-
cus on the pharmacodynamic potential of these biomarkers to be used in comparative clinical trials. To our knowledge, this is the first systematic review of biomarkers in leishmaniasis.

**METHODS**

**Literature search strategy.** Potential biomarkers for VL, CL, and PKDL were identified by a primary-literature search performed using PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines, querying the PubMed database, restricted to the English language, as follows: “(((Leishmaniasis-[title]) or Kala-azar[title]) or PKDL[title]) and (((((((((biomarker) or biomarkers) or marker) or markers) or level) or levels) or concentration) or activity) or profile).” This electronic search was performed from November 2013 to August 2014, and the date last searched was 19 August 2014. Results were screened manually to identify relevant publications based on title and/or abstract. Publications that did not focus on the identification or evaluation of biomarkers were excluded. Selected publications were then evaluated according to the exclusion criteria as described in Table 1. If the abstract did not clearly indicate whether a study met the initial inclusion criteria, the entire publication was assessed. Secondary literature was subsequently identified using references from the identified primary literature and related publications on PubMed and by specifically querying PubMed using the term of the identified biomarker in combination with “Leishmaniasis” and/or “Kala-azar.”

**Evaluation criteria.** The biological and clinical pharmacodynamic potential of biomarkers was evaluated based on five criteria: (i) time to normalcy, i.e., the time needed for the biomarker level to regress to healthy/control levels; (ii) specificity, in relation to concomitant (infectious) diseases, such as malaria and HIV; (iii) sensitivity, the marker’s quantitation in (treated) patients compared to that in healthy controls and its association with treatment cure or failure; (iv) additional sensitivity, i.e., further assessment of sensitivity by more in-depth association of the marker’s quantitation to standard clinical markers of disease, such as spleen and lesion size; and (v) geographical applicability. Biomarkers were given a score (−/+/++) for each criterion as further explained in Table 2.

**TABLE 1 Exclusion criteria**

<table>
<thead>
<tr>
<th>Exclusion criteria</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method uses <em>ex vivo</em> assays</td>
<td><em>Ex vivo</em> growth of cells is not feasible in practice, and the link with clinical relevance is unclear</td>
</tr>
<tr>
<td>Assay is nonquantitative</td>
<td>Quantitation is necessary for pharmacodynamic applicability</td>
</tr>
<tr>
<td>Sampling methods are invasive (e.g., splenic aspiration, high blood volumes)</td>
<td>Not feasible/cannot be done repetitively</td>
</tr>
<tr>
<td>Genetic markers are associated with drug resistance</td>
<td>Cannot be used to monitor treatment response during treatment</td>
</tr>
<tr>
<td>Genetic markers are associated with susceptibility to leishmaniasis</td>
<td>Not in scope of this article</td>
</tr>
<tr>
<td>No comparison with healthy controls</td>
<td>No information on “healthy levels”</td>
</tr>
<tr>
<td>Other</td>
<td>Not relevant to the topic for various reasons</td>
</tr>
</tbody>
</table>

**TABLE 2 Criteria to evaluate the pharmacodynamics potential of biomarkers**

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Reason for score of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression to normalcy</td>
<td>Occurs in 1 mo after treatment</td>
</tr>
<tr>
<td>Specificity</td>
<td>Occurs within 1 mo after treatment</td>
</tr>
<tr>
<td>Sensitivity (quantitative comparison of marker levels)</td>
<td>Occurs within treatment period</td>
</tr>
<tr>
<td>Markers show no correlation with clinical parameters</td>
<td>Occurs after treatment period</td>
</tr>
<tr>
<td>Additional sensitivity (correlation with clinical markers)</td>
<td>Occurs within 1 mo after treatment</td>
</tr>
<tr>
<td>Geographical applicability</td>
<td>Occurs within 1 mo after treatment</td>
</tr>
</tbody>
</table>

**TABLE 3 Exclusion criteria**

<table>
<thead>
<tr>
<th>Exclusion criteria</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method uses <em>ex vivo</em> assays</td>
<td><em>Ex vivo</em> growth of cells is not feasible in practice, and the link with clinical relevance is unclear</td>
</tr>
<tr>
<td>Assay is nonquantitative</td>
<td>Quantitation is necessary for pharmacodynamic applicability</td>
</tr>
<tr>
<td>Sampling methods are invasive (e.g., splenic aspiration, high blood volumes)</td>
<td>Not feasible/cannot be done repetitively</td>
</tr>
<tr>
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<tr>
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<td>Not in scope of this article</td>
</tr>
<tr>
<td>No comparison with healthy controls</td>
<td>No information on “healthy levels”</td>
</tr>
<tr>
<td>Other</td>
<td>Not relevant to the topic for various reasons</td>
</tr>
</tbody>
</table>
LITERATURE SEARCH

The primary-litterature search identified 1,875 studies for which the titles were screened and assessed for eligibility. One thousand five hundred forty-seven records were found to be nonrelevant and excluded. Thereafter, abstracts and, subsequently, the full text of the remaining studies were assessed for their eligibility; 133 articles were eventually included in this systematic review. Thirty-seven studies were additionally included through a secondary-literature search (Fig. 1).

IDENTIFIED BIOMARKERS

Fifty-three potential biomarkers were identified for VL, CL, and PKDL and are summarized in Table 3. The identified biomarkers were grouped into (a) direct markers of parasite biomass, such as parasite DNA/RNA detection and antigen-based detection, and (b) indirect markers, such as macrophage-related markers, cytokines, receptors, acute-phase proteins, and other biomarkers. Biomarkers are further discussed in this section only if they demonstrate promising potential based on the evaluation criteria (>4+). Antibodies were excluded from Table 3 because of their long elimination half-lives (see “Antibody detection” below).

Direct markers. (i) Parasite detection. Assessing the viable parasite load within a patient is probably the most direct marker of disease status for leishmaniasis, and assessing the reduction of the viable parasite biomass would allow for exact monitoring of the therapeutic response. Several target genes have been identified and used for the molecular identification and quantification of Leishmania in clinical samples, including kinetoplast DNA (kDNA, both mini- and maxicircles), small-subunit (SSU) RNA, such as 18S rRNA, and 7SL RNA. For VL patients, the measurement of the Leishmania parasite load in blood using quantitative PCR (qPCR) has been evaluated mainly for diagnosis but also as a proxy value of the overall parasite load and clinical response during and after treatment (14–26). The parasite load in blood rapidly decreases upon initiation of treatment, in parallel with clinical improvement (14–17). qPCR of blood of East African VL patients reflected differences in treatment responses to different AmBisome dosages (27); however, the sensitivity of the assay was lower than for Indian VL patients (28).

For CL, the parasite burden is localized and confined to the upper layer of the dermis, in which it is probably homogenously spread in the inflammatory zone that surrounds the necrotic ulcer (29). Confirmation of parasites by microscopy or, if available, PCR-based techniques from lesion biopsy specimens or scrapings is currently the diagnostic practice for CL (30–36). Quantitation of parasite RNA in repeated lesion biopsy specimens has been demonstrated as a technique to assess the parasite burden in CL lesions (35, 37). Treatment response was quantified in CL patients, demonstrating declines in Leishmania major parasite loads of ~1 log/week after initiation of miltefosine treatment, which paralleled clinical improvement (29). Swabbing of lesions, which is less invasive than biopsy, was performed to determine whether parasite DNA/RNA loads were diagnostic for CL, and the sensitivity was around 90% (38–40). The pharmacodynamic use of repeated swabbing has not yet been reported. Interestingly, the presence of parasites in CL has also been shown at (unaffected) extraleisional sites (38, 40), opening up other possibilities for less invasive sampling procedures. For PKDL, Leishmania DNA was also detected in lesion material before treatment; a significantly higher parasite burden was found in chronic lesions than in transient lesions, with burdens reduced to nondetectable levels post-treatment (17). The pharmacodynamic use of newer molecular tools (e.g., loop-mediated isothermal amplification [LAMP]) (41, 42) has not yet been investigated.

(ii) Antigen detection. Disease-specific antigen detection is regularly used as a predictive biomarker, e.g., for various cancer types (43), and is potentially useful for infectious diseases as well. For leishmaniasis, however, the application of antigen tests has been limited mainly to diagnostics making use of a urine-based latex agglutination assay (KAtex), which detects a heat-stable low-molecular-weight carbohydrate antigen found in the urine of VL patients (26, 44–46). The method has been successfully evaluated and compared to other methods for diagnosis of VL patients in various geographical areas, ranging from East Africa to South Asia (3, 26, 47–55). Even though specificity was consistently high (98% to 100%) in these studies, sensitivity appeared to be very low to moderate (48% to 95.2%), with a high discrepancy between studies. Studies from India and Sudan indicated that the urine antigen detection test became negative in cured VL patients at least 30 days after the end of treatment (48, 49), indicating a possible pharmacodynamic use of this assay.

Indirect markers. (i) Macrophage-related markers. Leishmania parasites reside and replicate inside the phagocytic cells of the reticuloendothelial system, mainly macrophages, increasing the overall macrophage biomass in the host. Since the macrophage load also decreases again with waning parasitic infection, soluble macrophage-related markers—specifically when produced by infected macrophages—are potential semidirect biomarkers. Neopterin is a heterocyclic pteridine compound which is synthesized by macrophages after gamma interferon (IFN-γ) stimulation (56). It is considered an indicator of activation of cellular immunity. Increased neopterin production is found in a broad range of diseases, e.g., in viral infections (HIV, hepatitis B and C) and infec-

![Study flow diagram](https://example.com/study-diagram.png)
<table>
<thead>
<tr>
<th>Marker category</th>
<th>Biomarker</th>
<th>Detection technique(s)</th>
<th>Matrix(es)</th>
<th>Region(s)</th>
<th>Clinical presentation(s) of leishmaniasis</th>
<th>Biomarker evaluation score</th>
<th>Time until normalcy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Sensitivity (quantitative comparison)</th>
<th>Additional sensitivity</th>
<th>Geographical applicability</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct markers for:</td>
<td>Parasite detection</td>
<td>(q)RT-PCR, NASBA(-OC), OC</td>
<td>Blood</td>
<td>India, France, Netherlands, Nepal, Italy, Sudan</td>
<td>VL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>14–25, 27, 28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parasite detection</td>
<td>qRT-PCR, QT-NASBA</td>
<td>Lesion biopsy specimen</td>
<td>Netherlands, India, Germany, Israel, Brazil</td>
<td>CL/PKDL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>17, 29–37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parasite detection</td>
<td>qRT-PCR</td>
<td>(Extra)lesional swab</td>
<td>Colombia, Ecuador</td>
<td>CL</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>38–40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen detection</td>
<td>Carbohydrate antigen</td>
<td>Latex agglutination (KAtex), ELISA</td>
<td>Urine</td>
<td>Bangladesh, Nepal, Sudan, Brazil, Yemen, Spain, Iran</td>
<td>VL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>26, 44–55</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Indirect markers for:

Macrophage-related markers:

IDO (HPLC) | Plasma | France | VL | – | ? | + | + | + | + | 157 |

ADA (colorimetric, substrate assay) | Serum | India, Nepal | VL | + | + | + | + | + | 59–62 |

MIF (colorimetric, substrate assay) | Serum | Turkey | CL | ? | + | + | + | + | 63 |

Neopterin (RIA) | Serum | Netherlands, Kenya, Brazil | VL | + | + | + | + | + | 57, 58 |

C1q (Radial immunodiffusion) | Serum | Kenya | VL | + | + | + | + | + | 158 |

Cytokines:

CCL3/MIP-1α | ELISA | Serum | Ethiopia | VL | + | + | + | + | + | 159 |

CCL4/MIP-1β | ELISA | Serum | Ethiopia | VL | + | + | + | + | + | 159 |

CXCL10/IP10 | ELISA | Serum | Ethiopia | VL | – | + | + | + | + | 159 |

IL-12 | ELISA | Serum | Ireland | VL | + | + | + | + | + | 159 |

IL-15 | ELISA | Serum | Israel, Brazil, Ethiopia | VL | + | + | + | + | + | 159 |

IL-17A | CBA | Serum | Sudan | VL | + | + | + | + | + | 159 |

IL-22 | CBA | Serum | India | VL | + | + | + | + | + | 159 |

IL-32 | qPCR | Biopsy specimen | Brazil | CL | ? | + | + | + | 159 |

IL-33 | ELISA | Serum | France | VL | ? | + | + | + | + | 159 |

IL-4 | ELISA | Serum | Brazil | VL | ? | + | + | + | + | 159 |

RT-PCR | Biopsy specimen | Tunisia, Mexico, Brazil | CL | ? | + | + | + | + | 159 |

IL-6 | ELISA | Serum | France | VL | + | + | + | + | + | 159 |

IL-8/CXCL8 | CBA | Serum | Bangladesh, Brazil | VL/CL | ? | + | + | + | + | 159 |
<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Test Description</th>
<th>Sample Type</th>
<th>Country/City</th>
<th>Parameter</th>
<th>Country/City</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>CBA/ELISA/multiplex biometric immunoassay</td>
<td>Serum</td>
<td>India/Brazil/Sicily/Ethiopia/Sudan</td>
<td>VL/PKDL</td>
<td>+ + + + + + + +</td>
<td>65–68, 73, 74, 77, 78, 83, 97, 99, 100, 160, 161</td>
</tr>
<tr>
<td></td>
<td>RT-PCR</td>
<td>Serum/biopsy specimen</td>
<td>French Guinea/Mexico/Tunisia/Spain</td>
<td>CL</td>
<td>? ? + – –</td>
<td>79, 89, 162, 164</td>
</tr>
<tr>
<td>sFas/sFasL</td>
<td>ELISA</td>
<td>Serum</td>
<td>India/Brazil/India</td>
<td>VL/PKDL</td>
<td>? – + + + +</td>
<td>70</td>
</tr>
<tr>
<td>TGF-β</td>
<td>RT-PCR/immunohistology</td>
<td>Biopsy specimen</td>
<td>India/Brazil/Mexico</td>
<td>CL/PKDL</td>
<td>? ! + + + + +</td>
<td>160, 162</td>
</tr>
<tr>
<td>sTNF-α</td>
<td>CBA/ELISA/immunoradiometric assay kit</td>
<td>Serum</td>
<td>Brazil/India/Ethiopia/Sudan</td>
<td>VL/PKDL</td>
<td>+ + + + + + –</td>
<td>74, 77, 83–86, 88, 90, 91, 99, 100, 160, 171</td>
</tr>
<tr>
<td>Cell surface molecules and circulating receptors</td>
<td>sHLA-G</td>
<td>ELISA</td>
<td>Serum</td>
<td>French</td>
<td>VL</td>
<td>? – + ++</td>
</tr>
<tr>
<td>β2-microglobulin</td>
<td>ELISA</td>
<td>Serum</td>
<td>Sicly</td>
<td>VL</td>
<td>– ? + + ?</td>
<td>174</td>
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<tr>
<td>sCD14</td>
<td>ELISA</td>
<td>Serum</td>
<td>Brazil</td>
<td>VL</td>
<td>? ? + + ?</td>
<td>72</td>
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<tr>
<td>sCD26</td>
<td>ELISA</td>
<td>Serum</td>
<td>Iran/India</td>
<td>VL/CL</td>
<td>? ? + + +</td>
<td>175–178</td>
</tr>
<tr>
<td>sCD30</td>
<td>ELISA</td>
<td>Serum</td>
<td>Iran</td>
<td>VL/CL</td>
<td>? ? + + + ?</td>
<td>175, 177, 178</td>
</tr>
<tr>
<td>sCD4</td>
<td>ELISA</td>
<td>Serum</td>
<td>Brazil/Sicily</td>
<td>VL</td>
<td>? ? + + +</td>
<td>57, 174</td>
</tr>
<tr>
<td>sCD8</td>
<td>ELISA</td>
<td>Serum</td>
<td>Brazil/Sicily</td>
<td>VL</td>
<td>? ? + + +</td>
<td>57, 174</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>ELISA</td>
<td>Serum</td>
<td>Brazil/Sicily</td>
<td>VL/PKDL</td>
<td>– + + + +</td>
<td>57, 70, 104–106</td>
</tr>
<tr>
<td>sIL-2R</td>
<td>ELISA</td>
<td>Serum</td>
<td>Kenya</td>
<td>VL</td>
<td>? + + ?</td>
<td>106</td>
</tr>
<tr>
<td>sIL-4R</td>
<td>ELISA</td>
<td>Serum</td>
<td>Sudan/Brazil</td>
<td>VL</td>
<td>+ ? + ++</td>
<td>86, 91</td>
</tr>
<tr>
<td></td>
<td>SAA</td>
<td>ELISA</td>
<td>Serum</td>
<td>Kenya</td>
<td>VL</td>
<td>+ + ? + +</td>
</tr>
<tr>
<td>Other proteins</td>
<td>Arginase</td>
<td>Colorimetric assay</td>
<td>PBMG/lesion biopsy specimen/skin biopsy</td>
<td>Brazil/India/Ethiopia</td>
<td>VL/CL/PKDL</td>
<td>+ + ? + +</td>
</tr>
<tr>
<td></td>
<td>Cortisol</td>
<td>Radioimmunoassay</td>
<td>Serum</td>
<td>Brazil/India/Ethiopia</td>
<td>CL</td>
<td>? ? + + +</td>
</tr>
<tr>
<td></td>
<td>CTLA-4 (CD152)</td>
<td>RT-PCR</td>
<td>Lesion biopsy specimen</td>
<td>Ind</td>
<td>PKDL</td>
<td>– ? + + +</td>
</tr>
<tr>
<td></td>
<td>DHEA-S</td>
<td>Automated enzyme immunoassay-based techniques/radi immunoassay</td>
<td>Serum</td>
<td>Brazil/Mexico</td>
<td>CL</td>
<td>? ? + + +</td>
</tr>
<tr>
<td></td>
<td>Foxp3</td>
<td>RT-PCR</td>
<td>Lesion biopsy specimen</td>
<td>India</td>
<td>PKDL</td>
<td>+ ? + + +</td>
</tr>
<tr>
<td></td>
<td>MMP2</td>
<td>RT-PCR</td>
<td>Lesion biopsy specimen</td>
<td>Brazil</td>
<td>CL</td>
<td>? ? + + +</td>
</tr>
<tr>
<td></td>
<td>MMP9</td>
<td>RT-PCR</td>
<td>Lesion biopsy specimen</td>
<td>Brazil/Bangladesh</td>
<td>CL</td>
<td>? ? + + +</td>
</tr>
<tr>
<td></td>
<td>NOs</td>
<td>Luminex assay</td>
<td>Serum</td>
<td>Greece/Turkey</td>
<td>VL</td>
<td>? ? + + ?</td>
</tr>
<tr>
<td></td>
<td>Prostaglandin</td>
<td>Luminex assay</td>
<td>Serum</td>
<td>Greece/Turkey</td>
<td>CL</td>
<td>? ? + + +</td>
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<tr>
<td></td>
<td>Prostatic</td>
<td>Automated enzyme immunoassay-based techniques</td>
<td>Serum</td>
<td>Brazil</td>
<td>CL</td>
<td>? ? + + +</td>
</tr>
<tr>
<td></td>
<td>SOD1</td>
<td>ELISA</td>
<td>Serum</td>
<td>Brazil</td>
<td>CL</td>
<td>+ + + +</td>
</tr>
</tbody>
</table>

IDO, indoleamine 2,3-dioxygenase; ADA, adenosine deaminase; MIF, migration-inhibitory factor; CCL, chemokin (C-C motif) ligand; MIP, macrophage-inflammatory protein; CXCL, chemokin (C-X-C motif) ligand; MIG, monokine induced by gamma interferon; CD40L, CD40 ligand; IL, interleukin; IFN-γ, gamma interferon; sFas, soluble Fas; FasL, Fas ligand; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; sHLA, soluble human leukocyte antigen; sCAM-1, soluble intercellular adhesion molecule-1; sTNFR, soluble tumor necrosis factor receptor; AGP, alpha-1-acid glycoprotein; CRP, C-reactive protein; SAA, serum amyloid A protein; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DHEA-S, dehydroepiandrosterone sulfate; MMP, matrix metalloproteinases; NO, nitric oxide; SOD1, superoxide dismutase 1; RT, reverse transcription; NASBA-4OC, nucleic acid sequence-based amplification (qPCR/quantitative PCR); QT, quantitative; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay; CBA, cytometric bead array.
tions due to intracellular bacteria (tuberculosis, malaria). Serum neopterin concentrations were elevated in VL patients compared to levels in controls and decreased to normal concentrations at the end of treatment in cured patients, whereas they were still significantly increased in refractory patients (57). Serum neopterin concentrations were not found to be elevated in CL patients (58).

Adenosine deaminase (ADA), found particularly in macrophages and lymphocytes, is a key enzyme in the breakdown of adenosine, a purine nucleoside that suppresses the inflammatory responses. For VL, serum ADA activity was increased at diagnosis and returned to almost normal concentrations at the end of therapy (day 30) in Nepalese and Indian patients (59–62). At diagnosis, activity appeared higher in VL patients than in malaria, leprosy, or tuberculosis patients (60). Also, in Turkish CL patients, adenosine deaminase was increased at the time of diagnosis (63).

(ii) Cytokines. Recovery from VL is linked mainly to the CD4+ T-cell-mediated cellular immune response. More specifically, the Th1-mediated response is generally associated with macrophage activation, host resistance, and protection against Leishmania parasites, leading to control of disease. Conversely, the Th2-mediated response is associated with downregulation of macrophage activation and eventually progression of disease. Unfortunately, this distinction between Th1 and Th2 activation is a simplified model, and many patients demonstrate a nonspecific immune response profile.

The most studied cytokines are the proinflammatory cytokines IFN-γ and tumor necrosis factor alpha (TNF-α) and the regulatory cytokine interleukin-10 (IL-10). Plasma IL-10 was found to be increased in Indian patients with active VL and could be detected in the keratinocytes and sweat glands of patients who eventually developed PKDL (64). The increase of IL-10 concentrations in VL patients was later confirmed in a range of countries, including, among others, India, Brazil, and Ethiopia (65–77). IL-10 levels were found to drop significantly after successful treatment (66, 70, 73, 78) to near-control levels 5 to 7 days posttreatment (74). Ansari et al. found no difference in pretreatment IL-10 levels between responsive and unresponsive patients (74). For CL, IL-10 might be a possible pharmacodynamic marker indicating treatment failure, as IL-10 mRNA levels in lesion biopsy specimens were found to be positively associated with unresponsiveness to treatment (79, 80). Cured mucocutaneous leishmaniasis (MCL) patients demonstrated a higher percentage of IL-10-expressing cells pretreatment than relapsing patients (81). Interestingly, IL-10 was found to be positively correlated with the parasite load in the blood of VL patients (17, 70) and lesional tissue of PKDL patients (82).

TNF-α is a cytokine produced mainly by activated macrophages. TNF-α levels were found to be significantly increased in patients with active VL (77, 83–85); they declined during treatment (85–89) and returned to healthy-control levels at the end of treatment (84). Unresponsive patients retained elevated levels of TNF-α (85). In contrast, other studies found minimal levels of this cytokine in Indian VL patients (74, 90, 91). Moreover, TNF-α was also present in asymptomatic VL patients (83), complicating the interpretation of TNF-α in cases of VL. For CL patients, studies of TNF-α serum levels are contradictory; some studies found elevated levels of TNF-α in the plasma of CL patients that decreased posttreatment compared to healthy controls (92–95), but others could confirm this only for MCL patients (85, 96). TNF-α mRNA levels in lesion biopsy specimens were found to be positively correlated with lesion size (80).

IFN-γ is a critical soluble cytokine for innate and adaptive immunity against intracellular infections and is involved in the activation of macrophages. IFN-γ levels were found to be significantly elevated in patients with active VL, which was confirmed in a wide range of countries: India (74, 75), Bangladesh (70), Brazil (70, 72, 73, 77), Ethiopia (67, 68), Sicily (66), and Iran (65). During and after successful treatment, IFN-γ levels were found to drop significantly but remained elevated compared to levels in healthy controls (66, 70, 73, 75, 78). In contrast, Cennini et al. (84) showed that IFN-γ levels returned to healthy-control levels at the end of treatment. Moreover, IFN-γ plasma concentrations appeared to be significantly higher after the end of treatment in patients unresponsive to therapy than in responsive patients treated with sodium stibogluconate (SSG) (74, 75). Discrepant results in asymptomatic VL patients indicated that IFN-γ was elevated in 48% of asymptomatic Brazilian patients but that it was undetectable in the vast majority of asymptomatic Ethiopian patients (67, 83). Additionally, a recent study of Brazilian pediatric VL patients showed that low levels of IFN-γ were associated with signs of severity, such as jaundice or hemorrhage (97). In CL lesion biopsy specimens, no significant difference in IFN-γ levels could be found between patients with favorable and unfavorable lesion evolutions (79).

For PKDL patients, the expression of the mRNA of the three cytokines IL-10, IFN-γ, and TNF-α in lesions was found to be significantly elevated compared to that in control tissues (74, 82). After treatment, these levels were restored to near-control levels (74). Ganguly et al. found that IL-10 and IFN-γ levels were significantly higher in patients with polymorphic PKDL than in patients with macular PKDL (98).

Concerning patients with HIV-VL coinfection, only TNF-α and IFN-γ serum levels were still significantly elevated in HIV patients when they developed VL coinfection, while IL-10 levels tended to decrease (99). Also, compared to Chagas disease, dengue fever, and tuberculosis patients, leishmaniasis patients showed high levels of TNF-α (70). TNF-α and IFN-γ levels increased significantly when malaria patients developed a VL coinfection (100).

The interleukins IL-6 (74, 75, 77, 84, 101) and IL-12 (65, 67, 69, 70) (often measured as the concentration of the subunit IL-12p40) were also found to be significantly increased in the sera of VL patients. In Sudanese and Ethiopian VL patients, IL-6 returned to normal concentrations within the treatment period (84, 101) and seemed indicative of relapse events (101). However, IL-6 was not correlated with spleen/liver size (73). Also IL-12 levels were found to drop significantly within 30 days of treatment (73) and was largely absent in cured and asymptomatic cases (67, 69). In contrast, in Bangladesh and Brazil, IL-12 was shown to be elevated in asymptomatic VL cases (83, 90). Both interleukins also showed pharmacodynamic potential in CL patients. IL-12 was correlated with unfavorable lesion evolution and lesion duration (80, 102). IL-6 mRNA from biopsy specimens was correlated with lesion size, and also IL-6 serum concentrations were found to be elevated in CL patients (80, 94).

IL-18 was also increased in patients with active VL compared to levels in healthy controls (67). Interestingly, a significant decrease in urinary IL-18 levels was detected during treatment (103). Uri-
nary detection of biomarkers would be ideal due to its noninvasive collection method.

Soluble CD40 ligand (sCD40L) (also called sCD154) was significantly decreased in VL patients at diagnosis compared to levels in controls in areas of endemicity (70, 87). During treatment, sCD40L levels increased toward healthy control levels. However, similar CD40L levels were found for Chagas disease and VL patients, which might cause specificity issues (87).

Cell surface molecules and circulating receptors. Levels of circulating soluble cytokine receptors for IL-2 and IL-4 (sIL-2R and sIL-4R, respectively) were elevated in patients with active VL, with higher concentrations of sIL4R than in patients with other local and systemic parasitic infections (57, 104–106). Serum sIL-2R concentrations correlated with *Leishmania* DNA serum levels (70) and significantly decreased during treatment (57, 70) but returned to normal only after several months (105). At the start of treatment, sIL-2R levels were also significantly higher in patients developing PKDL than in patients not developing PKDL (64). Additionally, mRNA levels of the IL-2R α-chain were significantly elevated in lesions of PKDL patients before treatment and returned to control levels posttreatment (82).

Circulating sCD4 and sCD8 were increased at the start of treatment and returned to levels comparable to those in healthy controls within several months after treatment (57, 105). sCD8 was significantly decreased posttreatment in responders to therapy compared to levels in nonresponders, making it a possible suitable pharmacodynamic marker (57).

Serum levels of the soluble receptors for TNF (sTNFRs) were significantly elevated in patients with active VL compared to levels in controls in areas of endemicity and nonendemicity (91). Responding patients showed a steep decrease in sTNFR levels already at day 15 during treatment, in contrast to nonresponders (86, 91).

Acute-phase proteins. Acute-phase proteins widely used as clinical markers of inflammation and infection, which increase during many (non)infective inflammatory diseases and malignancies, also increase during VL. C-reactive protein (CRP), serum amyloid A protein (SAA), and alpha-1-acid glycoprotein (AGP) were increased in Kenyan VL patients upon diagnosis and reached normal levels before or at the end of treatment (SAA and AGP) or at 60 days posttreatment (CRP) (107). Elevation of CRP levels was confirmed for Indian patients with active VL (75). Interestingly, pretreatment CRP levels were lower in patients responding fast to treatment than in slow-responders, with lower splenic parasite counts (107), which was confirmed in a large Indian pediatric VL cohort (108). An increased pretreatment CRP concentration in VL patients was associated with the development of PKDL (109), while CRP levels were not significantly elevated in PKDL patients. However, the specificity of acute-phase proteins in the monitoring of VL treatments is probably low, as they are increased in a myriad of other infectious and noninfectious inflammatory illnesses.

Other markers. Arginase catalyzes the metabolism of l-arginine into l-ornithine and urea. The resulting diminishing bioavailability of l-arginine is regarded as a potent mechanism of immune suppression and impairment of T-cell responses. In patients with active VL and CL, arginase activity in plasma was found to be significantly increased, and levels returned to control levels for VL patients during SSG treatment (110, 111). VL-HIV co-infection patients appeared to have increased arginase activity compared to VL patients, both in plasma and peripheral blood mononuclear cells (PBMCs) (112). In PKDL patients, arginase activity declined after miltefosine treatment but not after SSG treatment (113).

Antibody detection. All of the current first-line diagnostic serological tests for VL are antibody detection tests (114, 115). Two serological tests are currently being used in the field: the direct agglutination test (DAT), based on numbers of killed whole *L. donovani* promastigotes, and the recombinant K39 (rK39)-based immunochromatographic antibody detection test. Other antigen-based assays have been developed for *Leishmania* antibody detection, using (recombinant) proteins rK9, rK26, rK28, *Leishmania infantum* cytosolic tryptarexidox peroxidase (LicTXNPx), rgp63, rLepp12, recombinant open reading frame F (rORFF), BHUP2, rKLO8, rHSP70, guanylate binding protein (GBP), galactosyl-α(1-3)galactose, 9-O-acetylated sialic acids, recombinant peroxi-
doxin, and amastin (116–130). Unfortunately, antibodies against *Leishmania* parasites exhibit a long half-life and stay detectable for several months up to several years after an infection [tested by the DAT and for galactosyl-α(1-3)galactose, LicTXNPx, rK26, rK39, and BHUP2] (49, 120, 121, 131–141), which compromises the diagnosis of a relapse case and also the pharmacodynamic application of these markers. However, it was found that for some antibodies (against the recombinant *Leishmania* antigens rH2A, KMP11, the "Q" protein, and 9-O-acetylated sialic acids), the levels do decrease significantly 30 to 60 days after treatment (129, 142). Furthermore, 1 week posttreatment, only ~40 to 50% of patients gave a positive signal for rLepp12, compared to 100% for rK39 and for direct agglutination (125). Though not very sensitive (44%), *Leishmania*-specific immunoglobulin E (L-IgE) has been suggested to be a specific (98.3%) marker of active VL disease (*L. chagasi*), although it is undetectable at subclinical levels in VL patients, Chagas disease patients, and healthy controls (143–145). Moreover, increased L-IgE concentrations were demonstrated to regress to normal values during the time span of treatment (143, 145, 146). In cases of atypical cutaneous leishmaniasis, IgE levels were not significantly different from those of asymptomatic or healthy controls (147). Anam et al. (144) also hinted at a possible (diagnostic) role for *L. donovani*-specific IgG3 antibody isotype detection. While the IgG3 antibody level decreases significantly posttreatment (148, 149), the pharmacodynamic value of this marker is probably very low, as the time to normalcy for IgGs is longer than 3 months for both CL and VL patients (150–154).

Besides the drawback of the long half-lives of antibodies, antibody detection tests tend to be positive in a significant proportion of noninfected or otherwise asymptomatic individuals living in areas where VL is endemic (135, 155, 156). Due to these crucial limitations in the use of antibodies to monitor therapies, these markers are excluded from Table 3.

General issues pertaining to the pharmacodynamic potential of biomarkers

Our systematic literature review identified 53 biomarkers for VL, CL, and PKDL. Several general issues might limit their pharmacodynamic potential. First, the large majority of biomarkers were evaluated only for their diagnostic use. Leishmaniasis patients were generally compared to healthy controls before the start of their treatment. Only a few VL studies have focused on differentiating active, clinical disease from subclinical or asymptomatic disease, although this might potentially be an interesting approach to demonstrate the Th1/Th2 paradigm. When a biomarker was evaluated for its ability to monitor a treatment effect, it was almost
always done by comparison of pre- and posttreatment concentrations, without repeated longitudinal measurements. Therefore, the pharmacodynamic potential of most biomarkers remains difficult to assess based on the available literature.

Second, most identified biomarkers for leishmaniasis are indirect markers, i.e., universal markers of activation and the subsequent waning of cellular immunity. As a result, specificity may be low compared to that for patients suffering from common concomitant infections. Interestingly, a few biomarkers (TNF-α, CCL3, and CCL4) have been shown to be specific for HIV-VL coinfected patients rather than HIV patients. Other biomarkers (ADA and sIL4R) were elevated in patients with VL, but not malaria, indicating a possible value in malaria-VL coinfection. Despite these exploratory results, the majority of markers have not been tested against the most common VL coinfections, and further research is needed to establish their specificity as biomarkers.

Third, multiple studies focused on correlating biomarker levels to clinical features of CL (e.g., lesion size), while this correlation was generally ignored for VL. In general, more emphasis should be put in future clinical trials on establishing a correlation between the studied biomarkers and clinical parameters.

Fourth, the time needed to regress to normalcy for the biomarkers (characterized by their elimination half-lives) remains a concern. For instance, almost all of the antibody-related markers have a very long elimination half-life of up to several months and stay present in the body long after the actual parasitic infection has been resolved. Their potential for pharmacodynamic monitoring of antileishmanial treatment is therefore probably negligible. Leishmania antigen detection might be more promising in that respect; however, this has been investigated mainly in the context of a diagnosis of VL, with only limited attention paid to repeated quantitative measurement during and after treatment. The less specific indirect markers, for example, AGP and TNF-α, often show preferable time-to-normalcy values.

Lastly, the practical feasibility, in terms of cost, invasiveness, and laboratory requirements, is an additional concern. The preferred sample matrix for a biomarker should be noninvasive (e.g., urine or saliva). All identified biomarkers were measured in blood or biopsy specimens, except for IL-18 and KATex, both of which can be measured in urine. Though this review focused on biomarkers within the context of a clinical trial setting, it is important to note that equipment-free procedures not requiring a cold chain are required for the application of pharmacodynamic biomarkers in routine settings.

SELECTION OF POTENTIAL PHARMACODYNAMIC BIOMARKERS

In this section, we will highlight and critically appraise the application of a selection of potential pharmacodynamic biomarkers, with some recommendations for research priorities.

Direct biomarkers. Recently, the quantitative application of molecular parasite detection methods as a pharmacodynamic measure was demonstrated, both in VL and CL. While this method measures the parasite directly and therefore is theoretically the most promising biomarker, there are some issues. First, the sensitivity of this marker for VL is relatively low (~80%) and seems to vary between geographical regions (27, 28). The parasite loads appear to decrease with clinical cure but are undetectable before clinical cure can be established. The parasites reside within the spleen, bone marrow, and liver, and plasma is therefore only a proxy reservoir of the parasite. Additionally, it remains unknown what the predictive value is of blood parasite loads in relapsing patients and controls in areas of endemicity. Last, it is unsuitable for routine monitoring due to its high costs (considering the ~€30/sample material costs and the required state-of-the-art laboratory equipment and trained technicians, this tool can be used only in a clinical trial setting).

Another direct biomarker with potential is the Leishmania carbohydrate antigen, which forms the basis for the diagnostic KATex test. One of the biggest advantages of this biomarker is that it can be detected in urine. Its specificity is consistently high, but its sensitivity appears variable, which may make it suitable only in controlled settings. The Leishmania-specific antigen can be assessed quantitatively by enzyme-linked immunosorbent assay (ELISA), which makes it easier to adopt at primary health care facilities than the molecular detection methods.

Indirect biomarkers. Of the indirect biomarkers, the most promising are the macrophage-related markers, as these are directly related to parasitic infection of macrophages. ADA activity is increased in patients with VL and CL, returns to normal during treatment, and shows promising results in patients with HIV-VL coinfection. Unfortunately, this marker has no proven geographical applicability, and there are no data on the relation between ADA activity levels and clinical outcome.

Though most cytokines demonstrated a lack of specificity, a range of them showed promising results with regard to the other evaluation criteria. IL-10 correlated with the parasite load at the time of diagnosis, decreased during treatment, and was even associated with the occurrence of PKDL. However, IL-10 was increased as well in subclinical cases, which complicates its interpretation, certainly in the context of parasite recrudescence. Although studies from different regions contradict each other on its sensitivity, TNF-α shows the highest specificity in comparison to other cytokines, indicating that it might be applicable as a biomarker in certain regions of endemicity. Levels of other indirect markers (e.g., sTNFR, IL-6) appeared predictive for clinical outcome but require further evaluation with regard to the other criteria for us to be able to draw conclusions on their potential. A practical advantage of cytokines is that their ELISA kits are relatively low in cost and may be implemented on a large scale, even though a basic laboratory is still required.

CONCLUSIONS AND FUTURE PERSPECTIVE

The biomarkers identified in this systematic review have been evaluated mainly for diagnostic purposes and do not (yet) meet the requirements for monitoring of clinical outcome as surrogate endpoints in clinical trials. Most promising for the application in pharmacodynamic evaluations are the highly specific direct biomarkers (DNA/RNA or antigenic markers), which appear to have a good correlation with clinical outcome. However, future research should specifically focus on the identification of optimal molecular and antigenic targets to increase the sensitivity of these tools. Macrophage-related markers are theoretically the most promising of the indirect markers, as they are directly linked to macrophage (and possibly parasite) load. Though neopterin and ADA have shown high sensitivity and geographical applicability as biomarkers, more evidence is needed to confirm their potential in predicting clinical outcome. Indirect markers, such as IL-10 and TNF-α, have demonstrated high sensitivity and seem to indicate clinical outcome. Nevertheless, given the lack of specificity and the
complexity of the immunological response associated with VL infection, it is unlikely that a single immunological biomarker will be suitable to accurately monitor treatment response. These markers can still be of use in well-controlled trials with sufficient exclusion of concomitant diseases. However, they are not suitable for application in routine clinical care, as in that case, the biomarker should be able to discriminate clinical outcome at the level of an individual patient. Additional efforts are needed to investigate the applicability of combinations of cytokines as biomarker profiles to monitor treatment outcome at the patient level.

In general, future biomarker research should extend its focus to biomarkers’ pharmacodynamic potential by correlating longitudinal quantitative assessments of the marker (i.e., the marker concentration-time profile in response to therapy) to multiple clinical parameters.

The coming of age of new treatment options for leishmaniasis was long and eagerly awaited, but now that this moment approaches, we urgently need better and more accurate tools to evaluate their potential superiority over existing regimens and rationalize their dosing schedule. Evaluation of pharmacodynamic biomarkers is therefore of crucial importance to optimize and accelerate drug development for this neglected tropical disease.

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**REFERENCES**


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