Clinical pharmacology in leishmaniasis: treatment optimization of a neglected disease
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Biomarkers to monitor therapeutic response in leishmaniasis

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Abstract

Since a few years there is a renewed interest in the screening of new chemical entities and the development of new drugs and drug combinations for the treatment of leishmaniasis. This renewed interest propitiates the need for better options to monitor and compare therapies, and that allow for a more accurate comparison of endpoints between interventions. For instance, establishing clinical cure takes minimally 6 months for visceral leishmaniasis (VL) and 3 months for cutaneous leishmaniasis (CL), whereas primary recrudescence of parasites is a particularly long-term event which remains difficult to predict. There is thus an urgent need for pharmacodynamic biomarkers for the evaluation and comparison of new interventions for leishmaniasis, which may also play a crucial role in the (early) assessment of cure of patients, the identification of relapses, and possibly also the detection of emerging parasite drug resistance. In this systematic review, we identified 40 biomarkers for the diagnosis and monitoring of treatment response for VL and/or CL, with an emphasis on the (potential) use of these biomarkers in the evaluation of therapeutic interventions. The biomarkers that were identified can generally be grouped into: (1) direct markers of disease (parasite detection and antigen-based detection), (2) semi-direct markers (host-cell related biomarkers, such as macrophage-specific markers), (3) indirect markers (cyto- and chemokines, acute phase proteins and antibody-based detection). The large majority of these biomarkers constituted universal markers of activation and subsequent waning of cellular immunity or acute immunological response. Only very few studies evaluated their ability to assess or monitor treatment effect or differentiate ‘active’ from ‘asymptomatic’ disease. The pharmacodynamic potential of most biomarkers remains therefore difficult to assess. Future research should focus on the discovery and prospective evaluation of new pharmacodynamic biomarkers for leishmaniasis, already in early preclinical animal studies, to further rationalize drug development. To aid this process we here summarized the desired characteristics for a pharmacodynamic biomarker for leishmaniasis, leading to a benchmark for future research. Furthermore we highlight the current tools that may be regarded as the best potential pharmacodynamic biomarkers available for various purposes.
Introduction

Significant progress has been made in understanding 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 available to patients suffering from these neglected tropical diseases. Fortunately, there is renewed public interest and momentum in drug discovery and development for the leishmaniases, which is substantiated in the last decade for instance by the World Health Organization - Special Programme for Research and Training in Tropical Diseases, OneWorld Health and the Drugs for Neglected Diseases initiative (DNDi) [1–3]. This renewed interest stipulates the need for better modalities to compare and monitor therapeutic interventions, and for more accurate and precise endpoints.

Classical clinical features used to evaluate individual treatment response of VL include the reduction of spleen/liver size, defervescence and normalisation 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 re-epithelialization, crustation and a multiplicity of skin lesions complicate interpretation. These clinical features play, however, a very limited role in current comparative clinical 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 semi-quantitative technique which cannot be regularly repeated [4–8]. 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 oedema and/or hardening) and complete scarring or reepithelialisation of ulcerative lesions at 3 months follow-up [9–11]. For both VL and CL, confirmation of 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 to establish final cure. Currently, final cure is generally established at 6 months follow-up but even longer periods (up to 12 months) have been suggested to capture slow parasite recrudescence (Rijal et al., submitted). Parasite recrudescence is a rare event and is difficult to predict. Little is known about the causes or risk factors associated with recrudescence. These may include, amongst others, the causative parasite species, failure to achieve certain pharmacokinetic targets, the patient’s immune status and severity or extensiveness of initial disease. For interpretation of these sparsely occurring long-term events, large sample sizes are required for any controlled clinical trial and even then it remains difficult to compare primary outcome measures between interventions due to e.g. high numbers of patients lost-to-follow-up.

In addition to comparing the efficacy of treatment regimens, sensitive and specific endpoints would also be instrumental in detecting the emergence of drug resistant...
parasites. This is expected in areas with high anthroponotic transmission combined with e.g. increasing numbers of HIV-coinfection [12–16], and (too) liberal use of available therapeutics [17]. Drug resistance or increased insensitivity in vivo in the field is difficult to determine quantitatively, as it may not always be related to in vitro drug insensitivity or vice versa [18,19], and is therefore difficult to detect using the current clinical endpoints, e.g. increased failure rates at 6 months follow-up. Whereas for malaria the extended field trials to detect emerging resistance were introduced almost 50 years ago, for leishmaniasis this is not well established and even neglected. Pharmacodynamic biomarkers that are able to monitor the treatment effect are urgently needed, for the evaluation and comparison of interventions for leishmaniasis and for the early detection of emerging drug resistance.

The general definition of biomarkers, a neologism for ‘biological markers’, was previously established by the working group on biomarkers of the US National Institute 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” [20]. The application of biomarkers is highly valued in the complete drug development process. The use of biomarkers as surrogate endpoints in trials for leishmaniasis may have several possible advantages: (1) they can be used for additional (earlier) analyses because primary clinical endpoints are both sparse and only available after a very long period of follow-up; (2) they may allow the design of smaller, more efficient clinical studies, reducing the number of subjects exposed to a given experimental treatment and thereby speeding up the regulatory evaluation and approval of drugs [21]. And also in the earlier clinical stages of development of therapies or for regulatory purposes, the evaluation of biomarkers is regarded as an important and useful addition to the evaluation of primary clinical endpoints, and may be used as surrogate endpoints of clinical efficacy or toxicity [22]. Lastly, biomarkers also feature already prominently in the early stages of drug development, when confirmation of efficacy directly feeds the decisions about continuation of the development track. [23].

This systematic review focuses on the identification, use, status and application of biomarkers to monitor the treatment response of both VL and CL, with an emphasis on the pharmacodynamic potential of these biomarkers and their use in the evaluation of treatments and interventions.

**Methods**

Potential biomarkers for VL and CL were identified by a primary literature search, querying the PubMed/MEDLINE database with the following terms (both “all fields” as well as “Mesh Terms”): (leishmaniasis) AND ((biomarker) OR (biomarkers)
OR ((biological) AND ((marker) OR (markers))) OR (marker) OR (markers) OR (pharmacodynamics) OR (treatment monitoring)) NOT (resistance). This electronic search was performed in January and February 2012. Results were manually screened and relevant publications were identified. Publications which did not focus on the identification or evaluation, either pre-clinically or clinically, of biomarkers were excluded. Also publications that examined stimulated immunological biomarkers only in incubations of e.g. isolated host cells were excluded, since it was our aim to focus on the availability and detection of (circulating) biomarkers in vivo. Secondary literature was subsequently identified using references from the primary identified literature, related publications on PubMed or by specifically querying PubMed using the term of the identified biomarker in combination with (leishmaniasis). Data from the included studies was extracted individually.

**Results**

Forty potential biomarkers were identified for VL and/or CL and summarized in Table 1. Most identified papers focused on the diagnostic application of possible biomarkers, identifying associations between increased levels and active disease. Only very few papers also evaluated the prognostic, predictive or pharmacodynamic value of the biomarkers, e.g. by establishing abnormal values of the marker at time of diagnosis and following up the effect of the intervention or measuring the time period needed for regression to normal. Moreover, the terms *prognostic* and *predictive* were used interchangeably, whereas none of the biomarkers was actually truly evaluated as either *prognostic* or *predictive*, i.e. predicting the course of disease when a patient remains untreated (for a fatal disease such as VL this would be unethical), or predicting success of a certain treatment in an individual, respectively. We will therefore use the more appropriate term *pharmacodynamic* biomarker here to indicate that a specific biomarker was used to assess or monitor a therapeutic treatment effect. In Table 1 is also indicated the level of evaluation (preclinical or clinical) and some properties which are important for the pharmacodynamics potential (quantitative and the time needed for regression to normal) of the specific biomarker. The identified biomarkers were grouped into (1) **direct markers** of parasite biomass, such as (1.1) parasite detection (focusing on the detection of parasite RNA/DNA) and (1.2) antigen-based detection, (2) **semi-direct markers**, such as host-cell-related biomarkers (focusing on the detection of macrophage-specific markers), and (3) **indirect markers**, such as (3.1) cyto- and chemokines, (3.2) acute phase protein biomarkers, (3.3) antibody-based detection and (3.4) kidney toxicity.
### Table 1. Identified potential biomarkers for visceral and cutaneous leishmaniasis

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Detection techniques(^a)</th>
<th>Matrix</th>
<th>Level of evaluation</th>
<th>Properties</th>
<th>Clinical presentation of leishmaniasis CL/VL/ PKDL(^b)</th>
<th>Time until normal</th>
<th>Comment</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Direct marker</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>Parasites in blood</td>
<td>(q)RT-PCR, NASBA(-OC), OLIQO-C</td>
<td>Blood</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>2</td>
<td>Parasites in lesion biopsy</td>
<td>qRT-PCR</td>
<td>Lesion biopsy</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>EOT-3 months FU</td>
</tr>
<tr>
<td>3</td>
<td>Parasites in skin swab</td>
<td>qRT-PCR</td>
<td>(Extra) lesional swab</td>
<td>√</td>
<td>√</td>
<td>?</td>
<td>√</td>
<td>FU</td>
</tr>
<tr>
<td><strong>Antigen-detection</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>Carbohydrate antigen</td>
<td>Latex agglutination (KATEX), ELISA</td>
<td>Urine</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>–</td>
<td>√</td>
</tr>
<tr>
<td><strong>Semi-direct marker</strong></td>
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<tr>
<td><strong>Host cell related</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>Neopterin</td>
<td>RIA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>EOT</td>
</tr>
<tr>
<td>6</td>
<td>Chitotriosidase</td>
<td>Fluorogenic substrate assay</td>
<td>Serum/plasma</td>
<td>√</td>
<td>√</td>
<td>?</td>
<td>√</td>
<td>EOT</td>
</tr>
<tr>
<td><strong>Indirect marker</strong></td>
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</tr>
<tr>
<td><strong>Cyto- and chemokines</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>IL-2</td>
<td>ELISA, FCBA</td>
<td>Blood/Serum</td>
<td>√</td>
<td>√</td>
<td>–</td>
<td>–</td>
<td>CL/VL</td>
</tr>
<tr>
<td>8</td>
<td>IL-4</td>
<td>ELISA, FCBA</td>
<td>Blood/Serum</td>
<td>√</td>
<td>√</td>
<td>√/X</td>
<td>√</td>
<td>CL/VL</td>
</tr>
<tr>
<td>Biomarker</td>
<td>Detection techniques*</td>
<td>Matrix</td>
<td>Level of evaluation</td>
<td>Properties</td>
<td>Clinical presentation of leishmaniasis</td>
<td>Comment</td>
<td>Refs.</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>9</td>
<td>IL-6</td>
<td>ELISA, FCBA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>EOT</td>
<td>VL</td>
<td>Generally increased with active infection; not correlated with parasite load; decreased during treatment</td>
</tr>
<tr>
<td>10</td>
<td>IL-8</td>
<td>FCBA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>EOT</td>
<td>Only increased in 19% of patients at start Rx</td>
</tr>
<tr>
<td>11</td>
<td>IL-10</td>
<td>ELISA, FCBA</td>
<td>Blood/Serum/ Plasma</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>EOT</td>
<td>Increased in acute infection but also present in 'subclinical' infection; correlated with parasite load; predictive for PKDL and minor increase in PKDL; decreases during treatment</td>
</tr>
<tr>
<td>12</td>
<td>IL-12</td>
<td>ELISA</td>
<td>Blood/Serum</td>
<td>√</td>
<td>√/X</td>
<td>√</td>
<td>NR</td>
<td>Mainly associated with acute infection; decreased in cured and asymptomatic patients, contradicting results from Brazil &amp; Bangladesh</td>
</tr>
<tr>
<td>13</td>
<td>IFN-γ</td>
<td>ELISA, FCBA</td>
<td>Blood/Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>EOT</td>
<td>Increased with active disease; after Rx higher in unresponsive patients; discrepant results in asymptomatic patients</td>
</tr>
<tr>
<td>14</td>
<td>TNF-α</td>
<td>ELISA, FCBA</td>
<td>Blood/Serum</td>
<td>√</td>
<td>√/X</td>
<td>√</td>
<td>VL/PKDL</td>
<td>Higher levels in acute infection but not in all patients detectable; also present in asymptomatic patients; increased in PKDL</td>
</tr>
<tr>
<td>15</td>
<td>sIL-2 receptor</td>
<td>IFAT, ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>~6 months FU</td>
<td>Decreased during therapy in responders and not in refractory patients; returns to normal only after several months after therapy</td>
</tr>
<tr>
<td>16</td>
<td>sIL-4 receptor</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>VL</td>
<td>Increased at start of Rx</td>
</tr>
<tr>
<td>17</td>
<td>sCD4</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>VL</td>
<td>Increased at start of Rx</td>
</tr>
<tr>
<td>18</td>
<td>sCD8</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>EOT-1 month FU</td>
<td>Increased at start of Rx, decreased post-treatment</td>
</tr>
</tbody>
</table>
Table 1. Identified potential biomarkers for visceral and cutaneous leishmaniasis

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Detection techniques</th>
<th>Matrix</th>
<th>Level of evaluation</th>
<th>Properties</th>
<th>Clinical presentation of leishmaniasis</th>
<th>Comment</th>
<th>Refs.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clinical</td>
<td>Pharmacodiagnostics</td>
<td>Quantitative</td>
<td>Time until normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prognostic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>sICAM-1</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td></td>
<td>VL</td>
</tr>
<tr>
<td>20</td>
<td>CXCL9</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>EOT</td>
</tr>
<tr>
<td>21</td>
<td>CXCL10</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>EOT</td>
</tr>
<tr>
<td>22</td>
<td>CCL3</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td></td>
<td>VL</td>
</tr>
<tr>
<td>23</td>
<td>CCL4</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td></td>
<td>VL</td>
</tr>
<tr>
<td>24</td>
<td>Adenosine deaminase activity</td>
<td>Colorimetric, Substrate assay</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>EOT</td>
</tr>
</tbody>
</table>

**Acute phase proteins**

| 25        | C-reactive protein   | ELISA  | Serum               | √         | √                     | √          | √                 | 2 months FU | VL               | Possibly less PD use than other acute phase proteins, but not well investigated | [26,107,108,128] |
| 26        | Serum amyloid A protein | ELISA  | Serum               | √         | √                     | √          | √                 | EOT         | VL               | Increased at diagnosis, corresponding with parasite clearance within Rx-period | [26] |
| 27        | Alpha-1 acid glycoprotein | Radial immunodiffusion | Serum | √         | √                     | √          | √                 | EOT         | VL               | Increased at diagnosis, corresponding with parasite clearance within Rx-period | [26] |

**Antibody-detection**

| 28        | rK9                  | ELISA  | Serum               | √         | √                     |            | VL               | Lower diagnostic specificity and sensitivity than rK39 | [114,115] |
| 29        | rK26                 | ELISA  | Serum               | √         | √                     |            | VL               | Lower diagnostic specificity and sensitivity than rK39 | [110,112,114,115,129] |
| 30        | rK39                 | ELISA, dipstick, IFA, ICT, WB | Serum | √         | √                     |            | VL               | Good diagnostic, but remains positive for several months | [60,61,63,69,110–112,115,129–134] |
| 31        | rLepp12              | ELISA, WB | Serum | √         | √                     | -          | NR               | Similar specificity and sensitivity as rK39, but possibly more useful as a PD | [97,116,117] |
Table 1. Identified potential biomarkers for visceral and cutaneous leishmaniasis

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Detection techniques</th>
<th>Matrix</th>
<th>Clinical&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diagnostic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pharmacodynamic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Quantitative&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time until normal</th>
<th>Clinical presentation of leishmaniasis CL/CL/ PKDL&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Comment</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 rKMPII and rTRYP</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>~</td>
<td>√</td>
<td>1-6 months FU</td>
<td>VL</td>
<td>Insect-derived antigens evaluated in canine VL; predictive for 1 yr disease-free survival</td>
<td>[118]</td>
</tr>
<tr>
<td>33 Non-specific antibodies from whole parasites</td>
<td>Direct agglutination test (DAT)</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>~</td>
<td>VL</td>
<td>Good diagnostic, but remains positive for several months after Rx</td>
<td>[60,63,69,70,109,110,132,134–147]</td>
<td></td>
</tr>
<tr>
<td>34 L-IgE</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>~</td>
<td>VL</td>
<td>Increased in active disease, although possibly not with high specificity</td>
<td>[119,120]</td>
<td></td>
</tr>
<tr>
<td>35 L-IgG1</td>
<td>ELISA, nephelometer</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>VL/CL</td>
<td>Increased in active disease, elimination half-life too long for PD value; decreased in PKDL</td>
<td>[120,125,148–150]</td>
<td></td>
</tr>
<tr>
<td>36 L-IgG2</td>
<td>ELISA, nephelometer</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>VL</td>
<td>Increased in active disease, elimination half-life too long for PD value</td>
<td>[120,125,148,149]</td>
<td></td>
</tr>
<tr>
<td>37 L-IgG3</td>
<td>ELISA, nephelometer</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>VL/CL</td>
<td>Increased in active disease, with highest specificity and sensitivity of all antibody isotypes; elimination half-life too long for PD value; increased in paediatric VL</td>
<td>[120,125,150]</td>
<td></td>
</tr>
<tr>
<td>38 L-IgG4</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>PKDL</td>
<td>Increased in PKDL and paediatric VL</td>
<td>[125]</td>
<td></td>
</tr>
<tr>
<td>39 Amastin-binding IgG</td>
<td>ELISA</td>
<td>Serum</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>VL</td>
<td>Increased in active VL compared to asymptomatic and recovered VL</td>
<td>[151]</td>
<td></td>
</tr>
<tr>
<td>40 FARP1</td>
<td>ELISA, dipstick</td>
<td>Urine</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>VL</td>
<td>Related to kidney damage and observed proteinuria. Not extensively evaluated, pathophysiological mechanism unclear</td>
<td>[96]</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** CL: cutaneous leishmaniasis; CD4: ; ELISA: enzyme-linked immunosorbent assay; EOT: end of treatment; FCBA: flow cytometric bead assay; ICT: immochromatographic; IFA: ; L-IgE: Leishmania-specific immunoglobulin E; L-IgG1/2/3: Leishmania-specific immunoglobulin G1/2/3; LAMP: loop mediated isothermal amplification; NASBA (-OC): nucleic acid sequence based amplification (-oligochromatography); NR: not reported; OLIGO-C: oligochromatography; PD: pharmacodynamic marker; PKDL: post-kala-azar dermal leishmaniasis; qRT-PCR: quantitative (real-time) reverse-transcriptase polymerase chain reaction; RAI: radioimmunoassay; Rx: treatment; VL: visceral leishmaniasis; WB: western blot.

<sup>a</sup> Detection techniques used in the identified publications from the systematic literature search.

<sup>b</sup> These categories indicate whether the biomarkers and or techniques have been actually evaluated, e.g. a checkmark for ‘Prognostic’, indicates that the biomarker has been evaluated positively as a prognostic biomarker. It does not mean that the biomarker has been validated as such. An X indicates that the evaluation was not positive or not possible for other reasons.

<sup>c</sup> Includes veterinary (e.g. canine) evaluations.

<sup>d</sup> A pharmacodynamic biomarker indicates the longitudinal evaluation of this biomarker during the time span of treatment or follow-up after treatment. In the comments section is indicated the prognostic usefulness or value of the biomarker. A tilde (~) indicates that differences between active and cured disease were reported for that marker, but that it was unclear within which timeframe (e.g. when active patients were compared with historic cured controls).
Direct markers

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 therapeutic response. For VL, unfortunately, the main clinical residence sites of parasites are the spleen, liver, bone marrow and, to a lesser extent, distant lymph nodes, which require invasive aspirations to assess the local parasite burden. Confirmation of *Leishmania* parasites in aspirate material from these target organs (spleen or bone marrow aspirates) by microscopy is the classic confirmatory assay for diagnosis of VL [24,25], but these invasive and potentially life-threatening procedures cannot be repeatedly performed during treatment, although this was done in some historic studies [26]. 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. The measurement of the *Leishmania* parasite load in blood using quantitative PCR (qPCR) or NASBA, has been evaluated mainly for diagnosis, but also as a proxy value of the overall parasite load and clinical response during and after treatment [27–40]. The absolute parasite biomass in the blood compartment is relatively low compared to that in the spleen or bone marrow and this may limit its sensitivity, at least in East Africa, but it appears to correlate well with the overall parasite load in the bone marrow at the time of diagnosis (TPC Dorlo & GJ Schoone, in preparation). Moreover, the parasite load rapidly decreases upon initiation of treatment parallel with the clinical improvement [27–30]. For instance, for a *Leishmania infantum* VL infection it was demonstrated (NASBA targeting 18S *Leishmania* rRNA) that parasite levels in the blood decrease log-linearly with ~0.5 log/week after initiation of a 4-week miltefosine regimen, with a complete resolution of parasites from the blood in week 6 after initiation of treatment [29]. Despite a relatively low sensitivity of the qPCR assay on blood of East African VL patients, the response to therapy could also be demonstrated in these patients, reflecting differences in response to different AmBisome dosages (Hailu et al., under review). For VL in India (L. donovani) this technique has shown a higher sensitivity [152], possibly because of a higher parasite load in the blood compared to East African VL patients.

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 [41]. Confirmation of parasites by microscopy or, if available, PCR-based techniques from lesion biopsies or scrapings is currently the diagnostic practice for CL [24,42–46]. Quantitation of parasite DNA/RNA by qPCR in repeated lesion biopsies has been demonstrated as a technique to assess the parasite burden in CL lesions [46,47]. It was used to quantify the treatment response in CL patients, demonstrating decline of *L. major* parasite loads, of ~1 log/
week, after initiation of miltefosine treatment, parallel the clinical improvement [41]. More interestingly, parasite rRNA could be detected in the skin lesion biopsies for several weeks after end of treatment. Less invasively than biopsies, swabs, taken from lesions, and analyzed to determine parasite DNA/RNA were diagnostic for CL with a sensitivity of 88% [48]. The pharmacodynamic use of repeated swabs has not been reported yet. Interestingly, the presence of parasites in CL has also been shown at (unaffected) extralesional sites [48–50], opening up other possibilities for less invasive sampling procedures. For PKDL, Leishmania DNA was also detected in lesion material before treatment, with a significantly higher parasite burden in chronic lesions compared to active lesions [30]. Post-treatment, no parasites could be detected in the lesions [30], indicating a possible pharmacodynamic potential of this technique for PKDL also. New molecular tools intended for easier use and implementation of molecular diagnosis in the field are currently under evaluation. The loop-mediated isothermal amplification (LAMP) assay has been developed for molecular diagnosis of VL and CL at primary health care settings, with two different gene targets [51,52].

The optimal molecular target for the molecular detection of Leishmania parasites still needs to be clarified. To our knowledge, a formal evaluation is still lacking. There may be differences in the in vivo stability and thus circulation time of parasite (k)DNA versus RNA, which may complicate comparisons of different molecular targets and their correlation with the viable parasite load, also in terms of sensitivity of the various assays. Moreover, the differences in absolute abundance and variability in abundance of the various molecular targets per Leishmania parasite appear to be substantial, albeit not very well investigated. For instance, significant variability in copy number has recently been demonstrated for kDNA between species (up to 6x) and even between strains [53], which may specifically complicate comparison of results from different geographical regions or in regions with multiple species and strains. Similarly, comparison of results obtained for different target genes or even for the same target with different primer sets may not be assumed comparable without proper validation.

**Antigen-detection**

Disease-specific antigen detection is regularly used as a predictive biomarker, e.g. for various cancer types [54], and is potentially useful for infectious diseases as well because it might allow for differentiation between active and past infections and should be more specific than antibody-detection tests. For leishmaniasis, however, the application of antigen-tests has mainly been limited to diagnostics making use of a urine-based latex-agglutination assay (called Katex) which detects a heat-stable low-molecular-weight carbohydrate antigen found in the urine of VL patients [55–57]. The test was recently improved to increase specificity [58]. 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 [57,59–71].
Although specificity was consistently high (98%-100%) in these studies, sensitivity appeared very low to moderate (48%-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 end of treatment [64,69], indicating a possible pharmacodynamic use of this assay.

**Semi-direct markers**

**Host-cell related markers**

*Leishmania* parasites reside and replicate inside the phagocytic cells of the reticuloendothelial system, mainly macrophages. The parasitic infection recruits and activates additional macrophages causing a massive increase of the overall macrophage biomass in the host. The macrophage load decreases with waning parasitic infection. Soluble macrophage-specific markers, especially if these are being produced by infected macrophages, are therefore potential semi-direct biomarkers for intracellular parasitic infections. **Neopterin** is a heterocyclic pteridine compound, which is synthesized by macrophages after IFN-γ stimulation [72]. It is considered an indicator of activation of the cellular immunity. Increased neopterin production is found in a broad range of diseases, e.g. in viral infections (HIV, hepatitis B and C), infections due to intracellular bacteria (tuberculosis, *Borrelia* infections), malaria, autoimmune and cardiovascular diseases, but also various malignancies. Serum neopterin concentrations were elevated in VL patients compared to controls and had decreased to normal concentrations at the end of treatment in cured patients, whereas they were still significantly increased in refractory patients [73]. These findings were confirmed in a subsequent study that also included two HIV-coinfected patients [74]. Neopterin concentrations were not elevated in CL patients [74]. **Chitotriosidase** is an enzyme that hydrolyzes chitin and is specifically produced by a subset of activated phagocytes like macrophages and neutrophils [75,76]. It is currently successfully used in the therapeutic monitoring of Gaucher’s disease, a lysosomal storage disorder, employing a fluorogenic substrate assay [77]. Plasma chitotriosidase activity has been demonstrated to be substantially increased in VL patients at the start of treatment [78], although a rigorous evaluation remains lacking. In CL patients (*L. major*) plasma chitotriosidase activity was not increased (TPC Dorlo, P de Vries & JGHM Aerts, personal communication). Chitotriosidase is only increased in a subset of the intracellular infectious diseases, providing specificity: e.g. it was elevated in leprosy [79], but not in tuberculosis.

**Adenosine deaminase** is a key enzyme in the purine metabolism, that has an important physiological role in the proliferation and differentiation of lymphocytes and activation of macrophages. The enzyme is strongly implicated specifically in the cellular immunity, reflected by increased activity in infections associated with the cellular immune response [80]. For VL, serum adenosine deaminase activity
was increased at diagnosis [81–83] and returned to (almost) normal concentrations already at the end of therapy (day 30) in Nepalese and Indian patients [81–83]. At diagnosis, activity appeared even higher in VL patients when compared to malaria, leprosy or tuberculosis patients [83]. Also in Turkish CL patients, adenosine deaminase was increased at the time of diagnosis [84].

**Indirect markers**

**Cyto- and chemokines**

Recovery from VL is mainly linked to the CD4+ T-cell mediated cellular immune response. More specifically, the Th1-mediated response is generally associated with macrophage activation, eventually host resistance and protection against *Leishmania* parasites and finally control of disease. Conversely, the Th-2 mediated response is associated with downregulation of macrophage activation and eventually progression of disease. Typically Th-2 associated soluble serum cytokines (like IL-4, IL-6 or IL-10) are expected to be increased in the acute phase of disease and slowly decrease again in the wake of control of the disease (whether or not treatment-induced). Unfortunately, the distinction between Th-1 and Th-2 activation is not so clear. Many infectious diseases show a a switch from Th-1 to Th-2 during evolution of disease that is highly variable. This causes a highly non-specific immune response profile, variable regression to normal between VL/CL and other infectious diseases or even conflicting mechanisms in patients with multiple infections, which is not uncommon in the areas where VL and CL are endemic.

Only 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 Th-1/Th-2 paradigm. More studies have focused on circulating serum cytokine concentrations in active VL patients and compared this either to healthy controls or post-kala-azar dermal leishmaniasis (PKDL), with diverse results.

**Interleukins**

In general, IL-2 was not associated with active disease, present in asymptomatic patients and not related to parasite load [30,85]. IL-4 was increased in Brazilian patients and was correlated with parasite load and active disease in canine VL [85,86], but this was not confirmed in Indian human VL patients by Verma *et al.* [30]. In CL patients, on the other hand, IL-4 was correlated to parasite load [87]. IL-6 was increased in Indian, Ethiopian and Sudanese VL patients and also in canine VL [88–91], but not in Bangladeshi VL patients [92]. In Sudanese patients, IL-6 returned to normal concentrations within the treatment period, and seemed indicative for relapse events [89]. IL-8 was increased in 19% of active Bangladeshi patients [92].
More consistent data are available for the use of IL-10. Already in 1988, IL-10 was implicated in Indian VL patients and increased concentrations were associated with later development of PKDL: IL-10 concentrations were increased in plasma and could be detected in keratinocytes and sweat glands of patients who eventually developed PKDL [93]. This was later confirmed, when IL-10 was increased in “active” Indian VL patients when compared to healthy controls and PKDL patients [90], and in Brazilian patients [85]. Of various cytokines, only IL-10 was found to be correlated with the blood parasite load in Indian VL patients [30]. Both in India, Bangladesh and Ethiopia, IL-12 was significantly increased in active VL [92,94,95], whereas it was largely absent in cured and asymptomatic cases [95]. On the other hand in Brazil and Bangladesh it was shown to be mainly present in asymptomatic VL cases [85,92]. IL-15 and IL-18 were also increased in active VL, but not as much as other discussed interleukins [95]. Urinary IL-18 has been demonstrated to be related to disease activity, although a lot of overlap appeared between endemic controls and patients [96].

Other cytokines

Interferon-gamma (IFN-γ) is a critical soluble cytokine for innate and adaptive immunity against intracellular infections and involved in the activation of macrophages. It was generally shown to be increased in VL, both in terms of transcription and expression levels in CD4 T-cells, as well as circulating cytokines in the serum [86–88,90,95,97]. IFN-γ concentrations were found to normalize within a week after end of treatment [90]. Moreover, IFN-γ plasma concentrations appeared to be significantly higher after end of treatment in patients unresponsive to therapy compared to responsive patients, who where treated with sodium antimony gluconate [90]. Discrepant results in asymptomatic VL patients indicated that IFN-γ was elevated in 48% of asymptomatic Brazilian patients, while it was undetectable in the vast majority of asymptomatic Ethiopian patients [85,95]. As expected, tumor necrosis factor-alpha (TNF-α), which is mainly produced by activated macrophages, was also found to be increased in active VL [85,88,98]. However, a moderate number of studies also indicated that TNF-α was not increased or could not be detected in all active VL patients [90,92,99], nor in canine VL [91]. Moreover, TNF-α was also present in asymptomatic patients [85]. These combined findings complicate the interpretation of TNF-α concentrations.

Circulating cytokine receptors and cell surface markers

Circulating soluble cytokine receptors for IL-2 (sIL2R) and IL-4 (sIL4R) were investigated in active VL. Both sIL2R and sIL4R were elevated in active VL, with higher concentrations compared to other local and systemic parasitic infections [73,100–102]. Serum sIL2R concentrations significantly decreased during treatment [73], but only returned to normal after several months [102]. Two studies investigated
the serum concentrations of soluble concentrations of intercellular adhesion molecule-1 (sICAM1): Schriefer et al. found an increase of sICAM compared to controls, with higher pre-treatment concentrations in responding patients compared to non-responders to therapy [73]; while Sipsas et al. found a significant pre-treatment increase, comparable to concentrations in patients with AIDS [103]. Circulating soluble CD4 (sCD4) and CD8 (sCD8) were increased at start of treatment, whereas only sCD8 was significantly more decreased at ‘post-treatment’ in responders to therapy compared to non-responders [73], making it a possible suitable pharmacodynamic marker. In CL, corresponding to the cytokine release in VL, the association between expression of IL-4 and IL-10 with progression of disease, in contrast to the association between expression of IL-2 and IFN-γ with healing of disease, was demonstrated preclinically in a murine model of CL (L. major) [104]. Although, this pattern of cytokine release was corroborated by cytokine production from peripheral blood mononuclear cells isolated from healing and nonhealing CL patients [105] and by a correlation of intralesional IL-4 transcription and parasite load [87], no further specific reports on circulating cytokine concentrations in CL patients were identified.

Chemokines

Serum concentrations of the IFN-γ-induced chemoattractants CXCL10 and CXCL9 were increased in Ethiopian VL patients and decreased after therapy, although the difference between normal and increased concentrations was not very major and in general appeared to be quite variable [95]. CCL3 and CCL4 were significantly elevated in VL and HIV/VL patients, compared to both controls and the already increased concentrations in HIV patients [106], although nothing is known about the dynamics of these chemokines in VL.

Acute-phase proteins

Acute-phase proteins are a widely used clinical marker of inflammation and infection, which are increased in many (non-)infective inflammatory diseases and malignancies. The effect of treatment on the C-reactive protein (CRP), serum amyloid A protein (SAA) and alpha 1-acid glycoprotein (AGP) of Kenyan VL patients over time was monitored by Wasunna et al. [26]. All three markers were increased upon diagnosis, and reached normal concentrations already before or at the end of treatment (SAA and AGP) or at 60 days post-treatment (CRP). Interestingly, pre-treatment concentrations were lower in clinically fast responders to therapy, who also had lower splenic parasite counts [26], which was confirmed for CRP in a large set of paediatric Indian VL patients [107]. Also for canine VL, CRP appeared to be predictive for initial treatment response. SAA was not always increased at diagnosis [108]. In general, however, the specificity of acute-phase proteins both for diagnosis of VL and monitoring of VL treatments is probably low as they are increased in a
myriad of other infectious and non-infectious inflammatory illnesses.

**Antibody-detection**

The current first-line diagnostic serological tests for visceral leishmaniasis that are used in the field are all antibody-detection tests. Antibody-detection remains thus most important in the confirmation of clinical diagnosis of VL [25]. The antigens that are being used for these tests consist either of whole killed parasite material or a specific recombinant antigen. Unfortunately, antibodies against *Leishmania* parasites exhibit a long half-life and stay detectable for several months up to several years after an infection [109–111], which compromises the diagnosis of a relapse case and more importantly also any pharmacodynamic use of these antibody-detection assays. Besides the lack of prognostic value, antibody-detection tests tend to be positive in a significant proportion of non-infected or otherwise asymptomatic individuals living in areas where VL is endemic [112,113]. This might hamper their use for e.g. epidemiological screening studies, although seroconversion studies could be informative for incidence estimates. Two serological tests are currently being used in the field, the direct agglutination test (DAT), based on killed whole *L. donovani* promastigotes, and the rK39-based immunochromatographic antibody-detection test, of which the (diagnostic) value and application have been extensively reviewed elsewhere by Chappuis *et al.* [25]. Other antigen-based assays have been developed for *Leishmania* antibody-detection, such as rK9, rK26, rLepp12, rKMPII and rTRYP [114–118], but all of these generally suffer from the same lack of pharmacodynamic potential.

*Leishmania*-specific immunoglobulin E (IgE) has been suggested as a marker of active VL disease when it was shown to be specific for active VL (*L. chagasi*), and undetectable in subclinical infected patients, Chagas disease patients and healthy control [119]. Moreover, increased *Leishmania*-specific IgE concentrations were demonstrated to regress to normal values during the timespan of treatment [119]. This was partly confirmed in a screening of immunoglobulin isotype distribution in Indian VL patients, where IgE was shown to be specific (98.3%), but not very sensitive (44%), when compared to controls consisting of malaria, tuberculosis, leprosy and typhoid patients and healthy individuals [120]. In this study, the rank orders for various *L. donovani*-specific antibodies of VL patients were in terms of sensitivity: IgG = IgG1 = IgG3 = IgG4 > IgG2 > IgM > IgE > IgA; and in terms of specificity: IgM = IgG3 > IgE > IgG4 > IgG2 > IgG > IgG1 > IgA; which hints at a possible (diagnostic) role for *L. donovani*-specific IgG3 antibody isotype detection [120], although nothing is known about the pharmacodynamic value of this marker. Overall, the pharmacodynamic value of any of the IgG antibody isotypes in the evaluation of therapies is probably low, given their relatively long half-life in the human body (~21 days). In that respect IgE may be theoretically more promising as an immunoglobulin with an estimated serological half-life of 2 days [121].
**Kidney damage**

Based on the occasional reporting of proteinuria associated with VL by clinicians in areas of endemicity, various urinary markers of kidney injury are currently being investigated for their association with disease activity. Although results remain formally unreported, the biomarker currently under investigation is FABP1 [96]. Its exact pathophysiological role in VL is currently unknown.
Discussion & future perspectives

Our systematic literature review identified 40 (potential) biomarkers for VL and CL, which we divided into three categories: direct markers (parasite related markers and antigens), semi-direct markers (host-cell-related markers) and indirect markers (cyto- and chemokines, acute phase proteins and antibodies). The large majority of biomarkers were only evaluated for their diagnostic use, for which leishmaniasis patients were generally compared before the start of their treatment to healthy controls. Only a few studies also evaluated their applicability in differentiating active from asymptomatic disease, which may be complicated by the lack of a solid definition of what constitutes ‘asymptomatic’ in both VL and CL. When a biomarker was evaluated for its ability to monitor a treatment effect, it was almost always done by comparison of a pre- versus post-treatment concentration, often at a variable time point during the follow-up period, without a dynamic longitudinal assessment employing repeated measurements during and after the treatment period. Therefore, the pharmacodynamic potential of most biomarkers remains very difficult to assess based on the available literature.

Characteristics of an ideal pharmacodynamic biomarker

In Table 2 we propose a (non-exhaustive) list of ideal and minimally acceptable characteristics of pharmacodynamic biomarkers for VL and CL, related to their purpose of use. The ideal, and more stringent, characteristics would make the biomarker suitable for the monitoring of treatment of a single patient in the clinic, whereas the minimally acceptable characteristics relate to the use of the biomarker in a clinical trial setting to compare treatment arms or to detect emergence of parasite insensitivity in a population. Whereas most of the desired characteristics are similar for these two purposes, some of the minimally acceptable requirements are less strict. For instance, in a clinical trial-context costs and laboratory requirements are often not a limiting factor due to the controlled and well-defined setting in which the trial takes place. Moreover, requirements for specificity and sensitivity are less stringent in the context of comparing groups of individuals. For instance, co-infections which potentially interfere with the biomarker can be managed and controlled for. Although a biomarker with low diagnostic sensitivity may not be sufficient to monitor every individual patient, in a clinical trial setting with a population-based approach such a marker can be used if the reduced sensitivity can be compensated by enlarging the sample size. For example, this also applies to certain tumor markers which cannot be used for diagnosis because of poor sensitivity but which can serve as good markers of treatment response for those individuals with abnormal pre-treatment values. The ideal pharmacodynamic marker could eventually also be used to predict eradication or final cure of disease, which would make transform it additionally into a predictive biomarker. Admittedly, it is very unlikely that any one biomarker will fulfill all the characteristics listed in Table 2, but this biomarker
Table 2. Ideal and minimally acceptable characteristics for a pharmacodynamic biomarker for leishmaniasis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Ideal&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Minimally acceptable&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost</td>
<td>Low (e.g. &lt;1$/test)</td>
<td>No specific requirements</td>
</tr>
<tr>
<td>Clinical sample matrix</td>
<td>Urine or saliva</td>
<td>Blood or biopsy</td>
</tr>
<tr>
<td>Correlates with</td>
<td>Clinicopathological parameters (clinical measures of parasite biomass)</td>
<td>Overall severity/burden of disease</td>
</tr>
<tr>
<td>Laboratory requirements</td>
<td>Equipment-free, no cold-chain</td>
<td>No specific requirements</td>
</tr>
<tr>
<td>Diagnostic specificity</td>
<td>High, also against other (common) co-infections (e.g. malaria or Tb)</td>
<td>No specific requirements</td>
</tr>
<tr>
<td></td>
<td>Low variability in normal values (e.g. &lt;15% RSE)</td>
<td>Low variability in normal values (e.g. &lt;15% RSE)</td>
</tr>
<tr>
<td>Diagnostic sensitivity</td>
<td>&gt;95%</td>
<td>No specific requirements; low sensitivity would only increase required sample size</td>
</tr>
<tr>
<td>Pharmacodynamic specificity</td>
<td>Normalisation highly specific for adequate parasitological response to treatment</td>
<td>Normalisation specific for clinical improvement</td>
</tr>
<tr>
<td>Pharmacodynamic sensitivity</td>
<td>Normalisation within treatment period</td>
<td>Normalisation within follow-up period</td>
</tr>
<tr>
<td></td>
<td>Short half-life (&lt;2 days)</td>
<td>Short half-life (e.g. &lt;7 days)</td>
</tr>
<tr>
<td>Predictiveness</td>
<td>Predicting eradication of disease</td>
<td>No specific requirements</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ideal features would be for application in field settings for the treatment monitoring of an individual patient

<sup>b</sup>Minimally acceptable features would be for application in strictly controlled settings such as controlled clinical trials to compare treatment arms (e.g. for application in dose-finding studies or comparative drug trials)

Profile could serve as a benchmark for future research.

Pharmacodynamic potential

Ideally, the pharmacodynamic biomarker should be cheap, easy to assess, non-invasive, accurately reflect disease burden, respond rapidly to therapy and eventually predict initial and definite cure (Table 2). In addition to these requirements there are a few general issues which may restrict the pharmacodynamic application of the identified biomarkers.

Firstly, most identified biomarkers for leishmaniasis belong to the category of indirect markers, which constitute universal markers of activation and subsequent waning of cellular immunity or are related to the general immunological response to the acute phase of infection. As a result, often a high diagnostic specificity between leishmaniasis patients and healthy controls was found, but specificity may be much
lower when compared to patients suffering from (a combination of) other infections, as would be common in clinical practice in the various areas of endemicity. For instance, the elevated acute phase proteins reflect response to therapy and decrease substantially within the treatment period. They are probably the most frequently used treatment response of chronic infections (endocarditis, osteomyelitis a.o.). Nevertheless, these proteins are affected by many other (underlying) infections and might only be useful as a pharmacodynamic marker in a well-controlled trial with sufficient exclusion of concomitant diseases. Such diagnostic specificity issues would preclude the use of these biomarkers to monitor the treatment of each individual patient, the ideal application (Table 2), but it would not necessarily preclude its use in a controlled clinical trial context where these concomitant factors may be partly controlled by using appropriate exclusion criteria. On the other hand, direct and semi-direct markers (quantification of parasites and macrophage host cell markers) may be much more specific as they relate (semi-)directly to the parasite biomass and thus the disease burden, but their current methods of quantitation may be less sensitive (e.g. parasites are not detectable in blood of all patients). Again, this would not necessarily preclude their use in a clinical trial context, but it would probably increase the required sample size needed for these studies.

### Table 3. Selected potential pharmacodynamic biomarkers for leishmaniasis

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Research priorities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parasites in blood (VL) / lesion (CL)</strong></td>
<td>Directly related to parasite biomass, highly specific, direct response to treatment</td>
<td>Relatively high costs, RT-PCR equipment required, not very sensitive in VL</td>
<td>Establish correlation with clinical parameters, easier methods of sampling, reduce cost and lab requirements for analysis</td>
</tr>
<tr>
<td><strong>Carbohydrate antigen</strong></td>
<td>Directly related to parasite biomass, can be measured in urine samples, highly specific</td>
<td>Pharmacodynamic potential not well investigated, variable sensitivity, probably not useful in CL</td>
<td>Establish regression to normal in more detail, assess variability, establish correlation with clinical parameters</td>
</tr>
<tr>
<td><strong>Neopterin</strong></td>
<td>Probably high specificity, regresses to normal within treatment period</td>
<td>Limited number of studies performed, low absolute increase, high variability between patients; not useful in CL</td>
<td>Establish correlation with clinical parameters, assess correlation between variability and severity of disease, assess urine levels</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>Related to burden of disease (active VL), probably also to parasite biomass, ELISA widely available, regresses to normal within treatment period</td>
<td>Low specificity, also (less) increased in subclinical infection</td>
<td>Establish correlation with clinical parameters, assess mechanism of response to therapy</td>
</tr>
<tr>
<td><strong>C-reactive protein</strong></td>
<td>Related to severity of infection, probably high sensitivity, detection methods widely available</td>
<td>Very low specificity</td>
<td>Establish correlation with clinical parameters and parasite biomass, assess specificity in VL patients, establish time needed to regress to normal</td>
</tr>
</tbody>
</table>
Secondly, the time needed to regress to normal for the biomarkers (i.e. their elimination half-life) remains a concern. For instance, almost all of the antibody-related markers have a very long elimination half-life up to several months and stay present in the body long-after the actual parasitic infection has been resolved. As a result their potential use for pharmacodynamic monitoring of antileishmanial treatment is therefore probably very limited. Leishmania antigen detection might be more promising in that respect; however, this has mainly been investigated in the context of diagnosis of VL with only limited attention to the dynamic and quantitative application during and after treatment. In a similar direction, concerns have been ventilated about the quantitation of parasite DNA versus RNA, where parasite DNA was thought to be more stable and longer-lived than RNA. Nevertheless, a formal comparison of all these factors in clinical samples has not been performed.

**A selection of potential pharmacodynamic biomarkers**

In Table 3 is listed a selected group of potential pharmacodynamic biomarkers based on the available data from our literature review. We will highlight and critically discuss their application below, with some recommendations for research priorities.

Recent research has shown the quantitative application of molecular parasite detection methods as a pharmacodynamic measure, both in VL and CL. Sensitivity in CL appears to be high both in lesion biopsies as well as lesion swabs. In VL, sensitivity might be less since overall parasite load in the blood is generally low. Moreover, a subset of patients even appears PCR-negative targeting the 18S Leishmania rRNA, with apparent variability between geographical regions. On the other hand, the parasite load in the blood seems to be directly related to the burden in the target organs like spleen and bone marrow and decreases immediately after initiation of therapy (TPC Dorlo & GJ Schoone, personal communication). One of the disadvantages of this tool may be considered the relatively high costs of analysis probably around ~€30/sample (for material costs DNA/RNA extraction plus qPCR analysis). Moreover, state-of-the-art laboratory equipment is required and as such it can only be carried out in specialized laboratories with trained personnel onsite, all of which are still often not readily available in the countries of endemicity, or at least not in the vicinity of leishmaniasis foci. All these factors combined make this tool unsuitable for monitoring the treatment of individual patients under field circumstances. This does not, however, preclude its use in controlled clinical trials to compare treatment regimens or in trials undertaken to detect emerging drug tolerability, purposes for which this may even be the best biomarker tool available at the moment. Future research should be directed towards increasing the sensitivity of this tool to detect parasite loads in blood of all VL patients, e.g. by evaluating different (more abundant) primer targets. For CL, less invasive sampling techniques, such as lesion swabs, need to be evaluated for their quantitative and pharmacodynamic application. In general, more emphasis should be put in future
clinical trials on establishing a correlation between the biomarkers under evaluation and clinical parameters.

Another direct biomarker with pharmacodynamic 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 and as such it is the most non-invasive biomarker currently available. Its specificity is consistently high, but its sensitivity appears variable, which may make it only suitable in controlled settings. The *Leishmania*-specific antigen can be assessed quantitatively with ELISA, which makes it easier to adopt at primary health care facilities than the molecular detection methods. Nevertheless, no formal pharmacodynamic evaluation and adequate prospective validation has been performed yet and future studies should focus on the dynamics of the elimination of this antigen from the patient’s body and the time needed to regress to zero. Other research priorities would include the specific of variability of this marker in the population and further efforts to get a better understanding of the previously observed variability in sensitivity, which could be caused by the lack of a real golden standard for comparison.

**Neopterin** is one of the markers related to macrophage activity. In general these markers may be specific, although chitotriosidase is probably more specifically expressed in leishmaniasis compared to neopterin, provided its very selective expression profile. Nevertheless, currently more experience is available on the use of neopterin as a biomarker in leishmaniasis, although the number of studies available is still very small. Neopterin seems to regress to normal concentrations again within the treatment, although more evidence is needed on its dynamic profile within the treatment response. Moreover, the limited evidence available suggests high variability between patients and it should be further assessed in future studies whether this is correlated to disease severity or not. Interestingly, neopterin concentrations are generally detectable in urine, but this non-invasive assessment has not been evaluated yet in VL patients.

Of all the indirect markers, **IL-10** is probably the most investigated immunological marker and its role in active VL infection appears well supported. IL-10 correlates with the parasite load at the time of diagnosis, appears to decrease during treatment and even indicates the prediction of PKDL. Nevertheless, in view of the delicate balance and interplay between cytokines, the more general 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 effects in a clinical trial. Also, IL-10 has been demonstrated to be increased in subclinical cases as well, which complicates its interpretation, certainly during follow-up and in the context of parasite recrudescence. IL-10 ELISA kits are relatively low-cost and may be implemented on a larger scale for assessment, although a basic laboratory setting is still required.

**CRP** is one of the most frequently used markers to monitor chronic infections. Although it is generally used in the clinical evaluation of VL, relatively little has been reported on its pharmacodynamic potential or, more specifically, its time
needed to regress to normal in relation to the treatment provided. This marker has a high diagnostic sensitivity, but suffers from a low specificity (see above), and is therefore only useful in the comparison of clearly defined patient groups in clinical trials. Given the widespread availability of detection assays for CRP at health care facilities and its sensitivity, further formal evaluation of CRP could be warranted.

In general, most of the identified biomarkers were tested and evaluated clinically and not experimentally in animal models. This will be in part related to the lack of a general experimental animal model which accurately reflects all aspects of VL or CL, relating to problems with species differences, and more general problems in determining end-points in animal studies. This might potentially impede the extrapolation and translation of a drug and its dosage from preclinical studies to first-in-human studies. Taking into consideration the increasingly filled pipeline of new potential antileishmanial drugs (see e.g. http://www.dndi.org/index.php/portfolio.html), this aspect deserves more consideration in the future design of preclinical studies with new antileishmanial compounds.

**Conclusion & perspective**

Several new combination therapy strategies have been proposed for the treatment of visceral leishmaniasis and a few new chemical entities currently in the drug development pipeline are expected to proceed to Phase I/II clinical trials in the very near future. In view of these upcoming potential new treatment options for the leishmaniases, combined with the discussed difficulties in establishing clinical cure in both VL and CL and the sparsely occurring long-term failure events, there is an urgent need for better or at least more accurate pharmacodynamic assays: (1) to compare the new interventions to existing ones; and (2) to translate and evaluate the preclinical effects of the new chemical entities in animal models of leishmaniasis, to assist and support early dose finding studies in human. Future biomarker research should therefore extend its focus to the pharmacodynamic potential of the, mainly diagnostic, biomarkers under investigation. Moreover, we would like to argue here for a better integrated approach for the discovery and evaluation of biomarkers in leishmaniasis, extending from early preclinical discovery and evaluation of biomarkers in established animal models of CL and VL towards the assessment of their translational value in human to better support and rationalize dose-finding studies. The coming of age of new treatment options for leishmaniasis was long and eagerly awaited, but now that this moment is approaching we urgently need better and more accurate tools to evaluate their potential superiority over existing regimens, rationalize their dosing schedule, assess or even predict their effects on parasite recrudescence, and generally be able to compare their pharmacodynamic effects to the outdated repertory of current antileishmanial drugs.
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