Label-free quantitative proteomic analysis reveals potential biomarkers for early healing in cutaneous leishmaniasis

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Background. Leishmaniasis is a parasitic disease caused by more than 20 species of the Leishmania genus. The disease is globally distributed and is endemic in 97 countries and three territories in the tropical and subtropical regions. The efficacy of the current treatments is becoming increasingly low either due to incomplete treatment or resistant parasites. Failure of treatment is frequent, and therefore, the search for early biomarkers of therapeutic response in cutaneous leishmaniasis (CL) is urgently needed. Objective: The aim of this study was to compare the proteomic profiles in patients with CL before and after 7 days of treatment and identify early biomarkers of curative response. Methods. Four patients with a parasitological diagnosis of leishmaniasis with confirmation of species by PCR-RFLP were recruited. All patients had a single lesion, and a protein from the middle of the ulcer was quantified by liquid chromatography and mass spectrometry (LC-MS). Results. Twelve proteins showed differential expression in the comparative LC-electrospray ionization MS/MS (LC-ESI-MS/MS) triplicate analysis. Seven of them were up-regulated and five of them were down-regulated. Calcium binding proteins A2, A8, and A9 and hemoglobin subunits alpha-2 and delta showed high correlation with epidermis development and immune response. Conclusion. We identified changes in the profiles of proteins that had a positive therapeutic response to the treatment. The proteins identified with differential expression are related to the reduction of inflammation and increased tissue repair. These proteins can be useful as biomarkers for early monitoring of therapeutic response in cutaneous leishmaniasis.
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Short title: Biomarkers in cutaneous leishmaniasis

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ABSTRACT

Background. Leishmaniasis is a parasitic disease caused by more than 20 species of the Leishmania genus. The disease is globally distributed and is endemic in 97 countries and three territories in the tropical and subtropical regions. The efficacy of the current treatments is becoming increasingly low either due to incomplete treatment or resistant parasites. Failure of treatment is frequent, and therefore, the search for early biomarkers of therapeutic response in cutaneous leishmaniasis (CL) is urgently needed.

Objective: The aim of this study was to compare the proteomic profiles in patients with CL before and after 7 days of treatment and identify early biomarkers of curative response.

Methods. Four patients with a parasitological diagnosis of leishmaniasis with confirmation of species by PCR-RFLP were recruited. All patients had a single lesion, and a protein from the middle of the ulcer was quantified by liquid chromatography and mass spectrometry (LC-MS).
Results. Twelve proteins showed differential expression in the comparative LC-electrospray ionization MS/MS (LC-ESI-MS/MS) triplicate analysis. Seven of them were up-regulated and five of them were down-regulated. Calcium binding proteins A2, A8, and A9 and hemoglobin subunits alpha-2 and delta showed high correlation with epidermis development and immune response.

Conclusion. We identified changes in the profiles of proteins that had a positive therapeutic response to the treatment. The proteins identified with differential expression are related to the reduction of inflammation and increased tissue repair. These proteins can be useful as biomarkers for early monitoring of therapeutic response in cutaneous leishmaniasis.

INTRODUCTION
Leishmaniasis is a parasitic disease caused by more than 20 species of Leishmania genus. The parasite is transmitted by Diptera insects of the genus Phlebotomus (Old World) and Lutzomyia (New World) (1). The disease is distributed on five continents and is endemic to 97 countries and territories in the tropical and subtropical regions (2). Cutaneous leishmaniasis (CL), which is the most prevalent clinical form, manifests as a single or multiple ulcer with indurated edges, is painless, and presents a granular background that may or may not be covered by an exudate; the presence of plaques and nodules is also frequent (3).

Despite the high number of people affected, leishmaniasis control programs present difficulties not only with the diagnosis of cases and the control of vectors to reduce transmission but also with the treatment, given that the therapeutic response with the available drugs is becoming poorer due to incomplete treatments or the development of resistance in the parasites (4). Unfortunately, the confirmation of a definitive cure is complicated by the possibility of relapses after an initial cure, making necessary long follow-up periods (up to 6 or 12 months).

The prognosis of the treatment can be improved by detecting an early curative response or treatment failure. Such markers should also specifically correlate with the response to treatment and predict the long-term clinical outcome, by non-invasive sampling methods.
Up to now, the main advances have been made in visceral leishmaniasis (VL) where levels of IgG1 and IgG2 antibodies, TNFα, IL10, and parasite load were proposed as biomarkers of infection (5-7). IFN-γ, IL-2, monokine-induced-by-IFN-γ (MIG) and IFN-γ-inducible protein-10 (IP-10) could be used as markers of cure in immunocompetent patients with VL caused by L.infantum (8). In case of CL, the levels of IL4, IL8, INFγ, and MMP2 in skin biopsies and levels of TNFα, IL10, IL15, IL32γ, TGFβ, sCD26, sCD30, Cortisol, Prolactin, and SOD1 in serum, have been associated with infection and disease susceptibility (9-11). Nevertheless, these candidates are not fully validated as biomarkers of infection, and other biomarkers for treatment monitoring have not been identified or validated.

Analysis by liquid chromatography and electrospray ionization in tandem mass spectrometry (LC-ESI-MS/MS) has emerged as one of the most robust alternatives for the comparison of proteomic profiles through a quantitative approach based on labels (12). Quantitative analysis has been useful in clinical proteomics, proteomics of subcellular protein and high-resolution comparative proteomics (13), which has advanced the analysis of systems biology, particularly in the elucidation of the mechanisms that underlie the pathophysiology of several diseases.

The introduction of stable isotopes is the basis for the quantification in label-based approach and various protocols are now available that vary depending on the used methods for the incorporation of isotopes in the peptides/proteins. One of these methods is the isobaric tag for relative and absolute quantification (iTRAQ). The iTRAQ is a label-based method that allow the simultaneous analysis of eight samples in a single run of MS (multiplexing), resulting in reduced analytical variability. (14). With this method, the labeled peptides are shown as a single peak in the MS spectrum and, after fragmentation of peptides in the MS/MS analysis, the isotopes are released and peptides are identified according to their masses. This type of proteomic analysis results in data with high quality, sensitivity and a wide dynamic range (15).

The use of unlabeled protein profiles to examine differentially expressed proteins in tissue samples largely eliminates the variations and biases in replicate MS measurements and has allowed for the exploration of the mechanism of tumorigenesis and the discovery of biomarkers in various diseases (16,17).
Based on the advantages offered by this methodology, the aim of this study was to find and validate early biomarkers of therapeutic response in CL using proteomics without labeling in order to provide patients with adequate monitoring and identify early treatment failure, thus providing the possibility of changing the therapeutic scheme in a timely manner.

**MATERIALS & METHODS**

**Clinical samples**

In this study we included samples from patients with parasitological diagnosis of CL caused by *Leishmania panamensis* as confirmed by PCR-RFLP, with a single lesion, an evolution period of less than 4 months, and a lesion area less than 4 cm². The proteomic analysis was done with samples from 4 patients while the RT-qPCR analysis was carried out in samples from 5 patients.

**Sample preparation for proteomic analysis**

Granulation tissue from the center of the ulcer was collected before starting the treatment (TD0) and on day 7 of treatment (TD7). The material was placed in a vial containing 50 mM Tris, 100 mM NaCl, 20 mM DTT, protease inhibitor and 1X phosphatases, with pH 7.5 buffer. The samples were sonicated for 5 minutes at 4°C and subsequently centrifuged at 13,000 rpm for 20 min at 4°C; the supernatants were stored at -80°C until use. The proteins were quantified, and their integrity was confirmed by SDS-PAGE.

**Immunoaffinity depletion**

Protein extracts were diluted 5-fold with 1X dilution buffer (Tris-Buffered Saline (TBS) – 10 mM Tris-HCl with 150 mM NaCl, pH 7.4), filtered using a 0.45 µm pore-size spin filter to remove particulate materials and centrifuged at 9,000×g for 1 min. Each of the diluted and filtered samples (500 µl) were injected in a Seppro IgY 14 LC5 column with a constant flow rate of 0.5 ml/min for 20 min, followed by a Seppro washing step at a flow rate of 2.0 ml/min for 3 min. Non-bound proteins (depleted fraction) were collected in the flow-through fraction. Due to the large volume of collected fractions, depleted samples were concentrated using Amicon™ Ultra-15 Centrifugal Filter Units (Millipore). Concentrated samples were reconstituted in a chaotropic buffer containing 8 M urea, 2 M thiourea, and 100 mM.
triethylammonium bicarbonate (TEAB) at pH 8.5. Concentrated fractions were stored at –
80°C (18).

**In-gel protein digestion (Stacking gel)**

An aliquot concentrated flow-through fraction of each sample from four patients was diluted
with enough amount of sample loading buffer and then applied onto 1.2 cm wells of a
conventional SDS-PAGE gel (1 mm thick, 4% stacking, and 12% resolving). The run was
stopped when the sample front entered 1 cm into the resolving gel, to concentrate the whole
proteome in the stacking/resolving gel interface. The bands of unseparated protein were
visualized by Coomassie staining and then, bands were excised (19), cut into pieces (1 mm²),
deposited in 96-well plates, and processed automatically in a Proteineer DP (Bruker Daltonics,
Bremen, Germany). For digestion step, gel pieces were washed with 50 mM ammonium
bicarbonate (NH₄HCO₃) and then, with ACN prior to reduction with 10 mM DTT in 25 mM
NH₄HCO₃. The alkylation was carried out with 55 mM IAA in 50 mM NH₄HCO₃. Gel pieces
were then rinsed firstly with 50 mM NH₄HCO₃ and secondly with ACN and then were dried
under a stream of nitrogen. Proteomics grade Trypsin (Sigma Aldrich) at a final concentration of
16 ng/μl in 25% ACN/50 mM NH₄HCO₃ was added and the digestion took place at 37°C for 4 h.
The reaction was stopped by adding 50% ACN/0.5% TFA for peptide extraction. The tryptic
eluted peptides were dried by speed-vacuum centrifugation and were cleaned up/desalted using
Stage-Tips with Empore 3M C18 disks (Sigma-Aldrich).

**Liquid chromatography and mass spectrometer analysis**

One μg aliquot of the previously digested sample from four patients was subjected to 1D-nano
LC ESI-MS/MS analysis using a nano-liquid chromatography system (Eksigent Technologies
nanoLC Ultra 1D plus, SCIEX, Foster City, CA) coupled to high-speed Triple TOF 5600 mass
spectrometer (SCIEX, Foster City, CA) with a Nanospray III source. A silica-based reversed
phase Acquity UPLC M-Class Peptide BEH C18 Column, 75 μm × 150 mm, 1.7 μm particle size
and 130 Å pore size (Waters) was used as analytical column. The trapping column was a C18
Acclaim PepMap™ 100 (Thermo Scientific), 100 μm × 2 cm, 5 μm particle diameter, 100 Å
pore size. The loading pump delivered a solution of 0.1% formic acid in water at 2 μl/min. The
nano-pump provided a flow-rate of 250 nl/min and was operated under gradient elution
conditions. Peptides were separated using a 250 minute gradient ranging from 2% to 90% mobile
phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). Injection volume was 5 µl.

A TripleTOF 5600 System (SCIEX, Foster City, CA) was used for data acquisition with an ion spray voltage floating (ISVF) 2300 V, curtain gas (CUR) 35, interface heater temperature (IHT) 150, ion source gas 1 (GS1) 25, declustering potential (DP) 100 V. All data were acquired using information-dependent acquisition (IDA) mode with Analyst TF 1.7 software (SCIEX, USA). For IDA parameters, 0.25 s MS survey scan in the mass range of 350–1250 Da were followed by 35 MS/MS scans of 100 ms in the mass range of 100–1800 (total cycle time: 4 s). Switching criteria were set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1250 with the charge state of 2–5 and an abundance threshold of more than 90 counts (cps). Former target ions were excluded for 15 s. IDA rolling collision energy (CE) parameters script was used for automatically controlling the CE (20).

**Quantification and data analysis**

The mass spectrometry data obtained were processed using PeakView® 2.2 Software (SCIEX, Foster City, CA) and exported as mgf files. Proteomics data analysis were performed by using 4 search engines (Mascot Server v.2.6.1, OMSSA, X!Tandem, and MyriMatch) and a target/decoy database built from sequences in the Homo sapiens reference proteome at UniProt Knowledgebase. Search parameters included: trypsin as enzyme; 2 allowed missed cleavages; carbamidomethyl (C) as a fixed modification and acetyl (Protein N-term) and Oxidation (M) as variable modifications; peptide mass tolerance ± 25 ppm for precursors and 0.02 Da for fragment masses. Score distribution models were used to compute peptide-spectrum match p-values, and spectra recovered by a False Discovery Rate (FDR) <= 0.01 (peptide-level) filter were selected for quantitative analysis (21).

**Label-free quantification**

Differential regulation was measured using linear models and the q-values (FDR) were used for determining statistical significance. All analyses were conducted using Proteobotics software (Madrid, Spain) (16).
Pathway and interaction network analysis

The identified proteins were analyzed for biological function through the Panther Classification System website (http://www.pantherdb.org). The proteins were categorized according to their components, functions, and biological processes. The protein-protein interaction network (PPI) was constructed using the online STRING database (https://string-db.org) (22).

RNA isolation

Five patients were included in the validation assay. The RNA was extracted using High Pure RNA Tissue Kit (Roche, Mannheim, Germany), from scraping samples obtained on TD0 and TD7. Samples were placed in 400 µl of lysis/binding buffer and sonicated for 5 min in cold room. Then, 200 µl ethanol was added and each sample was transferred to a High Pure filter tube. After 30 min centrifugation at 13,000 x g, samples were treated with DNase for 15 min at 25 ºC. Five hundred µl wash buffer I were added and samples were centrifuged during 30 sec at 8,000 x g. Washing step was repeated using 300 µl of wash buffer II and centrifugation for 30 sec at 13,000 x g. Finally, the RNA was eluted with 30 µL of elution buffer. A reverse transcription reaction was performed in a 20 µl volume in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Quebec, Canada) using the Transcriptor First Strand cDNA Synthesis kit (#04379012001; Roche Diagnostics) according to the manufacturer's instructions, with certain modifications.

cDNA synthesis

A reverse transcription reaction was performed in 20 ng of RNA using the maximum first strand cDNA synthesis kit, following the manufacturer’s instructions: 4 µl master mix reaction, 2 µl RT enzyme mix, 2 µl RNA (DNase I treated), and 12 µl water were mixed and incubated in the PTC 100TM (MJ research) thermocycler for three cycles: 10 min at 25 ºC, 15 min at 50 ºC, and 5 min at 85 ºC, The obtained cDNA (20 µl) was stored at -20 ºC.

RT-qPCR

Each reaction mixture consisted of 2 µl reverse transcription reaction product as the template, 0.5 µl each of forward and reverse primers, 7 µl PCR grade water and 10 µl of QuantiTect SYBR Green PCR Kits (Qiagen, Hilden, Germany). The total volume of the reaction was 20 µl. The
RT-qPCR reaction was completed with an initial denaturation step (95 °C for 15 min) followed by 40 cycles of denaturation (95 °C, 10 sec), annealing (60 °C, 30 sec) and extension (72 °C, 30 sec). A final cooling step was conducted at 72 °C for 300 sec. The RT-qPCR reaction was performed using the LightCycler 96 Instrument. Each sample was analyzed in duplicate.

Three genes from up regulated proteins SERPINB1, S100A8, and S100A9 were selected for the measurement of relative expression. The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as constitutive gene. The primers were designed using Primer 3 software (bioinfo.ut.ee/primer3-0.4.0/). The following primer sequences were used: SERPINB1, Fwd 5′-ATGAAAGAAGCCACGACGAA-3′ and Rev 5′-GGTAAGGCAAGTTCCAGCACA-3′; S100A8, Fwd 5′-GCCAAGCCTAACCGCTAT-3′ and Rev 5′-CCCACCTGAAAAACAGAACC-3′; S100A9, Fwd 5′-GCACCCCCTAAGACCACAG-3′ and Rev 5′-CCCCACAGCCAAGACAGTT-3′; and GAPDH, Fwd 5′-GCTTCGCTCTCTGCTCCTC-3′ and Rev 5′-ACGACAAATCCGTTGACTC-3′.

Ethics Statement

All study subjects provided their informed written consent to be included in the study. The study was approved by the Human Bioethics Committee of Universidad de Antioquia – SIU (Act 007-2017).

RESULTS

All patients were treated with meglumine antimoniate. The cure was monitored for six months (until PTD 180). By this time all patients healed their lesion. No reactivation was detected (Figure 1).

Proteins detected in granulation tissue of CL

In total, 344 proteins were identified in the granulation tissue formed in the center of the ulcer. After analyzing the quality of the data obtained in MS, three of the four biological replicas were selected for label-free quantification by Q-values (FDR) using Proteobotics software (Madrid, Spain). The label-free quantification analyses yielded 267 proteins that were truly quantified in the three biological samples (p <0.05). Of the 267 proteins, 86 proteins were up-regulated.
(32.21% ratio > 0.67), 59 proteins were down-regulated (22.09% ratio < 0.717) and 122 proteins showed no changes (44.9%). The 267 proteins were grouped according to their cellular locations as follows: 63 intracellular, 4 intracellular membranes, 53 extracellular membranes, 6 extracellular matrices, 56 extracellular space, 43 macromolecular complex and 42 organelles (Figure 2A). The proteins were also classified according to their Class: 21 Calcium binding protein, 11 Chaperone, 13 Cytoskeletal protein, 14 Defense/immunity protein, 33 Enzyme modulator, 18 Hydrolase, 10 Membrane traffic protein, 13 Nucleic acid binding, 22 Receiver, 112 Other (Figure 2B). Finally, they were also classified according to their biological process: Antioxidant activity 7, Binding protein 89, Catalytic activity 59, Receptor activity 2, Structural molecule activity 13, Translation regulatory activity 1, Transporter activity 5 and Other 91 (Figure 2C).

Label-free quantification

Using the FDR analysis, 12 proteins were found with differential expression when comparing TD0 and TD7. Five were down-regulated (FABP5, S100A2, PRDX2, HBA1, and HBD) and 7 were up-regulated (KRT73, LAMP2, S100A9, S100A8, KRT10, SERPINB1, and ATP7A). All differentially expressed proteins were classified using the Panther Classification System website (http://www.pantherdb.org).

The classification according to their molecular function showed a distribution between fatty acid binding 3, Toll-like receptor 4 binding 2, arachidonic acid binding 2, RAGE receptor binding 2, and oxygen transporter activity 2 (Figure 3). The classification of the cellular components showed that all proteins are located at the extracellular level: extracellular region 10 genes, extracellular exosome 9 genes, extracellular region 10 genes, extracellular space 6 genes and membrane-bound vesicle 8 genes.

The classification according to their biological process showed that these proteins participate in neutrophil aggregation and degranulation, sequestering of zinc, peptidyl-cysteine S-nitrosylation, chemokine production, leukocyte migration during the inflammatory response, removal of superoxide radicals, oxygen transport, hydrogen peroxide catabolic process, astrocyte development, antimicrobial humoral response and epidermis development. The proteins showed...
a fold enrichment between 17.1 and 100%, the p-log10 value between 3.9 and 5.7 and an FDR <0.05 (Table 1).

Interaction network analysis

The proteins were analyzed to determine the functional interaction network that unites the 12 proteins differentially expressed between TD0 and TD7 using STRING database, obtaining a minimum required interaction score of 0.4 (medium confidence). The analysis showed that 5 of the 12 proteins had a good correlation and were classified in 2 groups. In the first group were S100A9, S100A8, and S100A2, all calcium-binding proteins. In the second group were HBD and HBA2, both components of hemoglobin (alpha and delta) (Figure 4).

Relative expression by RT-qPCR

The levels of expression of SERPINB1, S100A8, S100A9 at TD7 were compared with respect to the levels before treatment (TD0) using the ΔΔCT method. All 5 patients were cured after 6 months of follow-up. The expression levels increased 2.65 ± 1.22 times for SERPINB1, 3.2 ± 2.81 for S100A8, 1.38 ± 0.67 for S100A9 (Figure 5).

DISCUSSION

Leishmaniasis is one of the seven main tropical diseases and continues to be one of the major public health problems around the world (23). Reevaluation of treatment guidelines for cutaneous leishmaniasis is urgently needed because there is high risk of toxicity of pentavalent antimony and treatment failure is increasing (24, 25).

The major adverse effects associated with pentavalent antimonial treatments are local pain, arthralgia, myalgia, increases in hepatic enzymes, urea, and creatinine, and electrocardiographic alterations (inversion of the T wave, prolongation of the Q-T segment, depression of the S-T segment, and sinus bradycardia). Based on their severity, it is necessary to stop the treatment and modify the treatment scheme or change the drug (26).

The search for biomarkers in leishmaniasis has emerged as a need to not only identify markers of susceptibility and resistance to infection or the evaluation of vaccines effectiveness, but also to
monitor early and late therapeutic efficacy (5-7,9). However, none of the proposed biomarkers has been validated.

For monitoring of the therapeutic response of cutaneous leishmaniasis, various biomarkers were proposed. These biomarkers include the parasite load, levels of IL4, IL8, INFγ, MMP2 expression in skin biopsies and also levels of TNFα, IL10, IL15, IL32γ, TGFβ, sCD26, sCD30, cortisol, prolactin and SOD1 in serum (27). Nevertheless, the applicability of these proposed biomarkers is still debatable either because of the difficulty of collecting samples from invasive biopsies, or because the cytokines detected in serum cannot be attributed to a specific infectious process located on the skin as would be in the case of CL, where it can be influenced by conditions particular to each individual.

Little has been explored in the search for early biomarkers of therapeutic response to predict the effectiveness of treatment in the first week. The biomarkers mentioned above were detected in skin biopsies and serum samples before treatment and their previous levels correlated with the therapeutic response at the end of treatment. In our approach, we use a sample obtained of the center of the ulcer by scraping that does not generate pain or discomfort in the patient. Additionally, the material is obtained from the site where both the damage and repair process occur, during a curative therapeutic response.

The healing proteome has been studied in different animal models, especially in mice and pigs. These studies showed that proteins with differential expression correspond to receptors, hydrolases, enzymes, immunity, cytoskeletal proteins, transporters and oxidoreductases (28,29). Additionally, in pathogenic processes such as type 2 diabetes and chronic ulcers, where there is an increase in expression levels of proteins related to inflammation and tissue destruction, proteins with differential expression have been identified, most of which relate to angiogenesis, cell death and inflammation (30,31).

In CL, where scarring of the lesion is distorted by the presence of the parasite and the inflammatory phase is perpetuated over time, the healing process only begins with the disappearance of the antigenic stimulus triggered by the parasite.
Our results showed differential expression in 12 proteins when comparing expression levels on TD0 and TD7. Five were down-regulated and seven were up-regulated. The up-regulated proteins participate in biological processes such as neutrophil aggregation, response to zinc ion, sequestering of zinc ion, peptidyl-cysteine S-nitrosylation and cellular transition metal ion homeostasis, the down-regulated proteins participate in oxygen transport and hydrogen peroxide catabolism.

There was a decrease in the expression of FABP5 and S100A2. FABP5 is a fatty acid binding protein while S100A2 is a calcium transport protein. Both proteins show increased levels in psoriasis (32), revealing their role in the inflammatory process (33). Thus, the decrease in FABP5 and S100A2 in the case of CL would correlate with a regulation of the inflammatory stage. On the other hand, PRDX2 is a peroxidase which has been considered a biomarker in inflammatory processes such as multiple sclerosis (34). Its early decline in CL also confirms a regulation of the inflammatory process. Finally, HBA1 and HBD are subunits of hemoglobin, which is highly important in oxygen transport that favors oxidative response. Decrease of HBA1 and HBD levels in tissue can be correlated with a reduction of the oxidative response and therefore of the damage generated in the tissue by oxidative mechanisms. Else, the low levels of these proteins in skin could be related to their inability to regulate oxidative response similar to that observed in Steatohepatitis (35).

Change in the protein profiles with increased Keratin, type II cytoskeletal 73, a protein present in the hair follicle (related to keratinization and important in the development of the skin in mice (36), confirms that this protein would be participating early in the new tissue formation. On the other hand, LAMP2 is a glycoprotein that has been described as important in processes mediated by lysosomes. In the context of healing, it has been described in squamous cell carcinoma where its expression is related to cell adhesion and cell migration, generating a layer of glycocalyx that prevents the degradation of cells and the action of different proteases (37). Thus, in the case of CL it may be participating in the process of tissue repair, possibly in cell migration.
S100A9 and S100A8 are calcium- and zinc-binding proteins related to different processes such as the regulation of inflammation, cell cycle progression and cell differentiation, induction of neutrophil chemotaxis, phagocytosis, as well as degranulation (38). Additionally, they are overexpressed during the healing process (30), particularly in the differentiated keratinocytes and their participation in the reorganization of the keratin cytoskeleton in the injured epidermis is well-established (39). Based on their roles in healing, we believe that their increase in early response to treatment correlates with the beginning of the process of tissue repair.

KRT10 is a type of keratin that has been described as an early marker of daughter cell differentiation in the basal stratum that faces the epidermal surface and contributes to re-epithelialization. Studies in mice confirmed that the absence of cells expressing KRT10 correlates with deterioration in re-epithelialization (40). Therefore, in CL, the increase in the expression of KRT10 would be correlated with the beginning of the re-epithelialization process.

SERPINB1 belongs to the superfamily of serine protease inhibitors, a protein that has various functions. In the inflammatory process it has been shown to play an important role inhibiting apoptosis, activation of caspase 3 and the action of different proteases such as elastase, cathepsin G and D, and proteinase-3 (41). It has been demonstrated in models of corneal epithelium in cattle, that during cicatrization SERPINB1 participates in cell migration and its increased level is related to the closure of the wound; the levels are restored to normal values when the injury has healed (42). Both mechanisms would be important in the therapeutic response in CL since increased apoptosis has been described as one of the pathogenesis markers in proteome studies of pathogenesis in CL (43). On the other hand, cell migration and wound closure would be related to the start of the lesion healing.

Finally, ATP7A is a very interesting protein for the regulation of intracellular copper concentrations, fulfilling two very important functions: the elimination of the accumulated intracellular copper and the transportation of the copper necessary for the proper functioning of the copper-dependent enzymes (44). The human peptide Gly- (L-His) - (L-Lys) or GHK has a high affinity for copper as a carrier and once it binds copper, it may have an anti-inflammatory effect with suppression of free radicals, formation of thromboxane, release of iron oxidant and TGFβ, increase of superoxide dismutase and vasodilation, which would be essential for tissue repair.
repair in LC. It also has a chemoattractant effect on macrophages, mast cells and capillary cells (45). All these effects are essential for the entire process of tissue repair in CL.

The wound healing process includes homeostasis, inflammation, proliferation and remodeling. In the case of CL, once the infection is controlled by the treatment, the inflammatory phase ends and the oxidative response and the production of proinflammatory molecules involved in tissue damage disappear. The oxidative response is regulated by a decrease of HBA1, HBD and PRDX2. While HBA1 and HBD are essential for oxygen transportation, PRDX2 is essential for the oxidative response. The relationship between HBA1 and HBD with PRDX2 is also demonstrated in chronic inflammatory processes such as Bowel's disease (46). On the other hand, FABP5 can regulate the production of prostaglandin E2 (47) and S100A2 is associated with inflammatory processes such as periodontitis and constitutes a candidate for biomarker of inflammation in this context (48). The repair process begins with migration, adhesion, proliferation and cell differentiation. The GHK controls the migration of macrophages and the macrophages differentiate to M2 type to increase the production of TGFβ (45). SERPINB1 attract fibroblasts to the wound healing site (42) and LAMP2 promotes the extracellular matrix (37). Lastly, KRT10, S100A9 and S100A8 induce proliferation and differentiation of fibroblast (30, 40) and then, occur the re-epithelialization with the participation of keratin and type II cytoskeletal 73 protein.

The identification of these proteins with increased expression after healing are closely related to the beginning of the cell proliferation stage when the infection and inflammation is controlled. Although unexpected, we do not find proteins belonging to the parasite in the ulcer center, possibly because the proportion of infected cells in the ulcer is lower than in the active borders. The measurement by RT-qPCR showed an induction level of S100A8 > 2 in the cured patients suggesting that SERPINB1 and S100A8 are good candidates for an early biomarker of healing. In turn, the induction level of S100A9 was < 2, indicating that this marker is not a good candidate. Nevertheless, before accepting or ruling out any of these candidates for biomarkers, these results need to be validated in a larger group of patients.

**CONCLUSION**

In conclusion, our work allowed for two very important analyses. The first is the characterization of proteome of pathogenesis in the ulcer generated in CL and the second is the identification of
the changes in the protein profile in a positive therapeutic response to treatment, allowing to
identify pathways related to inflammation and a negatively regulated oxidative response, usually
associated with the reduction of inflammation and increased tissue repair. With these results, it is
possible to propose a multicentric study that allows the validation of the healing biomarkers by
RT-qPCR for the early prediction of the treatment. These results will allow clinicians to
implement, in a timely manner, an appropriate strategy to guarantee a successful treatment.

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Conflict of interest
We have no conflicts of interest to declare.

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Table 1. Classification of proteins with differential expression according to the complete biological process*

* Panther classification system
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| GO biological process                                      | Genes                  | - Log10 (p-value) | Fold Enrichment | FDR     |
|------------------------------------------------------------|------------------------|-------------------|-----------------|---------|
| Neutrophil aggregation (GO:0070488)                        | P06702, P05109         | 5,7               | > 100           | 0,00464 |
| Sequestering of zinc ion (GO:0032119)                      | P06702, P05109         | 5,4               | > 100           | 0,00696 |
| Peptidyl-cysteine S-nitrosylation (GO:0018119)             | P06702, P05109         | 5,1               | > 100           | 0,00683 |
| Chemokine production (GO:0032602)                          | P06702, P05109         | 4,7               | > 100           | 0,0122  |
| Leukocyte migration involved in inflammatory response (GO:0002523) | P06702, P05109         | 4,6               | > 100           | 0,0134  |
| Removal of superoxide radicals (GO:0019430)                | P32119, Q04656          | 4,5               | > 100           | 0,0152  |
| Oxygen transport (GO:0015671)                              | P69905, P02042          | 4,4               | > 100           | 0,0163  |
| Hydrogen peroxide catabolic process (GO:0042744)           | P32119, P69905          | 4,2               | > 100           | 0,0242  |
| Astrocyte development (GO:0014002)                         | P06702, P05109          | 3,9               | > 100           | 0,0426  |
| Antimicrobial humoral response (GO:0019730)                | Q04656, Q01469, P13473 | 4,6               | 52,08           | 0,0134  |
| Neutrophil degranulation (GO:0043312)                      | P06702, P05109, P30740 | 5,3               | 18,15           | 0,00643 |
| Epidermis development (GO:0008544)                         | Q04656, P13645, Q01469, Q86Y4 | 4,2 | 17,11 | 0,0233 |

* Panther classification system
Figure 1

Evolution of ulcer in patients.

The figure shows the appearance of the cutaneous leishmanaisis during follow-up. TD0: before the start of treatment; TD7: on day 7 of treatment; PTD180: 6 months after the end of treatment.
Figure 2

Global proteomic analysis of the granulation tissue.

Samples were obtained from a cutaneous leishmaniasis ulcer before treatment (TD0) and day 7 of treatment (TD7). The figure shows the gene ontology analysis of all the quantified proteins. (A) Proteins present in cellular components. (B) Protein classified in classes. (C) Molecular function of proteins.
Figure 3

Molecular function of differentially expressed proteins early.

The figure shows the classification of the 12 proteins with differential expression according to their molecular function. Pathway description.
Figure 4

Interaction network analysis of protein.

Functional interaction network linking the significantly differentially expressed proteins between TD0 and TD7. S100A9: S100 calcium binding protein A9. S100A8: S100 calcium binding protein A8 up-regulated. S100A2: S100 calcium binding protein A2. HBD: Hemoglobin, delta. HBA2: Hemoglobin, alpha 2 down-regulated.
Figure 5

Fold induction of S100A8, S100A9 and SERPINB1 by RT-qPCR.

The figure shows the induction of each gene at day 7 of treatment vs. before treatment calculated by the ΔΔCT method. S100A8 (circles). S100A9 (squares). SERPINB1 (triangle). Each point corresponds to one patient.