*Parasitology*

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# Comparative proteomic analysis of *Leishmania* parasites isolated from visceral and cutaneous leishmaniasis patients

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**Research Article**

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## Abstract

Leishmaniasis is an infectious disease in which different clinical manifestations are classified into three primary forms: visceral, cutaneous and mucocutaneous. These disease forms are associated with parasite species of the protozoan genus *Leishmania*. For instance, *Leishmania infantum* and *Leishmania tropica* are typically linked with visceral (VL) and cutaneous (CL) leishmaniasis, respectively; however, these two species can also cause other form to a lesser extent. What is more alarming is this characteristic, which threatens current medical diagnosis and treatment, is started to be acquired by other species. Our purpose was to address this issue; therefore, gel-based and gel-free proteomic analyses were carried out on the species *L. infantum* to determine the proteins differentiating between the parasites caused VL and CL. In addition, *L. tropica* parasites representing the typical cases for CL were included. According to our results, electrophoresis gels of parasites caused to VL were distinguishable regarding the repetitive down-regulation on some specific locations. In addition, a distinct spot of an antioxidant enzyme, superoxide dismutase, was shown up only on the gels of CL samples regardless of the species. In the gel-free approach, 37 proteins that were verified with a second database search using a different search engine, were recognized from the comparison between VL and CL samples. Among them, 31 proteins for the CL group and six proteins for the VL group were determined differentially abundant. Two proteins from the gel-based analysis, pyruvate kinase and succinyl-coA:3-ketoacid-coenzyme A transferase analysis were encountered in the protein list of the CL group.

## Introduction

Leishmaniasis is an infectious disease mostly affecting people who live in developing countries with a lack of sanitation. The disease rises from parasites of the genus *Leishmania*; however, sandflies can be accused of contraction since a bite suffices to transmit these obligate intracellular protozoans to vertebrates. Three main clinical forms, cutaneous (CL), mucocutaneous and visceral (VL) leishmaniases emerge regarding body location where parasites in amastigote form are accumulated. The life-threatening form, VL, affects the inner organs, especially the lymph nodes, spleen and liver. On the contrary, CL which may leave behind unpleasant permanent scars is diagnosed by skin lesions on the exposed part of the body. Muco-cutaneous form is more severe than cutaneous form since it may result in the mutilation of the nose, mouth or throat (Chappuis *et al.*, 2007; Steverding, 2017; Garrido-Jareño *et al.*, 2020). Among more than 50 *Leishmania* species defined, approximately 20 are pathogenic for humans and the disease forms are associated with those species. In this context, only *Leishmania braziliensis* is seen in the cases of muco-cutaneous leishmaniasis, *Leishmania donovani* and *Leishmania infantum* are primarily causative agents of VL and finally *Leishmania major*, *Leishmania mexicana*, *Leishmania tropica* and the others are generally responsible for CL. Apart from that, *L. infantum* can cause CL to a lesser extent and the same goes for *L. tropica* about VL (Akhoundi *et al.*, 2017).

Atypical case is quite a broad term and refers to abnormalities including the unusual appearance of lesions (Guimarães *et al.*, 2016; Espinoza-Morales *et al.*, 2017; Meireles *et al.*, 2017) and unfamiliar clinical indications due to the coinfections and concurrent occurrence of other diseases (Calvopina *et al.*, 2005; Da-Cruz *et al.*, 1999; Kouyialis *et al.*, 2005; Celesia *et al.*, 2014; Hashemi *et al.*, 2018; Martínez *et al.*, 2018). Apart from that, clinical manifestations caused by unexpected *Leishmania* spp. which has become widespread around the world are also considered as atypical cases (Mebrahtu *et al.*, 1993; Siriwardana *et al.*, 2007; Elamin *et al.*, 2008; Lopes *et al.*, 2013; Özbilgin *et al.*, 2017; Thakur *et al.*, 2018). Genomic studies demonstrated that mixed *Leishmania* infections and interspecies *Leishmania* hybrids which were promoted by climate change, mass migrations and travels (Dereure *et al.*, 2009; González *et al.*, 2010; Alawieh *et al.*, 2014; King *et al.*, 2015; Karakuş *et al.*, 2019; Lypaczewski and Matlashewski, 2021), render the disease more detrimental for this reason aforementioned complications supposedly have emerged under these factors (Akopyants *et al.*, 2009; Romano *et al.*, 2014; Ferreira *et al.*, 2015; Alves Souza *et al.*, 2019). Since these alterations somehow jeopardize disease control and prevention, to keep up with the biological



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alterations and to update our knowledge, *Leishmania* parasites should be investigated from all perspectives, especially involving the ones isolated from patients.

Differential proteomics enables scientists to reveal the alterations between two or more different conditions by comparing their overall protein expression. Two methodical approaches namely gel-based and gel-free proteomics have gained wide acceptance for the comparison. While the first is based on protein separation in a porous gel, the latter is built on the separation of complex peptide mixtures with liquid chromatography after single-shot digestion of the protein mixture (Lambert *et al.*, 2005). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was formerly the leading method, in which proteins are separated on thin square polyacrylamide gels, however, shotgun proteomics has become the method of choice today due to accurate and high-throughput data accumulation in a short time (Zhang *et al.*, 2013). Since only a limited number of proteins can be detected with the gel-based method, gel-free or shotgun proteomics techniques which keep evolving with technological advancements and improvements in auxiliary materials, dominate over gel-based techniques nevertheless seeing proteins isolated on the gel visible to the naked eye enables scientists a reassurance for the protein expression while providing simplicity during database search. Although these two approaches can give results at a completely different levels and perspectives, both of them can be used as complementary to each other. Therefore, to determine differential proteins on *Leishmania* isolates obtained from VL and CL patients, both gel-based and gel-free methods were applied to gain a broader insight into the *Leishmania* parasite tropism. The comparison was built on the species, *L. infantum* and *L. tropica* were partially incorporated since these two are the prevalent species in Europe and the Mediterranean region including our country (Ready, 2010).

## Materials and methods

### Parasite cultivation and total protein extraction

The *Leishmania* isolates obtained from patients who were admitted to dermatology clinics in different provinces of Turkey were genetically identified and cryopreserved as described in the previous study (Özbilgin *et al.*, 2017). Real-time ITS1-polymerase chain reaction (PCR) and PCR-based cysteine protease B methods were used for the species discrimination (Hide and Bañuls, 2006; Toz *et al.*, 2013). Parasite Bank in Manisa Celal Bayar University supplied these isolate samples which were cryopreserved in liquid nitrogen after cell cultivation and labelling. VL and CL promastigote cells were cultivated in enriched NNN medium at 25 °C and then transferred to RPMI-1640 medium containing 10% foetal calf serum (FCS), 1% penicillin/streptomycin and 1% gentamycin. When the population reached approximately $10^8$ cells $mL^{-1}$, the growth medium was centrifuged at 4400 rpm for 10 min at 4 °C. Pellets were incubated overnight in RPMI-1640 medium without FCS and harvested again by centrifugation at 4400 rpm (Özbilgin *et al.*, 2016). After washing gently with phosphate-buffered saline three times, 1 mL of Mammalian Cell Lysis Reagent (Fermentas Life Sciences; Thermo Fisher) was added for the cell lysis. The resuspended solution was incubated at room temperature on a shaker at 900 rpm and centrifuged at 14 500 rpm for 15 min. Finally, the supernatant was decanted gently to a new tube and stored at −20 °C for further processing. The protein solution was concentrated with centrifugal filters (Amicon Ultra 10K molecular weight cut-off 0.5 mL; Merck Millipore) and the slurry was resuspended in the rehydration buffer. Protein concentration was measured with Bradford protein assay (Coomassie Plus Bradford Assay Reagent; Thermo Scientific). Both gel-based and gel-free approaches were employed for differential protein expression analysis by using different mass spectrometers.

### Gel-based proteomics: two-dimensional gel electrophoresis and in-gel tryptic digestion

Protein was dissolved in the rehydration buffer containing 7 M urea, 2 M thiourea, 4% v/v CHAPS, 65 mM dithiothreitol (DTT) and 2.5% ampholyte solution pH 3–10 (40%, w/v). A total of 400 $\mu$g protein in 350 $\mu$L solution was loaded onto 17 cm, pH 3–10 nonlinear strip (ReadyStrip™ IPG Strips; BioRad). In addition, two pH 4–7 strips and one pH 5–8 strip were run with remaining samples to evaluate the degree of resolution in narrower ranges. After passive rehydration for 2 h and subsequent active rehydration at 50 V for 16 h, the first stage of two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D-SDS PAGE) (O'Farrell, 1975), isoelectric focusing (IEF) was started to run until a total of 60 000 Vh electrophoresis was completed (Protean IEF cell; Bio-Rad). Before mounting onto the polyacrylamide gels, strips were first equilibrated in a solution containing 6 M urea, 50 mM Tris-HCl pH 8.8, 2% w/v SDS, 30% w/v glycerol and 65 mM DTT for 15 min. Following this, they were kept in another equilibration solution in which 4% w/v 2-iodoacetamide (IAA) was substituted with DTT for 15 min more, this time in the dark. After the equilibration step, the strips were washed by immersing into electrophoresis running buffer Tris-glycine-SDS. The second stage, SDS PAGE, was performed placing the strips onto 12% polyacrylamide gels and applying 180 V for approximately 9 h. Gels were sealed with overlay agarose solution containing 0.5% agarose and 0.003% bromophenol blue in the running buffer to monitor the reaching of the dye to the bottom of the gel to terminate the current. After rinsing the gel with water, proteins were detected with silver staining (Gromova and Celis, 2006) and selected spots were subjected to in-gel digestion (Shevchenko *et al.*, 2006) with trypsin (proteomic grade porcine pancreas, Sigma Aldrich). No computational tool was utilized for the comparison and differential protein assignment therefore only explicit and repeating changes were taken into consideration. Peptide solution was desalted with the C18 ZipTip™ pipette tip (Millipore) and mixed with alpha cyano-4-hydroxycinnamic acid for the thin layer deposition onto a matrix-assisted laser desorption/ionization (MALDI) plate (Dai *et al.*, 1999). MALDI mass spectrometry (MS) measurements were performed on an Autoflex III Smartbeam MALDI TOF/TOF MS (Bruker).

### Gel-free proteomics: off-line high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS)

For in-solution tryptic digestion, the aforementioned 10 kDa MWCO filter tube was used to remove excess urea and detergent which interferes with trypsin and causes denaturation (Wiśniewski *et al.*, 2009). Before trypsin addition, 5 $\mu$L of 0.2 M reducing agent DTT has been added to 400 $\mu$g protein solutions and incubated for 1 h. Later, 20 $\mu$L of 0.2 M alkylating agent IAA was added and incubated for 1 h in the dark. Next, 20 $\mu$L of 0.2 M reducing agent DTT was added again and incubated for 1 h to neutralize the excessive IAA. Samples were centrifuged at 14 000 rpm for 10 min for the solvent exchange with Tris-HCl. Two washing steps were performed by adding 200 $\mu$L of 100 mM Tris-HCl buffer followed by centrifugation. Finally, 4 $\mu$g trypsin (enzyme to protein 1:100) was mixed in a solution containing 50 mM, pH 8.5 Tris-HCl and 10% acetonitrile. An enzyme solution of 200 $\mu$L was added to the concentrated protein mixture and incubated at 37 °C overnight. Since hundreds of thousands of peptides are produced from protein mixtures by a single tryptic

digestion step, this method is also called shotgun proteomics. Although shotgun proteomics seems labourless due to the absence of protein separation, this time the peptide mixture needs to be reduced to a measurable degree before the LC-MS/MS analysis. This task can be achieved with 2D-HPLC systems so-called online analysis or by collecting fractions from another HPLC, the off-line separation. For the off-line peptide separation, high pH reverse-phase chromatography (Shimadzu Prominence UFLC, LC Solution Version 1.25 SP3) with a fraction concatenation strategy was applied at a 0.5 mL $min^{-1}$ flow rate (Wang *et al.*, 2011). Peptide solution was evaporated with a speed-vac vacuum concentrator and 100 $\mu$L solution was injected to C18, 5 $\mu$m, 250 × 4.6 mm analytical column (C18-Teknokroma Mediterranea sea18) which was equipped with a 10 × 4.6 mm column protection cartridge (uniguard; Thermo). Eluent was collected in a 96-well plate with 35-s intervals (SunCollect micro fraction collector MALDI spotter; SunChrom). To decrease the 96 fractions, six wells were pooled into a new 1.5 mL tube by skipping 15 fractions each time and then all 16 fractions were completely evaporated. All 16 fractions belonging to one sample were resuspended, cleaned with the C18 ZipTip™ pipette tip and analysed by using capillary HPLC (DIONEX UltiMate 3000) coupled with an ESI linear ion trap MS (LTQ XL mass spectrometer, Thermo Scientific) twice. The outlet of the reversed-phase capillary column (15 cm × 500 $\mu$m; 2.7 $\mu$m; Sigma Supelco Ascentis) having a 5 $\mu$L $min^{-1}$ flow rate was connected to the ESI source whose spray voltage and the capillary temperature was set to 5 kV and 200 °C, respectively. MS/MS data were collected through data-dependent acquisition (Xcalibur software 2.0.7) that allows the fragmentation of the nine most abundant ions in positive mode unless the ion count is below 10 000, using 35% collision energy. Full spectra were collected from $m/z$ 400–1800 and dynamic exclusion was 30 s.

### Protein identification with peptide database search

In the gel-based part, MS and MS/MS spectra were collected with software flexControl 3.0 and transferred from software flexControl 3.0 to Biotools 3.0 for database search with the Mascot search engine (version 2.3; Matrix Science) (Perkins *et al.*, 1999). For the database search, carbamidomethylation (C) as fixed global modification and oxidation (M) as variable modification and allowance for up to one tryptic cleavage were set. The peptide mass tolerance was either 200 ppm or 1.0 Da and fragment ion mass tolerance was 0.5 Da. Charge state was 1+ and monoisotopic option mass was chosen. The mass range of the analyses was between 700 and 3500 Da. Data were searched against the NCBI database (NCBI Resource Coordinators, 2017) of *Leishmania* genus and alternatively UniProt (The UniProt Consortium, 2016) protein database of *L. infantum*.

In the gel-free part, 32 MS/MS raw files were converted to a mascot generic file (mgf) by Proteome Discoverer 1.4. for each sample. The combined data of all fractions were searched against the *L. infantum* proteome database obtained from UniProt using an in-house Mascot server version 2.3 as the first option. After post-processing with Mascot percolator (Brosch *et al.*, 2009), the results were exported and further analysed in MS Excel. Apart from that, all mgf files were submitted to software SearchGUI (Vaudel *et al.*, 2011) for multiple-search using the search engines Comet (Eng *et al.*, 2013), MS Amanda (Dorfer *et al.*, 2014), MS-GF+ (Kim and Pevzner, 2014) and xTandem (Craig and Beavis, 2004). Results were displayed and exported to excel files by PeptideShaker (Vaudel *et al.*, 2015). The following set-up parameters were entered to SearchGUI for all searches: enzyme: trypsin, fixed modifications: carbamidomethyl, variable modification: oxidation, peptide tolerance: 1.0 Da, MS/MS tolerance: 0.5 Da, peptide charge: 2+ and 3+, instrument: ESI-TRAP and experimental mass values: monoisotopic. The same settings were used for Mascot searches except for the peptide tolerance and MS/MS tolerance which were changed to default values 1.2 and 0.6 Da, respectively. Protein false discovery rates (FDR) of all searches were <1%. To determine the differential proteins, emPAI values (Ishihama *et al.*, 2005) of Mascot results and spectrum counting values obtained from PeptideShaker (Powell *et al.*, 2004) were transferred to Microsoft Excel spread sheet and normalized values (Shinoda *et al.*, 2010) were imported to Perseus software (Tyanova *et al.*, 2016) for the statistical analysis. Web-based Venn diagram tools were utilized when necessary (VIB/UGent, 2021; Bardou *et al.*, 2014).

## Results

Promastigote parasites isolated from different patients were examined in three groups which were designated with CLi for the CL group caused by *L. infantum*, CLt for the CL group caused by *L. tropica* and VLi or just VL for the VL group caused by *L. infantum*. To compare the protein expression of promastigote parasites caused to different disease forms such as VL and CL, both gel-based and gel-free proteomic approaches were applied. The first was studied on 15 samples while the latter were contained nine out of those 15 samples.

### Gel-based approach: 2D-SDS PAGE results

From a broad perspective, gel images of CL isolates, belonging to either *L. infantum* or *L. tropica* group, were quite similar whereas those of VL isolates were distinguishable from them, especially in particular samples. Main differentiation between CL and VL isolates was observed as downregulation in several proteins on the gels of VL samples.

Whole gel images of all samples and the list of identified protein spots are available in the Supplementary material_1; however, one gel from each group is given in Fig. 1 for representation purposes. For ease of viewing, specific regions of interest (ROIs) whose locations are shown by dashed rectangles in Fig. 1. Aligned version of all these gel sections for each ROI is given in Supplementary material_1. In those figures, all VL samples are laid on the top row while CL(i) samples follow them in the middle and CL(t) samples are aligned down on the third row. Gels of three samples are missing in the first ROI due to a technical problem during the electrophoresis, therefore sections from two pH 4–7 and one pH 5–8 gels are added on the left column for this region. Protein spots only identified with in-gel digestion and mass spectrometric analysis by MALDI-TOF/TOF MS were indicated with a number. Some of these spots which were identified more than once were like anchor points helping us to ensure about the positions during the visual inspection. A total of 40 protein spots were identified by using peptide mass fingerprinting and peptide fragmentation or both. Among them, nine protein spots seemed deserve special attention therefore they are flagged with a lower-case letter and gathered in Fig. 2. Our first observation was the absence of a spot in the VL group in the first ROI. As it is seen on the left side of Fig. 2, this protein was pinned with a blue -a letter and defined as superoxide dismutase. In this region, we have also determined another antioxidant protein namely peroxidoxin corresponding to a quite dark and large spot. As we verified with extra runs using narrow pH ranges (pH 4–7 to two samples and pH 5–8 to one sample), peroxidoxin and superoxide dismutase have protein variations whose molecular weight and pI values are too close.

Since the second ROI which was nearer to alkaline side of the gel was expanded by the addition of aligned sections from the

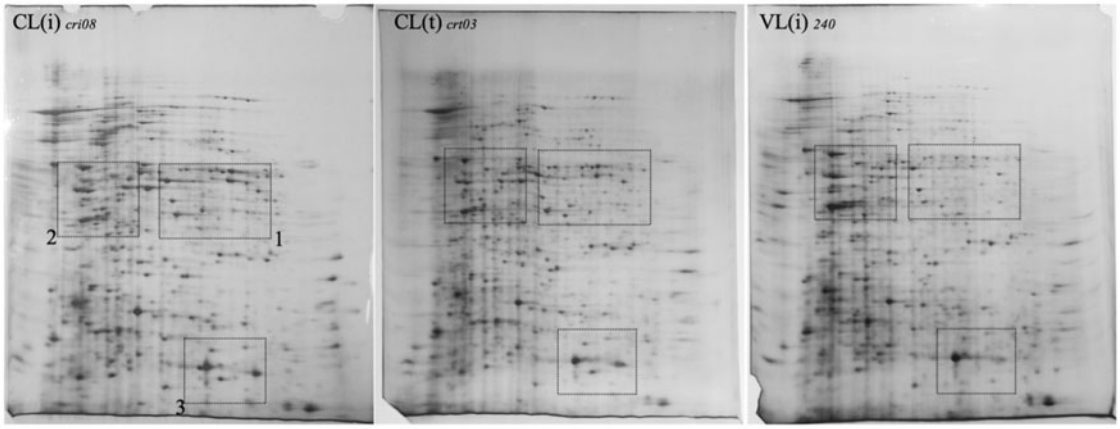


**Fig. 1.** One whole gel image from each group and locations of ROIs as rectangular frames.

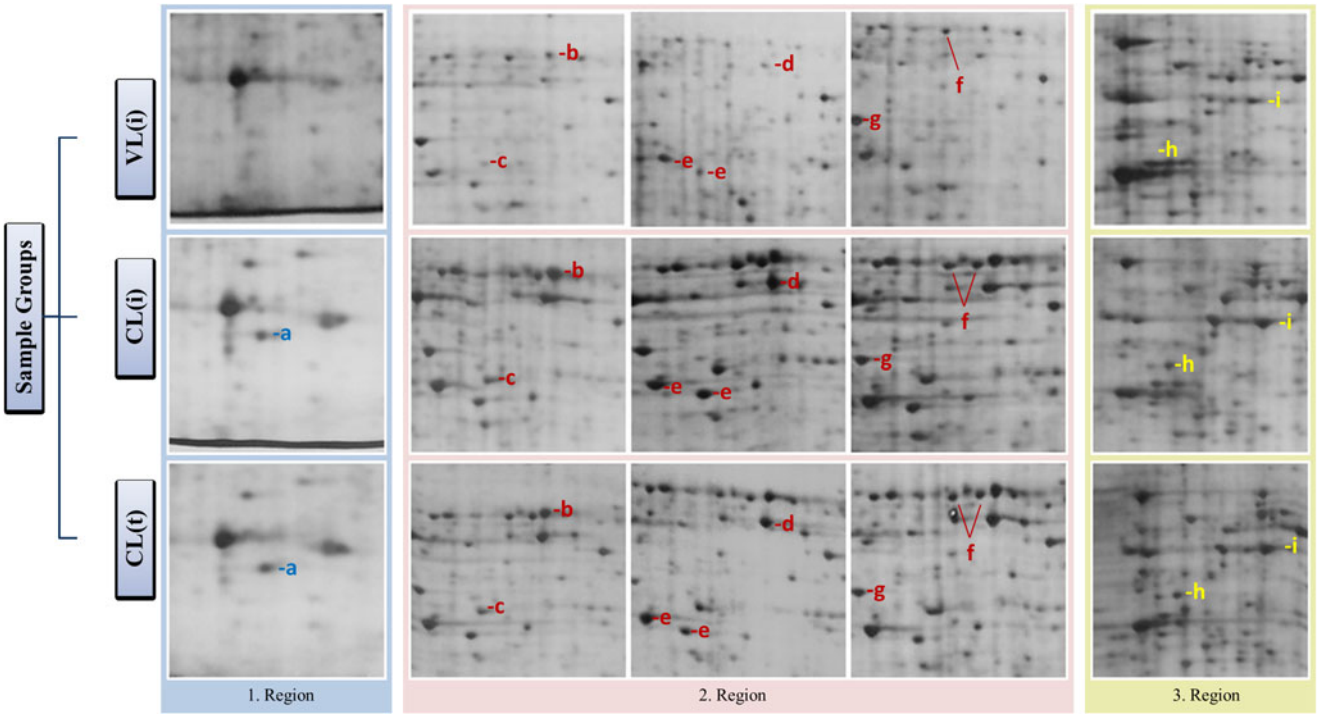


**Fig. 2.** Differentiated spots between the groups.

other samples (Fig. 2) to facilitate the tracking. Herein, letters from -b to -g are corresponding to pyruvate kinase, aspartate aminotransferase, putative succinyl-coA:3-ketoacid-coenzyme A transferase, putative aldose 1-epimerase, putative dihydrolipoamide dehydrogenase and putative eukaryotic initiation factor 4a, respectively. Except for the latter, all of these proteins seemed downregulated mostly in the samples of the VL group (top row).

In the third region, two collateral spots which were represented with the letter -h was identified as putative *N*-acyl-L-amino acid amidohydrolase and distinguished from a very close other spots below namely metallopeptidase (spots 28 and 31; Supplementary material_1). The other protein spot in this region which were downregulated in all VL samples and represented with the letter -i, was identified as enolase.

### *Gel-free approach: shotgun proteomic results*

All shotgun proteomic data were searched against the *L. infantum* proteome database using two different bioinformatic search engines for the cross-check therefore we did not apply the *two-peptide rule* (Gupta and Pevzner, 2009) for the reliable protein identification. After the elimination of contaminant proteins, both searches resulted in more than 1900 *Leishmania* proteins in total. Samples were grouped based on clinical disease forms (CL and VL) they caused, and moreover, samples of CL were divided into two namely CLi and CLt representing *L. infantum* and *L. tropica* to be able to make an interspecific and intraspecific comparison. Tables containing both search findings (the Mascot Search and the Search *via* Search GUI) and the results of statistical comparisons of the groups are given in Supplementary material_2 and Supplementary material_3, respectively. Table 1 shows the number of differentially expressed proteins obtained from all comparisons and the last column contains those of common proteins between the searches which are considered more reliable. To sum up, according to *t*-test with $P < 0.05$ in which the spectrum counting of clinical forms (CL and VL) was compared, a total of 127 proteins differentially abundant were detected in the first search by mascot and 126 proteins were in the second search

**Table 1.** Number of differentially expressed proteins between the clinical forms and the species

| Comparisons and groups (only in CL $n = 6$, for the rest $n = 3$) | No. of proteins in Search-1 | No. of proteins in Search-2 | No. of common proteins |
|---|---|---|---|
| CL *vs* VL | | | |
| CL | 87 | 96 | 31 |
| VL | 40 | 30 | 6 |
| CLi *vs* VLi | | | |
| CLi | 85 | 89 | 25 |
| VLi | 12 | 5 | 2 |
| CLt *vs* VLi | | | |
| CLt | 84 | 75 | 28 |
| VLi | 23 | 14 | 4 |
| CLi *vs* CLt | | | |
| CLi | 19 | 41 | 5 |
| CLt | 19 | 15 | 1 |

run by searchgui. Thirty-seven proteins common in these outputs are given in Table 2. Exceptionally, one protein called malate dehydrogenase was appeared twice with different entry names. In conclusion, among 36 proteins, six proteins were determined differentially abundant in the VL samples, and the rest 30 proteins were more expressed in the CL samples. Finally, a peptide centric comparison was carried out between the CL and VL groups which are provided in Supplementary material_4, in case a specific peptide sequence peculiar to a group might come out.

## Discussion

In the early days of proteomics, 2D gel electrophoresis was the leading method since protein separation or protein isolation was inevitable for the further analysis. With the advent of automatic LC-MS/MS data acquisition, it became possible to detect and identify thousands of protein in a complex mixture thus shotgun proteomic analysis has taken over the lead role gradually in association with the contributions of technological developments. Undoubtedly, the latter is superior to a gel-based method with regards to the number of proteins attained and keeps pushing the limits *via* expensive high-end mass spectrometers and powerful computers. On the contrary, highly abundant soluble proteins mostly get the chance to be examined in a gel-based method; therefore, it addresses to proteoform investigation rather than proteome-level scanning. Apart from that, visualized proteins in the gel with conserved integrity have an idiosyncratic persuasive effect on the expression of that protein.

Herein, proteomic comparison between the two clinical forms of leishmaniasis disease namely visceral and cutaneous has been performed using the abovementioned both gel-based and gel-free proteomic approaches. Although *L. donovani* and *L. infantum* are the main causative agents of the clinic form, VL, they can cause the cutaneous form to a lesser extent. Since *L. infantum* is more common than *L. donovani* in the Mediterranean region including our country, intra-species alteration between the clinical forms was investigated on *L. infantum*. In addition, samples of another common species, *L. tropica* which corresponds to the typical cases for CL in Turkey, was included to obtain an intra-species comparison. Gel-based and gel-free proteomic methods were applied to the promastigote parasites which were isolated from different patients. The first was studied on 15 samples while the latter were contained nine out of those 15 samples.

**Table 2.** Differentially abundant proteins obtained from the comparison of the gel-free analysis (Bold text indicates a significance level of $p < 0.01$)

| No. | Entry name | Protein name |
|---|---|---|
| *Differentially abundant proteins in CL group ($n = 6$)* | | |
| 1 | **A2TEF2** | **Putative lanosterol 14-alpha-demethylase (Sterol-14-alpha-demethylase)** |
| 2 | **A4HTA6** | **Flavoprotein subunit-like protein** |
| 3 | **A4I294** | **Proline dehydrogenase** |
| 4 | **A4I4S1** | **UDP-glucose:Glycoprotein Glucosyltransferase putative** |
| 5 | **A4I9I3** | **Malate dehydrogenase** |
| 6 | **A4ICR0** | **Zinc finger protein family member putative** |
| 7 | **A4HUY0** | **ATPase ASNA1 homologue (Arsenical pump-driving ATPase homologue)** |
| 8 | A4HRH1 | Beta eliminating lyase putative |
| 9 | A4HRR9 | Dipeptylcarboxypeptidase |
| 10 | A4HSK5 | *S*-Methyl-5′-thioadenosine phosphorylase (MTA phosphorylase) |
| 11 | A4HT18 | Coproporphyrinogen oxidase |
| 12 | A4HT47 | Uncharacterized protein family putative |
| 13 | A4HUS2 | Seryl-tRNA synthetase |
| 14 | A4HV24 | Eukaryotic release factor 3 putative |
| 15 | A4I4L8 | Glutamamyl carboxypeptidase putative |
| 16 | A4I4T3 | Aminopeptidase |
| 17 | A4IB12 | FAD:protein FMN transferase |
| 18 | E9AH43 | Fumarate hydratase |
| 19 | E9AH64 | RNA recognition motif putative |
| 20 | E9AHQ2 | Succinyl-CoA:3-ketoacid-coenzyme A transferase |
| 21 | A4IAQ1 | Pyruvate kinase |
| 22 | A4HY91 | Malate dehydrogenase |
| 23 | A4I3V7 | Glycosomal membrane protein putative |
| 24 | A4I6L2 | Mevalonate kinase |
| 25 | A4HRT1 | Multifunctional fusion protein (L-glutamate gamma-semialdehyde dehydrogenase) |
| 26 | Q25299 | Hypothetical protein conserved (ORF2) |
| 27 | A4I8F1 | Glucosamine-6-phosphate isomerase |
| 28 | A4HYZ8 | Putative soluble *n*-ethylmaleimide sensitive factor (SNAP protein putative) |
| 29 | A4HSS8 | Putative glutamine synthetase |
| 30 | A4I4I1 | Hsp70_protein/Tetratricopeptide_repeat putative |
| 31 | A4I3G6 | Ribonucleoside-diphosphate reductase |
| *Differentially abundant proteins in VL group ($n = 3$)* | | |
| 1 | **A4I005** | **I/6 autoantigen-like protein** |
| 2 | **A4I745** | **Metal-ion transporter putative** |
| 3 | A4HVE7 | SURF1-like protein |
| 4 | A4HYQ3 | MORN repeat putative |
| 5 | A4HUJ1 | Hypothetical_protein conserved |
| 6 | A4I7S3 | Guanine nucleotide-binding protein subunit beta-like protein |

The gel images of CL samples exhibited more similar picture regardless of the species while the gel images of VL samples were discriminable from them based on three specific locations according to our visual inspection. Among these regions, one distinct spot which was identified as superoxide dismutase protein was only detected in CL samples. In the gel-free results, we observed that VL samples shared a specific peptide sequence of superoxide dismutase while none of the CL samples were containing it. These findings from two different methods might indicate a structural regulation on this antioxidant enzyme regarding the tissue tropism in the *Leishmania*; therefore, targeted analysis focusing on this protein will provide a deeper understanding. In the second ROI, especially three spots which seemed faded and smaller in the VL samples, repetitively, were considered as downregulation. Two of them, pyruvate kinase and putative succinyl-coA:3-ketoacid-coenzyme A transferase were confirmed by a gel-free method; however, the third one, putative dihydrolipoamide dehydrogenase which, in fact, consists of two lateral spots, did not show up in any comparison results of the gel-free method. Apart from them, two spots identified as aspartate aminotransferase and putative aldose 1-epimerase which can be said partially supported by the gel-free method, looked as if they were downregulated in VL samples too. These two proteins emerged in the list of comparison result of the gel-free method which was based on Mascot database searching. Finally, a protein identified as putative *N*-acyl-L-amino acid amidohydrolase was prominently seen in the CL samples. This protein was observed in the third ROI and encountered in the list of comparison result of the gel-free method which was based on the other database searching with Search GUI.

In a comparative proteomic study which was conducted on promastigote *L. tropica* parasites caused to CL and VL in a dog (Hajjaran *et al.*, 2015), general downregulation in the proteins of energy metabolism and protein synthesis was observed in the VL sample too. This correlation does not rely on the same proteins nevertheless a particular hypothetical protein with the entry name Q4QFN8 was noted. This uncharacterized protein which corresponds to A4HW39 for *L.* infantum with 95% similarity is present in five of the six CL samples according to our shotgun proteomic results.

In the gel-free method, shotgun proteomic data of each sample were analysed by using two different bioinformatic search tools; thus, an ultimate protein list containing more dependable proteins was generated from the output in common. Since we acquired the LC-MS/MS data from quite an outmoded low-resolution mass spectrometer with microlitre LC flow, *two-peptide rule* would not be efficient for our data; therefore, we aimed to compensate these drawbacks partially by confirming the search results with another one. Numerically searching outcomes of the both bioinformatic tools were quite close to each other. Both searchings were resulted in total protein between 1900 and 1930 cumulatively and more than 125 of them were potentially related to clinical forms. The ultimate list of differentially abundant proteins which were constituted from the two searches contained 37 components with specific entry name. As an exception, malate dehydrogenase protein was emerged twice with two different entry names. Consequently, six proteins associated with VL and 30 proteins associated with CL were determined as more reliable differentially expressed proteins. Finally, this list was created in the order of importance by taking into account the *P* value of the *t*-test which means significance level for the first nine proteins was less than 0.01% and similarly *P* was less than 0.03% for the next 14 proteins (2 of them belong to VL).

There might be remarkable findings in this list judging by the studies in the literature of *Leishmania* research. For example, putative lanosterol 14-alpha-demethylase, a protein that participates in steroid biosynthesis deserves special attention due to its connection with the protozoan counterpart of cholesterol, ergosterol. In our results of the gel-free method, this protein was not detected in any of the VL samples while all the CL samples were containing it. In addition, another enzyme related to ergosterol biosynthesis namely mevalonate kinase which protects cells against oxidative stress (Shafi *et al.*, 2021) was observed downregulated in VL samples. Since major antifungal and antileishmanial drugs target the sterol metabolism to destroy the membrane integrity through ergosterol (Emami *et al.*, 2017), drug-resistant parasites seemingly figured out to avoid from this issue by being less predictable on the membrane content. Reduction of ergosterol coupled with alternative sterol formation as well as a mutation on the gene of sterol 14-alpha-demethylase in the amphotericin B-resistant cells supports this notion (Mwenechanya *et al.*, 2017). Moreover, lipid profile changes between *L. infantum* and *L. amazonensis* (Negrão *et al.*, 2017) indicate that alterations on cell membrane enable the parasite to escape from the host's immune system and treatments. A similar mechanism pertaining to ergosterol content can be anticipated between the viscerotropic and dermotropic strains of *L. infantum*.

Flavoprotein subunit-like protein and proline dehydrogenase proteins were never detected in any VL samples while expressed in all six CL samples. Up-regulation of the first was shown in some drug-resistant *L. infantum* strains (Vincent *et al.*, 2015). In addition to proline dehydrogenase, a more expression of a second proline degradation protein, multifunctional fusion protein (A4HRT1) might be related to the high proline content in the skin collagen (Shoulders and Raines, 2009). Similar to these, I/6 autoantigen-like protein was like a determinant protein for the VL group since none of the CL samples contained it while it was expressed in all VL samples. Considering the importance of glycosylation on protein recognition and cell adhesion (Mule *et al.*, 2020), UDP-glucose: glycoprotein glucosyltransferase putative (A4I4S1) might be noteworthy to understand the dermotropic strains in *L. infantum*. Similarly, ion transporters are crucial membrane proteins for nutrient uptake; therefore, occurrence of metal-ion transporter protein (A4ICR0) only in viscerotropic strains must have vital implications for the organism survival in an inner organ. Indeed, more evidence is needed to claim their relevance to clinical forms *per se*. Apart from the protein-centric comparison, a qualitative peptide-centric comparison was provided in the case of being used in an immunoassay or targeted proteomics someday.

To conclude, proteomic comparison in which capacity and perspectives of gel-based and gel-free proteomic analyses can be evaluated is presented in this study to gain more insight about *Leishmania* parasites once caused to VL and CL in people.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021001967

**Data.** All mass spectrometric data have been deposited at the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD016333 and 10.6019/PXD016333.

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