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2 **Profiling inflammatory response in lesions of cutaneous leishmaniasis patients using a non-**
3 **invasive sampling method combined with a high-throughput protein detection assay**
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31 Highlights

32 • A non-invasive sampling method combined with a multiplexed protein assay was
33 developed for evaluation of cytokine and chemokines in the lesions of cutaneous
34 leishmaniasis patients.

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37 • Various inflammatory cytokines, chemokines and other proteins were detected in the
38 lesions of cutaneous leishmaniasis patients.

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41 • This non-invasive sampling method combined with a multiplexed protein assay has
42 implications for evaluation of skin inflammatory responses in cutaneous leishmaniasis
43 and other skin diseases.

44

45

46 **Abstract:**

47

48 **Background:**

49 Cutaneous leishmaniasis (CL) is an infection caused by *Leishmania* (*L.*) protozoa transmitted
50 through the bite of infected sand fly. Previously, invasive sampling of blood and skin along with
51 low throughput methods were used for determination of inflammatory response in CL patients.

52 **Aims/Methodology**

53 We established a novel approach based on a non-invasive adhesive tape-disc sampling combined
54 with a powerful multiplexing technique called proximity extension assay for profiling 92
55 inflammatory cytokines, chemokines and surface molecules in the lesions of CL patients infected
56 with *L. tropica*. Sample collection was done non-invasively by using adhesive tape-discs from
57 lesion and normal skin of 33 *L. tropica* positive patients.

58 **Results**

59 Out of 92 inflammatory proteins, the level of 34 proteins was significantly increased in the
60 lesions of CL patients compared to their normal skin. This includes the chemokines CCL2,
61 CCL3, CCL4, CXCL9, CXCL10, CXCL11 and CXCL5, together with the interleukins
62 IL-18, IL-8, IL-6, LIF and OSM. The remaining significantly changed inflammatory proteins
63 include 7 surface molecules and receptors: CD40, CDCP1, 4E-BP1, TNFRSF9, CD5, IL-18R1
64 and OPG as well as 16 other cytokines and proteins: MMP-1, CSF-1, VEGFA, uPA, EN-RAGE,
65 LAP TGF- β 1, HGF, MMP-10, CASP-8, TNFSF14, STAMPB, ADA, TRAIL and ST1A1.
66 Further, 13 proteins showed an increasing trend, albeit not statistically significant, in the CL
67 lesions, including TGF- α , CCL23, MCP-2, IL-12B, CXCL6, IL-24, FGF-19, TNF β , CD6,
68 TRANCE, IL10, SIR2 and CCL20.

69 **Conclusion**

70 We herein report a novel approach based on a non-invasive sampling method combined with the
71 high-throughput protein assay for profiling inflammatory proteins in CL lesions. Using this
72 approach, we could profile inflammatory proteins in the lesions from CL patients. This new non-
73 invasive approach may have implications for studying skin inflammatory mediators in CL and
74 other skin disorders.

75 **Keyword**

76 Cutaneous leishmaniasis, non-invasive sampling, Proximity extension assay, Inflammation
77 biomarkers

78

79 **Abbreviation**

80 ADA: Adenosine deaminase

81 ARTN: Artemin

82 Beta-NGF: Nerve growth factor

83 CSF-1: Colony stimulating factor 1

84 CST5: Cystatin D

85 DNER: Delta/Notch Like EGF Repeat Containing

86 4E-BP1: Eukaryotic initiation factor 4E-binding protein 1

87 EN-RAGE LAP (TGF-beta-1): Advanced glycation end-products binding protein

88 FGF: Fibroblast growth factor

89 GDNF: Glial cell line-derived neurotrophic factor

90 HGF: Hepatocyte growth factor

91 LIF: Leukemia inhibitory factor; IL-6 family

92 MCP-1: Monocyte chemoattractant protein-1

93 MMP: Matrix metalloproteinase

94 NRTN: Neurturin

95 OPG: Osteoprotegerin, member of TNF family

96 OSM: Oncostatin M, IL-6 family

97 PDL-1: Program death legend 1

98 ST1A1: Sulfotransferase1A1
99 STAMPB: STAM binding protein
100 SIR2: SIR 2 like protein 2
101 TNFRSF: Tumor necrosis factor receptor superfamily member
TGF- β : Transforming growth
102 factor-beta
103 TRAIL: TNFSF10
104 TRANCE: TNF-related activation-induced cytokine
105 uPA: Urokinase-type plasminogen activator
106 VEGFA: Vascular Endothelial Growth Factor A
107

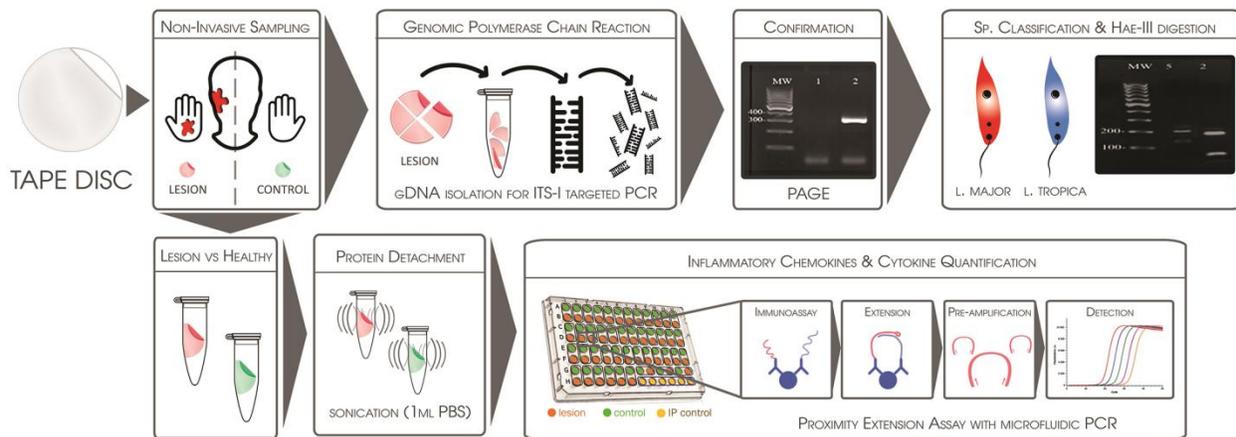
108 **1. Introduction**

109 Cutaneous leishmaniasis (CL) is a skin infection caused by *Leishmania* protozoa transmitted by
110 the bite of an infected sand fly. More than twenty different *Leishmania* species can cause the
111 disease [1]. As a multifaceted disease, CL has different clinical manifestations ranging from self-
112 healing papules to permanent non-healing lesions. Although CL is not a fatal disease, it causes
113 serious socio-economic burden in endemic areas. CL infections are mostly zoonotic with the
114 exception of the infection caused by *L. tropica*, which is often anthroponotic. Following the sand
115 fly bite, the outcome of the disease is dependent on the extent to which the parasite propagates,
116 the host immune response, and the sand fly characteristics. Studying the site of infection is
117 pivotal in understanding the underlying mechanism of the disease and the host immunity. Due to
118 restrictions associated with collection of skin biopsies from humans, including ethical limitations
119 and the risk of secondary infection, the majority of the previous studies were centered on the use
120 of mouse models of the disease [2-8]. However, some studies have limitedly investigated the
121 immune responses in the skin lesions of CL patients [8-13]. There is currently limited
122 information available on the immunological responses in the skin lesions of CL patients.

123 Upon injection of the metacyclic form of the parasite into the skin, various inflammatory cells
124 such as macrophages, monocytes, neutrophils and dendritic cells are considered to play roles in
125 immunity to infection and tissue damage [14]. *Leishmania* parasites are rapidly engulfed by
126 infiltrating neutrophils that are deemed to act as the first target cells. Macrophages are among the
127 first inflammatory cells that encounter the parasites, which may kill the parasites by production
128 of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [15]. Dendritic cells were
129 also shown to play a key role in the immunity to the parasite [16]. It is clear that the balance
130 between Th1 and Th2 responses is critical in the control of the disease. A Th1 response is

131 considered to contribute to clearing the infection, whereas a Th2 dominated response is deemed
 132 to be associated with non-healing cases [17, 18]. We have recently developed a new non-
 133 invasive tape-disc sampling method for the diagnostics of CL [19, 20]. Building on this
 134 approach, we herein established the potential of this tape-disc sampling method combined with a
 135 high throughput multiplexing protein detection assay (Proximity Extension Assay technology)
 136 [21, 22] for studying the host inflammatory response to *L. tropica* infection in human skin
 137 lesions. This novel non-invasive approach has implications for studying local immune responses
 138 in skin diseases. A schematic representation of the approach employed in the present work is
 139 summarized in Figure 1.

140



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142

143 Fig. 1: A schematic representation of tape-disc sampling method combined with Proximity
 144 Extension Assay and ELISA for assessment of inflammatory mediators and specific antibody in
 145 the lesions of CL patients, respectively.

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147

148 **2. Materials and Methods**

149 **2.1. Ethics**

150 This study was authorized by the Ethics committee of the Pasteur Institute of Iran 27/01/2019,
151 TP-9566. A written informed consent form was signed by the patients or their guardians (for
152 those with 18 years of age) before sampling.

153

154 **2.2. Study design, sample collection and diagnostics**

155 A cross-sectional study enrolling 33 CL patients (25 acute and 8 chronic cases) with total of 66
156 specimens (including lesions and their healthy skin counterparts) was conducted. The acute cases
157 were determined as those with active CL lesions for less than 12 months, while chronic cases
158 were those with active CL lesions for one year or more. Acute cases with no treatment and
159 treated chronic cases were selected for this study. Sampling was done in Abobargh health care
160 center for CL, North Khorasan province, Mashhad. This area is highly endemic for *L. tropica*,
161 and patients are referred to this center for diagnosis and receiving their free medication and
162 follow-up of their treatment. Sample collection from lesions and normal skins was done non-
163 invasively by using 22mm adhesive tape-discs (D-squame, CuDerm Corporation, Texas, USA)
164 as described previously [19]. Samples were stored at -20°C until further use. Genomic DNA
165 from the tape- discs was extracted using DNeasy Qiagen Blood and tissue kit (Qiagen,
166 Germany), and subsequently subjected to *L. tropica* specific PCR-RFLP as previously described
167 [19].

168

169 **2.3. Protein quantification using multiplex inflammation panel**

170 Supernatants retrieved from tape disc samples of CL lesion samples and their healthy skin
171 counterparts were analyzed using the Olink 92-plex Inflammation panel (performed by Olink
172 Bioscience, AB). This panel includes 92 chemokines, cytokines and surface proteins involved in
173 inflammation. Briefly, tape-discs from lesions and healthy skins were sonicated in 1ml of sterile
174 Phosphate Buffer saline (PBS, pH 7.4) for 10 minutes. The supernatant was collected and
175 divided into 100µl aliquots and stored at -20°C until further use. Protein suspensions (25µl) were
176 quantified using PEA and normalized with inter-plate controls and internal analyte controls to
177 generate normalized protein expression values (NPX) in a log₂ scale. PEA is based on a pair of
178 oligonucleotide strands coupled with specific antibodies which pairwise bind to the target
179 biomarker. Polymerase chain reaction (PCR) was carried out by adding DNA polymerase to the
180 PEA mixture [22].

181

182 **2.4. Antigen lysate preparation for ELISA**

183 *L. tropica* (MOHM/IR/Khamesipour-Mashhad) parasites were cultured at 26°C in Medium 199
184 (M199; Sigma), pH 7.2, supplemented with 10% heat-inactivated FCS (Gibco), 0.1mM
185 adenosine (Sigma), 40 mM HEPES (Sigma), and 50µl/ml gentamicin (Sigma). *Leishmania*
186 parasites were retrieved by centrifugation and washed in PBS (1000 g/10min), and 3x10⁸
187 parasite/ml were used for 10 times freeze-thaw (liquid nitrogen followed by 37°C water bath)
188 cycles. Pierce™ BCA Protein Assay Kit (Thermo fisher scientific) was used to measure the total
189 protein concentration of the parasite lysate.

190

191 **2.5. Detection of *L. tropica* specific IgG antibodies**

192 The sonicated tape-disc supernatant was used for detection of *L. tropica* specific IgG antibody
193 [23]. MaxiSorp flat bottom 96-well plate (Sarstedt) was coated with 10µg/ml of *L. tropica* lysate
194 in 100 µl/well and incubated at 4°C overnight. Un-bound leishmanial antigens were washed three
195 times with 300µl/well of 0.05% Tween 20 (Sigma) in PBS. To reduce the non-specific binding,
196 the wells were blocked with 300 µl/well of 1% IgG-free bovine serum albumin (BSA; Sigma) in
197 PBS for one hour at room temperature. The wells were washed three times, and 100 µl of 1:2.5
198 diluted disc supernatants with dilution buffer (0.05% Tween and 1% BSA in PBS) was added to
199 each well and incubated for one hour at room temperature. The plate was washed and then 100
200 µl/well of Horseradish Peroxidase (HRP) conjugate Goat-Anti-human IgG Fc antibody (diluted
201 1:20000 with blocking solution) (Sigma) was added and incubated in dark for 1 hour. The plate
202 was then washed 4 times, and 100 µl/well HRP substrate, TMB (ebioscience) was added. After
203 15 minutes, 100 µl/well of 1N H₂SO₄ was added to stop the reaction and the plates were read at
204 450 nm absorbance (BioTek, ELx800).

205

206 **2.6. Statistical analysis and data visualization**

207 Age distribution of patients was reported as mean ± SD. Statistical comparison of differences
208 between lesion and healthy skin was performed using Mann-Whitney U test (Graph Pad Prism
209 version 5.0) for both PEA and ELISA assays.

210 PEA analyses were performed separately for acute and chronic lesions compared to healthy skin
211 samples using Mann-Whitney U test in R version 3.6.0 (R Core Team; 2019). The PEA analysis
212 results were then visualized in a Circos plot generated using CIRCOS v 0.69-6 [24] to visualize

213 the similarities and differences between inflammation related proteins in the lesional samples and
214 the normal samples. *P*-values < 0.05 were considered statistically significant.

215

216

217 **3. Theory/Calculation**

218

219 Cutaneous leishmaniasis patients suffer from delayed diagnosis, mis-diagnosis and consequently
220 a postponed treatment which may cause severe tissue damage and the formation of hypertrophic
221 scars on the patient's skin for the rest of their lives. It could be due to the lack of knowledge
222 related to the immunological interactions. Nowadays, finding proper biomarkers as an early
223 diagnostic tool is a need for various diseases. Biomarkers are mostly found in blood and body
224 fluids, most likely due to the ease of sample collection, and in the case of CL they need to be
225 investigated in the lesions. In this work we report development of a non-invasive sampling
226 method combined with a high-throughput protein detection assay for profiling proteins involved
227 in the inflammatory response on CL lesions, which may be beneficial for studying inflammatory
228 proteins in other skin diseases alike.

229

230 **4. Results**

231

232 **4.1. Diagnosis determination of CL patients**

233 Twenty five acute patients, including 14 men and 11 women, with mean age of 27.5 (IQR: 7.5,
234 47) and median lesion duration of 4 months and 8 chronic patients, including 5 men and 3
235 women, with mean age of 44.5 (IQR: 11, 51.75) and median lesion duration of 29 months were
236 included in the study (Table 1). To differentially diagnose the *Leishmania* species, DNA samples
237 isolated from 33 non-invasively collected tape-discs obtained from acute and chronic patients
238 were subjected to *L. tropica* specific PCR-RFLP. All 33 collected samples were diagnosed and
239 characterized as *L. tropica* (Data not shown).

240

241

242 Table 1: Characteristic profile of *L. tropica* leishmaniasis patients

Type of lesion	Age, median, year (IQR)	Sample size n (% male)	Duration of lesion (median, month)	Lesion location	Number of Lesion	Number of cases
Acute	27.5 (IQR: 7.5, 47.5)	25 (56)	4	Head: 11 Hand: 15 Foot: 2	Single: 14 Multiple: 11	25
Chronic	44.5 (IQR: 11, 51.75)	8 (62)	21	Head:6 Hand:2 Foot:2	Single: 5 Multiple: 3	8

243

244 Table 1: Characteristic profile of *L. tropica* infected cutaneous leishmaniasis patients. Values are
245 presented as median (IQR 25- 75), IQR: Interquartile range.

246

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248 **4.2. Profiling inflammatory-related proteins in acute and chronic CL lesions**

249 To investigate the difference in expression of inflammatory proteins between lesions and their

250 normal skin, 92 soluble factors included in Olink® inflammatory panel were selected. The disc

251 supernatants obtained from lesion and normal skin samples were subjected to the PEA

252 inflammatory panel.

253 Out of the 92 measured proteins, 34 (37%) proteins showed significantly ($P<0.05$; Mann-

254 Whitney U test) higher levels in the acute lesions compared to their normal skin counterparts

255 (Fig 2 and Fig 3a). These proteins are involved in Apoptotic process, Cell activation involved in
256 immune response, Cell adhesion, Cellular response to cytokine stimulus, Chemotaxis,
257 Extracellular matrix organization, Inflammatory response, MAPK cascade, Regulation of
258 immune response, Response to hypoxia, Secretion, and other biological processes.

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285 Fig 2: CIRCOS plot depicting similarities and differences in the levels of the inflammation-
286 related proteins in lesions of acute and chronic CL patients compared to normal skin. Red lines:
287 $P < 0.01$, orange lines: $P < 0.05$ and yellow lines: $P < 0.1$. Outer circles represent the family group
288 of each protein, including Chemokines, Interleukins, other cytokines and proteins and also
289 Surface Molecules and Receptors.

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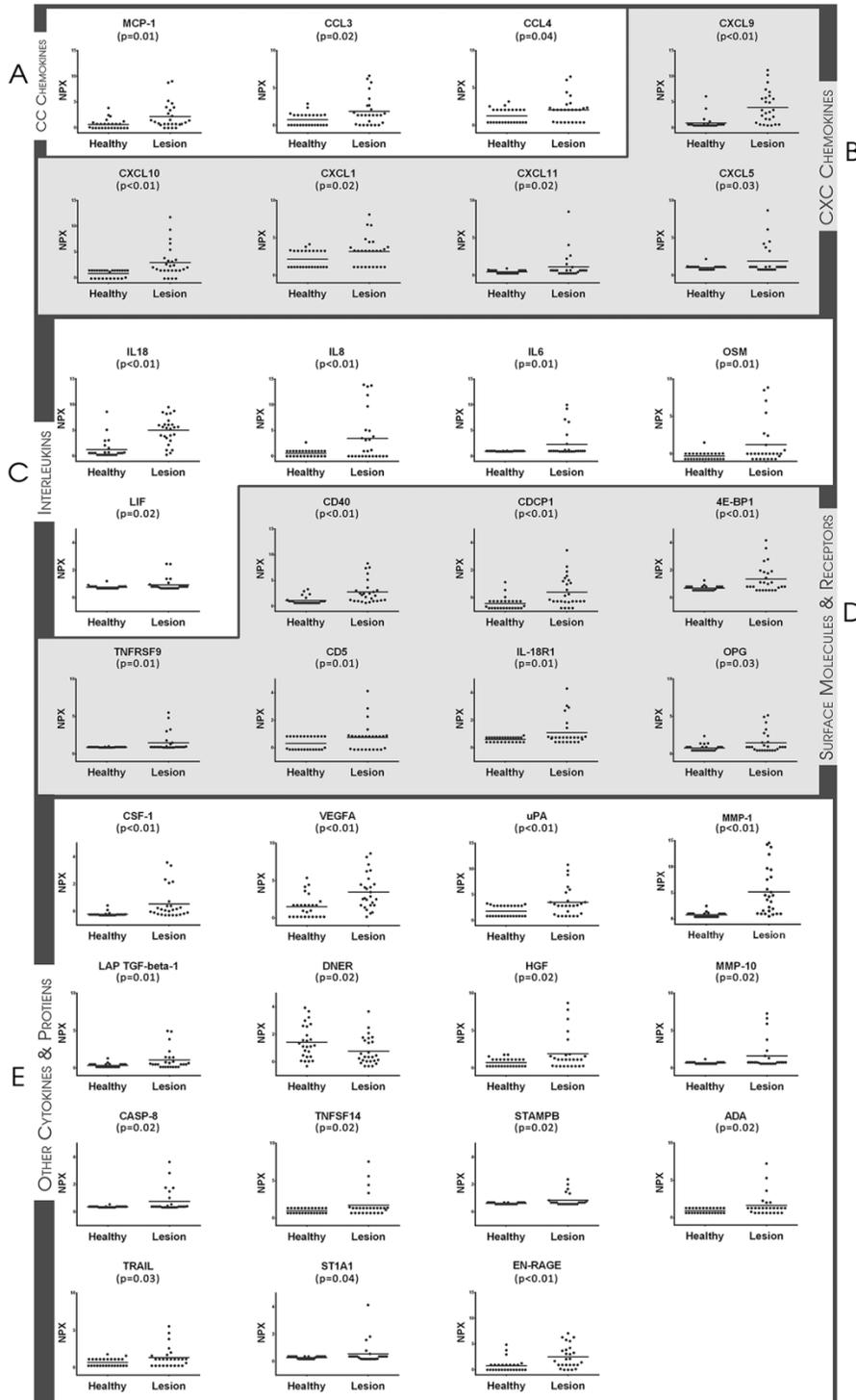
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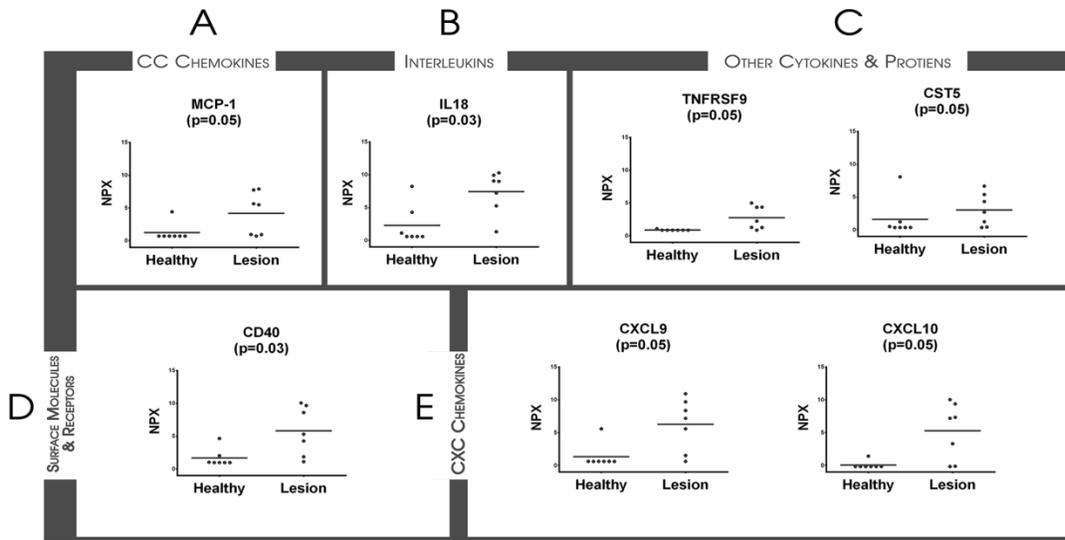
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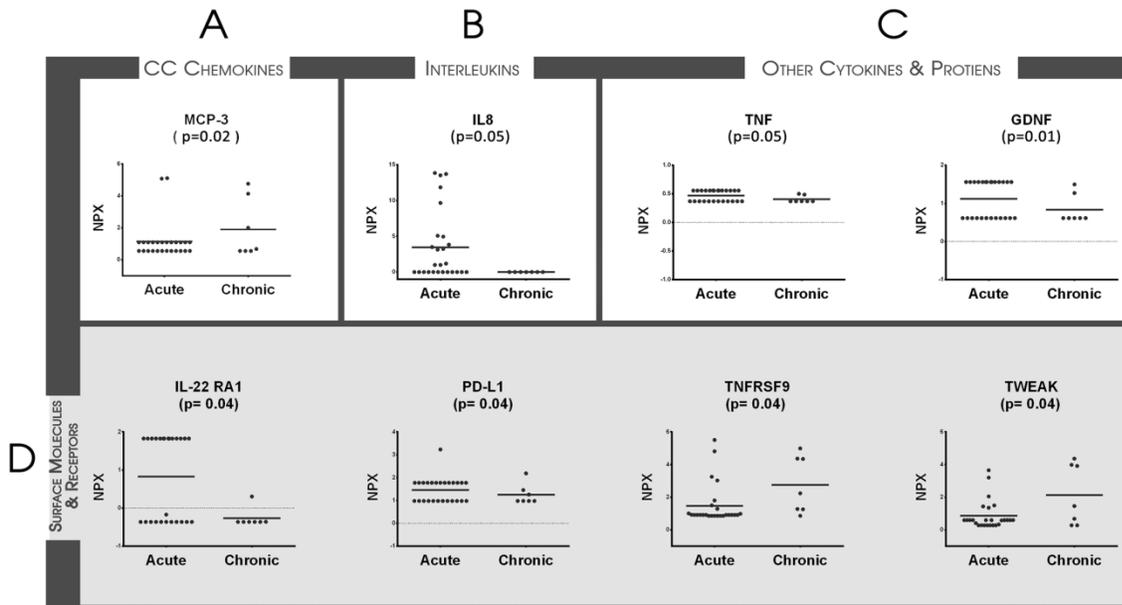


314 Figure 3b:



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316 Figure 3c:



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318 Figure 3: The inflammation-related proteins significantly changed in the lesions of the CL
319 patients compared to their healthy skin counterparts. Normalized protein Expression Units
320 (NPX) values of healthy skin compared to leishmaniasis lesion are plotted. Proteins are classified
321 into five groups based on their functions. A. CC Chemokines; B. CXC Chemokines; C.
322 Interleukins; D. Surface molecules and receptors; E. other cytokine and proteins. *P*-value of
323 $P < 0.05$ was considered statistically significant and the exact *p*-value is indicated in each graph.
324 a: Acute patients' (Lesion, n=25) inflammation-related proteins compared to their own healthy
325 skin (Healthy, n=25). Fig 3b: Chronic patients' (Lesion, n=8) inflammation-related proteins
326 compared to their own healthy skins (Healthy, n=8).
327 Fig 3c: Comparison of inflammation-related proteins in acute lesion-healthy skin (n=25) to
328 chronic lesion-healthy skin (n=8). Both lesion groups have been corrected by using respective
329 healthy skin.

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333 Among these proteins, eight were chemokines: MCP-1 (CCL2), CCL3, CCL4, CXCL9,
334 CXCL10, CXCL11 and CXCL5, together with five interleukins: IL-18, IL-8, IL-6, LIF
335 and OSM (Fig 2 and Fig 3a).

336 The other important groups of proteins which showed enhanced levels in the acute lesions
337 include the surface molecules and receptors CD40, CDCP1, 4E-BP1, TNFRSF9, CD5, IL-18R1
338 and OPG, together with 16 cytokines and other proteins consisting of MMP-1, MMP-10, CSF-1,
339 uPA, EN-RAGE, LAP TGF- β 1, HGF, VEGFA, CASP-8, TNFSF14, STAMPB, ADA, TRAIL
340 and ST1A1 (Fig 2, Fig 3a).

341 Further, 13 proteins showed an increasing trend, albeit not statistically significant: CCL20,
342 CCL23, CXCL6, MCP-2, IL-12B, IL-24, FGF-19, TGF- α , TNF- β , CD6, TRANCE, IL-10RB
343 and SIR2. DNER was the only marker with significant decrease in the lesions (Fig 2 and Fig 3a).

344 In chronic patients, 7 proteins had significantly higher levels of expression in the lesions as
345 compared to their healthy skin counterparts (Fig 2 and Fig 3b). These include CCL2, CXCL9,
346 CXCL10, IL-18, CD40, TNFRS9 and CST5 (Fig 2 and Fig 3b). CST5 was, however, exclusively
347 detected in the chronic lesions compared to healthy skin and acute ones. IL-8, IL-22 RA1, PD-

348 L1, TNF and GDNF showed significantly increased levels in the acute lesions compared to those
349 of the chronic lesions. MCP-3, TNFRSF9 and TWEAK were decreased significantly in the acute
350 lesions (Fig 3c).

351 It is worth mentioning that amongst 92 cytokines and chemokines measured, 15 of them
352 including interleukins IL-2, IL-5, IL-7, IL-13 and IL-33, some receptors such as IL-10RA, IL-
353 2RB and ILF-R, cytokines: IFN- γ , TSLP and a group of growth factor related proteins such as
354 ARTN, NRTN, Beta-NGF and FGF-5 were found to be undetectable in 99% of the samples in
355 both lesions and their healthy control counterparts.

356

357 **4.3. Evaluation of *L. tropica* specific IgG antibody in the tape-discs from CL lesions**

358 Next, we sought to evaluate the potential of the tape-disc sampling method in the retrieval of *L.*
359 *tropica* specific IgG antibodies from lesions of acute and chronic CL patients. To do this, the
360 supernatants of tape-discs collected from the lesions and their normal skin counterparts were
361 subjected to *L. tropica* specific IgG antibody ELISA. *L. tropica* specific IgG antibody was
362 detected in 17 out of 25 acute patients' samples, and in 6 out of 8 chronic cases (Fig 4). This
363 result shows the potential of the tape-disc sampling for evaluation of *L. tropica* specific IgG
364 antibody responses in CL lesions.

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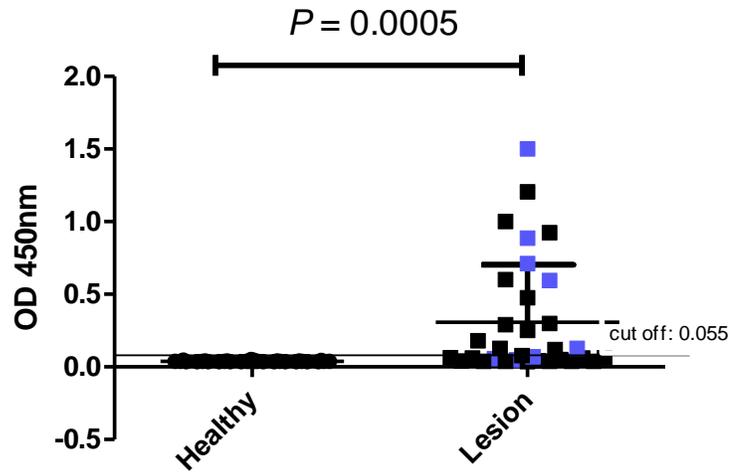
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371 Figure 4:

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375 Figure 4: *L. tropica* specific IgG antibody levels in the lesions of acute and chronic CL patients.
376 Tape-discs' supernatants retrieved from lesions and healthy skins of acute (n=25; colored in
377 black) and chronic (n=8; colored in blue) cutaneous leishmaniasis patients (n=33). Squares
378 represent the average mean and SD in duplicate. Cut- off value was adjusted three times higher
379 than the mean absorbance obtained from normal skins.

380

381

382 **5. Discussion**

383 We herein report, for the first time, the establishment of a non-invasive tape-disc sampling
384 method combined with a multiplexed protein detection assay for the assessment of inflammatory
385 mediators in the skin lesions of CL patients. Using this novel approach, we have observed
386 significant differences in the expression of five important groups of inflammatory proteins in the
387 lesions of acute CL patients compared to their normal skin. These include CC chemokines, CXC
388 chemokines, interleukins, surface molecules and immune receptors. This finding is in line with
389 previous reports on the recruitment of monocytes, macrophages, DCs, NK cells and T cells to the
390 site of CL infection [25]. The CXC chemokines CXCL1, CXCL9, CXCL10 and CXCL11
391 showed significantly higher levels in the acute CL lesions, CXCL1 acts as an activator and
392 recruiting agent of neutrophils into the CL lesion [26]. CXCL9, CXCL10 and CXCL11 have
393 previously been shown to be up-regulated in localized CL caused by *L. braziliensis*. Conversely,
394 these chemokines were reported to be down-regulated in diffuse CL caused by *L. amazonensis*
395 [27]. The CC chemokines CCL2, CCL4 and CCL5 were reported to serve as putative biomarkers
396 of high parasite load in the lesions of *L. infantum* infected dogs [28]. Interleukins whose
397 expression increased significantly in CL lesions include IL-6, IL-8, IL-18, OSM and LIF. The
398 skin level of IL-6 was previously reported to be elevated in CL [11], psoriasis and leprosy [29].
399 IL-8 has been shown to play a major role in tissue damage in CL [30]. However, the role of the
400 Th1/Th2 regulating cytokine IL-18 in the healing process of CL lesion is controversial [17, 30].
401 IL-18/IL-18R interaction promotes activation of NK cells, and induces the production of IFN- γ ,
402 and was shown to contribute to protection against *L. major* [31]. The members of IL-6 family,
403 including OSM, LIF and IL-6 were shown to be involved in skin inflammatory diseases such as
404 psoriasis and atopic dermatitis [32].

405 We could also show an increasing trend in the protein levels of several surface molecules and
406 receptors such as 4E-BP1, CD-40, TNFRSF9, OPG, CDCP1, CD5, and IL-18R1. 4E-BP1 has
407 been reported to promote the persistence and survival of the parasite in CL lesions [33]. CD40 as
408 a member of TNFR superfamily plays an important role in antigen presentation to control CL
409 [34]. CD5⁺ B cells have been shown to increase in the active phase of the disease [35]. The other
410 cytokines and proteins which showed significantly increased levels in the lesions of the CL
411 patients were CSF1, VEGFA, uPA, EN-RAGE, LAP TGF- β 1, DNER, HGF, MMP-1, MMP-10,
412 CASP-8, TNFSF14, STAMPB, ADA, TRAIL and ST1A1. The CSF1 and its receptor signaling
413 have been shown to play a crucial role in development of dermal macrophages [36]. The
414 expression of VEGFA and its receptor was reported in CL lesions [37]. The inflammatory
415 mediator TNFSF14 was shown to contribute to the control of cutaneous and visceral
416 leishmaniasis [38]. The pro-inflammatory antimicrobial EN-RAGE was reported in the brain of
417 mice infected with *L. amazonensis* [39]. MMP-1 has been reported as one of the most highly up-
418 regulated proteins in CL [40]. The serine protease uPA was found in tissue extracellular matrix,
419 and is expressed in the amastigote of *Leishmania* parasites [41]. TRAIL accompanied by FAS-
420 ligand was found to mediate keratinocyte damage, and was detected in the lesions of *L.*
421 *aethiopica* infection [42]. An increased ADA level was also reported in the lymphocytes of CL
422 patients [11].

423 Blockade of CASP-8 has been reported to enhance the immunity against *L. major* infection [43].
424 The activation of LAP TGF- β 1 has been reported in pathology of CL [44]. Of note, DNER
425 involved in Notch pathway [45] was the only mediator tested in our study with a significantly
426 decreased level in the lesions of the CL patients. The down-regulation of DNER may result in

427 the inhibition of Notch signaling pathway, which may contribute to the tissue damage observed
428 in the lesions of CL patients.

429 In the case of chronic patients, seven proteins including CCL2, CXCL9, CXCL10, IL-18, CD40,
430 TNFRS9 and CST5 (cystatin D) were shown to have significant increasing rate in the lesions
431 comparing with their normal skin counterparts.

432 By comparing the profile of inflammatory mediators in acute and chronic lesions, IL-8, IL-22
433 RA1, PD-L1, TNF and GDNF showed significantly increased levels in the acute lesions. On the
434 other hand, MCP-3, TNFRSF9 and TWEAK showed significantly decreased levels. .

435 Up-regulation of MCP-3/CCL7 was reported in the diffuse form of CL, and was suggested to be
436 involved in the recruitment of immune cells during different phases of the disease. IL-8, IL-22
437 RA1, PD-L1, TNF and GDNF were found to have significantly higher levels in the acute CL
438 lesions compared to those of the chronic. MCP-3, TNFRSF9 and TWEAK were found to have
439 significantly lower levels in the acute lesions. IL-22RA1 has been shown to play an important
440 role in wound healing [46]. PDL1 was shown to regulate immune response against leishmaniasis
441 caused by *L. mexicana* [47].

442 We observed an increase in the expression of OSM, TNFRSF9, OPG, CDCP1, MMP-10,
443 ST1A1, HGF, CST5, TWEAK, GDNF and IL-22RA1 in CL. We also demonstrated the potential
444 of our approach in detection of *Leishmania* specific IgG antibodies in the lesions of CL patients.

445

446 **6. Conclusion**

447 Herein, we report the development of a non-invasive sampling method combined with a high-
448 throughput protein detection assay for profiling proteins involved in the inflammatory response
449 in *L. tropica* caused lesions. Using this approach, we could profile inflammatory proteins in the

450 lesions from both active and chronic CL patient. Our data demonstrate that this non-invasive
451 sampling method could be useful for identifying biomarkers for CL, albeit this approach would
452 need to be evaluated and validated in a larger study in which control groups are included for
453 other inflammatory/ulcerative skin lesions.

454

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478 **References**

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*Declaration of Interest Statement

We confirm that the manuscript was approved by all authors and the authors declare no conflict of interest, and that the results presented in this manuscript have neither been published nor under consideration for publication elsewhere.

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