AAC Accepts, published online ahead of print on 31 March 2014
Antimicrob. Agents Chemother. doi:10.1128/AAC.02482-14
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1 **Host Biomarkers of Invasive Pulmonary Aspergillosis to Monitor Therapeutic**
2 **Response**
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19 **ABSTRACT**

20 Invasive pulmonary aspergillosis (IPA) is a life threatening disease of
21 immunocompromised patients that requires aggressive therapy. Detection and monitoring
22 therapeutic response during IPA is complex and current molecular diagnostics are not
23 suitably robust. Here, we explored proteomic profiles of bronchoalveolar lavage fluid
24 (BALF) specimens from a persistently neutropenic rabbit model of IPA. Three
25 experimental arms: uninfected control animals, infected untreated animals, and animals
26 infected and treated with ravuconazole/amphotericin B were studied. Total proteins were
27 evaluated by 2D gel electrophoresis, followed by matrix-assisted laser
28 desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) mass
29 spectroscopy (MS) and quantified by enzyme-linked immunosorbent assay (ELISA).
30 Host derived proteins: Haptoglobin (Hp), C-reactive protein (CRP) and Annexin A1 (Anx
31 A1) were prominently found in BALF during the IPA infection and showed significant
32 changes in response to antifungal therapy ($p < 0.0001$). In serum differences in Hp ($p =$
33 0.0001) were observed between infected and treated rabbits. Preliminary *in vitro* studies
34 revealed that *Aspergillus fumigatus* secreted proteases may contribute to the cleavage of
35 Anx A1 during IPA. In summary, host protein biomarkers Hp, CRP and Anx A1 may
36 have value to monitor therapeutic response to antifungal agents in IPA patients with
37 confirmed disease.

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39 **INTRODUCTION**

40 Invasive pulmonary aspergillosis (IPA) caused by *Aspergillus fumigatus* is a
41 devastating disease for immunocompromised patients. Successful management of
42 patients depends on early diagnosis, effective therapy and monitoring of therapeutic
43 response. Detecting IPA and monitoring response to therapy still remains very difficult
44 especially in early stages of the disease. Currently, according to the European
45 Organization for Research and Treatment of Cancer/Invasive Fungal Infections
46 Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses
47 Study Group (EORTC/MSG), diagnosis of IPA relies on a positive CT scan, culture
48 and/or microscopic evidence of disease, and detection of *A. fumigatus* antigens in a
49 susceptible host (1).

50 The use of surrogate markers of infection is an important adjunct to diagnosis of
51 IPA. The galactomannan immunoassay is commonly used in the diagnosis of this
52 infection in patients. Yet this test has limitations for monitoring therapeutic response and
53 it has a potential for false-positive results (2, 3, 4). In recent years, improved
54 methodologies have been developed for detection of *Aspergillus* nucleic acid in blood
55 and bronchoalveolar lavage fluid (BALF). Developing additional biomarkers could
56 complement existing diagnostic methods by helping to improve detection of IPA and
57 monitoring response of at-risk patients to therapy.

58 During the past several years, proteomic techniques have been used to study *A.*
59 *fumigatus* during invasive infection (5, 6). However, less is known about the host
60 proteomic profile in response to infection with *Aspergillus*. Proteomic analysis of primary

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61 specimens such as BALF and serum can provide an understanding of complicated host-
62 pathogen interactions during IPA.

63 In this pilot study, we profiled the proteome of BALF and serum samples from an
64 experimental rabbit model of IPA. Prominent host biomarkers were identified following
65 *Aspergillus* infection and were useful in assessing therapeutic response to antifungal
66 agents.

67

68 **MATERIALS AND METHODS**

69 **Animal model**. A well described (7) persistently neutropenic rabbit model of IPA
70 was used for the experiments. Three experimental arms consisted of uninfected control
71 animals (n=13), infected endotracheally with *A. fumigatus* untreated animals (n=17), and
72 infected rabbits treated intravenously with antifungal agents (n=16): liposomal
73 amphotericin (LAMB) at 5 mg/kg, deoxycholate amphotericin B (DAMB) at 1 mg/kg, or
74 ravuconazole (RVZ) at 5 mg/kg. Neutropenia was deliberately chosen for comparison of
75 host biomarkers response during the antifungal therapy to simulate prominent condition
76 for human infection.

77 **Bronchoalveolar lavage.** Bronchoalveolar lavage was performed postmortem on
78 each lung preparation by the instillation and subsequent withdrawal of 10 ml of sterile
79 normal saline twice into the clamped trachea with a sterile 12-ml syringe. The lavage was
80 then centrifuged for 10 min at 400 × *g*. The upper and lower (2 ml) portions of the
81 supernatant were transferred into centrifuge tubes, and stored at -80°C.

82 **Serum collection**. Serum specimens (1 ml) were collected from infected
83 untreated and treated rabbits on postinoculation days (PID): 0, 1, 4, 6, and 13 and stored
84 at -80°C. Serum specimens for uninfected control rabbit arm were collected from healthy
85 rabbits and stored at -80°C.

86 **2D gel electrophoresis and protein identification.** Total proteins in BALF
87 samples were evaluated by 2D gel electrophoresis as previously described (8). Protein
88 spots of interest were excised from the gel, trypsin digested and analyzed by mass
89 spectroscopy (MS) on a 4800 matrix-assisted laser desorption/ionization time-of-
90 flight/time-of-flight (MALDI TOF/TOF) mass analyzer. Protein identification was

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91 performed by searching the combined tandem mass spectra against the NCBI (National
92 Center for Biotechnology Information) mammals sequence database, using a local
93 MASCOT search engine (V. 1.9) on a GPS (V. 3.5, ABI) server.

94 **The enzyme-linked immunosorbent assay (ELISA).** ELISA was performed
95 using Human Haptoglobin ELISA kit (GenWay), Rabbit CRP ELISA kit (Immunology
96 Consultant Laboratory, Inc) and Rabbit Annexin A1 ELISA kit (Uscn Life Science Inc.)
97 following manufacture's protocol.

98 ***A. fumigatus* growth conditions.** *A. fumigatus* wild type R21 strain was grown
99 for 4 days at 37°C on potato dextrose agar (PDA; Becton Dickenson, Sparks, MD). At
100 the end of the incubation period conidia were harvested with 0.01% Tween-20 solution.
101 For degradation assays, 100 ml of *Aspergillus* minimal medium (AMM) were inoculated
102 with 1×10^8 conidia and incubated at 37°C with shaking (225 rpm) for 72 h.

103 **Annexin A1 degradation assay.** Five milliliters of 72 h-old *A. fumigatus* culture
104 were filtered through 0.45-µm filter (Fisher Scientific) to separate conidia and hyphae
105 from the culture supernatant. Two micrograms of purified Recombinant Human Annexin
106 A1 (RD Systems) or 10 µl of K-562 (Human erythromyeloblastoid leukemia cell line)
107 (Santa Cruz Biotechnology, Inc.) whole cell lysate were added to 20 µl of *A. fumigatus*
108 culture filtrate. The solutions were incubated at 37°C for 2 h and Western blotting of the
109 samples was performed.

110 **Western blotting**. Samples were separated by SDS-polyacrylamide gel
111 electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF)
112 membrane (Invitrogen) using iBlot Gel Transfer Device (Program 3) (Invitrogen). After
113 the transfer, cleavage products were detected by Western blotting using iBlot Gel

114 Transfer Device (Program 9) and iBlot Western detection kit (Invitrogen). Annexin A1
115 monoclonal antibodies (EMD Millipore) were used for the detection in a dilution of
116 1:1,000.

117 **Statistical analysis**. Comparisons between two groups were made by Fisher's
118 exact tests for categorical variables and Mann-Whitney test or unpaired t-test for
119 continuous variables. Multivariate analysis of variance (ANOVA) was performed to
120 compare continuous variables repeatedly measured at multiple time points among
121 different groups. Significance was defined as a P value ≤ 0.05 (two-tailed).
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123 ## RESULTS

124 **Identification of BAL host proteins responding to infection and therapy by**
125 **2D gel electrophoresis.** BALF samples from uninfected controls, infected untreated and
126 treated rabbits were analyzed by 2D gel electrophoresis in which the samples were first
127 separated on a pH 5-8 linear gradient followed by 12.5% SDS-PAGE in second
128 dimension. **Figure 1** shows a typical 2D gel after staining with SYPRO ruby. The images
129 of 2D gels were analyzed using PDQuest 2.3 to determine those prominent proteins
130 responsive to both IPA infection and subsequent therapy. Proteins corresponding to spots
131 1, 2 and 3 were absent in BALF from healthy animals, appeared with strong signals in
132 samples from infected rabbits, and disappeared in samples collected after the treatment.
133 In contrast, the protein level at spot 4 decreased following IPA infection and was partially
134 restored after therapy.

135 Protein spots of interest 1, 2, 3 and 4 were excised from the gel, trypsin digested
136 and analyzed by MALDI TOF/TOF MS. The MS analysis revealed that spot 1 was
137 Haptoglobin (Hp), spots 2 and 3 were found to be C-reactive protein (CRP), and spot 4
138 was identified as Annexin A1 (Anx A1). Spots 2 and 3 represented the same protein
139 (CRP) possibly due to the proteolytic cleavage of CRP subunits or various
140 posttranscriptional modifications.

141 Three proteins listed above appeared to respond to IPA infection, as well as to
142 treatment with antifungal drugs. Therefore, they were chosen as potential host-based
143 biomarkers of IPA useful in monitoring therapeutic response.

144 **Quantification of host biomarkers in BALF.** To quantify the amount of Hp,
145 CRP and Anx A1 in rabbit BALF samples, ELISA was performed for each protein

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146 biomarker (**Figure 2**). Hp abundance in BALF from infected untreated rabbits was highly
147 pronounced (10.8 ± 2.1 µg/ml), while it was minimal in BALF from uninfected and
148 treated animals (0.14 ± 0.10 µg/ml and 0.44 ± 0.19 µg/ml respectively). The level of Hp
149 in BALF from rabbits with IPA was significantly greater than either that of uninfected or
150 treated animals ($p < 0.0001$) (**Figure 2A**). There was no significant difference in Hp
151 between the uninfected control and treated groups ($p > 0.05$). CRP was detected in 16
152 (94%) of 17 samples of infected untreated rabbits (14.84 ± 2.41 µg/ml) and undetectable
153 in 100% of BALF from uninfected control animals (**Figure 2B**). In treated animals, CRP
154 was found in 1 (6%) of 16 samples (0.1 ± 0.06 µg/ml). The amount of CRP in BALF
155 from infected untreated rabbits was significantly higher than in the samples from
156 uninfected and infected-treated animals ($p < 0.0001$). Anx A1 level was significantly
157 lower in BALF from infected untreated rabbits (0.11 ± 0.02 µg/ml) than in the samples
158 from control (1.99 ± 0.25 µg/ml) and treated animals (1.83 ± 0.19 µg/ml), ($p < 0.0001$)
159 (**Figure 2C**). There was no significant difference found between ravuconazole and
160 amphotericin B treatment in terms of the three biomarkers profiles. Both drugs led to
161 reduced levels of Hp and CRP and restored level of Anx A1.

162 **Abundance of putative host biomarkers in serum.** The abundance of all three
163 biomarkers in serum was measured by ELISA and compared in cohorts of uninfected
164 control, infected untreated and treated animals (**Figure 3**). While serum was collected
165 from all treated rabbits at specified time points (PID 0, 1, 4, 6, 13), only a few samples or
166 none were collected from the untreated infected animals on or after PID 6 due to
167 euthanasia using humane endpoints.

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168 Anx A1 was undetectable in serum from all three groups of animals. Hp and CRP
169 were mainly found in serum from both infected untreated and treated rabbits at all time
170 points except day 0 (baseline). As shown in **Figure 3**, serum Hp levels appeared to
171 increase with the progression of infection and decrease in response to treatment. Serum
172 CRP levels showed an upward trend in 6 of 7 untreated rabbits and decreased with
173 antifungal therapy.

174 The time courses of each protein biomarker were compared between infected
175 untreated and treated groups. Examination of the estimated marginal means indicated that
176 serum Hp concentrations were not significantly different between infected untreated and
177 treated rabbits from PID 0 to 4. On PID 6, however, serum Hp concentration in treated
178 animals was significantly lower than that in untreated animals ($F = 17.831$, $p = 0.0001$).
179 Serum CRP showed no significant difference between infected untreated and treated
180 rabbits from PID 0 to 6. However CRP concentration in serum showed a downward trend
181 in response to antifungal treatment from PID 6 to 13.

182 **Effect of *Aspergillus fumigatus* secreted proteases on Anx A1.** To test weather
183 *A. fumigatus* plays a role in Anx A1 degradation, supernatant of a 5 ml 72-h-old *A.*
184 *fumigatus* liquid culture was filtered and subsequently incubated with purified Anx A1
185 (**Figure 4A**, lane 3), as well as with K562 cell line lysate (**Figure 4A**, lane 1) for 2 h at
186 37ºC. Cleavage of Anx A1 was visualized by Western blotting. The analysis showed that
187 after 2 h incubation Anx A1 was completely degraded (**Figure 4A**, lane 4). In the case of
188 K562 cell lysate incubation, low molecular weight products were visible (**Figure 4A**,
189 lane 2).

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190 Different volumes of *A. fumigatus* culture filtrate were incubated with K562 cell
191 line lysate to investigate if different cleavage products could be obtained depending on
192 the volume of culture filtrate added (**Figure 4B**). When a volume of culture filtrate higher
193 than 10 µl was added to K562 cell lysate, it resulted in cleavage of Anx A1 into four
194 major low molecular weight products (**Figure 4B**, lanes 3 to 5). Degradation of Anx A1
195 also was observed after incubation with 5 µl of culture filtrate but it was less prominent
196 (**Figure 4B**, lane 2). After incubation with 2.5 µl of culture filtrate the protein remained
197 intact (**Figure 4B**, lane 1).
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199 **DISCUSSION**

200 The development of host biomarkers of IPA may provide better understanding of
201 disease, improve diagnostics, and help monitor treatment response. In this pilot study, we
202 identified several biomarkers that responded to infection and subsequent therapy.

203 Hp levels were found to be high in BALF from rabbits with IPA infection and
204 significantly decreased after the treatment (Figure 2A). Hp is an acute phase protein,
205 which takes part in various processes of immune responses including activation of the
206 innate and adaptive immune response, tissue repair and regeneration. Hp release induces
207 leukocyte activation, modulation of cytokine patterns, prostaglandin synthesis, and tissue
208 repair (9). Hp has a bacteriostatic role by binding hemoglobin and preventing the
209 utilization of iron by pathogenic bacteria that require iron for their growth (10). Iron
210 uptake is crucial for *A. fumigatus* growth in the lung environment (11). Thus, high levels
211 of Hp during IPA suggest the host sequestration of iron to prevent its use by *A. fumigatus*
212 along with immunomodulatory response to the infection.

213 *A. fumigatus* secretes large quantities of hemolysin (12). During IPA,
214 angioinvasion results in hemorrhagic infarction and release of hemoglobin. The
215 importance of host response of releasing Hp to bind hemoglobin during invasive
216 aspergillosis was recently reported by Goetting *et al* who found that plasma iron was
217 significantly increased in infected animals (13). *Fusarium* infection also has been
218 associated with elevated Hp concentrations in the tears of patients with fusarial keratitis
219 (14). Given the increasingly recognized role of Hp in invasive mycoses, understanding its
220 role as a host-based diagnostic biomarker of therapeutic response is important.
221 Combining an organism-based biomarker, such as galactomannan, for measuring

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222 therapeutic response (15) with a pathophysiologically based host biomarker, such as Hp,
223 may further strengthen our ability to assess therapeutic efficacy and predict outcome.

224 The analysis of rabbit BALF specimens during IPA also revealed a substantial
225 increase of another acute phase protein CRP (Figure 2B). It is noteworthy that CRP levels
226 in BALF from treated rabbits were almost undetectable, which makes it a promising
227 marker to monitor response to antifungal therapy. At the molecular level, production of
228 CRP is induced by proinflammatory cytokines IL−1, IL−6, and IL−17 in the liver in
229 response to microbial infection, tissue injury, and autoimmune disorders. (16). Human
230 endothelial cells and murine macrophages exposed to CRP *in vitro* express chemokine
231 MCP-1 (17). CRP acts as an opsonin supporting ingestion of apoptotic cells by human
232 macrophages and plays a role in the clearance of apoptotic cells, especially during acute
233 phase reactions (18, 19). Our data is consistent with other studies that also detected high
234 level of CRP in BALF during the fungal infections (20, 21).

235 Finally, our study indicates that Anx A1 levels in BALF from infected rabbits
236 were significantly reduced compared to samples from uninfected and treated animals
237 (Figure 2C). Anx A1 is a member of a phospholipid and calcium binding family of
238 proteins. Large amounts of this protein were found in BALF from normal volunteers
239 while the degradation of Anx A1 was seen in cystic fibrosis patients (22, 23). Both Anx
240 A1 and its biologically active N-terminal peptide are considered to be modulators of
241 systemic anti-inflammatory processes (24, 25). It has been shown that the amounts of
242 Anx A1 increase in response to corticosteroid treatment (26), suggesting an essential role
243 during the inflammatory processes in the lungs.The major site of IPA infection is the
244 lungs, which contains abundant Anx A1 (27). Thus, inactivation of Anx A1 might be

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245 critical for *A. fumigatus* growth, considering its regulatory role during the anti-
246 inflammatory pathway. We demonstrated that culture filtrate form *A. fumigatus* possessed
247 proteolytic activity towards Anx A1 *in vitro* (Figure 4). Our study suggests that secreted
248 proteases of *A. fumigatus* may play role in Anx A1 cleavage. However, additional
249 experiments are needed to establish the specificity of the observed cleavage.

250 The fact that Anx A1 was undetectable in serum from all three groups of animals
251 (uninfected, infected untreated and treated) suggests that it is a biomarker of local
252 inflammation rather than circulating infection. Nevertheless, the state of Anx A1 might
253 still reflect treatment response during IPA using BALF. In contrast, Hp in serum
254 displayed time-dependent patterns associated with IPA development, as well as with
255 response to antifungal therapy, while in healthy normal rabbits it was undetectable
256 (Figure 3A). The level of circulating Hp was increasing with the progression of infection
257 and subsequently decreasing in treated rabbits with significant difference on PID 6 of the
258 study ($p=0.0001$). Therefore, further exploring Hp kinetics may provide additional
259 information on disease progression as well as therapeutic response to antifungals. CRP
260 was found in serum collected from both infected untreated and treated rabbits, but not
261 from uninfected healthy rabbits. There was no significant difference in CRP serum
262 concentration between untreated and treated groups from PID 0 to 6; however the level of
263 protein was decreasing in response to antifungal treatment during the remaining study
264 period (PID 6 to 13) (Figure 3B).

265 Our findings suggest that host proteins Hp, CRP and Anx A1, which play
266 important roles in host defense mechanisms during the pathogenesis of IPA, may have
267 value for monitoring therapeutic effect on patients who were diagnosed with IPA

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268 according to EORTC/MSG diagnosis criteria. Once a definite diagnosis of IPA is made,
269 these biomarkers could be useful in guiding antifungal treatment. However the response
270 of these biomarkers to both infection and antifungal therapy requires further study.

271 In conclusion, a proteomic approach was used to identify potential host protein
272 biomarkers of IPA that are responsive to therapy. Detection of the prominent host
273 proteins: Hp, CRP and Anx A1 may have value to monitor antifungal therapeutic
274 response in patients with IPA.

275

276 **ACKNOWLEDGMENTS**

277 This work was supported by NIH grant AI103636 to D.S.P., NINDS grant
278 P30NS046593 to H.L. and by internal funds provided by the Public Health Research
279 Institute.

280

281 **DISCLOSURES**

282 Dr. Perlin receives research support from Merck, Astellas and Pfizer, has a patent
283 application on assays for drug resistant fungal infections. We declare that there are no
284 conflicts of interest.

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386 **Figure 1.** 2D gels of bronchoalveolar lavage fluid (BALF) samples. An aliquot of 50 µg
387 of protein was subjected to 2-dimensional gel electrophoresis using strips of pI 5 to 8
388 followed by 12.5% SDS-PAGE gel in second dimension. The marked spots were excised,
389 trypsin digested, and analyzed by MS. Spot 1 – Haptoglobin (Hp), spots 2, 3 – C-reactive
390 protein (CRP), spot 4 – Annexin A1 (Anx A1). (**A**) BALF from infected untreated animal
391 demonstrates abandon presence of both Hp and CRP, whereas Anx A1 is almost
392 undetectable. (**B**) BALF from treated rabbit is characterized by the absence of Hp and
393 CRP with the presence of Anx A1 (similar to the uninfected control). (**C**) BALF from
394 uninfected control (healthy rabbit) shows the absence of Hp and CRP, while Anx A1 is
395 present. Gels were stained with SYPRO ruby for visualization.

396 **Figure 2**. Histogram of Haptoglobin (Hp), C-reactive protein (CRP) and Annexin A1
397 (Anx A1) ELISA results (mean of three tests) for bronchoalveolar lavage fluid (BALF)
398 from uninfected control (n=13), infected untreated (n=17) and treated (n=16) rabbits. (**A**)
399 Hp concentration is high in BALF from untreated rabbits and minimal in BALF from
400 uninfected and treated animals. (**B**) CRP level is abundant in BALF from untreated
401 rabbits while in BALF from treated and uninfected animals is almost undetectable. (**C**)
402 Anx A1 is present in BALF from uninfected animals, decreased following IPA infection,
403 and restored after therapy.

404 **Figure 3**. Kinetics of Haptoglobin (Hp) and C-reactive protein (CRP) concentrations
405 (mean of two tests) in serum of infected untreated (n=7) and treated (n=7) rabbits. (**A**) Hp
406 concentration in serum from untreated and treated rabbits measured on postinoculation
407 days (PID) 0, 1, 4, 6 and 13. (**B**) CRP concentration in serum from untreated and treated
408 rabbits measured on PID 0, 1, 4, 6 and 13.

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409 **Figure 4**. The effect of *A. fumigatus* culture filtrate on Annexin A1 (Anx A1) analyzed
410 by Western blotting. (**A**) Cleavage of purified Anx A1 (lane 4) and Anx A1 from K562
411 cell lysate (lane 2) after 2 h incubation (37ºC) with 72 h-old *A. fumigatus* culture filtrate.
412 In controls Anx A1 (lane 3) and K562 cell lysate (lane 1) were incubated with PBS
413 instead of culture filtrate. (**B**) K562 cell lysate was incubated with 2.5, 5, 10, 15, 20 µl
414 (lanes 1 to 5 respectively) of 72 h-old *A. fumigatus* culture filtrate for 2 h at 37ºC. In
415 control (lane 6) K562 cell lysate was incubated with 20 µl of PBS.

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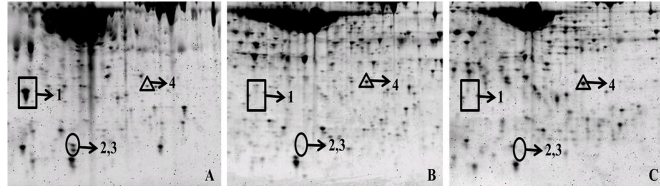

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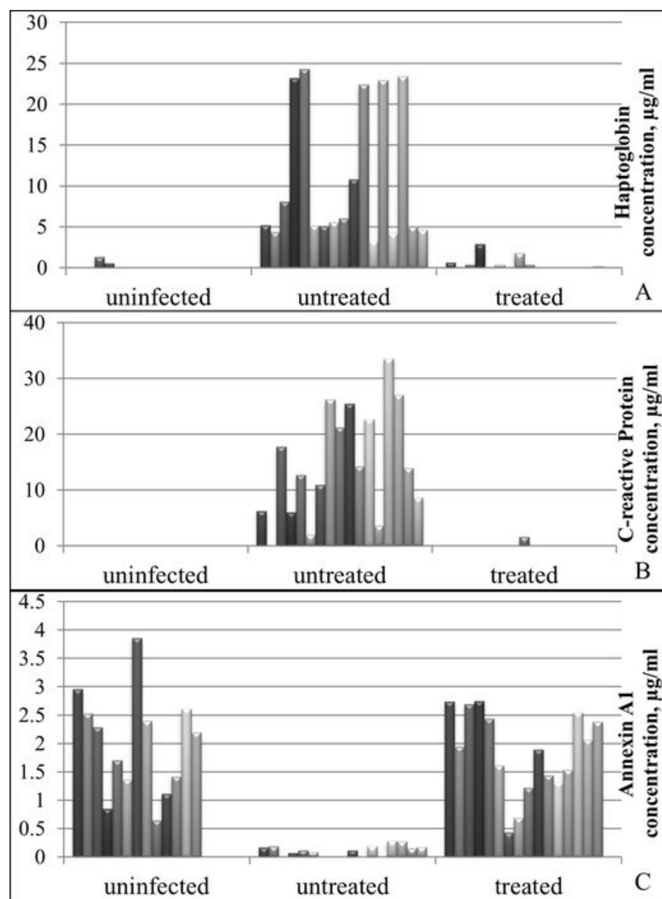

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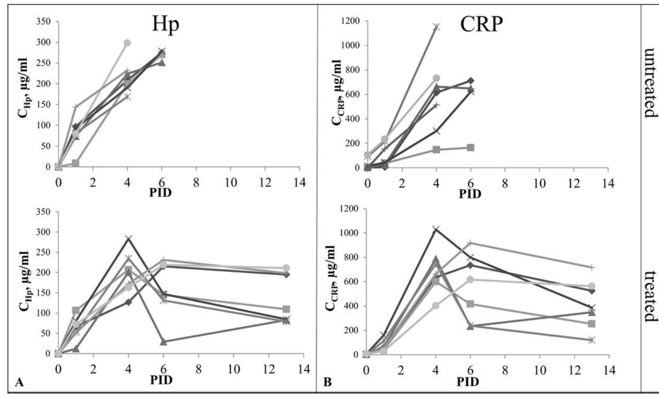

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