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Severity markers in severe leptospirosis: a cohort study

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21 **Summary:**

22 **Purpose:** We aimed at evaluating parameters for their value as severity markers in hospitalized
23 leptospirosis patients.

24 **Methods:** We recruited 47 informed adult consenting patients and assessed a number of clinical,
25 hematological, biochemical and biological variables. Patients were sorted according to severity based
26 on the fatality or the requirement of mechanical ventilation or dialysis; the parameters studied were
27 compared between groups on inclusion and the next day.

28 **Results:** Beside septic shock presentation or a high severity score SAPS II, increased lactate, total
29 bilirubin, lipase, and the AST / ALT ratio or a decreased cytokines IL-10 / TNF- α ratio were all
30 significantly associated with severity. The gene expression of the IL-1 receptor antagonist IL-1ra, the
31 IL-1 α and the long pentraxin PTX-3 were also transcribed at higher levels in most severe cases.
32 Patients could rapidly improve or deteriorate, highlighting the need of a new assessment the next
33 day.

34 **Conclusions:** Our results add to the limited body of knowledge about severity markers in
35 leptospirosis. They also suggest that patients should be re-assessed the next day before being
36 possibly discharged from the hospital. Further studies are needed to confirm relevant and reliable
37 prognostic parameters in leptospirosis that would be helpful for triage purpose.

38

39 **Keywords:**

40 Leptospirosis, Leptospira, Clinical signs, treatment, severity markers, Cytokines

41

42 **Introduction**

43 Leptospirosis is an infectious disease of global significance with a massive burden in tropical and
44 subtropical regions (1, 2). Symptoms are usually a flu-like syndrome with fever, headache and
45 myalgia and can resolve spontaneously. However, the disease can degenerate to severe forms
46 evolving to multiple organ failures with hemorrhages, hepatic, renal and pulmonary damages related
47 to septic shock. Case fatality rates ranging from 5% to 70% depending on clinical complications have
48 been reported (3). Beside its endemic burden, epidemics as well as massive outbreaks have been
49 increasingly reported over the past decades (4-8). These epidemics sometimes coincide with dengue
50 or other epidemics (9-11). Such contexts result in limited availability of intensive care. An appropriate
51 triage of “at high risk” patients would be helpful to optimize medical care, but is often impeded by
52 the few prognostic factors available and / or validated for leptospirosis patients. In this work, we
53 aimed at evaluating clinical, hematological, biochemical and other biological indicators in severe
54 leptospirosis, using questionnaires, medical files and laboratory results in confirmed leptospirosis
55 hospitalized patients. In a previous work, we evidenced differential gene expression patterns in
56 inflammatory mediators between surviving and non-surviving hamsters injected with an LD₅₀,
57 modeling the variability of leptospirosis severity (12). Therefore, besides biochemical and
58 hematological parameters, we also studied cytokine gene expression at the transcript and protein
59 levels.

60

61 **Patients, material and methods**

62 **Patient selection and case definitions**

63 Adult patients were recruited between March 2009 and February 2011 at Noumea Central Hospital
64 in New Caledonia, based on clinical suspicions of leptospirosis. Three units recruited patients:
65 Emergency (persons visiting as outpatients), Internal Medicine and Intensive Care Units. Confirmed
66 leptospirosis cases were defined as patients with a positive PCR (13) from blood or urine or with
67 serological evidence of leptospirosis: a four-fold increase or a seroconversion from nil to a titer of at
68 least 800 between acute and convalescent sera using the Micro Agglutination Test. When suspicions
69 were not confirmed, patients were excluded *a posteriori*. An additional 8 adult healthy volunteer
70 blood donors provided blood samples for normalization of gene expression values.

71 Severe cases were defined as patients with either a fatal outcome or a need for mechanical
72 ventilation or dialysis at any time of their hospital stay. Non severe patients were patients who
73 survived with neither a need for mechanical ventilation nor dialysis.

74 **Questionnaire and medical files**

75 Upon recruitment, an admission form was filled by medical staff with demographical data (sex, age),
76 date of symptom onset, clinical presentation, body temperature, current antimicrobial treatment
77 and possible underlying condition. A form with symptoms, treatment, care (notably mechanical
78 ventilation or dialysis) was also filled daily by medical staff for the duration of the hospital stay.

79 **Samples and analysis**

80 Consenting patients were sampled one 4.5 mL dry tube and one 2.5 mL PAXgene™ Blood RNA Tube
81 venous blood daily from inclusion to Day 2 in addition to the hematological or biochemical analyses
82 requested by the medical practitioner. Clinical scores (APACHE II and / or SAPS II) were recorded
83 when calculated in the medical file.

84 Serum was collected and used for quantifying (i) the following cytokines: Tumor Necrosis Factor- α
85 (TNF- α), Interleukin (IL-) 1 β , IL-1ra, IL-6, IL-10 and Interferon- γ (IFN- γ) and the long pentraxin 3 PTX-3
86 using Quantikine ELISA kits (RnDSystems); (ii) Procalcitonin (PCT) using VIDAS® (BioMérieux).
87 PAXgene™ venous blood tubes were used for RNA extraction with the PAXgene Blood RNA kit
88 (PreAnalytix), followed by additional DNA digestion with the TURBO DNase (Ambion); the resulting
89 RNA was used to analyse the relative normalized gene expression (relative target gene expression
90 normalized to the relative gene expression in healthy volunteers, using β -actin as a household
91 reference gene) of TNF- α , IL-1 α , IL-1 β , IL-1ra, PTX-3, IL-4, IL-6, the sub-unit 40 of IL-12 (IL-12p40), IL-
92 10 and the IFN- γ . Gene expression studies used RT-qPCR performed with the primers described in
93 Supplementary Table 1 and 50 cycles: 95°C for 8 seconds, 60°C for 5 seconds and 72°C for 10 seconds
94 on a LightCycler 480 using the LightCycler® 480 SYBR Green I Master kit (Roche). Sera and RNA were
95 stored at -80°C until batch analysis in duplicates.

96 **Statistical analysis**

97 Data were made anonymous and captured in a database. Statistical analyses were made using SPSS
98 12 software (SPSS Inc., Chicago, USA). Categorical variables were expressed as percentages, while
99 quantitative variables were described using median and percentiles. Exact Fisher's test was used for
100 categorical variables and Kruskal Wallis one-way ANOVA for continuous variables. A p-value < 0.05
101 was considered significant. Two-way categorical variables were created from significant continuous

102 variables, allowing the calculation of a univariate odds-ratio using logistic regression. The
103 relationships between severity and parameters were tested for data collected upon admission and
104 for data collected the day after.

105 **Ethics**

106 Written informed consent was obtained from all patients. The study was endorsed by Institut Pasteur
107 under number RBM 2008.34 after approval by Comité de Protection des Personnes Ile de France and
108 approved by the Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans
109 le domaine de la Santé (CCTIRS) with number 09.176. The database was declared to French
110 Commission Nationale de l'Informatique et des Libertés (CNIL).

111

112 **Results and discussion**

113 **Patients**

114 Despite a high incidence in New Caledonia, only 47 leptospirosis patients could be included in our
115 study over a two-year period. Similar studies have been conducted with either less (14-19) or
116 oppositely more patients (20-27). Patient demographic, clinical and selected biological data are
117 summarized in Table 1. Because the recruitment only relied on the Central Hospital, all cases could
118 be considered as severe leptospirosis, as milder forms would be treated in peripheral health centers.
119 However, even in hospitalized cases of leptospirosis, there is a difference in severity and in the
120 medical care to be implemented. We therefore categorized patients as severe or non-severe cases
121 based on operational criteria already used in another study in Guadeloupe (21): severe cases were
122 those who died or required dialysis and/or mechanical ventilation; oppositely non-severe cases
123 survived with neither dialysis nor mechanical ventilation at either time of their hospital stay. Using
124 these criteria, 22 patients were considered as “non-severe” and 25 as “severe” cases, including 4
125 fatalities, an 8.5% fatality rate; additionally, 4 patients initially classified as non-severe upon inclusion
126 later deteriorated to severe. In a recent study in Brazil, severe cases were split according to the fatal
127 outcome or recovery on one hand and to the presence of a severe pulmonary hemorrhage syndrome
128 (SPHS) on the other hand (20). In our study, the number of fatalities or SPHS did not allow this
129 distinction.

130 Patients were mostly males (31 versus 16 females) between 18.2 and 84 years old (mean 41.9,
131 median 39.1), representative of leptospirosis in adults in New Caledonia when compared to
132 surveillance data (8, 28). Neither the sex (Fisher $p=0.755$) nor the age (Kruskal Wallis $p=0.654$) were

133 related to severity. In other studies, older patients were at increased risk of severe forms or fatality
134 (22, 24, 29-31). None of the underlying condition observed: diabetes ($p=0.611$), chronic alcoholism
135 ($p=0.732$), obesity ($p=0.352$) or high blood pressure ($p=0.611$) proved to be significantly associated
136 with severity. High blood pressure was shown to be a risk factor for severity in a study in Guadeloupe
137 (21) and a risk factor for death in a study in Thailand (26). In our cohort, all 3 severe patients with an
138 underlying high blood pressure had a fatal outcome, reinforcing these earlier findings.

139 The time between onset of symptoms and hospitalization was in the range 1-10 days (median 4.0)
140 and was not associated with severity ($p=0.360$). Antimicrobial had been administered before
141 inclusion in the study, in both severe (52.0%) and non-severe (77.3%) cases, a non-significant
142 difference ($p=0.127$). Former studies as well as empirical knowledge consider a delayed antimicrobial
143 treatment as a risk for severity (21, 25), a fact however not confirmed in a systematic review of
144 clinical trials (32).

145 **Clinical examination**

146 Body temperature was not associated with severity ($p=0.704$, $n=42$). Most patients were febrile but 5
147 severe and 2 non-severe cases were hypothermic (in the range 35.4 – 36.5°C). Many classical
148 symptoms of leptospirosis: icterus, hemorrhage, conjunctiva injection and a meningeal syndrome
149 were not differentially noticed between severe and non-severe cases ($p=1.000$; $p=0.702$; $p=0.539$;
150 $p=1.000$, respectively). In a study in France, icterus was considered a prognostic factor for severity
151 (33). Cardiac involvement was a risk factor for mortality in a study in Guadeloupe (29) or for severity
152 in studies in Brazil (31) and France (33); similarly, it was observed in 18.2% non-severe and 50%
153 severe cases of our cohort ($p=0.032$). Interestingly, out of the 4 patients who deteriorated, 3 had
154 cardiac involvement upon admission. Patients with a septic shock presentation accounted for 31.8%
155 of the non-severe cases and 75% of severe cases ($p=0.007$), in agreement with other studies (17, 21,
156 26, 34, 35). Some clinical findings are the basis for our definition for severity and are therefore
157 confounding: as a result, normal breathing, normal pulmonary auscultation and normuria were all
158 more frequent in non-severe cases than in severe cases, mostly reflecting our severity criteria.

159 **Hematology and hemostasis**

160 Complete blood counts upon inclusion were not different between severe and non-severe cases
161 ($p=0.741$ for leukocytes; $p=0.558$ for red blood cells; $p=0.388$ for platelets). In the 4 patients who
162 later deteriorated, 3 had low platelet counts (10, 12 and 21G/L). Similarly, in the hemostasis
163 assessment, neither the prothrombin time ($p=0.621$) nor the fibrinogen concentration ($p=0.380$)
164 were related to severity. However, the following day, leukocyte count was higher ($p=0.037$) and red

165 blood cells lower ($p=0.001$) in severe cases. Similarly, the prothrombin time was higher ($p=0.035$) and
166 the fibrinogen concentration lower ($p=0.021$) in severe patients at Day 1. This finding is in agreement
167 with other studies which show that coagulation disorders are more frequent in severe cases, as
168 evidenced by the prothrombin time measurement (36) and that leukocyte counts were higher in
169 non-survivors (29). Interestingly, these severity factors were only evidenced at Day 1 in our study but
170 were not significant upon inclusion. Similarly in a cohort study in Indonesia, Wagenaar and
171 colleagues redefined the severity status at Day 2 (34), taking into account the possible deterioration
172 of patients during hospitalization. During the course of hospitalization, 10 patients in our study
173 required platelet transfusion (3 patients), packed blood cells (4 patients) or both (3 patients,
174 including one fatality). Though this criterion is independent of our severity criteria, these 10 patients
175 were actually recognized as severe cases.

176 **Biochemistry**

177 Biochemistry upon admission provided some discrimination: Lactate ($p=0.015$), LDH ($p=0.019$),
178 bilirubin ($p=0.016$) and Aspartate aminotransferase (AST) ($p=0.049$) were significantly higher in
179 severe cases, while Alanine aminotransferase (ALT) ($p=0.690$) or Gamma glutamyl transpeptidase
180 (GGT) ($p=0.473$) were not different. The AST / ALT ratio already reported as a risk factor in non-
181 survivors in a small cohort (16) was higher in severe (median 2.004) than in non-severe cases
182 (median 1.56), a significant difference ($p = 0.008$, see Figure 1). A ratio equal or above 2 was
183 associated with an odds ratio of 7.1 (95% CI = 1.8-28.1) for severity (see Table 2). An LDH value above
184 390 UI.L^{-1} was associated with an odds ratio of 5.8 (95% CI = 1.3-25.6) for severity, in agreement with
185 a former study (25). A bilirubin concentration above $35 \mu\text{mol.L}^{-1}$ was associated with an odds ratio of
186 5.0 (95% CI = 1.3-20.0) for severity, in agreement with other studies (21, 24, 33). Because low lactate
187 clearance was associated with increased mortality in severe sepsis (37), we compared the drop in
188 lactate concentrations between inclusion and Day 1, but failed to evidence any difference between
189 severe and non-severe cases (data not shown). In the patients deteriorating during their early
190 hospital stay, high AST / ALT ratios (circled in figure 1), high bilirubin, high Lactate concentration, high
191 AST or high LDH was each observed in 2 or more patients. Taken together, at least two of these
192 parameters had values that could be considered as alerting.

193 Procalcitonin, a recognized marker of sepsis (38) was above the $2.5 \mu\text{g.L}^{-1}$ threshold for all cases,
194 slightly (but not significantly, $p=0.477$) higher in severe cases (median 35 for severe, 22.6 in non-
195 severe), both higher than levels recorded in benign leptospirosis ($1.2 \mu\text{g/L}$) in a study in France (24).
196 The SAPS II score was only calculated for 16 patients (10 non-severe and 6 severe) but was

197 significantly higher in severe cases (median 56.5 and 28.5 respectively, $p=0.013$). It could prove
198 useful to further evaluate this simplified scoring system derived from APACHE (39) in leptospirosis.

199 **Cytokines and immune effectors**

200 Serum concentrations of cytokines could be assessed only in a subset of patients ($n=24-30$ depending
201 on the target), restricting the statistical power. The use of ELISA kits for measuring the concentration
202 of several cytokines requires large volume of serum. A recent study used a multiplex bead array, an
203 emerging technology with high analytical capacity using small volumes (20). In our study, no
204 significant difference in serum concentration of cytokines or other immune markers was found
205 between severe and non-severe cases, in marked contrast with other studies (14, 15, 20, 40). We
206 notably evidenced no difference in IL-6 concentration, a cytokine widely regarded as a marker for
207 severity in sepsis (38) and also identified as such in some (20, 23, 34), though not all (15) studies on
208 leptospirosis. The interleukins IL-1 β , IL-2 and IL-4 were all found to be at significantly higher
209 concentrations in severe than in mild leptospirosis in a study in Brazil (20). In our cohort a similar
210 trend, though not significant, was noticed for IL-1 β . Oppositely, IL-2 and IL-4 were low in both severe
211 and non-severe cases. A possible reason for our findings is that a number of patients were included
212 after they had already been treated, including massive fluid infusion that may result in an unknown
213 dilution of serum. Studying ratios allows neutralizing this dilution bias. Since the pioneering work of
214 Tajiki and colleagues (14), the IL-10 / TNF α ratio was frequently evaluated in leptospirosis. This IL-10
215 / TNF α ratio (Figure 1) was significantly lower in severe than in non-severe cases (median 0.64, $n=14$
216 and median 1.15, $n=10$ respectively, $p=0.016$). An IL-10 / TNF α ratio below 1 was associated with an
217 increased risk of severe forms (odds ratio 19.5, 95% CI = 1.8-213.9). In a very simplified view, this
218 suggests that if the anti-inflammatory activity (as reflected by IL-10 concentration) does not
219 counterbalance the pro-inflammatory (as reflected by TNF α), the evolution towards severe forms of
220 leptospirosis is more likely to occur. This hypothesis is also in agreement with experiments
221 comparing the kinetics of cytokine gene expression kinetics between susceptible and resistant animal
222 models (12, 41). Our result is in agreement with the pioneer work of Tajiki and colleagues, but differs
223 from a study in Greece (15). Similarly, Reis and collaborators in Brazil (20) evidenced a higher ratio in
224 fatal than in severe non-fatal cases. This discrepancy in the results between cohort studies could be
225 explained by the difference in the time of analysis with regard to the onset of symptoms, because
226 cytokine expression is a very dynamic and fluctuating phenomenon, as notably evidenced in animal
227 models (12), where kinetics are most different between susceptible and resistant models (41).
228 Interestingly, although considered as an anti-inflammatory cytokine, IL-10 can also exert a pro-
229 inflammatory activity enhancing the activation of natural killer (NK) cells and cytotoxic T lymphocytes

230 (CTLs) during endotoxemia (42). Of note, the antibiotic treatment can also trigger TNF- α over-
231 expression, in response to *Leptospira* killing.

232 Gene expression techniques evidenced significant differences between severe and non-severe cases.
233 These results are summarized in Table3. The IL-1 receptor antagonist IL-1ra gene was expressed at
234 higher levels in the blood of severe cases ($p=0.015$), a two-fold induction being associated with an
235 odds ratio of 6.8 (95% CI = 1.3 – 34.6). Similarly, PTX-3 gene was more expressed in severe cases
236 ($p=0.001$, see Figure 1), a two-fold induction corresponding to an odds ratio of 5.8 (95% CI = 1.1 –
237 29.9). This latter result reflects, at the transcriptional level, results formerly published by Wagenaar
238 and colleagues about the prognostic value of the PTX-3 for both severity and fatality (34). The IL-1 α
239 gene was also expressed at higher levels in severe cases ($p=0.025$), a two-fold increase corresponding
240 to an odds ratio of 13.00 (95% CI = 1.4-124.3). This result corroborates our former results in a
241 hamster model of leptospirosis, where non-survivors had a higher gene expression of this cytokine
242 (12). TGF- β gene expression was down-regulated in all cases, whatever severity (mean relative
243 expression ratio = 0.117), being slightly but not significantly more down-regulated in severe cases
244 ($p=0.062$). Because mostly regarded as anti-inflammatory, this significant down-regulation might be
245 related to our cohort, where all cases were acute leptospirosis requiring hospitalization. Lastly, no
246 significant difference was found in the gene expression levels of TNF- α , IL-1 β , IL-6, IL-4, IL-12p40, IL-
247 10, IFN- γ and Cox-2. The Cox-2 gene was formerly found expressed at higher levels in non-survivors
248 in a hamster model of leptospirosis. In our cohort, a similar trend was observed, however not
249 significant. IL-4 is known to have an anti-inflammatory activity suppressing overexpression of TNF- α ,
250 IL-1 β and IL-6 in human monocytes (43). Interestingly, although the IL-4 mRNA expression was
251 associated with survival in patients with severe sepsis, the plasma IL-4 levels in septic patients on the
252 day of admission to the hospital did not differ between survivors and non-survivors (44). Moreover,
253 specific IL-4 gene deficiency in mouse had no impact on the outcome in experimental leptospirosis
254 (45). The *Leptospira* burden was evaluated in our study using 16SrRNA quantification, as a ratio to
255 the host housekeeping gene transcripts, as formerly done in animal models (12). Though the highest
256 levels were observed in most severe cases, no significant difference was evidenced, mostly because
257 of a high variability. In a former study in New Caledonia, a high bacteremia was shown to be
258 associated with severity (25).

259

260 **Conclusions:**

261 We evidence the usefulness of using ratios for serum concentrations, as evidenced by the AST / ALT
262 and the IL-10 / TNF- α ratios in our study, especially when massive fluid infusion have been
263 implemented. Similarly, gene expression techniques which use ratios to normalize to reference
264 housekeeping genes can prove useful, though not yet used in medical routine. Another concern
265 raised in our study is a need to re-evaluate the patient the day after admission for severity, as
266 already suggested in a former study in Indonesia (34): patients identified as non-severe at the time of
267 admission should be kept for surveillance in a medical service before being possibly dispatched after
268 complete re-evaluation. From inclusion to the next day, 38 patients from our cohort remained in the
269 same severity category, only 2 improved, whereas 3 severe cases died and 4 initially regarded as non-
270 severe deteriorated to severe. These 4 patients with rapid deterioration upon admission had at least
271 four alerting criteria among cardiac involvement, low platelet counts, high lactate, LDH, lipase, AST,
272 AST / ALT ratio or bilirubin (Supplementary Table 2). As an example, a woman who was not severe at
273 the time of inclusion was kept for surveillance and required dialysis, mechanical ventilation and a
274 transfusion of platelets the next day, finally recovering within 10 days. Not only can patients
275 deteriorate quickly, but antimicrobial treatment can also trigger a Jarisch-Herxheimer reaction (46,
276 47) leading to hemodynamic support requirement. Our study provides a new independent cohort of
277 confirmed leptospirosis patients and identifies or confirms a number of parameters that can be
278 useful for the triage of leptospirosis patients requiring intensive care.

279

280 **Limitations:**

281 There are some limitations in our study. First, because the differential diagnosis of leptospirosis is a
282 real clinical challenge, the parameters evidenced in our study should also be assessed for a number
283 of other medical conditions. These conditions may vary depending on the regions but most notably
284 include bacterial sepsis, malaria, dengue and possibly influenza.

285 Because patients' recruitment was only in Central hospital, all are severe leptospirosis. This
286 recruitment strategy also restricted the number of patients informed and amenable to recruitment.
287 Taken together, these factors have restricted the statistical power. Therefore, possible biological
288 markers usable for prognosis may have been poorly informative in our cohort, when compared with
289 studies that included benign leptospirosis presentations (20, 24). However, there also is a need to
290 evaluate if a hospitalized patient can be kept under surveillance in a standard medicine unit or
291 should be transferred to an Intensive Care Unit, especially during concurrent outbreaks leading to
292 limited ICU availability. Thus, a better assessment of severity in hospitalized patients would also

293 prove useful for triage purpose. Additionally, some patients had been referred from peripheral
294 health centers, so that not all were included at the first medical presentation. Thus, some patients
295 were recruited after they had already been treated, including intravenous fluid infusion or
296 antimicrobial treatment, before being recruited. Though limiting our study, this again reflects the
297 actual medical need in hospitals, where patients may have already presented to peripheral health
298 centers, or when leptospirosis is initially not considered as a possible etiology.

299

300

301 **Competing interests:**

302 The authors declare that they have no competing interests

303

304 **Authors' contributions**

305 Study conception: CG, FVP. Study design: CG, FVP, PB, FL, MM, DB. Patient recruitment and care:
306 MM, FL, DB. Biological analyses: MESH, CM, LBO, YB, ACG. Data management: PB, MESH, CM, LBO.
307 Analysed the data: PB, CG. Wrote the paper: CG, with contributions from all authors who read and
308 approved the final version.

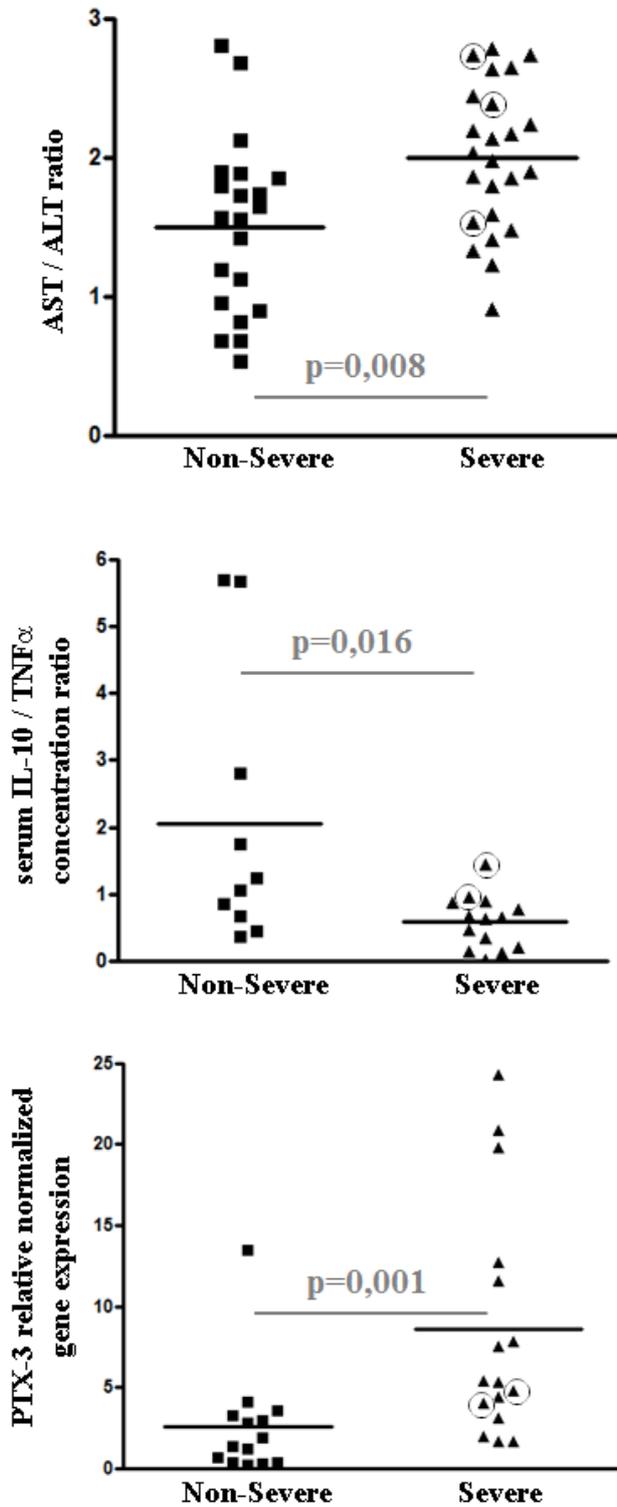
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314

315 **Figure 1:** AST / ALT transaminases ratio (top), IL-10 / TNF- α serum concentration ratio (center) and
316 PTX-3 relative normalized gene expression (bottom) in severe and non-severe leptospirosis cases.
317 Patients initially classified as non-severe but who deteriorated to severe at Day 1 are circled.



318

319

320 Table 1: Demographic, clinical and biological results for patients according to severity

	Numbers Non- severe / Severe	Normal value	Non severe			Severe			p value
			25th percentile	Median	75th percentile	25th percentile	Median	75th percentile	
Age (years)	22 / 25		32.01	40.55	50.91	24.06	38.97	57.86	0.654
Days since onset	21 / 22		2.00	4.00	4.50	3.00	4.00	5.00	0.360
SAPS II score	10 / 6		14.75	28.50	34.00	42.75	56.50	98.00	0.013
Lactate (mmol/L)	10 / 20	0.3-1.3	1.37	1.76	2.23	2.00	2.80	3.87	0.015
Total Bilirubin (μ mol/L)	20 / 24	3.4-17	25.25	40.00	172.75	56.75	146.50	328.25	0.016
AST (GOT) (IU/L)	21 / 24	5-34	47.50	76.00	167.00	87.50	127.50	213.50	0.049
ALT (GPT) (IU/L)	21 / 24	<55	40.00	64.00	92.50	49.00	62.00	92.75	0.690
AST / ALT ratio	21 / 24		0.95	1.565	1.86	1.58	2.004	2.40	0.008
Lipase (IU/L)	15 / 15	5.6-	13.00	55.00	112.00	24.00	154.00	436.00	0.050

		51.3							
LDH (IU/L)	19 / 23	100-350	188.00	277.00	368.00	276.00	395.00	515.00	0.019
Procalcitonin (µg/L)	14 / 15	<0.01	5.55	22.58	55.11	15.99	35.00	42.29	0.477
TNF-α (pg/mL)	13 / 14	<15.6*	9.70	81.60	153.60	29.58	112.55	197.70	0.325
IL-1β (pg/mL)	13 / 12	<3.90*	0.00	0.00	1.51	0.00	3.70	10.10	0.101
IL-1ra (pg/mL)	12 / 14	360 +/- 214*	2547.25	2706.10	3633.73	2555.25	2646.50	2710.85	0.347
PTX-3 (ng/mL)	13 / 14 19 / 15	0.65*	37.10 22.63	60.81 40.25	64.76 54.30	39.83 42.95	49.20 48.50	62.91 59.15	0.458 (0.023 at Day 1)
IL-6 (pg/mL)	12 / 13	1.77 (<10)*	7.43	17.31	22.90	1.80	10.34	17.60	0.087
IL-10 (pg/mL)	13 / 14	<3.47*	40.87	69.25	169.70	13.42	24.65	119.40	0.128
IL-10 / TNF-α ratio	10 / 14		0.62	1.15	3.52	0.19	0.64	0.88	0.016

321 * no normal value is clinically validated. Data indicated are values in sera of healthy volunteers according to the manufacturer.

322

323 Table 2: Categorical variables and corresponding univariate odds ratio where significant.

	Numbers Non-severe / Severe	Proportion in Non-Severe (%)	Proportion in Severe (%)	p-value (Fisher exact or * χ^2)	univariate Odds ratio	95% CI
Septic shock presentation	22 / 24	31.8	75.0	0.007	6.4	1.8 – 23.3
AST / ALT \geq 2	21 / 24	14.3	54.2	0.006	7.1	1.8 – 28.1
Total Bilirubin \geq 35	20 / 24	50.0	83.3	0.025	5.0	1.3 – 20.0
LDH \geq 390	19 / 23	15.8	52.2	0.023	5.8	1.3 – 25.6
serum IL-10 / TNF- α ratio $<$ 1	10 / 14	40.0	92.9	0.009	19.5	1.8 – 213.9
IL-1 α gene expression \geq 2	14 / 16	7.1	50.0	0.017	13.0	1.4 – 124.3
PTX-3 gene expression \geq 2	14 / 16	42.9	81.3	0.029*	5.8	1.1 – 29.9
IL-1ra gene expression \geq 2	13 / 16	30.8	75.0	0.027	6.8	1.3 – 34.6

324

325

326 Table 3: Gene expression of cytokines and other immune markers according to severity

	Numbers Non-severe / Severe	Non severe			Severe			p value
		25th percentile	Median	75th percentile	25th percentile	Median	75th percentile	
TNF- α	14 / 16	0.51	1.22	2.32	0.56	1.84	3.44	0.313
IL-1 β	13 / 16	0.42	0.92	1.62	0.36	0.78	1.64	0.846
IL-1α	13 / 16	0.58	1.29	3.05	1.66	4.10	6.98	0.015
PTX-3	14 / 16	0.38	1.62	3.38	3.38	5.40	12.43	0.001
IL-6	14 / 16	0.01	0.09	0.19	0.08	0.26	0.42	0.110
IL-1α	14 / 16	0.16	0.80	1.52	0.74	1.96	4.19	0.025
IL-12p40	14 / 16	0.22	0.33	1.03	0.22	0.55	1.41	0.179
IL-10	13 / 16	1.63	3.99	22.70	5.19	10.89	24.80	0.156
IFN- γ	13 / 14	0.02	0.10	0.59	0.12	0.37	1.21	0.105
TGF- β	13 / 16	0.06	0.11	0.12	0.05	0.06	0.07	0.062
Cox-2	13 / 16	0.40	1.06	1.26	0.43	1.43	2.12	0.170
<i>Leptospira</i> 16S to human household	12 / 14	0.01	3.95	802.25	0.00	54.77	3867.50	0.494

327 Results are relative normalized expression ratio as detailed in the text, where normal values are 1.

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