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► **To cite this version:**

S. Ruiz, D. Concordet, T. Lanot, B. Georges, P. Goudy, et al.. Hydroxychloroquine lung pharmacokinetics in critically ill patients with COVID-19. *International Journal of Antimicrobial Agents*, 2021, 57 (2), pp.106247. 10.1016/j.ijantimicag.2020.106247 . hal-03219720

HAL Id: hal-03219720

<https://hal.inrae.fr/hal-03219720>

Submitted on 13 Feb 2023

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1 **Hydroxychloroquine lung pharmacokinetics in critically ill patients infected**
2 **with COVID-19**

3
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24
25 **Running title:** Hydroxychloroquine concentration in lung epithelial lining fluid of COVID-19
26 patients.

27 **Abstract**

28 Different dosage regimens of hydroxychloroquine were used to manage COVID-19 patients,
29 with no information on the lungs' exposure in this population. The aim of our study was to
30 evaluate hydroxychloroquine concentrations in the lung epithelial lining fluid (ELF) in
31 patients infected with COVID-19. This study is a retrospective, observational, multicenter,
32 pharmacokinetics study of hydroxychloroquine in critically ill patients. No additional
33 interventions or additional samples compared to standard care of these patients were
34 conducted in our teaching hospital. We included all intubated COVID-19 patients treated with
35 crushed hydroxychloroquine tablets, regardless of the dosage administered by the nasogastric
36 tube. Blood and bronchoalveolar lavage (BAL) samples (n= 28) were collected from 22
37 COVID-19 patients and the total hydroxychloroquine concentrations in epithelial lining fluid
38 were estimated. Median hydroxychloroquine plasma concentrations were of 0.09 [0.06; 0.14]
39 mg/l and 0.07 [0.05; 0.08] mg/l for 400 mg x 1/day and 200 mg x 3/day, respectively. Median
40 hydroxychloroquine ELF concentrations were of 3.74 [1.10; 7.26] mg/l and 1.81 [1.20; 7.25]
41 for 400 mg x 1/day and 200 mg x 3/day, respectively. The median ratio of ELF/plasma
42 concentrations was of 40.0 [7.3; 162.7] and 21.2 [18.4; 109.5] for 400 mg x 1/day and 200 mg
43 x 3/day, respectively. Exposure in the ELF is likely to be underestimated due to the
44 concentrations of plasma hydroxychloroquine. In clinical practice, low plasma concentrations
45 should not induce an increase in drug dosage because the lung exposure may already be high.

46

47 **KEYWORDS:** COVID-19, hydroxychloroquine, plasma drug monitoring, bronchoalveolar
48 lavage (BAL)

49

50 1. Introduction

51 Based on the *in vitro* work carried out against SARS-CoV-2 and preliminary clinical data,
52 hydroxychloroquine is currently being used in the management of COVID-19 patients [1, 2].
53 Hydroxychloroquine may have an antiviral action through three main mechanisms: (1) viral
54 entry prevention, (2) impairment of viral replication and (3) pleiotropic action on the human
55 immune system through immuno-modulating activity [3]. The various *in vitro* studies have
56 shown that the EC₅₀ of hydroxychloroquine ranged from 0.72 to 4.4 μM (i.e. 0.241 to 1.4
57 mg/l) at 48 hours and 72 hours post-infection, respectively [3-5]. Pending the results of robust
58 clinical trials and due to the lack of pharmacokinetic-pharmacodynamic information in
59 COVID-19 patients, and in accordance with the National French Team, AC43-ANRS/STP-
60 SFPT, different dosage regimens were applied in Toulouse University Hospital (France) (200
61 mg x 3 / day; 400 mg x 2 on day 1 then 200 mg x 3 / day; 400 mg x 2 on day 1 then 400 mg /
62 day; and at least for ICU patients, 600 mg x 2 on day 1 then 400 mg/day) in order to reach
63 pharmacokinetic equilibrium as quickly as possible [6].
64 On 2 April 2020, the French Ministry of Health imposed a dosage regimen identical to the one
65 used in systemic lupus erythematosus (SLE; no loading dose and 200 mg x 3/day) for patients
66 treated outside the context of a clinical trial.
67 Regardless of the dosage regimen, plasma concentration was monitored in order to evaluate
68 individual drug exposure. It has been proven that the plasma concentrations measured in
69 COVID-19 patients tended to be lower than the values reported in SLE patients, in particular
70 for the standard regimen of “200 mg x 3/day” [7]. These preliminary results suggest that
71 hydroxychloroquine concentrations are unlikely to be adequately predicted using the
72 pharmacokinetic models derived from patients receiving hydroxychloroquine for SLE or
73 rheumatoid arthritis treatment [8].

74 As the apparent volume of distribution of hydroxychloroquine is so large in volunteers as well
75 as in malaria patients ($\approx 5\ 000\ \text{l}$ for the blood volume of distribution and $\approx 40\ 000\ \text{l}$ for the
76 plasma volume, at steady state) [9, 10], it can be suggested that the hydroxychloroquine gets
77 trapped in the red cells and granulocytes [9, 11, 12] and probably in various tissues [10]. It
78 could be assumed that the same occurs for COVID-19 patients. Consequently, it is natural to
79 question the concentration of hydroxychloroquine at the infectious site (i.e. the lung) [13].
80 Unfortunately, this information is not available. A lung biopsy is the most informative
81 approach. Even if a biopsy is a mixture of both intra- and extra-cellular matrices usually
82 homogenised so as to determine a mean concentration [14], hydroxychloroquine gets trapped
83 in the cells suggesting this drug is more likely present in the cells rather than outside.
84 However, this option would be highly intrusive and an alternative approach would be to
85 evaluate the hydroxychloroquine concentration in lung epithelial lining fluid (ELF) at the
86 bedside of intensive care unit (ICU) patients [15, 16].

87

88 Drug concentration in ELF can be inferred based on the concentration of hydroxychloroquine
89 in the bronchoalveolar lavage (BAL) fluid and the concentration of urea both in the plasma
90 and the BAL fluid [17]. This method is not new and is usually applied to explore the lung
91 diffusion of antibiotics in ICU patients. Consequently, when available, BAL fluid can be used
92 as a kind of “quality control” used to obtain information on the degree of impregnation in the
93 lung for a short period (10-15 days) of treatment.

94

95 The aim of our study was to evaluate hydroxychloroquine in the lung’s ELF in COVID-19
96 patients in order to estimate the level of lung exposure.

97

98 **2. Patients and Methods**

99 This study is a retrospective, observational, multicentre, pharmacokinetic study of
100 hydroxychloroquine in critically ill patients. We included all intubated COVID-19 patients
101 treated with crushed hydroxychloroquine tablets, regardless of the dosage administered by
102 nasogastric tube.

103 According to the guidelines established by the French National AC43-ANRS/STP-SFPT
104 Team in March/April 2020, blood samples were collected at different time points (from 48h
105 to 192h) during clinical management of COVID-19 patients after hydroxychloroquine
106 initiation and 30 minutes before drug administration (i.e. trough concentration). In plasma, the
107 steady state is supposed to be reached in 48 hours [18].

108
109 As part of our standard practice for monitoring patients with acute respiratory distress
110 syndrome (ARDS) who are at high risk for infectious complications, and in this particular
111 context of COVID-19, a mini-bronchoalveolar lavage (BAL) (twice 20 ml of physiological
112 saline) was systematically performed 7 days (+/-2 days) after treatment initiation or in the
113 event of a new respiratory degradation, for microbiological monitoring purposes
114 (bacteriology, mycology and viral replication of SARS-CoV-2). A leftover volume of
115 approximately 500 µl remained after these microbiological investigations, which was used to
116 determine the hydroxychloroquine concentration. BAL sampling was carried out between two
117 dose administrations, with no specific time imposed. Except for one case, only the BALs for
118 which plasma determinations were performed within 48 hours before or after collection were
119 included.

120
121 Hydroxychloroquine in plasma and BAL fluid concentrations were determined using a
122 chromatographic analytical method, validated as per FDA guidelines. All BAL sample

123 preparations included a protein precipitation and a virus inactivation step in a methanol
124 solution. The HCQ dosage method presents a lower limit of quantitation (LLOQ) of 0.05 mg/l
125 in the plasma and 0.01 mg/l in the BAL fluid, an upper limit of quantitation (ULOQ) of 2
126 mg/l in the plasma and the BAL fluid, and an intra- and inter-day variability of <4% and
127 <10% respectively. When plasma concentrations were <LOQ, the value was set to 0.025
128 mg/L (i.e. half the LLOQ). The plasma samples were stored at +4°C before analysis, for a
129 period of 24h maximum. The BAL fluid samples were stored at -80°C before analysis, for a
130 period of 30 days maximum. Previous studies have shown that hydroxychloroquine was
131 stable in the whole blood under these conditions [19]. As whole blood is a complex matrix, in
132 which xenobiotics tend to be less stable than other biological fluids, we considered that
133 hydroxychloroquine was also stable in plasma and BAL fluid in these conditions.

134 Urea is used as an endogenous marker of ELF because urea, a small and relatively nonpolar
135 molecule, can freely travel across membranes to reach the outer surfaces of alveoli. The
136 concentration of urea in ELF (Urea_{ELF}) is considered to be same as in the serum urea (Urea_{serum})
137 concentration, implying complete distribution. Therefore, the volume of ELF (V_{ELF}) is
138 adjusted for excess exogenous water using the following equation:

$$139 \quad V_{ELF} / V_{BAL} = \text{Urea}_{BAL} / \text{Urea}_{plasma}$$

140 Knowing (1) the concentration of hydroxychloroquine measured in the BAL (HCQ_{BAL}), (2)
141 the volume of BAL collected (V_{BAL}), the estimated ELF volume (V_{ELF}), it is then possible to
142 determine the concentration of hydroxychloroquine in ELF (HCQ_{ELF}) using the following
143 formula:

$$144 \quad \text{HCQ}_{ELF} = V_{BAL}/V_{ELF} \times \text{HCQ}_{BAL} = (\text{Urea}_{plasma} / \text{Urea}_{BAL}) \times \text{HCQ}_{BAL}$$

145 Plasma urea levels were determined using an automated enzymatic method, validated as per
146 FDA guidelines. This method presents a LLOQ of 0.5 mmol/l, an ULOQ of 15 mmol/l and an
147 intra- and inter-day variability < 2% and <3%, respectively. Safety practices require a greater

148 level of caution when handling respiratory specimen from SARS-CoV-2 positive patients
149 [20]. Thus, urea concentrations in BAL was assayed using a gas chromatography – a mass
150 spectrometry method which included a protein precipitation and virus inactivation step in a
151 methanol solution. A LLOQ of 0.1 mmol/l, and a ULOQ of 20 mmol/l were achieved.
152 Precision assays showed an intra-day variability < 9% and an inter-day variability < 10%.
153 BAL fluid samples were stored at -80°C before analysis, for a period of 60 days maximum.
154 Previous studies have shown that urea was stable in serum in these conditions [21].

155

156 Continuous data was expressed as median (25th-75th percentiles) and categorical variables in
157 numbers (percentages). The relationship between the plasma and ELF concentrations and the
158 other parameters was assessed by simple linear regression. The analysis was performed using
159 the MedCalc®15 statistical software program (Ostend, Belgium). $p < 0.05$ was considered to
160 be statistically significant.

161

162 This study is entered in the Toulouse University Hospital register of retrospective studies
163 (registration number: RnIPH 2020-33) and is covered by MR-004 (CNIL number: 2206723 v
164 0). This study was approved by Toulouse University Hospital and ethical requirements were
165 entirely respected.

166

167 **3. Results**

168 *3.1 Population*

169 Twenty-eight hydroxychloroquine plasma and BAL fluid concentrations from 22 patients
170 were measured (Table 1). The median patient age was of 60 [interquartile range (IQR) 53-70]
171 years, and 91% of the patients were male. The median body mass index (BMI) was of 28
172 [IQR 26-31] kg/m². The median SAPS II and SOFA scores pertaining to the included patients
173 were of 37 [IQR 32-46] and 6 [IQR 3-7] respectively, indicating a critically ill patient
174 population.

175

176 *3.2 Hydroxychloroquine trough concentrations*

177 The values of the BAL fluid hydroxychloroquine concentration were determined 7 to 12 days
178 after treatment initiation. For one point, the time from blood collection to BAL was 9.8 days,
179 but the plasma concentration was at steady-state.

180 The median hydroxychloroquine plasma concentrations were of 0.09 [0.06; 0.14] mg/l and
181 0.07 [0.05; 0.08] mg/l for 400 mg x 1/day and 200 mg x 3/day, respectively. The median
182 hydroxychloroquine ELF concentrations were of 3.74 [1.10; 7.26] mg/l and 1.81 [1.20; 7.25]
183 mg/l for 400 mg x 1/day and 200 mg x 3/day, respectively. The median ratio of ELF/plasma
184 concentrations were of 40.0 [7.3; 162.7] and 21.2 [18.4; 109.5] for 400 mg x 1/day and 200
185 mg x 3/day, respectively (Table 2).

186 The relationship between ELF and the plasma hydroxychloroquine concentration is presented
187 in Figure 1.

188

189 No relationship was observed between the measured hydroxychloroquine concentrations and
190 the biological parameters characterising renal and hepatic functions (Supplementary Table 1).

191

192 **4. Discussion**

193 As previously reported for many anti-infective drugs used to treat pulmonary infections, ELF
194 concentration gives information on the intra- and extracellular lung exposure [15, 16, 22].
195 However, this approach is essentially reserved for clinical research as the therapeutic
196 monitoring of anti-infective drugs in ELF is determined by practical and organisational
197 constraints. Firstly, performing a BAL requires that the operator be trained and that the patient
198 be stable enough to tolerate the serum injection in such way that it is only exceptionally
199 carried out on non-intubated patients. Secondly, drug quantification has to be performed in an
200 unconventional matrix (i.e. BAL) with a very sensitive analytical method (i.e. more often LC-
201 MS/MS). In the special case of COVID-19 patients, the BAL is contaminated by SARS-CoV-
202 2, thus imposing a specific and time consuming pre-analytical process.

203

204 We were able to gather all these conditions in order to assess whether all ELF concentrations
205 are higher than plasma concentrations, despite the variability of ELF values. The significant
206 variability in ELF concentrations may be explained in part by the BAL sampling. In fact, even
207 if the injection volume were standardised (2 x 20 ml), the dwell time and the aspiration
208 pressure cannot be strictly identical [23]. Cells can also be part of the ELF, especially
209 macrophages, and may be lysed when measuring the drug concentration. Depending on their
210 quantity, the lysis of these cells may induce an increase in the hydroxychloroquine
211 concentration [11, 17]. As no measurement of the cell burden in the BAL sample was
212 performed due to insufficient BAL volumes available for pharmacokinetic exploration, this
213 lack of information has to be considered as a limitation of our study. Indeed, the cell burden
214 in the BAL sample is likely associated with the ELF hydroxychloroquine concentration (i.e.
215 the more the cells, the higher the concentrations).

216 Collecting blood and BAL samples at a different moment (day and/or time) and the potential
217 post-dose discrepancy between the blood sample and the collection of BAL, could appear as
218 limiting the interpretation of the ELF/plasma concentrations ratios. However,
219 hydroxychloroquine presents a large volume of distribution with deep compartments (i.e.
220 lung, spleen, melanin-containing tissues... [3] leading to different kinetic profiles in the
221 plasma and lung tissue [24]. Indeed, half-life elimination is likely to be short in the plasma of
222 COVID-19 patients [7, 18] as opposed to deep compartments. The blood samples have always
223 been taken at steady state while the BALs could be collected always after the plasma
224 concentration peak. Indeed, the staff in charge of carrying out the BALs was warned of this
225 constraint. Furthermore, a flat kinetic profile was expected in the lung tissue [14]. As a
226 consequence, it seems reasonable to suppose that ELF/plasma concentrations ratios do not
227 change between administrations, once the steady state has been reached. In fact, the ideal
228 solution would consist in determining the area under the time-concentration curve (AUC) in
229 both plasma and BAL matrices at steady state and to calculate the AUC ratio. This option is
230 not feasible and not ethical for critically ill patients presenting ARDS because multiple BALs
231 would alter the gas exchanges between alveoli and capillaries. It would worsen PaO₂/FiO₂
232 ratios (the ratio of arterial oxygen partial pressure (PaO₂ in mmHg) to fractional inspired
233 oxygen (FiO₂ expressed as a fraction)).

234 However, as our data was retrospectively collected from a small population of ICU patients,
235 one limitation of our study is that the inter-individual variability of the plasma and ELF
236 hydroxychloroquine concentrations likely under/overestimates the actual inter-individual
237 value.

238 Our results show that hydroxychloroquine concentrations in the lung are higher than in the
239 ELF. Passage from the blood compartment to the ELF involves passing through the
240 pulmonary epithelial cells (i.e. prime target for the replication of SARS-CoV-2 [17, 25, 26])

241 in which hydroxychloroquine is most likely accumulated with pharmacokinetic hysteresis.
242 Hydroxychloroquine's mechanism of action is poorly elucidated, but includes, among others,
243 the increase in endolysosomal pH necessary for viral fusion. The initial fusion between the
244 viral and the cellular membranes (e.g. lung epithelial cells) requires an interaction between the
245 surface proteins of the two partners, and this interaction can only take place under particular
246 acidic conditions, through the phenomenon of endocytosis. The inability to obtain the ideal
247 pH can block this process, and it is probably through this means that hydroxychloroquine may
248 act. Other properties may be involved: modification in the glycosylation of angiotensin
249 converting enzyme-2, the receptor that SARS-CoV-2 uses to enter the cells and/or post-
250 translational modification of some viral proteins [27]. In addition, viral invasion may also
251 trigger a massive margination of the phagocytic cells to the infection site, which may deliver
252 increased amounts of hydroxychloroquine [28]. But, in the absence of clear information on
253 the influence of the inflammatory status reported in COVID-19 patients as to the
254 accumulation of hydroxychloroquine in lungs, this point should be considered as a limit of
255 our study.

256

257 Plasma concentration is not predictive of lung concentration, as shown in Figure 1. Therefore,
258 drug dosage determinations in studies assuming equilibrium between epithelium and plasma
259 concentrations may lead to overly high dosages [29]. Furthermore, in clinical practice, low
260 plasma concentrations should not induce an increase in the drug dosage because the lung
261 exposure may already be high. The various *in vitro* studies have shown that the EC₅₀ of
262 hydroxychloroquine ranged from 0.72 to 4.4 μ M (i.e. 0.241 to 1.4 mg/l) at 48 hours and 72
263 hours post-infection, respectively [3-5]. However, EC₅₀ could be determined in Vero cell
264 lines, but not in a human epithelial cell model [4]. This discrepancy may explain that
265 hydroxychloroquine has not shown efficacy in clinical trials [29]. Thus, whether for a dose of

266 400 mg x 1 /day or 200 mg x 3 /day, the median ELF concentration of hydroxychloroquine is
267 above the maximum EC₅₀ value. Therefore, both regimens lead to a median lung exposure
268 that could be sufficient to eradicate the virus. However, the heterogeneity of EC_{50s} raises the
269 problem of selecting the "right" threshold used to determine the dosage, in particular through
270 modelling techniques. In conclusion, despite all its imperfections, BAL fluid provides a rough
271 idea of lung exposure.

272

273 **Acknowledgements**

274 We would like to thank Dr Marion Grare and Dr Sophie Cassaing for their help in sampling
275 bronchoalveolar fluid, the technicians of the Pharmacokinetics and Toxicology Laboratory of
276 Toulouse University Hospital, Magali Centelles for her help in data collection, and Della Hoy
277 for her kind help proofreading the English.

278

279 **Declarations**

280 **Funding:** This study was supported by internal funding.

281 **Competing interests:** None to declare

282 **Ethical Approval:** This study was entered in the Toulouse University Hospital register of
283 retrospective studies (registration number: RnIPH 2020-33) and is covered by MR-004 (CNIL
284 number: 2206723 v 0). This study was approved by Toulouse University Hospital and all
285 ethical requirements were complied with.

286

287 **Authors' contributions:** SR, DC, JMC and PG conceptualised the research aims, designed
288 the study, and took responsibility for the integrity of the data and the accuracy of the data
289 analysis. PG, HV, DR, VM and BG contributed to the acquisition of data. TL, SB, ML and
290 CM performed HCQ concentration determinations in BALs and plasma. DC and JMC

291 performed the statistical analysis. SR, DC, JMC and PG wrote the first draft of the paper, and
292 other authors provided comments and approved the final manuscript.

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Table legends

Table 1: Socio-demographic and clinical data of 22 severe COVID-19 patients treated with hydroxychloroquine and for whom a bronchoalveolar lavage was performed (BMI: body mass index; CKD-EPI: Chronic Kidney Disease Epidemiology; IQ: interquartile; SAPS II: simplified acute physiology score; SOFA: Sepsis-related Organ Failure Assessment).

Table 2: Hydroxychloroquine (HCQ) plasma and epithelial lining fluid (ELF) concentrations (IQR: interquartile range).

Figure legend

Figure 1: Relationship between lung epithelial lining fluid (ELF) and plasma hydroxychloroquine (HCQ) concentrations. The red line is the identity line (i.e. the plasma and ELF concentrations are equal). The dots above the red line indicate that the concentrations in the ELF are higher than the plasma concentrations (ELF/plasma HCQ concentrations: 38.072 [8.338 to 138.521]). This figure shows that (i) even with small plasma concentrations, the ELF concentration can be high and (ii) the plasma concentration is a poor predictor of the ELF concentration.

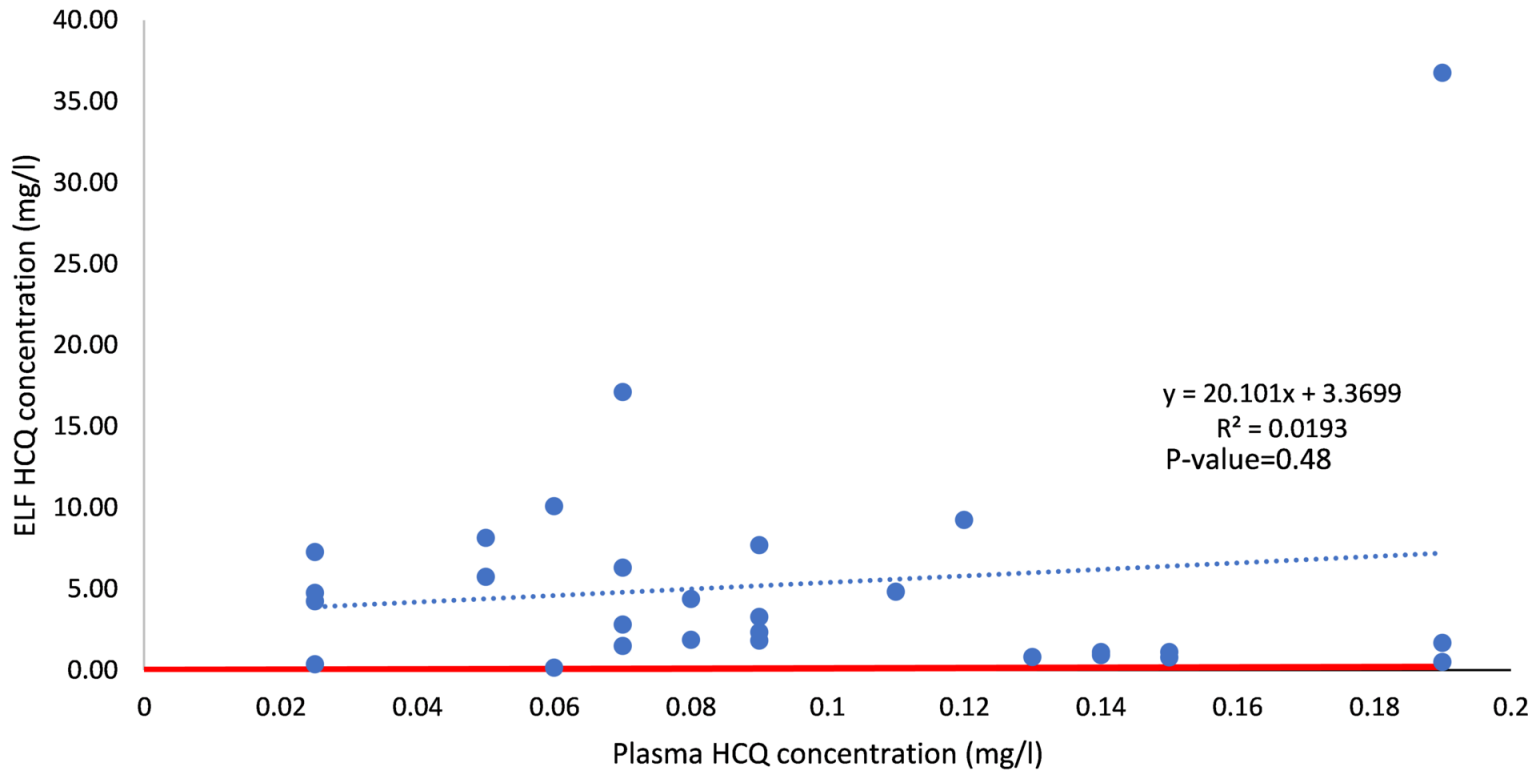


Table 1: Socio-demographic and clinical data of 22 severe COVID-19 patients treated with hydroxychloroquine and for whom a bronchoalveolar lavage was performed (ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: body mass index; CKD-EPI: Chronic Kidney Disease EPIdemiology; D0: day 0 of the hydroxychloroquine initiation, D7: day 7 after hydroxychloroquine initiation; IQR: interquartile range; SAPS II: simplified acute physiology score; SOFA: Sepsis-related Organ Failure Assessment).

All Patients (n=22)	Median [IQR]	Min - Max
Age	59.5 [53 to 70]	30 - 81
BMI	28.3 [26 to 31.3]	20.7 - 37
SAPS II	37 [32 to 46]	8 - 76
SOFA score	6 [3 to 7]	2 - 14
Proteinemia (g/L) D7	61 [59 to 68]	50 - 77
AST(U/L) D7	65[69 to 179]	28 - 135
ALT (U/L) D7	99 [69 to 179]	18 - 257
Bilirubin (μmol/L) D0	7.6 [5.15 to 11.2]	4 - 29
CKD-EPI D7 (mL/min/1.73 m²)	97 [60.5 to 105.8]	9 - 123
Duration of invasive ventilation (day)	19.5 [11 to 28]	0 - 22

Table 2: Hydroxychloroquine (HCQ) plasma and epithelial lining fluid (ELF) concentrations (IQR: interquartile range).

	All dosages		400mg x1/day		200 mgx3/day	
	Median [IQR]	Min - Max	Median [IQR]	Min - Max	Median [IQR]	Min - Max
Plasma HCQ concentrations (mg/L)	0.09 [0.06 to 0.14]	0.03 – 0.19	0.09 [0.06 to 0.14]	0.03 – 0.19	0.07 [0.05 to 0.08]	0.03-0.09
ELF HCQ concentrations (mg/L)	3.03 [1.10 to 6.78]	0.13 – 36.75	3.74 [1.1 to 7.26]	0.13 – 36.75	1.807 [1.2 to 7.25]	0.34 – 10.08
ELF/plasma HCQ concentrations	38.07 [8.34 to 138.52]	2.1 - 290.4	39.96 [7.33 to 162.66]	2.1 - 290.4	21.22 [18.41 to 109.49]	13.4 - 168