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A robust enzyme-linked immunosorbent assay to measure serum ramucirumab concentrations

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1 **A robust enzyme-linked immunosorbent assay to measure serum**
2 **ramucirumab concentrations**

3

4

5 **Abstract:**

6 **Introduction:** Ramucirumab, an anti-VEGFR2 monoclonal antibody, has been approved for the
7 treatment of metastatic gastric and colorectal cancer. An assay measuring ramucirumab
8 serum concentrations was needed to investigate its pharmacokinetics and concentration-
9 response relationship.

10 **Results:** An ELISA was developed and validated according to the international guidelines for
11 ligand binding assays. Ramucirumab calibration standards ranged from 0.125 to 40 mg/L. Low,
12 Middle and High Quality Controls were spiked at 0.2, 4 and 8 mg/L, respectively. The limits of
13 quantification were established to be 0.125 and 10 mg/L for LLOQ and ULOQ, respectively. No
14 cross-reactivity with anti-VEGF or anti-EGFR was detected.

15 **Conclusion:** This in-house-developed ELISA is sensitive, accurate, reproducible, and suitable
16 for pharmacokinetic studies of ramucirumab.

17

18 **Keywords:** Monoclonal antibody, ramucirumab, enzyme-linked immunosorbent assay,
19 Pharmacokinetics, Therapeutic drug monitoring.

20

21

22 Introduction

23 During the last two decades, the therapeutic arsenal against advanced malignancies was
24 strengthened by the use of anti-angiogenic monoclonal antibodies (mAbs) or fusion proteins
25 in addition to standard chemotherapy, as bevacizumab, aflibercept and later ramucirumab [1-
26 4]. Ramucirumab was approved in gastric cancer [5, 6], aggressive non-small cell lung cancer
27 [7], metastatic colorectal cancer [8] and hepatocellular carcinoma [9].

28

29 Ramucirumab, developed as IMC-1121B (ImClone Systems) and marketed as Cyramza®
30 (LY3009806, Lilly), is a 147 kDa fully human immunoglobulin G1 (IgG1 κ) mAb that targets
31 extracellular domain of vascular endothelial growth factor receptor 2 (VEGFR-2). VEGFR-2
32 ligands include VEGF-A, formerly known as vascular permeability factor (VPF) and one of the
33 most potent anti-angiogenic factors discovered and characterized in the 80s [10, 11]. The
34 intended target of ramucirumab is VEGFR-2 expressed on endothelial cells surface and its
35 expected effect is an inhibition of the VEGF-A/VEGFR-2 axis involved in pathological
36 angiogenesis. However, it is worth noting that the concept of direct effects of anti-angiogenic
37 therapies on tumor cells themselves was only recently highlighted and reviewed by Simon *et*
38 *al.* [12].

39

40 The administration of approved doses of ramucirumab leads to a large pharmacokinetic
41 variability, which is influenced by several individual sources of variability as body weight [13].
42 In addition, other factors, as serum albumin levels or tumor burden may influence
43 ramucirumab pharmacokinetics [14]. This variability is relevant, since ramucirumab
44 concentrations lead to better clinical response. Indeed, higher ramucirumab exposure was

45 associated with longer overall and progression-free survivals [15]. Thus, the interpretation of
46 clinical efficacy of ramucirumab might benefit from accurately defined pharmacokinetic
47 parameters [16, 17].

48

49 The present work focused on the development of an Enzyme Linked Immunosorbent Assay
50 (ELISA) that measures accurately and reproducibly ramucirumab concentrations for a large
51 range of concentrations. A high level of sensitivity (*i.e.* very low LLOQ - Low Limit Of
52 Quantification) is also required for future pharmacokinetic studies. This ELISA technique was
53 developed under both European Medicines Agency (EMA) and US Food and Drug
54 Administration (FDA) guidelines.

55

56 **Material and Methods**

57 Reagents

58 96-well plates were purchased from ThermoFisher Scientific (Nunc plates, Elancourt, France).
59 Phosphate-buffered saline (PBS) was provided by Immuno Concepts (Sacramento, CA). Tween
60 20 was purchased from Biosolve (Dieuze, France), Bovine serum albumin (BSA), a polyvalent
61 peroxidase-conjugated goat anti-IgG specific for Fc fragment (HRP-anti-human IgG; lot
62 number 067M4885V and 127M4800V), and FAST o-phenylenediamine dihydrochloride tablets
63 were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). A human recombinant
64 protein of 82.5 kDa, soluble form of VEGFR-2 from Clinisciences (Nanterre, France), was used
65 as the capture antigen. A stock solution of 10 mg/ml of ramucirumab (Cyramza®) was
66 purchased from Eli-Lilly (Indianapolis, IN, USA) through [REDACTED].

67

68 Reagent preparation

69 The calibration standards and quality controls (QCs) prepared from a 10 mg/ml ramucirumab
70 stock solution were aliquoted and stored at +4°C until use or at -20°C for long term.
71 Concentrations of standard calibrators were the following 0.125, 0.25, 0.625, 2.5, 12.5, 25 and
72 40 mg/l and the concentrations of QCs were 0.2, 4 and 8 mg/l. A 1:100 dilution in 1% (w/v)
73 PBS-BSA was prepared for all calibration standards, QCs and samples.

74

75 Assay procedure

76 Assay validation followed the procedure previously described [18-20]. Notably, coating was
77 performed overnight at +4°C, by incubating 100 µL/well of 2 mg/l sVEGFR-2 in carbonate-
78 bicarbonate buffer. Calibration standards, QCs and samples were incubated at 37°C during 1 h
79 under agitation. One hundred microliters per well of HRP conjugated-anti IgG antibody
80 (1:8000 dilution in 1% PBS-BSA) was used. The final absorbance was read at 490 and 630 nm
81 (for correction) on an ELx808 ELISA plate reader (BioTek, VT, USA). Non-specific background
82 was determined with carbonate-bicarbonate buffer only. Serum ramucirumab concentrations
83 were calculated with a 4-parameter logistic calibration curve ($\text{Response} = (\text{Min-Max}) / (1 +$
84 $(\text{Concentration}/\text{Inflection point})^{\text{Slope}} + \text{Max})$) implemented in Gen5 software (BioTek, VT,
85 USA).

86

87 Precision, accuracy, limit of quantification

88 The precision and accuracy were assessed as previously described [18-20]. Briefly, intra- and
89 inter-day precisions and accuracies were defined as coefficient of variation (% CV = Standard
90 deviation (SD)/mean) and absolute bias (% RE, where relative error (RE) = [measured value –
91 actual value]/actual value), respectively. The limit of detection (LOD) was determined as
92 3 times the mean SD of the concentration of 11 blank samples. The LLOQ corresponded to the

93 lowest calibration standard. The upper limit of quantification (ULOQ) was defined as the
94 highest amount of ramucirumab that could be quantified with SD and RE < 20%. The low QC
95 value was within three times the LLOQ, the mid QC value was the geometric mean of the curve
96 and the high QC was spiked at 80% of ULOQ.

97

98 Matrix effect and other parameters

99 To assess the matrix effect, we compared the precision and accuracy when QCs were prepared
100 in PBS-BSA or normal human serum (n = 4) against calibrators prepared in PBS-BSA. The
101 recovery percentage was calculated as (recovered concentration / spiked concentration) x
102 100. These results were compared using a non-parametric Mann-Whitney test. Dilution
103 linearity, prozone, parallelism and stability were determined in agreement with our published
104 methods without any particular modification [18-22].

105

106 Selectivity & specificity

107 Selectivity was assessed using serum from healthy controls (n=18) and serum from healthy
108 controls spiked with rheumatoid factor (55 UI/ml or 30 UI/ml or 15 UI/ml) (n=6 for each).
109 Analytical interference with other mAbs used in colorectal cancer patients was evaluated
110 using serum samples from patients treated with anti-VEGF-A bevacizumab (n=9), anti-EGFR
111 (Epidermal Growth Factor Receptor) cetuximab (n=9) or VEGF-targeting fusion protein
112 aflibercept (n=9). Identical samples (except for aflibercept) were spiked with ramucirumab at
113 a final concentration equal to 4 mg/L. Normal human serum was spiked with aflibercept and
114 ramucirumab at a ratio equal to 1:1 or 1:5. The impact of hemolyzed and lipemic samples
115 spiked or not with ramucirumab (= 4 mg/L) was also tested. In all cases, to consider there is
116 no cross-reactivity, the concentrations of these samples had to be below the LLOQ of

117 ramucirumab, ie 0.125 mg/l.

118

119 Detection of unbound ramucirumab

120 Ramucirumab is a bivalent molecule and is able to bind two molecules of VEGFR2. When

121 ramucirumab is binding to VEGFR2, it blocks activity and prevents fixation of VEGFA, VEGF-C

122 and VEGF-D. During ramucirumab treatment, three different species may be present in the

123 serum: free ramucirumab and ramucirumab bound to either 1 or 2 VEGFR2 molecules. Among

124 these three species, biologically active species are free ramucirumab and ramucirumab

125 monovalently bound to a molecule of VEGFR2. Therefore, we tested our in-house ELISA by

126 mixing serum samples containing ramucirumab (4 mg/L) with increasing

127 ramucirumab:sVEGFR2 ratios of: 0:25, 1:0, 1:1, 1:5 and 1:25. We also tested ramucirumab

128 (4 mg/L) mixed with increasing ramucirumab:VEGF-A ratios of: 0:100, 1:0, 1:1, 1:10 and 1:100.

129 All mixes were incubated for 30 minutes at 37°C. Each ratio was assayed on four occasions on

130 the same day.

131

132 Data interpretation: acceptance and rejection criterion

133 Measurements were accepted if differences between duplicates were lower than 20% for

134 more than 75% of the calibrators. Calibrators and QCs performances were validated when the

135 intra- and inter-day precision and accuracy were between $\pm 20\%$ ($\pm 25\%$ at LLOQ and ULOQ)

136 and the total error [ICVl + IREl] was between $\pm 30\%$ ($\pm 40\%$ at the LLOQ and ULOQ). In order to

137 be validated, the precision of each back-calculated concentration, had to be within $\pm 20\%$

138 (dilution linearity) and $\pm 30\%$ (parallelism) of the target concentration [23-29].

139

140 Patients and blood samples

141 Concentrations were measured in serum samples collected from two patients treated for
142 advanced colorectal cancer by 8 mg/kg ramucirumab infusions every 2 weeks. These samples
143 are part of a declared biological collection (N° [REDACTED]) and were in conformity with the
144 National Data Information and Freedom Commission (CNIL). Trough (n=9) and peak (n=9)
145 samples were collected just before infusion and 2 hours after the end of the infusion,
146 respectively. This study was an ancillary study performed after the constitution of the declared
147 biological collection.

148

149 **Results**

150 Enzyme-Linked Immunosorbent assay

151 The chosen coating concentration led to reproducible standard calibration. A mean curve from
152 six individual calibration curves on 6 different days is given on Figure 1. This latter was
153 generated using ramucirumab concentrations from 0 to 40 mg/l. The limits of quantification
154 (LOD, LLOQ and ULOQ) (table 1) of the assay are within the range of our previously published
155 ELISA methods [18-20].

156

157 Precision & accuracy of the assay

158 Intra-day and inter-day reproducibility, precision and accuracy of calibration standards and
159 QCs were within -/+ 15% of the nominal value (Table 2). The total error was below 30% for all
160 calibration standards.

161

162 Matrix effect and other parameters

163 Mean concentrations (CV, bias) of low (0.2 mg/l), medium (4 mg/l), and high (8 mg/l) QCs
164 prepared in PBS-BSA were 0.19 mg/l (3.63%, -3.45%), 3.92 mg/l (5.93%, -2.09%), and 8.12 mg/l

165 (4.77%, 1.52%), respectively. In human serum, mean concentration of QCs were 0.13 mg/l
166 (7.93%, -36.07%), 3.23 mg/l (3.92%, -19.30%), and 6.36 mg/l (3.63%, -20.56%), respectively.
167 Concentrations of low QC prepared in human serum were significantly lower than those
168 measured in PBS-BSA (Mann–Whitney test, $P < 0.05$). Therefore, the calibration standards and
169 QCs were prepared in the same matrix as the samples as previously recommended by our
170 team [19].

171

172 Dilution linearity & prozone

173 A serum sample was spiked with ramucirumab at a concentration of 200 mg/l and was diluted
174 1:10, 1:20, 1:50, 1:100, 1:500, 1:1000, 1:2000 and 1:5000 in normal human serum $\pm 20\%$,
175 which is in accordance to EMA guideline (Figure 2) [26]. Therefore, samples can be diluted up
176 to 1,000-fold. No prozone effect was observed until a ramucirumab concentration of
177 1,000 mg/l (Figure 3). The dynamic range of the assay is therefore between 0.125 – 10 mg/l.,
178 i.e. between the LLOQ and ULOQ of the assay.

179

180 Parallelism

181 Parallelism was assessed using different dilutions (1:25 to 1:1000) with a panel of three patient
182 serum samples ($n = 1$ for each dilution). Patient's serum concentrations were respectively
183 192.56, 153.25 and 145.10 mg/l. All measured concentrations were within the target
184 concentration of $\pm 30\%$ as recommended by guidelines on bioanalytical method validation [24,
185 26-28, 30], indicating that samples can be diluted up to 1,000-fold (Figure 4).

186

187 Selectivity

188 Selectivity of the assay was determined by using sera from healthy donors, from normal

189 human serum spiked with rheumatoid factor and from patients treated with bevacizumab,
190 cetuximab or aflibercept (Figure 5). All measured concentrations were below LLOQ. The same
191 samples (except for aflibercept) were spiked with ramucirumab and all concentrations were
192 equal to 4 mg/L ($\pm 20\%$). For normal human serum spiked with aflibercept and ramucirumab
193 at a ratio equal to 1:1 or 1:5, measured concentrations were also equal to 4 mg/L ($\pm 20\%$).
194 The same results were obtained with hemolyzed and lipemic samples spiked or not with
195 ramucirumab. These results revealed no analytical interference.

196

197 Ramucirumab concentrations after addition of sVEGFR2 or VEGF-A

198 Mean ramucirumab concentrations (SD) were 0.1 (0.01), 3.6 (0.28), 1.7 (0.19), 0.3 (0.01) and
199 0.2 (0.00) mg/L for 0:25 (negative control), 1:0 (positive control), 1:1, 1:5 and 1:25
200 ramucirumab to sVEGFR2 respectively.

201 Mean ramucirumab concentrations (SD) were <LLOQ (not calculated), 4.1 (0.27), 4.3 (0.24),
202 3.4 (0.33) and 2.0 (0.14) mg/L for 0:100 (negative control), 1:0 (positive control), 1:1, 1:10 and
203 1:100 ramucirumab to VEGF-A respectively.

204

205 Stability

206 The three concentration levels maintained at room temperature, +4°C and -20°C were all
207 stable up to 14 days, 28 days and 180 days, respectively (Supplemental figure 1). Samples were
208 stable up to 7 freeze-thaw cycles (Supplemental figure 2). In all cases, the relative error was
209 below 20%.

210

211 Patients samples concentrations

212 Ramucirumab serum concentrations were measured in 2 patients treated for metastatic

213 colorectal cancer and were both above LLOQ except for baseline concentration (0 mg/l).
214 Median (range) trough and peak concentrations during treatment were respectively 26.6 (9.3-
215 56.9) mg/l and 156.0 (93.3-243.0) mg/l.

216

217 **Discussion**

218 To our knowledge, this is not the first ELISA technique that was published to measure
219 ramucirumab concentrations. Three other techniques have been published so far, but present
220 considerable differences with our technique, including limits of quantification. The advantage
221 of our in-house ELISA is characterized by a lower LLOQ (0.125 mg/l vs 0.5 mg/l [31] and
222 2.5 mg/l [32]) and a higher ULOQ (10 mg/l vs 0.2 mg/l) [33]. Furthermore, we report here more
223 detailed information about intra- and inter-run variabilities, selectivity, interferences, and
224 dilution-induced inaccuracy or imprecision on our development and validation method.

225 This high sensitivity of our assay allowed measuring very low concentrations. To our
226 knowledge, the pharmacokinetics of ramucirumab was investigated in only one study using
227 population PK modelling. We therefore believe that this pharmacokinetic, notably the
228 elimination decay still has to be thoroughly investigated. This can only be done using an assay
229 able to measure low concentrations that are observed after single doses or when treatment
230 is discontinued. These measurements are needed for an accurate estimation of
231 pharmacokinetic parameters. A sensible assay is even more mandatory if a nonlinear target-
232 mediated elimination has to be studied. This level of sensitivity may not be useful for clinical
233 trials, but is mandatory for pharmacokinetic studies a feature needed to estimate
234 pharmacokinetic parameters in future studies.

235

236 An exposure-response relationship has been observed from randomized trials of ramucirumab

237 in lung cancer, gastric cancer and colorectal cancer [15, 34, 35]. Therefore, a fully validated
238 assay is required to thoroughly investigate ramucirumab pharmacokinetics (dose-
239 concentration relationship) and concentration-effect relationship, either in clinical trials or in
240 routine clinical use of this drug. Moreover, the understanding of the interindividual variability
241 of ramucirumab clinical response has to be made with respect to a sound assessment of its
242 concentration-effect relationship, in order to determine the optimal dosing regimen which
243 maximizes patients survival, while minimizing the risk of adverse events [36, 37].

244

245 In the context of daily clinical practice with samples from our registered biological collection,
246 concentrations ranged from 26.6 mg/l to 156 mg/l), corresponding to trough and peak
247 concentrations, respectively. This range of quantification is in accordance with the values
248 reported in clinical studies, i.e. 49.5 [6.3-228 mg/l] to 74.4 mg/l [13.8- 234 mg/l]) [38] or
249 49.7 to 81.1 mg/L for Cohn *et al.* [34] which confirms the relevance of measurements provided
250 by our ELISA technique.

251

252 To eliminate matrix effect for low QC concentration in human serum, the calibration standards
253 and QCs were prepared in human pooled serum. In the present study, we observed no
254 analytical interference with bevacizumab, cetuximab or aflibercept. This result reveals that
255 our ELISA may be also useful to measure serum ramucirumab concentration in patients
256 concomitantly or previously treated with an anti-VEGF or an anti-EGFR biopharmaceutical.
257 Moreover, since our ELISA is based on the capture of ramucirumab by sVEGFR2, i.e.
258 ramucirumab unbound or bound to one sVEGFR2. In order to confirm this hypothesis, we
259 tested ramucirumab mixed with increasing concentrations of sVEGFR2. Measured
260 ramucirumab concentration decreased when the amount of sVEGFR2 increased. When

261 ramucirumab and sVEGFR2 were equimolar, measured ramucirumab concentration
262 decreased to nearly 50% of that obtained in the absence of sVEGFR2. Our test may reflect
263 unbound ramucirumab, since almost total signal inhibition occurs for molecular ratio above
264 1:5. The influence of VEGFA was also tested by measuring concentrations in normal human
265 serum spiked with ramucirumab with or without VEGFA. The ramucirumab concentration is
266 not affected by for equimolar ratio and decreased by half for ratio 1:100. The difference
267 between the two targets is explained by a higher affinity of ramucirumab for VEGFR2. These
268 two experiments revealed that our concentration measurement reflects the unbound fraction
269 of ramucirumab.

270

271 **Conclusion**

272 Overall, we developed and validated an immunoassay that is suitable for measuring serum
273 concentrations of ramucirumab in treated patients according to guideline criteria. Our assay
274 is sensitive, reproducible and accurate and thus can be used in both pharmacokinetic studies
275 and future therapeutic drug monitoring of ramucirumab.

276

277 **Future perspective**

278 In the [REDACTED]-BLINDED-[REDACTED] clinical trial

279 [REDACTED]-BLINDED-

280 [REDACTED]

281 the measure of ramucirumab concentrations, will allow better understanding of the
282 relationship between ramucirumab concentrations and clinical outcomes in advanced gastric
283 cancer in elderly patients, which has never been evaluated before.

284

285 **Ethical conduct of research**

286 The authors state that they have obtained appropriate institutional review board approval or
287 have followed the principles outlined in the Declaration of Helsinki for all human or animal
288 experimental investigations. In addition, for investigations involving human subjects,
289 informed consent has been obtained from the participants involved.

Background

- Pharmacokinetic and concentration-response studies of ramucirumab, a full human anti-VEGFR-2 monoclonal antibody, require a validated and robust analytical method.

Experimental section

- An ELISA using sVEGFR-2 coated microtiter plates was developed.
- The assay was validated according to International guidelines (EMA & FDA).

Results

- The assay enables the reproducible measurement of ramucirumab concentrations in human serum.
- The range of quantification is 0.125 to 10 mg/l.

Conclusion

- The assay is sensitive and accurate, and suitable for both pharmacokinetic studies and, potentially, therapeutic drug monitoring of ramucirumab.

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- 414
- 415

416 **Table 1. Limits of detection and quantification of the assay.**

417

Parameter	Value (mg/l)
LOD	0.02
LLOQ	0.125
ULOQ	10.00

418

419

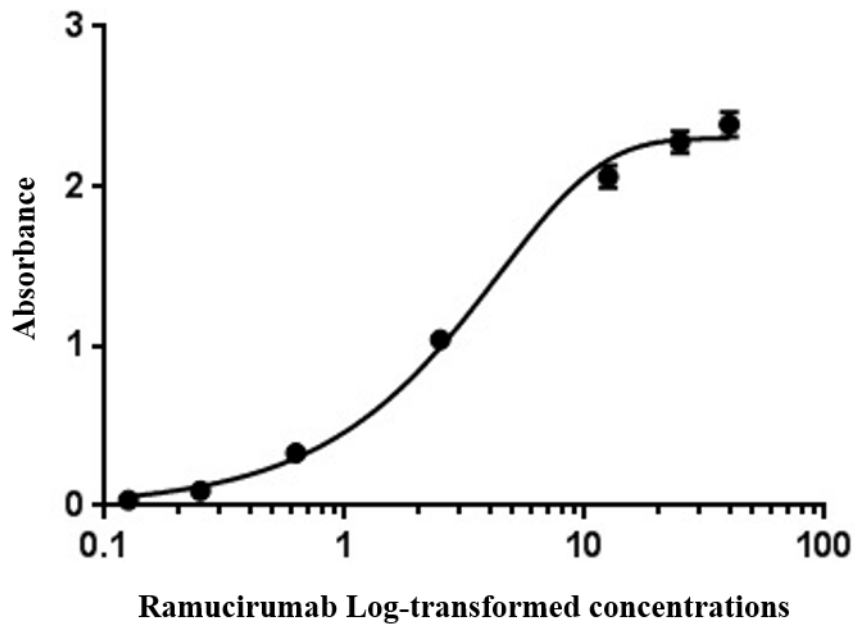
420 **Table 2. Intra and interday precision, accuracy and total error of calibration standards and**
 421 **quality controls.**

422

Spiked concentration	Measured concentration (mg/l)		Precision (CV%)		Accuracy (%RE)		Total error (%CV + %RE)		
	Intraday (n=9)	Interday (n=12)	Intraday (n=9)	Interday (n=12)	Intraday (n=9)	Interday (n=12)	Intraday (n=9)	Interday (n=12)	
Calibration standards (mg/l)									
40	40.50	42.59	6.97	13.60	1.25	6.49	8.22	20.08	
25	24.04	26.65	7.18	7.88	3.83	2.60	11.01	10.48	
12.5	12.96	12.42	2.67	3.25	3.67	0.61	6.35	3.87	
2.5	2.45	2.51	1.13	2.26	1.86	0.45	3.00	2.71	
0.625	0.67	0.65	2.21	5.04	7.71	4.03	9.93	9.07	
0.25	0.22	0.22	3.35	5.67	10.46	13.06	13.81	18.73	
0.125	0.11	0.12	6.34	10.44	10.37	5.90	16.72	16.34	
QCs (mg/l)									
8 (n=12)	7.58	7.80	7.92	8.96	5.29	2.49	13.21	11.45	
4 (n=12)	3.53	3.75	6.02	8.31	11.79	6.35	17.81	14.66	
0.2 (n=12)	0.20	0.22	3.48	5.57	1.40	10.63	4.87	18.20	

423

424



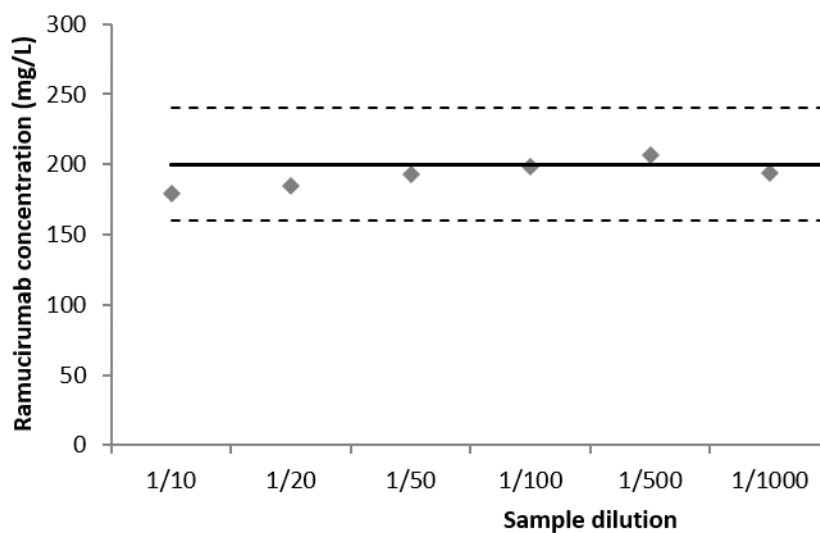
425

426

427 **Figure 1. Mean standard curve of ramucirumab ELISA.**

428 Six curves were obtained on 6 different days. Eight calibration standards were prepared in
 429 normal human serum in a wide range of concentrations (0 to 40 mg/l). Results were used to
 430 assess the four-parametric logistic standard curve. The error bars represent standard
 431 deviations.

432

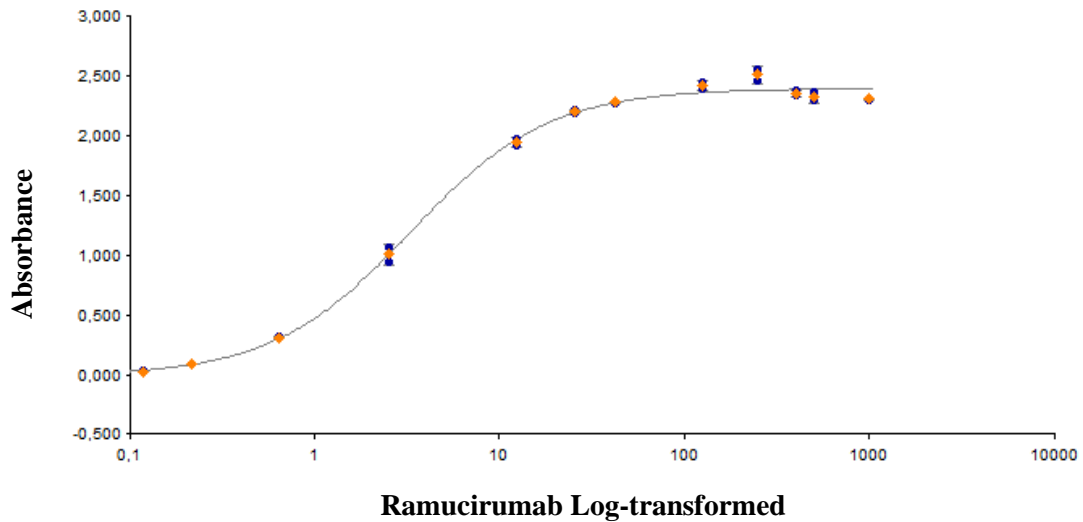


433

434 **Figure 2. Dilution linearity of ramucirumab ELISA.**

435 Samples containing 200 mg/l of ramucirumab were diluted up to 1,000 folds and analyzed in
 436 duplicate. Target concentration is represented as solid line and $\pm 20\%$ of target concentrations
 437 are displayed as dashed lines.

438

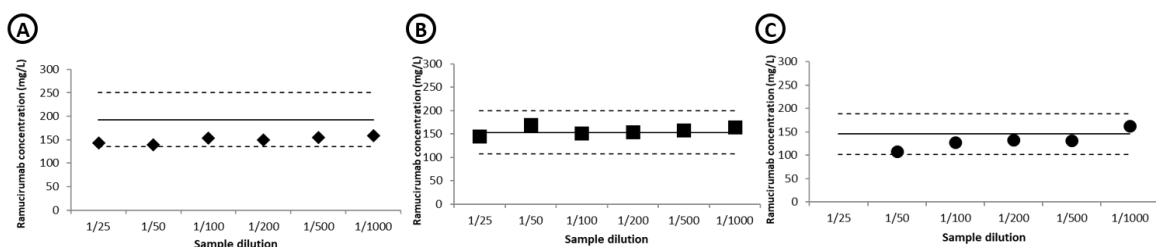


439

440

441 **Figure 3. Research of a prozone with ramucirumab ELISA.** A four parametric curve was
 442 produced using ramucirumab concentrations up to 1,000 mg/l.

443

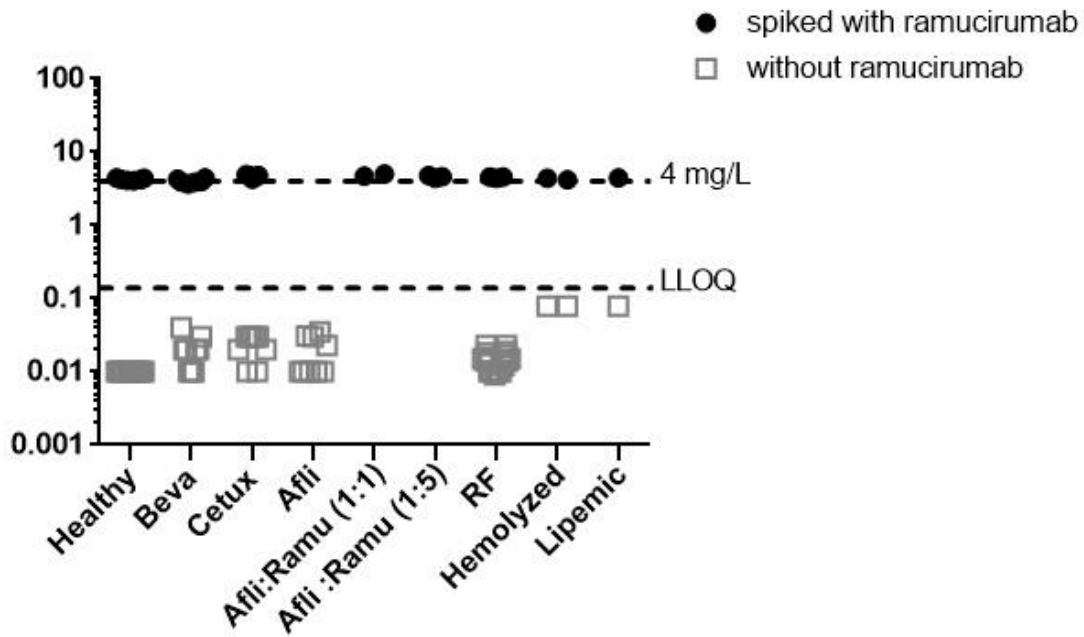


444

445 **◆ 192.56 mg/l ramucirumab ■ 153.25 mg/l ramucirumab ● 145.10 mg/l ramucirumab**

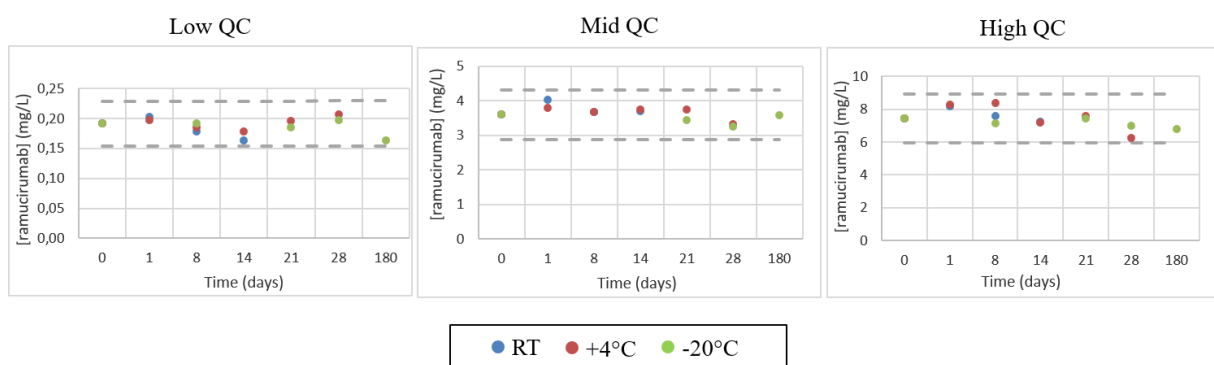
446

447 **Figure 4. Parallelism of ramucirumab ELISA.** Three different patient sera samples containing
 448 192.56 mg/l (A), 153.25 mg/l (B) or 145.10 mg/l (C) were diluted up to 1,000 fold. Target
 449 concentration is represented as solid line and $\pm 30\%$ are figured with dashed lines.



450
 451 **Figure 5. Assay selectivity.** Selectivity was tested with sera from healthy donors, sera from
 452 patients treated with bevacizumab, cetuximab, normal human serum spiked with aflibercept
 453 or rheumatoid factor (RF). Ramucirumab concentration was measured in all samples spiked
 454 or not with ramucirumab. The same tests were done in hemolyzed and lipemic samples.
 455 (LLOQ: Lower limit of quantification).

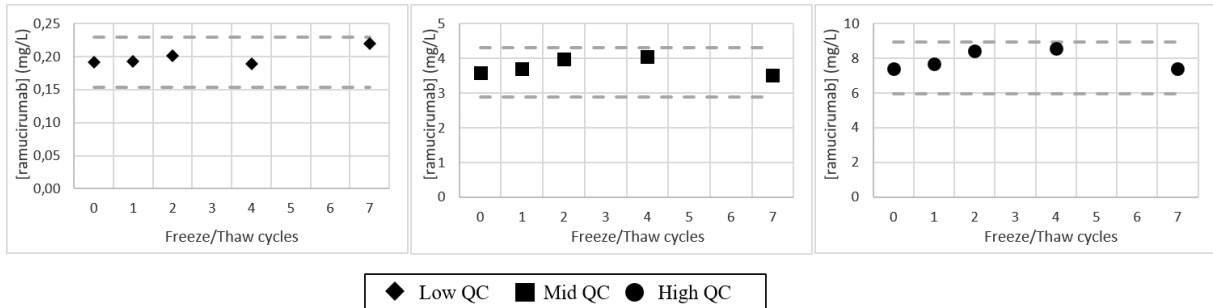
456



457
 458 **Supplemental figure 1. Stability of the samples according to storage temperature.** Quality
 459 controls were stored at different conditions for various durations. Ramucirumab

460 concentration was then measured. Dashed lines represent $\pm 20\%$ of the concentration of the
461 corresponding QC.

462



463

464 **Supplemental figure 2. Stability of the samples according to the number of freeze/thaw**
465 **cycles.** Samples were frozen at -20°C and thawed at room temperature. The freeze/thaw cycle
466 was repeated up to 7 times and ramucirumab concentrations was then measured. Dashed
467 lines represent $\pm 20\%$ of the concentration of the corresponding QC.

468

469