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1 **A multiplex liquid chromatography tandem mass spectrometry method for the**
2 **quantification of seven therapeutic monoclonal antibodies:**
3 **application for adalimumab therapeutic drug monitoring in patients with**
4 **Crohn's Disease.**

5
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21

22 Abbreviations

23 mAbs, therapeutic monoclonal antibodies

24 TDM, therapeutic drug monitoring

25 ADM, adalimumab

- 26 IFX, infliximab
- 27 SEC, secukinumab
- 28 TRZ, trastuzumab
- 29 TOC, tocilizumab
- 30 CET, cetuximab
- 31 RTX, rituximab
- 32 CD, Crohn's disease
- 33 SIL-ADM, stable isotope-labeled ADM
- 34 LC, liquid chromatography
- 35 PBS, phosphate-buffered saline, methanol (MeOH), acetonitrile (ACN),
- 36 IQC, internal quality controls
- 37 LOQ, limit of quantification
- 38 FDA, Food and Drug Administration
- 39 CRP, C-reactive protein
- 40 C_{min} , trough plasma concentration
- 41 Sp, specificity
- 42 Sn, sensitivity

43 **ABSTRACT**

44 The use of therapeutic monoclonal antibodies (mAbs) is steadily increasing. Previous
45 studies have reported the clinical interest of mAb therapeutic-drug monitoring (TDM),
46 including that of adalimumab, for patients with Crohn's disease (CD). Proof of concept
47 mAb-quantification studies by liquid chromatography mass spectrometry (LC-MS/MS)
48 have been published, but a specific and reliable routine-suited multiplex quantification
49 method is still needed to facilitate mAb TDM.

50 We describe an electrospray ionization LC-MS/MS method for the simultaneous
51 quantification of seven mAbs (adalimumab, cetuximab, infliximab, rituximab,
52 secukinumab, tocilizumab, and trastuzumab) in human plasma. Sample preparation
53 was performed using protein-G purification and trypsin digestion to obtain proteotypic
54 peptides. We retrospectively measured the adalimumab concentration in 65 plasma
55 samples from 56 CD patients and determined the adalimumab therapeutic cut-off
56 concentration associated with biological remission.

57 Calibration curves were linear from 1 to 100 $\mu\text{g mL}^{-1}$, except for rituximab (5 to 100 μg
58 mL^{-1}). This method was reproducible, repeatable, and accurate (coefficient of variation
59 and bias < 20%), with no cross contamination. Adalimumab concentrations were
60 significantly higher ($p=0.0198$) for patients with biological remission (median: 11.3 μg
61 mL^{-1} [4.6; 18.3]) than that for patients without a biological response (9.5 $\mu\text{g mL}^{-1}$
62 [3.94;17.0]). An adalimumab cut-off concentration of 8.0 $\mu\text{g mL}^{-1}$ correctly
63 discriminated patients with or without biological remission (sensitivity: 74.1%,
64 specificity: 57.9%).

65 This validated LC-MS/MS routine-suited method is the first allowing simultaneous
66 quantification of up to seven mAbs acting against different pharmacological targets. It
67 opens the field of TDM to numerous mAbs.

68

69 Keywords: adalimumab, Crohn's disease, liquid chromatography tandem mass

70 spectrometry, therapeutic drug monitoring, therapeutic monoclonal antibodies

71 **1. Introduction**

72 In recent years, therapeutic monoclonal antibodies (mAbs) have come to
73 occupy a prominent place in the development of new therapeutic strategies [1], mainly
74 for the treatment of solid cancers and chronic inflammatory diseases. The use of mAbs
75 represents a major financial burden for healthcare systems.

76 Compelling evidence has suggested that therapeutic drug monitoring (TDM) of
77 mAbs could help to improve clinical or biological response rates [2] and that a
78 personalized dose for each patient may improve efficacy [3]. ELISA methods have
79 been the most widely used analytical methods to quantify mAbs to date and thus most
80 studies that have proposed measuring the therapeutic index of mAbs have used these
81 methods. However, some of them present analytical limits, such as systematic bias [4-
82 6], lack of specificity due to cross reactivity [6,7] or small ranges of linearity. In addition,
83 each ELISA assay allows the quantification of only a single mAb.

84 Recent studies have demonstrated the proof of concept for the quantification of
85 mAbs using liquid chromatography mass spectrometry (LC-MS/MS) [8-13]. LC-MS/MS
86 methods are specific and reproducible, but require the determination of an updated
87 therapeutic index, as recommended whenever a new analytical method is used for
88 biotherapy TDM [14]. We previously demonstrated the feasibility of multiplexing the
89 quantitation of the anti-TNF α mAbs infliximab (IFX) and adalimumab (ADM) in a single
90 LC-MS/MS analysis [12]. Recently, Chiu *et al.* demonstrated the feasibility of
91 multiplexing the quantitation of three mAbs (bevacizumab, nivolumab, and
92 pembrolizumab) directed against two different therapeutic targets (EGFR for
93 bevacizumab for and PD-1 for nivolumab and pembrolizumab) in a single LC-MS/MS
94 run using protein-G trapping [15]. Given the increasing number of available mAbs with
95 different specific targets [1], there is an increasing need to develop a single and generic

96 analytical method that allows the quantification of several mAbs, irrespective of their
97 endogenous targets.

98 The first aim of this study was to describe a novel multiplex and easy-to-perform
99 LC-MS/MS method for the quantification of seven mAbs commonly used in various
100 clinical settings, such as inflammatory diseases or oncology: adalimumab and
101 infliximab (ADM and IFX, targeting TNF- α) secukinumab (SEC, targeting IL-17A),
102 tocilizumab (TOC, targeting the IL6 receptor), trastuzumab (TRZ, targeting HER2),
103 cetuximab (CET, targeting EGFR), and rituximab (RTX, targeting CD20). The second
104 aim was to determine the therapeutic cut-off for the ADM trough concentration (C_{\min})
105 in a cohort of Crohn's Disease (CD) patients using this LC-MS/MS method.

106

107 **2. Materials and methods**

108 *2.1. Multiplex LC-MS/MS method*

109 *2.1.1. Chemicals and reagents*

110 ADM (Humira®, AbbVie Inc., North Chicago, IL, United States), IFX
111 (Remicade®, Johnson and Johnson, New Brunswick, NJ, United States), SEC
112 (Cosentyx ®, Novartis, Basel, Switzerland), TOC (Roactemra ®, Roche, Basel,
113 Switzerland), TRZ (Herceptin ®, Roche), CET (Erbix® Merck KGaA, Darmstadt,
114 Deutschland), and RTX (Mabthera ®, Roche) were obtained from the institutional
115 pharmacy. These mAbs have an IgG1 structure and are either chimeric, humanized,
116 or human. Full-length stable isotope-labeled ADM (Arginine $^{13}\text{C}_6$ - $^{15}\text{N}_4$ and Lysine $^{13}\text{C}_6$ -
117 $^{15}\text{N}_2$) (SIL-ADM) was purchased from Promise Advanced Proteomics (Grenoble,
118 France). This internal standard has an estimated purity > 95% and isotope
119 incorporation of arginine $^{13}\text{C}_6$, $^{15}\text{N}_4$ and lysine $^{13}\text{C}_6$, $^{15}\text{N}_2$ > 99%. Sequencing-grade
120 modified trypsin was purchased from Promega (WI, USA). BioXtra urea, ammonium

121 bicarbonate, 30% hydrogen peroxide solution (H₂O₂), and tris (hydroxymethyl)
122 aminomethane (TRIS) Trizma® base were purchased from Sigma Aldrich (MI, USA).
123 LC-MS-grade methanol (MeOH), acetonitrile (ACN), and formic acid were purchased
124 from Honeywell, Sigma-Aldrich Co (St Louis, MO, USA), and Carlo Erba Reagents (Val
125 de Reuil, France), respectively. Ammonia solution (25%) was purchased from Merck
126 (Darmstadt, Germany). Ultrapure water (H₂O) with a resistivity ≥ 18.0 MΩ.cm was
127 produced with a Milli-Q Plus® system (Millipore, Molsheim, France).

128 Dulbecco's phosphate-buffered saline (PBS) solution and 96-well Pierce™
129 Protein-G Spin Plates for IgG Screening were purchased from Thermo Scientific
130 (Waltham, MA, USA). Drug-free human plasma and serum from volunteers was
131 provided by the French National Blood Service (Grenoble, France).

132

133 *2.1.2. Calibration curves and internal-quality control preparation*

134 Calibration curves and internal-quality controls (IQC) were designed according
135 to the therapeutic range of each mAb. Successive dilutions of a plasma stock solution
136 spiked with seven mAbs (100 µg mL⁻¹ each) were performed to obtain samples
137 containing 1, 5, 10, 25, 50, 75, and 100 µg mL⁻¹ to generate the calibration curve, except
138 for RTX, for which the lowest concentration was 5 µg mL⁻¹. Following the guidance of
139 the FDA [16], the lowest concentration of the IQC was three times higher than the
140 lowest concentration of the calibration curve. For IQC solutions, similar but
141 independent successive dilutions were performed to obtain three IQC concentrations:
142 3, 6, and 18 µg mL⁻¹ (ADA, IFX, TOC); 3, 30, and 90 µg mL⁻¹ (TRZ, CET, SEC); and 15,
143 30, and 90 µg mL⁻¹ (RTX).

144

145 *2.1.3. Sample preparation: protein-G purification and trypsin digestion*

146 PBS buffer (80 μL) and 3 μL 50 $\mu\text{g mL}^{-1}$ SIL-ADM were added to 20 μL sample
147 (calibrator, IQC, or clinical plasma sample). This mix was transferred to a well of a 96-
148 well plate containing protein-G resin, which was washed beforehand with 200 μL PBS.
149 After incubation for 1 hour at ambient temperature with smooth agitation, the
150 supernatant was vacuum-removed, and the protein-G resin washed twice with 100 μL
151 PBS buffer. Then, mAbs were eluted by two single applications of 100 μL 50/50 water-
152 acetonitrile 0.5% formic acid solution. This eluent was dried under N_2 . The dried eluent
153 was reconstituted with 5 μL 2 M urea, 1 μL 20 mM TRIS Base buffer, 45 μL 25 nM
154 ammonium bicarbonate buffer, and 5 μL trypsin at 200 mg L^{-1} . This mix was incubated
155 overnight at 37°C, as 14 h was previously shown to be the optimal duration for
156 digestion [15]. Digestion was stopped by the addition of 5.5 μL 1% formic acid solution.
157 Finally, the digested sample was oxidized with 15.6 μL 30% hydrogen peroxide and
158 transferred to a glass insert. The sample was then ready for analysis.

159

160 *2.1.4. LC-MS/MS analysis*

161 *2.1.4.1. Configuration of two-dimensional liquid chromatography (LC)*

162 The Shimadzu (Kyoto, Japan) LC system used consisted of an LC-20AD
163 quaternary pump (pump A) and two LC-20AD XR quaternary pumps (pumps B+C),
164 equipped with a SIL-20AC XR autosampler and a CTO-20AC column compartment.
165 Samples (10 μL injected) were cleaned up online with mobile phase A ($\text{H}_2\text{O}/\text{ACN}$ 98/2,
166 0.1% formic acid) and pump A, on a μ -Precolumn (C18 PepMap100, 5 μm , 100 \AA ,
167 Thermo Scientific, MA, USA). Chromatographic separation was performed in backflush
168 mode, on an XB C18 analytical column (Phenomenex Kinetex, 2.6 μm , 2.1 mm \times 50
169 mm, Aschaffenburg, Germany). The column temperature was maintained at 60°C.
170 Elution was performed at a flow rate of 0.4 mL min^{-1} under the following conditions:

171 mobile phase B from pump B (same composition as mobile phase A) and mobile phase
172 C from pump C (water/ACN 20/80 + 0.1% formic acid): 0 to 4 min: 8% C; 4 to 6.3 min:
173 8 to 60% C; 6.3 to 6.4 min: 60 to 90% C; 6.4 to 6.9 min: 90% C; 6.9 to 7 min: 90 to 8%
174 C, and 7 to 8.6 min: 8% C.

175

176 *2.1.4.2. Mass-spectrometry conditions*

177 Measurements were performed on an API 5500 QTRAP Tandem Mass
178 Spectrometer (Sciex, Toronto, Canada) equipped with a Turbo V® ion source,
179 operating in the positive electrospray ionization mode (ESI). The source-dependent
180 parameters were set as follows: ESI voltage, 5500 V; ion source temperature, 500°C;
181 turbo heater gas, 45 psi; nebulizer, 60 psi; curtain gas, 30 psi; and collision gas:
182 medium. Quantification was performed in the MRM mode. Proteotypic peptides were
183 selected from internal optimization of surrogate peptides following in-silico trypsin
184 digestion using version 4.1 Skyline software (MacCoss Lab Software, Washington,
185 USA). Proteotypic peptides and the MRM transition used for quantification are listed in
186 Table 1, as well as detailed MRM settings. Analyte peak areas were integrated using
187 Analyst software (version 1.6.3).

188

189 *2.1.5. Method validation*

190 *2.1.5.1. Within- and between-day accuracy (bias) and precision*

191 Within- and between-day accuracy (bias) and precision were examined by
192 performing replicate analyses (n = 6) for all IQC standards. Between-day accuracy and
193 precision were assessed with the same IQCs by repeated injection (n = 6) over six
194 days. The data were analyzed by several analysts and calculated as the percentage
195 deviation of the average calculated concentration from the nominal concentration.

196 Precision was expressed as the coefficient of variation (CV). The acceptance limits
197 were < 15% for precision and within $\pm 15\%$ of the nominal value for accuracy, except
198 for the lowest concentration of the IQC, for which < 20% for precision and within $\pm 20\%$
199 of the nominal value for accuracy were accepted.

200

201 2.1.5.2. *Linearity and limits of quantification*

202 Calibrator points were prepared from 1 to 100 $\mu\text{g mL}^{-1}$ (except for RTX, for which
203 the range was from 5 to 100 $\mu\text{g mL}^{-1}$) to generate standard curves. The lower limit of
204 quantification (LLOQ) was fixed at the lowest concentration of the calibration curve and
205 validated with accuracies < 20% and bias < $\pm 20\%$ ($n = 6$). The LLOQ was confirmed
206 by verifying that the signal-to-noise ratio of each analyte peak was > 10. The upper
207 limit of quantitation (ULOQ) was fixed at the highest concentration of the calibration
208 curve and validated with the same levels of precision and accuracy criteria as those
209 required for the medium and high levels of IQC.

210

211 2.1.5.3. *Selectivity & Carry-over*

212 Selectivity was tested by the analysis of six blank plasma samples, with and
213 without SIL-ADM, following the sample preparation procedure. Each mAb was
214 monitored to confirm the absence of interfering matrix compounds. Carry-over
215 interference was assessed by analyzing three plasma samples spiked with
216 concentrations of mAbs corresponding to the LLOQ of each after the injection of
217 samples spiked with concentrations of mAbs corresponding to the ULOQ of each, as
218 previously described [10].

219

220 2.1.5.4. *Matrix effect*

221 Matrix effects were determined by comparing peak areas after spiking blank
222 plasma samples or water with the compounds at the second concentration of the IQC
223 (n = 6). Matrix effects were then studied by comparing the variances obtained with
224 water or the matrix at the same concentrations. A Fisher variance test was used: p >
225 0.05 signified that the dispersions of the values were significantly equivalent, meaning
226 that the matrix effect of plasma did not affect the repeatability of the measures.

227

228 2.1.5.5. *Stability of prepared samples*

229 The stability of the prepared samples in the injection vials at 4°C for 24 and 72
230 h was evaluated for three concentrations (n = 6) to test conditions that might be
231 encountered during the analysis process. The values are expressed as the percentage
232 of the target concentration deviation measured in freshly prepared samples.

233

234 2.1.5.6. *Sample test dilution*

235 A dilution factor (four-fold) was tested by spiking blank plasma (n = 6) with all
236 mAbs at concentrations above the ULOQ (200 µg mL⁻¹).

237

238 2.2. *Adalimumab therapeutic cut-off in Crohn's Disease*

239 2.2.1. *Study design*

240 This was a monocentric retrospective study. Plasma samples (n = 90) from CD
241 patients (n = 71), followed from October 2016 to May 2018 for routine care in the
242 Division of Hepatology and Gastroenterology at the Grenoble-Alpes University
243 Hospital, were included (Supplemental Figure 1). Blood samples were collected at out-
244 patient care just before ADM administration. Demographic, clinical, and biological data
245 were collected from electronic medical records. Demographic data included age, sex,

246 weight, and height. Clinical data included disease localization, date of diagnosis, CD-
247 related surgery, date of treatment initiation, co-medication, last administered doses of
248 ADM, and time since the last dose. Biological data included the number of ADM C_{\min}
249 measurements per patient, plasma C-reactive protein (CRP) level, ferritin and albumin
250 concentrations, fecal calprotectin levels, and the presence of anti-drug antibodies
251 (ADA). The inclusion criteria were: patients treated for CD, on ADM maintenance
252 therapy (treatment for more than eight weeks since treatment initiation), and available
253 plasma CRP and/or fecal calprotectin concentrations (fecal calprotectin not necessary
254 if $CRP > 5 \text{ mg L}^{-1}$), determined concomitantly with the sample used for ADM
255 quantification. A total of 56 patients (65 plasma samples) fulfilled the inclusion criteria
256 (Supplemental Figure 1). ADM C_{\min} were measured using the LC-MS/MS method
257 described herein.

258

259 2.2.2. *Outcomes*

260 Patients were stratified based on the presence/absence of a biological response
261 to determine the association between ADM C_{\min} and biological response. Biological
262 remission was defined as a plasma CRP level $< 5 \text{ mg L}^{-1}$ and fecal calprotectin level $<$
263 $150 \mu\text{g g}^{-1}$ stool [17] and the absence of biological remission as a plasma CRP level $>$
264 5 mg L^{-1} and/or fecal calprotectin level $> 150 \mu\text{g g}^{-1}$ stool.

265

266 2.2.3. *Ethics*

267 Residual plasma samples were stored at $-80 \text{ }^{\circ}\text{C}$ in a biological sample collection
268 (DRC-2013-1983). The study and biobank were approved by the IRB 6705 (CPP Sud
269 Est 5, Grenoble, France).

270

271 2.2.4. Statistical analysis

272 Continuous data are expressed as the median (10th-90th percentiles) and
273 categorical variables as numbers (percentages). Non-parametric tests were used to
274 compare quantitative variables. Diagnostic performance was assessed using receiver
275 operating characteristics (ROC) curves [18]. The Youden index (sensitivity + specificity
276 -1) was computed to define the best threshold. The specificity was defined as the ratio
277 of patients in biological remission with an ADM C_{min} lower than the threshold to the total
278 number of patients not in biological remission. The sensitivity was the ratio of patients
279 in biological remission with an ADM C_{min} higher than the threshold to the total number
280 of patients in biological remission. The area under the ROC curve is given with its
281 confidence interval according to the DeLong method: the sensitivity and specificity of
282 each threshold with their confidence intervals. A p-value ≤ 0.05 was considered
283 statistically significant. ROC curve analysis was performed using Medcalc 18.6
284 (MedCalc Software, Ostende, Belgium) and Mann-Whitney tests using Statview 5.0
285 (SAS Institute Inc, Cary NC, USA).

286

287 **3. Results**

288 *3.1. Validation of the multiplex LC-MS/MS method*

289 *3.1.1 Chromatograms*

290 Figure 1 displays representative chromatograms of a calibration curve point at
291 $50 \mu\text{g mL}^{-1}$ for all mAbs (Figure 1a), a sample from a patient treated with SEC and
292 measured at $37.7 \mu\text{g mL}^{-1}$ (Figure 1b), a blank, revealing no interference from IFX,
293 SEC, TRZ, ADM, CET, RTX, or TOC MRM signals while monitoring SIL-ADM (Figure

294 1c), and a double blank, revealing no interference from IFX, SEC, TRZ, ADM, CET,
295 RTX, TOC, or SIL-ADM (Figure 1d).

296

297 *3.1.2. Accuracy and precision*

298 We determined the within-day and between-day precision and accuracy for the
299 three concentrations of the IQC samples (Table 2). All calculated concentrations were
300 within the ranges of the FDA Guidance [16]: $\pm 15\%$ for the low-IQC samples and $\pm 20\%$
301 for the middle- and high-IQC samples. We further assessed the reproducibility of
302 proteolytic peptide release from the mAbs by trypsin digestion by evaluating the
303 variability of the SIL-ADM proteotypic peptide peak area obtained from LC-MS/MS
304 acquisitions. We obtained a mean coefficient of variation of 8.3% (min 3.9%, max
305 11.9%) of the SIL-ADM peak area using seven calibrator points from six different
306 calibration curves.

307

308 *3.1.3. Linearity and limits of quantification*

309 We plotted the ratio of the mAb and ADM-SIL peak areas (y-axis) against those
310 of the mAb and ADM-SIL concentrations (x-axis) and calculated the calibration curves
311 by a least square regression with 1/x weighting with linear regression. All curves were
312 linear from 1 to 100 $\mu\text{g mL}^{-1}$, except that for RTX (5 to 100 $\mu\text{g mL}^{-1}$), with a coefficient
313 of determination (r^2) greater than 0.99 for all mAbs. Curve equation parameters are
314 available in Supplemental Table 1. The LLOQ and ULOQ were determined as the
315 lowest and highest concentrations of the calibration curve, respectively: 5 to 100 μg
316 mL^{-1} for RTX and 1 to 100 $\mu\text{g mL}^{-1}$ for the other mAbs. The LLOQ were validated with a
317 bias from 0 to 7.3% and CVs from 9.7 to 13% ($n = 6$). We also confirmed the LLOQ by
318 verifying that the signal-to-noise ratio of each analyte peak was greater than 10 (data

319 not shown). The ULOQ was validated with a bias from 0.1 to 3.2% and CVs from 3.3
320 to 7.6% (n = 6).

321

322 3.1.4. *Selectivity & Carryover*

323 Analysis of six blank plasma samples with or without SIL-ADM showed there to
324 be no interference between the seven mAbs or endogenous compounds from the
325 matrix after the purification process. Mean carryover effects (expressed as
326 percentages) were 0.04, 0.11, -0.05, 0.80, 0.48, 0.80, and -0.22 for ADM, IFX, CET,
327 RTX, SEC, TOC, and TRZ, respectively. These low contamination rates exclude the
328 possibility of low mAb concentrations being overestimated when measured just after
329 samples containing very high concentrations.

330

331 3.1.5. *Matrix effect*

332 The p-values of the F-tests performed for each mAb were between 0.06 and
333 0.70 (n = 6). This signifies that even if the matrix affects the sensitivity threshold of the
334 method, it does not significantly affect the variability of the results relative to those
335 obtained with water.

336

337 3.1.6. *Stability of prepared samples*

338 Stability values are expressed as the percentage of the target concentration
339 measured in freshly prepared IQC samples (low, middle, and high concentrations) of
340 all mAbs. The stability was good for ADM, IFX, CET, and RTX in extracted samples
341 stored for 24 or 96 h at + 4° in the autosampler. SEC showed a negative bias > 20%
342 after 24 and 96 h of storage. TOC and TRZ were stable for 24 h but showed a negative
343 bias > 20% after 96 h storage. Detailed results are available in Supplemental Table 2

344

345 *3.1.7. Sample test dilution*

346 During validation, we diluted plasma samples (n = 6) spiked with concentrations
347 of the analytes that were two-fold higher than the ULOQ. The resulting concentration
348 values calculated with an adapted dilution factor were $\pm 15\%$ of the target value for all
349 analytes.

350

351 *3.1.8. mAb concentration measures*

352 We also tested several plasma samples selected at random from patients
353 treated with IFX, CET, RTX, SEC, TOC, or TRZ. Patient characteristics and their
354 respective mAb concentrations are available in Table 3.

355

356 *3.2. Adalimumab trough concentration therapeutic cut-off in Crohn's Disease*

357 *3.2.1. Variability of the ADM C_{min}*

358 Characteristics of the study population (patients with CD) are presented in Table
359 4. The ADM C_{min} ranged from 1.0 to 34.4 $\mu\text{g mL}^{-1}$, with a median [10th-90th percentiles]
360 of 9.5 $\mu\text{g mL}^{-1}$ [3.94-17.0]. The inter-individual CV of the ADM C_{min} was 45.3%.

361

362 *3.2.2. Biological response*

363 Biological remission criteria were met in 27 of the 65 analyzed samples. ADM
364 C_{min} were significantly higher ($p = 0.0198$) for patients with biological remission (median
365 ADM C_{min} of 11.3 $\mu\text{g mL}^{-1}$ [4.6; 18.3]) than for patients without (median ADM C_{min} of 9.5
366 $\mu\text{g mL}^{-1}$ [3.94;17.0]) (Figure 2a). ROC-AUC analysis showed good performance of the
367 ADM C_{min} to discriminate between patients with or without biological remission (AUC
368 = 0.671; CI95 [0.543;0.782]; $p = 0.0143$) (Figure 2b). The Youden index showed the

369 ADM C_{\min} threshold of $8.0 \mu\text{g mL}^{-1}$ to provide the best balance of sensitivity (S_n) and
370 specificity (S_p) with a $S_n = 74.1\%$ (CI95 [53.7;88.9]) and a $S_p = 57.8\%$ (CI95
371 [40.8;73.7]).

372

373 **4. Discussion**

374 We show that it is possible to simultaneously quantify seven mAbs directed
375 against different pharmacological targets (TNF- α for ADM and IFX, IL-17A for SEC, IL-
376 6R for TOC, HER2 for TRZ, EGFR for CET, and CD20 for RTX). This was possible
377 due to single-calibration standard points that included the seven biotherapies, a
378 protein-G based purification strategy, and the use of a full-length stable isotope-
379 labelled antibody as internal standard. The Protein-G immunoaffinity procedure
380 enables the binding of all IgGs, including endogenous and target IgG-based drugs, to
381 protein G. This procedure enables the purification of all IgGs from the plasma sample.
382 A similar purification strategy was recently reported for the simultaneous quantification
383 of bevacizumab, nivolumab, and pembrolizumab [15]. However, the authors used two
384 internal standards: tocilizumab and a post-column infused internal standard procedure
385 to correct the matrix effect of their LC-ESI-MS method [15]. Matrix effects are a
386 significant problem when using electrospray ionization for mass spectrometry analysis
387 and, when possible, the use of an isotope-labeled internal standard is required. In our
388 study, we added a full-length stable isotope-labelled antibody to the plasma samples
389 at an early step of the protocol of sample preparation, minimizing any potential
390 variability that might arise during the sample preparation steps, as well as attenuating
391 the matrix effect that could occur during LC-MS/MS analysis. Such an approach
392 allowed the simultaneous quantification of all seven mAbs with high precision and
393 accuracy. Moreover, we demonstrate that the use of a single SIL-internal standard

394 (SIL-ADM) is suitable for the quantification of seven mAbs, showing the cost-
395 effectiveness of our method. The dynamic ranges of the calibration curves fitted the
396 concentrations of mAbs measured in plasma samples from patients treated with one
397 of the mAbs. Finally, this method meets the validation criteria of the FDA [16].

398 Several studies have focused on mAb TDM to set new cut-off concentrations for
399 LC-MS/MS. For CET, a pilot study performed on 25 head-and-neck cancer patients
400 showed higher residual concentrations in patients showing a clinical benefit than in
401 non-responding patients and proposed that a CET C_{\min} of $33.8 \mu\text{g mL}^{-1}$ is predictive of
402 a clinical response [19]. For IFX, we performed a study on 47 inflammatory bowel
403 disease patients that showed a higher C_{\min} in patients with biological remission than in
404 those without and proposed an IFX C_{\min} threshold of $6.2 \mu\text{g mL}^{-1}$ to be predictive of
405 biological remission [3]. The present study is the first to determine a cut-off of $8.0 \mu\text{g}$
406 mL^{-1} for the ADM C_{\min} determined by LC-MS/MS for CD patients associated with
407 biological remission. Numerous studies have suggested an association between the
408 ADM C_{\min} and therapeutic response, especially in patients with inflammatory bowel
409 diseases [20-22]. A cut-off for ADM levels of $5 \mu\text{g mL}^{-1}$ (Sn 80%, Sp 55.6%) has been
410 shown to predict clinical remission at week 52 [21]. Consistent with these findings,
411 higher concentrations of ADM were found in patients with disease remission, assessed
412 by measuring CRP levels ($< 5 \text{ mg L}^{-1}$), and asymptomatic patients and the authors
413 proposed a cut off ADM C_{\min} of $5.85 \mu\text{g mL}^{-1}$ for predicting remission (Sn 68%, Sp
414 70.6%) [20]. Measurement of ADM levels with the homogeneous mobility-shift assay
415 showed that a higher ADM concentration was associated with CRP normalisation in
416 CD patients ($C_{\min} = 11.8 \mu\text{g mL}^{-1}$ (Sn 69%, Sp 66%)) [23]. Finally, the American
417 Gastroenterological Association reported that ADM $C_{\min} \geq 5 \pm 1 \mu\text{g mL}^{-1}$ and $7.5 \pm 1 \mu\text{g}$
418 mL^{-1} were associated with 17 and 10% of patients not being in remission, respectively,

419 and therefore recommended an ADM $C_{\min} > 7.5 \mu\text{g mL}^{-1}$ [22]. In accordance with this
420 study, we determined a cut-off of $8.0 \mu\text{g mL}^{-1}$ for the ADM C_{\min} , associated with correct
421 Sn (74%) but lower Sp (57%), for predicting biological response. The retrospective
422 design and limited sample size of our cohort are limitations of our study, but it is the
423 first to determine an ADM C_{\min} cut off, measured by LC-MS/MS, associated with
424 biological remission in CD patients.

425

426 **5. Conclusions**

427 In conclusion, our original and validated LC-MS/MS method is compatible with
428 routine TDM due to its simple sample preparation and the use of single-calibration
429 standard points for all seven biotherapies. We show that an ADM cut-off C_{\min} of $8.0 \mu\text{g}$
430 mL^{-1} correctly discriminates between CD patients who are in biological remission from
431 those who are not. Further independent studies are required on larger cohorts to
432 confirm this threshold and determine the cut-off concentrations of other mAbs for their
433 respective indications.

434

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437

438 **References**

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Table 1

Mass spectrometry settings and proteotypic peptides: MRM transition and voltage settings: declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) for each analyte. Underlined ions were used as quantifiers and the second ions were used for confirmation.

mAb drug	mAb mass (Da)	Proteotypic peptide	Q1 m/z	Q3 m/z	DP (V)	EP (V)	CE (eV)	CXP (V)
Adalimumab	144,190.3	APYTFGQGTK	<u>535.3</u>	<u>738.4</u>	100	10	28.3	15
			535.3	499.8	100	10	25.2	15
Infliximab	144,190.3	YASEM[2Ox]SGIPSR	<u>658.8</u>	<u>866.4</u>	55	12	33	19
			658.8	1082.5	55	10	33	19
Tocilizumab	148,000.0	LLIYYTSR	<u>514.8</u>	<u>526.3</u>	100	10	25	11
			514.8	689.3	100	10	25	11
Secukinumab	147,940.0	HC.YYVGSVK	<u>408.2</u>	<u>652.4</u>	80	10	23.6	15
			408.2	489.3	80	10	23.6	15
Trastuzumab	145,531.5	IYPTNGYTR	<u>542.8</u>	<u>404.7</u>	90	10	22	15
			485.2	721.4	90	10	22	15
Cetuximab	145,781.6	YASESISGIPSR	<u>633.8</u>	<u>816.5</u>	80	10	30	15
			633.8	616.3	80	10	20	15
Rituximab	143,859.7	FSGSGSGTSYSLTISR	<u>803.9</u>	<u>676.4</u>	105	10	45	17
			803.9	476.3	105	10	40	11
SIL Adalimumab	-	[¹³ C ₆ - ¹⁵ N ₂] APYTFGQGTK	<u>539.3</u>	<u>746.4</u>	100	10	28.3	15

Table 2

Within- and between-day precision (expressed as coefficients of variation) and accuracy (expressed as bias) of the LC-MS/MS method.

Analyte	Within-day precision and accuracy (n = 6)				Between-day precision and accuracy (n = 6)		
	IQC nominal concentration ($\mu\text{g mL}^{-1}$)	Mean concentration ($\mu\text{g mL}^{-1}$)	CV (%)	Bias (%)	Mean concentration ($\mu\text{g mL}^{-1}$)	CV (%)	Bias (%)
Adalimumab	3	2.85	8.2	-5.1	3.11	5.7	3.7
	6	6.49	10.7	8.2	6.02	9.5	0.3
	18	19.92	6.6	10.6	18.93	8.2	5.2
Infliximab	3	3.43	14.4	4.7	3.00	15.2	0.0
	6	5.81	-3.2	5.7	5.73	12.0	-4.5
	18	16.00	-11.1	6.3	17.37	13.3	-3.5
Tocilizumab	3	3.08	8.0	2.6	2.82	7.3	-6.0
	6	5.51	4.9	-8.1	5.68	3.4	-5.4
	18	16.65	4.4	-7.5	16.73	7.8	-7.0
Secukinumab	3	3.16	5.4	5.2	3.20	7.1	6.7
	30	27.18	8.8	-9.4	30.30	11.5	1.0
	90	90.52	6.0	0.6	97.87	7.2	8.7
Trastuzumab	3	2.68	4.8	-10.8	2.77	12.8	-7.7
	30	33.65	4.6	12.2	28.00	13.9	-6.7
	90	89.67	5.1	-0.4	81.92	14.1	-9.0
Cetuximab	3	2.97	10.8	-0.9	3.20	16.0	6.7
	30	28.40	4.1	-5.3	30.68	15.0	2.3
	90	88.35	5.2	-1.8	93.05	4.0	3.4
Rituximab	15	13.83	6.0	-7.8	15.48	5.2	3.2
	30	28.50	9.3	-5.0	31.18	6.2	3.9
	90	89.65	9.2	-0.4	95.33	9.8	5.9

Table 3

Examples of mAb concentrations measured in plasma from patients treated with either infliximab, cetuximab, secukinumab, trastuzumab, tocilizumab, or rituximab.

mAb drug	Patient No	Gender	Age	Height	Weight	Dose	Administration route	Time since last dose (days)	Measured concentration ($\mu\text{g mL}^{-1}$)
Infliximab	1	F	58	170	100	10 mg kg ⁻¹	IV	14	6.89
	2	F	38	158	45	10 mg kg ⁻¹	IV	56	6.47
Cetuximab	3	M	56	169	58	250 mg m ⁻²	IV	22	12.1
Secukinumab	4	F	61	157	89.1	150 mg	SC	5	37.7
Trastuzumab	5	F	29	153	79	600 mg	SC	20	13.0
	6	F	81	162	76	6 mg kg ⁻¹	IV	3	32.8
Tocilizumab	7	M	62	175	84.8	8 mg kg ⁻¹	IV	7	89.6
	7	M	62	175	84.8	8 mg kg ⁻¹	IV	14	29.1
Rituximab	8	F	70	164	55	375 mg m ⁻²	IV	19	23.0
	9	M	75	169	62.5	375 mg m ⁻²	IV	151	34.6
	10	M	54	184	109	1,000 mg	IV	6	159

Table 4

Demographic, clinical, and biological characteristics of the study population. Continuous data are presented as medians (10th-90th percentiles). Qualitative data are presented as numbers (percentages).

Patients (N = 56)	
Age (years)	43.5 (22.0-58.5)
Gender, male	27 (48.2)
Weight (kg)	64 (51-91)
BMI (kg m ⁻²)*	22 (18.7-29.9)
Time since diagnosis (years)	9.2 (2.0-23.7)
Disease distribution	
Upper gastrointestinal	4 (7.1%)
Ileum	33 (58.9%)
Colon	4 (7.1%)
Ileocolon	12 (21.4)
Perianal	6 (10.7%)
Two-site disease extended	4 (7.1%)
Prior disease related abdominal surgery	24 (42.9%)
Plasma samples (N = 65)	
Duration of treatment (years)	2.6 (0.4-7.4)
Adalimumab dose	
40 mg every other week	47 (72.3%)
40 mg every week	17 (26.1%)
80 mg every other week	1 (1.5%)
Presence of anti-adalimumab antibodies	none
Concomitant immunosuppressive treatment	
Thiopurines	5 (7.7%)
Corticoids	1 (1.5%)
None	59 (90.8%)
Biological parameters	
CRP (mg L ⁻¹)	3 (3-11)
Fecal calprotectin (µg g ⁻¹ of stool)**	137 (14.6-816.8)
Albumin (g L ⁻¹)	43 (38-49)
Ferritin (mg L ⁻¹)	62 (12.4-298)

* available for 54 patients

** available for 63 samples

Figure legends:

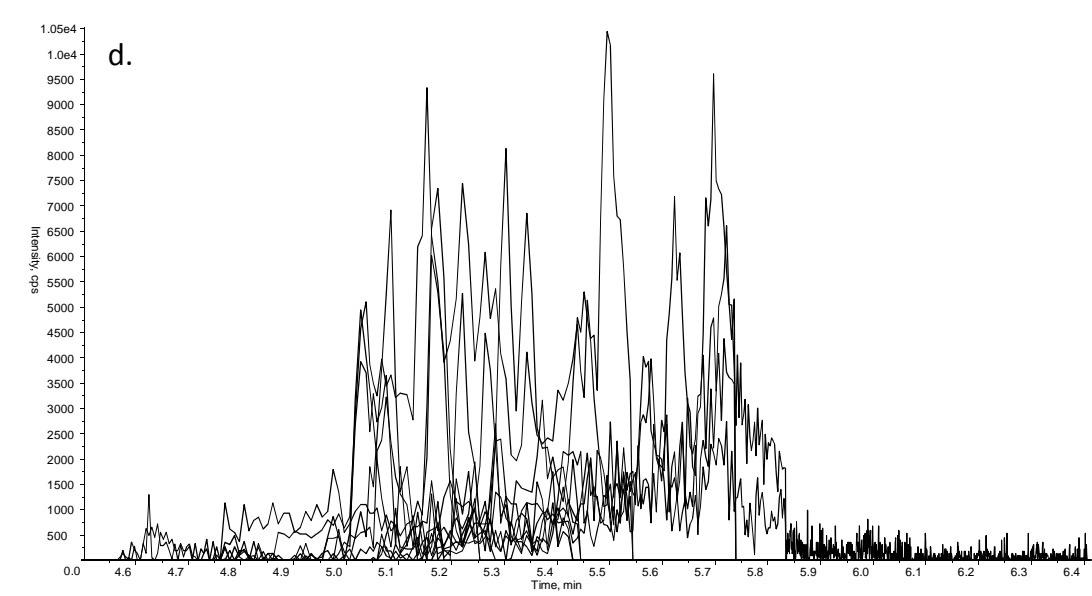
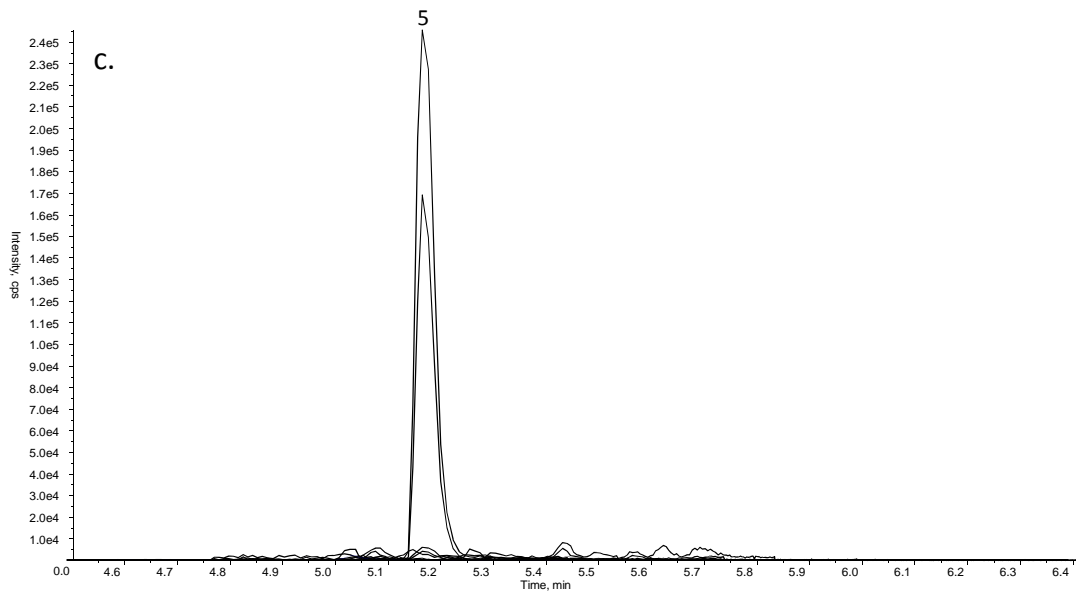
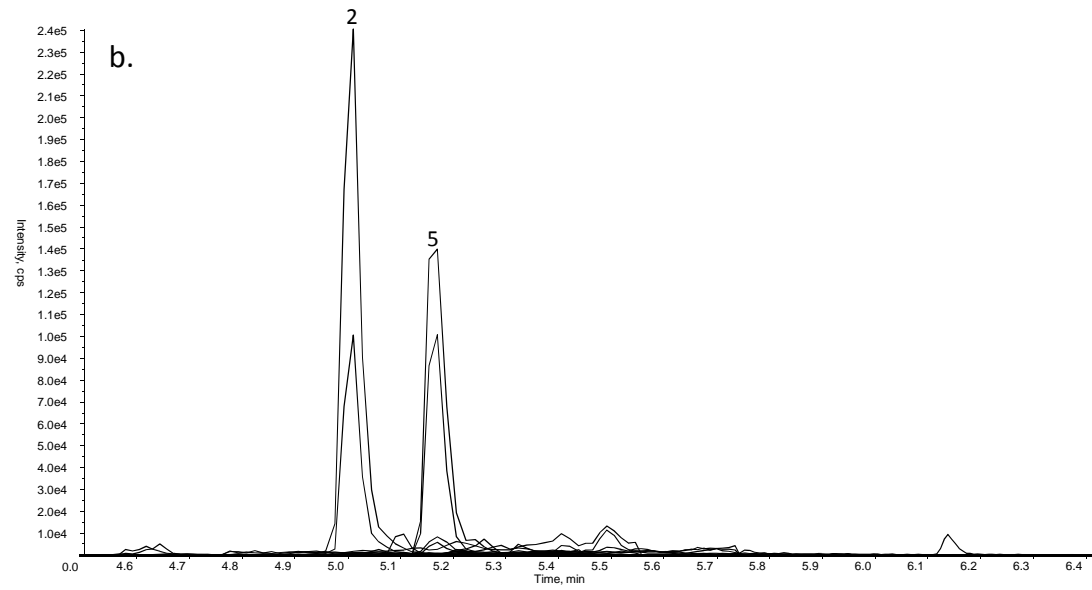
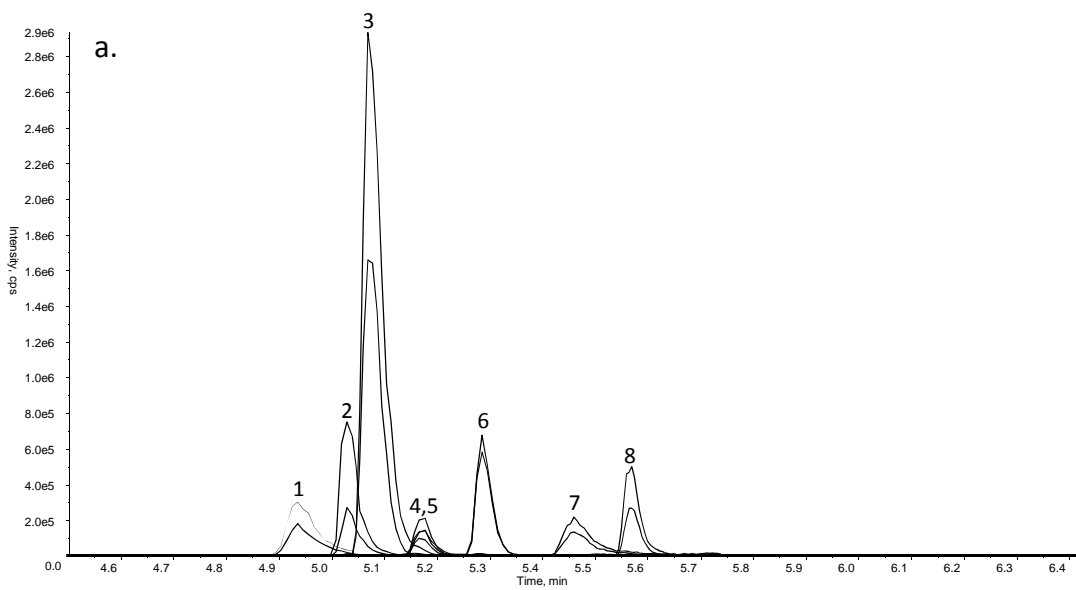
Figure 1.

Chromatograms illustrating the selected proteotypic peptides of adalimumab (ADM), infliximab (IFX), secukinumab (SEC), tocilizumab (TOC), trastuzumab (TRZ), cetuximab (CET), rituximab (RTX), and the full-length stable isotope-labelled adalimumab (SIL-ADM).

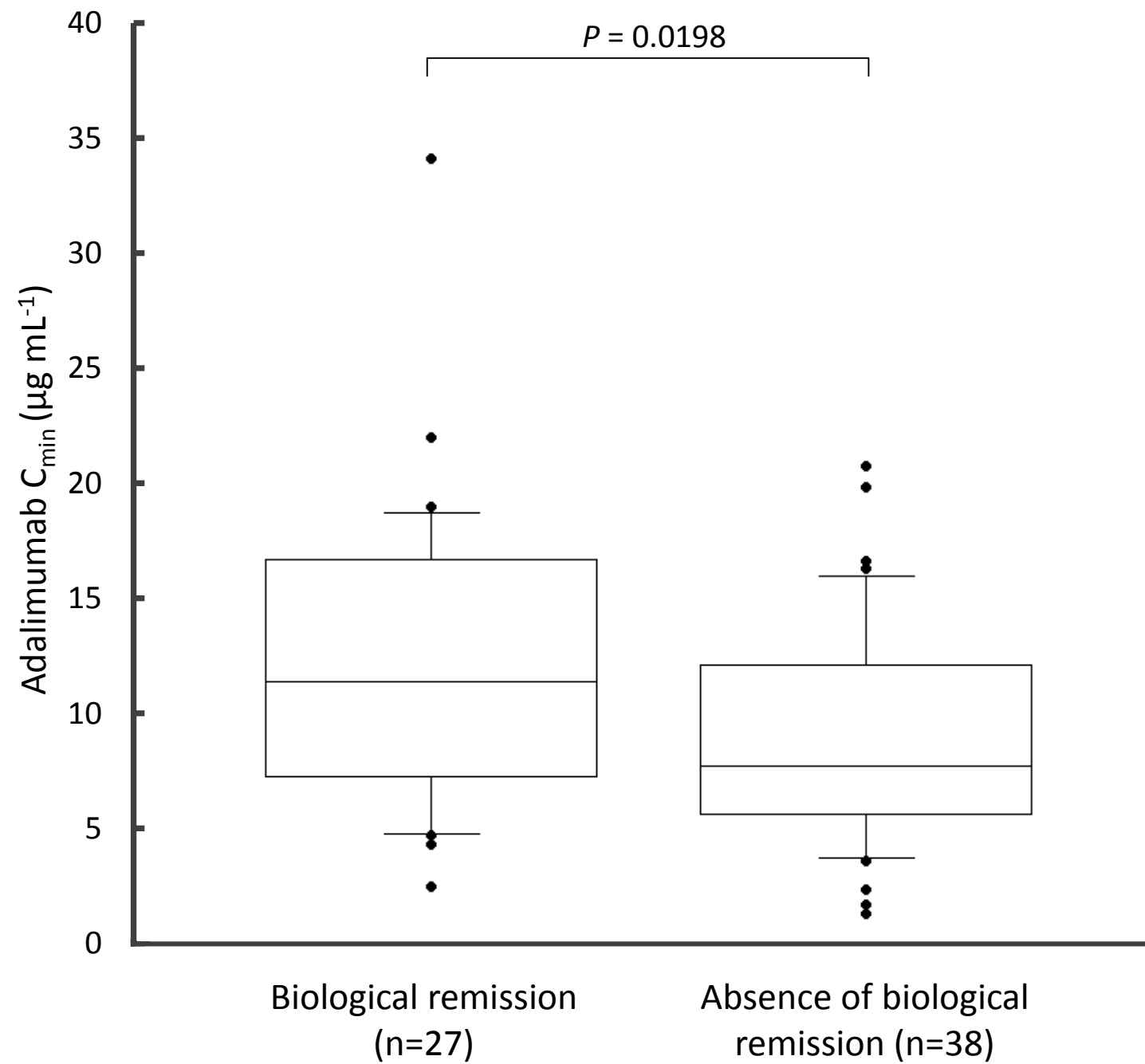
a. Calibration curve point at $50 \mu\text{g mL}^{-1}$ of IFX (1), SEC (2), TRZ(3), ADM (4), SIL-ADM (5), CET (6), RTX (7), and TOC (8). b. Sample from a patient treated with SEC (2) and measured at $37.7 \mu\text{g mL}^{-1}$, using SIL-ADM (5) as internal standard. c. Drug-free plasma sample with SIL-ADM (blank). d. Drug-free plasma (double blank).

Figure 2.

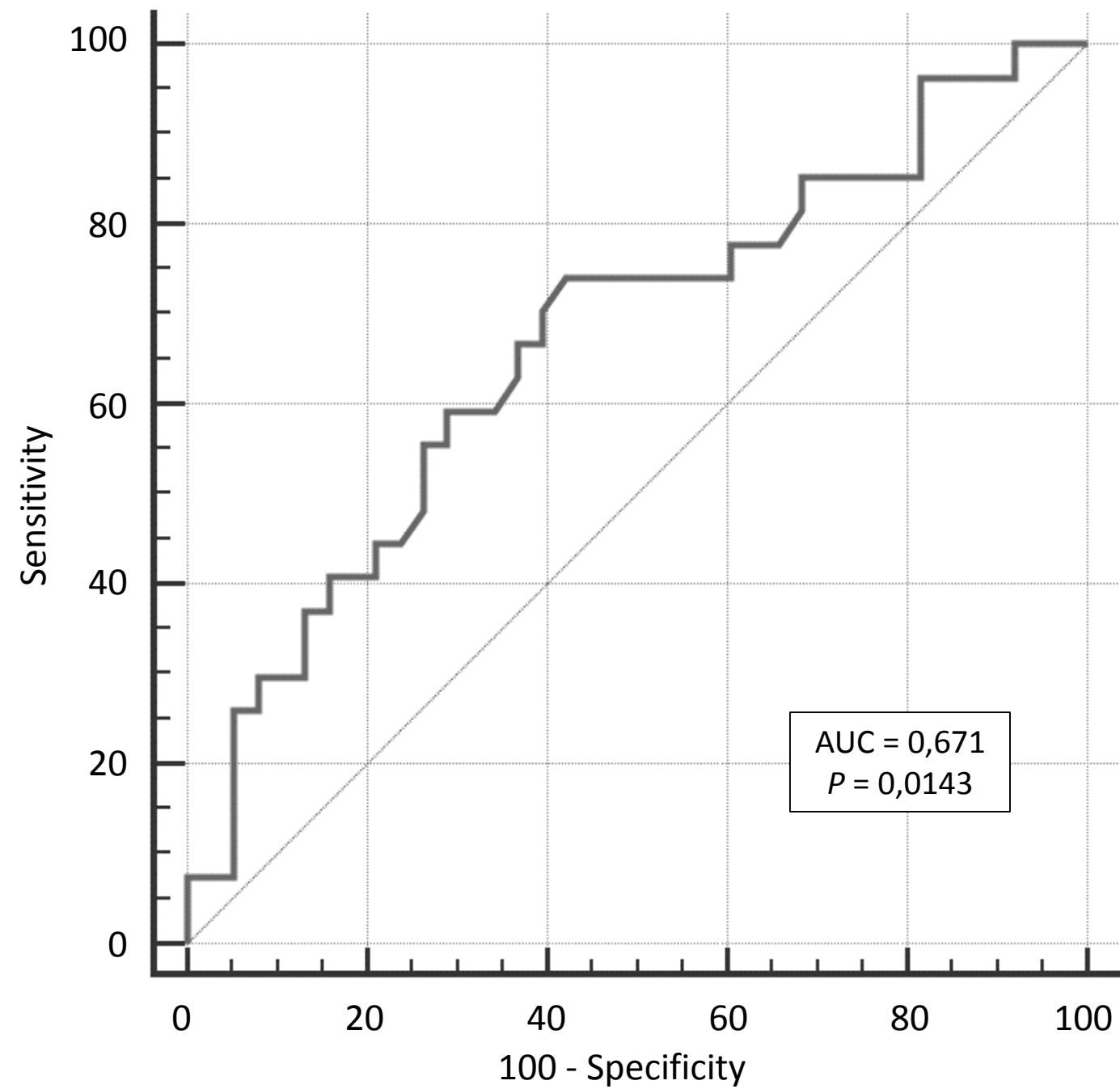
Association between adalimumab trough concentrations (C_{min}) determined by liquid-chromatography tandem mass spectrometry and biological remission. (a) Adalimumab C_{min} according to biological remission in patients with Crohn's Disease. (b) ROC curve for adalimumab C_{min} and biological remission.



a.



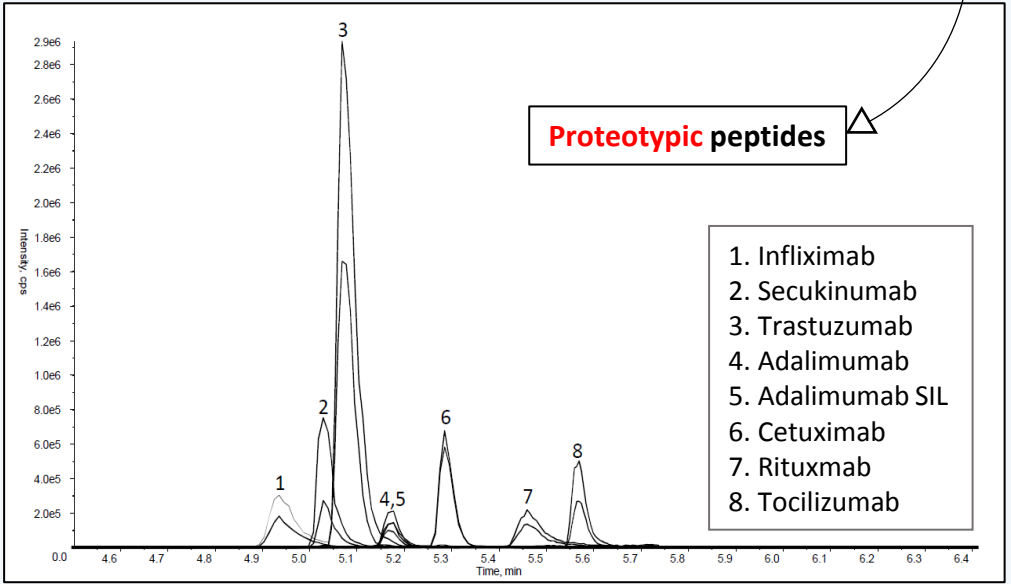
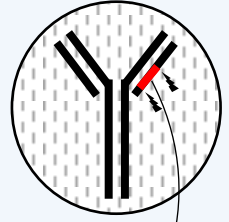
b.



Simultaneous quantification in human plasma of seven therapeutic monoclonal antibodies by LC-MS/MS for therapeutic drug monitoring.

From **Batch**...

Validated multiplex routine-suited method:
Patient plasma **Protein G** purification,
trypsin digestion and **LC-MS/MS** analysis.



...to **Bedside**.

Clinical application: **Adalimumab** plasma C_{min} therapeutic cut-off in Crohn's Disease

