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1 **Pig major acute-phase protein and haptoglobin serum concentrations correlate**
2 **with PCV2 viremia and the clinical course of postweaning multisystemic wasting**
3 **syndrome**

4
5 Grau-Roma L.^{1,2,*}, Heegaard P.M.H.³, Hjulsgaard C.K.³, Sibila M.¹, Kristensen C.S.⁴,
6 Allepuz. A.^{1,2}, Piñeiro M.⁵, Larsen L.E.³, Segalés J.^{1,2}, Fraile L.^{1,6}

7
8
9 ¹*Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat*
10 *Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

11 ²*Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona,*
12 *08193 Bellaterra, Barcelona, Spain.*

13 ³*National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-*
14 *1790 Copenhagen V, Denmark.*

15 ⁴*Danish Pig Production, Vinkelvej 11, DK-8620 Kjellerup, Denmark.*

16 ⁵*PigCHAMP Pro Europa S.A., Segovia, Spain*

17 ⁶*Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain.*

18
19 ***Corresponding Author:** e-mail: llgrau@cresa.uab.cat, Phone: +34 93 581 32 84 Fax:
20 +34 93 581 44 90.

23 **Abstract**

24 The aim of the present longitudinal study was to assess the evolution of two acute phase
25 proteins (APPs), pig-major acute phase protein (pig-MAP) and haptoglobin (HPT), in
26 serum from pigs that developed postweaning multisystemic wasting syndrome (PMWS)
27 in comparison to healthy and wasted non-PMWS affected pigs. In addition, evidence of
28 infection with other pathogens and its relation with variations in APPs concentrations
29 was also assessed. Fourteen independent batches of 100 to 154 pigs were monitored
30 from birth to PMWS outbreak occurrence in 11 PMWS affected farms. Pigs displaying
31 PMWS-like signs and age-matched healthy controls were euthanized during the clinical
32 outbreak. PMWS was diagnosed according to internationally accepted criteria and pigs
33 were classified as: i) PMWS cases, ii) wasted non-PMWS cases and iii) healthy pigs. At
34 the moment of PMWS occurrence, pig-MAP and HPT concentration in PMWS affected
35 pigs were higher than in healthy ones ($p < 0.0001$). No differences in APPs serum
36 concentrations between subclinically PCV2 infected pigs and healthy non-PCV2
37 infected pigs (based on quantitative PCR on serum results) were detected. Results
38 showed a significant correlation between PCV2 loads and both pig-MAP ($R = 0.487$ to
39 0.602 , $p < 0.0001$) and HPT ($R = 0.326$ to 0.550 , $p < 0.05$ to 0.0001) concentrations in
40 serum of PMWS affected pigs, indicating that the acute phase response in PMWS
41 affected pigs occurred concomitantly to PCV2 viremia. No other pathogen, apart from
42 PCV2, was consistently related with variations in APPs concentrations. A ROC
43 analysis, made to determine the capacity of discrimination of both APPs between
44 PMWS affected and non-affected pigs, showed higher sensitivity and specificity values
45 using pig-MAP compared to HPT. These results suggest that pig-MAP might be a better
46 indicator of PMWS status than HPT. Moreover, the fact that APR occurred some days

47 before the start of clinical signs suggests that APPs could provide valuable prognostic
48 information for PMWS development.

49

50 **Keywords:** Haptoglobin; pig-major acute phase protein (pig-MAP); acute phase
51 proteins (APPs); acute phase reaction (APR); porcine circovirus type 2 (PCV2);
52 postweaning multisystemic wasting syndrome (PMWS).

53

54 **1. Introduction**

55 The acute phase response (APR) is an innate, non-specific immune response which
56 occurs after many different stimuli such as infections, tissue damage, neoplastic growth
57 or immunological disorders (Baumann and Gauldie, 1994; Gruys et al., 2005a). This
58 complex reaction is mediated by pro-inflammatory cytokines and involves both local
59 and systemic reactions, including fever, increase in muscle protein catabolism,
60 alterations in sleep and appetite patterns, and changes in the concentration of a group of
61 plasma proteins which are called acute phase proteins (APPs) (Gruys et al., 2005a).
62 These proteins are mainly synthesised in the liver and are classified according to the
63 direction of change (positive APPs if their concentration increases, negative if it
64 decreases), and according to the extent to which their concentrations change (minor,
65 intermediate or major) during the APR (Petersen et al., 2004; Gruys et al., 2005a).

66

67 APPs have been described as useful for assessing health in humans and animals
68 (Petersen et al., 2004; Gruys et al., 2005b). In pigs, APPs concentrations have been
69 demonstrated to be increased after experimental infections with several bacteria, viruses
70 or parasites, as for instance *Streptococcus suis* (Sorensen et al., 2006), porcine
71 reproductive and respiratory syndrome virus (PRRSV) (Asai et al., 1999) and

72 *Toxoplasma gondii* (Jungersen et al., 1999). Moreover, increases in APPs
73 concentrations have also been detected in pigs affected by tail- and ear-biting or after
74 stress induced by transport conditions or changes in the pattern of food administration
75 (Parra et al., 2006; Piñeiro et al., 2007; Salamano et al., 2008). Specifically, haptoglobin
76 (HPT) and pig-major acute phase protein (pig-MAP) have been suggested as the most
77 robust APPs as indicators of infection in pigs (Sorensen et al., 2006).

78

79 Porcine circovirus type 2 (PCV2) is considered the essential infectious agent for
80 postweaning multisystemic wasting syndrome (PMWS) development, but it is also
81 known that other triggering factors are necessary for the full expression of the clinical
82 disease (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés et al., 2005). The
83 internationally accepted criteria for diagnosis of PMWS include the presence of
84 compatible clinical signs (mainly wasting and respiratory distress), moderate to severe
85 lymphocyte depletion with granulomatous inflammation in lymphoid tissues, and
86 detection of moderate to high amounts of PCV2 within these lesions (Sorden, 2000;
87 Segalés et al., 2005). Although it is known that APPs serum concentrations are
88 increased in PMWS affected pigs (Segalés et al., 2004; Parra et al., 2006), few field data
89 are available in the literature. Moreover, the longitudinal evolution of APPs in animals
90 that finally develop the disease has not been assessed.

91

92 The aim of this study was to assess the evolution of two APPs, pig-MAP and HPT, in
93 serum throughout the productive life of pigs that developed PMWS in comparison to
94 healthy pigs and pigs that developed wasting without fulfilling PMWS diagnosis.
95 Moreover, the infection dynamics of PCV2 and several other pathogens was monitored
96 by quantitative PCR, nested PCR and/or serological techniques to determine potential

97 associations between studied APPs concentrations and the moment of infection for each
98 pathogen.

99

100 **2. Materials and methods**

101 *2.1. Study design*

102 Two longitudinal case-control studies in PMWS affected farms, one in Spain and one in
103 Denmark, were performed as previously described (Grau-Roma et al., 2009). Briefly,
104 both studies were carried out using similar designs, and were performed on 13
105 independent farm batches (6 in Spain and 7 in Denmark) of 100 to 154 animals per
106 batch, coming from 10 different farms (3 from Spain and 7 from Denmark). Studied
107 piglets were ear-tagged at 1 week of age and monitored until the occurrence of the
108 PMWS outbreak. Nasal and rectal swabs as well as blood samples were serially
109 collected from those piglets at established weeks of age (1, 3, 7 and 11 in Spain, and 1,
110 4, 6 and 9 in Denmark) and at the time of PMWS outbreak occurrence (necropsy).
111 When the PMWS compatible clinical picture (Segalés, 2002) appeared at the studied
112 farms, pigs displaying PMWS-like signs and healthy age-matched pigs were selected,
113 euthanized and necropsied. At necropsy, sections of lymphoid tissues were collected
114 and fixed by immersion in neutral-buffered 10% formalin to assess the pathological
115 status of both clinically healthy and diseased animals.

116

117 All treatments, housing, husbandry and slaughtering conditions followed the European
118 Union Guidelines and Good Clinical Practices.

119

120 *2.2. Histopathology*

121 Formalin fixed tissues were dehydrated and embedded in paraffin blocks. Two
122 consecutive 4 μm thick sections containing collected lymphoid tissues from each pig
123 were cut from each block. One section was processed for haematoxylin and eosin stain,
124 while the other was used for PCV2 nucleic acid detection by *in situ* hybridization (ISH)
125 (Rosell et al., 1999) for Spanish cases, and for immunohistochemistry (IHC) for PCV2
126 antigen detection (Jensen et al., 2006) for Danish cases. Pathological evaluation and
127 PMWS diagnosis was carried out using a previously described scoring system
128 evaluating the PCV2 amount and the intensity of lymphoid depletion and
129 granulomatous infiltration present in lymphoid tissues (Grau-Roma et al., 2009). Three
130 different categories of pigs were established: i) PMWS cases: corresponding to pigs
131 showing clinical wasting and with moderate to severe lymphoid lesions and moderate to
132 high amount of PCV2 antigen/nucleic acid; ii) wasted non-PMWS cases: corresponding
133 to pigs showing clinical wasting but without or with slight PMWS characteristic
134 histopathological lesions and no or low amount of PCV2 antigen/nucleic acid within
135 lymphoid tissues; iii) Healthy pigs: corresponding to pigs showing good clinical
136 condition, without or with slight PMWS characteristic histopathological lesions and no
137 or low amount of PCV2 antigen/nucleic acid within lymphoid tissues.

138

139 *2.3. Acute phase proteins determination in serum*

140 Two APPs, HPT and Pig-MAP, were measured in serum of studied pigs. Both APPs
141 were determined in all longitudinally collected sera from Spanish necropsied pigs (107
142 pigs coming from 3 different farms). For the Danish pigs, serum samples collected at 1,
143 6 or 9 weeks of age and at necropsy from only those pigs with available serum at the
144 moment of necropsy were analyzed.(53 pigs coming from 7 different batches).

145

146 Pig-MAP concentration was determined by a sandwich enzyme-linked immunosorbent
147 assay (ELISA), using a commercial kit based on two anti-Pig-MAP specific monoclonal
148 antibodies (PigCHAMP Pro Europa S.A.), according to the manufacturers' instructions.
149 This kit was based on an ELISA previously developed and validated (Piñeiro et al.,
150 2009b). HPT concentration was determined by a sandwich ELISA as previously
151 described (Sorensen et al., 2006). Pig-MAP and HPT results were expressed as mg per
152 millilitre of serum (mg/ml).

153

154 *2.4. Quantitative PCV2 PCR*

155 PCV2 real time quantitative PCR (qPCR) was performed on all longitudinally collected
156 serum samples. DNA was extracted from 200 µl of serum from Spanish (Nucleospin®
157 Blood, Macherey-Nagel, GmbH & Co KG, Düren, Germany) and Danish samples
158 (QIAamp DNA Mini Kit, Qiagen® GmbH, Germany), according to the manufacturers'
159 instructions. DNA was eluted in 100 µl and 200 µl of elution buffer in Spain and
160 Denmark, respectively.

161

162 PCV2 DNA was quantified using two previously described qPCR techniques on
163 Spanish (Olvera et al., 2004) and Danish (Hjulsager et al., 2009) samples, respectively.
164 The performance of both qPCRs was compared in a previous study based on field
165 samples (Hjulsager et al., 2009). This latter work showed a highly significant linear
166 association between results of the two qPCR assays. However, the method performed
167 by the Danish laboratory had higher sensitivity and yielded systematically higher PCV2
168 load values than the one used in the Spanish laboratory. All PCV2 qPCR results were
169 expressed as \log_{10} (number of PCV2 copies per ml of sera).

170

171 2.5. Serology

172 Serological analyses were performed on all longitudinally collected serum samples. In
173 Spain, ELISAs were used to detect antibodies against porcine reproductive and
174 respiratory syndrome virus (PRRSV) (HerdCheck PRRS virus antibody test, IDEXX,
175 Inc., USA), Aujeszky's disease virus (HerdCheck anti-PRV gpI, IDEXX, Inc., USA),
176 porcine parvovirus (PPV) (Ingezim PPV, Ingenasa, Spain), swine influenza virus (SIV)
177 (Civtest Suis Influenza, Laboratorios Hipra, Spain), *Mycoplasma hyopneumoniae*
178 (Civtest Suis *Mycoplasma Hyopneumoniae*, Laboratorios Hipra, Spain), and *Salmonella*
179 spp. (*Salmonella* covalent mix-ELISA, SvanovirTM, Svanova, Sweden). In Denmark,
180 assays used were previously described immunoperoxidase monolayer assays (IPMAs)
181 to detect antibodies against European and American PRRSV strains (Sorensen et al.,
182 1998), ELISAs to detect antibodies against PPV (Madsen et al., 1997) and *Lawsonia*
183 *intracellularis* (Boesen et al., 2005), and haemagglutination inhibition (HI) test to detect
184 antibodies against H1N1 and H3N2 subtypes of SIV, which was essentially carried out
185 according to the OIE manual (OIE manual of Diagnostic Tests and Vaccines for
186 Terrestrial Animals 2008). A seroconversion against studied pathogens was considered
187 to be present when an increase in the antibody titres in two consecutive samples or
188 when a positive result preceded by a previous negative result was detected.

189

190 2.6 *Mycoplasma hyopneumoniae* nested PCR

191 It is known that the appearance of antibodies to *M. hyopneumoniae* can be delayed as
192 much as 8 weeks after infection (Sitjar et al., 1996). Thus, in order to avoid
193 underestimating its potential prevalence in studied animals, a nested PCR (nPCR) assay
194 to detect *M. hyopneumoniae* was performed in the Spanish nasal swabs collected at the

195 moment of necropsy (Calsamiglia et al., 1999). For 1 out of 107 Spanish pigs the nasal
196 swab sample was lacking.

197

198 2.6. Statistical analyses

199 For statistical evaluation, the SAS 9.1.3 software was used (SAS institute Inc., Cary,
200 North Carolina, USA). A repeated measurement analysis of variance according to the
201 general linear model procedure (PROC MIXED) and LSMEANS follow-up test was
202 used to evaluate APPs mean concentrations differences between: i) PMWS cases,
203 wasted non-PMWS and healthy pigs at each sampling time and within them at different
204 samplings points; ii) healthy PCV2 subclinically infected pigs (PCV2 qPCR positive)
205 and healthy non-infected pigs (PCV2 qPCR negative) at different sampling points; iii)
206 seroconverted and non-seroconverted pigs against studied pathogens at each sampling;
207 and iv) *M. hyopneumoniae* infected (nPCR positive) and non-infected (nPCR negative)
208 at necropsy. Constant correlation was assumed between repeated measurements. Post-
209 hoc multiple comparisons were addressed using the Tukey test. PCV2 qPCR data were
210 log₁₀ transformed prior to graphic representation, and negative results were coded as 0.
211 A Pearson's chi-square test was used to compare the proportions of seroconverted
212 animals to each studied pathogen at the moment of necropsy between the three
213 categories of pigs studied; same comparison was also established using *M.*
214 *hyopneumoniae* nPCR results. The level of significance for all analyses (α) was set to
215 $p < 0.05$.

216

217 To determine the capacity of discrimination of both Pig-MAP and HPT between PMWS
218 affected and healthy pigs, a Receiver Operating Characteristic (ROC) analysis from
219 samples collected at necropsy was carried out using Statsdirect (version 2.6.6,

220 CamCode, Ashwell, UK). Moreover, ROC analysis was also performed to determine the
221 diagnostic performance of both APPs on clinically affected animals. For this later
222 analysis, only wasted pigs were considered, considering PMWS as “diseased” and
223 wasted non-PMWS as “non-diseased” animals. Taking into account that APPs
224 concentration was determined by the same laboratorial tests in both studies, all samples
225 collected at necropsy coming from Spain and Denmark were analysed together.

226

227 **3. Results**

228 *3.1 Studied animals, histopathology and acute phase proteins*

229 The number of pigs from which APPs concentrations were analysed and their
230 pathological classification (PMWS, healthy and wasted non-PMWS) is detailed in Table
231 1. Mean±SD of weeks of age at necropsy for PMWS, healthy and wasted non-PMWS
232 pigs were 14.0±2.1, 14.7±2.5, 15.2±2.7, respectively in Spain, and 11.2±1.5, 10.0±3.5,
233 10.9±1.9, respectively, in Denmark.

234

235 The longitudinal evolution of pig-MAP serum concentrations is displayed in figure 1.
236 Pig-MAP concentration in PMWS affected pigs was higher than in non-PMWS affected
237 ones (healthy and wasted non-PMWS) either in Spain ($p<0.0001$) and Denmark
238 ($p<0.001$) at the moment of necropsy. Although no statistically significant differences
239 were found between healthy and wasted non-PMWS pigs, the mean pig-MAP serum
240 concentration at the moment of necropsy in wasted non-PMWS was higher than in
241 healthy ones (Spain: $p=0.0715$; Denmark: $p=0.1472$). Moreover, the Pig-MAP
242 concentration in PMWS pigs at the sampling prior to necropsy in Spain was higher than
243 its concentration in non-PMWS affected pigs (healthy and wasted non-PMWS)
244 ($p<0.01$). Accordingly, in the Spanish pigs, the increase of pig-MAP concentration in

245 PMWS affected pigs was already significant at the sampling prior to necropsy,
246 compared to basal pig-MAP concentrations detected at weeks 1 to 7. In Denmark, a
247 significant increase in Pig-MAP concentration was only detected at necropsy in PMWS
248 pigs. No statistical significant changes in pig-MAP concentration were found among all
249 samplings in healthy and in wasted non-PMWS pigs from both countries.

250

251 The longitudinal evolution of HPT serum concentrations is displayed in figure 2. HPT
252 concentration at the moment of necropsy was higher in PMWS than in healthy pigs
253 either in Spain ($p<0.0001$) and Denmark ($p<0.01$). On the other hand, wasted non-
254 PMWS could not be distinguished from the other two groups in Denmark, but had
255 significantly lower HPT concentration than PMWS ($p<0.001$) in Spain. Regarding the
256 longitudinal evolution of HPT concentration within each group, HPT increased
257 progressively in both PMWS and wasted non-PMWS pigs in Spain from 11 weeks of
258 age ($p<0.01$) to the necropsy moment ($p<0.0001$). In Denmark, only PMWS affected
259 pigs gave significant increase in HPT concentrations with respect to the basal
260 concentrations detected at the 1st week of age ($p<0.01$). No statistically significant
261 variation was found among the HPT concentrations of healthy pigs neither in Spain nor
262 Denmark.

263

264 Pig-MAP concentrations obtained at the moment of necropsy were statistically
265 correlated with the global average scoring (histopathology plus PCV2 detection in
266 lymphoid tissues) in Spain ($R=0.622$; $p<0.0001$) and also in Denmark ($R=0.524$;
267 $p<0.0001$). On the other hand, significant correlation between HPT concentration at the
268 moment of necropsy and the global average scoring was detected in Spain ($R=0.443$;
269 $p<0.0001$), but only a tendency was observed in Denmark ($R=0.254$; $p=0.066$).

270 Moreover, pig-MAP and HPT concentrations were significantly correlated (Spain:
271 $R=0.462$, $p<0.0001$; Denmark: $R=0.576$, $p<0.0001$).

272

273 3.2 PCV2 qPCR

274 PCV2 loads in sera are schematically displayed together with APPs results in figures 1
275 and 2. PCV2 loads increased progressively from the 1st week of age until necropsy in
276 Denmark and during the last two samplings in Spain. Maximum PCV2 loads were
277 observed at the moment of disease outbreak (necropsy) in both countries.

278

279 Significant correlations were observed between pig-MAP serum concentrations and
280 PCV2 loads in serum (Spain: $R=0.278$, $p<0.0001$; Denmark: $R=0.47$, $p<0.001$).
281 Considering the 3 groups of pigs separately (figure 3), PMWS affected pigs
282 corresponded to the group with higher correlation between PCV2 load and pig-MAP
283 (Spain: $R=0.487$, $p<0.0001$; Denmark: $R=0.602$, $p<0.0001$), followed by the
284 correlations observed in wasted non-PMWS pigs (Spain: $R=0.218$, $p<0.01$; Denmark:
285 $R=0.437$, $p<0.01$). On the contrary, no significant correlation was found between pig-
286 MAP concentrations and PCV2 loads when only healthy pigs were considered (Spain:
287 $R=-0.152$, $p=0.07$; Denmark: $R=0.2$, $p=0.194$) (figure 3).

288

289 Significant correlations were also found between HPT serum concentrations and PCV2
290 loads in serum (Spain: $R=0.443$, $p<0.0001$; Denmark: $R=0.326$, $p<0.001$). Considering
291 the 3 groups of pigs separately, the group of pigs with PMWS had again the highest
292 correlation (Spain: $R=0.550$, $p<0.0001$; Denmark: $R=0.326$, $p<0.05$), followed by the
293 correlations observed in wasted non-PMWS pigs (Spain: $R=0.437$, $p<0.0001$; Denmark:
294 $R=0.295$, $p=0.057$). The lowest correlation between HPT concentration and PCV2 load

295 was found in healthy pigs, being significant in Spain ($R=0.186$, $p<0.05$) but not in
296 Denmark ($R=0.273$, $p=0.73$).

297

298 Taking into account that more than half of healthy Spanish pigs gave negative results at
299 all samplings by PCV2 qPCR, mean APPs concentrations in healthy PCV2 infected and
300 non-infected pigs were also compared. No statistical differences were seen between
301 both groups of pigs neither in pig-MAP nor HPT concentrations. Such comparison was
302 not done in any other group of pigs due to the low number of negative PCV2 qPCR
303 results.

304

305 *3.4 Serology and PCR against different swine pathogens*

306 The number of pigs that had seroconverted against studied pathogens and PCV2 qPCR
307 results at the moment of necropsy in both countries, and *M. hyopneumoniae* nPCR in
308 Spain, are displayed in Table 2.

309

310 No serological evidence of infection to ADV was detected. Evidence of *M.*
311 *hyopneumoniae*, PPV or PRRSV infections in Spain and SIV infection in Denmark
312 were found in less than 10% of analysed pigs. Prevalence of SIV and *Salmonella spp.* in
313 Spain and PPV, PRRSVe, PRRSVu and *L. intracellularis* in Denmark was detected in
314 25 to 55% of the studied pigs.

315

316 No differences in the proportions of infected and/or seroconverted animals to studied
317 pathogens between PMWS, wasted non-PMWS and healthy pigs were observed.
318 Moreover, no significant differences in APPs concentrations between seroconverted and
319 non-seroconverted pigs to studied pathogens were detected. However, the 5 pigs that

320 gave positive nPCR results for *M. hyopneumoniae* in Spain had higher pig-MAP mean
321 serum concentrations (mean±SD=4.47±4.49) than the 101 pigs that gave negative nPCR
322 (2.35±2.13) ($p<0.05$).

323

324 3.5 ROC analysis

325 Optimal cut-off values for both studied APPs maximizing sensitivity (Sn) and
326 specificity (Sp) to discriminate diseased and non-diseased pigs are displayed in Table 3.

327

328 Sp and Sn values were higher when comparing PMWS versus healthy pigs than
329 comparing PMWS versus wasted non-PMWS animals. Moreover, Sn and Sp values
330 obtained using pig-MAP concentrations were always higher than when HPT was used.
331 Further exploration of the data showed that, when comparing PMWS and healthy pigs,
332 Sp values were slightly improved when the presence of PCV2 (detected by qPCR) was
333 considered together with the APPs concentration. Thus, a potential pre-mortem
334 diagnosis of PMWS could be established only when APP concentrations were above
335 optimal threshold and qPCR gave a positive result. Following this condition, pigMAP
336 and HPT maintained their Sn at 85.7% (73.8-93.6) and 67.9% (54.0-79.7), respectively,
337 and Sp increased to 93.5% (82.1-98.6) in both cases.

338

339 4. Discussion

340 The present study represents the first longitudinal determination of pig-MAP and HPT
341 concentrations in pigs that subsequently developed PMWS. Both APPs increased in
342 parallel to the increase of PCV2 load in sera and reached the maximum values at the
343 moment of disease manifestation. Pig-MAP and HPT concentrations were higher in
344 PMWS affected pigs than in healthy ones, which is in agreement with previous reports

345 (Segalés et al., 2004; Parra et al., 2006). This situation is probably a reflection of the
346 systemic inflammatory status suffered by PMWS affected pigs (Rosell et al., 1999).

347

348 It is known that APPs serum concentrations might vary with the age (Piñeiro et al.,
349 2009a). However, in the present study, samples from all pigs were collected at
350 previously established weeks of age to accomplish a longitudinal study (Grau-Roma et
351 al., 2009). Moreover, at the moment of necropsy, pigs displaying PMWS-like signs and
352 healthy pigs were euthanized in a age-matched way. Therefore, the differences observed
353 between the three studied groups of pigs should not be influenced by a potential age-
354 effect.

355

356 The higher correlation between PCV2 load and both APPs detected in PMWS affected
357 pigs, together with the absence or weak correlation detected in healthy pigs, indicates
358 that the APR in PMWS affected pigs occurs concurrently with PCV2 infection, further
359 supporting the idea that PCV2 is the essential infectious agent for PMWS development
360 (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés et al., 2005). Based on qPCR
361 PCV2 results, an important percentage of healthy and wasted non-PMWS pigs were
362 also infected by PCV2. However, significant increase in APPs concentrations was only
363 clearly observed in PMWS affected animals. It has been reported that infections with
364 pathogens like *Streptococcus suis* produce an increase of APPs in both clinically and
365 subclinically infected pigs (Sorensen et al., 2006). However, present data showed that,
366 in accordance with previous results (Segalés et al., 2004), no differences between
367 subclinically PCV2 infected pigs (healthy PCV2 qPCR positive pigs) and healthy non-
368 PCV2 infected pigs (healthy PCV2 qPCR negative pigs) were detected. These findings
369 suggest that sole infection with PCV2 does not imply a systemic inflammatory status

370 causing a detectable APR; only when the disease develops, HPT and pig-MAP
371 concentrations increase significantly. Taking into account that PMWS affected pigs
372 have higher serum viral loads than non-affected pigs (Brunborg et al., 2004; Olvera et
373 al., 2004; Grau-Roma et al., 2009), a certain level of serum PCV2 load might be
374 associated with the increase of HPT and pig-MAP serum concentrations. Therefore, it is
375 likely that when the animal limits the PCV2 replication to certain levels and/or the virus
376 is not able to overcome the defences of the host, the APR becomes much less evident or
377 even non-detectable using HPT and pig-MAP determinations in sera.

378

379 It is difficult to collectively interpret the results of the pigs suffering wasting but
380 without a PMWS diagnosis (wasted non-PMWS animals). This group of animals might
381 include pigs suffering from wasting due to PMWS-unrelated causes, such as other
382 infectious or non-infectious diseases and/or management conditions (Harding, 1997),
383 but might also include PMWS convalescent pigs or pigs that were able to overcome or
384 limit the disease expression (Segalés, 2002; Krakowka et al., 2005). The obtained pig-
385 MAP concentration profile in wasted non-PMWS pigs was similar to the one obtained
386 in healthy pigs. However, an increase in HPT concentration was detected at the two last
387 samplings in Spain. Moreover, in Denmark, HPT concentrations at the moment of
388 necropsy in wasted non-PMWS animals could not be distinguished from those in
389 PMWS affected pigs. These results, together with the wider APPs concentration
390 variation observed in wasted non-PMWS pigs compared to healthy ones, suggest that at
391 least a proportion of wasted non-PMWS animals mounted an APR around the PMWS
392 outbreak. On the other hand, this group of pigs had lower PCV2 serum loads than
393 PMWS-affected ones and higher than healthy pigs during PMWS outbreak (Grau-Roma
394 et al., 2009). Moreover, a correlation between PCV2 viral load and APPs concentration

395 was observed. Taken together, these findings suggest that the APR detected in the
396 groups of wasted non-PMWS affected pigs might also be associated with PCV2
397 infection.

398

399 The fact that an increase of both pig-MAP and HPT proteins was seen in Spanish pigs
400 prior to the PMWS outbreak suggests that the detectable APR occurred some days
401 before the start of clinical signs. Therefore, those APPs could provide valuable
402 prognostic information if proper timing of sampling is assured (Murata et al., 2004).
403 These increased values before the PMWS outbreak were not detected in the Danish
404 animals, probably due to the low number of samples analyzed at week 9 in each group.

405

406 Despite it is known that APPs are generic markers of inflammation and therefore are not
407 specific of any disease (Petersen et al., 2004; Gruys et al., 2005a), the previously
408 described potential use of APP as markers of health (Gruys et al., 2005b; Parra et al.,
409 2006) prompted us to evaluate their diagnostic performance for PMWS. Both HPT and
410 pig-MAP serum concentrations were significantly correlated. Nevertheless, pig-MAP
411 gave a better diagnostic value of PMWS, since this protein displayed higher Sp and Sn
412 values than HPT in the ROC analysis. Moreover, pig-MAP was able to differentiate
413 PMWS from non-PMWS affected pigs (comprising wasted non-PMWS and healthy
414 pigs) even at the sampling prior to PMWS development (at least in Spain). The
415 previously described individual higher variation in HPT concentrations (Piñeiro et al.,
416 2009a) could partially explain the lower diagnostic value of HPT. In any case, as
417 previously indicated, the APPs values of single reactants are not sensitive enough to
418 detect a special subject in a population livestock (Gruys et al., 2005a). It has been
419 suggested that the acute phase signal situation obtained for an individual animal can be

420 enhanced when the values of positive APPs are combined with negative APPs as an
421 index. Such an index could enhance Sn and Sp remarkably in comparison to single
422 APPs determination in the search for unhealthy subjects among a population of
423 apparently normal animals (Gruys et al., 2005a).

424

425 PMWS is considered a multifactorial disease in which the occurrence of PCV2 infection
426 is necessary but not sufficient (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés
427 et al., 2005). Present results showed that, among all studied pathogens, PCV2 was the
428 only one systematically present in PMWS affected farms and pigs. This fact suggests
429 that none of the other studied pathogens are necessary for the PMWS development
430 under field conditions. Moreover, no significant differences in APP serum
431 concentrations between those pigs showing evidences of infection for pathogens
432 different from PCV2 at the moment of clinical PMWS outbreak were detected, with the
433 only exception of *M. hyopneumoniae* in Spain. Specifically, the 5 pigs that gave
434 positive PCR results for *M. hyopneumoniae* had higher pig-MAP concentration than the
435 101 pigs with negative results. In fact, the two pigs with higher pig-MAP concentration
436 (10 and 9 mg/ml) corresponded to 2 out of the 5 *M. hyopneumoniae* nPCR positive,
437 suggesting that PCV2 co-infection with other pathogens could increase the APR,
438 aggravating the severity of the disease (Ellis et al., 2004). The potential influence of
439 other not studied pathogens on the observed clinical signs and, therefore, on the APR of
440 the PMWS affected animals, can not be ruled out.

441

442 In conclusion, the present results indicate that the acute phase response in PMWS
443 affected pigs occurred concomitantly to PCV2 viremia. In addition, present work
444 supports the hypothesis that the increase of APPs levels depends on the development of

445 PMWS and not on infection with PCV2 alone, as previously suggested (Segalés et al.,
446 2004).

447

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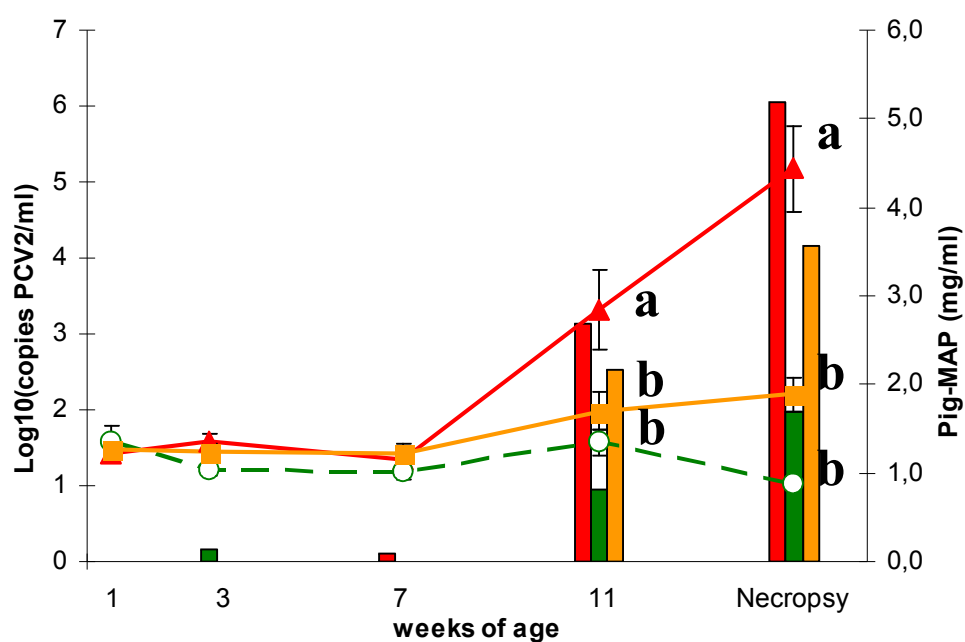
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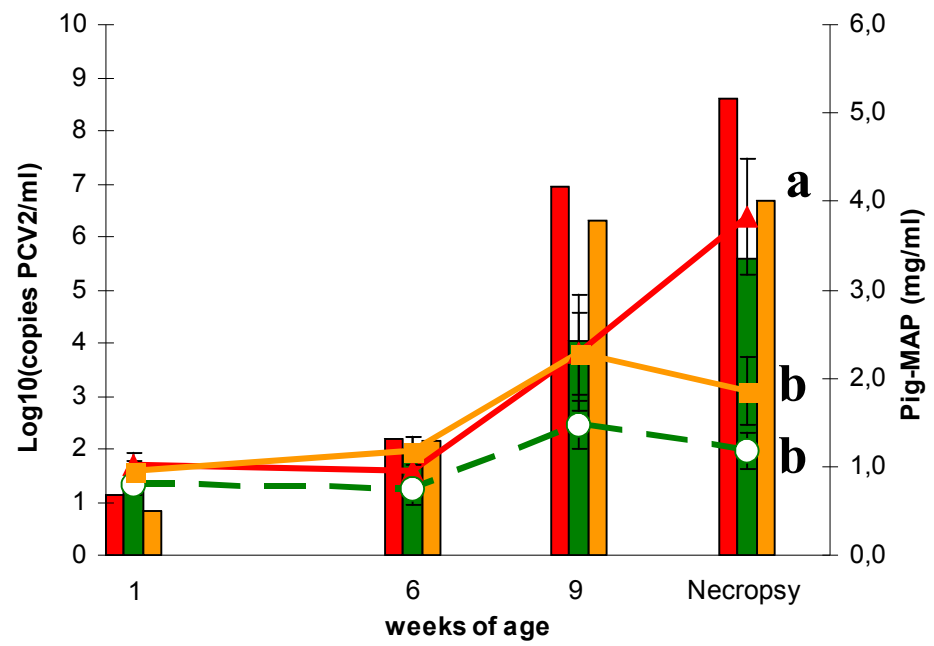
Figure 1. Pig-MAP concentrations (mg/ml) (lines) and PCV2 loads (copies of PCV2/ml of sera) (columns) in PMWS (\blacktriangle , \blacksquare), healthy (\circ , \square) and wasted non-PMWS (\square , \square) pigs at the different sampling points from Spanish (1A) and Danish (1B) farms. **a,b,c** indicate statistical significant differences in APP concentration between animal groups in a given sampling time. **a'b'c'** indicate statistical significant differences in APP concentration within each animal group along the study period ($p < 0.05$). Error bars represents standard error of the mean.

1A.



PMWS	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>b'</i>	<i>c'</i>
Healthy	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>
Wasted non-PMWS	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>

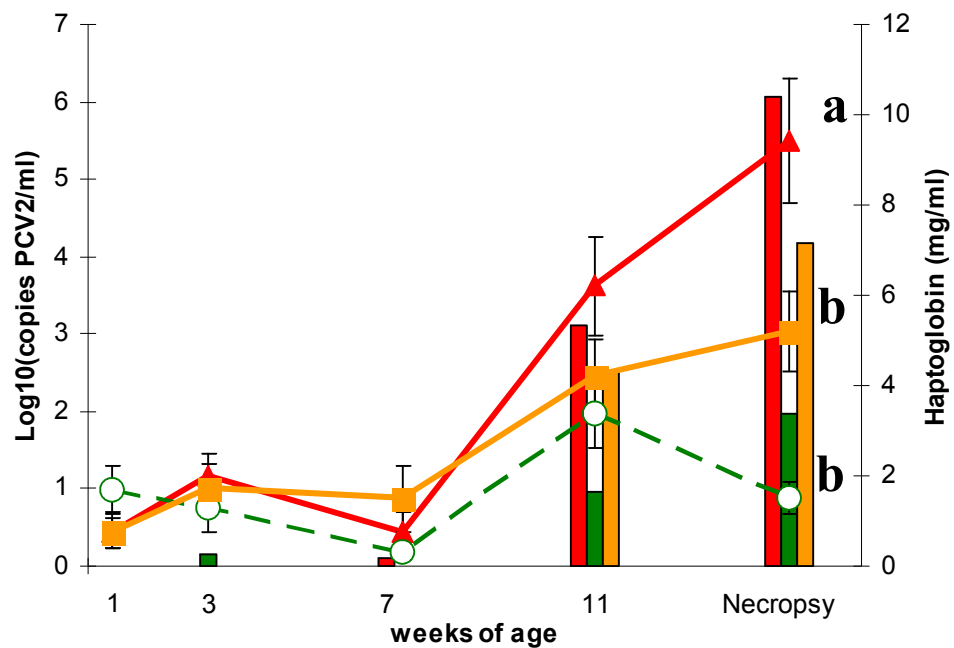
1B.



PMWS	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>b'</i>
Healthy	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>
Wasted non-PMWS	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>

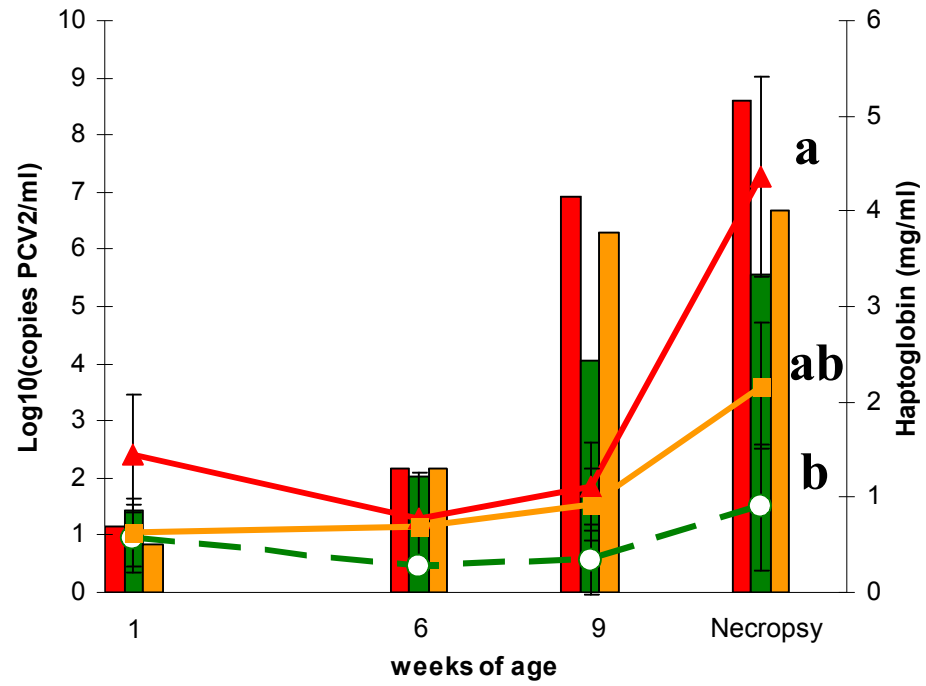
Figure 2. Haptoglobin concentrations (mg/ml) (lines) and PCV2 loads (copies of PCV2/ml of sera) (columns) in PMWS (\blacktriangle , \blacksquare), healthy (\circ , \square) and wasted non-PMWS (\blacktriangle , \blacksquare) pigs at the different samplings from Spanish (2A) and Danish (2B) farms. **a,b,c** indicate statistical significant differences in APP concentration between animal groups in a given sampling time. **a'b'c'** indicate statistical significant differences in APPs concentration within each animal group along the studied period ($p < 0.05$). Error bars represents standard error of the mean.

2A.



PMWS	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>b'</i>	<i>c'</i>
Healthy	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>
Wasted non-PMWS	<i>a'</i>	<i>a'</i>	<i>ab'</i>	<i>bc'</i>	<i>c'</i>

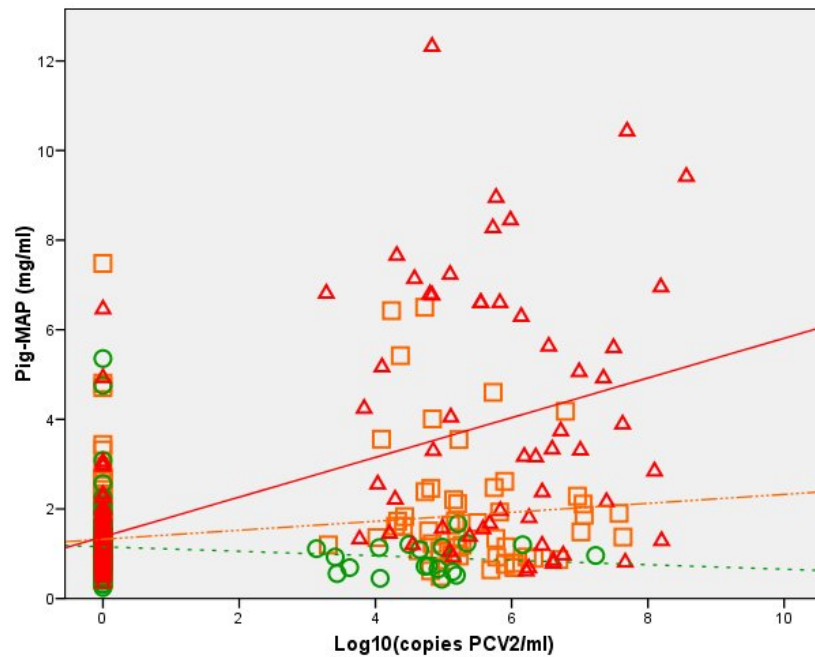
2B.



PMWS	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>b'</i>
Healthy	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>
Wasted non-PMWS	<i>a'</i>	<i>a'</i>	<i>a'</i>	<i>a'</i>

Figure 3. Correlation between pig-MAP concentration and PCV2 load in each studied category of pigs: PMWS (Δ , —), wasted non-PMWS (\square , - - -) and Healthy (\circ ,) in Spain (A) and Denmark (B).

3A.



3B.

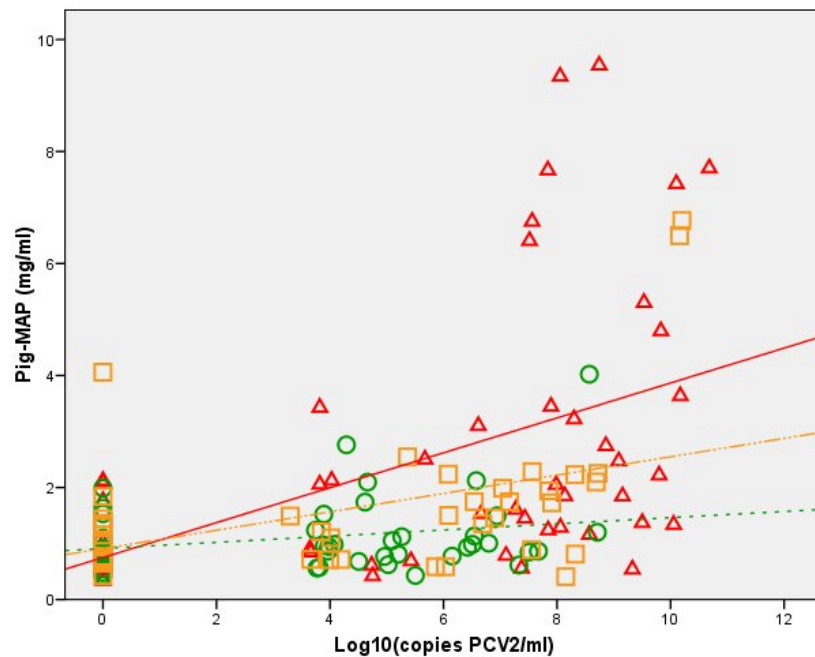


Table 1. Number of pigs within each pathological category from which haptoglobin and pig-MAP concentrations were determined in each country.

	PMWS	Healthy	Wasted non-PMWS	Total
Spain	35	29	43	107
Denmark	21	17	15	53
Total	56	46	58	160

Table 2. Number of seroconverted pigs against studied pathogens, and nPCR and/or qPCR results at the moment of necropsy in Spain (A) and Denmark (B). Results are indicated as the number of seropositive (serology) or positive (qPCR and nPCR) pigs at euthanasia during the PMWS outbreak respect the total of studied pigs. Percentage is given between brackets

A.

	Serology						nPCR	qPCR
	PPV*	PRRSV	SIV	ADV	Salm	Myco	Myco	PCV2
PMWS	2/35 (6)	1/35 (3)	16/35 (46)	0/35 (0)	8/35 (23)	0/35 (0)	3/35 (9)	35/35 (100)
Healthy	3/29 (10)	0/29 (0)	17/29 (59)	0/29 (0)	9/29 (31)	0/29 (0)	1/29 (3)	12/29 (41)
Wasted non-PMWS	2/43 (5)	1/43 (2)	26/43 (60)	0/43 (0)	19/43 (44)	0/43 (0)	1/43 (2)	34/43 (79)
Total	7/107 (7)	2/107 (2)	59/107 (55)	0/107 (0)	36/107 (34)	0/107 (0)	5/107 (5)	81/107 (76)

B.

	Serology						qPCR
	PPV	PRRSVe	PRRSVu	SIV_H1N1	SIV_H3N2	Law	PCV2
PMWS	7/21 (33)	9/21 (43)	10/21 (48)	1/21 (5)	3/21	5/21 (24)	21/21 (100)
Healthy	11/17 (65)	7/17 (41)	10/17 (59)	3/17 (18)	1/17	5/17 (29)	15/17 (88)
Wasted non-PMWS	4/15 (27)	8/15 (53)	9/15 (60)	0/15 (0)	0/15	3/15 (20)	13/15 (87)
Total	22/53 (42)	24/53 (45)	29/53 (55)	4/53 (8)	4/53 (8)	13/53 (25)	49/53 (93)

*Porcine parvovirus (PPV), porcine reproductive and respiratory virus (PRRSV), Aujeszky disease virus (ADV), swine influenza virus (SIV), *Salmonella* spp. (salm), *Mycoplasma hyopneumoniae* (Myco), porcine circovirus type 2 (PCV2), European and American PRRSV strains (PRRSVe, PRRSVu), *Lawsonia intracellularis* (law).

Table 3. Optimal cut-off, sensitivity and specificity values of ROC analyses for pig-MAP and Haptoglobin serum concentrations considering PMWS pigs as “diseased” and healthy or wasted non-PMWS as “non-diseased animals”.

APPs	PMWS vs Healthy			PMWS vs Wasted non-PMWS		
	Optimal cut-off	Sensitivity (CI _{95%})	Specificity (CI _{95%})	Optimal cut-off	Sensitivity (CI _{95%})	Specificity (CI _{95%})
Pig-MAP	≥1,3	85,7 (73,8-93,6)	87,0 (73,7-95,0)	≥2,7	60,7 (46,7-73,5)	87,7 (76,3-94,9)
Haptoglobin	≥2,7	67,9 (54,0-79,7)	84,8 (71,1-93,7)	≥2,7	67,9 (54,0-79,7)	55,2 (41,5-68,3)