Accepted Manuscript

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Please cite this article as: F.Bonelli, V.Meucci, T.J.Divers, A.Boccardo, D.Pravettoni, M.Meylan, A.G.Belloli, M.Sgorbini, Plasma procalcitonin concentration in healthy calves and those with septic systemic inflammatory response syndrome (2010), https://doi.org/10.1016/j.tvjl.2018.02.003

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Original Article

Plasma procalcitonin concentration in healthy calves and those with septic systemic inflammatory response syndrome

F. Bonelli [a,*], V. Meucci [a], T.J. Divers [b], A. Boccardo [c], D. Pravettoni [c], M. Meylan [d], A.G. Belloli [c], M. Sgorbini [a]

[a] Department of Veterinary Sciences, via Livornese snc, 56122, San Piero a Grado (PI), Italy.
[b] College of Veterinary Medicine, Cornell University, Vet Box 25, Ithaca, NY 14853, USA.
[c] Department of Veterinary Medicine, via dell'Università 6, 26900, Lodi, Italy.
[d] Clinic for Ruminants, Vetsuisse-Faculty, University of Bern, Bremgartenstrasse 109a, 3012 Bern, Switzerland.

* Corresponding author. Tel.: +39 050 221 0115.
  E-mail address: francesca.bonelli@unipi.it (F. Bonelli).

Highlights

- Plasma procalcitonin (PCT) level was evaluated in healthy calves and those with septic systemic inflammatory response syndrome (SIRS).

- Plasma PCT concentration was higher in the septic SIRS group than in the healthy group.

- An increase in PCT concentration in the septic SIRS group was confirmed, as in humans and other species.

- PCT could be used as marker for septic SIRS in calves.

Abstract

The diagnosis of sepsis in calves is challenging. Blood culture and clinical signs combined with a complete blood count have been used for the diagnosis of sepsis. Recent literature in humans and animal species has been focused on sepsis-specific biomarkers, such as procalcitonin (PCT), that may more accurately and efficiently diagnose sepsis. The aim of this study was to evaluate plasma PCT concentrations in healthy and septic calves. Twenty healthy control calves and 58 sick calves with septic systemic inflammatory response syndrome (SIRS) based on SIRS score and clinical findings were included. Calves with septic SIRS were further divided in septic SIRS survivors (SSS) and non-survivors (SSNS). Plasma PCT concentrations were measured with a commercial ELISA assay for cattle. A receiver operating characteristic curve was used to determine cut-off values and corresponding sensitivity and specificity for the diagnosis of sepsis. Differences in plasma PCT concentration between groups (control vs. SSS vs. SSNS) were evaluated.

Plasma PCT concentrations in healthy calves and those with septic SIRS were 33.3 pg/mL (0 - 44.3 pg/mL) and 166.5 pg/mL (85.9 - 233.0 pg/mL), respectively ($P<0.001$). The optimal cut-off value to predict septic SIRS was 67.39 pg/mL (81.0% sensitivity, 95.0% specificity). Plasma PCT concentrations were 127.4 pg/mL (72.2 – 216.0 pg/mL) and 234.3 pg/mL (204.5 – 309.4 pg/mL) in the SSS and SSNS subgroups, respectively. Statistically significant differences were found among groups (control vs. SSS and SSNS, $P<0.0001$; SSS vs. SSNS, $P >0.05$). These results confirmed an increase in plasma PCT concentrations in calves with septic SIRS, as previously reported in humans and other species.

Introduction

Since the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference in 1991, the term sepsis has been defined as the 'systemic inflammatory response to infection' (Bone et al., 1992). The expression 'systemic inflammatory response syndrome' (SIRS) describes a clinical condition that represents the culmination of the activation of a complex network of acute endogenous mediators, which lead to uncontrolled and widespread inflammation (Alberti et al., 2005). SIRS can be associated with many different factors, including hypoxia, burns, trauma, immunologic reactions, bacterial and viral infections (Bone et al., 1992; Alberti et al., 2005). Confirmation of microbial infection in the presence of SIRS is required for a diagnosis of sepsis (Zabrecky et al., 2015).

Sepsis in calves is either sporadic or epidemic, reaching a prevalence of 30% if predisposing factors for the development of neonatal septicemia are present (Aldrige et al., 1993; Fecteau et al., 2009). Many factors predispose calves to sepsis, such as failure of passive transfer, management deficits, adverse environmental conditions, cold stress, protein-energy malnutrition, micronutrient deficiencies, or bacterial colonization of a local site, such as the umbilicus, gastro-intestinal tract or respiratory system (House et al., 2015). The pathophysiologic changes associated with inflammatory activation in sepsis are dehydration and alterations in heart rate, respiratory rate, body temperature, mucous membrane status and capillary refill time, as well as leukopenia, hypotension and generalized weakness (House et al., 2015). The definitive diagnosis of sepsis is based on a blood culture. However, the sensitivity of blood culture can be low and a negative result must be interpreted with caution (Fecteau et al., 2009). In veterinary medicine, clinical signs combined with a complete blood

count (CBC) and scores are considered useful in the diagnosis of sepsis. However, sepsis-specific biomarkers have become a recent focus of research in both humans and veterinary species because they could potentially increase diagnostic accuracy and efficiency (Fecteau et al., 2009; Fielding and Magdesian, 2015; Ercan et al., 2016).

Procalcitonin (PCT) has been investigated as a biomarker of sepsis in humans (Riedel, 2012; Deliberato et al., 2013; Afsar and Sener, 2015), and in horses (Toribio et al., 2003; Pusterla et al., 2006; Rieger et al., 2014; Bonelli et al., 2015a; Bonelli et al., 2015b; Barton et al., 2016; Bonelli et al., 2017), cattle (Ercan et al., 2016) and dogs (Giunti et al., 2006; Yilimaz et al., 2008). Healthy individuals have very low serum PCT concentrations due to the restriction of the CALC-I gene transcription by the neuroendocrine cells in the thyroid gland and in the lungs (Riedel, 2012; Afsar and Senar, 2015). The expression of the CALC-I gene is up-regulated in many cell types in septic humans (Riedel, 2012; Afsar and Senar, 2015) and in septic animals (Toribio et al., 2003; Giunti et al., 2010); thus, PCT is released into the circulation from many cell types. In septic human patients, PCT rises rapidly (within 3-6 h) and decreases by half within 24 h when the infection and/or host immune response is controlled (Riedel, 2012; Afsar and Senar, 2015). PCT also remains stable in blood specimens at room temperature, unlike other markers of sepsis (Carrol et al., 2002). In veterinary medicine, studies have reported plasma PCT concentrations in septic and non-septic foals (Bonelli et al., 2015a), adult horses (Rieger et al., 2014; Bonelli et al., 2015b; Bonelli et al., 2017), and dogs (Giunti et al., 2006; Yilmaz et al., 2008). The aim of this study was to evaluate and compare plasma PCT concentrations in healthy and septic calves.

Materials and methods

Animals

This in vivo multicentric experimental trial was conducted in a clinical setting and was approved by the Italian Animal Care (DL 116/92) and by the Institutional Animal Care and Use Committees of the University of Pisa (Approval Number 2825; Approval Date 28 January, 2014) and the University of Milan (Approval Number 2; Approval Date 15 February, 2016). At Cornell University, residual blood from specimens routinely collected from calves on hospital admission was used. Written consent was obtained from the owners of all calves included.

During the research period, a total of 260 calves were admitted to the veterinary teaching hospitals involved in the study. However, the authors excluded cases that did not fit the inclusion criteria, resulting in the inclusion of 78 calves aged 9.6±4.3 days. Twenty of 78 were healthy Holstein calves (n=11/20 females and n=9/20 males) aged 8.4±3.9 days and were housed at the university dairy farm of the University of Pisa. Fifty-eight of 78 (n=35/58 females and n=23/58 males), aged 10.6±4.2 days, were sick, client-owned calves referred to three participating veterinary teaching hospitals providing secondary health care (the Veterinary Teaching Hospital 'M. Modenato', University of Pisa, Italy; the 'Nemo' Farm Animal Hospital, Cornell University, Ithaca, NY, USA; the Clinic for Ruminants and Swine, University of Milan, Italy). Sick calves were Holstein Friesian (n=50/58) or mixed dairy breeds (n=8/58).

Collection of clinical and clinical pathological data

A complete history was recorded, when possible, for each calf at admission time, especially information concerning previous antimicrobial treatment. All calves were subjected to a complete physical examination and blood was collected for CBC with differential white cell count and blood culture at admission time. Calves needed only manual

restraint for all procedures. A 1 mL blood sample for CBC was collected from the jugular vein in an EDTA tube (FL Medical) and analyzed by a cell counter (ProCyte Dx, IDEXX) within 5 min of collection. The EDTA blood was also used for blood smears, which were air-dried and stained using an automatic stainer (Aerospray 7150 Hematology Slide Stain-Cytocentriguge). The differential white cell count was performed by microscopic examination at 400 × and 1000 × magnification, counting 100 cells. A sample for blood culture was collected aseptically and a commercial culture system (OXOID SIGNAL Blood Culture System, Oxoid) was used as previously reported (Daley et al., 1990; Rohner et al., 1995). The outcome was recorded for all the sick client-owned calves as 'discharged' or 'died/euthanased'.

Inclusion criteria

Calves < 3 weeks old were enrolled. Each calf was scored according to a SIRS scale as previously described (Trefz et al., 2016). Only calves with a positive SIRS score were included in the septic SIRS group. SIRS positivity was based on the presence of two or more of the following criteria (Trefz et al., 2016): (1) presence of an abnormal leucocyte count, i.e. leukopenia or leukocytosis (reference interval, $5–12 \times 10^9$/L); (2) hyperthermia or hypothermia (reference interval, 38.5– 39.5 °C); (3) tachycardia (> 120 beats/min); and (4) tachypnea (> 36 breaths/min). Calves with SIRS were considered septic if there was clinical or necropsy evidence of septicemia (Lofstedt et al., 1999). In particular, the ante-mortem criteria were as follows: (1) positive blood culture; (2) culture of the same bacterial agent from at least two body fluids; or (3) culture of a bacterial agent from a single joint in a calf with joint effusion involving multiple joints. The post-mortem criteria were as follows: (1) morphologic changes, such as multiple disseminated abscesses of similar size, purulent vasculitis and intravascular identification of bacteria, or fibrin in multiple body cavities; (2)

bacterial isolation from heart blood; or (3) recovery of the same bacterial isolate from at least two tissues (excluding intestine). No calves included in the control group had any signs of SIRS (SIRS score, 0), or clinical signs of septicemia.

Evaluation of plasma PCT concentration

A 2.5 mL aliquot was collected at admission time in heparinized tubes (FL Medical) and immediately centrifuged at 2.100 *g* for 10 min. The harvested plasma was placed in sterile tubes, frozen at -18 °C, and analyzed for PCT in a single batch within 3 months. The concentrations of PCT were determined using a commercial kit for cattle (Bovine Procalcitonin ELISA kit, MyBiosource.com). The intra-assay coefficient of variation was determined from 10 replicates of calf plasma samples with known low and high PCT concentrations. These samples were obtained by adding standard amounts of PCT provided with the ELISA kit in blank samples of calf blood. The inter-assay coefficient of variation was determined by repeating the analysis of duplicate samples with low and high PCT concentrations in five different assays. Samples were measured in 10 replicates in a single assay and in five different assays. The intra- and inter-assay coefficient of variations were both < 15%; the limit of detection of the method was 10 pg/mL. To establish the detection limit for bovine plasma PCT, we performed repeated PCT measurement using bovine samples with low PCT concentrations (<10.0 pg/mL). Results less than the limit of detection were expressed as 0.

Statistical analysis

Kolmogorov-Smirnov tests were applied to verify data distribution. Descriptive data are reported as median and $25^{th}/75^{th}$ percentile for both the control and septic SIRS groups. Mann-Whitney U tests for unpaired data were performed to verify differences in plasma PCT

concentrations between the healthy controls and the septic SIRS groups. Receiver operating characteristic (ROC) analysis was performed to obtain specificity and sensitivity of the test at various cutoff values with a confidence interval (CI) of 95%. Likelihood ratio was calculated for each cut-off value. Results from animals in the septic SIRS group were further divided in two subgroups, depending on the outcome of disease, i.e. whether they recovered (septic SIRS survivors subgroup, SSS), or were euthanased or died (septic SIRS non-survivors subgroup, SSNS). Kolmogorov-Smirnov tests were applied to verify data distribution for plasma PCT concentrations in the SSS and SSNS subgroups. The results did not show a Gaussian distribution for plasma PCT concentrations in either subgroup, so descriptive data are reported as median and $25^{th}/75^{th}$ percentile. Kruskal-Wallis tests for unpaired data and Dunn's multiple comparisons post-hoc tests were performed to verify differences in plasma PCT concentrations among groups (control vs. SSS vs. SSNS).

Statistical significance was set at $P < 0.05$ and commercial statistics software was used (GraphPad Prism 6).

Results

Collection of a complete history was not possible for every calf included. All 20 healthy control calves remained healthy for the entire study period. All 58 sick calves had a SIRS score ≥ 2 and clinical or necropsy evidence of septicemia. The most common diagnoses in the sick calves were septicemia, pleuritis, pneumonia, diarrhea with signs of dehydration and electrolyte imbalance, peritonitis, omphalitis, polyarthritis. Blood cultures were performed in five cases (8.6% of all blood cultures were positive). Only *E. coli* was cultured from each of the calves with positive blood culture. Culture of the same pathogen from at least two body fluids antemortem (e.g. peritoneal fluid, bronchoalveolar lavage fluid) was

used to diagnose 45 cases: *E.coli* was cultures in 30/45 cases (67%); other bacteria isolated were *Campylobacter* spp. (*n*=6), *Pasteurella haemolytica* (*n*=4), *Clostridium* spp. (*n*=3), and *Enterococcus* spp. (*n*=2). A positive antemortem joint culture from calves with polyarthritis was used to diagnose septicemia in two cases. In both cases, *E. coli* was cultured. Regarding post mortem evaluation, morphologic lesions such as multiple abscesses, purulent vasculitis, intravascular identification of bacteria, and fibrin exudation in multiple body cavities, were present in six calves. All 58 sick calves satisfied the criteria for the diagnosis of septicemia and were included in the septic SIRS group.

Plasma PCT concentration was 33.3 pg/mL (0 - 44.3 pg/mL) in the control group and 166.5 pg/mL (85.9 - 233.0 pg/mL) in the septic SIRS group (Fig.1; *P*<0.0001). The resulting area under the ROC curve was 0.92 (95% CI: 0.86-0.98; *P*< 0.001; Fig. 2), and the optimal cut-off value to differentiate healthy calves from calves critically ill with clinical evidence of SIRS and septicemia was 67.39 pg/mL (mean sensitivity 81%, 95% CI, 68%–90%; mean specificity value 100%, 95% CI 83% – 100%; Table 1).

Fifty-two of 58 (89.7%) calves with septic SIRS recovered and were included in the SSS subgroup; 5/58 (8.6%) calves with septic SIRS were euthanased on economic grounds, and 1/58 (1.7%) died; thus 6/58 calves were included in the SSNS subgroup. Plasma PCT concentrations were 127.4 pg/mL (72.2 – 216.0 pg/mL) and 234.3 pg/mL (204.5 – 309.4 pg/mL) in the SSS subgroup and in the SSNS subgroup, respectively. Kruskal-Wallis tests for unpaired data indicated that there were statistically significant differencese in plasma PCT (*P* < 0.001) among the three groups of calves (SSS, SSNS, control). Post-hoc Dunn's multiple comparisons tests demonstrated differences between the control group and the SSS subgroup

(P<0.0001), between the control group and SSNS subgroup (P<0.0001), and between the SSS and SSNS subgroups (P<0.02).

Discussion

Despite the fact that neonatal sepsis is the third most common cause of mortality in large animal neonates, early recognition of clinical signs is challenging for farm managers and early diagnosis remains challenging for veterinarians (Fecteau et al., 1997; Lofstedt et al., 1999; House et al., 2015). In the present study, plasma PCT concentrations were statistically lower in healthy calves than in calves with septic SIRS. Our results confirmed that plasma PCT increases during septic SIRS in calves, as previously reported in the horses, dogs and humans (Yilmaz et al., 2008; Riedel, 2012; Rieger et al., 2014; Afsar and Senar, 2015, Bonelli et al., 2015a; Bonelli et al., 2015b; Bonelli et al., 2017). Moreover, mean plasma PCT concentrations in both healthy calves and those with septic SIRS were similar to those reported in horses (Bonelli et al., 2015a; Bonelli et al., 2015b; Bonelli et al., 2017), lower than those reported in human newborns (Altunhan et al., 2011) and children (Lacour et al., 2001), and higher than in dogs (Yilimaz et al., 2008), but these dissimilarities could be related to differences in the assays used. The results obtained for the control group were similar to those reported for healthy calves by others (Ercan et al., 2014). Recently, Ercan and colleagues evaluated some biomarkers for healthy cattle and Ercan et al., 2016 investigated the diagnostic value of determining the serum concentrations of PCT and other markers (neopterin, tumor necrosis factor alpha, prostaglandin E2, malondialdehyde, interleukin 8 and IFN-γ) in neonatal calves diagnosed with septicemic colibacillosis (Ercan et al., 2016). Serum PCT concentrations were four times higher in septicemic calves with colibacillosis than in healthy calves (Ercan et al., 2016). Plasma PCT concentrations in calves with septic SIRS in our study were similar to those described by Ercan and colleagues (2016). However, we

included a calves with SIRS and septicemia caused by a variety of conditions, while Ercan and colleagues (2016) enrolled only calves with septicemic colibacillosis.

Plasma PCT concentrations were statistically different when the control group was compared with the SSS and the SSNS subgroups and plasma PCT concentrations were statistically higher in the SSNS subgroup than in the SSS subgroup. This result suggests a possible role for plasma PCT in predicting unfavorable outcome in calves with septic SIRS, as reported in humans for sepsis, severe sepsis and septic shock (Huang et al., 2016; Ko et al., 2016; Liu et al., 2016; Poddar et al., 2016). However, the small group size in the SSNS subgroup compared to the SSS subgroup limits the conclusions that can be drawn concerning case outcome. The prognostic value of plasma PCT should be addressed in future studies.

Our cut-off value for plasma PCT (67.39 pg/mL) to differentiate septic from non-septic calves is lower than that reported for human adults (Riedel, 2012), neonates and children (Lacour et al., 2001; Altunhan et al., 2011), but similar to that reported for foals (Bonelli et al., 2015a). To the best of authors' knowledge, such cut-off values have not previously been reported in literature for calves or adult cattle.

The survival rate in our present study appears to be unusually high for a population of septic calves. At admission, few calves were bacteremic, but most had signs of localized sepsis. Although historical findings were not available for all cases, some calves had already received treatment before presentation to a veterinarian, which might explain the low number of calves that died or were euthanased.

A limitation of the present study is the low number of blood culture-positive calves. A negative blood culture result must be interpreted with caution because many factors can interfere with bacterial isolation from blood culture specimens. Prior antibiotic therapy, the presence of opsonizing antibodies, low numbers of circulating bacteria, relatively low specimen volume and disease stage have been described as possible factors affecting the sensitivity of blood culture (Wilson and Madigan 1989; Fecteau et al., 2009). In the present study, history of previous antimicrobial treatment was incomplete in some calves. Additionally, blood culture was always performed at admission and it is possible that not all calves were bacteremic at that time. More complete historical record collection is strongly recommended for further studies.

Conclusions

Plasma PCT measurements allow healthy calves to be differentiated from critically ill calves with clinical evidence of SIRS and septicemia. Further research could be performed to investigate the role of plasma PCT in reducing antimicrobial course length, as proposed in human medicine. Moreover, survival analysis using plasma PCT data should record the time to complete recovery, not just the final outcome. Finally, plasma PCT testing should be performed in calves with non-septic SIRS to better understand the role of this biomarker in the diagnosis of SIRS.

Conflict of interest statement

None of the authors has any financial or personal relationship that could inappropriately influence or bias the contents of the paper.

Acknowledgments

Preliminary results were presented as an Abstract at the European College of Bovine

Health Management Congress, Maribor, SLO, 10-13 June 2015.

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Figure legends

Fig. 1. Box and whisker plot showing median and 10-90 percentiles for plasma procalcitonin (PCT) concentrations in the septic systemic inflammatory response syndrome (SIRS) group (*n*=58) and in the control group (*n*=20). *** P<0.0001.
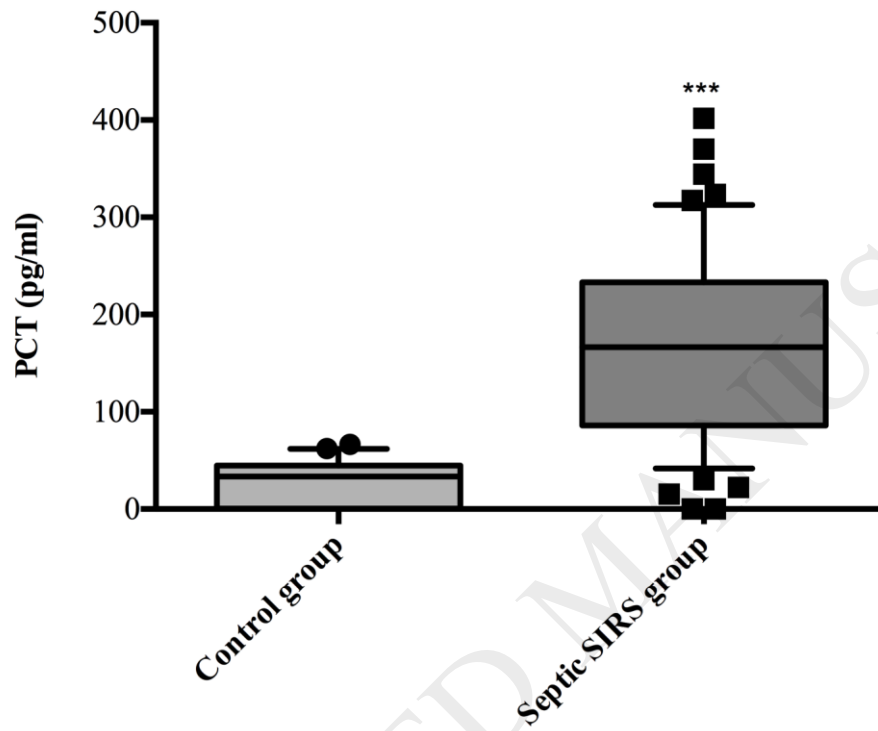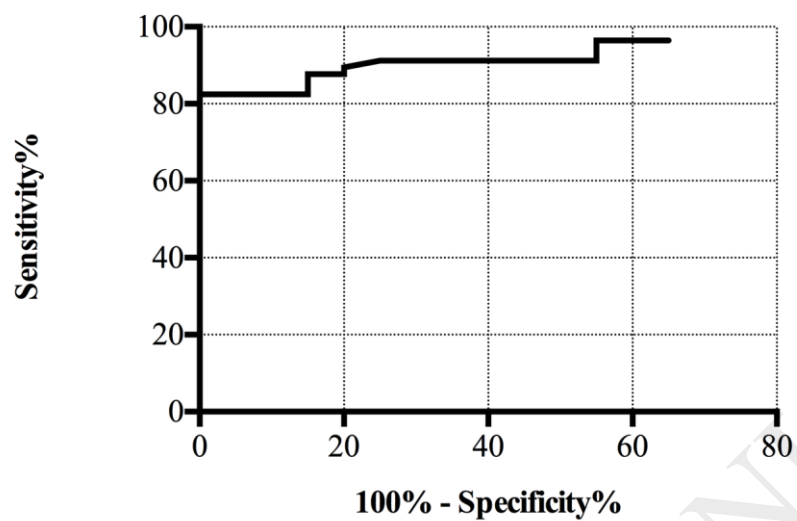
Fig. 2. Receiver operating characteristic curve for the defined septic systemic inflammatory response syndrome analysis performed to obtain specificity and sensitivity of plasma procalcitonin at various cut-off values with a confidence interval of 95%.

Table

Table 1.

Receiver operating characteristic curve analysis data as assessed for the definition of the optimal cut-off value for plasma procalcitonin concentration to discriminate healthy calves from calves critically ill with clinical evidence of systemic inflammatory response syndrome and septicemia (*n*=78).

Cut-off (pg/mL)	Sensitivity % (95% CI)[a]	Specificity % (95% CI)	Likelihood ratio
> 27.72	62 (35-85)	97 (85-100)	21.87
> 32.82	69 (41-89)	97 (85-100)	24.06
> 38.75	75 (48-93)	97 (85-100)	26.25
> 55.60	81 (54-96)	97 (85-100)	28.44
> 64.00	81 (69-90)	95 (73-100)	16.20
> 67.39	81 (68-90)	100 (83-100)	-
> 70.09	81 (66-88)	100 (83-100)	-

[a] 95% CI, 95% Confidence interval