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Significance of bioindicators to predict survival in irradiated minipigs

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Abstract

The minipig is emerging as a potential alternative non-rodent animal model. Several biological markers e.g. blood counts, laboratory parameter and clinical signs have been proposed for rapid triage of radiation victims. Here, we focus on the significance of bio-indicators for prediction of survivors after irradiation and compared it with human data; relationship between these biomarkers and radiation dose is not part of this study. Male Gottingen minipigs (age 4–5 months, weight 9–10 kg) were irradiated (or sham-irradiated) bilaterally with gamma-photons (Cobalt-60, 0.5–0.6 Gy/min) in the dose range of 1.6 – 12 Gy. Peripheral blood cell counts, laboratory parameters, and clinical symptoms were collected up to 10 days after irradiation and analyzed using logistic regression analysis and calculating ROC curves. In moribund pigs parameters such as decreased lymphocyte/granulocyte counts, increased C-reactive protein, alkaline phosphatase values as well as increased citrulline values and body temperature significantly ($p<0.002$ up to $p<0.0001$) discriminated non-survivors from survivors with high precision (ROC ≥ 0.8), but most predictive within the first three days after exposure was a combination of decreased lymphocyte counts and increased body temperature observed as early as 3 h after radiation exposure (ROC: 0.93–0.96, $p<0.0001$). Sham-irradiated animals (corresponding to “worried wells”) could be easily discriminated from dying pigs, thus pointing to the diagnostic significance of our analysis. These data corroborate with earlier findings performed on human radiation victims suffering from severe hematological syndrome and provide further evidence for the suitability of the minipig model as a potential alternative non-rodent animal model.

Keywords

minipig; non-rodent animal model; radiation; dosimetry

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Introduction

The minipig is emerging as an alternative non-rodent model besides NHP and dogs, to be used for radiation countermeasure testing under the FDA animal rule. Such a rule requires that the model is well characterized and predictive of the human condition. We have undertaken a step-by-step approach to evaluate the suitability of the minipig to replicate the Hematopoietic Acute Radiation Syndrome (H-ARS) observed in humans. During this process we established study feasibility in terms of animal housing and handling, dosimetry, blood sampling and animal care (Moroni et al, 2011a). Next, we irradiated animals at doses bracketing the H-ARS and observed that clinical signs and symptoms, kinetics of blood element loss and recovery, occurrence of infection, hemorrhages, lethality, cardiac- and respiratory-complications replicated the natural history of ARS in humans (Moroni et al, 2011b and 2011c). Finally, we proved that the minipig has the potential to predict the efficacy of drugs to be used in humans, by confirming that administration of G-CSF, the standard cytokine treatment for the ARS, improved survival and hastened recovery from neutropenia, as observed for humans (Moroni et al, 2013a).

Potential applications of the model are the development of tools for rapid assessment of exposure and guidelines for triage and prognosis. It has been proposed that estimation of consequence for radiation victim in large scale accidents can be achieved based on clinical signs, symptoms and blood counts. The Medical Treatment Protocols for Radiation Accident (METREPOL) system has provided guidelines for triage of victims within the first 3–6 days after exposure, based on the damage to the neurovascular, hematopoietic, cutaneous and gastrointestinal systems, the severity of which is reflected in grading of response categories (RC) and likelihood of survival (Fliedner et al, 2001). Along those lines, a pattern of changes in blood cell counts within the first week after exposure has been proposed as indicator for severity of damage to the hematopoietic stem cell pool (Fliedner et al, 2007). The METREPOL approach has been followed to assess severity of hematological syndrome for hospitalization and medical management for several patients following recent radiation accidents in Belgium and Senegal (Gourmelon et al, 2010), and Bulgaria (Djounova 2012).

Here we performed a discriminative analysis for several parameters (clinical signs, symptoms and routine laboratory parameters) in irradiated minipigs (1.6–12 Gy) as well as sham-irradiated, and evaluated the prognostic potential of such parameters collected up to 10 days after irradiation to discriminate survivor from non-survivor. The purpose of this study was (1) to continue to validate the model, (2) to establish similarities as well as differences of irradiated minipigs with irradiated humans regarding parameters predicting survivors and non-survivors after radiation exposure and (3) to systematically examine how well different parameters alone and in combination might predict survivors and non-survivors in order to improve early and rapid triage of radiation victims.

Materials and Methods

Animals and irradiation

The current study is a retrospective analysis of parameters collected from previous studies done in our laboratory, where animals were irradiated in the dose range 1.6 – 12 Gy (Table

1) (Moroni et al, 2011b, 2011c, and unpublished) spanning hematopoietic and gastrointestinal syndromes; doses ≥ 2 Gy are 100% lethal. Animal housing and care, irradiation procedure, blood collection and sample processing are reported in detail elsewhere (Moroni et al, 2011a and 2011b). Briefly, all animals were male Gottingen minipigs (Marshall Bioresources, Upstate NY), approximately 4–5 months old at the time of irradiation, and 9–10 kg in weight. Irradiation was bilateral, total body (Cobalt-60, 0.5–0.6 Gy/min). Procedures were done in accordance with the AFRRI IACUC. AFRRI is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. A subset of the raw data obtained from animals irradiated in the range 1.6–5.0 Gy has been used in previous publications (Moroni et al, 2011b, 2011c), but not for discriminative and multivariate analysis as described below.

Parameter examined

We examined (i) peripheral blood cell counts (lymphocytes, granulocytes (neutrophils), thrombocytes and white blood cell counts), (ii) clinical symptoms (body temperature, petechiae) and (iii) blood chemistries (C-reactive protein [CRP], alkaline phosphatase [AP] and citrulline) at different points in time (3 h, 1 d, 2 d, 3 d, 7 d and 10 d) for most of the variables in Gottingen mini-pigs either surviving up to 60 days (n=19) or moribund (n=70) after exposures ranging from 1.6–12 Gy, single whole body doses. A group of sham-irradiated animals was also included in the study (Table 1). From these 53 variables we excluded those variables with less than 8 entries in the group of the survivors and less than 10 entries in the group of pigs which did not survive, leaving 47 variables eligible for analysis.

Statistics

Descriptive statistics of variables and comparison of mean values (t-test) were performed using SAS (release 9.2, Cary NC, USA, 2010). In order to discriminate groups of pigs which survived over non-survivors we employed logistic regression analysis. According to this approach, parameters enter a prediction model and for each parameter a weighting factor (maximum likelihood estimate) is then calculated and the contrast for discriminating both groups sharpened. Predictions are compared with the known groups and concordance, discordance and tied pairs of predicted probabilities, and observed responses computed (Hosmer and Lemeshow Goodness-of-fit test). Concordances of 100% indicate complete discrimination of both groups. Odds ratios (OR) and 95% confidence intervals (95% CI) are also calculated.

Additionally ROC (receiver-operator characteristic) curves were computed using SAS. A ROC curve is a plot of the sensitivity versus (1-specificity) of a screening test, where the different points on the curve correspond to different cutoff points used to designate test positives from test negatives (Rosner, 2006). The area under the ROC curve is a reasonable summary of the overall diagnostic accuracy of the test. ROC areas of 1.0 indicate complete separation of both groups. ROC areas between 0.8–0.9 (80–90%) or 0.9–1.0 (90–100%) indicate a good or excellent discrimination ability, respectively. Based on the ROC models we calculated corresponding positive predictive values ([PPV=true positives/(true positives + false positives)], the probability that an animal with a positive test result will die) and

negative predictive values ([NPV=true negatives/(true negatives + false negatives)], the probability that an animal with a negative test result will survive).

After examining the discrimination ability separately for each variable (univariate analysis) we examined whether a combination of variables might improve our results (multivariate analysis). Only variables contributing significantly in univariate analysis during the first three exposure days and containing >15 cases per group were chosen. We ran a forward and backward selection procedure which finally led to the identification of the same combination of variables.

Graphs were created using Sigma Plot 9.0 (Jandel Scientific).

Results

Peripheral blood cell counts

Lymphocytes counts rapidly dropped following irradiation, while neutrophil granulocytes showed the classic transient increase after exposure prior to progressive decline (Figure 1). Unexposed animals could be discriminated almost completely from the exposed animals based on their lymphocyte and neutrophil granulocyte counts (Figure 1). We observed significant associations of lymphocyte and neutrophil granulocyte counts with survival, starting 3 h after radiation exposure (Table 2). For instance, mean lymphocyte counts at 3 h were 3.4/nl (+/− 0.9, range: 1.5–5.0) in the surviving animals, and 2.0/nl (+/− 0.9, range: 0.7–4.6) in non-surviving animals. These lower lymphocyte counts correspond to a 4-fold decreased (OR 0.24) likelihood to survive which further decreases up to 25-fold (OR 0.04) at ≥ 2 days after exposure. Discrimination of both groups during the whole period corresponded with ROC areas between 0.83–0.90.

In particular for the first three days after exposure we observed stronger associations, risks and greater ROC area for lymphocyte counts compared to the neutrophil granulocytes (table 2). Neutrophil granulocyte counts differed significantly between survivor and non-survivor for all time points except 2 days after exposure (table 2). For instance, at 7 days after exposure and later neutrophil granulocyte counts of non-surviving pigs dropped to one third of the values found for the survivor group corresponding to a 3.8–5 fold decreased likelihood to survive (OR 0.2–0.3). ROC areas ranged between 0.88–0.92 at ≥ 7 days after exposure, but appeared lower (ROC: 0.70–0.75) at 3 days after exposure and earlier (Table 2). Changes in total white blood cell counts behaved like the granulocyte counts with significant associations and similar discrimination abilities for the same time points after exposure (Table 2). Thrombocytopenia at 7 and 10 days after exposure (and not earlier) were significantly associated with survival and almost completely discriminated both groups with ROC areas close to 1 (0.94–0.97).

Clinical symptoms and laboratory parameters

Body temperature but not petechiae were significantly associated with the two groups, survivors versus non-survivors. Body temperature on average peaked at about 1 °C over control in animals that did not survive values at 3 h as well as 7 and 10 days after exposure, but remained unaltered between these time points (Figure 1 and Table 2). The corresponding

likelihood to survive decreased up to 8.6-fold and allowed a good separation of the groups (ROC area up to 0.87, table 2).

Laboratory parameters such as CRP (1, 3 and 7 days), AP (1 day) and citrulline (7 days) showed significant associations with ROC areas ranging between 0.77–0.83 (Table 2).

Combining variables for improved group separation within the first 3 days after exposure

Combinations of lymphocyte counts at 3 h or 2 d with body temperature at 3 h after exposure proved to be the most promising combinations of two variables and increased the ROC area from 0.87 in separate analysis for either lymphocyte counts at 3 h or body temperature at 3 h to 0.93 as early as 3 h after exposure. For lymphocyte counts at 2 d ROC area of 0.90 increased up to 0.96 in combination with body temperature at 3 h. This converts into an almost complete separation of both groups with positive predictive value (PPV) and negative predictive value (NPV) ranging between 90–100% (figure 2).

Discussion

We examined the potential of radiation induced changes in peripheral blood cell counts, clinical symptoms and laboratory parameters to discriminate between surviving and non-surviving minipigs. In the surviving group, we included sham-irradiated animals, mimicking what would be a real case scenario where worried-well must be separated from victims with poor prognosis. Survivors and non-survivors could be discriminated almost completely within the first three days after exposure based on lymphocytopenia and increased body temperature. The discrimination potency of radiation induced lymphocytopenia has already been shown in accidentally exposed humans using the METREPOL approach on historical cases collected in the database SEARCH (*S*ystem for *E*valuation and *A*rchiving of *R*adiation Accidents based on *C*ase *H*istories) (Friesecke et al 2000). Twenty-four clinical parameters, including blood count changes, fever and others (Fliedner et al, 2001) taken during the follow up of each exposed individual allow for grading of severity of damage in the absence of physical dosimetric measurements. In particular, in previous work, lymphocytopenia in combination with granulocytosis measured within the first three days after radiation exposure allowed us to successfully (ROC > 0.90) discriminate surviving from dying radiation victims (Knie et al., 2012) which is very much in line with our results of irradiated minipigs.

Increase in temperature during the prodromal phase is also considered an indicator of severity of symptoms; transient increases to 38–40°C, higher than 40°C for less than 24 hours and higher than 40°C for more than 24 hours are considered Degree 2, Degree 3 and Degree 4, respectively (Fliedner et al, 2001). These examinations performed in irradiated humans corroborate with our analysis on minipigs and again underline the similarity between our minipig model and biological processes going on in irradiated humans.

Beside the neutrophil granulocyte counts we also analyzed white blood cell counts (WBC) and found as expected similar discrimination characteristics, indicating that WBC could be used for prediction of moribund animals in the absence of neutrophil granulocyte counts. Similar to the human prediction model for survival, thrombocytes are of less significance

within the first 3 days after exposure, but at later points in time in the minipig model thrombocyte values discriminated both groups with high accuracy. Validity of thrombocytes counts as a prognostic indicator in minipigs has been suggested by the authors in a previous article, using a subset of data which did not restrict the analysis of the diagnostic significance to the first 3 days after exposure (Moroni et al, 2011c).

Considering limited clinical resources (e.g. ICU) we believe it to be of importance, that based on our prediction model our unexposed minipigs could be completely discriminated from the exposed with an NPV of 100%, which coincides with our findings in irradiated humans (Knie et al. 2012).

C-reactive protein (CRP), citrulline and alkaline phosphatase (AP) are among the suggested biomarkers used to determine exposure to radiation. CRP is an acute phase protein and marker of inflammation. It is found transiently increased within the first days after irradiation and then again during the later stages of ARS both in human and in animal models (Mal'tsev et al. 2006; Koc et al, 2003; Cengiz et al, 2001; Wood, 1960; Blakely et al, 2010). Levels of CRP in the blood during the first days post-exposure and again during the latent phase have been suggested as prognostic markers for humans (Mal'tsev et al, 2006). Similarly, in the minipig, blood concentrations of CRP 1 day after irradiation were significantly associated with survival.

Citrulline is a biomarker for viable small bowel enterocytes and decreased plasma citrulline levels indicate loss of the small bowel enterocyte mass. Citrulline has been proposed as candidate surrogate marker for GI-specific radiation damage. A significant dose-response relationship has been suggested at 4 days after total body irradiation (Lutgens et al, 2007); rapidity of citrulline loss did not appear to be dependent on the dose, but recovery was more rapid for lower doses and slower or incomplete for higher doses. Accordingly, we observed in the minipig that citrulline levels were inversely associated with survival at day 7, and surviving animals had much higher citrulline in the plasma with respect to non-surviving animals, reinforcing again the resemblance of radiation injury between humans and the minipig model.

AP is a marker for liver damage, and it is significantly increased after total body irradiation. In rats, already at day 1 after total body irradiation, AP activity is increased and remains elevated for several days (Anwar et al, 2013; Auda et al 1987; Manu et al, 2007). Altered levels of AP and long term consequences on hepatic and renal function have been demonstrated also in the NHP, even though morphological changes were only mild (Niemer-Tucker et al, 1995). In humans, hepatic dysfunction can occur as a delayed consequence of radiation therapy (Tanaka et al, 2013; Khozouz et al 2008). In the minipig, we showed here that elevated levels of AP on day 1 were predictive of survival; elevated levels of AP were present also at necropsy in morbid animals (Moroni et al, 2011b).

Study limitations

There are several limitations to this study, including the modality of exposure (total body irradiation and dose range) and the limited persistence overtime of some of the bioindicators (i.e. body temperature). Partial body irradiation is expected to represent a more realistic

scenario of exposure than total body, yet very few animal models have been developed to address this point (Hérodin et al, 2011; MacVittie, 2013). The translation of our results to a partial body scenario needs to be tested. Noteworthy, our approach links bioindicators to survival and not to absorbed dose. Different pattern of partial body irradiation (or whole body exposure with lower doses) might lead to similar changes in e.g. lymphocyte counts which then are predictive for survivor or non-survivor. Therefore, it is an advantage of our approach that we are less dependent on sophisticated dose estimations of body parts being exposed and issues e.g. related to homogenous or heterogeneous exposure. Instead, we are predicting the effect based on radiation-induced changes of bioindicators, e.g. changes in blood cell counts leading to comparable health effects. Furthermore, along the causal pathway starting with radiation exposure and leading finally to adverse health effects, changes in blood cell counts can be thought of as an intermediate which is closer to the effect making bioindicator of effect a robust and meaningful approach. The dose range used for this study spans from sub-lethal to supra-lethal doses, with 60% of the animals irradiated at supra-lethal doses (>2 Gy). Potential for introduction of biases was therefore checked. Restricting the analyses to a dose range of 1.6–2.0 Gy (with 19 survivors including 8 sham-irradiated animals and 18 non-survivors), however, still demonstrated that survivors can be completely discriminated from non-survivors using the model as shown in figure 2 (data not shown). Body temperature increased soon after exposure and returned to control values at 1 or 3 days after exposure. Hence, the prognostic significance of increased body temperature is restricted in time.

Conclusions

In large scale radiation accidents, clinical resources (i.e. ICU) are limited. It is crucial that actual exposure to radiation be assessed and confirmed, and that the worried well (unexposed individuals who believe they have been exposed) be separated from the actual radiation victims. We have shown here that the minipig may provide a suitable model for the investigation of parameters predictive of the condition in humans. In particular the lymphocyte, granulocyte and body temperature data show surviving and non-surviving animals can be clearly separated, leading to a useful prediction and excellent NPVs of the outcome prediction models.

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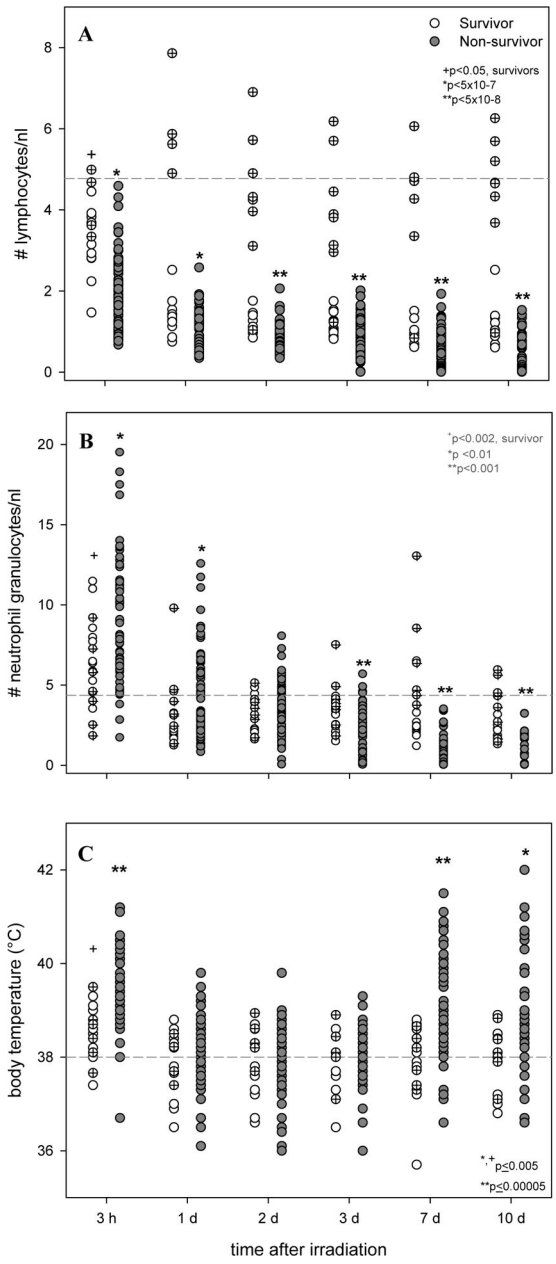

Figure 1.

Individual counts on lymphocytes (A) and granulocytes (B) as well as measurements of body temperature (C) are depicted for both groups, namely surviving (circles with white fills) and moribund animals (circles with gray fills) after whole body radiation exposure. Unexposed animals are identified by white crossed circles. Significant differences of mean values between groups at the same time are presented by asterix, but the + indicates significant differences within the survivor group at different points in time. We employed t-test and Mann-Whitney rank sum tests for mean/median comparison where appropriate. Horizontal dashed lines represent corresponding mean values of unexposed animals.

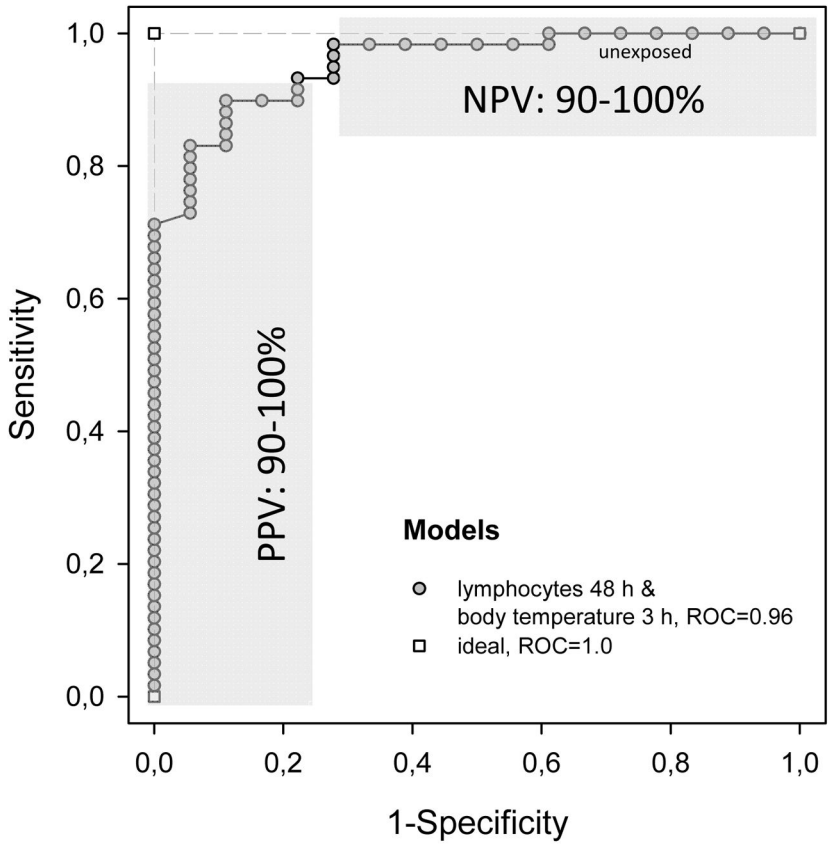

Figure 2.

A receiver-operator characteristic (ROC)-curve was calculated based on a logistic regression model comprising lymphocyte counts measured 1 d and body temperature measured 3 h after irradiation. PPV and NPV values exceeding 90% are shown for ROC-regions with corresponding sensitivity and specificity values. The 8 unexposed animals in our model are predicted with an NPV of 100%.

Table 1

The number of survivors and non-survivors are shown depending on the radiation dose.

Radiation dose (Gy)	survivor (19)	non-survivor (70)
0	8	0
1.6	5	1
1.7	4	2
1.8	2	4
1.9	0	6
2	0	5
2.2	0	1
2.4	0	2
2.6	0	1
2.8	0	1
3.8	0	3
4.1	0	2
4.2	0	2
4.4	0	2
4.6	0	2
4.7	0	2
5	0	5
6	0	2
7	0	4
8	0	6
9	0	5
10	0	6
11	0	4
12	0	2

Table 2

Variables significantly associated with death are presented in three categories, namely changes in the peripheral blood, clinical symptoms and laboratory parameter. Descriptive statistics are presented for surviving and non-surviving animals (left part) and odds ratios (OR), 95% confidence intervals (95%CI), chi-square p-values as well as concordance and the calculated area under the ROC curve are depicted to the right.

	survivor					non-survivor					log regression				
Variable	**n**	**mean**	**Stdev**	**min**	**max**	**n**	**mean**	**Stdev**	**min**	**max**	**OR**	**95% CI**	**chi-square**	**concordance**	**ROC**
peripheral blood															
lymphocytes (#/nl)															
3 h	15	3.4	0.9	1.5	5.0	69	2.0	0.9	0.7	4.6	0.24	0.117–0.493	<.0001	86.5	0.87
1 d	15	2.6	2.3	0.8	7.9	67	1.0	0.5	0.4	2.6	0.24	0.085–0.675	0.0069	82.4	0.83
2 d	19	2.5	1.9	0.9	6.9	65	0.8	0.3	0.4	2.1	0.04	0.006–0.284	0.0012	90.3	0.90
3 d	19	2.4	1.7	0.8	6.2	63	0.8	0.5	0.01	2.0	0.11	0.027–0.433	0.0016	84.9	0.85
7 d	16	2.1	1.8	0.6	6.1	60	0.5	0.5	0.01	1.9	0.10	0.023–0.455	0.0027	87.2	0.87
10 d	18	2.6	2.0	0.6	6.3	42	0.5	0.5	0.01	1.5	0.06	0.009–0.393	0.0035	90.2	0.90
neutrophil granulocytes (#/nl)															
3 h	19	6.3	2.6	1.8	11.5	68	9.2	3.7	1.7	19.5	1.34	1.100–1.633	0.0036	73.3	0.73
1 d	18	2.7	1.1	1.2	4.7	67	4.4	2.7	0.8	12.6	1.56	1.097–2.231	0.0135	70.2	0.71
3 d	19	3.4	1.4	1.5	7.5	64	2.0	1.4	0.01	5.7	0.50	0.325–0.780	0.0021	75.2	0.75
7 d	16	3.6	2.0	1.2	8.5	59	0.8	1.0	0.01	3.5	0.26	0.134–0.514	<.0001	91.6	0.92
10 d	18	2.8	1.4	1.3	5.9	41	0.8	1.0	0.01	3.2	0.22	0.091–0.511	0.0005	87.5	0.88
white blood cell counts (#/nl)															
2 d	17	6.4	2.6	3.2	11.8	66	4.6	1.7	0.4	9.4	0.62	0.461–0.843	0.0021	70.1	0.70
3 d	19	6.8	2.6	3.7	13.0	64	3.1	1.8	0.03	7.1	0.41	0.268–0.641	<.0001	89	0.89
7 d	17	6.3	3.7	2.4	15.4	59	1.5	1.6	0.01	5.8	0.40	0.247–0.657	0.0003	91.4	0.92
10 d	18	5.8	3.0	2.1	11.1	41	1.4	1.5	0.03	5.0	0.33	0.161–0.664	0.002	91.3	0.91
platelets (#/nl)															
7 d	16	350	191.17	110	881	59	107.0	73.86	7	278	0.98	0.962–0.988	0.0002	94.1	0.94
10 d	18	225	201.8	20	666	41	12.66	11.43	1	55	0.90	0.834–0.965	0.0035	96.6	0.97
clinical symptoms															

		survivor				non-survivor					log regression				
Variable	**n**	**mean**	**Stdev**	**min**	**max**	**n**	**mean**	**Stdev**	**min**	**max**	**OR**	**95% CI**	**chi-square**	**concordance**	**ROC**
body temperature (°C)															
3 h	18	38.5	0.5	37.4	39.5	66	39.5	0.7	37	41.2	8.59	2.800–26.354	0.0002	86.1	0.87
7 d	18	37.9	0.8	35.7	38.8	62	39.1	1.1	37	41.5	3.88	1.774–8.466	0.0007	79.7	0.81
10 d	18	37.9	0.6	36.8	38.9	41	38.9	1.2	37	42.0	2.96	1.381–6.339	0.0053	76.3	0.77
laboratory parameter															
c-reactive protein (ng/ml)															
1 d	13	26.6	5.0	14.9	37.2	56	81.3	77.0	17	319.2	1.14	1.018–1.267	0.0224	82.8	0.83
3 d	14	26.0	8.9	15.6	48.1	49	58.4	60.3	1.8	259.2	1.07	1.004–1.149	0.0375	77.4	0.78
7 d	13	21.5	7.2	12.9	37.3	52	117.2	137.2	8.2	727.1	1.07	1.001–1.148	0.0471	82	0.82
alkaline phosphatase (U/l)															
1 d	8	142.1	14.5	120.0	166.0	55	182.9	51.1	93.0	385.0	1.03	1.004–1.054	0.0241	81.8	0.82
citrulline (μM)															
7 d	9	72.4	13.8	47.9	90.0	47	51.2	25.6	2.4	112.3	0.96	0.926–0.996	0.0298	76.6	0.77