HUMANE ENDPOINTS IN SEVERE IRRADIATION EXPERIMENTS USING NON-HUMAN PRIMATES: A RETROSPECTIVE ANALYSIS.

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Humane endpoints in severe irradiation experiments using non-human primates: a retrospective analysis

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Summary

A retrospective statistical study has been performed in order to identify the biological, clinical and behavioural variables that could potentially predict the survival status of irradiated non-human primates (NHP) and to refine the future use of humane endpoints (HEP). The available data come from experiments that were initially designed and implemented to evaluate the efficacy of various treatments of the radiation-induced haematopoietic syndrome. A group of 15 NHPs was selected on the basis of similar irradiation and treatment protocols. Among those 15 NHPs, 3 animals died during the study despite efforts developed to prevent intense suffering and death. Among individual variables collected during the study, the ones retained for the present analysis were Flt3-ligand concentration in plasma, body temperature, alimentary behaviour and general behaviour score. A Bayesian predictive analysis showed that, although some of these variables were of potential interest, none of them alone proved valuable in predicting the death of the three animals. A refined analysis showed that a rapid decrease in body temperature during 3 consecutive days of more than 2.5°C was predictive of the death of two out of three animals. Moreover, a combination of several variables including decrease in body temperature and general behaviour score, improved the prediction of death. These results confirm that a HEP cannot only rely on the use of a single variable, suggesting using a combination of variables, including behaviour, as a composite HEP in NHP experiments using high doses of irradiation.

Introduction

The study of past accidents has shown that human victims of accidental irradiation develop an acute radiation syndrome (ARS). As a part of this ARS, the haematopoietic syndrome appears as a consequence of radiation doses as low as 0.5 Gy and is lethal in humans with whole body radiation doses higher than 4-5 Gy. Manifestations of this haematopoietic syndrome are pancytopenia, thrombocytopenia and anaemia with haemorrhages and opportunistic infec-

tions of graded severity depending on the radiation dose (Goans and Waselenko 2005). The haematopoietic syndrome remains difficult to treat and is the first cause of victim death following accidental irradiation (Densow et al. 1997; Coeytaux et al. 2015). Thus, alternative therapeutic strategies have to be developed and tested, such as the *ex vivo* expansion of bone marrow cells (Herodin and Drouet 2002;

Thierry et al. 2005) or cytokine injection (Herodin et al. 2007).

These alternative therapeutic strategies could not be tested directly on humans for obvious ethical reasons. Furthermore, *in silico* and *in vitro* methods were not relevant to address such complex questions. Although rodents were also used to address preliminary questions on irradiation-induced conditions (Prat et al. 2005; Prat et al. 2006), it should be noted that the physiology of these rodent models is not relevant enough for human physiology. Therefore, we developed a non-human primate (NHP) model (*M. fascicularis*) of bone marrow aplasia after irradiation in a heterogeneous, high dose field of gamma rays (Bertho et al. 2002). This model proved to be the most relevant and reproducible among other species to mimic the complexity of human accidental irradiation (Cui et al. 2016; Williams et al. 2010; Farese et al. 2012).

Various experimental therapeutic strategies were applied to NHPs immediately after irradiation, including autologous bone marrow transplantation, *ex vivo* expanded autologous haematopoietic cells and/or cytokine injections. These experimental therapeutic strategies have been extensively described and studied previously (Bertho et al. 2002; Bertho et al. 2005a; Bertho et al. 2005b). The clear-cut objective was to define the best possible treatment to apply in relation to the kinetic and severity of the bone marrow aplasia in order to foster the haematopoietic recovery. During the course of the performed experiments, the survival of animals was the main objective, through the use of experimental treatments, nursing and supportive care. Survival was defined as animals remaining alive for at least 60 days after irradiation with a full recovery of haematopoietic parameters. Expectedly, such protocols required intensive clinical support (antibiotics, fluid perfusion, platelet and red blood cell transfusion) along with a detailed monitoring of numerous parameters and the evaluation of the severity of the condition induced by high-dose irradiation. The definition of humane endpoints (HEP) for these experiments was an absolute requirement that was discussed with the animal care and use committee during the prospective ethical evaluation.

Although at the time of these experiments the definition of HEP was not a legal requirement, provisional HEPs were set based on biological, clinical and behavioural parameters that could be collected easily on a daily basis for each animal during experimentation. These parameters are the number of white blood cells (WBC), number of platelets (plt), number of red blood cells (RBC), Flt3-ligand (FL) concentration in plasma (a cytokine used as a biological indicator of bone marrow aplasia) (Bertho et al. 2001; Huchet et al. 2003; Prat et al. 2006), body temperature (BT), and a few alimentary and general behavioural features. These various parameters are thereafter referred to as "variables" or "covariates". For BT and behaviour, some provisional scores and thresholds were empirically defined as provisional HEP. Some of these have been already used by other groups in various experimental settings either with rodents (Olfert and Godson 2000) or with NHPs (Fante et al. 2007; Menon et al. 2016) and have shown to warrant some interest. All possible actions were taken to keep the NHPs alive in order to attain the experimental endpoint with all possible constraints to animal suffering thereby safeguarding as well as possible animal well-being and welfare. A few animals died suddenly at various points in time, despite the attempts to maintain their clinical status. Anticipated euthanasia could not be done because of the very rapid deterioration in the health of the animal. This demonstrated that the provisionally defined HEPs were not adequately predictive.

The aim of the present analysis was to perform a retrospective statistical assessment of these experimental data to identify biological, clinical and behavioural covariates that could potentially better predict the survival status of irradiated NHPs and to refine the definition of HEPs in similar experiments using NHPs. This study should be relevant in the context of the new French regulation (Ministry of agriculture Act No 2013-118, 2013) according to the European directive 2010/63 (2010) in which the retrospective assessment of the actual procedural severity is mandatory for all severe projects and for all projects using NHPs regardless of severity, with the objective of refining future animal use. Identification of the most predictive factors of NHP survival, an observed variable that is uncertain by nature in such experimental conditions, can be easily formalized as a problem of statistical prediction. The aim was then to compare the ability of several probability models, including one or more potentially predictive covariates, to reproduce plausible survival status. This approach was performed under the Bayesian statistical framework whose main advantage, in a predictive context, is to account for the uncertainty associated with unknown variables encountered in the prediction process, especially when the sample size is reduced.

Material and methods

Animals and housing conditions

Fifteen male crab-eating macaques (*Macaca fascicularis*, n=15), originating from a breeding colony (former Centre de recherches en primatologie Ltd, Le Ferney, Mauritius) were used in this study. On arrival, they were quarantined for 8 weeks during which clinical and blood analysis were conducted. Animals were all tested negative for common pathogenic and interfering viruses such as Herpes B virus, Filovirus, STLV-1, SIV, EBV, CMV and Hepatitis B virus and were in good health.

The macaques were housed in a specific animal facility with temperature control (22°C ± 1°C) and a day/night light cycle of 12 hrs. They received controlled quantities of sterilised primate chow (Safe, France) and fresh fruits and/or vegetables every day, and free access to water. Immediately after arrival in the animal care facility, the animals were housed in pairs using two connected cages. During experimental procedures, cages were placed in a laminar air flow to protect animals from opportunistic infections. In this manner, they were housed individually, but maintaining visual, auditory and olfactory contact between pairs. All animals were trained for restrain and manipulation (Including BT measurement at the armpit and blood sampling at the arm) before the experimental procedure was applied. Mean age and body mass at the start of the experiment were 40 ± 4.5 months (range 32.6-47.7) and 3.54 ± 0.58 kg (range 2.75-4.70 kg), respectively (Table 1).

For the purpose of platelet or red blood cells transfusion of irradiated animals and after approval of the animal care committee, a colony of healthy NHP housed in the same facility was used as blood donors. In order to prevent risks of anaemia of the donor animals, the volume of blood drawn was limited to 0.5% of the body mass per month. This represents approximately 50 ml of blood for a 10 kg donor animal.

All experimental procedures and all use of animal were subjected to and approved by the animal care and use committee of IRSN (protocols # CE-03-20 and CE-01-05) and in compliance with French regulations in application at the time of these experiments (Ministry of agriculture Acts No. 87-848, 1987 and No. 2001-464, 2001).

Experimental procedures

Experimental procedures have been described previously in detail (Bertho et al. 2005a; Bertho et al. 2005b). Here follows a concise overview. Animals were irradiated under general anaesthesia using a mixture of ketamine (Imalgene, 100mg/kg, Merial, Lyon, France) and Xylazine (Rompun, 2 mg/kg, Bayer Healthcare, Puteaux, France) in a homogeneous high-dose gamma ray field using ^{60}Co sources. The mean dose rate along these experiments was 0.181 ± 0.018 Gy/min. Heterogeneity of irradiation, which was used to reproduce accidental irradiation patterns in humans, was obtained by protecting the right arm with a lead shield during a part of the irradiation procedure. According to the experimental design, animals received one out of five different treatment protocols: *ex vivo* expanded autologous haematopoietic stem cells collected either before or after irradiation (Bertho et al. 2002; Bertho et al. 2005a; Bertho et al. 2005b), granulocyte-colony stimulating factor injection (G-CSF, Lenograstim, kindly provided by Chugai Pharma, France), or a combination of G-CSF treatment and irradiated expanded autologous haematopoietic cells (Bertho et al. 2005a). Five treatment groups were then constituted in these studies: Untreated control, G-CSF treatment, irradiated or not irradiated expanded haematopoietic cells (I-EHC and EHC), and a combination of I-EHC and G-CSF. In the entirety of these experiments, only one animal was irradiated but not treated, neither with cytokine nor with haematopoietic cells to serve as a negative control. However this animal received the same level of clinical care as the other animals. All other experimental groups were composed of at least three animals (Table 1). It should be noted that irradiation protocols may vary within treatment groups. Such intra-group variability was accounted for with relevant probabilistic models. In this manner all available animals were included in the study in order to optimize the statistical power of this study (see results section).

All animals received an intensive clinical support during the experiment with a single *i.v.* injection of Dexamethasone (5 µg/kg of body weight, Dexazone®, UVA, Ivry sur Seine, France) immediately after the end of irradiation to limit the initial acute inflammatory reaction. Animals also received antibiotic treatment starting two days after irradiation (oral administration of colistine 100 mg/kg/day (Acti-coli B®, Biové, Arques, France) and systemic treatment with gentamicin 40 mg/kg/day and ampicillin 160 mg/kg/day (Bi-genta®, Shering-Plough, Segré, France)), fluid perfusion, platelet and red blood cell transfusion as necessary (according to the following thresholds: Plt counts less than 20×10^9/L and RBC counts less than 3×10^{12} RBC/L, respectively) and special nutriments

to stimulate appetite (fruit juice, dry fruits, hyper-energetic food if required). This clinical support continued until the animals recovered normal blood variables and normal alimentary behaviour.

Follow-up of animals and scoring

Starting 10 days before irradiation and continuing until full haematopoietic recovery, a daily (increased to several times a day during critical periods) follow-up of animals was performed. Body temperature at the armpit using a paediatric electronic thermometer was recorded every morning. Daily clinical support (as described above) was then carried out according to the blood variables analysis. A blood sample (1 ml) was drawn into EDTA coated tubes (BD, le pont de claix, France) daily in the morning and was used to monitor cell count and formula, FL, and other biochemical variables. The amount of food, water consumption, quantity and quality of faeces, appearance of petechiae and small haemorrhages on the skin and general behaviour and appearance status were recorded every morning as well. Each of these five variables was graded on a scale of 1 to 5 or 6, 5 or 6 being the worst, with possible graduation by step of

0.5 (Table 2). At each observation time, the five scores were summed to obtain a general score accounting for behaviour, haematological and digestive clinical signs. This total score together with body temperature and plasma FL concentration were used as HEP, with provisionally defined thresholds for each of the parameters.

Group comparison

In a preliminary statistical analysis, the data were split into two groups, according to the survival status of animals (dead or alive). These two groups were then compared using Student's t tests. The level of statistical significance was set as $p < 0.05$.

Statistical prediction method

Standard logistic regressions were assumed to predict the binary survival status of each primate i (i=1,…,15), defined as its survival status (i.e., dead/alive) 60 days after irradiation, given one or several potential predictive factors. Univariate regressions were tested first. Then, specific bivariate and trivariate regressions were considered depending on the results obtained with the univariate models.

Table 1. Summary of animal age and body mass at the time of irradiation, irradiation procedure and following treatments of the haematopoietic syndrome.

Animal id.	Age, months	Body mass, kg	Radiation dose, Gy [a]	Treatment[b]	Survival[c]	Reference
AB470	44.3	4.70	8/3.4	Untreated	Yes	Bertho et al 2005a
AA870	35.7	3.45	10/4.4	EHC	No (31)[d]	
AA809	32.6	4.05	8/3.4	EHC	Yes	
AB413	44.7	3.25	8/3.4	EHC	Yes	
AA208	38.2	3.80	10/4.4	I-EHC	Yes	
AA592	36.6	3.15	10/4.4	I-EHC	No (24)	
AA295	46.5	4.15	8/3.4	I-EHC	Yes	
AA900	44.3	4.40	8/3.4	I-EHC	Yes	
AA864	47.7	3.60	8/3.4	I-EHC	Yes	
AF382	37.4	2.85	8.5/4.5	G-CSF	Yes	Bertho et al 2005b
AF373	39.9	2.75	8.5/4.5	G-CSF	No (20)	
AF569	39.6	3.30	8.5/4.5	G-CSF	Yes	
AF270	36.7	3.05	8.5/4.5	I-EHC + G-CSF	Yes	
AF384	36.5	3.00	8.5/4.5	I-EHC + G-CSF	Yes	
AF477	39.3	3.60	8.5/4.5	I-EHC + G-CSF	Yes	

[a] A heterogeneous irradiation configuration was used by shielding the right arm during a part of irradiation. Values indicate the dose to the whole body/the dose to the shielded arm. Please see details in (Bertho et al, 2005a).

[b] Haematopoietic treatments were detailed in (Bertho et al, 2005a, Bertho, et al, 2005b, Bertho et al, 2002). EHC: Expanded haematopoietic cells; I-EHC: irradiated then expanded haematopoietic cells. G-CSF: Granulocyte-colony stimulating factor daily s.c. injection at 5 μg/kg.

[c] Survival was defined as animal still alive 60 days after irradiation with a full recovery of haematopoietic parameters.

[d] Time of death after irradiation is indicated in brackets (days).

All potential predictive factors were centred and standardized to avoid numerical problems. Due to the small number of available data, more complex models were not considered. Working under the Bayesian paradigm, prior probability distributions were assigned to all the unknown regression parameters to reflect the uncertainty of these quantities. For all parameters, due to a lack of information, centred normal priors with high variance 10^3 were assumed. All the considered models are summarized in Table 3.

Table 2. Clinical/behavioural score. Score scale for the evaluation of clinical and behavioural status of animals during the course of experiment. This evaluation was made daily in the morning from 10 days prior irradiation until the end of experiment.

Category	Description	Score
Food intake[a]	Eat normally the food ration (2x/day: fruits and chow)	1
	Eat normally either fruits or chow, leave partly (<75%) the other	2
	Eat normally one of the two components	3
	Eat partially (<75%) one of the two components	4
	Eat very little (<25%) or do not eat	5
Beverage intake[b]	Drink 100% (ad libitum)	1
	Drink 75-100%	2
	Drink 50-75%	3
	Drink 25-50%	4
	Drink 0-25%	5
Faeces as compared to control period[c]	Normal in appearance, quantity similar	1
	Smooth formed faeces, quantity similar	2
	Unformed faeces, higher quantity	3
	Diarrhoea	4
	Highly liquid diarrhoea	5
	Haemorrhagic diarrhoea	6
Cutaneous Haemorrhages[d]	No skin haemorrhagic sign	1
	Few cutaneous petechiae	2
	Numerous cutaneous petechiae	3
	Small cutaneous haemorrhages Impaired coagulation after blood puncture Marks on skin after restraint	4
	Substantial cutaneous haemorrhages Suspicion of internal haemorrhage	5
Behaviour[e]	Usual behaviour/interaction	1
	Normal behaviour/interaction but appears quiet	2
	Reacts to stimulation but tendency to prostration	3
	Sleepy animal, show prostration with slow reactions	4
	Complete prostration, no reaction to stimuli	5

[a] The normal food ration consisted of two fruits and 500 g of primate chow, distributed in two portions each day. The score was based on an estimate of the eaten fraction of the daily ration.

[b] Drinking water was provided ad libitum. The individual normal intake was evaluated during the 10-days control period before irradiation and the score was then based on the estimated percent of water intake as compared to the control period.

[c] Animals were followed for all variables (including clinical, behavioural and blood variables) during a 10-days control period before irradiation.

[d] Petechiae are defined as visible cutaneous haemorrhages of less than 1 mm diameter and cutaneous haemorrhages are defined as more than 1 mm diameter. "Few" refers to less than 10-20 petechiae visible on the face and on parts of the body without fur. "Numerous" refers to more than 20-50 visible petechiae. A suspicion of internal haemorrhage is linked to clinical manifestations such as abnormal skin swelling.

[e] The behaviour score is based on comparing the behaviour during the experiment with behaviour before the experiment, *i.e.* during normal housing.

Bayesian model fitting was performed using the software OpenBUGS for Bayesian analysis of statistical models using Markov Chain Monte Carlo (MCMC) techniques (Gelman, 2013, Lunn, 2012). For each model, 2 Markov chains were run for 70,000 cycles with a burn-in period of 10,000 cycles. As a consequence, the posterior samples associated to each unknown parameter included 120,000 values. The convergence of MCMC algorithms was checked by visual inspection of the simulated Markov chains and by computing intra-chain autocorrelations and Gelman-Rubin Statistics thanks to the R package Coda.

A cross-validation method (Geisser and Eddy 1979; Bernardo 2008; Gelman 2013) was implemented to gauge the predictive ability of each considered logistic regression using a sample of 120,000 values (directly derived under OpenBUGS) from the posterior predictive distribution (PPD) of the survival status of each primate. Each PPD_i represented the uncertainty associated with the prediction of the survival status of primate i (i=1, ..., 15) after fitting a given logistic regression on the available dataset but excluding the survival status of primate i (to avoid double use of the same data). The observed survival status y_i of primate i was compared with the associated posterior predictive sample (PPS) using plots and numerical scores. The aim was to identify the logistic regression(s) for which the obtained PPS look the most plausible (i.e., both close to the observed y_i and precise), given the observed survival data.

Three PPS, averaged over 15 primates, were computed to evaluate the predictive ability of each logistic regression: a) the average Brier Score (BS) (Brier 1950): this score measures the mean squared difference between the predicted probabilities of death and the observed survival status; b) the average logarithmic score (LogS) (Good 1952): this score is closely related to the value of the posterior predictive probability assessed at the observed vital status and which *should* be close to 1; and c) the average zero-one score (01S): this score corresponds to the success rate in predicting the primates' survival status (Gneiting and Raftery 2007).

Results

Data description

The fifteen animals were chosen for this study based on very similar irradiation protocols with the exception of three animals that received a higher radiation dose: 10 Gy vs. 8 Gy heterogeneous protocol, as previously described (Bertho et al. 2005a; Bertho et al. 2005b) (Table 1). Out of these fifteen animals, three (Animal Id: AA870, AA592, AA373) died during the experiment, between day 20 and 31 post-irradiation (Table 1). Two of them received a 10 Gy irradiation dose; the third one received an 8 Gy radiation dose. A detailed analysis of findings at autopsy was given in (Bertho et al. 2005a; Bertho et al. 2005b).

Figure 1 shows the post-irradiation temporal evolution of white blood cell (WBC) numbers (Figure 1A) and Flt3-ligand concentration (Figure 1D) in the blood of the three deceased animals and in three animals that survived after receiving similar irradiation and treatment (Figures 1C and 1F). Both the decrease in WBC numbers and the increase in FL concentration are characteristics of an acute haematopoietic syndrome induced by high-dose irradiation, as can be encountered in radiation accidents (Hirama et al. 2003; Bertho et al. 2008). The NADIR (the time in days after irradiation at which the minimal value for WBC numbers is reached) varied between day 15 and 20 and the number of WBC at the NADIR varied between 0.04×10^6 and 0.44×10^6 $WBC.L^{-1}$ (Figures 1A to 1C). Additionally, important variations are observed in the FL concentration in plasma according to the time post-irradiation (Figures 1D to 1F). This concentration is inversely correlated to the severity of the haematopoietic syndrome, and a decrease in FL concentration is a sign of recovery (Bertho et al. 2008). However, as can be seen from figure 1 D-F, the maximal value of FL concentration varied between 2257 $pg.mL^{-1}$ and 5495 $pg.mL^{-1}$ and the duration of the plateau phase was between 15 and 30 days. No significant difference between surviving and dying animals were observed according to the WBC NADIR (Figure 1B) or the maximal FL concentration in the blood (Figure 1E) (Student's t test).
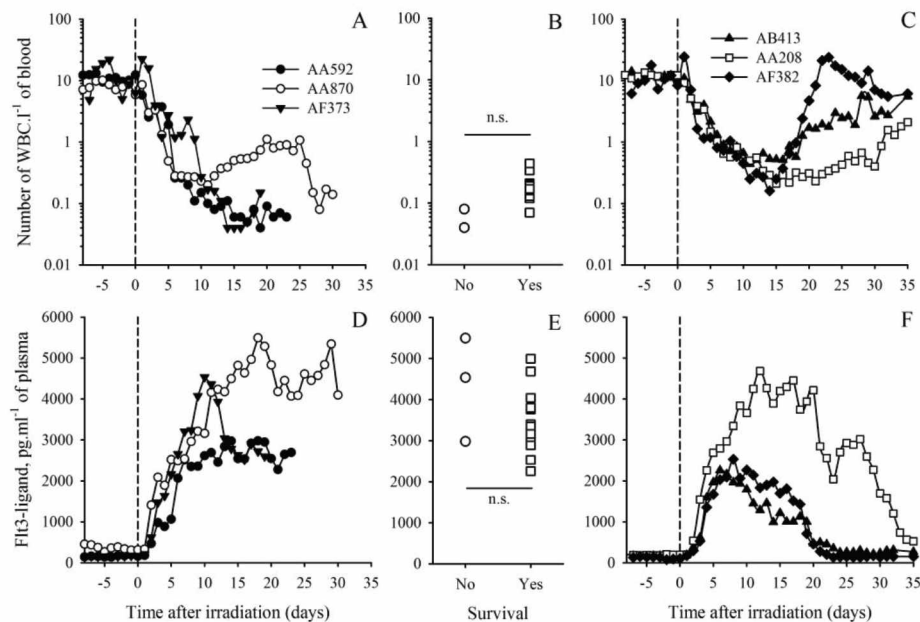
Figure 1. Variations in white blood cell numbers (upper row) and in plasma Flt3-ligand concentration (lower row) in animals deceased during the experiments (**A, D**) and in three surviving animals with similar protocol (**C, F**). Each of these animals showed a different pattern of variation. Minimal values of WBC (**B**) and maximal FL concentrations (**E**) observed for animals deceased (circles) and alive (squares) showed no significant difference (n.s.) between the two groups (Student's t test).
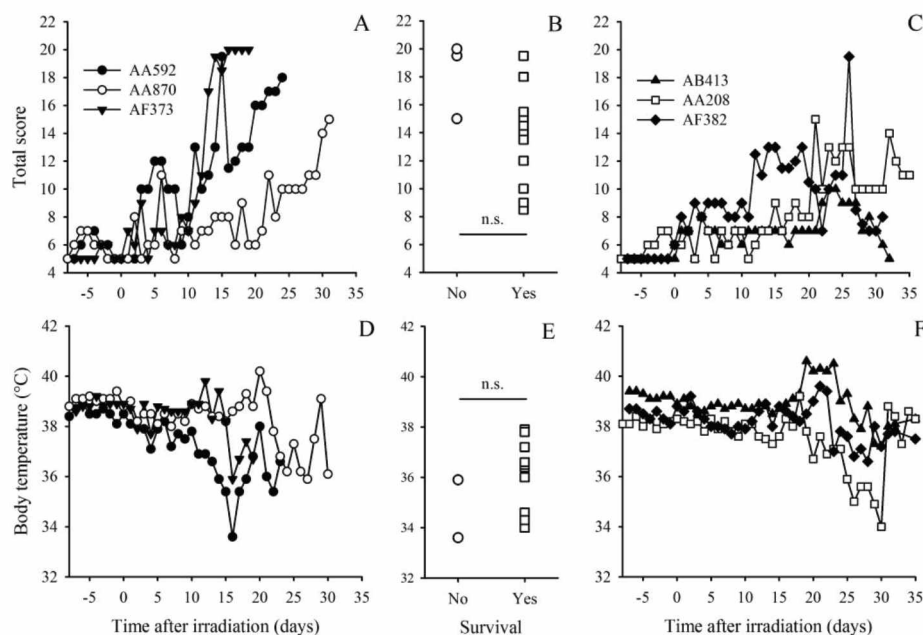

Figure 2. Daily record of behaviour score (upper row) and body temperature (lower row) for animals deceased during the experiments (**A, D**) and for three surviving animals with similar irradiation and treatment protocol (**C, F**). Each of these animals showed a different pattern of variation. The comparison of maximal values for the behaviour score (**B**) and minimal body temperature (**E**) observed for each individual animal either deceased (circles) or alive (squares) did not show significant difference (n.s.) between these two groups (Student's t test).

Figure 2 shows the development of the behaviour score, including feeding and drinking behaviour, clinical signs of haematological and digestive disease and general behaviour (Table 2), for the three deceased animals (Figure 2A) and for three animals receiving a similar treatment protocol out of the twelve that survived (Figure 2C). The general score showed day to day variations with the exception of animal AF373 that stayed for 4 days at a high value (20 as compared to 26 worst score) before dying.

During this period this particular animal showed other signs of a possible recovery, as suggested by decreasing FL concentration (from 4535 pg.mL^{-1} on day 10 down to 2520 pg.mL^{-1} on day 16, see Fig 1D) and slight increase in body temperature (From 35.9 °C on day 16 up to 37.8 °C on day 18, see Figure 2D). The positive development of these two variables justified the continuation of the experiment. The comparison of the individual maximal behaviour scores (MBS) observed for each of the three deceased animals with the MBS observed for each of the twelve animals that survived (Figure 2B) showed no significant difference between the two groups (Student's t test). Based on these results, the MBS value did not seem to be predictive of the death of animals.

The progression of body temperature (BT) values for the three deceased animals is presented in Figure 2D and for three of the twelve animals that survived in Figure 2F. BT exhibited day-to-day variations during these experiments with a different pattern for each animal. Hypothermia has been suggested as a possible HEP in rodents (Becker et al. 1998; Olfert and Godson 2000), and in fact, hypothermia was observed in the three deceased animals, close to 36°C for two animals and down to 34°C for one animal (Figure 2D). When comparing these three animals with the 12 animals that survived, other cases of hypothermia were observed, in the range of 34-35°C (Figures 2E and F). As expected, the comparison of minimal BT in the three deceased animals with the 12 animals that survived did not allow for the conclusion that the two groups of animals differ significantly (Student's t test, p>0.05). Hypothermia even down to 34 °C, and although critically informative, appears not to be sufficiently predictive for the animal demise.

We also examined the decrease in body temperature (BT) during 3 to 7 consecutive days (whatever the initial BT) by calculating the difference in BT between the 1st and the last of these 3, 4, 5, 6 or 7 days. In fact, when looking at Figure 2D, all deceased animals presented BT decrease of 1.5 to 4 °C over a two to three days period during the few days preceding death. We also considered 3-days BT decrease before the haematological NADIR, since the NADIR is a critical, characteristic point during the development of haematopoietic syndrome. Ultimately, none of these variables showed a significant difference between surviving and dying animals (Student's t test, data not shown).

Posterior predictive results: univariate logistic regressions

Table 3 shows the Bayesian point estimates (*i.e.* posterior median), the 95% credible intervals (CI) and the estimated values for the three (average) PPS (*i.e.*, LogS, BS and 01S) for 14 univariate logistic regression models, and the simplest model without any covariate (called "Intercept"). For a given model, the lower the BS or the LogS score are, the more plausible the prediction is. Inversely, the higher the 01S is, the more plausible the prediction is. The analysis first focused on individual primate-specific covariates that may influence the outcome directly, namely treatment of the haematopoietic syndrome, radiation dose, age and body mass of animals at the time of irradiation. Univariate logistic regressions accounting for each of these covariates provided higher LogS and BS than the intercept model, meaning that the predictions of survival status are less accurate and precise when accounting for these covariates than when assuming that all the primates have the same probability of death. This in turn suggests that the outcome is not significantly influenced by these covariates, thus allowing for the analysis of other covariates as potential HEP independently from the above-mentioned covariates (Table 3).

We analysed the predictive ability of other univariate logistic regressions including covariates such as minimal body temperature (BT) before the NADIR, BT during the overall experiment, maximal Flt3-ligand concentration in the blood, maximal slope of BT decrease (defined as the difference in BT during 2, 3, 4, 5, 6 or 7 consecutive days), and maximal clinical/behavioural score (MBS). The variation in BT appears as the most relevant covariate to consider in terms of predictive performances. The univariate model based on the decrease of BT during 3 consecutive days provided a lower LogS, a lower BS and a higher 01S score than the intercept model meaning that the predictions of survival status were more plausible. Moreover, this model also clearly provided the lowest LogS, among all considered univariate regression models, meaning that the posterior probability to predict the observed vital status from this model was higher on average over the 15 primates. It should also be noted that, even if its LogS is higher than the intercept model and the model based on the 3-days BT decrease, the models based on the 5-day and 7-day BT decrease also gave plausible predictions. In particular, the 3-day BT decrease provided the lowest BS and the highest 01S among all considered univariate regression models. In con-

Table 3. Bayesian estimates (i.e., posterior median), 95% credible intervals (CI) of the regression coefficients, predictive abilities (summarized with the average predictive scores: BS, LogS and 01S) of the different logistic regressions and number of failed predictions relative to the 3 deceased animals. The Intercept model corresponds to the model without any covariate, the probability of death is assumed to be the same for all the primates. Bold numbers indicate the lowest value for LogS and BS, the highest value for 01S.

	Model	LogS	BS	01S	Number of failed predictions	Posterior median 95% CI
UNIVARIATE	Intercept	0.58	0.18	0.80	3/3	-1.47 [-3.02 ; -0.28]
	Treatment (ref= EHC)	1.22	0.29	0.80	3/3	- 1.73 [-5.19; 0.37] (EHC) - 20.0 [-69.96; 1.56](Untreated) 0.90 [-3.17; 5.08] (I-EHC) 0,90 [-3.19; 5.07] (G-CSF) -21.08 [-70.8; -0.02] (I-EHC + G-CSF)
	Age	0.65	0.21	0.73	3/3	-1.38 [-3.88 ; 0.39]
	BT min_30 days[1]	0.63	0.21	0.67	3/3	-1.07 [-2.87 ; 0.35]
	FL max	0.70	0.19	0.73	3/3	1.21 [-0.27 ; 3.21]
	Body mass	0.61	0.19	0.73	3/3	-1.99 [-5.51 ; 0.01]
	MBS	0.71	0.16	0.73	2/3	0.67 [0.12 ; 1.61]
	2days BT ↓ max	0.68	0.16	0.80	2/3	2.65 [0.40 ; 6.02]
	Radiation dose	0.66	0.17	0.80	2/3	1.83[0.31 ; 4.16]
	4days BT ↓ max	0.66	0.12	0.87	1/3	2.36 [0.62 ; 5.48]
	5days BT ↓ max	0.63	0.11	**0.93**	1/3	2.98 [0.79 ; 7.21]
	7days BT ↓ max	0.62	**0.09**	**0.93**	1/3	2.53 [0.67 ; 6.49]
	6days BT ↓ max	0.56	0.10	0.87	1/3	3.23 [0.88 ; 8.86]
	BT min NADIR	0.65	0.13	0.87	1/3	-6.9 [-19.31 ; -1.64]
	3days BT ↓ max	0.50	0.12	0.87	1/3	5.01 [1.45 ; 13.36]
BIVARIATE	3days BT ↓ max + radiation dose	0.67	0.17	0.73	1/3	6.16 [1,29; 16,02] 1.57 [-1,98; 6,04]
	BT min NADIR + radiation dose	0.97	0.15	0.87	1/3	-12.02 [-39.53 ; -1.80] 1.57[-6.50 ; 1.79]
	BT min NADIR + MBS	0.41	0.13	0.80	2/3	-34.93[-77.95 ; -8.71] 5.69 [1.19 ; 13.47]
	3days BT ↓max + MBS	**0.26**	**0.09**	0.80	2/3	15.6 [4.60 ; 32.19] 4.03 [0.80 ; 9.39]
TRIVARIATE	max 3days BT ↓ + MBS + radiation dose	0.39	0.13	0.80	2/3	15.0 [2.63 ; 37.88] 5.04 [1.38 ; 10.91] 2.40[-13.69 ; 19.9]

Abbreviations: BT min_30 days: Minimal body temperature over the 30-day period post-irradiation; FL max: Maximal FL concentration over the 30-day period post-irradiation; (x)days BT ↓ max: Maximal body temperature decrease during (x) consecutive days; BT min NADIR: Minimal body temperature before the NADIR; MBS: Maximal behavior score.

clusion, it should be noted that, even if different from zero, the number of failed predictions relative to the three deceased animals is also the lowest (i.e., 1/3) for the models based on the 3-day, 5-day and 7-day BT decrease. The univariate regression including only the maximal clinical/behavioural average score or only the maximal FL concentration in the blood showed very poor predictive performances. This suggests that none of these covariates whether they rely on clinical score, behaviour, or physiological variables can be considered alone as a HEP.

Posterior predictive results: multivariate logistic regressions

In order to improve the survival prediction, bivariate and trivariate logistic regression models were also fitted, including several combinations of minimal BT, radiation dose, maximal clinical/behavioural score, and 3-day maximal BT decrease. Table 3 shows that the inclusion of radiation dose, either with the 3-day maximal BT decrease or with the minimal BT before the NADIR, decreased the predictive ability of the two BT-associated covariates tested in univariate logistic regression. This tends to confirm that primate-specific covariates do not significantly influence the outcome and cannot be used as HEPs, and that only covariates measured during the course of the acute radiation syndrome are of interest for the definition of HEPs at least in the present dataset.

Table 3 also shows that the smallest values obtained for the average logS and BS are for the bivariate logistic regression accounting for both the maximal 3-day BT decrease and the maximal behaviour score. Two predictive scores among three are in agreement with the superior predictive performance of this model to reproduce both accurate and precise primate survival status. The inclusion of the maximal behaviour score increases the predictive ability of these bivariate models, although this behaviour score gave poor results when tested in an univariate logistic regression. The best predictive model is therefore the bivariate model including the maximal behaviour score and the maximal 3-day BT decrease. This result underlines the potential of using a combination of covariates as an HEP to predict the death of animals.

In comparing the predictive scores (Table 3) and the predicted probabilities of death (Figure 3) for the univariate (Figure 3A), bivariate and trivariate (Figure 3B) regressions accounting for the 3-day BT decrease max, one can see that the maximal behaviour score contributes to the reduction of the prediction uncertainty. For the primates alive at
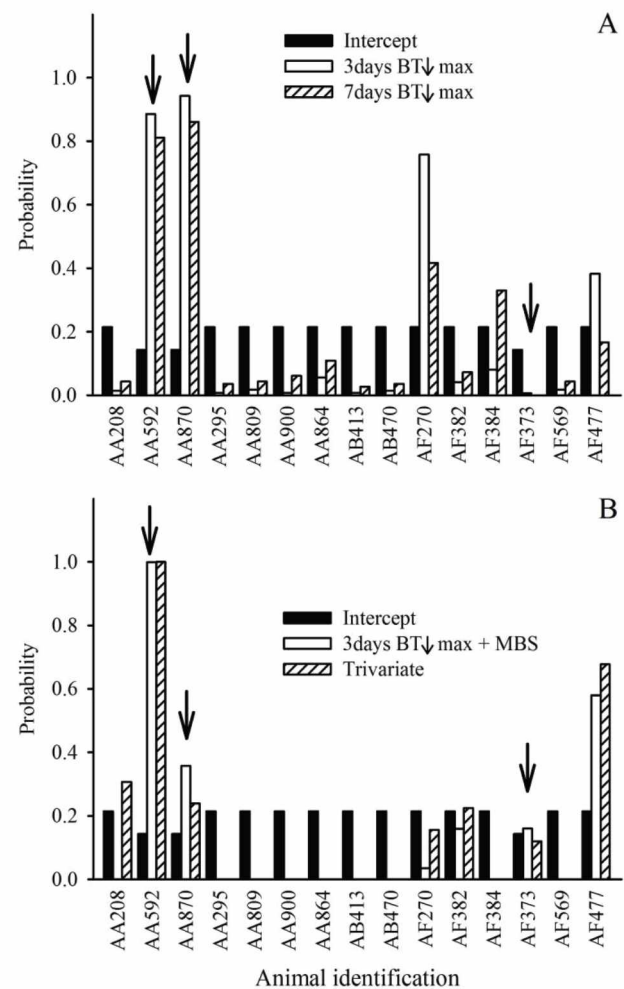

Figure 3. Predicted probabilities of death before 60 days after irradiation for the 15 studied primates provided by **A**: Intercept model not taking into account any covariate and univariate regression models accounting for the maximal BT decrease during 3 and 7 consecutive days. **B**: Intercept model not taking into account any covariate, bivariate regression model accounting for both the maximal 3 days BT decrease and the maximal behaviour score, and trivariate regression accounting for the maximal 3 days BT decrease, radiation dose and maximal behaviour score. The arrows indicate the 3 deceased primates.

the end of the experiment, the predicted probability of death is generally closer to zero for the bivariate and the trivariate as compared to the Intercept model and the univariate regressions. For two of the three deceased primates, the predicted probability of death is closer to one for the bivariate and the trivariate than for the univariate regression. In particular, the bivariate regression accounting for both the maximal 3-day BT decrease and the maximal behaviour score attributes a probability of death of 0.16 to primate

AF373 whereas it is 0.006 and 0.001 respectively for the univariate regression only accounting for the 3- or 7-day BT decrease max.

When comparing the bivariate and the trivariate regressions, it appears that for the three deceased animals, the predicted probability of death is similar. However, when looking at the surviving animals, the trivariate regression gave higher probability of death as compared to the bivariate regression. We can conclude that the trivariate model is less efficient in predicting the survival of animals, confirming the interest of the bivariate model as a predictor of survival.

The average 0-1 score does not confirm the results obtained with the Brier and the LogS scores. The best predictive models according to this 0-1 score (*i.e.* with the highest 01S = 0.93) are the univariate logistic regressions only accounting for the maximum decrease in BT during 5 or 7 days. Nevertheless, Table 3 shows that this predictive score is not discriminative enough between the models. It assigns the same score (equal to 0.80) to the intercept model (model without any covariate) and the bivariate regression accounting for both the max 3 days BT decrease and the maximal behaviour score. Figure 3 shows that the intercept model provides less plausible predictions, especially for surviving animals. Even if the above bivariate regression leads to two failed predictions (score=0.80) compared to one failed prediction for the univariate logistic regression only accounting for the maximal body temperature decrease during 5 and 7 consecutive days (score=0.93), the provided predictions are more accurate and precise, explaining why the logarithmic and Brier scores are lower for the former than the latter model.

Discussion

The irradiation protocol used in these experiments induced a severe ARS with haematopoietic, digestive, liver, kidney and possible lung damage, called a radiation-induced multiple organ disease syndrome (Bertho et al. 2005b; Fliedner et al. 2005). In humans, such radiation-induced syndrome is dramatically difficult to reverse and the treatment of the haematopoietic syndrome, even combined with strong clinical support, does not always prove to be sufficient to ensure the survival of patients (MacVittie and Monroy 1990; Coeytaux et al. 2015). In this study, this is exemplified by the fact that each of the three animals that died received a different haematopoietic treatment and that the unique control animal (AB470) receiving only clinical support – without any cell graft or growth factor treatment - did survive the irradiation. The number of animals considered in this retrospective study was low (*i.e.* 15) due to the initial experimental design. In fact, in each experiment, the number of animals was restricted to the minimum judged necessary and the number of control was restricted to one in accordance with one of the three Rs principle, the reduction (Russell and Burch 1959). Based on this, the statistical analysis of such a limited sample size, with numerous different experimental conditions, was hampered by a very limited statistical power. In this case, Bayesian statistical framework presents several advantages to identify the most predictive factors of animals' survival. First, it allows accounting for parameters estimation uncertainty (which may be large for samples of small size) in the prediction of survival status. This prevents overconfident predictions. Moreover, Bayesian approach can accommodate small-sized samples as it allows integrating additional information (i.e., expert knowledge or previous similar experimental studies) in the logistic regression models through informative prior distributions on the unknown regression coefficients. That being said, we did not use this latter feature due to the sparse description and analysis of HEP in non-human primates after total body irradiation.

A first analysis based on univariate logistic regression models strongly suggested that, despite the heterogeneity of body mass and age at the time of irradiation, the heterogeneity of radiation dose (two different protocols) and the heterogeneity of haematological treatment (five different protocols including one untreated animal), these primate-specific covariates did not allow for predicting the three observed deaths in the group of 15 animals. This suggested that the death of animals was independent from these four covariates and especially from the treatment in these studies. In fact, the treatment efficiency was not measured by the mean of survival but mainly by the monitoring of haematological variables such as duration of aplasia and time to normalization of neutrophil counts (Bertho et al. 2002; Bertho et al. 2005a; Bertho et al. 2005b). The animal survival was an absolute requirement as an experimental endpoint in these experiments, which in turn explained the low number of deceased animals and the absence of influence of these primate-specific covariates on the PPD of the survival status. On the basis of this first univariate analysis, we assumed that this group of animals was sufficiently homogeneous to conduct a global analysis of covariates measured after irradiation and potentially useful as HEPs.

The analysis then focused on univariate regression models based on some specific covariates that were easy to follow during the course of these experiments: a mean behaviour score and the minimal BT. The minimal BT was described as a reliable predictive indicator of death in septic shock model in rodents (Nemzek et al. 2004). Additionally, tracking BT was frequently proposed as an indicator of animal status (Kort et al. 1998; Morton 1998). In our model, the minimal BT alone was proven not sufficiently predictive of the death of animals to be used as an HEP. Our results are in accordance with a previous study in irradiated NHP which used hypothermia at <34.6°C as a HEP and in which no animal was euthanized according to this criteria (Menon et al. 2016). Moreover, the visual inspection of individual BT data showed important variations in BT during the critical phase of ARS. This suggested that the rate of BT decrease could be also an important covariate to consider as an HEP. In order to consider this, we studied the predictive ability of univariate logistic regressions based on the maximal BT decrease during 2, 3, 4, 5, 6 or 7 continuous days. The results suggested that the maximal 3-day decrease in BT was one of the most interesting covariate to consider for death prediction, especially when considering the average BS and LogS scores. Even if the associated logistic regression model allowed for the correct prediction of survival status in 14 out of 15, it failed to predict the death of the NHP AF373. This is also in accordance with studies in a rodent model of septic shock in which large variations in BT were observed but were not an accurate and precise predictive indicator of death (Krarup et al. 1999) as well as in irradiated NHPs (Menon et al. 2016). In an attempt to improve the predictive ability of this model, one or two additional covariates were included in the logistic regression models. The results clearly indicated that the combination of the maximal clinical/behaviour score with the maximal 3-day BT decrease lead to the most efficient model to predict survival outcome. This result highlights the value of using a combination of variables as an HEP rather than one single variable, as suggested in previous works (Wolfensohn et al. 2015; Fante et al. 2007; Graham et al. 2012).

Although not mandatory at the time the initial experiments were performed, this retrospective study was conducted in the context of the new French regulation (Ministry of agriculture Act No 2013-118, 2013) according to the European directive 2010/63 (2010) in which the retrospective analysis of the protocol severity is mandatory for refining animal use. During the experiments, most of the single HEPs

provisionally defined before these experiments were changed during the course of the experiments, due to increasing level of nursing, allowing the animals to survive with minimally acceptable welfare conditions. This was also due to the estimated general status of the animals which could appear better than what the composite score alone would have implied. For instance, animal AF373 that stayed for 4 days at a high composite score value before dying also showed signs of haematopoietic recovery (through decrease in FL concentration and increasing numbers of WBC) and a rise in BT. Thus when looking at these haematopoietic variables, it was justified to postpone preventive euthanasia. Accordingly, a discrepancy appeared in several cases between the general status of the animals and the results of the composite score. This in turn suggests that the use of a fixed HEP might induce non adequate decisions regarding the termination of the experiment. Moreover, these results suggest that other variables, either physiological or behavioural, should be included to catch clinical signs coming from physiological systems other than the haematopoietic system, the main interest in this set of experiments. Overall, this study shows that a multi-variable approach is mandatory, and that this is in accordance with previous studies (Graham et al. 2012; Fante et al. 2007; Wolfensohn et al. 2015), even if in the course of this study, not all deaths and all survivals were accurately predicted by the multivariate analysis.

Two variables stand out in importance when used in combination: the maximal slope of body temperature decrease and the mean clinical/behaviour score. The initial development of the provisionally defined HEPs with these two covariates showed the lowest LogS and BS scores, suggesting that nursing adaptation is essential to improve animal conditions, facilitating animal survival despite the elevated scores observed in some cases. However, when looking at individual results this combination of two variables fails to predict the death of one NHP (AF373) and the survival of another NHP (AF477), and predicted the death of a third NHP (AF870) with a low probability. The small number of animals in the present study limits the accuracy of predictions.

The study also brought to light that the clinical/behaviour score is based on a limited number of criteria. It would be desirable to refine this clinical/behaviour score through the inclusion of other criteria such as the use of enrichment tools by NHP, the measurement of spontaneous ambulatory activities, or by quantifying already used criteria with more precision. Other physiological variables could also

be used such as cardiac frequency or breathing rate, although these are more difficult to measure with NHPs without putting more constraints on the animals (such as telemetry probes).

Another limitation of this retrospective study is the inability to determine a threshold of action for each of the putative endpoints used. This limitation is in part due to the small number of animals included. The threshold of action could be defined by using the results of other studies with animals under similar experimental conditions. Another way to try to define the threshold of action might be through the specification of informative prior distributions on the unknown coefficients in the logistic regression models. Ultimately, a combination of these two different approaches may lead to a more accurate definition of potential threshold of action. Nevertheless this Bayesian multi-variable approach is an interesting tool for assessing reliable humane end points and demonstrated the merit of a multi-criteria analysis of the clinical status of NHPs. This multi-criteria approach should be adapted to each experimental situation and to each kind of stressor and should also be adapted during the course of the experiment through the frequent oversight of animals to detect slight changes in their behaviour. It is therefore essential to have the best possible knowledge of the clinical/behaviour of each individual animal entering this kind of experiment with a high level of potential suffering.

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Conflict of interest

The authors declared no potential conflict of interest.

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