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PART I: MINIMUM QUALITY THRESHOLD IN PRE-CLINICAL SEPSIS STUDIES (MQTiPSS) FOR STUDY DESIGN AND HUMANE MODELING ENDPOINTS.

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ABSTRACT

Pre-clinical animal studies are mandatory before new treatments can be tested in clinical trials. However, their use in developing new therapies for sepsis has been controversial because of limitations of the models and inconsistencies with the clinical conditions. In consideration of the revised definition for clinical sepsis and septic shock (Sepsis-3), a Wiggers-Bernard Conference was held in Vienna in May 2017 to propose standardized guidelines on pre-clinical sepsis modeling. The participants conducted a literature review of 260 most highly cited scientific articles on sepsis models published between 2003 and 2012. The review showed, for example, that mice were used in 79% and euthanasia criteria were defined in 9% of the studies. Part I of this report details the recommendations for study design and humane modeling endpoints that should be addressed in sepsis models. The first recommendation is that survival follow-up should reflect the clinical time course of the infectious agent used in the sepsis model. Furthermore, it is recommended that therapeutic interventions should be initiated after the septic insult replicating clinical care. To define an unbiased and reproducible association between a new treatment and outcome, a randomization and blinding of treatments as well as inclusion of all methodological details in scientific publications is essential. In all pre-clinical sepsis studies, the high standards of animal welfare must be implemented. Therefore, development and validation of specific criteria for monitoring pain and distress, and euthanasia of septic animals, as well as the use of analgesics are recommended. A set of four considerations is also proposed to enhance translation potential of sepsis models. Relevant biological variables and co-morbidities should be included in the study design and sepsis modeling should be extended to mammalian species other than rodents. Additionally, the need for source control (in case of a defined infection focus) should be

considered. These recommendations and considerations are proposed as “best practices” for animal models of sepsis that should be implemented.

INTRODUCTION

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). It is the most important cause of morbidity and mortality in patients admitted to intensive care units (ICU) with a significant cost impact in health care worldwide (2). Over the last decade, basic science research has identified fundamental molecular processes that are involved in the pathophysiology of the dysregulated metabolic, inflammatory and immune responses. However, despite this growth in fundamental knowledge and large investments in drug development and multiple clinical trials, no new effective therapies have been introduced to clinical practice. The treatment approach of the patient still relies on supportive care and antimicrobial agents (3). While we cannot underestimate inherent issues within clinical trials (4, 5), there is increasing skepticism about the usefulness of animal models for predicting responses in clinical sepsis (6). One prominent reason of the inability of industry- and government-sponsored clinical trials to validate results from the majority of animal studies should be attributed to methodological challenges in pre-clinical study design that poorly correlates with the clinical condition of sepsis.

An important step to develop guidance on how to improve the quality and efficiency of pre-clinical studies was undertaken by the international Wiggers-Bernard Conference, which was held in May 2017 in Vienna. Prior to the meeting, participants conducted a literature review of the 260 most highly cited scientific articles on sepsis models published between 2003 and 2012 as the basis for the conference discussions. The objective of the conference was to identify limitations of pre-clinical sepsis models and to propose a set of guidelines, defined as the "*Minimum Quality Threshold in Pre-Clinical Sepsis Studies*" (MQTiPSS; 7), to enhance translational relevance of the models. The main aim of this article is to propose a set of

standardized guidelines for study design and humane modeling endpoints, with a major emphasis on clinical relevance and animal welfare. Concrete examples of experimental design, procedures and ethical endpoints are provided throughout these guidelines. It is expected that these guidelines will be used in conjunction with the more general and mandatory rules of a national legislation of a country in which research is conducted. It is important to note that these recommendations and considerations are proposed to assist in the design of the most appropriate animal sepsis model(s) and should be tailored to the specific hypothesis of the investigation.

Overall, the Wiggers-Bernard initiative has led to the creation of three joint publications (8, 9) to serve as a MQTiPSS guideline for establishing the basic conditions in modeling of sepsis to improve their translational relevance. The current Part I paper makes specific recommendations preclinical models of sepsis within the areas of study design and humane modeling endpoints. The goal of the conference was to create quality thresholds for future studies so that findings in those two particular areas are more clinically applicable and the studies themselves are better comparable across laboratories and/or species.

METHODS

The Wiggers-Bernard Conferences on Shock, Sepsis and Organ Failure is an expert opinion exchange platform for international scientists organized by the Ludwig Boltzmann Institute of Experimental and Clinical Traumatology in the AUVA Research Center (LBI Trauma), Vienna, Austria (http://trauma.lbg.ac.at/en). The conference series was named after two outstanding scientists, one from the "New World" (Dr. Carl Wiggers) and one from the "Old World" (Dr. Claude Bernard) who devoted their careers to critical care medicine and experimental sciences. LBI Trauma is responsible for the topic selection while the Austrian Society of Advancement of Research in Shock and Tissue Engineering provides sponsorship for each Wiggers-Bernard conference.

To address the deficits regarding management guidelines and standardization in the field of pre-clinical sepsis research, in May 2017 LBI Trauma organized the 9th iteration of the Wiggers-Bernard Conferences titled: "*Pre-clinical Modeling in Sepsis: Exchanging Opinions and Forming Recommendations*". The key goal of the conference was to create publishable material that identifies essential elements that should be included in pre-clinical sepsis studies and defined by the MQTiPSS descriptor (10). A total of 31 experts from 12 countries, including five members of the Sepsis-3 definitions task force (1), were invited to participate in the initiative based on their experience in experimental, clinical and translational research.

The initiative consisted of three phases: a) three-months preparatory phase where participants performed a systematic review of the 260 top cited publications from 2003-2012 and identified the key modeling topics to be discussed, b) discussions in Vienna (two days), during which the participants drafted a list of guidelines and c) post-conference refinement of the created works.

The preparatory phase review was conducted using ISI Web of Knowledge database (using the query: "*sepsis model*"). The 260 most cited papers (the citation range 50-743; referenced over 29,000 times in aggregate) featuring a total of 374 animal studies were identified. The time frame was subjectively defined as 10 consecutive years beginning with 2003 as the year of publication of the second iteration of sepsis definitions (11, 12). The results of that survey pertinent to the topics covered in this paper are collated in Tables 1 and 3. Since the first analysis showed that mice were used in 79% of the 2003-2012 papers, a secondary smaller search was performed and included all 2013-2017 studies (total of 190; irrespective of the number of citations) with mouse sepsis models only (using the query: "*sepsis AND mice*"); to compare to selected endpoints reviewed in the main review that spanned 2003-2012. Both analyses were used during the meeting. Overall, the preparatory phase aimed at identification of the most important concepts in animal sepsis modeling to be addressed at the Viennese Wiggers-Bernard Conference. All participants were allocated into six specific thematic Working Groups (WGs): 1) Study Design, 2) Humane Modeling, 3) Infection Type, 4) Organ Failure/Dysfunction, 5) Fluid Resuscitation and 6) Antimicrobial Therapy Endpoints.

During the conference phase, each WG separately drafted a set of guideline points that were subsequently subjected to general discussion and streamlined either for further refinement in WGs or dismissal (day 1). After improvements, the proposed points were subjected to voting by all participants to reach consensus (day 2). Overall, the Wiggers-Bernard Conference participants reached consensus on 29 points; 20 at "recommendation" strength and 9 at "consideration" strength (the WG-1/2 points are listed in Tables 2 and 4). Following the format used by the Sepsis-3 task force (13), at least 2/3 (over 65%) of the votes were required for approval of a proposed point. All consensus points were reached either unanimously or with no

more than 2 abstentions per point (i.e. Recommendation 8). The “recommendation” strength indicates virtually unanimous agreement among the 31 participants, regarding both the content as well as the need for rapid implementation. Issues that require additional discussion (in the opinion of the participants) before final recommendations could be made were classified as considerations.

During the post-conference phase, the arguments to be included in the final MQTiPSS publications were finalized through teleconferences and electronic-based discussion among WGs using a modified Delphi method. Finally, a writing committee (formed at the conference) together with all participants developed an Executive Summary for MQTiPSS (7) and three full-size publications (8, 9). Each (of the three) publication focuses on two related WGs; the current Part I paper provides detailed discussion on the guideline points for Study Design and Humane Modeling Endpoints.

CHAPTER 1: STUDY DESIGN

An ideal pre-clinical animal model should accurately reproduce the human disease. While the complexity of human sepsis and its phenotypes precludes creation of a single ideal model, a standardization of defined model systems appears feasible and should be considered (14). We are convinced that adequately designed animal models of sepsis and other diseases can be useful tools, including the discovery and development of new therapeutic interventions (15). Therefore, it is important to elucidate the criteria, which must be fulfilled to obtain meaningful animal results for human translation.

The pre-meeting review of the top-cited experimental animal studies (2003-2012) provided evidence of bias and numerous methodological limitations of animal research in sepsis. As summarized in Table 1, we identified several challenges in the study design, experimental conduct and reporting that can impede successful translation of the findings from animal research to human patients. For example, although survival was reported as a primary endpoint in 43% of the animal sepsis studies, the vast majority of experiments had a brief follow-up. Given the frequent late mortality and long-term sequelae in septic patients (16), such a brief monitoring in many pre-clinical studies is not justified by the prolonged course of clinical sepsis. Another design shortcoming is the mismatch of therapeutic interventions between animal studies and septic patients. Only in 36% of the animal studies the experimental therapy was given after the onset of sepsis. In most cases, the timing of those interventions was chosen subjectively and not dictated by symptoms and/or disease severity. Of concern is also a low inclusion of biological variables and co-morbidities in the study design; only 5% of the reviewed studies featured any type of comorbidity. While the choice of healthy, inbred animals of same sex, age and weight limits the baseline variability in pre-clinical models, it simultaneously prohibits

replication of the heterogeneity encountered in the patient population. Promising pre-clinical findings obtained in those simplistic models should be validated in more complex experiments that take into consideration modifying risk factors of morbidity and lethality.

The above study design shortcomings are additionally aggravated by yet another hindrance: insufficient reporting of the methodological details. In the reviewed top-cited papers, we identified several inadequacies in describing details on animals, methods and materials employed in the experiments - all of which can potentially confound the interpretation of the study results and impede experimental reproducibility. Inadequate reporting also prohibits verification whether proper tools for reducing bias were employed, i.e., randomization for group allocation and blinding of outcome assessment. Scientific rigor demands that scientific reports provide accurate and sufficient details on the methodology to enable replication of the findings by other investigators to prove their validity (17). For a successful translatability of animal models, it is paramount that rigor is observed in pre-clinical sepsis research.

Specific recommendations for Study Design

The conference discussed several specific recommendations for pre-clinical models of sepsis. In the current Part I paper, the recommendations and considerations from the Study Design Endpoints working group are numbered consecutively beginning with recommendation 1 and continue in the next chapter (Humane Modeling Endpoints) and the two subsequent companion papers (Part II and III).

Recommendation 1: Survival follow-up should reasonably reflect the clinical time course of the sepsis model.

Although animal models will never fully recapitulate human illness, it is paramount to adapt them to reflect the changing nature of the studied disease. Historically, animal sepsis models employed a relatively high mortality rate (18), which reflected the high lethality of multiple organ failure (MOF) (19). As a result of multiple international initiatives to improve the implementation of evidence-based medicine in the ICU, the epidemiology of sepsis has evolved: early in-hospital mortality has decreased and many high-acuity patients survive, generating a new patient phenotype of "chronic critical illness" (CCI) (20). For example, recent prospective longitudinal cohort studies of sepsis/septic shock revealed that early inpatient mortality from refractory shock and MOF is now below five percent (21, 22). Other studies have demonstrated that the initial 28-day sepsis mortality is approximately 20 percent, but this increases to nearly 35 percent at 6 months (16). Additionally, the long-term CCI morbidity can be dismal - after 6-months, a significant portion of these patients have poor function and cognition and are discharged to non-hospital inpatient facilities rather than home (16, 21, 22). Of the latter, mortality is almost 40 percent at 6-months (21, 22).

The clinical need for a long-term focus is poorly met by typical short-term animal sepsis studies. For example, only 10% of the studies we reviewed employed monitoring exceeding 14 days (Table 1). Thus, we recommend that the survival follow up should reasonably reflect the clinical time course of the infectious agent used in the model or the patient population being studied. For example, a shorter monitoring period for meningitis would be appropriate given the acute clinical course of the human disease (23). There are appropriate abdominal, urinary, and pulmonary models with high early mortalities that can study important aspects of the early

mammalian response to severe infection (18). However, if the research goal is to replicate the clinical trajectory of most sepsis patients in developed nations, a longer monitoring period is more appropriate as acute mortality with sepsis is becoming rare. Researchers need to develop models of persistent chronic immuno-dysregulated conditions after sepsis, as this is now the predominant human phenotype (24-26). Although rarely performed, due to complexity and costs, modeling of chronic sepsis that features persistent immuno-inflammatory deficits and late mortality is achievable (27, 28). Such a modeling shift, however, requires a concurrent development of humane endpoints appropriately tailored for that type of animal sepsis research (see Chapter 2).

It is still unclear what the equivalent number of hours or days in a rodent (most commonly used species) are when compared to human time points, as there is a temporal mismatch between the species (18). For example, 1 hour in mice/rats is equivalent to approximately 40 hours in humans (assuming the lifespan of 2 *versus* 80 years). However, the 1 hour *versus* 40 hours recalculation formula should not be used reflexively as acute response between rodents and man bear many temporal similarities. For example, intravenous lipopolysaccharide (LPS) stimulation in human volunteers (29, 30), mice (31), rats (32) and pigs (33) leads to a virtually identical time-based response in the acute release of circulating inflammatory cytokines. Although some researchers have determined certain equations to relate different species (i.e. mouse with man) (34, 35), variations persist when considering weaning, puberty and senescence (34, 36). In addition, there are other differences in the timing and magnitude of the sepsis response in both species (18). Therefore, researchers may never be able to definitely state, *"one week after cecal ligation and puncture (CLP) in a mouse is the same as one month for a septic human in the ICU."* However, it is unlikely that a murine model with 80-

100% mortality in the first 48-72 hours accurately represents the biology of patients who become CCI, or subsequently develop the Persistent Inflammation Immunosuppression Catabolism Syndrome (21, 22).

Recommendation 2: Therapeutic interventions should be initiated after the septic insult replicating clinical care.

In order to mimic the clinical scenario, it is instrumental to administer any therapeutic agent to be tested after the induction of sepsis. Animal models have been utilized for the initial testing of potentially effective therapies for decades but one can conclude that there is a large inconsistency between animal and human trials (37, 38). The application of pretreatment instead of posttreatment has been an important shortcoming in numerous studies that has hindered the extrapolation of animal data to the patient population (37, 38). While the onset of experimental sepsis is known in animal models, patients never present at "time zero" and their infection develops for a significant time prior to clinical identification. Furthermore, it should be emphasized that sepsis itself will have a profound impact on the metabolic, cardiovascular, immunological, and other responses in an animal model (24, 26, 37). These, in turn, can have considerable impact on the therapeutic intervention that is evaluated. In this respect, it is essential to extrapolate treatment-related findings from the specific models towards the specific clinical scenario they attempt to recapitulate (e.g., CLP representing polymicrobial peritonitis).

There are some points of contention concerning this recommendation. There are many examples of pharmacological agents, which have been positively evaluated in an animal model of sepsis using a proper post-treatment approach, but which yielded negative results in large human clinical trials (39). Well documented examples include the use of recombinant tissue factor pathway inhibitor (tifacogin) (40, 41), anti-Toll like receptor (TLR)-4 strategies (42-44)

and interleukin-1 receptor blockade (45-46). Also, the issue of post-treatment is not simply time, but more the evolution of organ dysfunction and the processes that could result in death. Compared to patients, these trajectories can be divergent in animal models. Thus, a therapeutic intervention should not be solely based on the vector of time but should also account for the phenotype of the sepsis pathophysiology at the time of the intervention.

This further emphasizes the point that this specific recommendation is just part of the presented broader set of recommendations aiming to better mimic the clinical scenario and enhancing the translational power of the sepsis model used. In fact, it would not be entirely surprising if the above-mentioned treatment strategies will eventually be demonstrated to work in more precisely defined subsets of septic patients (37, 47). In addition, it should be acknowledged that - depending on the objective of any particular study - pretreatment can be reasonable, e.g. in multi-hit models simulating secondary infections or in a setting of prophylaxis. Also, pre-treatment is valuable if the goal is to understand disease pathogenesis rather than to predict treatment efficacy. These points, however, should be explicitly mentioned in the description of the methods.

In summary, from a clinical translation standpoint, when testing novel therapies, treatments should mimic the clinical management of the patients, i.e. given after the onset of sepsis. Efficacy of experimental drugs should be compared to or used in addition to minimum standard care of sepsis, i.e. fluid resuscitation and antibiotics, as is outlined in Part III paper (9).

Recommendation 3: We recommend that the treatment be randomized and blinded when feasible.

Methodological shortcomings in animal experimentation introduce bias and may distort study conclusions. Systematic analyses of pre-clinical studies demonstrate shortcomings in randomization and blinding. For example, in 2009, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) reported that only 12% of studies performed randomization and 14% used blinding (out of 271 surveyed studies) (48) and similar deficiencies were reported by others (49, 50). While we did not determine randomization/blinding in our review of pre-clinical sepsis studies, others reported difficulty in appraising the risk of bias secondary to lack of randomization, allocation concealment and blinding (51). In the critical care field, Ramirez et al. (50) demonstrated presence of randomization only in 22% and blinding in 33% of examined cardiovascular model studies. Although it cannot be excluded that some of those studies used randomization/blinding without disclosing it, the lowest use of randomization (17%) was reported in mouse-based experiments (50) – the species whose size enables repetitive high-power testing.

Lack of proper randomization and blinding in critical care studies may overestimate treatment benefits both in human trials (52) and animal models (53). An analysis of abstracts presented at the annual emergency medicine meetings revealed that non-randomized and/or unblinded studies were more likely to report positive outcomes (odds ratio 5.2; 95% confidence interval, 2.0-13.5) (54). Failure to randomize and/or blind were key factors behind the inability to reproduce landmark pre-clinical findings in anti-cancer, cardiovascular and amyloid lateral sclerosis therapies (55-56).

Both randomization and blinding can be implemented, although some constraints in blinding are unavoidable and must be recognized in some study types, e.g. when testing new devices. Promising animal treatment studies often constitute a launching platform for human

testing. To eliminate dissemination of misleading data, the methodological rigor of randomization and blinding should be applied in pre-clinical experiments, whenever study design allows that. As an ultimate goal, to strengthen the translation bridge between bench and bedside, the pre-clinical testing should approach the quality of clinical trials. The recent multicenter pre-clinical randomized controlled trial verifying efficacy of anti-CD49d treatment against stroke (57) demonstrates that this is feasible and could be adapted for sepsis research.

Recommendation 4: Provide as much information as possible (e.g. ARRIVE guidelines) on the model and methodology, to enable reproducibility.

Although experimental reproducibility is key in evidence-based science, pre-clinical studies (17) are burdened by methodological under-reporting with estimates for irreproducibility ranging from 75% to 90% (58). Holman *et al.* (59) showed that under-reporting precluded identification of animal attrition in over 60% of articles in stroke and cancer. Publication analysis of three high-impact critical care journals revealed poor methodological reporting of study design and ethical intervention in animal studies (60, 61). Poor transparency impedes replication and cross-comparison of animal studies including sepsis. The NIH has recently launched a training initiative to address this problem (www.nih.gov/research-training/rigor-reproducibility).

Our recommendation may appear redundant in view of various existing guidelines promoting transparency reporting, e.g., Animals in Research: Reporting *In Vivo* Experiments (ARRIVE) (62), journal checklists (e.g. EMBO Press Checklist) (63) and Transparency Openness Promotion (TOP; https://cos.io/top) initiative by *Nature*. Compliance to these guidelines is key for reproducibility. Unfortunately, using the ARRIVE example, the recent analysis of pre-clinical studies demonstrated their low compliance with the ARRIVE and poor improvement in reporting quality (64, 65). The underlying reasons are mixed:

disinclination/neglect of the authors to provide complete methodology, space limitations by the journals, and lax enforcement at the stage of peer-review process and publication. It is possible that guidelines specifically tailored to individual research areas and endorsed by their professional bodies will have more impact and enable better enforcement. Sepsis research should follow the existing examples: the American Heart Association released recommendations on design, execution and reporting of animal studies in atherosclerosis (66) and the *Stroke* journal has recently issued a second checklist edition for experimental stroke models (67). The latter example demonstrates a success of such a focused approach: reporting standards improved in animal studies submitted to *Stroke* after implementation of the first checklist (68). Implementation of best reporting practices should also include full disclosure of the originally posited study objective(s) to communicate the rationale behind the experimental design. Use of the journals' supplementary section to provide a detailed methodological description should be encouraged. This absence of detail is not trivial; despite widely endorsed CONSORT guidelines (www.consort-statement.org), analysis of 67 clinical trials by COMpare Trial Project identified severe discrepancies between final clinical trial reports and their entry protocols (e.g. 357 unplanned outcomes added, 354 planned outcomes not reported) (69). The pre-clinical field is much less controlled for such inconsistencies and more efforts should be made to improve the reporting practices. The high-quality reporting standards will not be achieved without strong enforcement mechanisms.

Consideration a) Consider replication of the findings in models that include co-morbidity and or other biological variables (i.e., age, gender, diabetes, cancer, immunosuppression, genetic background and others).

Advanced age, chronic obstructive disease, cancer, chronic renal disease, chronic liver disease, diabetes and immunosuppression constitute known risk factors in sepsis (70) and influence the degree of infection/injury as compared to the same insults in healthy young adults (70-76). While young mice are valid for specific types of basic science sepsis research, the results of such works are limited in their ability to be directly translated to septic humans (4, 18). In pre-clinical studies, the use of healthy inbred animals of the same sex, age and weight is frequent as it limits baseline variability (18). However, human patients are 'outbred,' have variable ages, gender and weight, individual comorbidities, and have different causes of sepsis. All of these will affect the host response and influence the morbidity and mortality of the septic patient. Host genetic factors are also relevant to the variability in sepsis susceptibility and outcomes (70).

Sepsis pathophysiology is extremely complex (26, 37, 77, 78). Although there is value in studying the mammalian response to severe infection in standardized rodent models, researchers should consider repeating their work in modified animal models that more closely recapitulate the human condition/variability prior to directly translating their findings to patients (18, 37). We encourage development of a large family of sepsis models that represent options in which sepsis phenotypes may present and fluctuate, for example, validating the work in a model that features a modifying risk of morbidity and lethality (e.g., aging, gender, diabetes, cancer, immunosuppression, genetic background). This also includes two-hit models in which sepsis is modified by a defined critical care condition (e.g., trauma) and/or secondary infection (79-81). For sepsis, age constitutes one of the key modifiers given the demographic characteristic of septic patients (typically >65 years old) and associated age-related comorbidities (82). Yet, a review of pre-clinical sepsis studies reported that less than 1% of studies employed appropriately aged animals (83). Regarding biological variables, outbred mice feature an immune system that

is more comparable to humans, providing a tool to improve the translatability of sepsis research to human patients (84). Furthermore, non-rodent sepsis models in species whose biology is more similar to humans can be conducted to determine how applicable rodent work is to human biology (18). Based on our current understanding of sepsis pathophysiology, failure to properly integrate the above-mentioned factors into experimental designs of animal studies likely limits the translational potential of the pre-clinical results.

Consideration b) In addition to rodents (mice and rats), consider modeling sepsis also in other (mammal) species.

Due to the varied nature of sepsis, it is unlikely that models that involve one species will be able to mimic all aspects of the clinical and biological complexity of the disease that are encountered in humans (37). Therefore, the authors believe researchers should consider modeling sepsis in other mammalian species in addition to rodents. This consideration does not intend to compel investigators into performing repetitions of their studies across multiple species, especially if studies in rodents are well validated and government agencies (e.g., United States Food and Drug Administration (FDA) and European Medicine Agency (EMA)) do not require such a step. However, as for most basic science research, it is important to note that rodents are by far the most commonly used species for modeling sepsis (85). The reasons for robust use of mice and rats include: high fecundity; accelerated life cycle; low maintenance; well-characterized genome; inbred, outbred and transgenic strains; widespread availability as well as reagents used to study them; and the creation of ‘humanized’ mice (18). Our literature review (Table 1) shows that 79% of the studies on sepsis used mice and 94% used rodents. However, we need to consider that rodent models have several significant inherent limitations (85, 86). For example, mice have a

higher resistance to the systemic inflammatory response associated with infection (18); due to the high resilience of mice against infections, $10E^{7-9}$ *E. coli* or *S. aureus* are needed to affect mice; this would correspond to $3.5x10E^{12}$ bacteria in humans (87, 88). In this context, a rabbit model may be more appropriate for *S. aureus*-induced sepsis (89). Another example is the blunted response of mice to bacterial products, such LPS – the lethal dose in mice is approximately 1,000 times greater than the estimated lethal dose in humans (18). Other differences between humans and mice also need to be taken into consideration. The composition of murine leukocytes in whole blood is dissimilar to that of adult humans, for both innate and adaptive immunity (18) and the two species have mismatching temporal response patterns to infection (6).

Thus, a mouse does not necessarily represent the complex systemic background of the septic response in humans and narrow (i.e. in a single species) pre-clinical testing of given phenomena could mask numerous effects (18). Given these concerns, validation of pre-clinical sepsis findings in more than one single species can enhance its translational potential. This can include rabbit, porcine, bovine and non-human primate models (85, 90). Furthermore, other species may be more appropriate in specific sepsis models due to their physiology and pathophysiology being more similar to humans. It should be noted, though, that to date these animals have also not been successful in the clinical application of biological response modifiers in humans (18). Finally, cost and the absolute necessity for the humane treatment of these research animals can limit what can be conducted by individual laboratories regarding animal sepsis research (18). However, those issues should not preclude attempted advancement or optimization of animal sepsis research modeling.

Consideration c) Consider need for source control.

"*Ubi pus, ibi evacua*"; when there is a collection of pus in the body causing sepsis the evacuation of it is the most important aspect of its management. The Surviving Sepsis Campaign guidelines recommend that source control should be implemented as soon as medically and logistically practical after the diagnosis is made (3). Consequently, prompt removal of intravascular access devices that are a potential source of sepsis is recommended after other vascular access has been established (3). In humans, source control within 6 to 12 hours after diagnosis seems to be sufficient in most cases (3, 91, 92). The sepsis guidelines mention the following foci of infection readily amenable to source control: intra-abdominal abscesses, gastrointestinal perforation, ischemic bowel, cholangitis, cholecystitis, pyelonephritis associated with obstruction or abscess, necrotizing soft tissue infection, other deep space infection (e.g., empyema or septic arthritis), and implanted device infections (3). How do these insights translate to the design of a pre-clinical model of sepsis?

Most literature on this subject is derived from the CLP model. The ligated and punctured cecum can be excised at various intervals to serve as a source control model of sepsis (93). Source control measurements in that model have been associated with resolution of the inflammatory process (94). Nonetheless, it should be acknowledged that source control adds complexity to the model which could interfere with other endpoint parameters. In addition, the timing of excision and drainage is of importance. An early excision for source control can be associated with no mortality, while delayed intervention can lead to increased mortality or no effect on the clinical course. In summary, the committee recommends researchers to consider the use of source control in an animal model of sepsis, when appropriate, in order to be consistent with the management of human sepsis.

CHAPTER 2: HUMANE MODELING

Our desire to establish humane endpoints relates to our aim to promote good care and welfare practices in pre-clinical sepsis experimentation worldwide. The current rules for experimental animal welfare in sepsis studies differ among countries, although these differences are declining as more U.S. and international organizations are voluntarily seeking accreditation by Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Furthermore, many journals are going beyond local ethics committee approval and are adhering to animal welfare recommendations promulgated by the Guide for the Care and Use of Laboratory Animals (8th edition), published by the National Research Council (USA) (95). Given that animal sepsis models display a relative high burden of suffering, precise monitoring, effective analgesic control and death-as-an-endpoint are frequently discussed. The present paper aims to instigate a long-term process that eventually leads to an optimal standardization of humane practices in animal sepsis modeling. For example, the emphasis should be on improving the ability to detect indicators of sepsis-related morbidity and mortality, and on challenging assumptions that mortality as an endpoint is 'inevitable', yet retaining compatibility with human sepsis studies, which continue to rely on the death endpoint and include patients with comorbidities. Numerous humane endpoints can be refined and perceptions about the ability to predict impending death are constantly changing due to technological improvements (e.g., determinations of biomarkers from small blood samples, non-invasive microchip monitoring of vitals).

Humane endpoints in sepsis are best used in conjunction with prospective planning for their use (i.e. not *ad hoc* to address welfare concerns as they arise). In designing an experiment, the researcher should a) clearly specify the expected experimental outcome (and efficacy

endpoints for drug intervention studies), b) adequately justify the needs for a given outcome to prove the hypothesis and c) precisely delineate all tools employed for eliminating/reducing animal suffering. Our review of the 260 pre-clinical sepsis studies demonstrates that the welfare-related elements are typically not reported (Table 3). For example, in over 90% of the reviewed studies no euthanasia criteria were defined/mentioned, while in less than 10% of the studies the use of analgesia was disclosed. Specific criteria that will allow recognition of when the experimental outcomes have been met should be identified when planning the study and non-invasive techniques including imaging, behavioral or physiological monitoring (e.g., via biotelemetry) can be useful in reaching this goal. It is also important to recognize that it is not always necessary for an animal sepsis model to share all features of the human sepsis pathophysiology. It may be sufficient that the animal model recapitulates one specific but relevant element of the human disease (e.g., cardiac dysfunction, acute lung or kidney injury). Overall, development of uniform and justifiable humane endpoint guidelines for pre-clinical sepsis experiments would aid in facilitating approval for necessary and clinically translatable studies with professional regulators as well as public opinion concerned with the ethical use of animals in research.

Specific recommendations for Humane Modeling

The conference discussed several specific recommendations for pre-clinical models of sepsis to advance the use of these models. The following recommendations and considerations from the Humane Modeling Endpoints working group are numbered consecutively from the preceding chapter and start with recommendation 5.

Recommendation 5: The development and validation of standardized criteria to monitor the well-being of septic animals is recommended.

A laboratory animal should be able to exercise natural behavior without experiencing distress. Such an environment can be provided for rodents and rabbits (96, 97), but not as easily for large mammals (98) and non-human primates (99). Majority of experimental procedures impair the animal well-being, but sepsis studies produce a significant degree of suffering. To classify the magnitude of the impact on well-being, development and validation of monitoring criteria for septic animals is necessary (100, 101). The selected well-being criteria need to be frequently monitored and should encompass animal behavior as well as clinical examination (100, 102). Few scientific publications propose specific evaluation protocols (103, 104); they focus on the assessment of the sepsis severity rather than the animal welfare itself. For example, a mouse clinical assessment score for sepsis (M-CASS) allows staging the severity of pneumonia (103) by evaluating several clinical and behavioral parameters. Our literature search failed to reveal any standardized scores for the monitoring of animal well-being and/or sepsis progress in large mammals.

Modern technology can enhance the non-invasive monitoring capability in animal experimentation. Implantable biotelemetry devices are used for monitoring physiology and, in sepsis, disease severity (e.g,. heart rate, body temperature and mobility) in mice (105-107). Recently, Lewis et al. (108) demonstrated a large-scale utility of biotelemetry monitoring for fluid resuscitation and antimicrobial treatment in CLP mice. *In vivo* wireless monitoring of cardiovascular endpoints has been successfully tested in pigs (109), dogs and non-human primates (110). Continuous body temperature monitoring using non-invasive infrared light is another alternative (111). However, disadvantages exist: biotelemetry-devices are relatively

expensive, require a surgical intervention, which may alter the response to the sepsis insult, and might necessitate specific housing (112). Alternative monitoring techniques such as echocardiography (113), blood pressure and heart rate assessments (114) are also possible but require expertise and additional procedures for the animals, such as anesthesia and restraint.

Initial well-being assessment criteria can be simple; it is important to first instigate a positive reception for such practices and create a framework that enables its quality standardization and further technical development. As the first step, we recommend focusing on systematic recordings of a) behavioral changes (e.g., food intake, vocalization, mobility, social interactions) and b) clinical symptoms (e.g., body weight, respiratory and temperature changes) (103, 104, 115). Pre-clinical laboratories already employ many of these endpoints and their arrangement into a standardized well-being protocol should not be arduous. The next step will require adjustments of the evaluation criteria to meet the 'welfare demands' of more complex sepsis models (e.g., co-morbidity, two-hit models, chronic sepsis). Comorbidities typically alter both behavior and clinical parameters: e.g., an overweight diabetic mouse is less active compared to a healthy mouse (116) and weight gain in a chronically septic mouse is a sign of recovery, not deterioration as in acute sepsis (117). A routine use of standardized well-being scores in septic animals can serve as additional efficacy or adverse effect variable to complement non-mortality secondary endpoints such as assessment of organ dysfunction. Thus, monitoring of well-being scores can be tailored to the respective sepsis model to account for variations in pathophysiologic responses secondary to changes in environment, strain, gender and co-morbidities.

Recommendation 6: The development and validation of standardized criteria for euthanasia of septic animals is recommended (exceptions possible).

Current legislation for animal experimentation in the United States (95), Japan (118) China (119), and European Union (EU) (120) allows but discourages inclusion of death as an endpoint. Some EU countries (e.g., the United Kingdom) and individual research institutions (e.g., Vlaams Instituut for Biotechnologie, Belgium) have voluntarily implemented ban on using death as endpoint. In critical care animal and human studies, death remains a frequently used parameter; its replacement with surrogates is not always justified and may be misleading. The key concern is that "preemptive" euthanasia (i.e. dictated by the ban) or euthanasia based on commonly utilized humane endpoints is either uninformative or the outcome assumptions can be imprecise. For example, Nemzek et al. (121) demonstrated that only 56% of CLP mice with a body temperature below 30°C died. Thus, liberal euthanasia cut-offs in pre-clinical sepsis can distort data subsequently impairing translatability of animal findings to patients (115, 121). Additionally, predictive imprecision of surrogate endpoints may preclude identification of unexpected life-saving effects by some therapies. Currently, many investigators assume their favorite surrogate endpoint, such as reduction of organ dysfunction predicts an increase in survival; such an approach should not be followed until a given surrogate marker has been validated to precisely predict death or long-term survival.

The above controversies underline the need for developing precise and standardized criteria for euthanasia in sepsis research (122) to ensure an acceptable combination of experimental design quality and ethical practices. Development of defined cut-off(s) for euthanasia in septic animals is inherently linked with R-5 given that the animals must first deteriorate (i.e. decreased well-being) to the moribund state (defined by R-6). The two R-5 and R-6 recommendations should be viewed as a continuous monitoring paradigm transitioning from a) a set of behavioral/clinical descriptors (R-5) in non-lethal sepsis to b) a precise decision-

making tool (R-6) for euthanasia in animals approaching the moribund state. Thus, the key welfare issue predominantly arises from the distress preceding the moribund state; it is currently unclear to what extent (if at all) unresponsive and/or comatose animals experience pain (122). Euthanasia of animals that reach "a dying state" eliminates spontaneous deaths but does not eliminate their distress experienced during progression to that state (123, 124). In the ethical context, endpoints identifying moribund animals can never be considered humane enough (115); they constitute a trade-off between a need for investigative confidence and relief from unnecessary suffering. A tight synchronization/use of both R-5 and -6 should enhance the latter without jeopardizing the former. A reliable R-5 (well-being) score will automatically strengthen the informational quality and precision of R-6. This, in turn, will potentially facilitate identification and implementation of the irreversible "dying state" cut-offs at earlier stage(s) of sepsis.

In the technical context, the existing criteria for euthanasia in septic rodents (103, 104, 121) and pigs (124) are typically based on changes in behavior, body weight and temperature; telemetric devices can further refine the above approach (105, 109, 110). Blood biomarker measurements are another alternative as outcome predictor in septic mice (125, 126). Compared to the clinically-based criteria, biomarkers are advantageous as relatively early predictors of mortality, thus preventing the deterioration of animals to the moribund state. However, they require repeated blood sampling (127), which produce distress (128). Additionally, current biomarker-based assays are not completely precise (126, 129), are technically challenging and preclude the monitoring of late sepsis.

Several elements require consideration for creation of effective pre-clinical euthanasia criteria. First, non-invasive clinical and behavioral descriptors (as discussed in R-5) appear to be

a good starting platform. Recent works in mice provide several candidates for defining the moribund state (121) and demonstrate how their combinations can be effectively applied for 'euthanasia decision-making' (103, 104). For example, body temperature changes are indicative of sepsis severity (130, 131) and are an independent predictor of outcome (132, 133). In large septic mammals, the blood glucose monitoring was reported as useful outcome predictor (134). Second, disease-specific parameters should be integrated into the euthanasia criteria depending on the type of the sepsis model used (e.g., severe dyspnea, respiratory alterations in pneumosepsis) (103, 104). Finally, the chosen euthanasia criteria have to be validated before use in each laboratory separately, for every sepsis model, species/strains, age and gender. Strong inter-laboratory and animal variability precludes automatic adaptation of euthanasia protocols across research laboratories.

Recommendation 7: Analgesics recommended for surgical sepsis should be consistent with ethical considerations.

A principle of animal welfare is that any procedure expected to cause pain in humans is considered likely to cause pain in animals and should be alleviated through appropriate care and pain management (e.g., analgesia). Regardless of international regulatory differences, surgical sepsis is always rated as causing the most severe grade of distress, assuming the animal recovers consciousness after surgery.

The use of pre-and/or post-operative pain medicine is rarely reported in pre-clinical sepsis studies (115, 135). In our Wiggers-Bernard review, analgesics were used in 30 experiments, not used in 19 and not reported in 329 experiments, despite CLP being the leading sepsis model. This is consistent with a recent report where only 15% of the analyzed publications used analgesics in experimental sepsis (61). While some (or many) of the manuscripts not

reporting actually used analgesics, it is likely that many studies did not use analgesics in surgical sepsis. There are two possibilities for why investigators have historically withheld analgesia in surgical sepsis – a) the belief that animals cannot feel pain and/or do not feel pain based upon lack of signs such as vocalization, and b) the concern that analgesics would alter critical endpoints. The first supposition is incorrect; mice and rats demonstrate pain via changes in facial expression (136) and they also have subtle behavioral changes following surgery (137). This can be alleviated by analgesics, and trained personnel can distinguish rodents that received post-surgical pain medicine compared to those that did not. However, mice do not typically have a vocal response to a painful procedure, and mice may vocalize at frequencies above the range of human hearing (138).

The often-quoted reason for withholding analgesia to laboratory animals is that analgesics cause alterations in the inflammatory response and coagulation (139-141). It is important to acknowledge that different analgesics have different side effects, which may limit the utility of some classes of drugs in sepsis research. For instance, non-steroidal anti-inflammatory drugs are associated with anti-inflammatory effects via inhibition of prostaglandins and may also be associated with renal impairment and bleeding (142). Similarly, mu-agonists such as morphine have immune-modulating effects and can cause respiratory depression (143). However, opioids that act as kappa-receptor agonists and mu-receptor antagonists have been demonstrated to be safe and effective without causing significant immunomodulation. Specifically, numerous studies have demonstrated the efficacy/safety of buprenorphine and tramadol (144, 145). While buprenorphine adversely may impact mortality in male (but not female) septic mice, this can be prevented by dose reduction (146). Furthermore, buprenorphine treatment results in minimal differences in inflammatory parameters although neutrophil counts

are transiently decreased in male mice (146). In addition, continuous infusion of nalbuphine, another opioid agonist/antagonist, has been used in rodent models of CLP and fecal slurry (147, 148).

Importantly, while analgesics can alter the inflammatory profile, pain, in and of itself, may also affect disease outcome and experimental variability (115). Thus, from both an ethical and experimental standpoint, the use of analgesics should be standard and reported in surgical sepsis models. Exceptions – if they exist – should be rare, should be experimentally demonstrated and cannot be justified by the catch-all phrase that "analgesics alter the host response".

Consideration d) Consider analgesics for nonsurgical sepsis.

The data are less clear for animals in which sepsis is induced via a non-surgical approach. Sepsis clearly causes encephalopathy in patients. Many septic patients in the ICU appear to be in pain, although it is difficult to separate the impact of the underlying disease from interventions meant to support septic patients (mechanical ventilation, pressor support via large bore invasive catheters). Guidelines for the management of critically ill patients state that "adult medical, surgical and trauma ICU patients routinely experience pain, both at rest and with routine ICU care" and recommend intravenous opioids as the first-line drug class of choice to treat non-neuropathic pain in critically ill patients (not specific to sepsis) (149). In the absence of clear data suggesting the degree to which laboratory animals with sepsis from a non-surgical source experience pain, investigators should weigh the benefits of analgesia versus the potential side effects of analgesia. Implementation of rigorous pain-oriented monitoring of septic rodents (and larger species) subjected to non-surgical sepsis protocols may likely provide the necessary evidence regarding the absence/presence of pain as well as its potential magnitude. It is possible

that the emerging evidence will support a uniform implementation of analgesics in all septic models regardless of the experimental origin of sepsis.

SUMMARY

This Part I manuscript details the recommendations and considerations of the two working groups from the Wiggers-Bernard conference on pre-clinical models of sepsis. Analysis of the top-cited pre-clinical sepsis papers showed substantial shortcomings regarding both the use and reporting on the study design and humane modeling elements. Due to multiple inconsistencies with the clinical conditions, inadequate modeling protocols are at least partly responsible for failures in developing effective therapies for septic patients. Given the disease burden, the highest standards of animal welfare must be implemented in all pre-clinical sepsis studies. The two working groups made specific recommendations about the rigors of study design and adequate humane modeling of sepsis in animals. We hope that these recommendations and considerations will serve to bring a level of standardization to pre-clinical models of sepsis and ultimately improve the translation of pre-clinical findings. We acknowledge that new challenges based on new information from the clinical and bench studies will continue to arise. A close collaborative work between basic scientists and clinicians is critical for a thoughtful (re)interpretation of any existing and newly posited principles.

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Table 1. Study Design Endpoints in Sepsis Models (2003-2012*)

Species	**Presence of comorbidity**	**Mortality as endpoint**	**Follow-up**	**5-day mortality**	**If present, experimental treatment given as**
mouse: 295	yes: 18	yes: 160	<5 days:	high (≤70%): 142	pre-treatment: 96
rat: 56	no: 356	no: 214	143	(>30%<70%):	co-treatment: 59
pig: 7			>5 days <14:	77	post-treatment: 95
sheep: 5			154	low (≤30%):	not stated: 13
NHP: 4			>14 days:	43	
rabbit: 3			37	not stated: 74	
cat: 1			not stated:		
dog: 1			13		
hamster: 1					
guinea pig: 1					

*Collated data is obtained from review of the 360 most-cited papers (featuring total of 374 animal experiments) identified with ISI Web of Knowledge database (using the query:"*sepsis model*"). NHP: non-human primate.

Table 2. Study Design Endpoints Working Group (WG): Recommendations (R) and Considerations (C)

Study Design (WG-1)	**1.** Survival follow-up should reasonably reflect the clinical time course of the sepsis model **2.** Therapeutic interventions should be initiated after the septic insult replicating clinical care **3.** We recommend that the treatment be randomized and blinded when feasible **4.** Provide as much information as possible (e.g. ARRIVE guidelines) on the model and methodology, to enable replication	**R**
	a. Consider replication of the findings in models that include co-morbidity and/or other biological variables (i.e., age, gender, diabetes, cancer, immuno-suppression, genetic background and others) *b. In addition to rodents (mice and rats), consider modeling sepsis also in other (mammal) species* *c. Consider need for source control*	**C**

Table 3. Humane Modeling Endpoints in Sepsis Models (2003-2012*)

Defined criteria for euthanasia given[&]	**Analgesics used**	**If analgesics used: frequency of application**	**Full anesthesia throughout the duration of experiment**
yes: 33 no: 341	yes: 30 no: 19 not stated: 329	1x: 14 2-4x: 4 >5x: 4 continuous i.v.: 4 not stated: 4	yes: 19 not used/not stated: 355

*Collated data is obtained from review of the 360 most-cited papers (featuring total of 374 animal experiments) identified with ISI Web of Knowledge database (using the query:"*sepsis model*"). [&]irrespective of mortality as an endpoint. i.v.: intravenous.

Table 4. Humane Modeling Endpoints Working Group (WG): Recommendations (R) and Considerations (C)

Humane Modeling (WG-2)	**1.** The development and validation of standardized criteria to monitor the well-being of septic animals is recommended **2.** The development and validation of standardized criteria for euthanasia of septic animals is recommended (exceptions possible) **3.** Analgesics recommended for surgical sepsis should be consistent with ethical considerations	**R**
	a. Consider analgesics for nonsurgical sepsis	**C**