UNIVERSIDADE D
COIMBRA

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A NOVEL APPROACH FOR NON-INVASIVE MONITORING AND SIGNALLING OF HUMANE ENDPOINTS USING INFRARED THERMOGRAPHY, TESTED IN A MURINE MODEL OF SEPSIS

Dissertação no âmbito do Mestrado em Patologia Experimental orientada pela Professora Doutora Salomé Pires e pelo Doutor Nuno Henrique Franco e apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre.

Novembro de 2020

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“Humanity's true moral test, its fundamental test (…) consists of its attitude towards those who are at its mercy: animals.“

Milan Kundera

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Abstract

Body temperature is an important tool for assessing animal health and welfare. However, there is still limited information on temperature variation in animal models of human pathologies. This study aims to assess whether infrared thermography can be used to follow body temperature variations in a mouse model of sepsis induced by cecal ligation and puncture and compare it with the readout from passive integrated transponder (PIT) tags.

We report data retrieved from 18 Female C57BL/6 wildtype (WT) that underwent either severe cecal ligation and puncture or sham surgery, as well as 8 mice subjected to a less severe form of the intervention: 4 knockout (KO) mice on a C57BL/6 background (2 male and 2 female), and 4 WT mice (2 male and 2 female). These correspond to a partial sample of an ongoing larger study. All mice were monitored 4 times per day in the severe model and 3 times per day in the mid-grade CLP by reading of a thermosensitive passive integrated transponder (PIT) tag and by infrared thermography for 10 days post-surgery, or until reaching a humane endpoint.

There was an observable decrease in mean body surface temperature (MBST) and subcutaneous temperature (SCT) after surgery with time in both CLP models, for both animals that survived and those reaching the humane endpoint. The surface temperature assessed by infrared thermography and subcutaneous temperature were correlated, albeit not strongly. Receiver operating characteristic curves (ROC) demonstrate that the lowest SCT (AUC = 0,65; 95% CI), weight loss (AUC = 0,635; 95% CI) the lowest MBST (AUC = 0.43; 95% CI) do not appear to be sufficiently satisfactory models to predict non-recovery stages

MBST, SCT or percentage of weight loss do not appear to be reliable markers for assessing disease severity and predicting death in the CLP model, though subcutaneous temperature shows some promise. This is quite preliminary data, so further studies with a larger sample are warranted.

Key words: Temperature variation ; infrared thermography ; mouse model ; sepsis ; hypothermia

Resumo

A temperatura corporal é uma ferramenta importante para inferir saúde e bem-estar animal. Contudo, a informação sobre a variação da temperatura em modelos animais de patologias humanas é limitada. Este estudo visa testar termografia de infravermelhos para acompanhar variações térmicas num modelo murino de sepsis, induzida por ligação cecal e punção (CLP), comparando-a com a leitura de um *passive integrated transponder* (PIT) *tag*.

Reportamos dados de 18 murganhos fêmea C57BL/6 *wildtype* (WT) submetidos a uma variante severa de CLP ou cirurgia *sham*, bem como de uma variante menos severa em 8 murganhos, 4 *knockout* (KO) (2 fêmeas e 2 machos) e 4 wildtype (2 fêmeas e 2 machos). Estes dados correspondem a uma amostra parcial de um estudo maior a decorrer. Todos os animais foram monitorizados 4 vezes por dia no modelo severo e 3 vezes por dia no CLP de grau médio através da leitura de *PIT tags* e termografia de infravermelhos durante 10 dias após a operação, ou até atingirem o seu *endpoint*.

Houve uma diminuição observável da temperatura média da superfície corporal (MBST) e da temperatura subcutânea (SCT) após a cirurgia, ao longo do tempo e em ambos os modelos CLP, quer para os animais que sobreviveram atingiram o *endpoint*. Como esperado, a MBST e SCT estão correlacionadas, embora não fortemente. As curvas de Característica de Operação do Receptor (ROC) demonstram que a SCT mais baixa (AUC = 0,769; 95% CI), a perda de peso (AUC = 0,703; 95% CI) a MBST mais baixo (AUC = 0,534; 95% CI) não parecem ser modelos suficientemente satisfatórios para prever a fases de não-recuperação

MBST, SCT e a percentagem de perda de peso não parecem ser marcadores fiáveis para avaliar a gravidade da doença e prever a morte no modelo CLP, embora a temperatura subcutânea demonstre algum potencial. Os dados obtidos são dados preliminares, pelo que se justifica a realização de um estudo com uma amostra maior.

Palavras-chave: Variação de temperatura; termografia de infravermelhos; modelo murino; sépsis; hipotermia

Abbreviations

CLP- Cecal ligation and puncture

FIP- Fecal-induced peritonitis

IRT- Infrared thermography

KO- Knockout

LPS- Lipopolysaccharide

MBST- Mean body surface temperature

MSS- Murine sepsis score

PIT- Passive integrated transponder

ROC- Receiver operating characteristic

SCT – Subcutaneous temperature

SIRS- Systemic inflammatory response syndrome

WT- Wildtype

Introduction

1. Laboratory animal welfare

The use of animal models in biomedical research played a central role in most medical achievements, since the dawn of scientific medicine (Franco, 2013). The welfare of laboratory animals has also for a long time been a matter of controversy, with concerns being raised about the harms endured by them for the sake of scientific enquiry and about its justifiability when compared to the benefits of the research (Hubrecht *et al.*, 2019).

An approach to ease the ethical harm-benefit dilemma of animal experimentation is offered by the 3Rs principles of Replacement, Reduction and Refinement proposed by William Russell and Rex Burch, in their *Principles of Humane Experimental Technique* in 1959 (Russell & Burch, 1959). Replacement is understood as the substitution of sentient animals for non-sentient alternatives, whenever possible, such as systematic reviews, computer models, *in vitro* tests, organoids or use of invertebrates (the latter deemed *relative replacement*). Reduction refers to reducing sample sizes to the minimum number of animals necessary to obtain information 'of a specified amount and precision' without compromising the objectives of the research or collecting more scientific information without need for increasing the number of animals. Reduction can be achieved by means of improved experimental design and statistical analysis, choosing methodological approaches that dispense killing animals at each time-point (e.g. imaging techniques, using larger animals (to allow taking sufficient biological samples without need to killing at each sampling event), sharing of biological samples (e.g. someone studying the mouse brain donating livers to colleagues needing that organ), reusing animals (e.g. for education or other experiments, when possible and ethical) or using the same animals for different research projects, simultaneously, among other strategies. Refinement is the principle applied upon exhaustion of replacement and reduction option and aims at minimising – or indeed prevent – suffering from procedures, by adopting appropriate measures to alleviate pain or distress (Russell & Burch, 1959; Hubrecht & Carter, 2019). These principles provide a scientifically-sound ethical framework for animal experimentation and are now deeply rooted in the legislation regulating animal research (Franco *et al.*, 2018).

The laboratory mouse has become the preferred animal model. Statistical reports on animal use for scientific purposes in the European Union show a clear preference for the use of these species in research (Figure 1) (Commission to the European Parliament and the Council, 2020). The small size, resistance to inbreeding, short-life span, and high reproductive rate were the primary reasons for the prevalence of mice use in experimental research (Franco, 2013), to which can be added the high investment in new mouse models in recent decades and the development of countless genetically modified strains of mice (Gurumurthy & Lloyd, 2019).

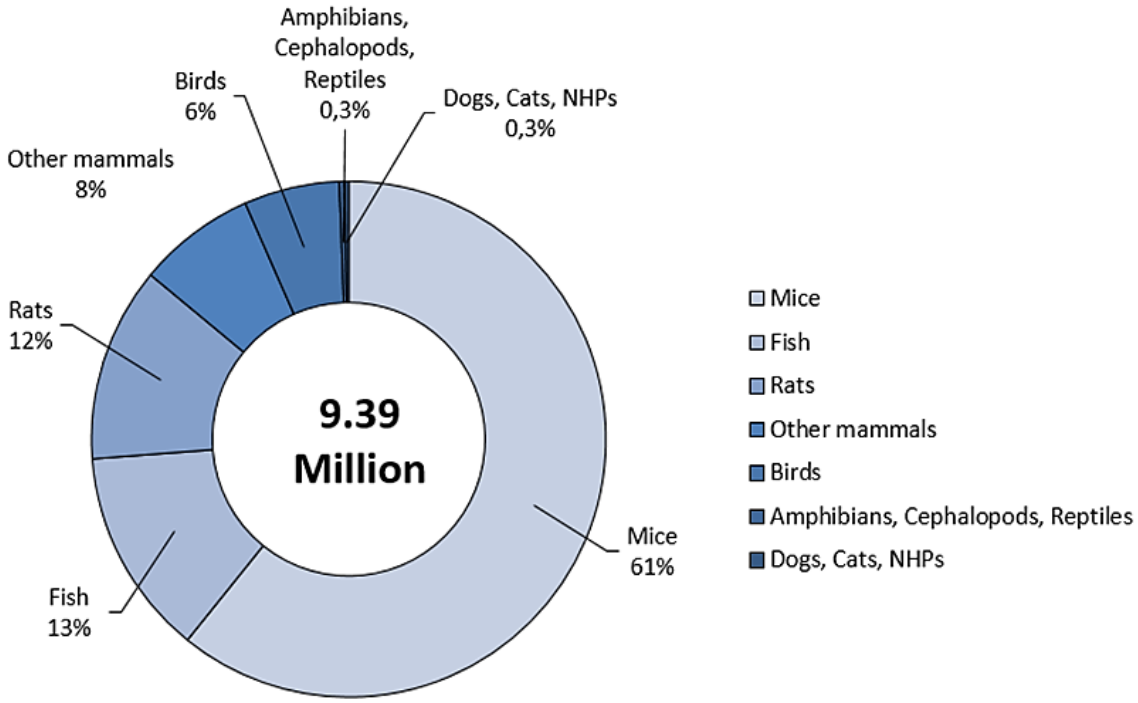

Figure 1. Numbers of animals used for the first time by main classes of species in 2017 (Source: Commission to the European Parliament and the Council, 2020).

Researchers and animal facility staff have a legal and moral obligation to minimize animal discomfort and uphold high animal welfare standards. This moreover favours both animals and experimental outcomes (Baumans 2005), as stress and discomfort during an experiment can cause non-specific biochemical changes, jeopardizing the reliability of the results (Beynen *et al*., 1989).

Researchers, caretakers, animal care technicians and the animal facility veterinarian have the obligation of monitoring the health and well-being of mice. Animals are observed in their home-cage and a variety of information on their health and welfare status is collected, to detect early warning signs (Burkholder *et al*., 2012). Physical and psychological health can be accessed by animals' level of activity, their interaction with cage-mates and general appearance (Burkholder

et al., 2012). A hands-on physical examination is also important to provide information such as level of hydration, body condition and the existence of overt abnormalities. Monitoring schemes are study- and species—specific, varying also with the procedures performed (Burkholder *et al*., 2012).

2. Body temperature

Among the physiological parameters used for measuring health and welfare in laboratory animals, body temperature is one of the most informative (Hunter *et al*., 2014; Mai *et al.*, 2018; Mei *et al.*, 2018). Core body temperature, along with other thermobiological parameters, can provide valuable information on the physiology – and pathophysiology – of homeotherms, including laboratory mice (Gordon *et al.*, 2012). Body temperature is also effective and clinically relevant to evaluate sepsis progression. It has furthermore been reported – and hypothermia in particular – to be a suitable criterion for applying humane endpoints for experimental models of sepsis. (Mai *et al.*, 2018). For this project, we focused on the monitoring of thermal parameters to follow disease progression in a murine model of septic shock and compare their informative value for predicting death from septic shock.

2.1. Core body temperature estimation

Although the definition of core body temperature can be straightforwardly defined as the temperature of the thermal core of the body, the criteria for defining what 'thermal core' actually means (especially considering how heterogeneous temperature is across different organs and tissues in an organism, even within it) and how it can be estimated may vary. Indeed, there may not be a 'pure' measure of body temperature, but rather local temperatures in different parts of the body – internal or external – each of them with its particular bias (Franco *et al*., 2019).

The core temperature in mice fluctuates pronouncedly over a 24 h period. Even when housed under ideal conditions, it can vary between 2-4 °C within one hour. Rats also present acute variations, although not as high as those found in mice (Gordon, 2012a). Thermal physiologists generally agree that normothermic regulation appears to be better developed in rats when compared to mice (Gordon, 2012b). Specifically, since the rat possesses a bodyweight of about ten times of a mouse, it is expected to maintain a more stable core temperature (Gordon, 2009).

This theory is difficult to test because the long-term stability of the core temperature of rodents is easily affected by a wide variety of aforementioned factors. Nonetheless, a study by Gordon (2009) supplied evidence supporting the premise that rats are better thermoregulators than mice and, depending on the strain, and that temperature regulation of mice can be approximately 50% more unstable when compared to rats.

2.1.1. Core body temperature estimation by rectal probe

Rectal and colonic thermometry are common methods for measuring core body temperature, in several species. In laboratory mice, body temperature is also typically estimated in the rectum or colon by a rectal thermometer probe. This method involves inserting a lubricated probe connected to a thermometer, through the anus, and offers a simple, inexpensive method for estimating core temperature in conscious mice. However, this method can raise technical and welfare complications. Rectal probes are particularly aversive because they can cause abrasion lesions to the mucosa and potentially life-threatening bacterial infections (Newsom *et al*., 2004). This assessment may also produce an experimental artefact consisting of increased core temperature due to stress-induced hyperthermia (Clement *et al*., 1989), a phenomenon observable in most mammals, including laboratory rodents (Adriaan *et al.*, 2007). Indeed, in group-housed mice, animals removed last typically presents higher rectal temperatures than those removed first (Hartinger *et al*., 2003). This is likely to result from alarm calls from the first mice eliciting stress responses in cage-mates, and can be interpreted as a conditioned response caused by anticipatory fear of being handled and/or the insertion of the rectal probe (Borsini *et al*.,1989). According to Borsini *et al.* (1989) steady-state temperature of a mouse during a fever is approximately 37 ºC and due to the hyperthermic state may rise to as high as 38,5 ºC. Therefore, the simple handling of mice can quickly lead to a hyperthermic state exceeding that of most fevers. This, along with other factors – such as depth of probe insertion affecting readings (Meyer *et al.,* 2017) – is cause of inter-individual variability.

2.1.2. Core body temperature estimation by radiotelemetry

Telemetry is a useful tool to monitor physiologic parameters such as heart rate, blood flow, blood pressure, body temperature, respiratory rate, and locomotor activity in conscious free-ranging animals throughout the circadian cycle (Baumans *et al*., 2001).

Radiotelemetry eliminates the need for stressful interventions – handling, restraint or probe insertion – and thus allows more reliable measurements, and least influenced by chemical, and psychological factors (Kramer *et al*., 2003). Transponders enable the experimenter to monitor changes over time, rather than picking optimum points for measurement. In consequence of the circadian variation of body temperature, if probes are used, a significant variation might be missed, and incorrect conclusions reached due to missing data-points. Nowadays radiotelemetry and data logger technology can also be used to monitor minute-to-minute changes in core body temperature (Gordon, 2012a). Therefore, telemetry allows the researcher to continuously record responses, and get a more accurate representation of body temperature without the added stress of repeated handling (Clement *et al*., 1989).

Although this approach emerged as the ideal method of collecting physiological data from undisturbed laboratory rodents, their surgical implantation is still associated with some challenges, since it demands general anaesthesia, which in turn warrants a recovery period and adds workload. Moreover, the size of the transmitter was proven to affect normal physiologic and behavioural health (Baumans *et al*., 2001). The most commonly used transmitters, usually placed inside the abdominal cavity, are still considerably large for a mouse and can delay recovery from surgery, cause inflammation, and decrease levels of activity and voluntary exercise (Helwig *et al*., 2012).

2.1.3. Core body temperature estimation by implanted transponders

The use of passive integrated transponder (PIT) thermosensitive tags is rising, as these can not only measure body temperature but also provide a unique digital identification number for each animal. PIT tags are typically implanted subcutaneously. Comparatively to traditional battery-operated telemetry devices, PIT tags are considerably smaller and less aversive. However, the handheld reader needs to be positioned in close range (<10 cm) to the transponder to give an immediate reading of the tag. This means that each animal needs to be picked up to avoid reading tags from cage mates, defeating the purpose of contactless measurement. PIT tags also only inform of subcutaneous temperature at one single point, which can vary both between and within (from migration under the skin) individuals. It may also differ from rectal temperature (Hartinger *et al*., 2003). Hence, interpreting temperature data from PIT tags implanted into different body regions must be done with care (McCafferty *et al*., 2015). Tags can also be lost (Mei *et al*., 2018). In addition, and as mentioned above, in telemetry there are disadvantages associated with the tag implantation (such as requiring anaesthesia and a short recovery period).

2.2. Body Surface temperature

2.2.1. Non-contact infrared thermometry

Infrared thermometers use infrared radiation naturally emitted from the surface of an animal and have been proposed as a method for non-invasive temperature measurement in rodents. Despite a few advantages, it is not completely reliable, as it relies on targeting a single spot of the body's surface, and aiming for the same exact surface point is hard to achieve consistently. In some body surface sites, such as the tail and the sole skin, the temperature varies widely, not being an accurate representation of the animal temperature (Saegusa & Tabata, 2003). Moreover, temperatures obtained are often too low. These non-physiological temperatures probably result from the isolating proprieties of the fur at the measuring spot (Vogel *et al.*, 2016).

2.2.2. Infrared Thermography

Infrared thermography (IRT) uses a thermal imager to detect radiation originated from the surface of an animal, estimating its temperature (Figure 2). It is a promising approach for non-invasive thermal assessment., and in laboratory animal science it has been applied in the identification of housing problems (David *et al.*, 2013), following neonates development (Harshaw *et al.*, 2012), measuring stress (Luzi *et al.*, 2010), and monitoring infection (Vadlejcha *et al.*, 2010), among other applications.

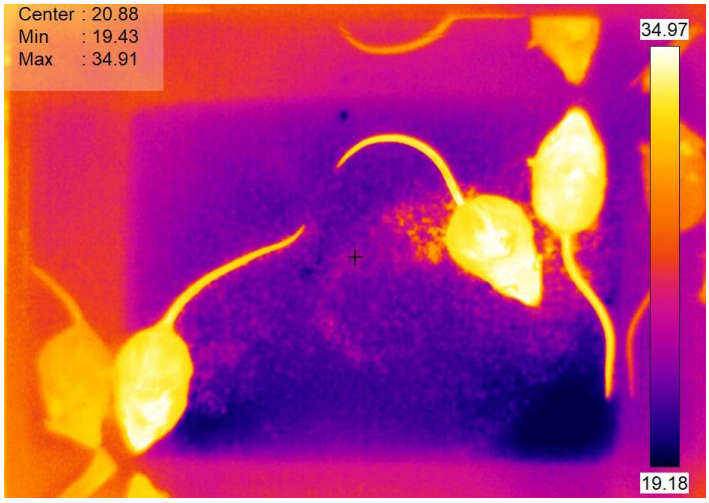

Figure 2. Thermal image of mice in their home cage.

These developments have made IRT receive more attention, in animal research. A recent study by Fiebig *et al*. (2018) concluded that IRT is a reliable parameter for measuring body temperature in nude mice, and comparable to rectal and subcutaneous temperature measurement. However, it might be less reliable in furred mice (Fiebig *et al*., 2018), because in spots devoid of fur (e.g. tail) temperature varies substantially in response to stress, cold, or heat (Vogel *et al*., 2016). Eye temperature has been shown to correlate with rectal temperature (Vogel *et al*., 2016) but cannot be reliably assessed in freely moving animals, as shown by Gjendal *et al*. (2018), who established mean body surface temperature (MSBT) as a more robust parameter for measuring body temperature variations in mice using thermography, when compared to the eye or tail temperature.

Franco *et al*. (2019) developed especially-devised software capable of identifying individual mice and automatically assess their MBST, as well as capable of batch analysis of a virtually unlimited number of images, yielding group mean MBST, or temperature of each animal in a group. Using this software for automated data collection removes observer errors and biases and speeds up data processing. In general, IRT has great potential as a non-invasive, animal-friendly method, with vast opportunities for automation in both data collection and analysis, minimizing operator bias and the impact of stress on the readout.

3. Experimental sepsis models

Sepsis is a response to microbial infection, characterized by the activation of inflammatory and coagulation pathways (Mai, 2012). According to a recent global study, 49 million cases and 11 million sepsis-related deaths were reported in 2017 (Rudd *et al.*, 2020). It is a devastating condition that can lead to multiple organ failure, septic shock, and death. The symptoms of sepsis are often non-specific and highly variable. The most common symptoms include hypothermia, hyperthermia, heart rate above the normal value for age, tachypnea, altered mental status, significant oedema or hyperglycaemia in the absence of diabetes (Lever & Mackenzie, 2007). Advances in the comprehension of the pathogenesis of sepsis led to the development of improved diagnostic methods and currently available nonspecific anti-sepsis treatments (Cohen *et al.*, 2015). However, successfully developing new and improved treatments remains a challenge, and sepsis remains a major public health threat.

One of the challenges in therapeutics developments is that successful new treatment strategies in mouse sepsis models often fail when reaching human clinical trials. One possible cause is

mouse models not replicating all the clinical features of human sepsis, as there are inherent differences between the immune response between the two species (Efron *et al.*, 2015).
Several experimental sepsis models have been developed and improved over the last eight decades (Wichterman *et al*., 1980; Marshall *et al*., 2005; Mai *et al.*, 2012). However, presently there is no perfect model that can mirror all aspects of the progression and pathophysiology of clinical sepsis in humans. Nonetheless, each model can answer distinct questions on sepsis pathophysiology, and thus the 'ideal' model will depend on the research question being answered or therapy being tested (Marshall *et al*., 2005).

3.1. Lipopolysaccharide injection models

The first attempts to develop animal models of sepsis relied on the administration of endotoxins such as lipopolysaccharides (LPS), lipoprotein and carbohydrate complexes present in the outer membrane of Gram-negative bacteria. These components are recognized by the innate immune system. (Heine *et al*., 2001), inducing the production of inflammatory cytokines. LPS models are hence particularly suited for modelling Gram-negative bacterial infections (Männel, 2007). The model entails the injection of LPS, most often intraperitoneally and in mice, inducing sepsis-like symptoms. As referred above, some immune properties affecting the pathogenesis of sepsis differ between species, and rodents are relatively more resistant to toxins, such as LPS, when compared to humans (Korneev, 2019). Thus, the dose of LPS injected needs to be high enough to induce a physiological response resembling Gram-negative bacterial infections in humans (Copeland *et al*., 2005). Nevertheless, this model presents some advantages. It is both simple to accomplish and reproducible, the dose of LPS is easily regulated or changed, and clinical symptoms – such as hypothermia – develop within a few hours. However, this model and human sepsis differ in a variety of key-points, especially in the kinetics and amplitude of cytokine release (Remick *et al.*, 2000). Septic shock also develops fairly quickly in this model, rapidly leading to death.

3.2. Cecal ligation and puncture models

The cecal ligation and puncture model (CPL) is the current gold-standard in sepsis research. It is considered a reliable model for polymicrobial infection of the peritoneum, resulting in the induction of bacteremia, systemic inflammatory response syndrome (SIRS) polymicrobial sepsis, and septic shock (Rittirsch *et al*., 2007). This model consists of the ligation of the distal

end of the cecum, below the ileocecal valve, followed by needle puncture of the cecum (Figure 3). Perforation of the cecum causes bacterial peritonitis, caused by an endogenous source of bacterial contamination. The enteric bacteria translocate into the blood compartment, resulting in systemic activation of the inflammatory response. These rodent models present clinical signs similar to typical symptoms of sepsis or septic shock such as hypothermia, tachycardia, and tachypnea (Rittirsch *et al.*, 2009).

In this model, the percentage of cecum ligated is a major factor influencing severity (Ruiz *et al.*, 2016; Singleton *et al.*, 2003) and it is a useful feature, as it makes it possible to induce sepsis with a range of severity levels, allowing to adapt disease severity for investigating either acute or chronic sepsis. Therefore, it is important to maintain high consistency, to obtain reliable and reproducible results. Other factors that may impact the reproducibility are: the calibre (gauge) of the needle used, the number of punctures, fluid resuscitation, and antibiotic treatment. The interplay between these factors offers considerable flexibility to models, allowing to model different manifestations and severities of the disease.

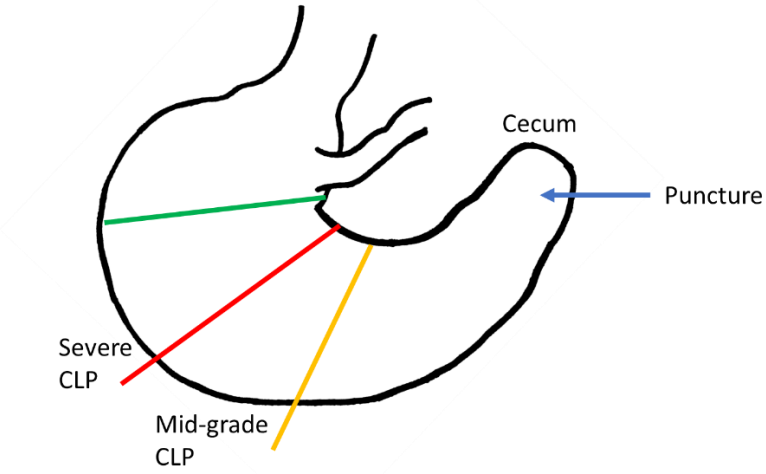

Figure 3. Illustration of the locations for cecal ligation of a mouse. The green line represents the basis of the cecum. The yellow line represents the ligation position to induce mid-grade sepsis and the red line represents the ligation position to induce high-grade sepsis (Adapted from Rittirsch *et al.*, 2009).

4. Humane endpoints

Humane endpoints are one of the most important refinement measures to prevent unnecessary suffering in laboratory animals (Franco *et al.*, 2012). The Guide for the Care and Use of Laboratory Animals defines a humane endpoint as “the point at which pain or distress in an

experimental animal is prevented, terminated, or relieved." (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

According to the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011): "the use of humane endpoints contributes to refinement by providing an alternative to experimental endpoints that result in unrelieved or severe animal pain and distress, including death.". When successfully implemented, humane endpoints can allow terminating experiments before animals experience severe harm, and without compromising scientific objectives. According to EU Directive 2010/63/EU, "Death as an endpoint to a procedure shall be avoided as far as possible and replaced by earlier and humane endpoints". Therefore, other markers and clinical signs predictors of death should be used as surrogate endpoints, whenever possible (Sneddon *et al.* 2017; Franco *et al*., 2012).

The field of sepsis research is often in conflict with animal welfare. The severity of the models is a primary welfare concern, due to the rapid onset of clinical signs (within hours) and death (within a few days). The lack of clear markers of death also presents a challenge. In fact, when it comes to experimental sepsis, specifically the cecal ligation and puncture model, clear endpoint markers have not been established (Mai *et al*., 2018). The lack of an objective endpoint marker might cause the premature termination of the research, which results in an incomplete number of observations and the use of more animals (Drechsler *et al*., 2015). For this reason, surrogate markers of death have been proposed (Franco *et al*., 2012; Morton, 2006; Shrum *et al*., 2014), which typically rely on clinical assessment, pain scales or other estimates of suffering to assess if an animal should be euthanized (Shrum *et al*., 2014). These are based on semi-quantitative scoring of the physical appearance or behaviour of an animal, being such an example the murine sepsis score (Shrum *et al*., 2014), further explained.

Improvements on finding biomarkers signalling non-recovery points that can be used as humane endpoints are essential to achieve a more ethical treatment of animals used in sepsis research. Recently, some studies have suggested using body temperature monitoring to both assess sepsis progression and to predict death in a mouse model of CPL-induced sepsis (Mai *et al*., 2018).

4.1. Modified Murine Sepsis Score

The murine sepsis score (MSS) was first validated by Shrum *et al*. (2014) in a fecal-induced peritonitis (FIP) model. A modified version of MSS was later validated in a CLP model (Mai

et al., 2018). This score focuses on six criteria: appearance, level of consciousness, activity, response to a stimulus, eyes, and respiration quality (Table 1). It is a reliable score to be applied consistently and independently to animal models of sepsis. The high sensitivity for predicting the onset of severe sepsis and death makes it an ethical alternative to death as an endpoint in the experimental model of sepsis. However, while it appears to be a clinically relevant method for monitoring endpoints, it is not an ideal method as it heavily relies on a subjective assessment and the on individual opinion of the researcher.

Table 1. Modified Murine Sepsis Score parameters. (adapted from Mai *et al*., 2018)

Murine Sepsis Score (MSS)				
Score	**0**	**1**	**2**	**3**
Appearance	Smooth Coat	Slightly ruffled fur	Majority of fur on back is ruffled	Piloerection, puffy appearance
Level of consciousness	Active	Active, avoids standing upright	Active only when provoked	Non-responsive, even when provoked
Activity	Normal	Supressed eating, drinking or running	Stationary	Stationary, even when provoked
Response to stimulus	Normal	Slowed response to auditory or touch stimuli	No response to auditory, slowed response to touch	No response to touch stimuli
Eyes	Open	Not fully open, potentially secretions	Half closed, potential secretions	Mostly or completely closed
Respiration quality	Normal	Periods of laboured breathing	Consistently laboured breathing	Laboured breathing with gasps

4.2. Humane endpoint determination by temperature monitoring

In a clinical setting, and similarly to what is observed in all deregulations of systemic inflammation, sepsis is often accompanied by pronounced changes in body temperature, either as hyperthermia (fever) or hypothermia. However, whether hypothermia can serve as a predictor of the outcome in clinical sepsis remains unknown (Rumbus *et al.*, 2019). A similar scenario is found in experimental sepsis in mice. After CLP sepsis induction, animals present

signs of progressively severe hypothermia within a few hours, the same occurring in a model of a high injection of an endotoxin (Remick *et al.*, 2000).

Body temperature measurements have been shown to be an effective, clinically relevant method for monitoring disease progression and signalling non-recovery stages (Mei *et al*., 2018) and thus potentially applicable to identify CLP-induced sepsis (Mai *et al*., 2018; Laitano *et al*., 2018). Body temperature monitoring might hence be a reliable replacement for more subjective endpoints in future mouse CLP studies.

Aim

The purpose of this study was to investigate if a low-temperature cut-off point could predict non-recovery stages, which can be used as a proxy to spontaneous death or moribund stage, in a murine surgical model of sepsis.

Materials and methods

1. Animals and care

This study involved two different animal models of septic shock by cecal ligation and puncture, of different severity. The most severe model was carried out in both male and female C57BL/6 WT (wildtype) mice, while the less severe model was carried out on knockout (KO) mice on a C57BL/6 background. Spα knockout models were generated by CRISPR/Cas9 engineering through insertion of three stop codons in one of the first exons of *CD5L* gene) and control wild-type mice. Overall, 51 C57BL/6 mice were intended to be used during the experiment: 27 mice for the mid-grade severity sepsis model and 24 for the severe sepsis model. However, due to time restrictions and other constraints caused by the 2020 covid-19 pandemic, only partial results from 8 mice for the mid-grade severity sepsis model and 18 for the severe sepsis model are included for the purpose of this dissertation.

All animal work was performed at the i3S Animal Facility. The procedures were performed by trained people duly licensed by the Direcção Geral de Alimentação e Veterinária (DGAV). The project ran under a project license approved by the i3S Animal Welfare and Ethics Body and the Competent Authority (DGAV project license 009951/2018-05-17), following the Decree-law 113/2013, which transposes the 2010/63/EU Directive.

As a measure to promote transparency and higher reproducibility of results, this study was pre-registered on the Animal Study Registry (www.animalstudyregistry.org) (DOI: 10.17590/asr.0000206), time-stamped on January 27th 2020 (and currently under embargo until publication of full results).

Animals were group-housed in single-sex groups in type II polycarbonate cages (268 x 215 x 141 mm; floor area: 370 cm2), with absorbent autoclaved bedding, nesting material, and a cardboard tube. The temperature at the animal facility was maintained between 21-22ºC and the humidity between 50-60%. The animals had *ad libitum* access to autoclaved food pellets and water. Mashed humid food was provided to animals unable to reach the food hopper and fortifying supplement (Amina-Strath®) was supplied to animals presenting overt anaemia.

2. Experimental Design

This experiment places great emphasis on the 3Rs principle of *Refinement*. Moreover, in adherence to the principle of *Reduction*, rather than animals being used for the sole purpose of this scientific objective, we gathered data from animals that were already planned to be used on an ongoing research project lead by the Cell Activation & Gene Expression group, at the i3S. The experimental design for this project had therefore to be compatible with the scientific objectives of the project in which the immunobiology of sepsis was studied, namely:

a) To compare the response to mid-grade severity model of experimental sepsis between WT and KO mice;

b) To compare the response to the high-grade severity model of experimental sepsis between untreated WT mice and WT mice treated with an experimental compound (which cannot be revealed for confidentiality reasons, and to which our group was blinded).

This project was therefore also divided in two separate studies, each to follow the disease progression in each of the experiments of the i3S Cell Activation & Gene Expression Group project. Given that a CLP is a surgical model, few animals can be involved at a time, for practical reasons. Hence, each experiment was divided into three cohorts of animals, following a randomised block design, with each cohort corresponding to a block.

Experiment A- In this experiment, 18 mice were used. These animals underwent surgical induction of high-severity experimental sepsis. Three animals per condition per block were used (N= 9 animals per block):

- 3 CLP, treated mice (N=3);
- 3 CLP, untreated mice (N=3);
- Sham-operated mice, wherein the cecum was exteriorized and returned to the abdominal cavity neither ligated nor punctured (N=3).

Experiment B- In this experiment, 8 mice were used. These animals underwent the mid-grade experimental sepsis model. Two animals per sex per genotype per block were used (N=8 animals per block), all undergoing CLP. For each block:

- Male wild-type mice (N=2);
- Male knockout mice (N=2);
- Female wild-type mice (N=2);
- Female knockout mice (N=2).

We followed disease progress for each cohort/block, by assessing body temperature (by three different methods, and later just two), weight variation, general locomotor activity, and other clinical signs, registered into a clinical score sheet for surgical models in use at the i3S Animal Facility.

3. Surgical protocol

3.1. Anaesthetic induction

Each mouse was placed inside the induction chamber (Figure 4). Once the animal was in the chamber, the oxygen flow was initiated (1.0 L/min) and the vaporizer control was turned to an induction level of 5% isoflurane. Once anaesthesia was achieved (as identified by loss of righting reflex), the isoflurane flux in the chamber was stopped and the camera was flushed with oxygen before the animal was removed to prevent exposure to anaesthetic gases.
The anaesthetized animal was placed on a pad with an anaesthetic face mask, with 0.5 L/min Oxygen and 3% isoflurane concentration. Anaesthetic depth was identified by absence of response to a pinch in a hind foot (e.g. no flexion of an extremity).

3.2. Tags implementation

Glass-coated, 2.1mm x 13mm thermosensitive PIT tags, (Biomark® BioTherm13), shown in Figure 5B, were preloaded into a 12 Gauge needle, and implanted subcutaneously in anaesthetised animals prior to CLP, while in a supine position. The tags were implanted into the loose skin over the interscapular region (Figure 5A). The tag reader (Biomark® GPR Plus) was used to scan the transponders to ensure they were placed correctly. The puncture site was sealed with a small drop of cyanoacrylate-based surgical glue (Vetbond®).

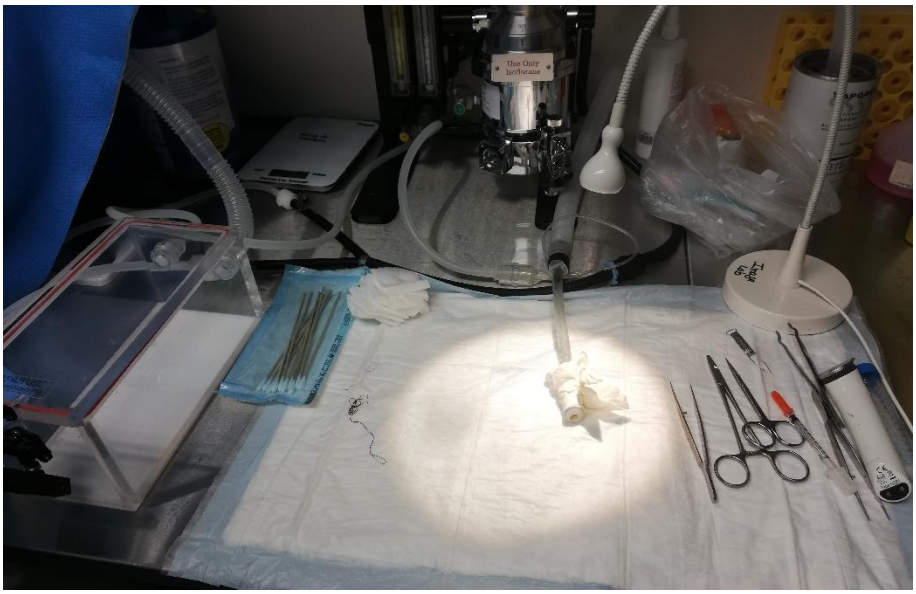

Figure 4. The surgical setup.

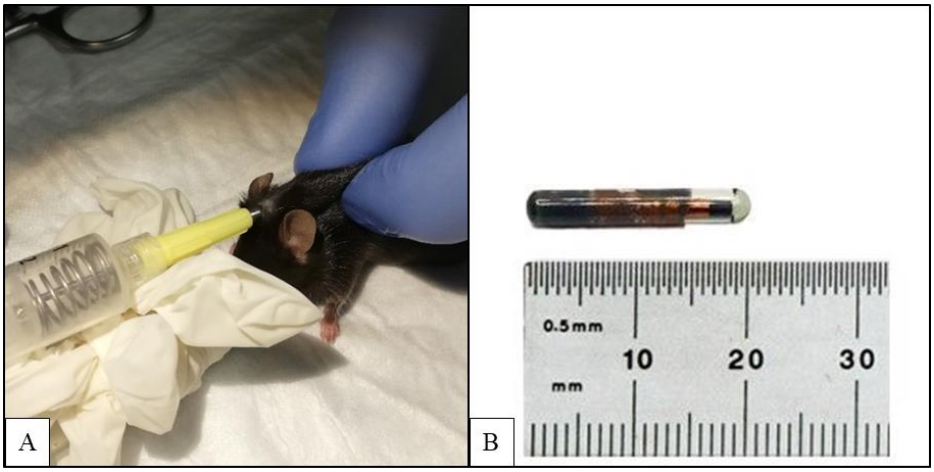

Figure 5. Implementation of the passive integrated transponders (PIT tags). A- Transponder implanted subcutaneously; B- BioTherm13 tag (Source: "BioTherm13." Biomark, www.biomark.com/biotherm13.)

3.3. Cecal ligation and puncture model

The CPL procedure was performed every time by Dra. Liliana Oliveira from the Cell Activation & Gene Expression group, to prevent variability of the data, and according to guidelines by Rittirsch *et al*. (2008). Under anaesthesia, mice were placed on their back over a surgical pad on top of a heating pad. To prevent corneal drying and eye trauma, an ophthalmic ointment was applied on the open eyes of the mice. For preoperative analgesia, buprenorphine was injected subcutaneously (0.08 mg/Kg). The lower quadrants of the abdomen were shaved using an

electric trimmer. The shaved area was disinfected with Povidone-iodine and alcohol at 70%. The last procedure was repeated two more times to ensure the area was adequately disinfected (Figure 6 and 7A).

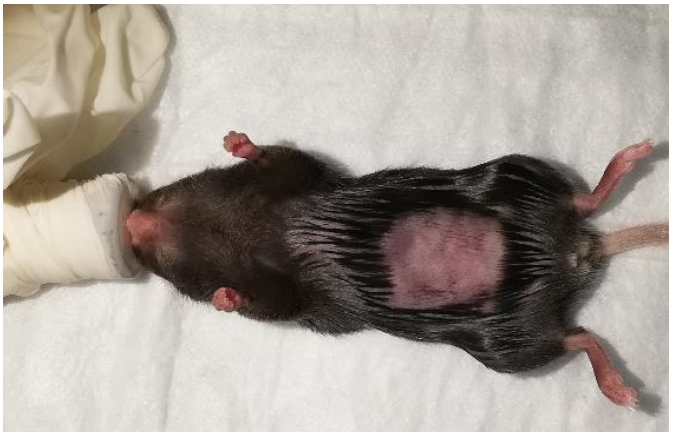

Figure 6. Animal prepared for surgery.

To minimize trauma, a small longitudinal skin midline incision with approximately 1.5–2 cm was made using dissection scissors, with care to avoid reaching the peritoneal cavity. When the skin incision was completed, a midline white fascia structure, the *linea alba*, was grasped with forceps and elevated slightly to separate the abdominal wall from the internal organs. A vertical midline incision was made through the translucent *linea alba* (Figure 7B), which does not bleed due to the scarcity of blood vessels in connective tissue (when compared to the vascularized muscle tissue). This enables a less painful recovery from surgery.

After the intermuscular, fascial, and peritoneal layers were sectioned using blunt forceps, the cecum was located and exteriorized (Figure 7C) with care to avoid moving the small and large bowel more than necessary. With the assistance of a swab, the cecal contents were pushed gently toward the distal cecum to ensure there was no air or gases trapped inside the cecum (Figure 7D) before ligation. The severity grade is dependent on the position of cecal ligation. In the high-grade severity model, the distance between the distal pole and the ligation basis of the cecum was approximately 70-75% of the cecum (Figure 7E), while for the mid-grade model the portion of ligated cecum was of about 40-50% (Figure 7F). The cecum was then perforated with a single through-and-through puncture equidistant to the tip of the cecum and the ligation site, with a 21 Gauge needle (Figure 7G), being perforated with caution to avoid puncturing blood vessels. Finally, the bowel was returned to the abdominal cavity. The peritoneum, fasciae, and abdominal musculature were closed with a simple continuous polyglycolic acid suture. The exterior skin layer was closed with metallic clips (Figure 7H).

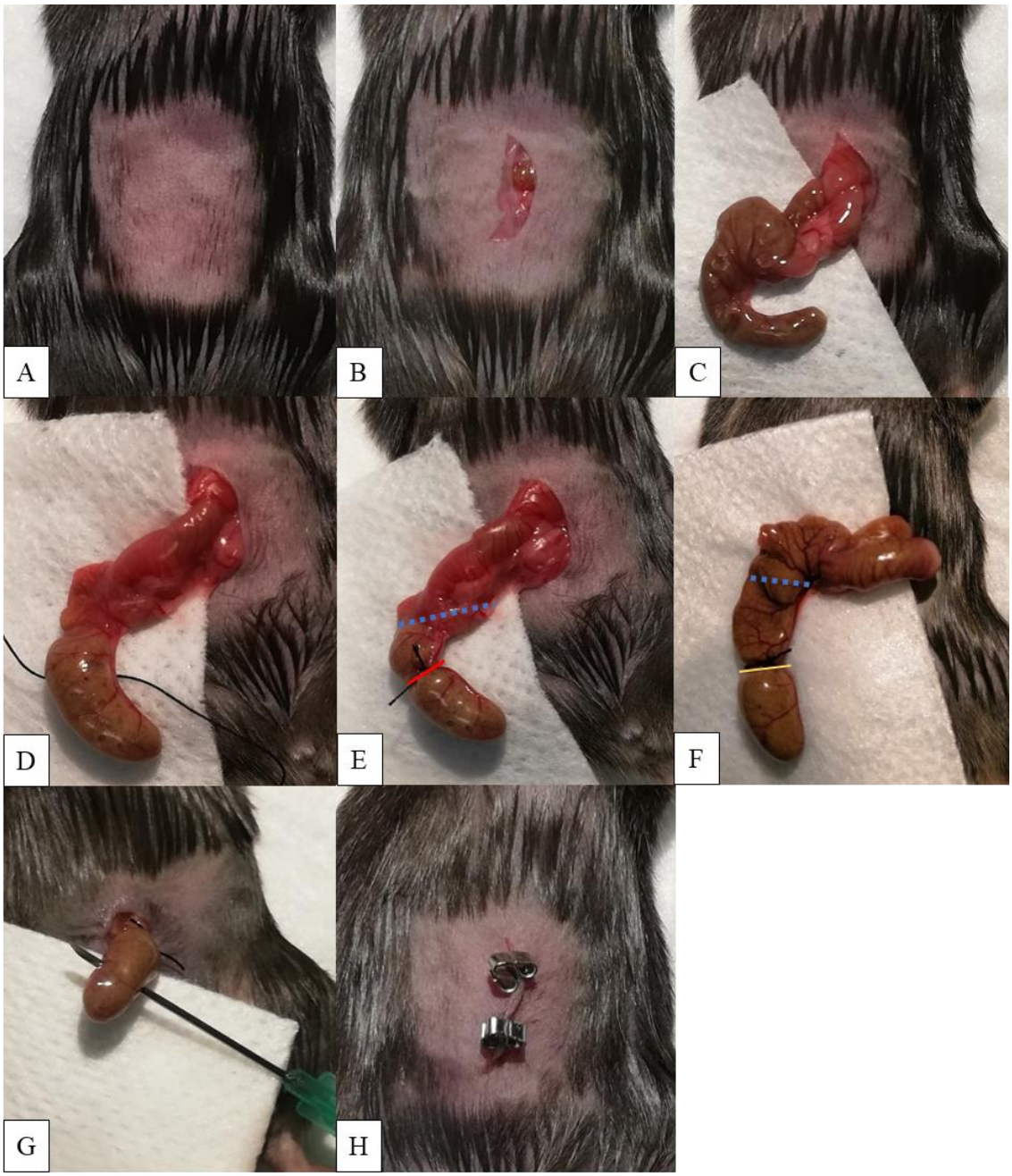

Figure 7. Experimental induction of sepsis by cecal ligation and puncture. A- Disinfected surgical area; B- Longitudinal skin and *linea alba* midline incision; C- Exteriorized cecum; D- Cecal contents pushed toward the distal cecum (indicated by the dotted blue line); E- In the high-grade severity model seventy to seventy-five percent of the cecum was ligated (indicated by the orange line); F- In the mid-grade severity model 40% to 50% of the cecum was ligated (indicated by the yellow line); G- Cecum perforated with a needle; H- Cecum returned to the abdominal cavity and incision site closed.

Pre-warmed saline solution (0,9% NaCl) was injected (5ml/100g of bodyweight) subcutaneously to resuscitate the animals. For postoperative care, buprenorphine analgesia was repeated every 12 hours for 2 days after the surgery. The mice were placed inside a cage over

a heating pad with sheets of paper instead of corn cob bedding until they fully recovered from the anaesthesia. Afterwards, animals were placed in their original cage. The cages were kept on a heating pad for the whole experiment.

4. Animal Monitoring

4.1. Monitoring scheme

After surgically-induced sepsis, body temperature was monitored at fixed time-points. The animals induced with the severe model were monitored four times a day, whereas the mice induced with the less severe model were monitored three times a day. In both cases, they were monitored for 10 consecutive days. Monitoring frequencies were higher for the first batch of animals to have an understanding of variation dynamics, and afterwards defined for the aforementioned schedule as result of observed temperature change patterns. Three different methods were originally used to assess the course of the disease progression (Figure 8):

- Thermal image acquisition of animals in the cage;
- Assessment of subcutaneous dorsal temperature from thermosensitive PIT-tags;
- Tail temperature assessed by a single-point infrared thermometer.

Due to the imprecision of tail temperature measurements, and obvious impact of restraining on temperature, this method was later interrupted. Weight, body condition score, and other clinical signs (e.g. posture, activity) were assessed at each monitoring session. For the clinical score, the i3S animal facility clinical score sheet for surgical models was used. The parameters considered for the clinical surgery score sheet are:

- Bodyweight;
- Appearance;
- Behaviour;
- Hydration;
- Respiratory Movement;
- Wound status;
- Auto-mutilation.

The animal facility does not allow death as an endpoint. The humane endpoint protocol was thus based on a predefined score (scoring 10 points or higher on the surgical score sheet) or reaching bodyweight loss higher than 20%, regardless of clinical score. Animals reaching the humane endpoint were euthanized by CO_2 asphyxiation.

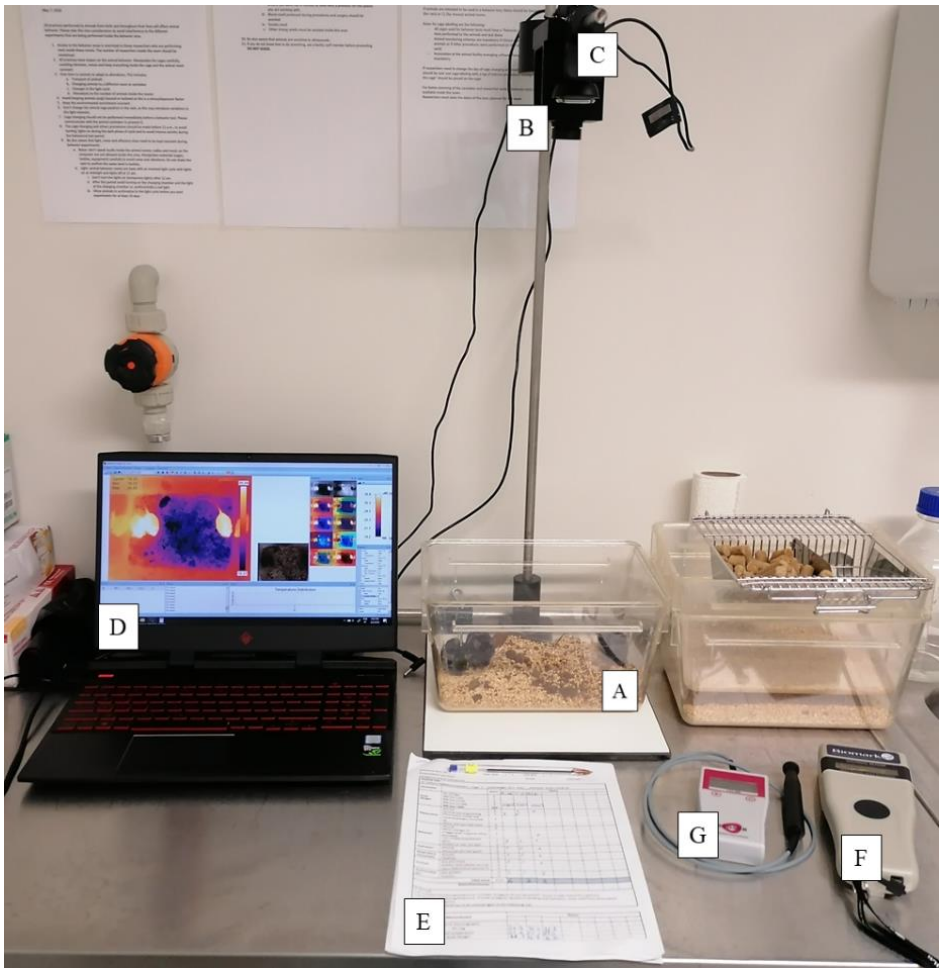

Figure 8. Set-up for the animal monitoring. A- Cage with the animals; B- Thermal camera; C- Visible camera; D- Thermal images captured; E- Score sheets; F- PIT-tags reader; G- Infrared tail thermometer.

4.2. Thermal camera accuracy assessment

Before using the thermal camera, it was necessary to confirm its accuracy. To accomplish that, a source of infrared radiance, continuous and predictable, *i.e.* a perfect blackbody, was used as an ideal radiator for that purpose. This instrument was kindly provided by Prof. Joaquim Gabriel, from the Faculty of Engineering of the University of Porto.

The thermal camera was placed in front of the irradiating blackbody, at a constant temperature of 30°C. One image (Figure 9) per minute for one hour was obtained for three separate tests. Images were uploaded into the Thermal Expert® Analysis Tool Software to obtain an average of the temperature in the centre of the circle (Figure 10).

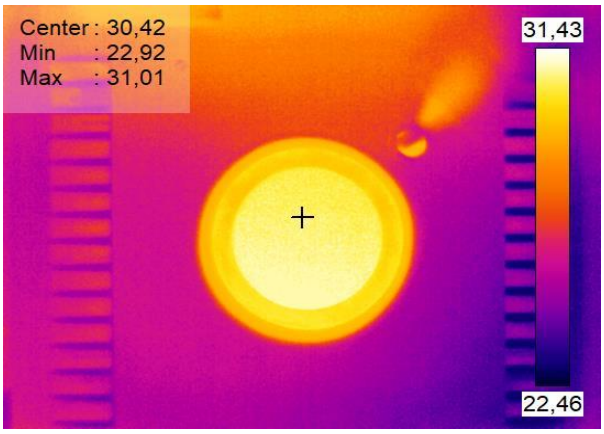

Figure 9. Thermal image obtained from the blackbody.

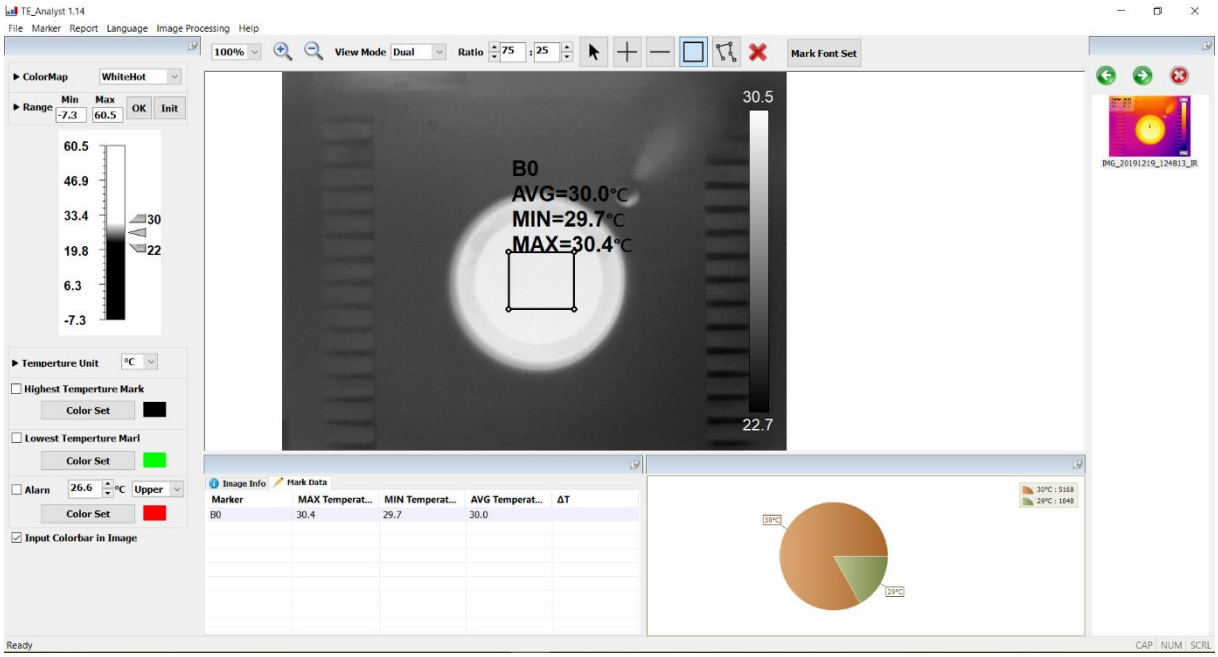

Figure 10. Thermal Expert® Analysis Tool interface.

A scatter plot was obtained from the averages of each image (Figure 11). The graphs obtained specified the amount of time, after the camera was switched on that it took for read temperature to stabilize at 30°C. It was determined that a ~45 min warm-up period was necessary for readings to stabilize, and that the camera is offset from target temperature ~0.5 ºC.

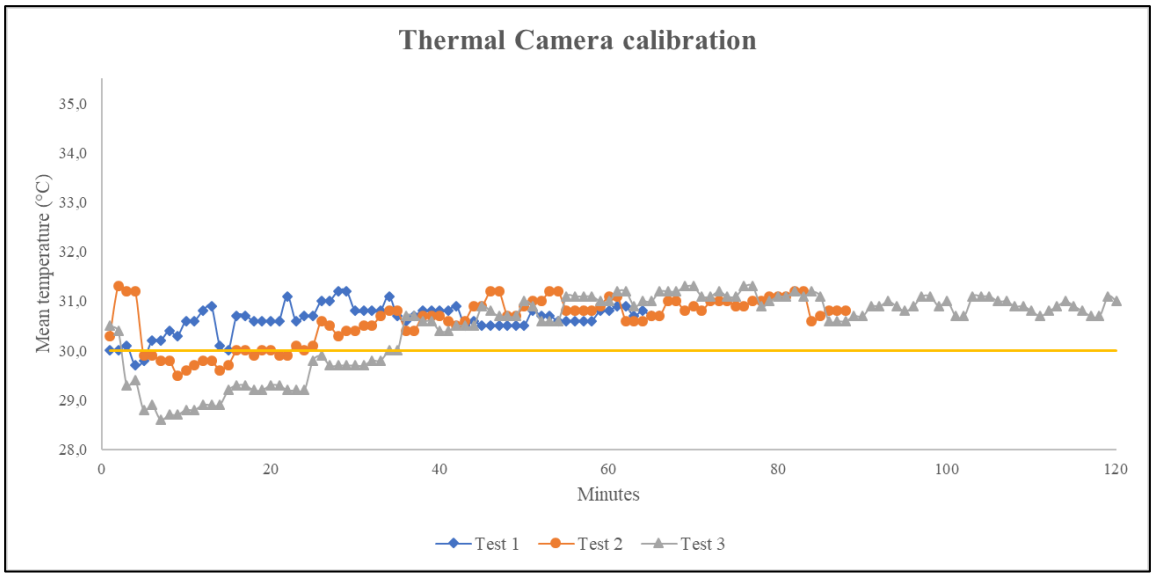

Figure 11. Mean temperature of each image during the warm-up period.

4.3. Thermal images acquisition

An infrared thermal camera ('Thermal Expert' TE-EV1 Camera) and a visible camera (Microsoft LifeCam HD-3000) were fixed on a specially devised structure, for a birds-eye perspective (Figure 12). Both cameras were set-up 59.4 cm from the table. The camera was switched on in the morning, approximately 40 min before the first measurement. It was left running during the whole day. Thermal Expert Q1 1.8.1. software was used to capture the thermal images.

Figure 12. Equipment setup for the infrared and visible camera

One single mouse was removed from the home cage and placed in a clean cage, placed under the cameras, for thermal image collection (Figure 14A and 14B). Three images were captured for each measurement.

The thermal images obtained were analysed by a dedicated software for automatic, high-throughput mean body surface temperature (MBST) estimation (Figure 14C) developed by the Neuroengineering and Computational Neuroscience group: *ThermoLabAnimal* (Figure 13). The images were analysed with automatic segmentation.

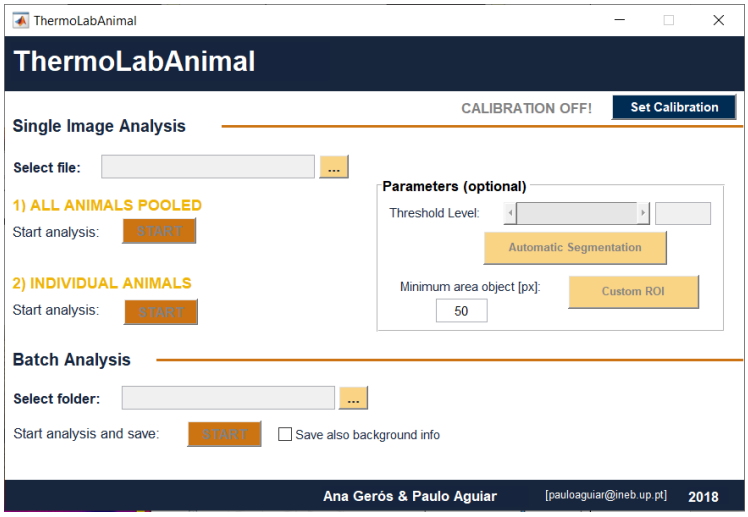

Figure 13. Graphical user interface of the ThermoLabAnimal software.

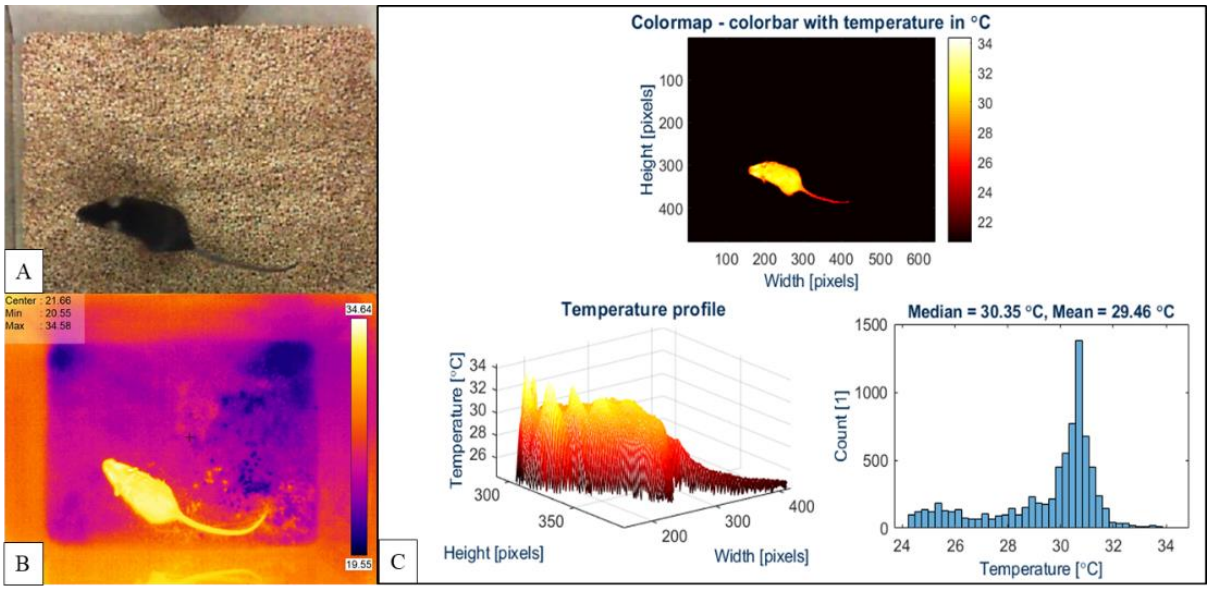

Figure 14. Analysis of the thermal images obtained. A- Image of an individual animal obtained with the visible camera; B-Thermal image of an individual animal; C-Thermal analysis for the individual animal.

4.4. Subcutaneous dorsal temperature from thermosensitive PIT-tags

The aforementioned implanted PIT-tags were read (Figure 15) with a digital tag scanner (Biomark® GPR Plus). These measurements were taken immediately after the individual thermal images. The scanner was kept as close to the animal as possible to ensure it was within range (<10 cm). Once a tag was detected, an audible beep along with the ID number and temperature were displayed on the screen signalling tag was successfully scanned.

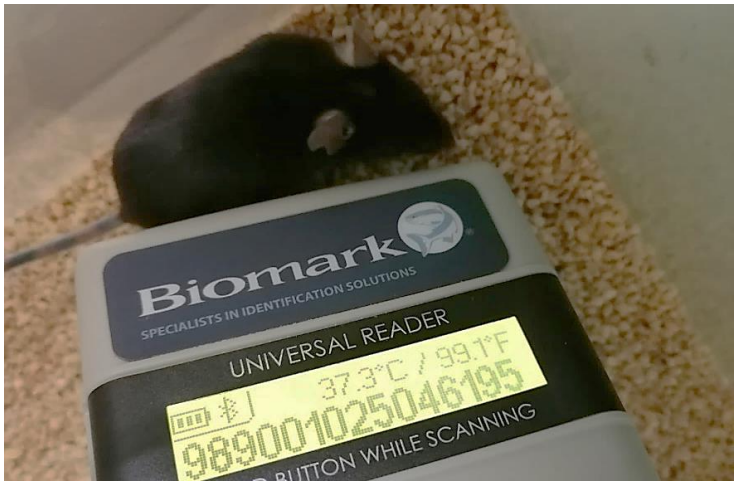

Figure 15. Subcutaneous dorsal temperature from thermosensitive PIT-tags reading.

4.5. Tail temperature assessed by a single-point infrared thermometer

Tail temperature was assessed after the aforementioned individual measurements. The single point infrared thermometer (153-IRB Bioseb® Infrared Thermometer) (Figure 16A) was placed on the lateral side of the tail, near the lateral tail vein to obtain tail temperature (Figure 16B), as temperature would rise substantially within seconds of containing the animal, likely due to a hyperthermic stress response (Adriaan *et al.*, 2007; Gordon *et al.*, 2012; Hankenson *et al.*, 2018). This method was hence found to be too inaccurate and unreliable and its use was thus discontinued.

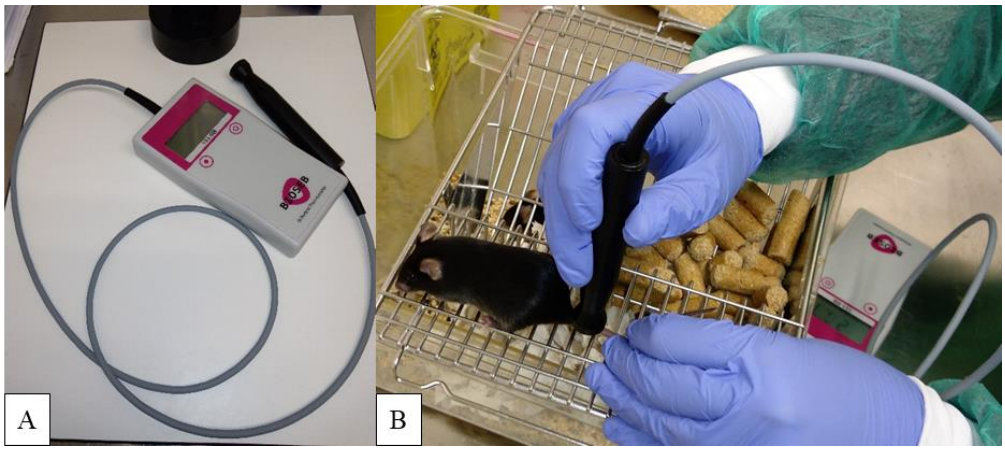

Figure 16. Tail temperature assessment by a single-point infrared thermometer. A- Infrared thermometer used for the measurements; B- Tail temperature measurement

5. Statistical Analysis

5.1. Sample size calculation:

Since the study is exploratory, the Resource Equation for sample size estimation was considered an acceptable approach, as further explained below, for our randomized block design. The overall sample size of N=24 for the more severe model and N=27 for the study on the less severe model allow identifying differences between a 75% sensitivity (or specificity) and a sensitivity of 50% or below as significant, by a signed ranks test, with 80% power (β=0.2) and α=0.05. A sensitivity/specificity of 50% (or below) corresponds to the null hypothesis, since it means cut-off points would not have better predictive value than the toss of a coin. In any case, the expected high prevalence of the condition (in this case, animals dying of sepsis) allowed relatively small samples to be sufficient.

The Resource Equation, for a blocked design, is as such:

$$E = (N-1)-(B-1)-(T-1)$$

E represents error degrees of freedom, "N" is the number of experimental units (in this case animals), "B" the number of blocks, and "T" the number of treatments. According to Mead (1988), cited in Festing (2014), E should be between 10 and 20. For the "less severe model" study, we have two treatments (genotype and sex), three blocks (three cohorts of animals done in different weeks). If we use 2 animals per sex per genotype (i.e. 2 male wildtype, 2 male

knockout, 2 female wildtype, 2 female knockout) per block, we have an N=24, so the Resource equation becomes:

$E = (N-1)-(B-1)-(T-1) \Leftrightarrow E = 23 – 2 – 1 \Leftrightarrow E = 20$

For the study on the more severe model, there were also three blocks/cohorts (B=3), three treatment groups (T=3), and three animals per group, in a total of 27 animals.

$E = (N-1)-(B-1)-(T-1) \Leftrightarrow E = 26 – 2 – 1 \Leftrightarrow E = 23$

It is relatively high (> 20), but still reasonable, since early deaths are common.

5.2. Primary statistical analysis

The predictive value of each of these parameters was compared, based on their ability to signal non-recovery, for each of the CLP models. Different tentative cut-off points were compared, for each method, as regards their sensitivity, specificity, precision, and accuracy following the method by Hendriksen (2011). Different tentative thresholds for number of survival days are defined (to determine which could indeed be deemed “surviving animals”), as animals were likely to die from secondary causes, such as severe anaemia, if not treated. The ideal cut-off point is then determined by ROC (receiver operator curve) analysis, based on the higher value for Youden’s J index.

$J = \text{sensitivity} + \text{specificity} – 1 \Leftrightarrow J = TP/(TP+FN) + TN/(TN+FP) – 1$

TP – True positives (mice reach cut-off value and die or have to be euthanized)
TN – True negatives (mice do not reach cut-off value and survive)
FN – False negatives (mice do not reach cut-off value but die or have to be euthanized)
FP – False positives (mice reach cut-off value but do not die or need to be euthanized)

Results

1. Mortality

During the 10-day study period (Figure 17) the severe model of CLP resulted in 58,3% mortality and the mid-grade severity model of CLP resulted in 50% mortality. During that same period sham surgery resulted in 0% mortality.

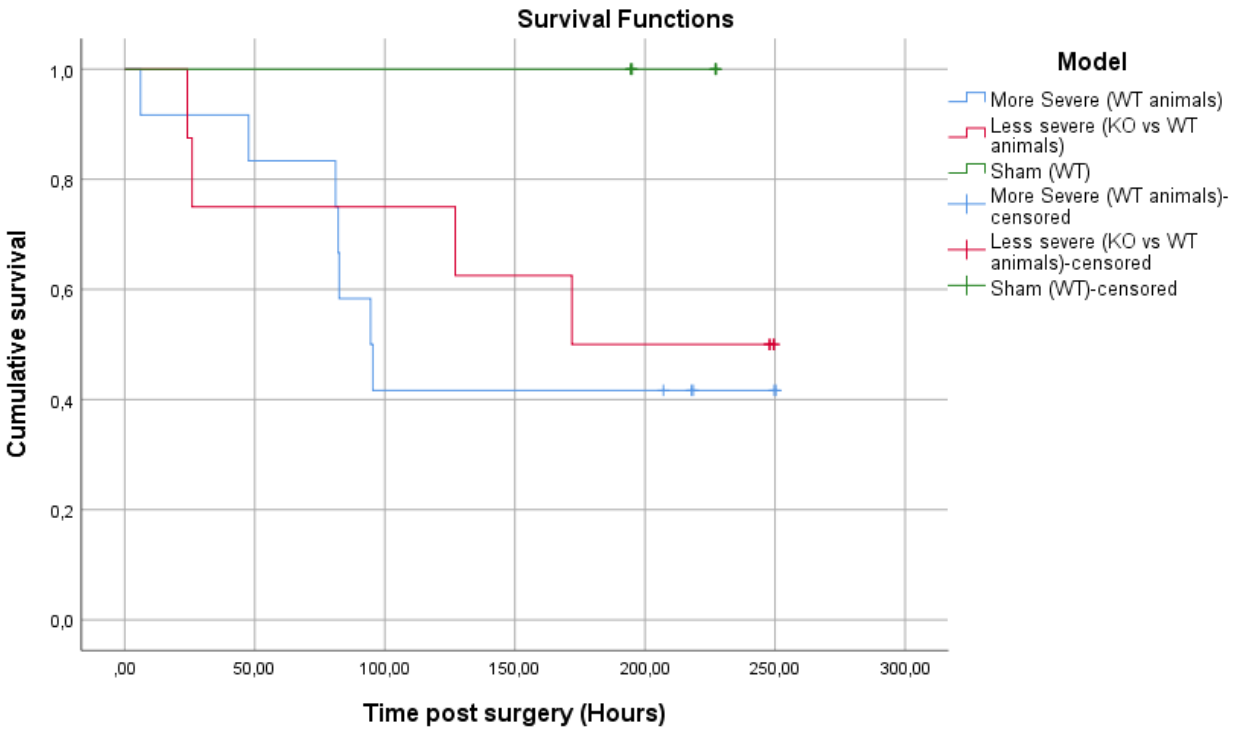

Figure 17. Kaplan-Meier survival curves for wildtype (WT) and knockout (KO) mice submitted to severe CLP, mid-grade CLP and sham surgery, 10 days after surgery. WT Mice that underwent severe cecal ligation and puncture (CLP) received treatment with a saline solution (n= 6) or an undisclosed experimental compound (n=6). WT mice submitted to sham surgery (n=6) received no treatment. KO (n=4) and WT (n=4) mice that underwent Mid-grade CLP received no treatment.

2. Body temperature monitoring

2.1. MBST and SCT - comparison by outcome

A decrease in both mean body surface temperature (MBST) and mean subcutaneous temperature (SCT) was observable in CLP-induced animals to which a humane endpoint was applied, as compared surviving up to the 10-days observation period (Figure 18), in particular in the last measurements before endpoint. The mean MBST of severe CLP-induced animals (Experience A) was the lowest at the 17th measurement (27.2°C; 48 hours post-surgery), with SCT also being the lowest (below 30°C, the lowest end of the PT tag reading range) for the same time-point. Overall, temperature of the animals reaching the humane endpoint appears to be lower than that of the animals that survived, although apparently more prominent for subcutaneous temperature. Statistical significance of mean differences was not calculated for any of the parameters, as only a subset of the sample size is presented, and statistical power had been calculated for the whole sample.

A similar trend appears to be observable for the mid-grade CLP model (Experience B). The mean temperature of the animals reaching the humane endpoint was lower than for the surviving animals, for both SCT and MBST, despite these animals recovered after a decrease in mean temperature, unlike what was observed for the severe CLP models.

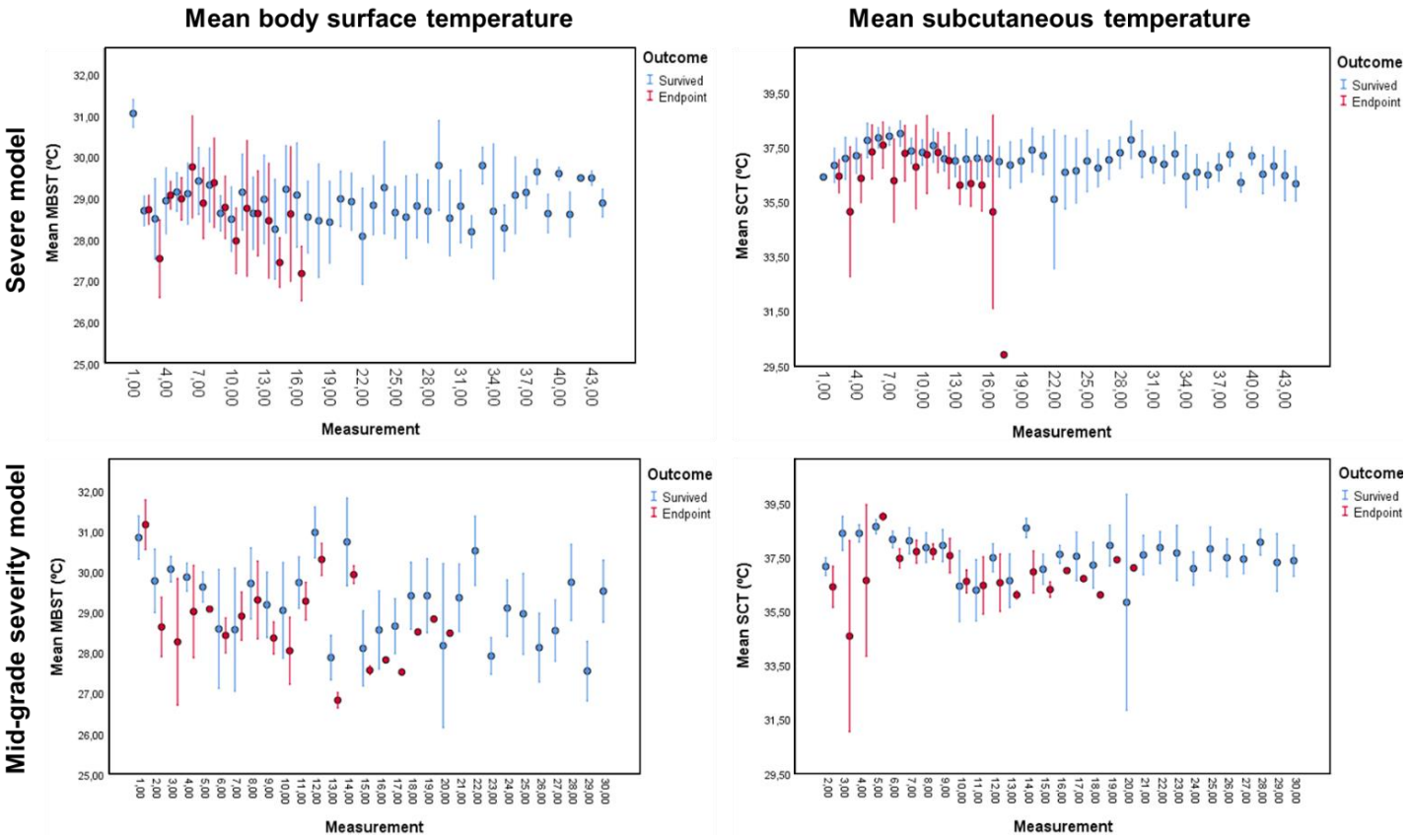

Figure 18. Mean body surface temperature (MBST) assessed by a thermal camera (left column) and subcutaneous temperature (SCT) from PIT-tags readout (right column), after severe model of CLP (top row) and mid-grade severity model of CLP (bottom row), according to survival outcome: survivors *vs.* endpoint (animals reaching humane endpoint). Wildtype (WT) mice (n=12) underwent severe CLP and sham surgery (n= 6). Measures of MBST and SCT were obtained four times per day for 10 days. From all these animals, 11 survived and 7 reached their endpoint. Knockout (KO) mice (n=4) and WT mice (n=4) underwent mid-grade CLP. Measures of MBST and SCT were obtained three times per day for 10 days. From all these animals, 4 survived and 4 reached their endpoint. Data is presented as mean ± SD.

2.2. MBST and SCT - comparison by group

A decrease in both mean body surface temperature (MBST) and mean subcutaneous temperature (SCT) was observable in CLP-induced animals when compared to animals submitted to the sham surgery (Figure 19).

Across the whole experiment, the animals subjected to the CLP model of sepsis presented lower mean temperatures and more abrupt variations than the animals subjected to sham surgery.

The animals subjected to the mid-grade CLP model did not have a sham group to be compared to, although the abrupt variations of temperature are still clear in the graphs, across both measurement modalities.

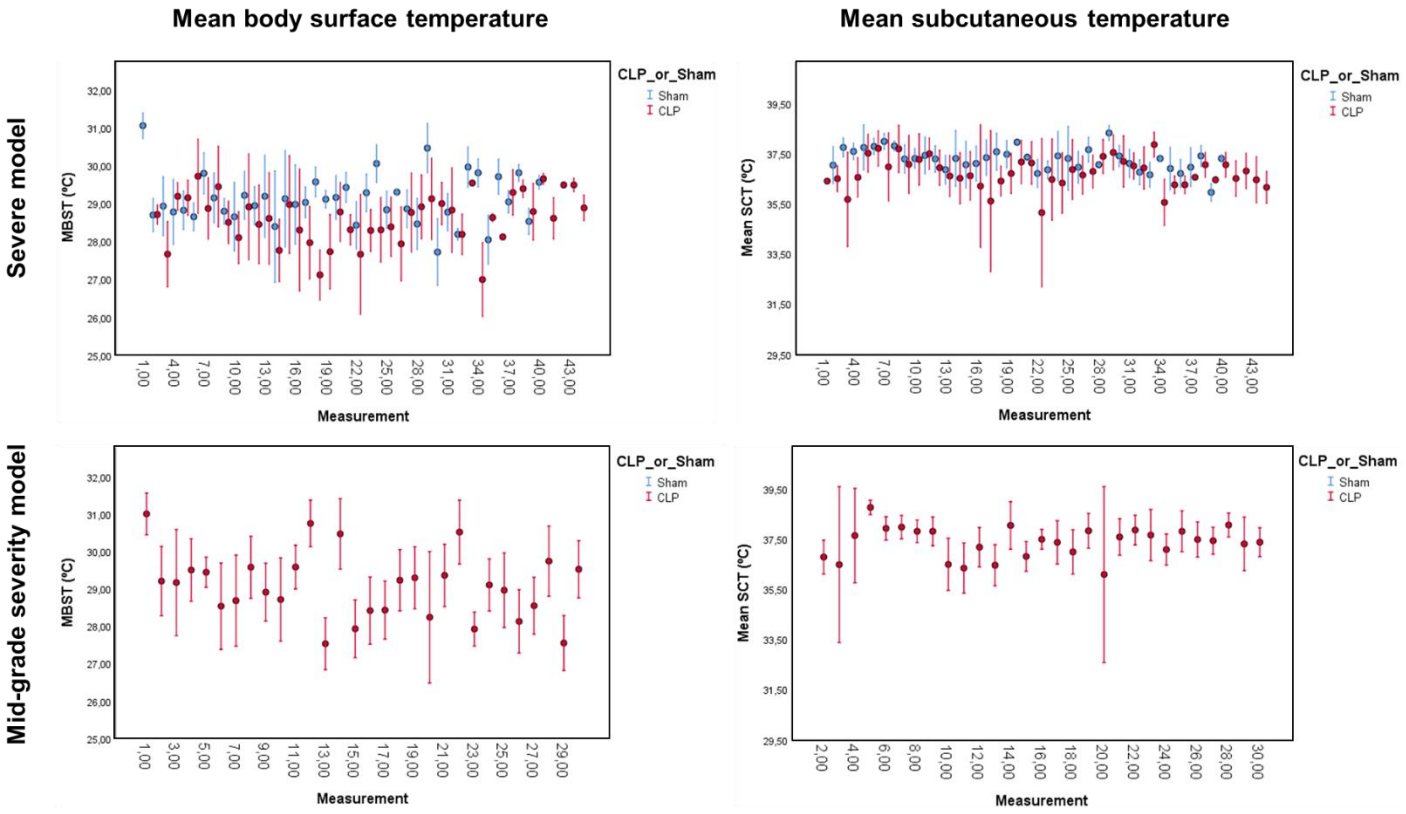

Figure 19. Mean body surface temperature (MBST) assessed by a thermal camera (left column) and subcutaneous temperature (SCT) from PIT-tags readout (right column), after severe model of CLP (top row) and mid-grade severity model of CLP (bottom row), according to the group. Wildtype (WT) mice (n=12) underwent severe CLP and sham surgery (n= 6). Measures of MBST and SCT were obtained four times per day for 10 days. Knockout (KO) mice (n=4) and WT mice (n=4) underwent mid-grade CLP, although in this group no animals underwent sham surgery. Measures of MBST and SCT were obtained three times per day for 10 days. Data is presented as mean ± SD.

2.3. Correlation between thermal assessment measures

As expected, MBST and SCT were found to correlate and predict each other significantly (linear regression analysis: Z=202.5; p<0.001), albeit not strongly (R = 536; R^2 = 2.87). The regression curve is presented on Figure 20.

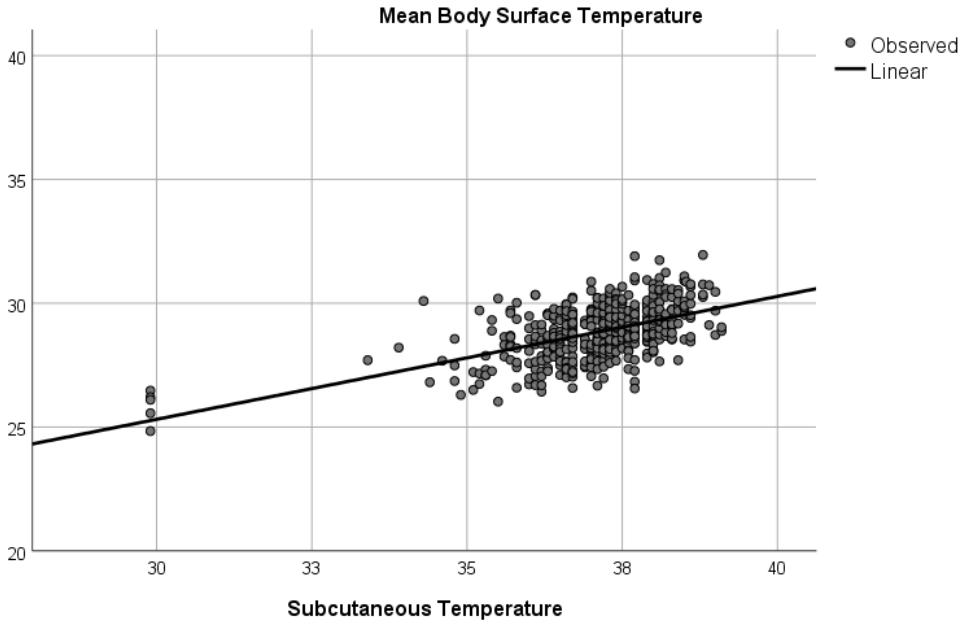

Figure 20. Linear regression curve for subcutaneous temperature (SCT) from PIT-tags readout and mean body surface temperature (MBST) assessed by a thermal camera MBST.

3. Body weight loss

3.1. Body weight loss - comparison by outcome

For the severe model, a progressive and substantial mean weight loss following surgery was observable for CLP-induced animals that ultimately reached the humane endpoint (Figure 21), and as expected the lowest value corresponded with the predetermined threshold (21% loss). Surviving animals also lost weight, though a recovery was observable. The lowest values coincided for both survivors and non-survivors at the 17th measurement (48 hours post-surgery).

As expectable, the lowest mean bodyweight loss for survivors (-9%) was still considerably higher than that of animals reaching the humane endpoint.

For the mid-grade severity model, there was also a steep mean loss of bodyweight for animals reaching the endpoint, with bodyweight being consistently lower than for survivors from 24 hours after surgery, though a few animals non-survivors recovered some of the weight loss before reaching the humane endpoint. There was also a mean loss in body weight (12% loss) in surviving animals until the 11th measurement (80 hours post-surgery), after which they recovered, overall. The lowest mean bodyweight loss (-12%) was never below the one observed in animals reaching the endpoint (Figure 21).

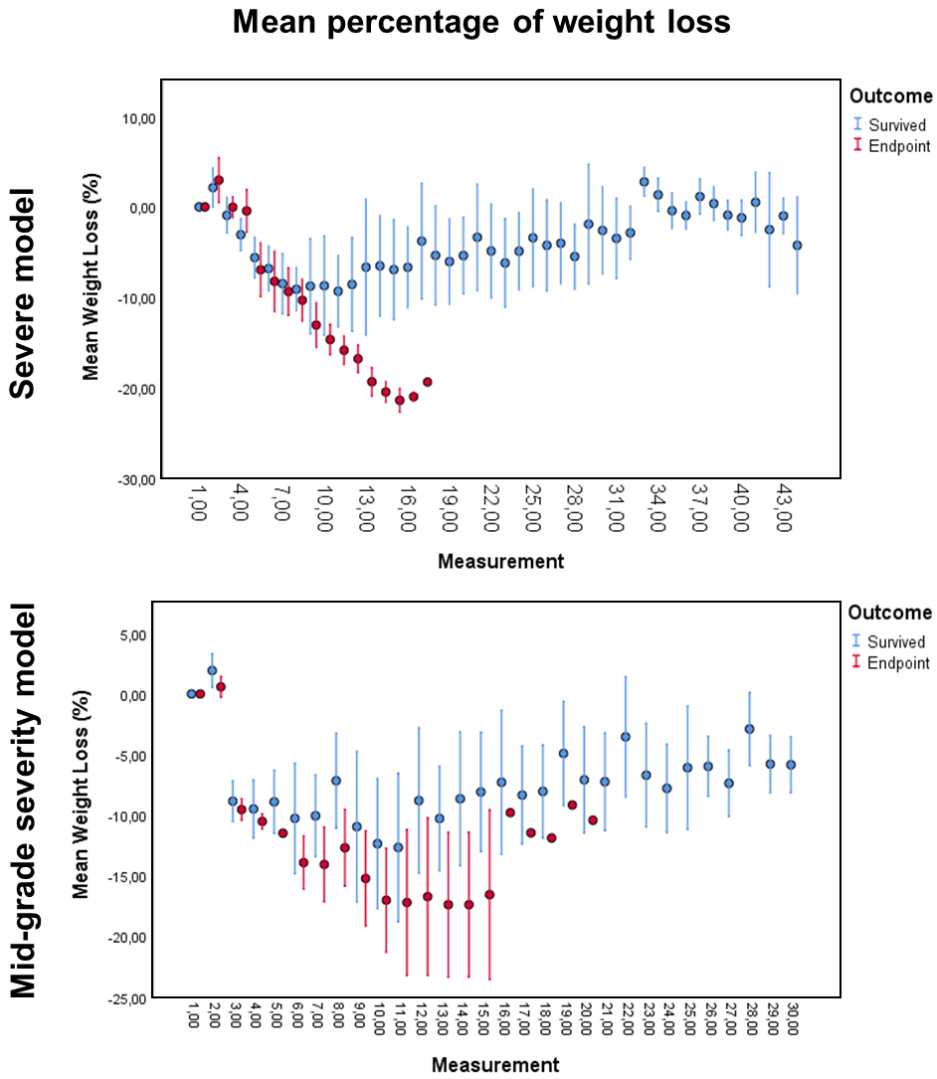

Figure 21. Mean percentage of weight loss after severe model of CLP (top panel) and mid-grade severity model of CLP (bottom panel), for animals with diverging outcomes: survivors *vs.* endpoint (animals reaching humane endpoint). Wildtype (WT) mice (n=12) underwent severe CLP and sham surgery (n= 6). Measures of body weight were obtained four times per day for 10 days. From all these animals, 11 survived and 7 reached their endpoint. Knockout (KO) mice (n=4) and WT mice (n=4) underwent mid-grade CLP. Measures of body weight were obtained three times per day for 10 days. From all these animals, 4 survived and 4 reached their endpoint. Data is presented as mean ± SD. Data is presented as mean ± SD.

3.2. Body weight loss - comparison by group

For the severe model, a progressive and substantial mean weight loss following surgery was observable for CLP-induced animals (Figure 22). Sham animals also lost weight after surgery, though a recovery was observable. The lowest mean bodyweight loss for sham induced mice (-8%) was still considerably higher than that of CLP induced (-15%).

For the mid-grade severity model, there was also a steep mean loss of bodyweight for animals induced with CLP until the 11th measurement (80 hours post-surgery) of 14% after which they recovered, overall.

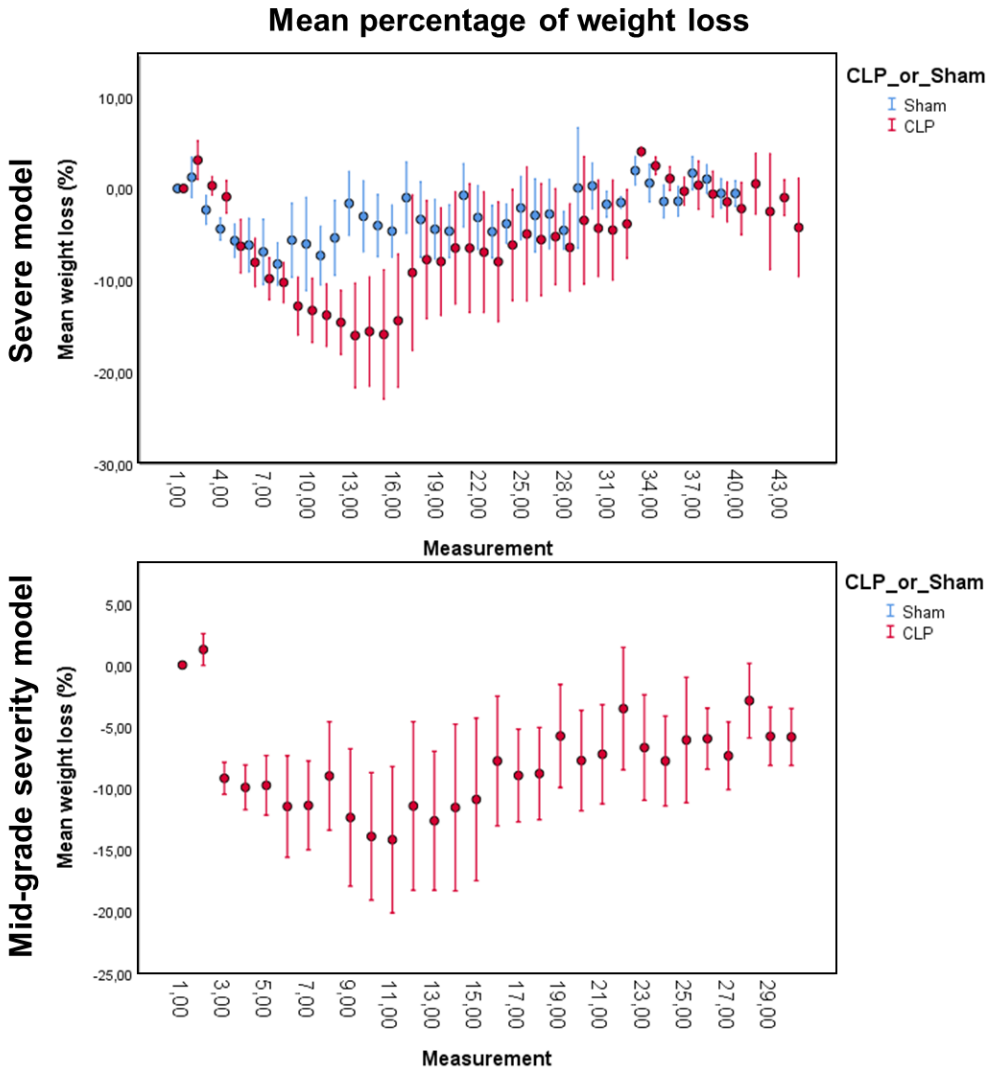

Figure 22. Mean percentage of weight after severe model of CLP (top panel) and mid-grade severity model of CLP (bottom panel), for animals with diverging outcomes: survivors *vs.* endpoint (animals reaching humane endpoint). Wildtype (WT) mice (n=12) underwent severe CLP and sham surgery (n= 6). Body weight was measured four times per day for 10 days. Knockout (KO) mice (n=4) and WT mice (n=4) underwent mid-grade CLP, although in this group no animals underwent sham surgery. Body weight was measured three times per day for 10 days. Data is presented as mean ± SD.

4. ROC Curve analysis

To investigate whether MBST could be used as a surrogate marker of death in the CLP model of sepsis, we carried out a Receiver Operating Characteristic (ROC) curve analysis for both severe and mid-grade severity CLP animals (total N=26), excluding sham-operated mice, as death is not an expected outcome. The criteria were the lowest recorded MBST, the lowest

recorded SCT and the lowest drop in bodyweight, in percentage of initial weight for each animal (Figure 23).

For weight loss, the highest Younden's index was of J= 0,5375, for -20.6% (60% sensitivity, 94% specificity). For MBST, the highest Younden's index was of J=0,250 (50% sensitivity, 75% specificity, for MBST lower than 26,55ºC). For SCT, setting the threshold for 34 ºC (Younden's index J=0.475) for deciding on when to euthanize animals would have a sensitivity of 60% with a specificity of 87,5%. Values for area under the curve are presented in Table 2.

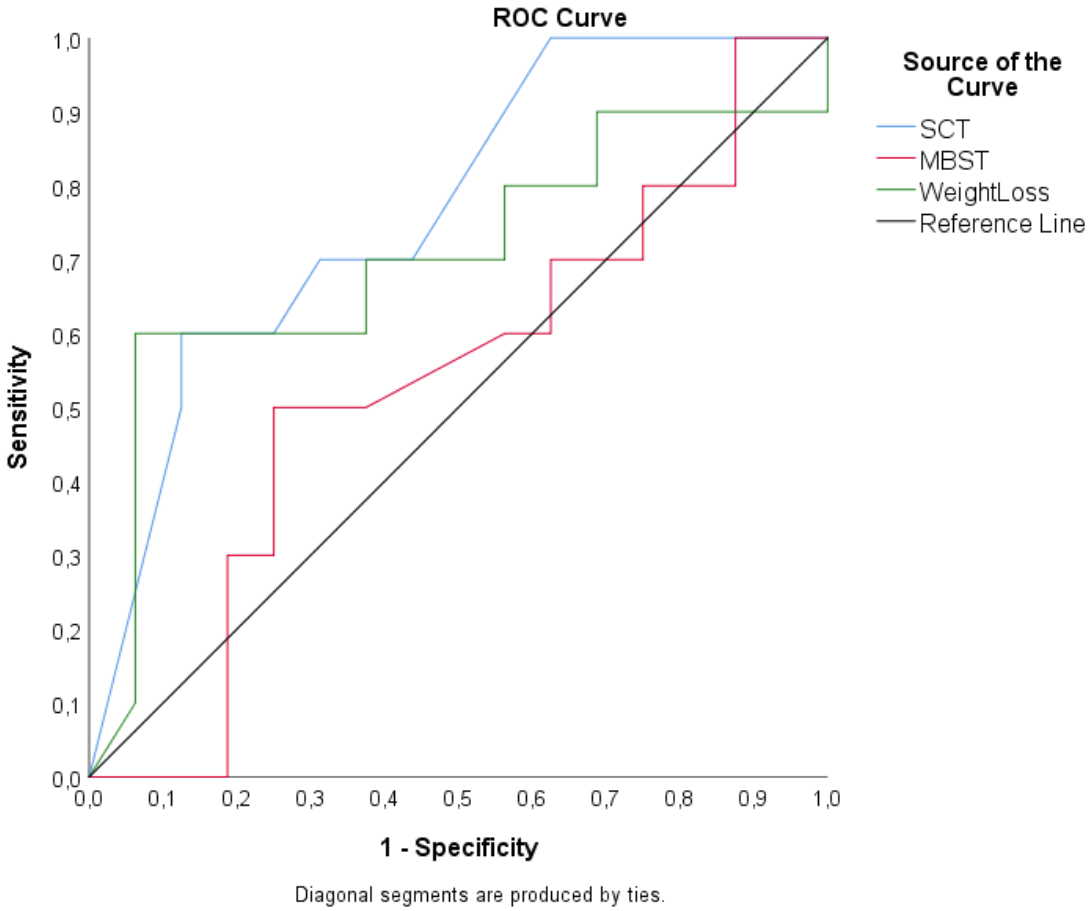

Figure 23. ROC analyses of lowest MBST, lowest SCT and lowest weight as predictors of death in CLP-induced septic mice. The red line represents the reference line for statistical comparison in ROC analyses. The ideal cut-off point was determined by ROC analysis, based on the higher value for Youden's J index.

Table 2. Area under the curve calculated for each of the putative predictors tested. A predictor with an area under 0.5 is typically deemed uninformative.

Variables	***Area***
SCT	0,769
MBST	0,534
Weight Loss	0,703

Discussion

The purpose of this study was to investigate if a low-temperature cut-off point could predict non-recovery stages, which in turn could be used as a proxy to spontaneous death or moribund stage, in a murine surgical model of sepsis. It is worth noting that these results are preliminary, and thus any interpretation of the partial data here presented should be taken with caution.

During the 10-day study period, the severe model of CLP resulted in 58,3% mortality. During that same period, the mid-grade severity model of CLP also resulted in 50% mortality. These results differ from the expected mortality of severe sepsis from the literature. Rittirsch *et al*. (2009) stated that 100% of the mice subjected to severe CLP-induced reached their endpoint within 4 days after the induction, although these authors did not report the humane endpoint applied. According to the same authors, the mid-grade CLP model results in a mortality rate of 60% within 7 days. The differences found in mortality rate, at least for the severe model induced in wild-type animals only could be partly explained by this study incorporating experimental groups receiving an undisclosed putative therapeutic drug. This possibility cannot be further explored without access to the information on which were the control and the treated groups, which will only be made available after analysis of the full sample of all cohorts of animals. As expected, sham-surgery animals had a 0% mortality, in accordance with the literature (e.g. Rittirsch *et al*., 2009).

In animals subjected to CLP, there was an observable decrease in MBST and SCT after surgery, in both models, and for both outcomes. This is consistent with the literature, as Li *et al.* (2018) reported that hypothermia was observed 4 to 32 hours after induction of CLP, with Granger *et al.* (2013) and Mai *et al*., (2018) obtaining similar results, though reporting slight differences in the CLP surgeries (e.g. different needle sizes and percentage of ligated cecum).

Overall, across all the measurements, on both models, the temperature of surviving animals appeared to be higher than the temperatures of the animals reaching the pre-established humane endpoint. Mai *et al.* (2018) also found similar results, with animals reaching the endpoint exhibiting significantly lower body temperature. However, the differences between surviving mice and mice reaching the humane endpoint were not as pronounced. A possible explanation might be found on the heating pad kept under the cage for the whole duration of the experiment. The cage temperature is a contributing factor in differences in temperature between an animal surface temperature and core temperature. Meyer *et al*., (2014) reported that subcutaneous temperature can vary widely, so it can be strongly affected by the ambient temperature. Mei *et*

al. (2018) found that surface temperature measurements have higher degrees of variation and that a significant difference in temperature between core and surface temperature results from cage temperature. As a result, the higher the ambient temperature, the higher the surface temperature, in mice. Variations in surface temperature between studies can result from variations in the ambient temperature. The heating pad kept throughout the whole experiment might have maintained the temperature stable and might even have concealed significant variations in temperature. Although there are small differences, these could be amplified if the heating pad was not kept there through the whole experiment.

Mice subjected to the severe CLP procedure presented lower body temperature across all the measurements when compared to mice undergoing the mid-grade CLP model. A previous study by Mai *et al*., (2018) reported similar findings. Mice subjected to severe CLP had significantly lower body temperature when compared to mice subjected to mid-grade severity CLP.

Animals subjected to CLP presented lower mean temperatures and more abrupt variations than sham-operated animals. In both cases, there was a slight decrease in the first measurements after the surgery, but the sham-treated mice quickly recovered. Ebong *et al.*, (1999) and more recently Safiah *et al*., (2018) obtained similar results. The temperature in animals submitted to sham surgery remained relatively stable, aside the expected circadian variations. Gordan *et al*., (2012) also reported this variability in telemetry and infrared thermography data across the circadian cycle, displaying periods of relative stability over limited times of this cycle. One of the limitations of the measurements from sham-operated animals is that some of the animals removed their PIT Tags. In fact, 67% of these animals had removed their tags by the end of the experiment.

After CLP, there was a decrease in percentage of bodyweight loss in animals submitted to CLP when compared to sham-operated animals. Pugh *et al*. (2017) also reported a similar weight variation. Several animals submitted to the CLP surgery in the present study appeared to recover from the weight loss. This data is consistent with a report from Nemzek *et al.*, (2004) that presented findings from weight loss 9 days after BALB/c mice being submitted to CLP with a 21-gauge needle. The graphs presented in their study showed a similar percentage of weight loss followed by a gradual recovery.

The surface temperature assessed by infrared thermography and subcutaneous temperature were correlated, albeit not strongly. Those findings are contrary to previous studies by Mei *et al*., (2018) and Nemzek *et al*., (2004) that found a correlation between core and surface temperatures using infrared thermometers.

Based on this yet limited data, we found that none of the models appears to be a sufficiently satisfactory model to predict non-recovery stages. However, the 60% sensitivity and 87.5% specificity for subcutaneous temperature below 34ºC suggests this parameter should be further explored. This is further highlighted by Nemzek *et al.* (2004) findings suggesting that temperature could be an early indicator of impending death. Recently Mai *et al.*, (2018) confirmed these findings, suggesting that body temperature measurements are effective, clinically relevant methods for monitoring and predicting non-recovery stages. In any case, our limited sample size does not currently allow making such a categorical affirmation. Also, subcutaneously-implanted PIT tags have limitations, since they cannot read temperatures below 30ºC, which was registered for some animals. This fact possibly affected the correlation analysis, given that 29.9 ºC had to be used as a surrogate value for SCT. Other factors may have affected our data, given that clinical assessment and all the measurements were made in a different room from the one the animals were housed. Carrying the cages in between rooms might have caused increased stress, resulting in stress-induced hyperthermia. Indeed, and as proposed by Zethof *et al.* (1994), stress can cause an increase in temperature from 1 to 1.5°C higher than the baseline within 10 minutes.

Another limitation of the study is the high potential for bias in using weight loss as a predictor, given bodyweight loss higher than 20% is itself one of the criteria of the humane endpoint protocol used, determining that animals reaching that point had to be humanely euthanized, regardless of other clinical signs. Indeed, some animals were euthanized for this reason alone, as they otherwise were in acceptable condition, in terms of posture and behaviour. As such, the level of reliability of this predictor is potentially susceptible to this artefact.

Conclusion

In summary, for the time being mean body surface temperature (MBST) does not appear to be a reliable method to access temperature if cages are to be kept on top of a heating pad for the whole duration of the experiment, *i.e.*, after the animals recovered from surgery. Our limited data does not allow us to conclude that any of the parameters measured can reliably predict (with high sensitivity *and* specificity) non-recovery stages, though subcutaneous temperature shows some promise. The upcoming analysis of the whole dataset may allow a clearer picture of their informative value.

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