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Characterization of cardiovascular autonomic mechanisms during shock resuscitation for an improved functional hemodynamic monitoring

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To Marco and Maria,
my amazing family

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Abstract

Circulatory shock is one of the major complication in critically ill patients with a mortality rate reaching 40%, a high-risk of second line treatments and long term physical and cognitive impairments in survivors. Currently, clinical guidelines are mainly concerned to restore homeostasis and to prevent multiple organ failure, but, despite significant improvements, clinicians are still far to have found the optimal therapy. Patients are commonly treated with fluids and vasopressors to restore a physiological blood pressure, blood volume and oxygenation, thus, the benefit of the resuscitation strategies is evaluated only based upon global hemodynamic, clinical and metabolic end-points. However, the understanding of the root causes is the main issue in order to find new therapy targets, improve drugs administration, and tailor therapies to each individual patient. From this perspective, a different approach to patients monitoring is needed in critical care.

Derangements in cardiovascular control by the autonomic nervous system typically occur during shock, but the restoration of these alterations is not commonly targeted by the clinical resuscitation protocols, although it is determinant for the return to a physiological condition of cardiovascular control.

The present thesis bases on the idea that combining the traditional mean values, as proposed by the current guidelines, with non-invasive indices of cardiovascular function, such as baroreflex sensitivity and heart rate variability, and integrating them into bedside monitoring of shock patients, may be of clinical importance; the aim is to have a functional hemodynamic monitoring to predict a possible catastrophic deterioration of the patient, thus allowing clinicians to act beforehand. The autonomic nervous system operates in the short term range (seconds to minutes) and clinical signs of an ongoing failure could be a wake-up call of an imminent decline, before any deterioration of mean values and global markers, which could occur too late for any effective intervention.

This hypothesis has been explored in three different clinical and experimental databases of septic and hemorrhagic shock collected under

the project ShockOmics. Standard and advanced mathematical indices and models were exploited to investigate the role of the autonomic nervous system in cardiovascular regulation during shock and standard resuscitation. In particular, standard indices include baroreflex sensitivity, heart rate variability, and blood pressure variability; the advanced and novel approaches proposed are based on black-box mathematical models to describe the different contributions in peripheral resistance control and ventricular contractility regulation, and on the 2-element Windkessel model to investigate the characteristic time constant of the arterial tree.

The results showed how the resuscitation maneuvers were able to restore global hemodynamic variables, such as mean blood pressure and circulating volume, but this was not accompanied to a recover of a physiological condition, as highlighted by the cardiovascular indices and models. The shock subject may be considered resuscitated according to clinical guidelines, but the combined information derived from autonomic cardiovascular indices may reveal a persistent autonomic disarray, that, if not appropriately treated, could lead to development of multi organ failure, cardiovascular collapse and death.

In conclusion, clinical protocols for shock resuscitation should give importance to the trends of these proposed indices, in order to make a step further in the optimization and personalization of the therapy.

Sommario

Lo shock circolatorio è una delle maggiori complicanze in terapia intensiva, con un tasso di mortalità che può raggiungere il 40%, un alto rischio di riospedalizzazione e importanti danni fisici e cognitivi a lungo termine nei sopravvissuti. Attualmente, in clinica, le linee guida mirano principalmente al ripristino di una condizione di omeostasi, nel tentativo di prevenire la sindrome di insufficienza multiorgano; tuttavia, nonostante significativi miglioramenti, non si è ancora arrivati ad una terapia ottimale. Nella normale pratica clinica ai pazienti vengono tipicamente somministrati fluidi e vasopressori per ripristinare una condizione fisiologica di pressione sanguigna, volume circolante e ossigenazione; di conseguenza, il beneficio della rianimazione viene valutato solo sulla base di marker emodinamici, clinici e metabolici globali. Tuttavia, la conoscenza dei meccanismi alla base della risposta fisiologica allo shock e alla rianimazione rappresenta la chiave principale per la scoperta di nuovi marker terapeutici, per il miglioramento e la personalizzazione delle terapie per ogni singolo paziente. Da questo punto di vista, diventa necessario un diverso approccio di monitoraggio dei pazienti in terapia intensiva.

Tipicamente durante shock circolatorio si verificano alterazioni nei meccanismi di controllo cardiovascolare mediati dal sistema nervoso autonomo; tuttavia, il ripristino di queste disfunzioni, pur essendo determinante per il ritorno ad una condizione fisiologica di controllo cardiovascolare, non rientra tra i comuni target considerati dai protocolli di rianimazione clinica.

Il presente lavoro di tesi si basa sull'idea che l'integrazione dei tradizionali marker di rianimazione con indici non invasivi di funzionalità cardiovascolare, come per esempio il guadagno di baroriflesso e la variabilità cardiaca, possa rappresentare un importante sviluppo clinico nel monitoraggio dei pazienti in shock. L'obiettivo ultimo è quello di un monitoraggio emodinamico funzionale che possa prevedere un possibile imminente deterioramento del paziente, permettendo così ai medici di agire in anticipo. Infatti, il sistema nervoso autonomo agisce in finestre temporali di breve durata (da secondi a minuti), pertanto una disfunzione

autonomica durante rianimazione potrebbe essere un campanello d'allarme di un imminente declino della condizione del paziente, prima di qualsiasi deterioramento dei valori medi e dei marker globali, che potrebbero verificarsi troppo tardi impedendo un intervento tempestivo ed efficace.

Questa ipotesi è stata esplorata in tre diversi database clinici e sperimentali di shock settico ed emorragico, raccolti nell'ambito del progetto ShockOmics. Indici standard e modelli matematici avanzati sono stati utilizzati per studiare il ruolo del sistema nervoso autonomo nella regolazione cardiovascolare durante shock e rianimazione. In particolare, tra gli indici standard sono stati considerati il guadagno di baroriflesso, la variabilità cardiaca e la variabilità della pressione sanguigna; gli approcci avanzati e innovativi proposti si basano su modelli matematici black-box, utilizzati per studiare il contributo dei diversi meccanismi nel controllo della resistenza periferica e nella regolazione della contrattilità ventricolare, e sul modello Windkessel a due elementi, usato per studiare la costante temporale di decadimento esponenziale caratteristica dell'albero vascolare arterioso.

I risultati ottenuti mostrano come, a seguito delle manovre di rianimazione, le variabili emodinamiche globali, come la pressione sanguigna media e il volume circolante, siano effettivamente ripristinati; tuttavia, questo miglioramento globale non è accompagnato da un reale recupero di una condizione fisiologica, come evidenziato dagli indici e dai modelli cardiovascolari. Il soggetto potrebbe essere considerato rianimato secondo le linee guida cliniche, ma le informazioni aggiuntive derivanti dagli indici cardiovascolari rivelano una disfunzione autonomica persistente, che, se non adeguatamente trattata, potrebbe portare allo sviluppo di insufficienza multiorgano, collasso cardiovascolare e morte.

In conclusione, i protocolli clinici per la rianimazione da shock circolatorio dovrebbero tenere in considerazione l’andamento degli indici qui proposti, cosi da poter fare un passo avanti nell’ottimizzazione e personalizzazione della terapia.

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List of abbreviations

ABP	Arterial Blood Pressure
AC	Arterial Compliance
AKI	Acute Kidney Injury
ANS	Autonomic Nervous System
BE	Base Excess
BP	Blood Pressure
BPV	Blood Pressure Variability
BRS	Baroreflex Sensitivity
CN	Cranial Nerve
CNS	Central Nervous System
CO	Cardiac Output
CO_2	Carbon dioxide
CRP	C Reactive Protein
CSNA	Cardiac Sympathetic Nerve Activity
cTnT	Cardiac Troponin T
CVLM	Caudal Ventrolateral Medulla
CVP	Central Venous Pressure
DAP	Diastolic Arterial Pressure
DAMPs	Damage-Associated Molecular Patterns
DMN	Dorsal Motor Nuclei
ECG	Electrocardiogram
EF	Ejection Fraction
EGDT	Early Goal Directed Therapy
eNOS	endothelial Nitric Oxide Synthase
FB gain	Feedback gain
FF gain	Feedforward gain
Fi_{O2}	Fraction of Inspired oxygen
GCS	Glasgow Come Scale
HP	Heart Period
HR	Heart Rate
HRV	Heart Rate Variability
hs-cTnT	High sensitive Cardiac Troponin T
Ht	Hematocrit
ICU	Intensive Care Unit
iNOS	inducible Nitric Oxide Synthase
LBNP	Lower Body Negative Pressure
LF	Low Frequency
LPS	E. Coli Lipopolysaccharide

MAP	Mean Arterial Pressure
MDL	Minimum Description Length
MODS	Multi Organ Dysfunction Syndrome
MOF	Multi Organ Failure
MSNA	Muscle Sympathetic Nerve Activity
NA	Nucleus Ambiguous
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NST	Nucleus of the Solitary Tract
$PaCO_2$	Arterial partial pressure of carbon dioxide
PAMPs	Pathogen-Associated Molecular Patterns
PaO_2	Arterial partial pressure of oxygen
PEEP	Positive End-Expiratory Pressure
PNS	Parasympathetic Nervous System
PP	Pulse Pressure
PPV	Pulse Pressure Variation
PRRs	Pattern Recognition Receptors
QSE	Quadratic Sample Entropy
RAP	Right Atrial Pressure
RMSSD	Root Mean Square of Successive Differences
ROS	Reactive Oxygen Species
RRI	RR interval
RSNA	Renal Sympathetic Nerve Activity
RVLM	Rostral Ventrolateral Medulla
SAP	Systolic Arterial Pressure
SNS	Sympathetic Nervous System
$ScvO_2$	Superior vena cava oxygenation saturation
SD	Standard Deviation
SDSD	Standard Deviation of Successive Differences
SOFA	Sequential Organ Failure Assessment
SV	Stroke Volume
SvO_2	Mixed venous oxygen saturation
T	Temperature
TNF	Tumor Necrosis Factor
TP	Total Power
TPR	Total Peripheral Resistance
UO	Urine Output
VLF	Very Low Frequency
VP	Vasopressors

Summary

Circulatory shock affects about one third of patients in the Intensive Care Unit (ICU) for a total of more than 1 million victims a year in United States and Europe. Among the different types of shock, septic and hemorrhagic shock are the most frequent in ICU, with an incidence of about 60% and 20%, respectively[1]. Septic shock is defined as a complication of sepsis characterized by a life-threatening organ dysfunction caused by a dysregulated host response to infection, which involves circulatory, cellular and metabolic abnormalities[2]. It still remains a major complication of critically ill patients, due to its elevated mortality (40%), high-risk of second lines treatments and long-term physical and cognitive impairments in survivors, with a 5-year mortality rate of 75%[3]. Hemorrhagic shock is a form of hypovolemic shock, in which an acute reduction in central blood volume causes organ hypoperfusion and inadequate oxygen supply at the cellular level. It still represents the leading cause of mortality after trauma in both civilian and military settings, with more than 60'000 deaths per year in the United States and an estimated 1.9 million deaths per year worldwide[4].

Although the pathophysiology of septic and hemorrhagic shock is very different, the pathophysiological pathway in response to the insult is similar, leading to collapse of circulation, hypoperfusion, tissue anoxia, organs failure and finally death. The initial hypoperfusion and hypotension trigger the activation of different physiological mechanisms, including neural, hormonal and bio-chemical processes, in the attempt to restore the lost homeostasis. If not promptly and efficiently treated,

[1] J.-L. Vincent and D. De Backer, "Circulatory Shock," N*EJM*, vol. 369, no. 18, pp. 1726–1734, 2013.

[2] M. Singer *et al.*, "The third international consensus definitions for sepsis and septic shock (sepsis-3)," *JAMA*, vol. 315, no. 8, pp. 801–810, 2016.

[3] T. Iwashyna, E. Ely, D. Smith, and K. Langa, "Long-term cognitive impairment and functional disability among survivors of severe sepsis," *JAMA*, vol. 304, no. 16, pp. 1787–1794, 2010.

[4] R. Lozano *et al.*, "Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010 : a systematic analysis for the Global Burden of Disease Study 2010," *Lancet*, vol. 380, pp. 2095–2128, 2012

shock evolves to a stage where all the compensatory mechanisms begin to fail in their effort to maintain homeostasis, and the exacerbated cellular hypoxia leads to tissues deterioration and organs failure. This condition is known as multiple organ failure (MOF), i.e. organs not directly injured by infection become dysfunctional due to systemic disorders involving immunoregulation and endothelial dysfunction. When MOF occurs, the damage to tissues is already so extensive that the patient is destined to die, even with an adequate medical intervention.

Current treatments for shock are mainly devoted to restore homeostasis and to prevent MOF, trying to reduce the mortality risk and the generation of secondary pathologies. However, clinicians are still far to have found the optimal therapy and clinical guidelines keep on being revisited and updated. Recently, many studies and research trials are shedding light on critical aspects of the existing resuscitation protocols. For example, the *Early Goal-Directed Therapy (EGDT)*, proposed by Rivers and colleagues, who demonstrated its efficacy in reducing morbidity and mortality among septic shock patients[5], was recently questioned by three large clinical trials[6][7][8]. Although the complexity and heterogeneity of shock population could be a possible explanation for these discrepancies, the lack of a pathophysiological approach to hemodynamic monitoring could also contribute to the failure of generalized resuscitation protocols.

Currently, the benefit of the therapeutic approach is evaluated based upon global hemodynamic, clinical and metabolic end-points, such as mean blood pressure (BP), oxygen saturation and lactate. Patients are treated with fluids, mostly crystalloids, to restore arterial BP, circulating volume, and oxygenation during the first hours after development of shock; moreover, vasopressors, mainly noradrenaline, are also

[5] E. Rivers *et al.*, "Early goal-directed therapy in the treatment of severe sepsis and septic shock," *NEJM*, vol. 345, no. 19, pp. 1368–1377, 2001

[6] ARISE Investigators *et al.*, "Goal-Directed Resuscitation for Patients with Early Septic Shock," *NEJM*, vol. 371, no. 16, pp. 1496–1506, 2014

[7] P. R. Mouncey *et al.*, "Trial of early, goal-directed resuscitation for septic shock," *NEJM*, vol. 372, no. 14, pp. 1301–1311, 2015

[8] T. P. Investigators, "A Randomized Trial of Protocol-Based Care for Early Septic Shock. Process trial," *NEJM*, vol. 370, no. 18, pp. 1–11, 2014

administered in case of prolonged hypotension[9]. However, the understanding of the root causes is the main issue in order to find new therapy targets, improve drugs administration, and tailor therapies to each individual patient. From this perspective, a different approach to patients monitoring is needed in critical care.

The autonomic nervous system (ANS) is directly involved in the maintenance of cardiovascular homeostasis, through its sympathetic and parasympathetic branches, and it is crucial both in physiological and in pathological conditions. The sympathetic nervous system (SNS) innervates most of the blood vessels, except for capillaries, and the heart. A sympathetic stimulation increases the resistance of arteries to blood flow, leading to an increase of mean BP; at the heart level, a sympathetic stimulation increases both the firing rate of the sinoatrial node and the contractility of the myocardium. The parasympathetic nervous system (PSN) innervates the heart though the vagus nerve, whose stimulation decreases the heart rate (HR) and the cardiac contractility. The short-term autonomic cardiovascular regulation is modulated through several afferent pathways and reflex mechanisms, which provide appropriate responses to rapid changes in cardiovascular homeostasis, in order to maintain arterial BP in a physiological range of values and to provide adequate blood flow. In particular, the baroreflex is the most important feedback mechanism responsible for BP homeostasis.

Many previous works have documented an altered ANS activity, also mediated by an altered baroreflex activity, with the progression of shock severity. In septic shock, although the exact pathophysiological mechanism of this autonomic imbalance is not clear yet, there is evidence that it is often due to an uncontrolled or prolonged sympathetic activation, or to an inappropriate regulation of the PSN[10]. During hemorrhagic shock, a decoupling between the oscillations in sympathetic activity and the oscillations in BP has been reported during high levels of

[9] A. Rhodes *et al.*, "Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016," *Crit. Care Med.*, vol. 45, no. 3, pp. 486–552, 2017.
[10] J. A. Ferreira and B. D. Bissell, "Misdirected sympathy: The role of sympatholysis in sepsis and septic shock," *J. Intensive Care Med.*, vol. 33, no. 2, pp. 74–86, 2018.

lower body negative pressure (LBNP), leading to decompensation and shock[11]. However, little attention has been given so far to the short term cardiovascular assessment of shock patients during the resuscitation phase.

Clinically, powerful established tools to characterize autonomic dysfunction include heart rate variability (HRV), blood pressure variability (BPV) and baroreflex sensitivity (BRS), and a reduction in their values has been demonstrated to be directly correlated with shock severity and mortality in several literature works[12][13].

The present thesis bases on the idea that combining the traditional mean values, as proposed by the current guidelines, with these non-invasive cardiovascular indices, and integrating them into bedside monitoring of shock patients, may be of clinical importance in the understanding of the responsiveness of the patients to the combination of sympathomimetic drugs and fluid therapy; the hypothesis is that the restoration of the autonomic cardiovascular control mechanisms in shock patients should be of primary importance combined to the resuscitation targets, since it can guarantee an effective return to physiological homeostasis. Given that the role of the ANS has been proven to be crucial during the compensatory phase of shock, similarly we can hypothesize that it is determinant in the recovery phase too.

The ultimate aim is to have a functional hemodynamic monitoring to predict a possible catastrophic deterioration of the patient, thus allowing clinicians to act beforehand. The ANS operates in the short term range (seconds to minutes) and clinical signs of an ongoing failure could be a wake-up call of an imminent decline, before any deterioration of

[11] A. M. Schiller, J. T. Howard, and V. a. Convertino, "The physiology of blood loss and shock: New insights from a human laboratory model of hemorrhage," *Exp. Biol. Med.*, vol. 242, no. 8, pp. 874–883, 2017.

[12] E. Salomão *et al.*, "Heart rate variability analysis in an experimental model of hemorrhagic shock and resuscitation in pigs," *PLoS One*, vol. 10, no. 8, 2015.

[13] R. Ramchandra, L. Wan, S. G. Hood, R. Frithiof, R. Bellomo, and C. N. May, "Septic shock induces distinct changes in sympathetic nerve activity to the heart and kidney in conscious sheep," *Am J Physiol Regul Integr Comp Physiol*, vol. 297, no. 5, pp. R1247–R1253, 2009.

mean values and global markers, which could occur too late for any effective intervention.

We explored this hypothesis in different datasets, including a clinical dataset of 21 severe septic shock patients and two experimental datasets, one consists of pigs undergoing a protocol of polymicrobial septic shock and resuscitation and the other includes pigs undergoing a protocol of hemorrhagic shock and resuscitation. All the datasets were collected under the project ShockOmics (NCT02141607)[14]. We exploited standard and advance mathematical indices and models to investigate the role of the ANS in BP and HR regulation, how it changes when shock occurs and if it is restored during the resuscitation phase.

Recently, the clinical interest in monitoring the ANS activity is rapidly growing and its implications into clinical practice is more and more recognized. Innovative technologies in the field of bioelectronics medicine are advocated as the future medicine for a vast majority of diseases, including sepsis and bleeding, and they are based on the direct stimulation of the ANS, in particular of the vagus nerve[15][16]. The contribution of the present thesis is an improved knowledge of the autonomic regulation of the cardiovascular system during shock that could be also beneficial for a future extension of the vagus stimulation technique in a clinical setting.

In the first study, presented in **chapter 2**, we examined the early response to therapy in a homogeneous subset of 21 severe septic shock patients recruited at Geneva University Hospital. Patients were followed during the first 48 hours of therapy after development of septic shock (time point T0) and they were analyzed at two relevant time points: within 16 h from T0 when the inflammatory cascade has been just activated (T1), and within 48 h after T0 time point (T2). SOFA score was

[14] F. Aletti *et al.*, "ShockOmics: multiscale approach to the identification of molecular biomarkers in acute heart failure induced by shock.," *Scand. J. Trauma. Resusc. Emerg. Med.*, vol. 24, no. 1, p. 9, 2016.
[15] K. Tracey, "The inflammatory reflex," *Nature*, vol. 420, pp. 853–859, 2002.
[16] L. V Borovikova *et al.*, "Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin," *Nature*, vol. 405, no. 6785, pp. 458–462, 2000.

used to classify patients into two groups according to their responsiveness to early therapy: responder patients (R, n=14) consisted in patients with a positive response to initial treatment. Those patients, who decreased their SOFA score of at least 5 points between T1 and T2 ($\Delta SOFA_{T2-T1} \geq 5$) or reached a SOFA score at T2 lower than 8 were classified as R. Non-responder patients (NR, n=7) consisted in patients who still had a SOFA score at T2 higher than 8 or $\Delta SOFA_{T2-T1} < 5$.

Mathematical indices and models were adopted in order to study the autonomic-mediated variability in BP and HR and the interactions between the two biological systems. The baroreflex mechanism was studied by means of a bivariate model method[17]; BPV and HRV were investigated by spectral analysis. A multi-input single-output causal black-box model was implemented and spectral decomposition of the beat-to-beat variability of the diastolic component of peripheral BP was performed, in order to better investigate the different mechanisms involved in peripheral resistance control.

Our results highlighted a sympathetic activation in response to fluids and vasopressors only in those patients who significantly improved their organ function, although all patients reached an adequate increase in mean BP. Moreover, we interestingly found out that NR patients received a higher dosage of vasopressors and fluids with respect to R patients. Thus, common mean targets routinely used to guide hemodynamic optimization and fluid therapy in acute shock patients, such as mean BP, are not sufficient alone to explain the evolution of patient's organ dysfunction. Variability indices and baroreflex trend can add information to individual vital signs and can help in understanding the responsiveness to the combination of sympathomimetic drugs and fluid therapy.

Such considerations are at the basis of the animal experiments and the next studies illustrate the investigation of autonomic control mechanisms in the septic and hemorrhagic shock experimental datasets. The animal experiments offer a great opportunity to have a controlled

[17] R. Barbieri, G. Parati, and J. P. Saul, "Closed- versus open-loop assessment of heart rate baroreflex.," *IEEE Eng. Med. Biol. Mag.*, vol. 20, no. 2, pp. 33–42, 2001.

condition without confounding factors, and to track changes in the physiological response at each step of shock and resuscitation evolution.

Chapter 3 and 4 illustrate the results of the analyses performed on a population of 6 pigs undergoing a protocol of polymicrobial septic shock and resuscitation. The experiments were performed in the Experimental Laboratory of Intensive Care at the Université Libre de Bruxelles. In particular, chapter 3 focuses on the heart autonomic regulation during septic shock and resuscitation, whereas chapter 4 focuses on the arterial tree autonomic modulation.

In the first study we proposed a new black-box autoregressive model to investigate the autonomic control of ventricular contractility (VC). The two mechanisms of interest are the baroreflex-mediated control of VC and the force-frequency autoregulation mechanism. The identified transfer functions related to these mechanisms were characterized in terms of their impulse and step responses. VC was indirectly measured by computing the maximum of positive time derivative of left ventricular pressure, the so-called dP/dt max. Moreover, we assessed similar indices as in the clinical study in order to characterize the cardiovascular autonomic dysfunction.

The animals were studied at baseline condition, after the development of septic shock through intraperitoneal instillation of autologous feces, and after resuscitation maneuvers were administered, such as fluids and vasopressors to restore BP and volemia.

Our results highlighted an autonomic-mediated increase in VC during shock in order to cope with the hypovolemia and hypotension typical of shock state. Interestingly, after resuscitation the physiological control of heart contractility was not restored and all the animals still exhibited high HR and elevated VC. Moreover, indices of autonomic dysfunction, such as HRV and BRS, were suppressed both in shock and after resuscitation.

To conclude, a condition of cardiovascular inefficiency and autonomic disarray was triggered by septic shock, and it was not resolved after resuscitation. The ANS-related indices and the VC model were able to highlight and track this inefficient condition, and, thus, they could be

useful to evaluate the effectiveness of treatments, combined with standard clinical measures. In addition, our results support the emerging evidence that heart contractility should be considered a new therapeutic target for sepsis resuscitation.

The results here presented question other mechanisms which could be involved. Indeed, sepsis induces changes in the hemodynamics and mechanical properties of the vascular system and these could also contribute to the observed alteration of cardiac functionality.

Chapter 4 presents the results of the analyses on the alterations of arterial tree vascular properties and their relationship with autonomic dysfunction during septic shock and resuscitation obtained from the same experimental model of porcine polymicrobial septic shock. The objective of this work was to assess the effectiveness of standard resuscitation, i.e. fluids and noradrenaline, at vascular level and to verify if there was any association between alterations in vascular properties and ANS activity. In particular, we wanted to verify if a phenomenon of vascular decoupling, such as an inversion of pulse pressure (PP) amplification from central to peripheral arterial sites, already reported in a porcine model of endotoxic shock[18], is still present after the resuscitation maneuvers or if a physiological condition of the arterial tree was restored. From this perspective, markers of vascular functionality could be helpful as novel therapeutic targets to guide the administration of fluids and vasopressors.

The animals were studied at the same time points as previously described, i.e. at baseline, after development of septic shock, and after full resuscitation. The arterial vascular tree was studied at three punctual sites, such as in the central aorta, and in the peripheral radial and femoral arteries.

A 2-element Windkessel model was used to estimate the characteristic time constant τ of the arterial tree, which takes into account both the resistive and the elastic properties of the vessels.

[18] F. Hatib, J. R. C. Jansen, and M. R. Pinsky, "Peripheral vascular decoupling in porcine endotoxic shock," *J. Appl. Physiol.*, vol. 111, no. 3, pp. 853–860, 2011.

Moreover, independent estimates of total arterial compliance (AC) and total peripheral resistance (TPR) were also performed. Similar indices of autonomic dysfunction were performed as in the previous studies (HRV, BPV and BRS).

Our results confirmed that septic shock induced a severe vascular disarray, decoupling the usual pressure wave propagation from central to peripheral sites; this phenomenon appeared as an inversion of the physiological PP amplification, with a higher PP in the central aorta than in the peripheral arteries during shock. The time constant τ was decreased, together with AC and TPR. ANS dysfunction was described by a reduced BRS, decreased BPV, and suppressed HRV. This compromised condition was not resolved by administration of fluids and noradrenaline, although global hemodynamic markers were restored. Thus, a persistent vascular and autonomic dysfunction were observed also in the resuscitated animals, and they were found to be significantly correlated.

These results suggest that measures of vascular properties should be taken into account in future researches, and, together with measures of ANS activity, they may help in having a more comprehensive picture of the therapy effectiveness and represent a useful end-point to guide the resuscitation strategy, combined to standard hemodynamic and clinical markers. Moreover, the present results highlight the importance of considering the heart and the circulation as coupled systems, to be evaluated jointly and not as independent systems in order to optimize therapies for a better management of shock patients. For example, increased vascular stiffness caused by septic shock can directly affect the heart workload and the administration of vasopressors[19] should be adjusted to balance the restoring of MAP with modification in arterial elastance. Indeed, a more effective vasopressor therapy should be tailored according the different characteristics between central and peripheral arteries and further studies are fundamental to understand

[19] M. I. Monge García *et al.*, "Noradrenaline modifies arterial reflection phenomena and left ventricular efficiency in septic shock patients: A prospective observational study.," *J. Crit. Care*, vol. 47, pp. 280–286, 2018.

how to restore not only mean arterial BP, but also a physiological condition of arteries and BP control.

The last study, reported in **chapter 5**, presents the analyses performed on a population of 6 pigs undergoing a protocol of hemorrhagic shock and resuscitation. The experiments were performed at Istituto di Ricerche Farmacologiche Mario Negri IRCCS.

We were interested in verifying if there are common hemodynamic patterns in septic and hemorrhagic shock, and, for this purpose, we applied similar analyses as in the previous studies, and we studied the trend of related autonomic indices.

The animals were studied at different time points: at baseline, after the massive blood withdrawal, after different resuscitation maneuvers were administered, such as after fluids and vasopressors infusion and after the shed blood infusion.

Our results highlighted that after the first resuscitation with fluids and vasopressors, mean BP reached the target value, but all the cardiovascular indices were not fully restored, hinting at a partial recovery of the autonomic mechanisms. Only after shed blood was reinfused, all the indices returned to the baseline level. The trends of autonomic indices obtained in hemorrhagic shock animals show similarities to those observed in septic shock, highlighting how the ANS is similarly elicited and how it plays an important role in the recovery.

In conclusion, both septic and hemorrhagic shock studies showed how the resuscitation maneuvers were able to restore global hemodynamic variables, such as mean BP and circulating volume, but this was not accompanied to a recover of a physiological condition, as highlighted by the cardiovascular indices and models. The shock subject may be considered resuscitated according to clinical guidelines, but the combined information derived from autonomic cardiovascular indices may reveal a persistent autonomic disarray, that, if not appropriately treated, could lead to development of MOF, cardiovascular collapse and death. Thus, clinical protocols should give importance to the trends of

these indices, in order to make a step further in the optimization and personalization of the therapy.

Even if the results of the current work are very promising, some limitations must be discussed. An important limit of the study is represented by the small sample size, mostly in the experimental studies. This may be the source of a high variability in the results and this could affect the statistical tests. Moreover, the most important limit of the experimental protocols consists in the limited time window; thereby, the duration between one time point and the other does not properly reflect what happens in clinical settings and only the short-term effects of shock and resuscitation protocols can be investigated. However, common patterns in the hemodynamic variables were observed which allow to draw similar conclusions, as previously discussed. Finally, another limitation of the present works is the unavailability of direct measures of autonomic outflow which limited our conclusions to speculation about autonomic activity at the heart level and in the periphery. Although a comparison with published researches reinforces our findings, we may suggest to include the direct microneurographic measurements of sympathetic activity in future studies.

Moreover, we suggest to include, in the study of sympathetic and vagal outflow, other physiological districts, not only the heart or the peripheral circulation, as it is known that, during shock, the sympathetic nerve activity is differentially modulated across the human body. A complete characterization of the changes in autonomic activity at multiple organs and their correlation with development of MOF in shock could represent an intriguing field of research, paving the way for novel therapeutic targets to be used in critical care.

Finally, the use of the indices proposed in this thesis can be exploited to test the efficacy of new drugs on the regulation of the target organ and on the overall control of cardiovascular homeostasis. Current studies are ongoing in our group, focusing, in particular, on the beneficial effect of two cardioselective drugs in septic shock, ivabradine and esmolol. While esmolol is a β1-selective blocker, ivabradine selectively inhibits only the pacemaker ionic current, leading to a reduction of HR without affecting cardiac contractility. Both the drugs have been already

studied in the context of septic shock and MOF, demonstrating their beneficial effect on cardiac chronotropic dysfunction. However, it would be interesting to investigate their influence also on peripheral circulation, as a secondary mechanical effect of a reduced HR. Preliminary results about these analyses are presented in the appendix.

To conclude, to the best of our knowledge, no previous scientific work has extensively focused on the short term cardiovascular assessment in shock patients during the resuscitation phase. Thus, the obtained results represent a novel approach in the field, and the proposed indices may represent new possible non-invasive tools to guide shock resuscitation protocols in critical care.

1 INTRODUCTION

Circulatory shock affects about one third of patients in the Intensive Care Unit (ICU) for a total of more than 1 million victims a year [1] in United States and Europe.

Although shock is generally recognized as a health burden, the amount of funding received for the research are very few. For example, sepsis, which often lead to septic shock, is one of the most common disease but sepsis research receives the lowest funding compared to other pathologies, as reported in Figure 1.1.

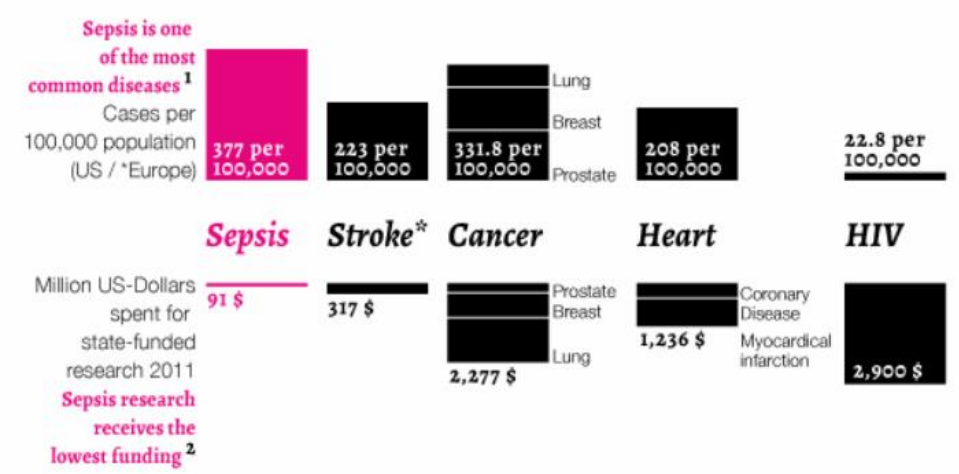

Figure 1.1. Statistical data of 2011 reporting the incidence of major diseases and the funding received for research (http://www.wfpiccs.org/projects/sepsis-initiative/)

However, many scientific and non-scientific initiatives became established in the recent years, trying to create networks among researchers worldwide, promote research in the field of shock and raise a global awareness of shock. Examples are the American Shock Society (http://shocksociety.org/) and the European Shock Society (http://www.europeanshocksociety.org/), the Surviving Sepsis Campaign (http://www.survivingsepsis.org) or the Global Sepsis Alliance (https://www.global-sepsis-alliance.org/) with the world's sepsis day initiative.

This global effort in shock research has led to an advancement of critical care medicine in the last decades, thus allowing the improvement of survival rate of critically ill patients. However, besides the success of an increased survival rate, the long term effects in surviving shock patients are still high. The so called post intensive care syndrome (PICS) has been established and it includes all the long-term disabilities persisting after ICU patients discharge, which could be cognitive, psychiatric and/or physical. The exact incidence of the syndrome is still unknown, but PICS is now recognized as a public health burden, affecting not only the survived patients but also their families [2].

In the current clinical practice, treatments are mainly devoted to restore homeostasis in the first hours after shock development to prevent multiple organ failure (MOF), trying to reduce the mortality risk and the generation of secondary pathologies. However, clinicians are still far to have found the optimal therapy and clinical guidelines keep on being revisited and updated. Up to now, most of the studies are devoted to find association with mortality or comorbidities incidence, mainly with the purpose to find new drugs which have a more beneficial impact on long-term outcomes [3], [4]. For example, the beta-blockers, such as esmolol, have recently gained an increasing interest in the field of septic shock therapies following consistent experimental findings of attenuation of inflammation and improved survival with β1 selective antagonist [5]–[7]. However, the current therapy targets are associated to symptoms only, e.g. hypotension, and the understanding of the root causes is the main issue in order to find new targets and to improve drugs administration. From this perspective, a different approach to patients monitoring is needed in critical care.

In this introductory chapter, definition, incidence, pathophysiology and clinical management of circulatory shock has been described in paragraph 1.1. In particular, as this thesis focuses on the study of two types of shock, i.e. septic shock and hemorrhagic shock, paragraph 1.2 and paragraph 1.3 provide a detailed description of these two specific conditions. The rationale for this choice lies in the scientific interest of investigating the *cardiovascular regulatory mechanisms* which are typical of shock syndrome and which, therefore, are common to both septic or

hemorrhagic shock. Moreover, these two types of shock have the highest incidence among ICU patients and, therefore, are responsible for the highest socio-economic burden. Among all the physiological mechanisms and reflexes which are activated during shock progression and resolution, the objective of this thesis is to give a contribution in the study of those mechanisms which are mediated by the autonomic nervous system (ANS). Indeed, ANS is the main player in maintaining cardiovascular homeostasis in both physiological and pathological conditions and its role during shock has been extensively demonstrated to be crucial, paving the way for innovative therapies based on the direct stimulation of autonomic nerves. Paragraph 1.4 provides a description of the functional anatomy of the ANS, with a detailed picture of the autonomic-mediated mechanisms for cardiovascular regulation. Moreover, an overview of the current cutting-edge technologies based on nerves stimulation is outlined at the end of the paragraph. Paragraph 1.5 summarizes the current knowledge about the role of the ANS both in septic and hemorrhagic shock. Finally, the motivations and objectives of the study are detailed in the last paragraph of this chapter.

1.1 SHOCK

1.1.1 Definition, description and causes

Shock is a syndrome that can be defined as a state of organ hypoperfusion which causes an imbalance between oxygen delivery and oxygen consumption to tissues with resultant cellular dysfunction and death [1]. It affects about one third of ICU patients, with an increasing epidemiological trend [1], [8], and the related mortality is still very high, from about 20% to 50%, depending on different factors such as the type of shock, the underlying cause and the extent of coexisting morbidities [1], [8]–[11].

Shock results from four potential, and not necessarily exclusive, pathophysiological mechanisms, which delineate the following classification [1], [12]:

- **Hypovolemic shock**. It is due to massive loss of fluids from the circulation which leads to a critical decrease in intravascular volume. It is commonly generated by severe external or internal bleeding, for example after surgery or traumatic injuries, and in this case it bears the name of *hemorrhagic shock*. In addition to actual blood loss, also the loss of body fluids can lead to a decrease in intravascular volume; this can occur in cases of excessive dehydration (e.g. after acute vomiting or diarrhea), severe burns or in the presence of disease which cause excess urination (e.g. diabetes or kidney failure).
- **Cardiogenic shock**. It results from a severe reduction in cardiac output (CO) caused by cardiac failure. Myocardial infarction with left ventricular failure remains the most common cause of cardiogenic shock, but other important factors include: end-stage cardiomyopathy, advanced valvular heart disease, myocarditis, mass or fluid accumulation and cardiac arrhythmias.
- **Obstructive shock**. It is caused by mechanical factors (usually a physical obstruction) that interfere with filling or emptying of the heart or of great vessels. Common causes are pulmonary embolism, i.e. blockage of an artery in the lungs, atrial tumor or clot and pericardial tamponade, i.e. accumulation of fluid in the pericardium resulting in a compression of the heart.
- **Distributive shock**. It is the result of excessive vasodilation, mostly of peripheral blood vessels, and pooling of blood in the intravascular space. *Septic shock* is the most common form of distributive shock and it occurs as a complication of sepsis, i.e. a life-threatening condition that arises when the body's response to infection causes injury to its own tissues and organs. Other causes include anaphylaxis, i.e. a sudden and severe allergic reaction, or severe damage to the central nervous system (e.g. after brain or spinal cord injury).

The first three types of shock are characterized by low CO and, hence, inadequate oxygen transport to the organs. In distributive shock, the main deficit lies in the periphery with decreased systemic vascular resistance and, thus, altered oxygen extraction; in these cases, CO is typically high, unless associated to myocardial depression.

The relative incidence of the different types of shock is highly different: septic shock is the most common among ICU patients (~60%), followed by cardiogenic and hypovolemic shock (~20%); obstructive shock is relatively rare (~2%) [1].

1.1.2 Pathophysiology

Regardless of the different causes triggering shock, a common pathophysiological pathway ensues leading to collapse of circulation, hypoperfusion, tissue anoxia, MOF and finally death (Figure 1.2) [1], [12], [13].

Depending on its severity, shock can be clinically classified into three stages: (1) non-progressive or compensated, (2) progressive, and (3) irreversible. Early stages of shock are reversible and treatable; however, once serious organ failure ensues, shock becomes irreversible.

During the initial phase, the state of hypoperfusion causes hypoxia, i.e. inadequate oxygen supply at tissue level, which generates a shift from aerobic to anaerobic cellular metabolism. As a consequence, the organism sees an increase in carbon dioxide (CO_2) production and accumulation of lactic acid, leading to a condition of acidosis and reduced energy production for cellular functions. At systemic level, this initial phase is characterized by the body's recruitment and activation of different physiological mechanisms, including neural, hormonal and bio-chemical processes, in the attempt to restore the lost homeostasis (compensated shock). Common clinical manifestations of these physiological compensatory mechanisms are hyperventilation, trying to get rid of the extra CO_2, peripheral vasoconstriction and tachycardia, in order to counterbalance hypotension and hypoperfusion. At the beginning, vasoconstriction is selective, shunting blood to the heart and brain and away from the splanchnic circulation. With shock progression, vasoconstriction involves the renal blood vessels, also through the activation of the hormonal renin-angiotensin axis, which causes the release of an anti-diuretic hormone (arginine vasopressin) in order to conserve fluids via the kidneys. This results in renal hypoperfusion and in a decreased glomerular filtration rate; low urine output and even anuria are typical of this stage.

If not promptly and efficiently treated, shock evolves to the progressive stage, when all the compensatory mechanisms begin to fail in their effort to maintain homeostasis. Anaerobic metabolism is exacerbated by the worsening of hypoxia and this has a depressive effect mostly on the heart, which undergoes a failure of its pumping functionality. Heart insufficiency generates a fall in blood pressure (BP) to very low levels and raises intrapulmonary venous pressure, causing stagnation of blood in the pulmonary circulation; this favors the formation of pulmonary edema and affects the alveolo-capillary functional units. Lungs cannot function properly, and this further contributes to general hypoxia. As BP further decreases, blood begins to clot in the small vessels. At the same time, if the bowel becomes sufficiently ischemic, toxins and bacteria are released and they may enter the bloodstream, resulting in increased complications due to infection. Moreover, tissue hypoxia results in the release of numerous inflammatory cytokines which cause vasodilation and promote fluid loss by increasing the permeability of peripheral blood vessels.

In response to these events, an intense tissue deterioration begins and, without adequate medical intervention, progressive shock evolves in irreversible shock. This last phase of shock is characterized by the decreasing of heart functionality and by the progressive dilation of peripheral blood vessels; brain damage and cell death occur and patient's death could imminently follow. These events are not reversible anymore at this stage, regardless of the amount or type of medical treatments, due to the arise of MOF syndrome. Under this condition, organs not directly injured by the original trauma become dysfunctional due to systemic disorders involving immunoregulation and endothelial dysfunction. The damage to tissues, including cardiac muscle, is so extensive that the patient is destined to die, even if adequate blood volume is reestablished and BP is restored to its normal value.

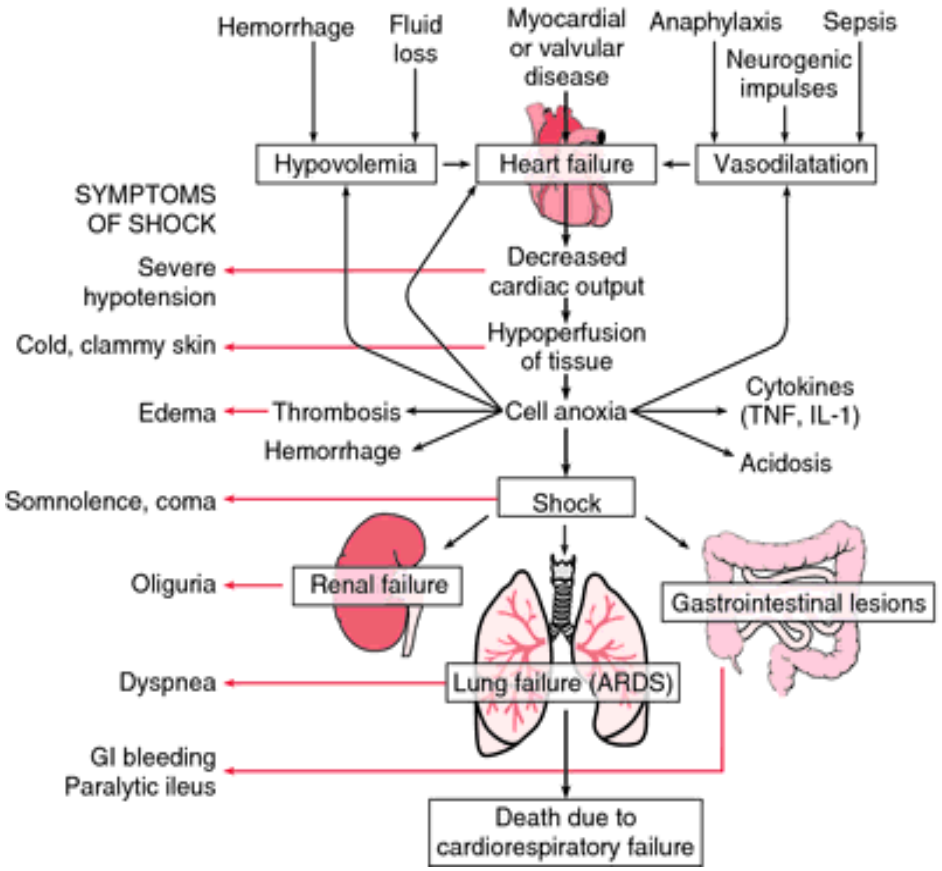

Figure 1.2. Pathogenesis of shock. Top row: possible causes of shock resulting in heart failure. Left: shock symptoms. Hypoperfusion of vital organ is the crucial effect in shock. Its consequences at cellular level are the shift to anaerobic metabolism and, if shock persists, cell anoxia and cell death. At systemic level, shock triggers neurohormonal mechanisms which eventually leads to multiple organ failure. From Damjanov, (2000) [12].

1.1.3 Clinical guidelines for resuscitation of shock patients

The current treatment for shock patients is mainly devoted to restore hemostasis and to prevent MOF [1]. Prompt identification is essential so that aggressive management can be started, and appropriate treatments have to be chosen based on the underlying pathophysiological mechanisms involved. Generally, treatment should include correction of the cause of shock (e.g. antimicrobial therapy in

case of septic shock) and hemodynamic stabilization, taking into account the actual response of the patient to the administered therapy.

Fundamentally, the resuscitation phase of shock patients is characterized by four stages: (1) salvage, (2) optimization, (3) stabilization and (4) de-escalation. During the first phase, the main goal is to achieve a minimum BP and CO compatible with immediate survival, together with a rapid correction of the triggering cause. Patients are typically intubated to receive ventilatory support and they are given fluids (mostly crystalloids) to improve microvascular blood flow, restore mean arterial BP (typically to values around 65-70 mmHg) and increase CO. If hypotension is severe or if it persists despite fluid expansion, the use of vasopressors is indicated. In particular, norepinephrine is recommended as first choice, given its predominantly α-adrenergic properties and its modest β-adrenergic effect which helps in maintaining CO despite the rise in the afterload.

During the optimization phase the target is to increase cellular oxygen availability in order to reduce inflammation, decrease mitochondrial dysfunction and restore an adequate cellular metabolism. After correction of hypotension and hypoxia, CO is the principal determinant of oxygen delivery to tissue and, during this phase, it has to be monitored and continuously optimized; inotropic agents (e.g. dobutamine) can be considered to achieve the goal. Moreover, measurements of mixed venous oxygen saturation (SvO_2) and lactate levels may help guide the therapy and monitor CO.

Once hemodynamically stabilized, patients usually undergo a stabilization phase, whose goal is to prevent further organ dysfunction; oxygen supply to the tissues is no longer the target at this stage, and organ support becomes more relevant (e.g. blood product administration, mechanical ventilation, renal replacement therapy).

Finally, when possible, de-escalation of therapy is started (fourth stage) in order to avoid the emergence of resistant organisms and to minimize the risk of drug toxicity; patients are gradually weaned from vasoactive agents and they are given diuretics to reduce possible fluid overload and to restore a physiological fluid balance.

In spite of the efforts made in the understanding of the underlying mechanisms of shock, clinicians are still far to have found the optimal clinical treatment. ICU mortality associated to shock syndrome is still very high, ranging from 20% to 50%, and survived patients are usually affected by long-term comorbidities and disabilities which can dramatically reduce their quality of life [2]. New knowledge about different patients' responses to therapy is necessary in order to tailor the therapies to each single patient, and a continuous update and optimization of the current guidelines can still be considered a hot topic in the critical care.

1.2 SEPTIC SHOCK

1.2.1 Definition and incidence

The definition of sepsis and septic shock has been continuously revised during the last two decades [14], [15] and recently a new update has been achieved by the *Third International Consensus Definitions for Sepsis and Septic shock (Sepsis-3)* [10]. According to this new definition, sepsis should be defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, and septic shock can be considered a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone.

Septic shock patients can be clinically identified by two main criteria: 1) vasopressor requirement to maintain a mean arterial BP of 65 mmHg or greater, i.e. persistent hypotension despite adequate resuscitation, and 2) serum lactate level greater than 2 mmol/L in the absence of hypovolemia. Together these criteria are associated with hospital mortality rates greater than 40%.

Usually in clinical practice, the severity and progression of organ failure is assessed with various scoring systems that quantify abnormalities according to clinical findings, laboratory data, and therapeutic treatments. The predominant score in current use is the *Sequential Organ Failure Assessment (SOFA)*, originally named Sepsis-related Organ Failure Assessment, which estimates the degree of dysfunction of six sub-systems such as the liver, the kidneys, the

coagulation system, the cardiovascular system, the respiratory system and the nervous system. Thus, it is composed of six sub-scores graded from 0 to 4, where the worst condition is defined by the highest sub-score. The clinical and laboratory variables involved in the calculation of the score are bilirubin, creatinine and urine output, platelets, mean arterial BP and dosage of catecholamines, the ratio between partial oxygen pressure and fraction of inspired oxygen Pa_{O2}/Fi_{O2} and the Glasgow Coma Scale (GCS) score, for each physiological sub-system, respectively. A higher SOFA score is associated with an increased probability of mortality and either the variation, the mean and the highest SOFA scores are predictors of outcome. Specifically, an increase of 2 points or more in the score is related with in-hospital mortality greater than 10% and maximum SOFA greater than 15 points is associated with a mortality rate above 90% [10], [16], [17].

Severe sepsis and septic shock occurs as a result of both community-acquired and health care-associated infections. Among the most common causes there is pneumonia, accounting for about half of cases, followed by intra-abdominal and urinary tract infections [18]. Risk factors for severe sepsis development include pre-existing chronic diseases, such as cancers or immunodeficiency syndrome, and the use of immunosuppressive agents [19]. Moreover, age, sex and ethnicity all influence the incidence of the syndrome [20], and, recently, the genetic cause has gained a lot of attention, given the strong evidence of inherited risk factors [21].

The incidence of sepsis and septic shock is continuously increasing, likely reflecting aging populations with more comorbidities or greater recognition [22], [23], and, although the true incidence is unknown, conservative estimates indicate sepsis as the leading cause of mortality and critical illness worldwide [24], [25]. In Europe, severe sepsis affects 90.4 cases per 100 000 adults per year and an overall hospital mortality is 36%, as described in the last Sepsis Occurrence in Acutely ill Patients study [26]. Moreover, patients who survived sepsis and septic shock often have long-term physical and cognitive impairment and they are at risk for early death within 5 years, with mortality rates as high as 75% [2], [27]. Thus, the socio-economic impact of the syndrome is one of

the highest among critical diseases; as reported by the Healthcare Cost and Utilization Project, sepsis accounted for more than $20 billion, corresponding to 5.2% of total United States hospital costs in 2011 [28]. In 2017, the World Health Organization (WHO) recognized sepsis as a global health priority, and was committed to improve the prevention, diagnosis, and management of sepsis [29].

1.2.2 Pathophysiology

Although the pathophysiology of septic shock is not precisely understood, it has become evident that it involves complex interactions between the pathogen and the host's immune system. More precisely, the initial host response to an infection triggers subsequent compensatory anti-inflammatory mechanisms, which on the one hand contribute to the clearance of infection and tissue repair, on the other are implicated in organ injury and in susceptibility to secondary infections [18]. When microorganisms invade the host, pathogens are recognized by pattern recognition receptors (PRRs), such as toll-like receptors, which are proteins expressed by cells of the innate immune system and are specifically responsible for the identification of the pathogen-associated molecular patterns (PAMPs). The binding between PAMPs and PRRs activates an intracellular cascade resulting in a series of concatenated events including the release of reactive oxygen species (ROS) and inflammatory mediators, such as cytokines (e.g. tumor necrosis factor α (TNF-α) or interleukin-1), local vasodilation [30], increased endothelial permeability, and activation of coagulation pathways. In sepsis and septic shock, the physiological inflammatory response is overwhelming and not adequately compensated by the anti-inflammatory mechanisms, which could even lead to an impairment of the immune system. This generates collateral tissue damage and necrotic cells death, which, in turn, results in the release of endogenous molecules from injured cells, the so-called damage-associated molecular patterns (DAMPs) or alarmins, that perpetuate inflammation at least in part by acting on the same PRRs that are triggered by pathogens [18]. If untreated, this exaggerated inflammation damages cellular proteins, lipids and DNA, compromises mitochondrial function and alters the coagulation cascade, frequently

leading to disseminated intravascular coagulation (DIC). DIC syndrome is a central event in the pathophysiology of sepsis and is characterized by massive thrombin production and platelet activation, which promotes the formation of microvascular thrombi and fibrin deposition, thus causing microcirculatory alterations and poor tissue perfusion [31]. This impaired tissue oxygenation is known to play a key role in organ failure during septic shock, and it is further exacerbated by vasodilation, hypotension, vascular endothelium dysfunction and impaired cellular oxygen use due to mitochondrial damage caused by oxidative stress [18], [32].

1.2.3 Septic shock management

Septic shock management protocols have been subjected to constant revisions and updates in the last decades, in the wake of the newest research findings on the pathogenesis of the syndrome and the continuous changes in its clinical definition (paragraphs 1.2.1 and 1.2.2).

In general, treatments are mainly devoted to restore homeostasis and prevent MOF. The guidelines currently used in clinical practice derive from the most recent updates proposed by the international consortium of the Surviving Sepsis Campaign in 2016 [33], and they are organized into two “bundles” of care: an initial protocol to be accomplished within 6 hours after the patient’s presentation, and a management bundle to be accomplished in the ICU. The rational of the initial management is based on the evidence that early treatment, i.e. in the initial hours after sepsis development, improves outcomes [34]. For patients with hemodynamic instability, as defined by either hypotension (systolic arterial pressure SAP < 90 mmHg, mean arterial pressure MAP < 70 mmHg, or decrease in SAP > 40 mmHg from baseline) or elevated lactate concentration (≥ 4 mmol/L), the guidelines recommend a rapid fluid resuscitation to be initiated within the first hour, with the administration of crystalloids. The goals of this initial care include 1) restore of central venous pressure (CVP) in the range 8-12 mmHg, 2) restore of MAP higher than 65 mmHg, 3) restore of urine output of at least 0.5 mL·Kg·h, and 4) superior vena cava oxygenation saturation ($ScvO_2$) or SvO_2 higher than 70% or 65%, respectively [33]. Following this initial resuscitation, additional fluids

should be given when necessary under the guidance of frequent assessments of the hemodynamic status, through clinical examinations and physiological variables evaluation, such as heart rate (HR), BP, oxygenation, respiratory rate, temperature, urine output and others, as available. Vasopressor therapy should be added to fluids administration in case of prolonged hypotension, being norepinephrine the first-choice drug.

Early source control and antimicrobial therapy are also of primary importance in the treatment of septic shock and the administration of intravenous antimicrobials should be initiated as soon as possible; empiric broad-spectrum antimicrobials should be given shortly to cover all likely pathogens, whereas antimicrobial therapy should be narrowed once pathogen is identified after appropriate microbiologic cultures. This initial resuscitation, targeting both the restoration of hemodynamic stability and the mitigation of the effects of uncontrolled infection, is followed by the so-called supportive therapy, which aims at supporting organ function, avoiding complications, and de-escalating when possible. Thus, patients usually receive blood product, mechanical ventilation and renal replacement therapy.

Although we now have an improved understanding of the pathophysiology underpinning the sepsis process, this knowledge has not been translated yet into a useful intervention that can change the course of the disease. Indeed, the current clinical strategies are not without controversy and the optimal therapy has not been found yet.

1.3 HEMORRHAGIC SHOCK

1.3.1 Definition and incidence

Hemorrhagic shock is a form of hypovolemic shock, in which an acute reduction in central blood volume causes organ hypoperfusion and consequently an inadequate oxygen supply at the cellular level. If hemorrhage continues uncontrolled, death quickly follows. Clinical signs of this condition are severe hypotension and hypoxia, pronounced tachycardia and tachypnea, diffused coagulopathy, hypothermia and metabolic acidosis [9]. The causes of hemorrhage resulting in shock vary

widely and include trauma, with the highest number of deaths per year globally (data from the Global Health Estimates 2015: Global Deaths by Cause, Age, Sex, by Country and by Region, 2000-2015), maternal hemorrhage, gastrointestinal hemorrhage, perioperative hemorrhage, and rupture of an aneurysm [9].

Hemorrhage and unresolved hemorrhagic shock still represent the leading cause of mortality after trauma in both civilian and military settings, with the majority of deaths occurring due to the inability to control bleeding and to effectively resuscitate hemorrhage patients [35], [36]. Thus, death from hemorrhage still represents a substantial global problem, with more than 60'000 deaths per year in the United States and an estimated 1.9 million deaths per year worldwide, 1.5 million of which result from physical trauma [37]. Moreover, people who survived the initial hemorrhagic insult have poor functional outcomes and significantly increased long-term mortality [38].

1.3.2 Pathophysiology

The complexity of the cascade of events leading to shock after severe hemorrhage has been elucidate only recently and it affects at the cellular, tissue, and whole-organism levels [9].

At the cellular level, the severe blood loss leads to inadequate oxygen delivery which causes cells transition to anaerobic metabolism. Typical symptoms of this phase are the increasing production of circulating lactate and the accumulation of oxygen radicals, as the result of the mounting oxygen debt [39]. As the cellular energy supplies continue to decrease, cell death ultimately ensues through necrosis from membrane rupture or apoptosis. The consequent release of DAMPs incites the inflammatory response, exacerbating the critical condition.

At the tissue level, the induced hypoperfusion generates end-organ damage in secondary organs such as kidneys, liver, intestine, and skeletal muscle, which inevitably leads to MOF. When noble organs, such as the brain and the heart, are also affected by the hypoperfusion process, this could lead to cerebral anoxia and severe arrhythmias, which can be fatal within minutes. Hemorrhage also induces profound changes at the level of the vascular endothelium and this has been demonstrated

to contribute to the so-called trauma-induced endotheliopathy (TIC). In particular, the activation of endothelial cells immediately results in an acute procoagulant action at the site of hemorrhage, with the activation of the clotting cascade and platelets to form a hemostatic plug. However, after a severe blood loss, the organism must cope with the competing interests of both reducing further blood loss and limiting microvascular thrombosis to assure end-organ perfusion. This, in turn, generates a massive anticoagulation and fibrinolysis in the blood, remote from the site of hemorrhage, which often leads to pathologic hyperfibrinolysis, diffuse coagulopathy and increased risk of death [40]. Moreover, other factors besides hypoxia may induce the activation of endothelial cells after trauma, including vasoactive catecholamines and inflammatory mediators (e.g. TNF-α) [41]; iatrogenic factors can further exacerbate coagulopathy, hypothermia and acidosis [9].

1.3.3 Bleeding control and shock management

The timing of intervention during hemorrhagic shock is vital in order to control the source of hemorrhage, restore the patient's intravascular volume, and pay the oxygen debt before shock becomes irreversible.

Prehospital care is essential as first approach before definitive hospitalization in the attempt to minimize blood loss (e.g. tourniquet application) and to provide limited fluid resuscitation. Once arrived at the hospital, a rapid evaluation and identification of hemorrhagic shock is necessary in order to guide resuscitation. Complications in this assessment phase could arise from the fact that clinical symptoms of hemorrhagic shock, especially from occult bleeding, could be often subtle as robust compensatory mechanisms (i.e. vasoconstriction and tachycardia) hide in most patients the presence of hypotension until more than 30% of blood volume has been lost. For this reason, laboratory measures are recommended, including lactate and base deficit values, hemoglobin, platelet count and fibrinogen levels, together with specific exams to identify the cause of bleeding (e.g. radiographs). The main goal of initial resuscitation is to repair oxygen supply to organs and restore intravascular volume, as part of the so-called damage-control

resuscitation paradigm [42]. Fluids are usually administered to achieve the hemodynamic target, but recent evidences suggest that massive fluid infusion is not always beneficial for hemorrhagic shock patients [43] and limiting crystalloids infusion to 3 liters in the first 6 hours after arrival at the hospital is now recommended as part of the clinical practice [44]. After the initial course of resuscitation and definitive homeostasis, patients should be monitored for evidence of ongoing hemorrhage, unpaid oxygen debt, anemia, coagulopathy and other complications arising from over- or under-resuscitation. Useful end points for a successful resuscitation are suggested in the clinical routine as lactate values and base excess [9].

1.4 THE AUTONOMIC NERVOUS SYSTEM

1.4.1 Functional anatomy

The ANS [45] is part of the central nervous system (CNS) and it provides the innervation of blood vessels, the airways, the heart, the intestines, the stomach and the urogenital organs, thus controlling physiological functions such as respiratory rate, cardiac frequency, vasomotor activity, gastrointestinal motility and other reflex actions such as coughing, sneezing or vomiting. Within the brain, the ANS is regulated by the hypothalamus, positioned just above the brain stem, which acts as an integrator for autonomic functions. The two efferent branches of the ANS system are the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). Both the systems are characterized by two types of neurons involved in the transmission of signals: the pre-ganglionic and the post-ganglionic fibers.

The *sympathetic pre-ganglionic neurons* originate from the thoracolumbar region of the spinal cord, specifically in the section between T1 and L2; they travel to a ganglion where they synapse with post-ganglionic neurons, which are usually longer and extend across most of the body (Figure 1.3). The pre-ganglionic neurons are called *cholinergic* as they release *acetylcholine* at the synapses within the ganglia, a neurotransmitter that activates the nicotinic acetylcholine receptors on post-ganglionic neurons. On the contrary, the post-ganglionic neurons

are typically *adrenergic*, apart from a few rare exceptions (e.g. neurons innervating the sweat glands), and they release *norepinephrine,* which activates the adrenergic receptors on the peripheral target tissues.

The PNS is composed by several cranial nerves (CN), i.e. the oculomotor nerve (CN III), the facial nerve (CN VII), the glossopharyngeal nerve (CN IX), the vagus nerve (CN X), and by three spinal nerves originating in the sacrum, between S2 and S4, commonly referred to as pelvic splanchnic nerves. Unlike the SNS, all the pre-ganglionic and post-ganglionic nerves of the PNS are cholinergic.

Thus, the acetylcholine is principally a parasympathetic neurotransmitter, and, in particular, it stimulates two types of receptors: (1) the muscarinic receptors, present on the cells of the organs target of the parasympathetic post-ganglionic neurons, and (2) the nicotinic receptors, present at the level of the synapses between pre- and post-ganglionic neurons of both SNS and PNS. On the contrary, the adrenergic receptors are characteristic only of the SNS and they can be divided into two types: (1) the alpha receptors, excited by both the norepinephrine and epinephrine, and (2) the beta receptors which are stimulated only by norepinephrine.

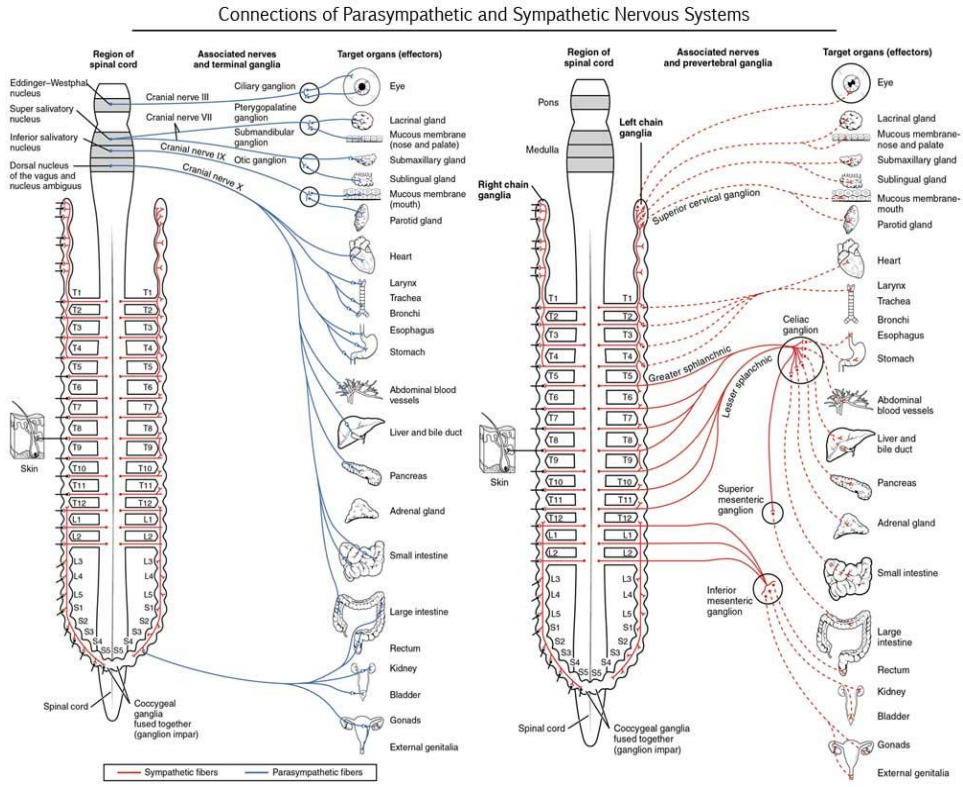

Figure 1.3. Left panel: parasympathetic or vagal autonomic nervous system. Right panel: sympathetic autonomic nervous system.

1.4.2 The autonomic cardiovascular regulation

The principal effects of SNS and PNS on cardiovascular regulation are typically opposite and complementary, and their combined effect is essential in order to maintain a condition of cardiovascular homeostasis.

SNS innervates most of the blood vessels, except for capillaries, and the heart. In the case of arterioles and small arteries, sympathetic stimulation could increase the resistance to the blood flow, mainly by the reduction of the diameter, leading to an increase of mean arterial BP and a deviation of blood to specific body areas. When acting on the veins, SNS could decrease their compliances, reducing blood volume and pushing more blood into the heart [46]. Finally, a sympathetic stimulation on the heart increases both the firing rate of the sinoatrial node and the contractility of the myocardium [45], [47]. *Alpha receptors* are mostly

located on the blood vessels and when activated they are responsible for the vasoconstriction of the vessels; *beta receptors* are mostly present on the heart where they mediate a positive chronotropic (i.e. increase in HR), dromotropic (i.e. increase in the conduction velocity) and inotropic effect (i.e. increase in contractility). At the level of vessels, the stimulation of the beta receptors causes vasodilation.

PNS system innervates the heart though the vagus nerve, whose stimulation decreases the HR and the cardiac contractility (negative chronotropic and inotropic effect). Although PNS actively influences only the activity of the heart, it could modify the level of BP as a secondary effect due to the regulation of CO [45], [47].

The circulatory regulation is placed in the vasomotor center which is part of the medulla oblongata located in the brainstem. It includes the nucleus of the solitary tract (NST), the rostral ventrolateral medulla (RVLM), the caudal ventrolateral medulla (CVLM), the dorsal motor nuclei (DMN) and the nucleus ambiguous (NA). RVLM is the main source of vessel vasoconstriction, projecting fibers to the spinal cord, in the pre-ganglionic neurons, and then to the vessels, passing through the sympathetic chain ganglia (located next to the vertebral column). The vagus nerve projects to the heart from the DMN through the NA, acting directly on the sinoatrial node. In contrast to the sympathetic branch, the PNS presents the pre-ganglionic neuron located in the brain medulla and the post-ganglionic neuron next to the effector organ. The NTS receives sensory nerve signals through the glossopharyngeal and the vagus nerve and directly projects on the medullary area, thus providing reflex control of many circulator functions.

The autonomic cardiovascular regulation is modulated through several afferent pathways and reflex mechanisms [45]. These mechanisms provide appropriate responses to rapid changes in cardiovascular homeostasis in order to maintain the arterial BP in a physiological range of values, to provide adequate blood flow to privileged regions (e.g. coronaries, kidneys and brain) and to redistribute it to specific regions according to the metabolic demand. For these reasons they are known as short-term reflex mechanisms (from seconds to minutes) and they include the arterial or cardiac baroreflex, the

cardiopulmonary baroreflex and the chemoreceptor reflex. Along with the these short-term mechanisms, the cardiovascular homeostasis is also regulated by long-term mechanisms (from minutes to hours or days) such as the renin-angiotensin system, the control of blood volume and pressure by the kidneys or the capillary fluid shift effect (Figure 1.4). As the present thesis is focused on the short-term regulation of BP and cardiovascular homeostasis, the long-term mechanisms will not be further discussed.

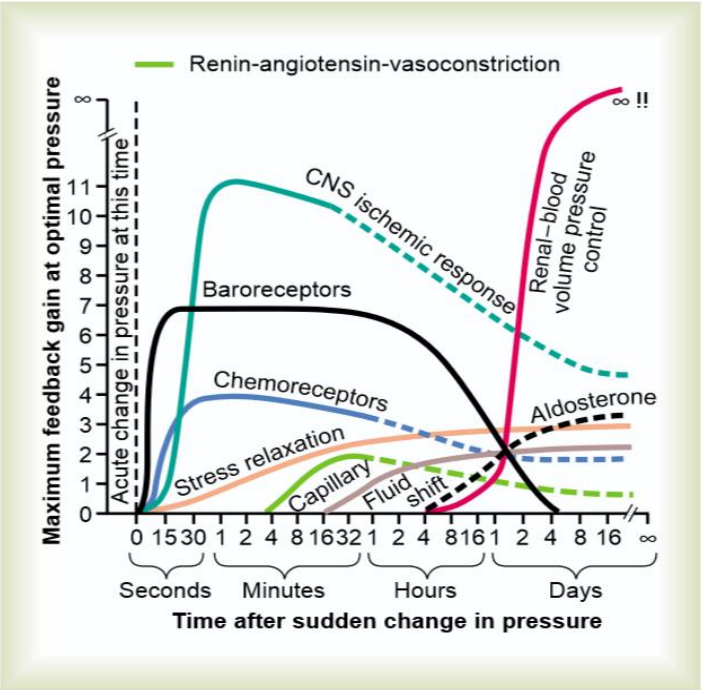

Figure 1.4. A synopsis of long-term and short-term regulatory mechanisms of arterial blood pressure regulation are reported from [45].

1.4.3 The short-term autonomic control of blood pressure

1.4.3.1 The baroreceptor reflexes

The baroreflex is the most important feedback mechanism responsible for BP homeostasis. It is initiated by the baroreceptors which are mechanosensitive nerve endings that respond to deformation or strain of the vessels walls generated by the fluctuations of BP.

Mechanosensitive ion channels are present on baroreceptor nerve endings, and the influx of sodium and calcium through these channels is responsible for the depolarization of baroreceptors during increased BP [48]. The generated neural signal is transmitted to the vasomotor center through the NST and secondary signals are thus sent to the sympathetic and vagal centers which can be stimulated or inhibited based on the physiological need. In particular, an increase in BP induces the inhibition of the sympathetic center, with a decreased outflow to heart and blood vessels, and the stimulation of the parasympathetic center, resulting in a raised vagal outflow towards the heart. The immediate consequence is an adjustment of BP through the action of the heart by CO modulation and of the circulation by modifying its resistance.

The sensitivity of the baroreceptors is not constant but it depends on the level of BP; in particular, their sensitivity becomes maximal in the range of BP values near the physiological ones, when even small variations of BP induce a strong reflex response. Moreover, also the rate of response of the baroreceptors is not constant: the more rapid is BP variation, the more rapid is the response of the receptors, regardless of the absolute value of pressure.

The arterial baroreflex

The arterial baroreceptors, usually known as high pressure baroreceptors, are mainly located in the aortic arch and carotid sinuses. A variation of arterial BP set point induces a modification in the tension of the arterial wall according to Laplace's law,

$$T = P * R \tag{1.1}$$

where P is the transmural pressure [N/m^2] and R is the lumen radius, that induces a change in the arterial wall stress as shown by the following equation

$$\sigma = \frac{T}{h} \tag{1.2}$$

where *h* is the wall thickness. The resulting modification in the shear stress elicits variations in the baroreceptors afferent discharge. Neural signals from arterial baroreceptors are transmitted to the NST in the medullary area of the brainstem through two pathways: (1) signals generated by the carotid baroreceptors are transmitted through the Hering's nerve and the glossopharyngeal nerve; (2) signals produced by the aortic baroreceptors reach the brain stem through the vagus nerve. At this level, neurons project to the medullary vasomotor center that mediates the sympathetic and parasympathetic outflows to the heart and the circulation. Thus, in response to an increase in arterial BP, the vagal centers are excited whereas the sympathetic pathway is inhibited. The net effects are a vasodilation of the veins and the arterioles and a reduction in HR and heart contractility. A decrease in arterial BP generates the opposite effects. A scheme of the arterial baroreflex functioning is reported in Figure 1.5 and Figure 1.6.

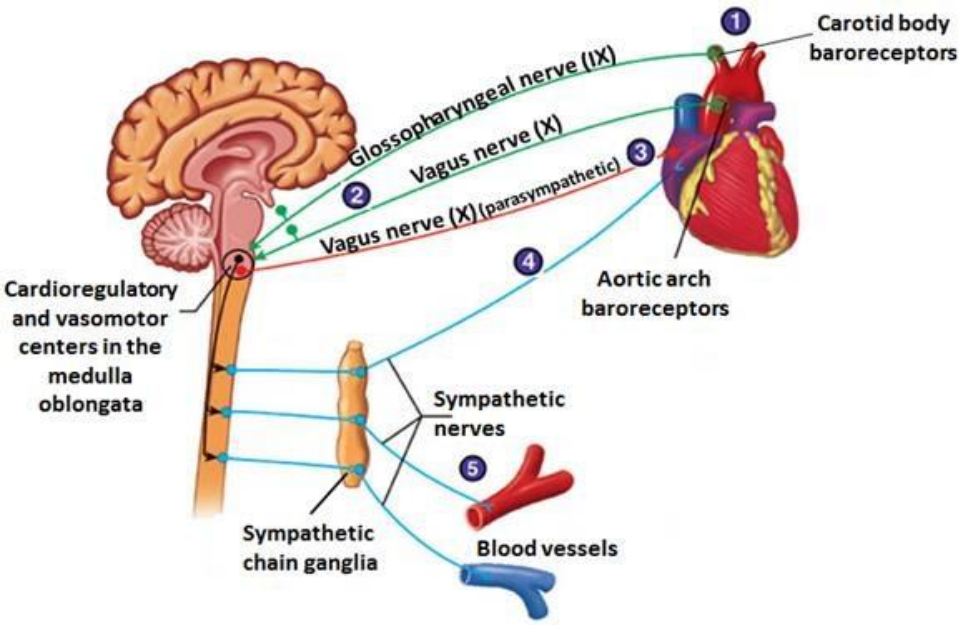

Figure 1.5. The anatomic scheme of baroreflex. In particular, the afferents are highlighted in green, while the efferent are illustrated in red and blue.

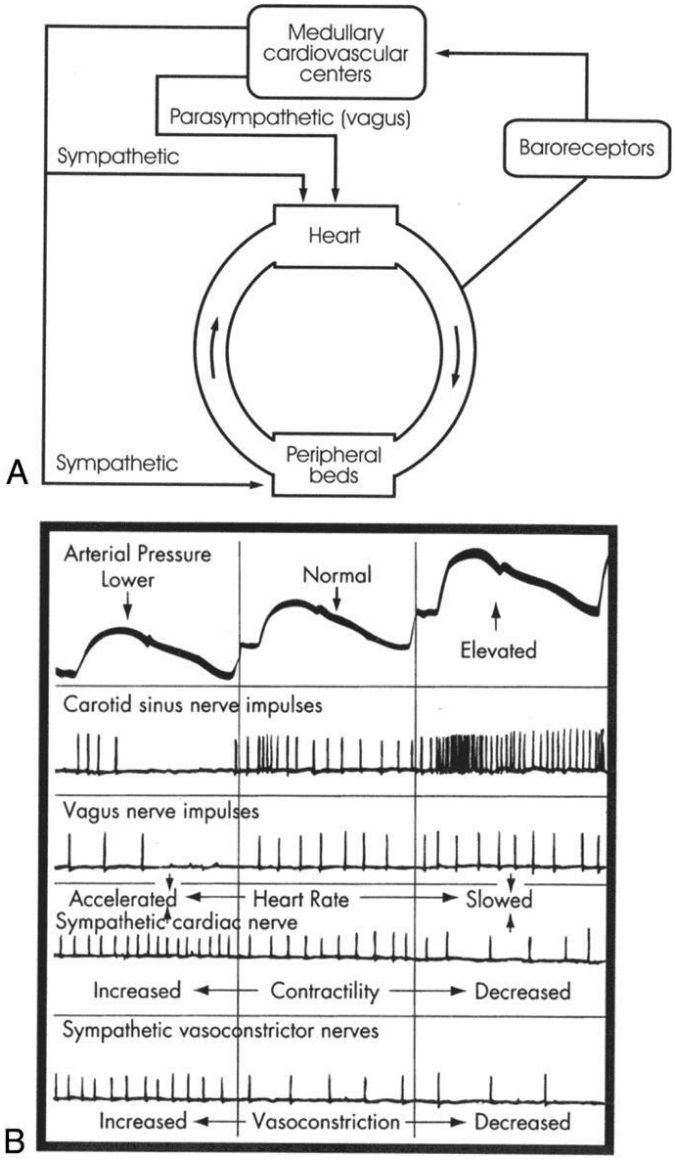

Figure 1.6. Baroreflex control of circulation. A) Principal components of the baroreceptors reflex: i) afferent branch, composed of arterial baroreceptors, located in the carotid sinuses and in the aortic arch, and afferent nerves, such as the glossopharyngeal and vagus nerve; ii) medullary cardiovascular centers which receive and integrate the neural information; iii) efferent branch, composed of the sympathetic fibers innervating the heart and the peripheral vessels, and the parasympathetic fibers, innervating the heart. B) A rise in BP generates an increased firing rate of the arterial baroreceptors resulting in sympathetic inhibition to heart and vessels and an increased vagal stimulation of the myocardium; the net effect is vasodilation, bradycardia and reduced CO, which allows the BP to return to physiological values. A fall in BP has the opposite effects [49].

The cardiopulmonary baroreflex

Cardiopulmonary baroreceptors, also known as volume receptors or low-pressure baroreceptors, are located in the cardiac atria and ventricles, vena cava and pulmonary vessels. They behave similarly to the arterial baroreceptors as they sense the mechanical stretch on the vessels or atria walls and they attempt to minimize the changes in BP due to changes in blood volume, mostly contained in the veins. The physiological variable which is actually sensed by the cardiopulmonary mechanoreceptors is the CVP, whose changes are elicited by volume shifts. Thus, in response to a reduction in CVP, the baroreceptors discharge is transmitted to the NST through the vagus nerve and the autonomic reflex mechanism produces an increase in the sympathetic outflow to heart and vessels and a decrease in the vagal outflow to the heart, with a net effect resulting in increase of HR and vasoconstriction. On the opposite, an increase in central pressure leads to a stimulation of the parasympathetic center and an inhibition of the sympathetic center which induces vasodilation for the absence of efferent discharge towards vessels as well as decrease in HR for the direct vagal stimulation on the heart [45]. However, with respect to the changes induced by the arterial baroreflex, the cardiopulmonary variations in HR have a much smaller extent [50].

It is important to notice that the direct effect of cardiopulmonary reflex on cardiac contractility is not clarified yet; moreover, the Bainbridge reflex is still a matter of debate. It consists in a transient increase of HR in case of an increase of central blood volume, leading to a transient tachycardia concomitant to high right atrial pressures, in contrast with the two types of baroreflex discussed above. The Bainbridge reflex has been proved in animals like dogs and rats, but not fully understood in humans; nonetheless, some results hint a possible role in cardiovascular regulation in case of large variations of venous return [49], [51].

1.4.3.2 The chemoreceptor reflex

Another mechanism involved in BP regulation is the control of respiratory activity by the chemoreceptors. These receptors are located both peripherally (carotid bodies and aortic arch) and centrally

(respiratory center of brain medulla), and they contribute in maintaining the arterial pH, PaO_2 and carbon dioxide ($PaCO_2$) within appropriate physiological ranges. Differently from baroreceptors, chemoreceptors respond to chemical stimuli. In particular, an increase in $PaCO_2$ (hypercapnia) detected by central chemoreceptors or a decrease in PaO_2 (hypoxia) sensed by peripheral chemoreceptors lead to an increase of breath rate and depth of respiration mediated by the neural respiratory center. Anatomically, the chemoreceptors transmit afferent signals through the vagus nerve to the NST where the respiratory center is located. Furthermore, the respiratory center stimulates also the vasomotor center; the net effect is a concomitant increase of arterial BP by means of neurally mediated vasoconstriction, although this reflex has a limited extent with respect to the arterial baroreflex [45], [52].

The chemoreceptor reflex has been largely studied and reviewed [53]–[55] and it can be summarized as a feedback process that closely interacts with the baroreflex with an inhibitory effect [56], [57].

1.4.4 The inflammatory reflex

In the last years a new field named bioelectronic medicine is rapidly evolving towards the discovery and development of innovative nerve stimulating and sensing technologies in order to diagnose and regulate biological processes and treat diseases. At the core of this new discipline is the electrical signal used by the nervous system to communicate information; the idea is that manipulating the neural signals it will be possible to change the way physicians treat disease conditions including autoimmune disorders (e.g. rheumatoid arthritis), inflammatory pathologies (e.g. Crohn's disease, sepsis), diabetes, paralysis, bleeding and even cancer [58].

The crucial role of the ANS in the inflammatory response modulation was proved by recent scientific evidences. The so called *inflammatory reflex* highlights the acquired awareness that the nervous system reflexively regulates the inflammatory response in real time, just as it controls HR and other vital functions [59]. In particular, the role of the vagus nerve is under intense investigation and, recently, researches have demonstrated that the electrical stimulation of the efferent vagus

nerve inhibits the pro-inflammatory cytokines cascade through the so called *cholinergic anti-inflammatory pathway* [59]–[62]. Briefly, efferent activity in the vagus nerve leads to acetylcholine release in target organs such as the liver, heart, spleen and gastrointestinal tract; acetylcholine interacts with α-nicotinic receptors on tissue macrophages and it inhibits the release of TNF, interleukin-1, high mobility group B1 and other pro-inflammatory cytokines. This mechanism has been exploited in numerous studies which showed the beneficial effect of vagal stimulation in treating sepsis and septic shock [60], [63], [64], in protecting against excessive inflammatory response in hemorrhagic shock [65], and in preventing intestinal dysfunction after traumatic brain injury [66]. Moreover, a direct influence of efferent vagal outflow in the treatment of hemorrhage has been demonstrated [58]. The neural tourniquet is a new technique based on vagal stimulation which is able to reduce blood loss and duration of hemorrhage, and to reduce coagulopathy in hemorrhagic shock. Briefly, the basic mechanism includes the stimulation of the efferent vagus nerve which conveys a signal to the spleen where the platelet blood cells, that are in charge for clotting, receive their instructions; this vagal signal "primes" the platelets, prepping them to form clots if they encounter a wound anywhere in the body. Clinical trials on human patients are currently ongoing [58].

The development of these new cutting-edge technologies are paving the way for innovative therapies based on selective stimulations of the nervous system. The contribution of the present thesis to understand the autonomic regulation of the cardiovascular system during shock could be also beneficial for a future extension of the vagus stimulation technique in a clinical setting.

1.5 THE ROLE OF THE AUTONOMIC NERVOUS SYSTEM IN SHOCK

1.5.1 The ANS in septic shock

Septic shock, as already reported in §1.2, can be described as a failure of the defense mechanisms which are in charge of counteracting

the uncontrolled septic immune response and the consequent hypotensive stress. The normal compensatory response to hypotension includes an increased sympathetic outflow to heart and peripheral vessels to restore BP to normal. In this respect, the function of the ANS during sepsis and septic shock is of ultimate interest.

Several previous studies tried to characterize the role of the ANS, both the sympathetic and vagal branch, during septic shock, but many determinants of its function remain to be elucidated. The majority of studies in literature describe a dysfunction of the sympathetic branch of the ANS, i.e. a maladaptive response to stress in septic shock subjects, which leads to an impaired autonomic control of heart and vessels, thus contributing to circulatory failure. The exact pathophysiological mechanism of this autonomic imbalance is not clear yet, but there is evidence that it is often due to an excessive, uncontrolled or prolonged sympathetic activation, or to an inappropriate regulation of the parasympathetic branch of the nervous system [67], [68].

Life-threatening illness, such as septic shock, is one of the most potent stimuli of the SNS and a massive sympathetic activation is documented by elevated concentration of endogenous circulating catecholamines, i.e. plasma epinephrine and norepinephrine, during shock and persisting during the post-resuscitation phase. However, persistently high plasma catecholamines level was demonstrated to be significantly associated with increased morbidity and mortality in critically ill populations [69], [70] and, in particular, in septic shock patients [71]–[74]. Protracted and overwhelming adrenergic stress in critical illness may exceed in time and scope the beneficial short-term compensatory effects and it may cause adverse effects. The entity of damage depends on the vulnerability of the different organs to adrenergic overstimulation and to the presence of coexisting chronic diseases; for example, the heart, which is abundant of β-adrenergic receptors, seems to be most susceptible to sympathetic overstimulation with detrimental consequences such as impaired diastolic function, tachyarrhythmia, myocardial ischemia, stunning, apoptosis and necrosis. Other organs are also affected with associated consequences such as pulmonary edema, with subsequent right heart dysfunction/failure,

increased thromboses formation, gastrointestinal hypoperfusion, immunosuppression, increase in cell energy expenditure, anemia, and microvascular dysfunction [67], [68].

Aside from massive stimulation, the SNS is widely disturbed during severe sepsis and septic shock with deficiencies in afferent, central and efferent pathways; for example, proinflammatory cytokines and overproduction of nitric oxide (NO) have been shown to induce a down-regulation of adrenergic receptors [75], [76], which could probably explain the loss in vascular response typical of septic shock patients. This deficiency condition is generally referred to as autonomic dysfunction.

Clinically, powerful established tools to characterize autonomic dysfunction includes heart rate variability (HRV), blood pressure variability (BPV), baroreflex sensitivity (BRS) and, less frequently, chemoreflex sensitivity [77]–[81]. A reduction in physiologic HRV, BPV and BRS has been demonstrated to be directly correlated with septic shock severity and mortality in several researches [82], [83], [92], [93], [84]–[91].

A basic feature of the healthy human body is a continuous communication among all vital organs through neural signals mediated by the ANS, which allows a constant adaptation to the different physiological and pathological conditions. Bacterial toxins and inflammatory mediators of sepsis can strongly alter neural reflexes [94] and consequently cause an uncoupling of these organs interconnections, advancing single organ dysfunction into MOF, which is a common cause of death of septic shock subjects [82].

However, the exact mechanism responsible for this autonomic disarray has not been disentangled yet and, in particular, scarce information is available about the level where the sympathetic ANS is altered, i.e. whether it is a reduction in central vasomotor activity, or at the level of peripheral neuroeffector transmission, or a depressed end-organ responsiveness. The role of the arterial baroreflex has also been hypothesized as a possible mechanism responsible for the observed autonomic anomalies. The autonomic-mediated baroreflex system is a key physiological mechanism for BP regulation and it is deputed to maintain homeostasis and blood flow to vital organs (see also §1.4.3.1).

Therefore, it's role during cardiovascular stress conditions is crucial and there is evidence that it becomes impaired after septic shock onset [82], [83], [88], [91]. Moreover, a direct relation between BRS and survival time has been reported in septic shock animal experiments, with a diminished survival time in the presence of a reduced baroreflex function [85], [86], [90]. Such sepsis-related dysfunction of BP regulation might involve the baroreflex arch at several levels such as, i) a reduction of baroreceptors sensitivity, ii) a shift of the baroreflex set point to lower levels of BP with impaired efferent sympathetic activity, or iii) a reduced responsiveness of the target organ.

A possible confounding factor which could play a role in the reduction of BRS observed in septic shock is the deactivation of baroreceptors caused by the severe drop in BP. However, a recent study on E. Coli Lipopolysaccharide (LPS) toxin-treated rats demonstrated that the impairment of the baroreflex occurs almost immediately after the induction of sepsis and in absence of BP changes, and it persisted after E. Coli infusion was stopped [87]. Thus, it was hypothesized a direct effect of the toxin through the production of NO and ROS, which have a direct negative effect on BRS [95]; circulating cytokines released after E. Coli infusion could also play an important role since they could induce an inflammation of the carotid body with a consequent reduction in arterial distensibility and, therefore, of baroreceptors engagement [96].

The limited knowledge about the role of sympathetic branches on cardio-circulatory control during septic shock is partly due to the difficulty to directly assess sympathetic activity. Indeed, the indirect measures of autonomic activity, as the HRV previously described, do not allow to discriminate whether the sympathetic outflow is compromised at the central level or at the level of peripheral neuroeffector transmission. The possibility to obtain direct measures, in animal experiments, of the sympathetic outflow through microneurographic measurements of muscle, cardiac, or renal sympathetic nerve activity (MSNA, CSNA, RSNA, respectively) is essential to deeply investigate the role of baroreflex in cardiovascular response to sepsis and septic shock and to directly unravel changes in centrally regulated sympathetic outflow to different peripheral districts [97]–[99]. The results of these

studies highlighted a general increase in RSNA and CSNA after the induction of sepsis, supporting the idea that the observed autonomic imbalance is associated with an uncontrolled and prolonged sympathetic activation. Ramchandra et al. [98] studied the changes in sympathetic activity following infusion of LPS in conscious sheep and reported an increase in CSNA highly correlated with the increasing HR and a transient inhibition of RSNA during the first 3 hours after infusion followed by a prolonged stimulation; in addition, they found differential changes in the range of the arterial baroreflex activity, as it was depressed for HR, increased for CSNA, and unchanged for RSNA. The high correlation between the changes in HR and CSNA support the notion that increased sympathetic drive to the heart contributes to the tachycardia during septic shock, although other mechanisms could also play a role [98]. Vayssettes et al. [99] also reported a strong correlation between marked increased RSNA and tachycardia in anesthetized rats after infusion of LPS; moreover, they showed that arterial baroreceptor denervation minimally affected RSNA increase induced by LPS. On the contrary, the study of Sayk et al. [97] demonstrated a suppressed MSNA, concomitant to an increased HR and a blunted MSNA baroreflex-mediated response in a population of healthy human volunteers after injection of endotoxin. Moreover, the accelerated HR did not react to any BP modulation, indicating that HR was uncoupled from baroreflex regulation. This points to the possibility that the septic immune response directly suppresses sympathetic outflow to the muscle vascular bed via central nervous mechanisms leading to a blunted BRS [97].

Together these studies highlighted an altered baroreflex control driving the sympatho-excitation elicited by sepsis, supporting previous researches which demonstrated that the sympathetic activation observed in septic shock was greater than what expected with the simply baroreceptors unloading due to hypotensive stress [100], [101].

Finally, these findings would explain the reduction in physiological variability described by indirect measures of autonomic activity, such as the HRV, as previously reported. A reduced variability in the HR implicates a strongly reduced baroreflex activity, until a possible complete uncoupling between HR and BP regulatory mechanisms, and a

compromised vagal activity at the heart level, both of which are documented in septic shock subjects. Moreover, also the high level of circulating catecholamines reported in septic shock are found to be inversely correlated to indices of HRV. A proposed speculation is that, as in heart failure patients, high sympathetic drive may lead to saturation of low frequency oscillatory systems [102], or that excessive concentrations of circulating catecholamines may compromise central autonomic controls [91], [97].

Although the concept of "adrenergic toxicity" in septic shock and in general acute illness is well established in the scientific literature, no clear clinical recommendations are currently available, and clinicians still rely on limited therapeutic options as proposed in protocolized guidelines (see §1.2.3). For example, supplemental catecholamines administration, as norepinephrine, is still a cornerstone of septic shock therapy even if it could further deteriorate adrenergic stress in these patients. However, other clinical options are currently under investigation and the available data are promising [67]. To date, the most robust clinical evidence involves the use of the cardioselective β-blocker esmolol, as documented both in patients with persistent tachycardia secondary to catecholamine use [5] and in animal experiments [103], [104]. A possible mechanism explaining the beneficial effect of this β1 selective antagonist on cardiac function is an indirect effect on vagal nerve activity, as recently proposed by Aboab and colleagues [6], supporting the concept of vagal stimulation as a potentially future therapeutic approach (see §1.4.4).

1.5.2 The ANS in severe bleeding and hemorrhagic shock

Acute hemorrhage, similarly to the sepsis, is a strong enhancer of the ANS and during progressive blood loss an autonomic-mediated compensatory response is usually elicited trying to maintain the physiological homeostasis. Specifically, a raised sympathetic activity is usually achieved by the organism in order to elicit peripheral vasoconstriction, tachycardia, increased myocardial contractility and CO, in the attempt to preserve BP and organs perfusion [105]. Direct measurements of sympathetic activity in nerves supplying muscle, renal, hepatic, adrenal, splenic, and cardiac vascular beds in different animal

experiments provided strong evidence of this initial phase of sympathoexcitation [106]–[110].

With the introduction of lower body negative pressure (LBNP) experimental model in the 1960s, it was possible to investigate the response to central hypovolemia in healthy human volunteers and it is now widely accepted that LBNP is a reliable model of simulated hemorrhage [111].

As in the animal models, it was found that in humans one of the early physiological compensatory mechanisms during the course of progressive LBNP is a baroreflex-mediated increase in MSNA, generated by deactivation of both cardiopulmonary and arterial baroreceptors [112]. Moreover, the same relationship between progressive hypovolemia and RSNA was also reported in anesthetized sheep [113]. In addition to an increase in absolute MSNA, it was also observed a change in the pattern of bursting activity [114]. In physiological conditions and homeostatic compensation, MSNA is characterized by low frequency (LF, 0.04-0.15 Hz) oscillatory patterns, which are strictly related to LF oscillations of BP [115]. In particular, a decrease in arterial pressure quickly initiates an increase in traffic of sympathetic nerve impulses by decreasing inhibitory afferent activity to the NST; the subsequent arterial vasoconstriction results in increased vascular tone and compensatory elevation in arterial BP, activating a baroreflex-mediated feedback reduction in sympathetic outflow. This baroreflex-mediated phenomenon of oscillatory coupling between arterial BP and sympathetic activity represents an important compensatory mechanism under severe hypovolemic conditions and it is designed to maintain tissue oxygenation despite compromised blood flow. At high levels of LBNP, right before decompensation occurs, an interesting phenomenon of bursts fusing has been reported both in MSNA and RSNA, as described in [113], [114], and a decoupling between oscillations in sympathetic activity and BP arises [105]. Several possible mechanisms responsible for the onset of cardiovascular collapse have been investigated. A sympathetic neural withdrawal was first suggested [111], [116], but, this hypothesis was corrupted by recent findings that not all subjects experienced this sympathetic inhibition at the point of hemodynamic decompensation

[114], [117]. Another possible mechanism is the central resetting of baroreflexes, resulting in a loss of synchrony between arterial BP and compensatory mechanisms such as sympathetic activation [118], [119]. Finally, there is evidence that an increase in the LF oscillatory patterns of both MSNA and BP could have a protective role during progressive central hypovolemia and a loss of this dynamic coupling might precipitate hypotension and decompensatory shock [120]. Moreover, this oscillatory coupling also translates into a protected cerebral perfusion. In a study of progressive LBNP by Kay and Rickards [121], a continuous transcranial Doppler measurement provides a measure of mean cerebral artery velocity (MCAv), commonly used as an index of global cerebral blood flow. Similar to arterial BP recordings, few oscillatory patterns are visible in MCAv at the time of decompensation.

Although LBNP is not an appropriate model to study the effects of prolonged hemorrhage and circulatory shock, with accompanying inflammation and metabolic acidosis, it turned out to be useful in the understanding of severe hemorrhage physiology and individual variability in the physiological compensatory responses. In particular, the possibility to collect a large database of LBNP human experiments [105] has revealed the classification of individuals into two main categories, such as two-thirds of the population have an high tolerance to central hypovolemia, i.e. > -60 mmHg LBNP level, whereas the remaining one-third displays a low tolerance to reduced central blood volume, i.e. they reached presyncope symptoms at LBNP $\leq$ -60 mmHg. These results lead to the definition of the so-called “compensatory reserve” which can be quantified as the difference between a baseline state and maximal physiological response [122], [123]. From the hemodynamic point of view, high tolerance individuals are characterized by a greater reserve for elevated HR and peripheral vascular resistance, which are associated with a greater cardiac baroreflex-mediated vagal withdrawal, sympathetic nerve activity and circulating neuroendocrine vasopressors [124].

These data highlight the physiological importance of measuring dynamic relationships, such as the oscillatory patterns in BP and flow and their coherence, for an accurate diagnosis of hemorrhage patients rather than relying on average responses. To date, standards of resuscitation for

prehospital trauma patients include the administration of fluids to stabilize BP and vascular volume before blood transfusion (for details see §1.3.3). However, there is an increasing evidence that a mean BP-targeted resuscitation is not always effective and that mean BP may be an inappropriate parameter to follow during fluid resuscitation, as it could lead to fluid overload and increased mortality [125].

Given the crucial role of the ANS during the compensatory phase of severe bleeding, a reasonable hypothesis is that these autonomic mechanisms are needed to be reestablished during the resuscitation phase, and the search for non-invasive surrogates of sympathetic nerve activity to be applicable in clinical monitoring practice is still open.

1.6 MOTIVATION AND OBJECTIVES OF THE STUDY

Despite significant improvements in clinical care, accurate diagnosis and effective therapies for shock patients still remain an open challenge. In fact, the assessment of patients' severity can be complicated by the multiple comorbidities often present in critical patients and by the high inter-subject variability observed either in the disease's symptoms and in the response to therapy. Moreover, the benefit of the therapeutic approach is evaluated based upon global hemodynamic, clinical and metabolic end-points (e.g. mean BP, oxygen saturation and lactate), thereby therapies are not optimized for individual patients. This could explain, at least partially, the inefficiency of current guidelines for resuscitation of shock patients; as already reported in §1.1, the mortality related to the syndrome is still very high (50% in some cases) and the recovery of patients is often only partial.

Recently, many studies and research trials are shedding light on critical aspects of the existing resuscitation protocols. Overzealous resuscitation with crystalloids in transfused hemorrhagic trauma patients, as indicated by the current resuscitation strategy, has been demonstrated not to be as effective as it claims to be; in fact, by diluting the oxygen-carrying capacity and clotting factor concentrations it can exacerbate coagulopathy, inflammation and hypoxia, leading to a deterioration of the patient's health status [9], [126].

Septic shock resuscitation guidelines have been extensively revisited during the past years [14], [33], [34], [127] but a definitive clinical strategy has not been achieved yet. In 2005, Rivers and colleagues proposed the so called *Early Goal-Directed Therapy (EGDT)* [34], proving its effectiveness in reducing morbidity and mortality. However, its benefit was questioned by three recent large trials. The ARISE trial [128] (Australiasian Resuscitation in Sepsis Evaluation) was conducted at 51 centers (mostly in Australia or New Zealand) and 1600 early septic shock patients were enrolled in the study. They were randomly assigned to receive either EGDT or usual care, and the primary outcome was all-cause mortality within 90 days after randomization. The analyses showed that EGDT did not reduce all-cause mortality at 90 days and no significant differences were found in survival time, in-hospital mortality, duration of organ support or length of hospital stay between the two groups. Interestingly, the EGDT group was shown to have larger mean volume of intravenous fluids and vasopressors, and it is known that fluid overload and persistent vasopressors need is positively related to increased morbidity and mortality [129]–[132]. Similar conclusions were reached also by the ProMISe trial [133] (Protocolised Management in Sepsis) which enrolled 1260 septic shock patients in 56 different hospitals in England, and by the ProCESS trial [134] (Protocolized Care for Early Septic Shock) which included 1341 septic shock patients of 31 emergency departments in the United States. Although the complexity and heterogeneity of shock population could be a possible explanation for these discrepancies [135], [136], the lack of a pathophysiological approach to hemodynamic monitoring could also contribute to the failure of generalized resuscitation protocols.

The role of the ANS during stress conditions such as circulatory shock is crucial in the attempt of the organism to preserve homeostasis, as specifically detailed in §1.5. Many works documented an altered sympathetic activity with the progression of shock severity, either by means of direct measures of sympathetic activity [111]–[113] such as CSNA, MSNA, or RSNA, through surgical or pharmacological interventions on the ANS in animal models [89], [137], [138], or by means of indirect estimations of autonomic activity, such as HRV, BPV or BRS [82], [83],

[88], [93], [139], [140] (for details see §1.5).

The idea of integrating these non-invasive cardiovascular indices into bedside monitoring of shock patients may be an expected conclusion of the results of these studies, with the aim of a functional hemodynamic monitoring to predict a possible catastrophic deterioration of the patient, thus allowing clinicians to act beforehand. As already illustrated in §1.4, the ANS operates in the short term range (seconds to minutes) and clinical signs of an ongoing failure could be a wake-up call of an imminent decline, before any deterioration of mean values and global markers, which could occur too late for any effective action.

The idea of a continuous hemodynamic monitoring which could include also cardiovascular indices together with mean values traditionally displayed on the monitor has already been proposed as a valuable tool in critical settings [141]–[144]; a valiant example is the HeRO (Heart Rate Observation) monitor which was developed to analyze a new cardiovascular index, the so called HR characteristics [145], and display to clinicians a score which indicates the risk of an infant deteriorating from sepsis in the next day [146], so to direct the actions of nurses and clinicians. Display of this HeRO score resulted in a 22% relative reduction in mortality in a large randomized clinical trial of very low birthweight neonatal ICU patients [147].

However, little attention has been given so far to the short term cardiovascular assessment of shock patients during the resuscitation phase. Clinical guidelines themselves recommend the supervision of global hemodynamic mean variables, such as mean arterial BP, or macroscopic clinical indices, such as lactate, thereby they do not point at short term cardiovascular monitoring; thus, clinicians usually keep on administering fluids and vasopressors to reach the target and to maintain the patient in a stable condition, without any kind of information about the functionality of autonomic mechanisms which are known to be the authors of physiological BP regulation and control. Thus, if the patient effectively recovered to a physiological status or if the restoration of BP was only induced by external pharmacological maneuvers is not taken into account during resuscitation. Moreover, an interesting review has recently highlighted the tendency of clinicians and nurses to exceed the

minimum BP target, as a consequence of the erroneous perception that "more is better" [132]. Thus, shock patients are often exposed to unnecessary and prolonged catecholamines and fluids infusion, which further worsen their condition [129]–[131].

The hypothesis of this thesis comes from the idea that the restoration of the autonomic cardiovascular control mechanisms in shock patients should be of primary importance combined to the resuscitation targets, since it can guarantee an effective return to physiological homeostasis; in other words, as the role of the ANS has been proven to be crucial during the compensatory phase of shock, similarly we can hypothesize that it is determinant in the recovery phase too.

This hypothesis has been explored in three different datasets: (1) a cohort of 21 severe septic shock patients recruited at Geneva University Hospital and followed during the first 48 hours of therapy after development of septic shock; (2) an experimental group of 6 pigs undergoing a protocol of severe septic shock and resuscitation, performed at Bruxelles Erasme University Hospital; (3) an experimental group of 6 pigs undergoing a protocol of severe hemorrhagic shock and resuscitation, performed at Istituto di Ricerche Farmacologiche Mario Negri IRCCS. All the datasets were collected under the project ShockOmics (NCT02141607) [148].

Standard and advanced mathematical methods and modelling have been developed in order to investigate the autonomic-mediated mechanisms of physiological regulation, how they change when shock occurs and if they are restored during the resuscitation phase. The primary focus of the study was given to the autonomic regulation of BP and HR, given the importance of the heart and the circulatory system in the progression of shock syndrome. The developed models enabled to highlight the importance of these ANS-mediated mechanisms for a full recovery of the patients, together with standard resuscitation indices, such as mean BP and lactate. The novel idea proposed by this research thesis is that the integration of such hemodynamic indices of BPV and HRV to the current clinical guidelines could help understanding the responsiveness of the patients to the combination of sympathomimetic drugs and fluid therapy and, thus, pave the way for a tailored therapy to

reduce mortality and increase the quality of life in survived patients.

1.6.1 Thesis outline

This thesis is organized into six chapters, including an introduction and a conclusion chapter with future directions and clinical impact of the work. Two appendixes complete the dissertation showing some ancillary studies.

The current chapter 1 illustrates the pathophysiological background of shock, in particular septic and hemorrhagic, the structure and functions of the ANS, with a particular emphasis on short-term cardiovascular regulatory mechanisms, and it summarizes the current knowledge about the role of the ANS both in septic and in hemorrhagic shock. Chapter 2 presents the analyses performed on a cohort of septic shock patients from ShockOmics clinical trial (NCT02141607) [148]. Chapters 3 and 4 show the analyses performed on experimental data obtained from a pig model of peritonitis-induced septic shock and resuscitation, with a particular emphasis on the regulation of cardiac contractility and on the hemodynamic characterization of arterial vascular properties. Chapter 5 describes the analyses performed on the experimental data obtained from a pig model of hemorrhagic shock and resuscitation. Chapter 6 summarizes the results, illustrates the clinical impact of the work and outlines the future steps. The appendixes contain additional results about ancillary studies. Appendix A presents the results of metabolomics profile of cardiac tissue in hemorrhagic shock animals associated to the hemodynamic findings. Appendix B describes preliminary findings on septic shock animals treated with ivabradine versus sham animals. The final appendix reports the complete list of the publications of the PhD candidate.

2 AUTONOMIC INDICES CAN HELP IN UNDERSTANDING THE DIFFERENT RESPONSE TO THERAPY DURING ACUTE PHASE OF SEPTIC SHOCK

In this study we examined the early response to therapy in a subset of 21 severe septic shock patients enrolled in the multicenter prospective observational trial Shockomics (NCT02141607). This work was partly presented at the 40th Annual Conference on Shock[1], and at the 16th Annual International Conference on Complex Acute Illness (ICCAI)[2], and it was published as journal paper on SHOCK journal[3].

Our purpose was to study the response of the ANS to standard resuscitation maneuvers during the acute phase of the syndrome testing the hypothesis that mathematical indices of ANS activation may help clinicians in understanding the actual effectiveness of the administered fluids and vasopressors therapy, in combination with standard biological measures.

Patients were divided into two groups, i.e. those who recovered during the first two days of intensive care and those who did not improve their condition, based on SOFA score changes. Advanced mathematical indices and models were adopted in order to study the autonomic-mediated variability in BP and HR and the interactions between the two biological systems.

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[1] M. CARRARA, B. Bollen Pinto, G. Baselli, K. Bendjelid, M. Ferrario, *"Different response to therapy in septic shock: an autonomic perspective"*, 40th Annual Conference on Shock, Fort Lauderdale, Florida (USA), June 3-6 2017 (abstract published in SHOCK, Vol. 47, No. 6, pp. 36-36, 2017)

[2] M. CARRARA, B. Bollen Pinto, G. Baselli, K. Bendjelid, M. Ferrario, "*The role of fluid accumulation in early septic shock: differences in ANS-driven response to therapy*", 16th Annual International Conference on Complex Acute Illness (ICCAI), Milan, Italy, July 27-29 2017 (abstract published in Journal of Critical Care, Volume 42, pp 379, 2017)

[3] M. CARRARA, B. Bollen Pinto, G. Baselli, K. Bendjelid, M. Ferrario, "*Baroreflex sensitivity and blood pressure variability can help in understanding the different response to therapy during acute phase of septic shock*", SHOCK, Vol. 50, No. 1, pp. 78-86, 2018

Our results highlighted a sympathetic activation in response to fluids and vasopressors only in those patients who significantly improved their organ function, although all patients reached an adequate increase in mean BP. Moreover, we interestingly found out that fluid accumulation was higher in non-improving patients.

Details about rationale, methodology, results and conclusions of the study are described in the following paragraphs. They are based on the related journal paper. A final remark on the main findings is reported at the end of the chapter.

2.1 INTRODUCTION

It is well known from literature that EGDT is not as effective as it initially advocated. Often aggressive fluid resuscitation during the first hours may cause fluid overload that subsequently affects patients' outcome (see §1.6). Persistent fluid overload during the first days increases the risk of cardiac, renal, and pulmonary dysfunction. Moreover, a positive fluid balance is associated with increased morbidity–mortality [128], [129], [133], [134].

Indeed, since the first positive study of Rivers et al. [34], three recent large trials were not able to replicate these results and none of these studies demonstrated a benefit of EGDT protocol [135]. The reasons for these inconsistencies in the medical literature are not fully clear: the complexity of the population under study and the lack of a pathophysiological approach to hemodynamic monitoring could be some possible explanations [136]. In this regard, a high inter-subject variability in response to treatment suggests the need for personalized approaches, i.e., patient-targeted therapy and not absolute thresholds.

The aim of EGDT is to restore BP, i.e., mean BP, CVP, and $ScvO_2$. However, the patient response to the initial hemodynamic optimization therapy cannot be evaluated solely by absolute values of vital signs, as they do not imply a restoration of the cardiovascular autonomic control. In other words, the restoration of the short term physiological mechanisms for BP and HR regulation cannot be inferred only from mean values of the classical hemodynamic variables.

In this study, the authors wanted to test the hypothesis that the changes in sympathetic ANS activity and in autonomic-mediated BP control mechanisms may help to understand the actual effectiveness of fluids and vasopressor therapies and to correctly interpret the trend in mean arterial BP values [149]–[151]. In particular, we analyzed patients' response to therapy in early phase of septic shock and examined possible associations among BP recovery, BP autonomic control, and fluid accumulation.

2.2 MATERIALS AND METHODS

2.2.1 Study design and patients' population

The present work is an ancillary study from the multicenter prospective observational trial "ShockOmics: Multiscale Approach to the Identification of Molecular Biomarkers in Acute Heart Failure Induced by Shock" (ClinicalTrials.gov Identifier NCT02141607). More details about the protocol can be found in the original paper [148].

A subset of 21 septic shock patients enrolled at Geneva University Hospital from October 2014 till December 2015 was included in this study, after approval of the Geneva regional research ethics committee (*Commission cantonale d'éthique de la recherché*, President: Prof. Bernhard Hirschel study number 14-041). All participants gave prior informed consent. Strict inclusion and exclusion criteria were observed during the recruitment phase, to avoid a too high inhomogeneity within the population [148]. In particular, patients were included in the study if the shock was severe enough, based on SOFA score, i.e. SOFA > 5, and if the first blood sample and first hemodynamic measurements were available within 16 hours from ICU admission. Exclusion criteria were the following:

- Risk of fatal illness and death within 24 hours
- More than 4 units of red blood cells transfused
- Transfusion of plasma or whole blood
- Active haematological malignancy
- Metastatic and/or active cancer

- Immunodepression and presence of immunodeficiency virus (HIV +)
- Pre-existing end stage renal disease needing renal replacement therapy
- Cardiac surgery in the previous days
- Cirrhosis or acute liver failure
- Terminal illness

The patients received initial therapy according to the standards [127] immediately after shock diagnosis (time T0). Patients were analyzed at two relevant time points: within 16 h from T0 when the inflammatory cascade has been just activated (T1), and within 48 h after T0 time point (T2). SOFA score was used to classify patients into two groups according to their responsiveness to early therapy: responder patients (R, n=14) consisted in patients with a positive response to initial treatment. Those patients, who decreased their SOFA score of at least 5 points between T1 and T2 ($\Delta SOFA_{T2\text{-}T1} \geq 5$) or reached a SOFA score at T2 lower than 8 were classified as R. Non-responder patients (NR, n=7) consisted in patients who still had a SOFA score at T2 higher than 8 or $\Delta SOFA_{T2\text{-}T1} < 5$.

2.2.2 Clinical and therapy data

Information on therapy and sedation administered to the patients were available and the overall dosage (mg/kg) of vasopressors and sedation drugs was calculated at T1 and T2. Vasopressors consisted in noradrenaline and adrenaline, whereas sedation drugs included dexmedetomidine, fentanyl, midazolam, and propofol. Fluid balance (mL) was also retrieved at T1 and T2 for each patient.

Other clinical variables considered at T1 and T2 were: lactate (mmol/L), C-reactive protein (CRP, mg/L), $ScvO_2$ (%), CO (mL/min), stroke volume (SV, mL/beat), and ejection fraction (EF, %). Parameters related to specific organ systems were included: PaO_2/FiO_2 ratio (mmHg) and positive end-expiratory pressure (PEEP, mmHg) value for respiratory system together with the number of intubated patients, bilirubin (mg/dL) for the hepatic system, platelets count (103/mm3) for coagulation system, creatinine (mg/dL) together with urine output (mL/d), and the presence of acute kidney injury (AKI) for renal system, GCS score as an

overall indication of the level of consciousness and sedation of the patients.

All patients except one had additional data provided by the hospital ICT system relating their entire ICU stay: minute-by-minute average measure of invasive systolic, diastolic, mean arterial pressure (SAP, DAP, MAP), and HR, annotations of any change in the therapy (i.e., start, stop, or change of infusion rate for every administered drug). Information on daily fluid input, daily fluid output, and daily fluid balance of the entire ICU stay was also available for each patient. Mean and standard deviation (SD) were calculated every hour for SAP, DAP, MAP, and HR series; total cumulative amount of drugs administered was evaluated every 2 h. All the series were aligned with respect to time point T1. We focused on the first 10 ICU days as most of the patients in R group had an ICU length of stay less than 1 week before moving to another hospital ward.

2.2.3 Signal processing

The ICU is equipped with the Philips Intellivue MP70 monitor system. A laptop computer was connected to the monitor to synchronously download the waveforms by means of a dedicated software (iXtrend, ixellence GmbH, Germany).

The arterial blood pressure (ABP) waveform was continuously recorded at 125 Hz at T1 and T2 during a stable condition of the patient, when no maneuvers or changes in the therapy occurred. The duration of ABP recordings spans from about 1 to 30 min (average time 13 min). All the mathematical analyses were performed into Matlab® software framework. First, the onsets of each ABP pulse were located by means of the algorithm described in [152]. Briefly, the algorithm is composed of three steps: a low-pass filtering to suppress the high frequency noise that might affect the onset detection; a windowed and weighted slope sum function derived from the ABP waveform; a decision rule, consisting of adaptive thresholding and local search strategies for ABP onset annotations to be placed in close proximity of the actual pulse onset [152]. Once the onsets were identified, we extracted beat-to-beat time series of SAP, i.e. the maximum value between two consecutive onsets,

DAP, i.e. the minimum value between two consecutive onsets, MAP, i.e. the mean value between two consecutive onsets, and heart period (HP), i.e. the time difference between consecutive onsets of ABP pulses, considered a surrogate of the RR-intervals time series. The beat-to-beat series were successively checked for abnormal values based on physiological criteria [153] and filtered using a physiological adaptive filtering algorithm, to remove outliers and irregularities [154]. Temporal relationships were maintained among the time series: given onset(i) as the onset of the current beat, HP(i) designated the difference between onset(i+1) and onset(i). SAP(i) follows onset(i) and is followed by DAP(i), which often coincides with onset(i+1).

We subdivided each vital sign series into 2-minute long windows and, to increase the number of windows, we adopted a 50% overlapping segmentation. Each window was resampled at 2Hz by means of zero-order hold techniques, to obtain evenly spaced time series. Time and frequency indices were computed for each window and then averaged.

2.2.4 Hemodynamic analyses

Indices in time and frequency domain

We computed mean and SD of each time series at T1 and T2. Spectral indices obtained from power spectra, as computed via autoregressive spectral analysis [155], included LF power, which is the area under the spectrum in the range of frequencies between 0.04–0.15 Hz; total power (TP), which represents the total area under the spectrum and it is a measure of the overall variability of the series; LF%, which represents the relative power in LF band, computed as LF/(TP-VLF)%, where VLF is the very low frequency band, between 0 and 0.04 Hz. Each time series was preprocessed for the spectral analysis as follows: first, it was detrended using a 10^{th} order polynomial function and divided by its SD in order to have zero-mean normalized oscillations; then, the stationarity of the obtained time series was verified by means of the Dickey–Fuller test.

Baroreflex sensitivity analysis

The cardiac baroreflex control mechanism was investigated through BRS analysis to assess the interrelationship between HP and SAP

short-term oscillations. We adopted the bivariate closed-loop model method [156], which allows to evaluate the causal relationship both from SAP to HP, also called feedback gain (FB) or α gain, and from HP to SAP, also named feedforward gain (FF). The relationship SAP→HP stands for the arterial baroreceptor reflex mediated by the ANS, whereas the relationship HP→SAP represents the direct influence of HP on SAP, which is not mediated by autonomic control and consists in a perturbation mechanism based on the Starling law, i.e. an increased SV with a longer HP, and on the diastolic runoff. The last one represents a larger decay of DAP with a longer HP that decreases SAP and keeps constant other variables like SV.

The autoregressive bivariate model of order *p* can be expressed in the following matrix form

$$\mathbf{X}(i) = \sum_{k=1}^{p} \mathbf{A}(i) * \mathbf{X}(i-k) + \mathbf{W}(i) \tag{2.1}$$

where

$$\mathbf{A}(k) = \begin{bmatrix} a_{11}(k) & a_{12}(k) \\ a_{21}(k) & a_{22}(k) \end{bmatrix}$$

$$\mathbf{X}(i) = \begin{bmatrix} HP(i) \\ SAP(i) \end{bmatrix}$$

$$\mathbf{W}(i) = \begin{bmatrix} W_{HP}(i) \\ W_{SAP}(i) \end{bmatrix}$$

The coefficients a_{ij} are then used to calculate the gains of the two transfer functions in the frequency domain

$$G_{SAP \to HP}(f) = \frac{A_{12}(f)}{1 - A_{11}(f)} \tag{2.2}$$

$$G_{HP\rightarrow SAP}(f)=\frac{A_{21}(f)}{1-A_{22}(f)} \tag{2.3}$$

where $A_{ij}(f)=\sum_{k=1}^{p}a_{ij}(k)e^{-j2\pi fk}$ are the Fourier transform of the a_{ij} coefficients. $\mathbf{A}(k)$ matrices and the variances of the input noises $\mathbf{W}(i)$ are estimated through the Yule-Walker equations through an extended version of the Levison-Wiggins-Robinson algorithm [156]. The reference scheme of the model is reported in Figure 2.1.

The bivariate method allows to estimate the interrelationship between SAP and RR-intervals oscillations in a closed-loop configuration, which is a more realistic representation of the real physiological interactions between the two systems of heart and vasculature. It has been demonstrated that this closed-loop identification of the arterial baroreflex gain is highly correlated with the indices obtained by the open-loop approaches, but it has significantly lower values; this may be intuitively explained by the fact that the closed-loop identification separates the SAP influence on RR (or HP) variability from the influence of RR (or HP) variability on SAP [156].

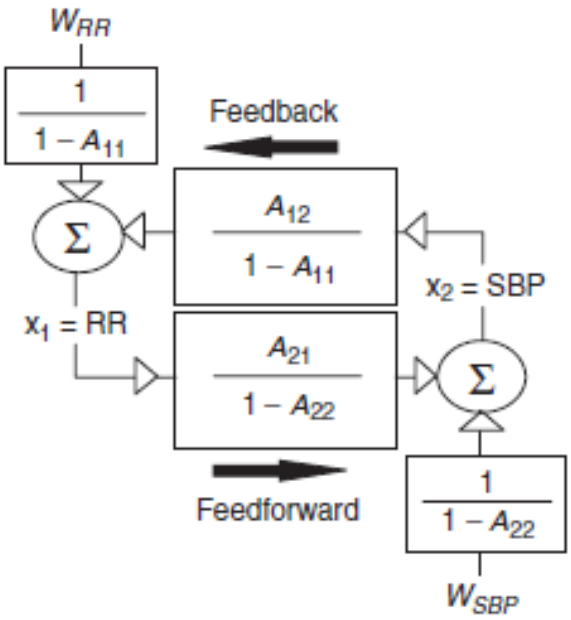

Figure 2.1 Closed-loop bivariate model diagram [156]. ($A_{21}(f)/1 - A_{22}(f)$) and ($A_{12}(f)/1 - A_{11}(f)$) represent the two transfer functions which denote the baroreflex mediated by the ANS and the mechanical influence of RR or HP on systolic BP through the heart and vasculature. W_{RR} and W_{SBP} are the two noise sources. RR=RR-intervals, SBP=systolic blood pressure

Black-box identification model of diastolic blood pressure variability

We implemented a multi-input single-output causal black-box model for the prediction and spectral decomposition of beat-to-beat variability of the diastolic component of peripheral BP, in order to better investigate the different mechanisms involved in peripheral resistance control. Assuming that DAP variability is a surrogate measure of peripheral vascular resistance [157], [158], the current DAP value is influenced both by the arterial baroreflex control mechanism and by the mechanical coupling between heart and circulatory system, the so-called runoff effect and feedforward mechanism, as previously introduced. A prolongation of the RR (or HP) interval produces an immediate reduction in DAP [159] but, due to the increased time for cardiac filling, the subsequent SV and PP increases via the Frank–Starling effect; the net result, therefore, is an increase in SAP in the next beat sensed by baroreceptor. This is followed subsequently by a more sustained

decrease, reflecting the effect of a reduction in CO produced by the lowered HR [160]. To disentangle these mechanisms and to highlight their different contributions in DAP variability, a black-box modelling approach was used both at T1 and T2 in the two groups of patients. The idea of a spectral decomposition method based on multivariate modelling analysis has been introduced by Baselli et al. [161]. Since parametric spectral analysis permits only the recognition and quantification of the oscillatory components in a single signal, the proposed approach of a spectral decomposition based on multivariate modelling allows to combine a multivariate analysis, i.e. information about the causal interactions among signals, and the spectral information, in order to understand how the interactions between different physiological systems influence the oscillations of a particular signal [161].

The implemented model is described by the following equation

$$DAP(i) = \sum_{j=1}^{n} h_{sap}(j) * SAP(i-j) + h_{hp} * HP(i-1) + e(i) = DAP_{/sap} + DAP_{/hp} + DAP_{/noise} \quad (2.4)$$

where the order *n* was fixed at 12 and the parameters $h_{sap}(j)$ and h_{hp} were determined by least-squares minimization procedure. We limited the effect of the mechanical runoff to a single gain parameter h_{hp} for physiological reason. The black-box input-output relationships in Equation 2.4 can be assumed to be representative of the following mechanisms of DAP variability control:

- SAP→DAP ($DAP_{/sap}$) represents the black-box model for the arterial baroreflex-mediated sympathetic control of vasomotor tone
- HP→DAP ($DAP_{/hp}$) represents the mechanical effect of diastolic runoff

The residual error ($DAP_{/noise}$) includes all the remaining sources of variability not measured, such as the influence of DAP past values, the autoregulation-mediated control of peripheral resistance, the cardiopulmonary baroreflex, possible errors, or noise.

To quantify the amount of DAP variability explained by the arterial baroreflex control or the mechanical runoff effect, a spectral

decomposition was performed. In particular, we performed the spectral analysis of the model component series ($DAP_{/sap}$, $DAP_{/hp}$) and then we assessed the ratio between LF power of $DAP_{/sap}$ component over LF power of DAP (LF $DAP_{/sap}$/LF DAP) and the ratio between LF power of $DAP_{/hp}$ component over LF power of DAP (LF $DAP_{/hp}$/LF DAP).

2.2.5 Statistical analyses

We adopted the Mann–Whitney U test, also known as Wilcoxon rank-sum test, to verify significant differences in the indices values between the two groups (R and NR patients) separately at T1 and T2, whereas we used the Wilcoxon signed-rank test to assess significant changes from T1 to T2 within the same group of patients. The variations from T1 to T2 in the indices values, i.e., the deltas, were compared between the two groups by means of the Mann–Whitney U test. For categorical parameters, i.e., data of incidence, we used the Fisher exact test. Significance was considered with a p-value < 0.05.

2.3 RESULTS

2.3.1 Population and therapy

R and NR patients had similar clinical characteristics at admission, as reported in Table 2.1. NR stayed longer in the ICU and had higher mortality rate after 28 days from development of shock. The source of infection and the comorbidities were balanced between R and NR underlying that the different outcome of the two groups of patients was not due to pre-existing pathologies, but it was highly dependent on patients' response to therapy.

Clinical variables and laboratory examinations values at time points T1 and T2 are shown in Table 2.2. According to inclusion criteria, all patients had a SOFA score higher than 8 at T1 and this was meant to ensure a similar severity of shock among patients at the enrolment. However, SOFA score at T1 was significantly higher in R than NR. This could be due to the limit number of patients and to a larger variance in the SOFA values in R group. For instance, in our clinical cohort two patients had a SOFA score of 16 at T1, but one improved ($SOFA_{T2}$ = 10)

and the other did not ($SOFA_{T2}$ = 16). Moreover, CRP and lactate were comparable between the two groups at T1 confirming that the severity of inflammation was similar at the admission.

Figure 2.2 describes the total dosage of vasopressors (noradrenaline, adrenaline) and sedation drugs (dexmedetomidine, fentanyl, midazolam, and propofol) administered. We observe that NR had a significantly higher dosage of vasopressors at T2 with respect to R and a large increase from T1 to T2, in contrast to R. We also report the total cumulative dose of vasopressors and sedation drugs, computed every 2 h, administered during the first 10 days of ICU stay (Figure 2.2): note that R patients received on average a lower dosage of vasopressors after T1 with respect to NR patients, whereas both groups received a similar sedation therapy.

In addition, we analysed the fluids administered as reported in Figure 2.3 where the daily trends of fluid input, fluid output, and fluid balance for the first 10 days of ICU stay are illustrated. NR received a similar initial fluid therapy at ICU admission (T0), but their fluid balance then became strongly positive at T1, in contrast to R patients, due to a higher dosage of fluid input and lower fluid output. The values of fluid balance at T1 and T2 and the delta between these two time points are also reported in Table 2.2.

Table 2.1. General characteristics of the two populations. Numerical data are reported as median (25^{th}, 75^{th}) percentile; categorical data are expressed as number of cases (percentage)

	Responder	Non Responder	p-value
Number of patients	14	7	
Age [years]	67.2 (62,75)	68.2 (61,77.7)	n.s.
Weight [Kg]	85 (75,90)	75 (61.8,78.8)	n.s.
Body Mass Index	26.5 (24.1,27.8)	25.9 (20.4,28.5)	n.s.
Males	12 (85.7%)	4 (57.1%)	n.s.
Total days in ICU	4.5 (3,6)	10 (9.3,19.8) *	0.016
Total days in hospital	13.5 (11,30)	23 (17.5, 39)	n.s.
Mortality at 28 days [#pts (%)]	2 (15.4%) $^{n=13}$	5 (71.4%) §	0.022
Mortality at 100 days [#pts (%)]	2 (20%) $^{n=10}$	5 (71.4%)	n.s.
Source of infection			
Respiratory	3 (21.4%)	3 (42.9%)	n.s.
Abdominal	3 (21.4%)	3 (42.9%)	n.s.
Urinary tract	5 (35.8%)	1 (14.3%)	n.s.
Others	3 (21.4%)	0 (0%)	n.s.
Comorbidities			
Chronic organ insufficiency	13 (92.9%)	6 (85.7%)	n.s.
Arterial hypertension	5 (35.7%)	3 (42.9%)	n.s.
Diabetes	0 (0%)	1 (14.2%)	n.s.
Coronary disease	1 (7.1%)	0 (0%)	n.s.
Systolic/diastolic disease	0 (0%)	1 (14.3%)	n.s.
Cerebrovascular disease	1 (7.1%)	1 (14.3%)	n.s.
Peripheral vascular disease	1 (7.1%)	0 (0%)	n.s.
Chronic lung disease	2 (14.3%)	2 (28.6%)	n.s.
Inflammatory bowel disease	0 (0%)	0 (0%)	n.s.
Liver disease	1 (7.1%)	1 (14.3%)	n.s.
Chronic kidney disease	0 (0%)	0 (0%)	n.s.
Acute heart failure	8 (57.1%)	4 (57.1%)	n.s.
Prolonged arrhythmias	1 (7.1%)	1 (14.3%)	n.s.

Comparisons between R and NR: * p-value<0.05 (Mann-Whitney U-test)
Comparisons between R and NR: § p-value<0.05 (Fisher Exact test)

Table 2.2. Clinical data, laboratory data, total dose of vasopressor drugs VP (noradrenaline, adrenaline) and of sedation drugs (dexmedetomidine, fentanyl, midazolam and propofol) for responder and non responder patients at time points T1 and T2. Numerical data are reported as median (25^{th}, 75^{th}) percentile; categorical data are expressed as number of cases (percentage)

	Responder			Non Responder		
	T1	**T2**	**Delta**	**T1**	**T2**	**Delta**
SOFA	12.5 (11,14)	6 (6,8)	-5 (-9,-4)§§§	16 (13.5,16)*	15 (13.5,15.8)***	0 (-1.8,0)°°°
Lactate [mmol/L]	3.8 (2.8,5.6)	1.3 (1,1.5)	-2.5 (-3.3,-1.4)§§§	4.7 (3.4,7.8)	2.7 (1.4,3)*	-1.7 (-6.3,-0.4)
CRP [mg/L]	271.5 (132.3,378)	212.8 (171.7,257.7)	-34.6 (-104.2,40.6)	273.4 (96.8,350.8)	243.7 (154,341.1)	32.1 (-27.5,103.2)
$ScvO_2$ [%]	76 (72,78)$^{n=10}$	71.5 (64.5,72.5) $^{n=8}$	-6 (-10.3,-2.5)	71 (61.8,77.8)$^{n=5}$	70 (61.5,79.5)$^{n=4}$	-1.5 (-7,1)
CO [L/min]	5 (4.7,6.9)$^{n=11}$	5.7 (4.3,6.1)$^{n=13}$	-0.4 (-1.1,1.1)	5.6 (3.7,6.8)	4.2 (3.5,5.3)$^{n=6}$	-1.2 (-2.2,-0.3)
SV [mL/beat]	64.9 (57.2,74.9)$^{n=11}$	58.6 (50.7,75.2)	-0.18 (-2.4,3.9)	58.5 (45.5,78.2)$^{n=13}$	63.9 (54.8,86.6)$^{n=6}$	3.4 (-3.7,7.8)
EF	55 (40,60)	47.5 (40,60)	0 (-5,5)	45 (22.5,57.5)	50 (35,55)$^{n=6}$	2.5 (-5,5)
Fluid Balance [mL]	1466 (413,2181)	-259 (-891,557)	-1379 (-2400,-813)§	4075 (2552, 5039)**	1488 (-133, 2837)	-3449 (-3888,-464)§
VP dosage [mg]	4.2 (0.6,7.6)	2.2 (1.7,2.9)$^{n=10}$	-2.7 (-7.2,1.4)$^{n=10}$	11.9 (5.1,16.5)	15.7 (11.6,17.9)***	3.8 (-0.7,8.1)°°
Sedation dosage [mg]	284.2 (160.6,832)$^{n=12}$	225 (1.4,2435.7) $^{n=11}$	-83.4 (-151,1264.4) $^{n=10}$	203.9 (122.5,749.9)	125.5 (2.5,242.2)$^{n=10}$	-212 (-606.4,0.3)$^{n=6}$
Respiratory System						
PaO_2/Fi_{O2} [mmHg]	183.7 (119,265)	272.4 (226.7,316.7)	72.8 (-7.9,129.5)§	148.6 (132.1,418.5)	180 (171.9,225.9)	35.8 (-199.2,47.2)
Tracheal Intubation [#]	13 (92.9%)	8 (57.1%)	-5 (-35.7%)#	6 (85.7%)	6 (85.7%)	0 (0%)
PEEP [mmHg]	8 (7,10)$^{n=13}$	8.5 (7.5,9.5)$^{n=8}$	-0.5 (-2.5,0)	10 (7,12)$^{n=6}$	8 (8,10)$^{n=6}$	0 (-2,0)

	Responder			Non Responder		
	T1	**T2**	**Delta**	**T1**	**T2**	**Delta**
Hepatic System						
Bilirubin [mg/dL]	1.3 (0.7,1.9)	0.65 (0.4,1.5)	-0.4 (-0.9,-0.2)§§	1.3 (0.9,14.1)	1.4 (0.9,14.2)*****	0 (0,0.4)
Coagulation System						
Platelets [10^3/mm^3]	194 (101,218)	175 (77,236)	-5 (-31,20)	112 (31.5,138.3) *****	39 (17.5,109.5) *****	-8 (-41.8,0)
Renal System						
Creatinine [mg/dL]	1.65 (1.1,2)	1.1 (0.6,1.3)	-0.5 (-0.9,-0.2) §	1.7 (1.3,2)	1.3 (1.1,2.4)	-0.1 (-0.5,0.2)
Urine output [L/day]	1.8 (0.8,2.1)	2.3 (1.4,3.1)	0.8 (-0.5,1.3)	0.8 (0.4,1.5)	0.6 (0.3,1.7) ******	-0.1 (-0.4,0.2)
AKI [#]	3 (21.4%)	2 (14.3%)	-1 (-7.1%)	5 (71.4%)	4 (57.1%)	-1 (-14.3%)
Nervous System						
GCS	3(3, 4)	11(8, 14)	6(3, 11)§§	3(3, 4.5)	4(3.3,7.5)*	1(0, 2.5)

SOFA=Sequential Organ Failure Assessment Score, CRP=C-Reactive Protein, ScvO2=central venous oxygen saturation, CO=Cardiac Output, SV=Stroke Volume, EF=Ejection Fraction, VP=vasopressors, PaO2=arterial partial oxygen pressure, FiO2=fraction of inspired oxygen, PEEP=positive end expiratory pressure, AKI=Acute Kidney Injury, GCS=Glasgow Coma Score.
Comparisons between R and NR: *p-value<0.05, **p-value <0.01, ***p-value <0.001 (Mann-Whitney U-test)
Comparisons between T1 and T2: §p-value<0.05, §§p-value<0.01, §§§p-value<0.001 (Wilcoxon signed rank test)
Comparison between Delta R and Delta NR: °°p-value<0.01, °°°p-value<0.001 (Mann-Whitney U-test)
Comparisons between R and NR: #p-value<0.05 (Fisher Exact test)

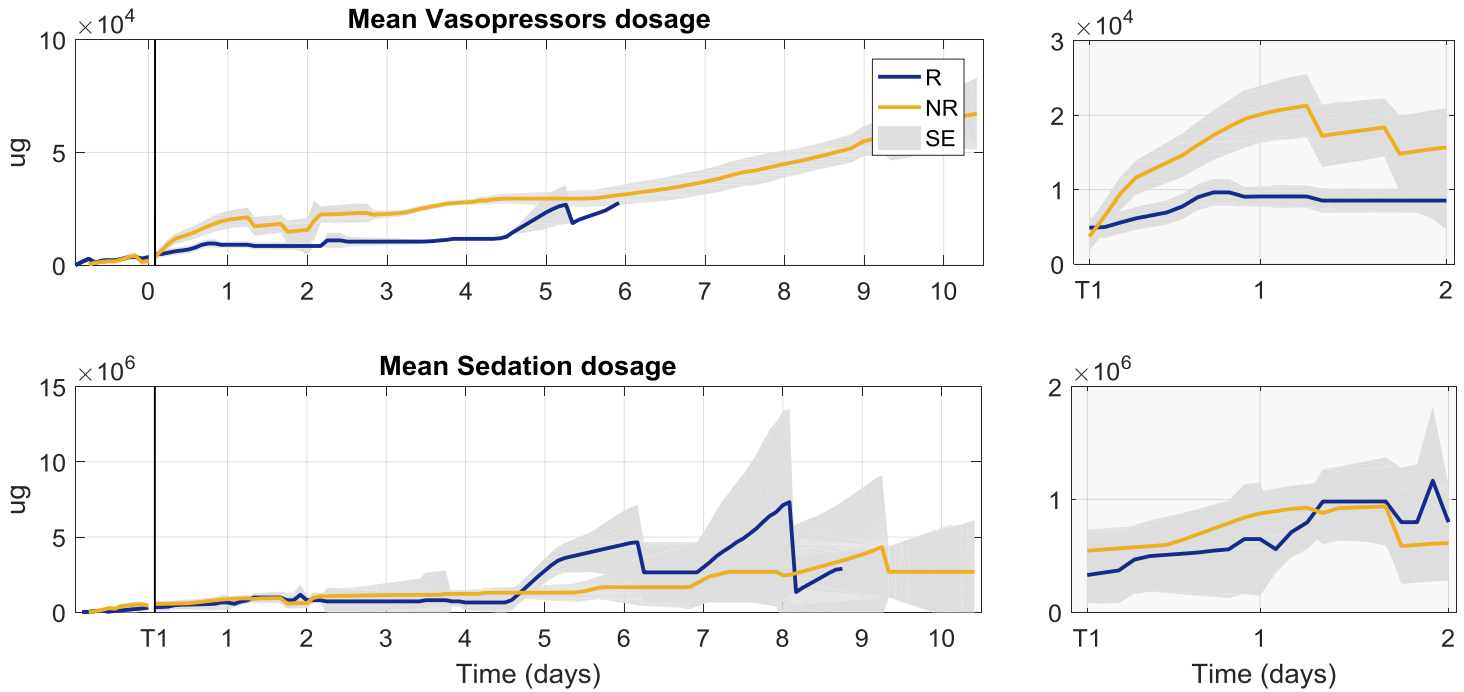

Figure 2.2. Trend of cumulative total dosage of vasopressors and sedation drugs administered during the first 10 days of ICU stay for responder (R) and non-responder (NR) groups. Total dosage is computed as the sum of all the drugs administered every 2 h. The vertical black line marks T1 time point. The grey shaded area represents the standard error (SE). The two panels on the right show a detail of the trends from T1 and the next 2 days.

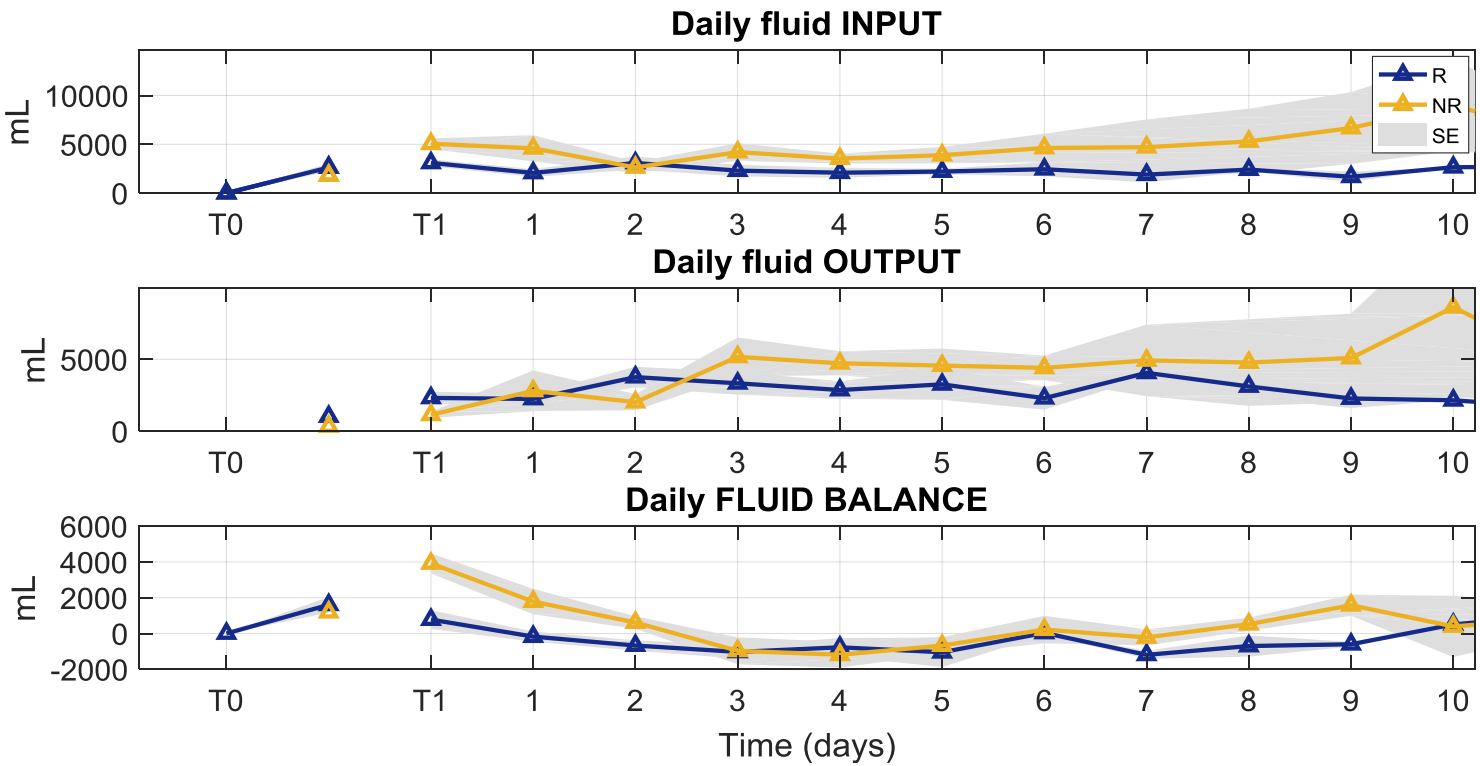

Figure 2.3. Trends of daily measures of fluid input, fluid output, and fluid balance during the first 10 days of ICU stay for responder (R) and non-responder (NR) groups. The grey shaded area represents the standard error (SE) of the mean estimation. T1 was used as time reference for all patients.

2.3.2 Hemodynamic variables and ANS indices

Figure 2.4 shows the distribution of mean values at each time point for SAP, DAP, MAP, and HR time series in both groups of patients. The upper right box displays the distribution of delta values computed as the difference between values at T2 and values at T1. The horizontal black line marks the zero value that means no change from T1 to T2.

Mean value of BP showed an increase between T1 and T2 in responders; moreover, at T2 R patients had significantly higher values than NR for MAP and SAP. All mean values of BP components were slightly decreasing in NR patients between the two time points. A significant decrease of HR mean value from T1 to T2 was observed in NR.

Figure 2.5 displays the trend of the average of minute-by minute MAP and HR values during the first 10 ICU days. The trend of MAP during the initial days was very similar in the two groups and, in particular, at T1 and T2 both groups maintained the target MAP value of 65 mmHg. The trend of HR showed instead a decrease in NR group with respect to a more stable trend in R group.

Figure 2.6 shows the distribution of SD and LF% values at each time point for SAP, DAP, MAP, and HR time series in both groups of patients. The overall variability in NR group was significantly lower with respect to R group: the standard deviation of SAP, DAP, and MAP was significantly lower at T2 in NR, and the standard deviation of HR was significantly lower at T1. In addition, at T2 the LF component of DAP (LF% and LF absolute power, reported also in Table 2.3) was significantly lower in NR group.

Figure 2.6 shows also a significant increase in LF% from T1 to T2 in responders for MAP, SAP, DAP, and HP, which was not reported in NR group.

With regard to BRS (Table 2.3), NR patients had significantly lower values of BRS feedback gain at T1 than R, but they showed a significant increase from T1 to T2 so that the gain became significantly higher in NR group at T2.

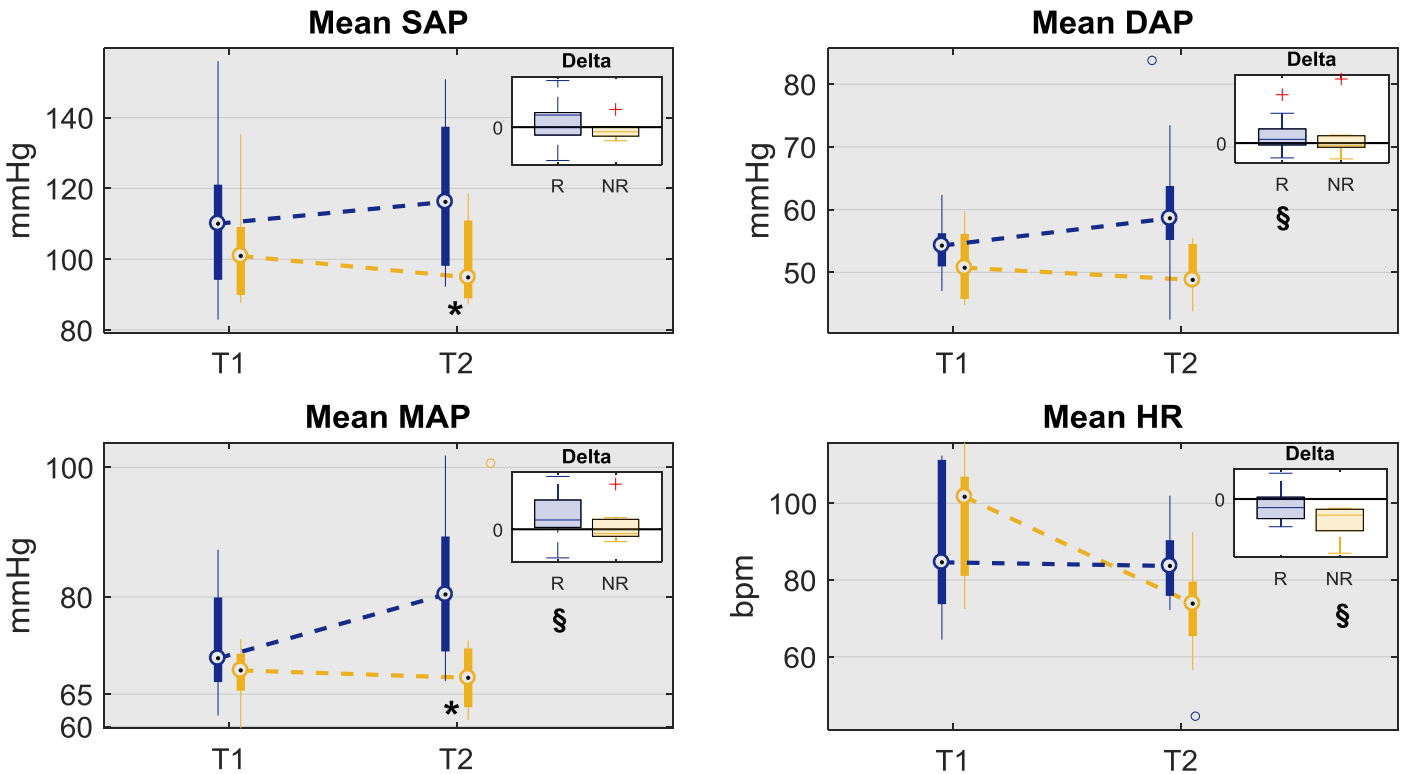

Figure 2.4. Boxplot distribution of mean values for systolic (SAP), diastolic (DAP), mean (MAP) arterial pressure, and heart rate (HR) at time points T1 and T2 for responder (R) and non-responder (NR) patients. The upper right box displays the distribution of delta values computed as the difference between values at T2 and values at T1 for both groups. The horizontal black line marks the zero value that means no change from T1 to T2. *P < 0.05Mann–Whitney U test between R and NR §P < 0.05 Wilcoxon signed-rank test between T1 and T2.

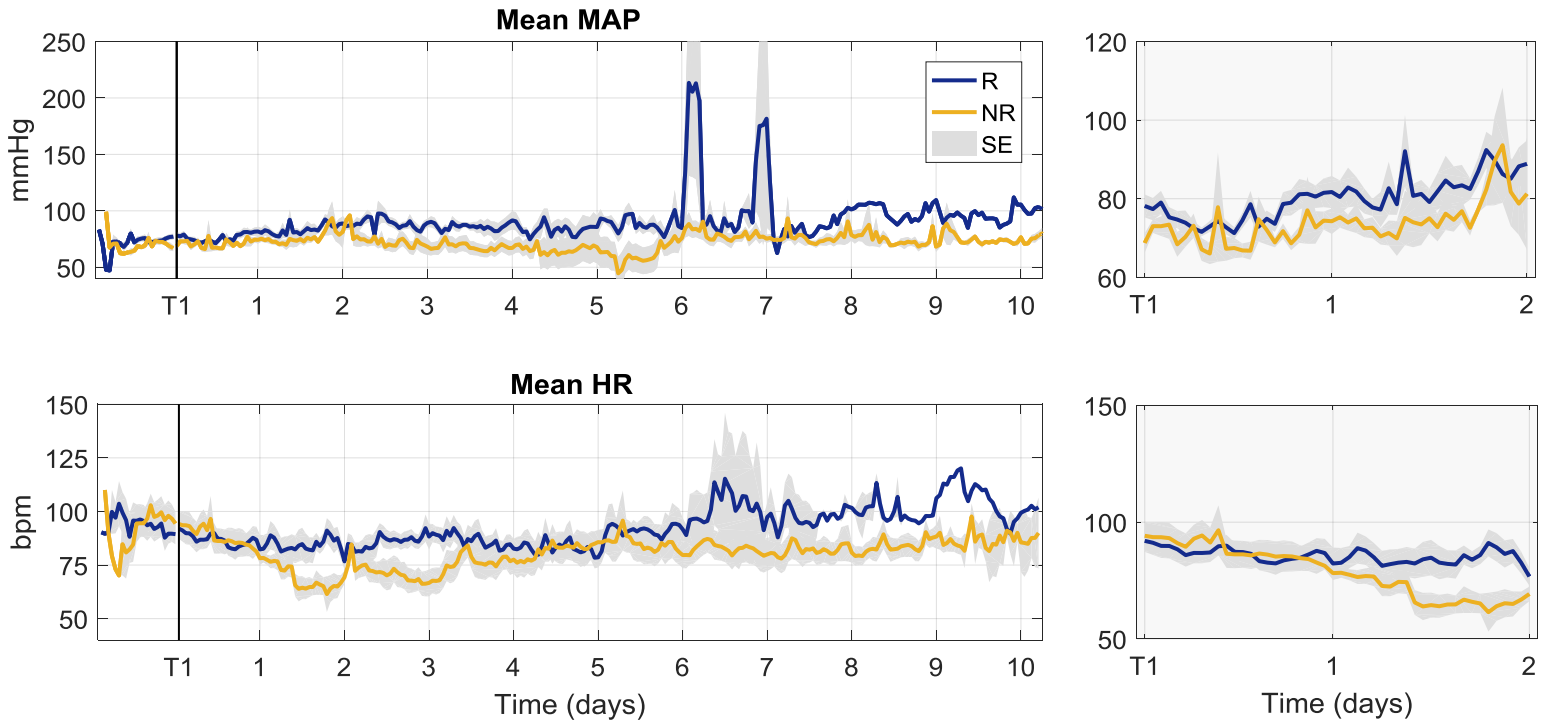

Figure 2.5. Trends of mean arterial pressure (MAP) and heart rate (HR) series during the first 10 days of ICU stay for responder (R) and non-responder (NR) groups. The reported values refer to hourly averaged values of the series. The vertical black line marks T1 time point. The grey shaded area represents the standard error (SE) of the mean estimation. T1 was used as time reference for all patients. The two panels on the right show a detail of the trends from T1 and the next 2 days.

Table 2.3. Baroreflex sensitivity and frequency indices for responder and non-responder patients at time points T1 and T2. Values ad reported as median (25^{th},75^{th}) percentile

		Responder			Non Responder		
		T1	**T2**	**Delta**	**T1**	**T2**	**Delta**
SAP	LF power [a.u.]	121.8 (90,173.4)	246.3 (108.5,278.4)	51.5 (-2.2,133)	114.4 (60.6,257.3)	105.6 (81.3,210)	47.9 (-68.3,62.7)
	TP [a.u.]	525.7 (509,626)	534.6 (507.9,549.9)	-0.9 (-72,16)	531.9 (520.5,544)	525 (498.7,544.9)	-2.7 (-33.5,31.6)
DAP	LF power [a.u.]	148.6 (94.7,189)	202 (156.1,257.1)	57.3 (4.3,117.4) §	105.7 (80.5,229.4)	91.9 (74.8,104.8) *	-13.8 (-91.5,20.2)°
	TP [a.u.]	521 (509.4,549.5)	526.1 (513.9,546.8)	-1.5 (-24.1,24.2)	538.4 (516.2,559.8)	510.3 (446,557.3)	-45.8 (-70.5,-3.3)
MAP	LF power [a.u.]	130.3 (79,222.6)	243.2 (106.5,327.5)	44.4 (-29.9,245.3)	119.9 (52.3,225.1)	99.3 (82,223.2)	44.3 (-86.5,74.7)
	TP [a.u.]	522.9 (502,578.2)	535.6 (525,555.7)	7 (-65.8,25.4)	546.9 (501.5,557)	533.2 (488,558.5)	11.5 (-67.3,44.1)
HP	LF power [a.u.]	209 (127,297.7)	260.8 (185.6,370.2)	55.3 (21.9,147.2)	143.5 (113,168.1)	188 (131.3,309.2)	44.6 (6.7,102.5)
	TP [a.u.]	519.6 (511,533.4)	528.4 (514.7,541.6)	8.6 (-9,36.2)	529.6 (510.4,546.9)	536.6 (500.6,545)	24 (-60.8,37.8)
BRS	FB [msec/mmHg]	2.2 (1.4,3.3)	3.2 (2.1,7.5)	0.9 (-0,3)	1.3 (0.5,1.7)	5.2 (2.4,8.1)	3.5 (1.3,6.1) §
	FF [mmHg/msec]	0.1 (0.05,0.19)	0.08 (0.04,0.15)	-0.05 (-0.08,0.05)	0.15 (0.08,0.3)	0.06 (0.06,0.1)	-0.04 (-0.29,0.03)

SAP=systolic arterial pressure, DAP=diastolic arterial pressure, MAP=mean arterial pressure, HP=heart period, BRS=baroreflex sensitivity, LF=low frequency power, TP=total power, FB=feedback, FF=feedforward.
Comparisons between R and NR: *p-value<0.05 (Mann-Whitney U-test).
Comparisons between T1 and T2: §p-value<0.05 (Wilcoxon signed rank test).
Comparison between Delta R and Delta NR: °p-value<0.05 (Mann-Whitney U-test)

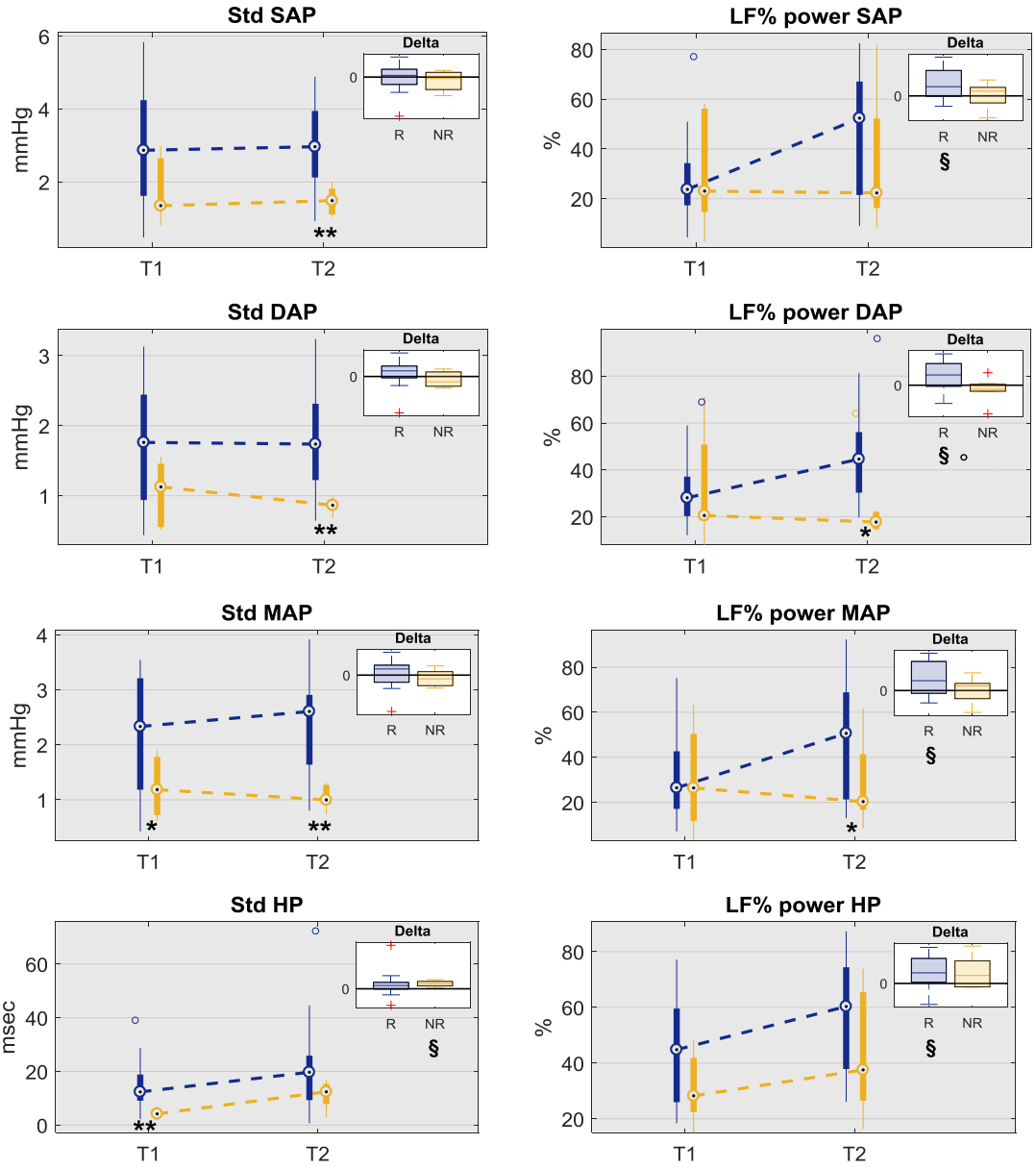

Figure 2.6. Boxplot distribution of standard deviation (SD) and LF relative power (LF%) values for systolic (SAP), diastolic (DAP), mean arterial (MAP) pressure, and heart period (HP) at time points T1 and T2 for responder (R) and non-responder (NR) patients. The upper right box displays the distribution of delta values computed as the difference between values at T2 and values at T1 for both groups. The horizontal black line marks the zero value that means no change from T1 to T2. *P < 0.05, ** P < 0.01 Mann–Whitney U test between R and NR §P < 0.05 Wilcoxon signed-rank test between T1 and T2 °P < 0.05 Mann–Whitney U test between delta R and delta NR

2.3.3 Diastolic blood pressure variability decomposition

Figure 2.7 illustrates the ratios distribution of DAP model components (LF $DAP_{/sap}$/LF DAP and LF $DAP_{/hp}$/LF DAP). In particular, in both groups the ratio computed between LF power of $DAP_{/sap}$ component over LF power of DAP, reflecting the portion of DAP variability mediated by the arterial baroreflex mechanism, was predominant with respect to the ratio between LF power of $DAP_{/hp}$ component over LF power of DAP, indicating the mechanical influence of the heart on the circulatory system (runoff effect). Moreover, we can notice that at T1 the component of DAP variability explained by the mechanical effect of the runoff was significantly lower in NR group.

Finally, Table 2.4 reports the LF power values of $DAP_{/sap}$ and $DAP_{/hp}$ components, showing that the LF oscillations of the model component related to the HR were significantly lower in NR than in R group.

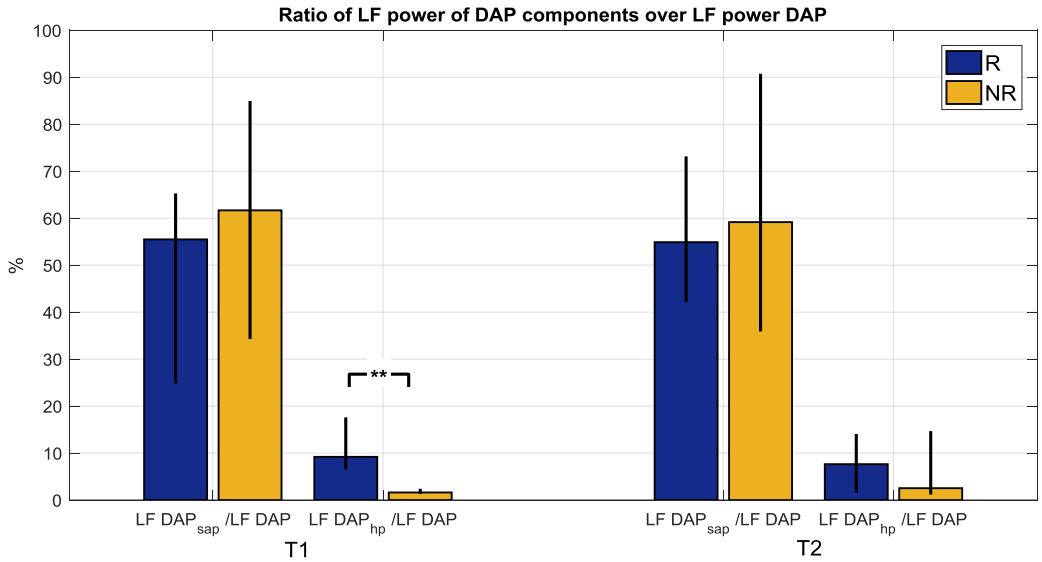

Figure 2.7. Ratio between LF absolute power of each predicted component and LF absolute power of diastolic blood pressure (DAP) at time points T1 and T2 for both groups of responder (R) and non-responder (NR) patients. The height of the bar is the median value of the population; black bars indicate values of 25th and 75th percentile. **P < 0.01 Mann–Whitney U test between R and NR.

Table 2.4. Low frequency (LF) power of DAP components variability for responder and non-responder patients at time points T1 and T2. Values ad reported as median (25^{th},75^{th}) percentile.

	Responder			Non Responder		
	T1	**T2**	**Delta**	**T1**	**T2**	**Delta**
LF DAP*/sap* [a.u. x10^{-3}]	5.82 (1.8,11.6)	8.38 (2.3,17.3)	1.47 (-2.7,10.5)	2.08 (0.4,8.6)	1.38 (0.6,2) **	0.16 (-7.5,0.5)
LF DAP*/hp* [a.u. x10^{-3}]	1.65 (0.6,4.4)	1.86 (0.2,4.6)	-0.03 (-0.5,1.2)	0.06 (0,0.1) **	0.06 (0,0.4) *	0.04 (-0,0.2)

Comparisons between R and NR: *p-value<0.05, **p-value<0.01 (Mann-Whitney U-test)

2.4 DISCUSSION

In the present study, the early response to standard therapy in 21 septic shock patients was investigated by means of several hemodynamic indices. Our major finding is that common mean targets routinely used to guide hemodynamic optimization and fluid therapy in acute shock patients, such as mean arterial BP, are not sufficient alone to explain the evolution of patient's organ dysfunction.

Specific BP targets have been recommended for septic shock patients, for instance, sepsis guidelines recommend vasopressors administration to achieve and maintain a MAP of at least 65 mmHg in patients not responding to initial fluid resuscitation. In this regard, the actual BP targets and their implementation are the main determinants of the patient's exposure to fluids and vasopressors [132]. Interestingly, the cumulative vasopressor administration is independently associated with morbidity and mortality, whereas reduction of exposure to vasopressors has the potential of improving outcomes. For these reasons, the key point is to understand if new hemodynamic markers and targets can be defined for a more effective therapy with a real benefit on organ recovery and outcome.

In this study, patients were stratified as R or NR to therapy according to change in SOFA score within 48 hours, between T1 and T2 time points. All patients had similar severity of shock at the enrolment and they received the same initial therapy, but the R group improved within the first two days after ICU admission, whereas the NR patients did not improve or even worsened.

NR patients displayed a higher fluid balance during the first days after shock development due to a larger fluid administration and a reduction in urine output (Figure 2.3). There are various possible explanations for this phenomenon, for instance, a higher capillary leak in these patients, or a prolonged vasoplegic state, which required a prolonged infusion of vasopressors with concomitant fluid infusions. Moreover, we must remind that vasopressors are intravenously administered and a prescription of a higher vasopressor dosage usually translates into a higher infusion rate with a consequently additional fluid

infusion. The additional administration of fluids to maintain hydration should take into account this portion, but in the acute phase the decision on fluid therapy is not straightforward and the recovery from hypotension is of primary importance.

Interestingly, fluid accumulation is known to affect ANS response to sympathetic stimuli. In fact, previous studies reported that the large increase in the power of LF oscillations in RR series observed during active or passive standing in healthy subjects was not observed during other sympathetic activation activities in which central volume did not change, like the prolonged handgrip [162]. Moreover, the magnitude of LF oscillations observed during exercise was reported to be influenced by body position, i.e., standing or supine positions—both of which are characterized by sympathetic activation [163]. Another study reported that the LF variability component of RR intervals and MSNA decreased in heart failure patients despite an overall increased sympathetic activation; one can interpret this result as being due to the increased central volume present in this pathological condition [102]. Finally, a recent study compared the changes in CSNA during volume loading in two groups of normal and heart failure sheep, showing that the decrease in CSNA due to the fluid infusion in the normal sheep was not present in the heart failure group [150].

Given these premises, we hypothesized that the fluid overload in NR could have been one of the possible mechanisms that prevented the sympathetic outflow to the heart and to the peripheral resistance in these patients. In fact, a different trend in the overall variability and LF oscillations was observed: R patients increased LF% values of SAP, DAP, MAP series (Figure 2.6), whereas NR patients did not despite a higher dosage of vasopressors (Table 2.2), which mainly act on the sympathetic outflow to the periphery. It is widely accepted that changes in LF oscillations of BP can be related to the changes in the outflow of SNS and spectral analysis of BP has been proved to be a powerful tool for the identification of the different cardiovascular control mechanisms that regulate BP [77], [164]. From this perspective, the absence of LF increase in NR patients can be read as a sign of non-responsiveness to the vasopressor therapy or let think to a mechanism that prevented SNS

outflow. Moreover, the large decrease in mean value of HR between T1 and T2 in the non-responders further supports this hypothesis: although an increasing trend in LF power of HR oscillations in NR patients, this did not translate into an increase in cardiac frequency. This could be a sign of a possible activation of vagal outflow to the heart mediated by the cardiopulmonary baroreflex. Cardiopulmonary baroreceptors are low pressure receptors located in the walls of the pulmonary artery and the cardiac chambers that sense the changes in central volume pressure and regulate ANS outflow to the heart and vessels.

The interaction between cardiopulmonary and arterial baroreflex has been widely demonstrated in literature: reflex forearm vascular resistance in response to carotid neck suction/pressure was augmented when the cardiopulmonary baroreceptors were unloaded [165]; the reflex control of total peripheral resistance mediated by cardiopulmonary baroreflex almost doubled in seven conscious dogs after chronic arterial baroreceptor denervation [166].

In our study the feedback gain of the baroreflex mechanism, which quantifies the relationship between SAP and HR mediated by the nervous system, increased significantly at T2 in NR. If cardiopulmonary afferent activation indirectly affects the arterial baroreflex control of heart and peripheral resistance, then the elevated loading (or stretching) of CP baroreceptors, due to large central volume pressures, could be a possible explanation of the different trend in BRS feedback gain observed in NR patients with respect to R patients, whose fluid balance significantly decreased at T2.

Commonly, the rising of BRS feedback gain is interpreted as a sign of recovery in various pathological conditions [90], [167]. However, in NR patients the increase in BRS gain is accompanied by a fall in HR and by a slight decrease in mean BP from T1 to T2. The model of DAP variability decomposition can help in understanding this paradox: the portion of LF DAP modulated by the HR (LF $DAP_{/hp}$) was highly reduced in NR compared to R (Table 2.4), meaning that the regulatory mechanism of DAP based on the mechanical effects of the runoff became negligible in NR patients. These results hint that the rising of BRS feedback gain observed in NR is not a sign of recovery, but a compensatory mechanism to a condition of

diminished HR and reduced sympathetic control of heart and vessels' resistance.

We want to highlight that the decrease in HR and increase of BRS in NR patients could be also interpreted as a prevalence of vagal tone. In fact, many studies demonstrate that the increase in baroreflex control of HR is, in part, mediated by the enhancement of the vagal nerve [167].

All patients were able to maintain their MAP over the targeted threshold of 65 mmHg (Figure 2.4), even if the trend between T1 and T2 in NR patients was slightly decreasing and they displayed lower values with respect to R patients both at T1 and T2. Looking at the international guidelines for acute shock resuscitation all these patients seemed to be responsive to the therapy. However, the hemodynamic analyses allowed to discriminate those patients who actively responded restoring the ANS regulation of BP and HR, and those who recovered from hypotension without a marked improvement in the cardiovascular autonomic control.

Finally, it is not unusual for septic shock patients to receive excess fluids even after they are weaned from vasopressors [130]. In our opinion, one of the reasons to explain this common practise is that indices relating to cardiovascular autonomic control are not commonly used to guide fluid therapy and our work is meant to support their introduction into clinical practice: this type of indices offers non-invasive options to guide fluid therapy and permit to assess the likely hemodynamic response of patients to the therapy.

2.4.1 Limitations

The main limitation of this study consists in the limited size of the population, collected in a single centre. The small sample size did not allow to take into account all possible confounding factors, such as comorbidities or previous history of vasoactive therapy. Further studies should be conducted on a larger population and by taking into account the response to changes in vasopressor dosage or fluid administration in a tighter way so to better infer patient conditions.

2.5 CONCLUSIONS AND REMARKS

In conclusion, BPV, HRV, and baroreflex trends can add information to individual vital signs such as MAP, and can help in understanding the responsiveness to the combination of sympathomimetic drugs and fluid therapy. Indeed, static values of BP give only an approximate overview of the hemodynamic status of shock patients, as they do not carry information about the short-term cardiovascular regulatory mechanisms of HR and BP, which are at the basis of the recovery of organs dysfunction.

The results of this study highlight the limitations of the current clinical guidelines for septic shock management, in line with the recent literature (§1.6), and they draw attention to new non-invasive tools to guide resuscitation protocols, paving the way for a new concept of continuous short-term hemodynamic monitoring. The therapeutic approach should include the monitoring of the ANS activity at the heart and vessels level, since the autonomic-mediated reflexes and mechanisms for HR and BP control have been documented as crucial in recovering from shock and supported by our results too.

Such considerations are at the basis of the animal studies. The next chapters illustrate the study of autonomic control mechanisms in two different experimental datasets: the first one consists of septic shock pigs undergoing to an experiment of polymicrobial sepsis and resuscitation; the second one consists of hemorrhagic shock pigs undergoing to severe bleeding and resuscitation. In the septic shock experimental studies, we aim at better understanding these preliminary results by investigating the role of the arterial tree autonomic modulation (chapter 4) and the heart autonomic regulation (chapter 3), which is still considered as an open issue in patients who recovered from shock.

Finally, we test the same indices and investigate the role of the ANS in a hemorrhagic experimental model, in order to understand if there are common patterns in the physiological mechanisms activated to respond to shock syndrome (chapter 5).

The animal experiments offer a great opportunity to have a controlled condition eliminating confounding factors, e.g. comorbidities or differences in severity of infection/bleeding, typical of shock patients'

populations, and to track changes in the physiological response from the injury (polymicrobial sepsis or bleeding) and the shock onset, before any resuscitative maneuver. It is also possible to disentangle the effects of the different resuscitation maneuvers, when fluids are administered or when sympathomimetic drugs or blood transfusions are given.

3 A MATHEMATICAL STUDY TO EVALUATE THE DYNAMIC CONTROL OF HEART CONTRACTILITY IN SEPTIC SHOCK

In this study we proposed a new mathematical model to investigate the autonomic control of ventricular contractility in a population of pigs undergoing a protocol of polymicrobial peritonitis-induced septic shock and resuscitation. This work was published as a journal paper on IEEE Transactions on Biomedical Engineering[1].

It is known that septic shock patients often show elevated HR despite resuscitation and this condition is considered an early manifestation of myocardial dysfunction, probably due to a malfunctioning of the sympathetic branch of the ANS. However, at present, most scientific research into cardiac impairment in septic shock focuses on myocardial depression due to bacterial toxins and inflammatory mediators, without reserving the proper attention to the autonomic modulation of HR and contractility which is more and more recognized as a crucial issue in sepsis and shock.

The animals were studied at baseline condition, after the development of septic shock through intraperitoneal instillation of autologous feces, and after resuscitation maneuvers were administered, such as fluids to restore blood volume and vasopressors to restore BP.

Our results highlighted an autonomic-mediated increase in ventricular contractility and HR during shock in order to cope with the hypovolemia and hypotension typical of shock state. Interestingly, after resuscitation the physiological control of heart contractility was not restored and all the animals still exhibited high HR and elevated cardiac contractility. Moreover, indices of autonomic dysfunction, such as HRV and BRS, were suppressed both in shock and after resuscitation.

[1] M. CARRARA, A. Herpain, G. Baselli, M. Ferrario, "*A mathetmatical model of dP/dt max for the evaluation of the dynamic control of heart contractility in septic shock".* IEEE Trans Biomed Eng, 2019 [in press]

Details about rationale, methodology, results and conclusions of the study are described in the following paragraphs. They are based on the related journal paper. A final remark on the main findings is reported at the end of the chapter.

3.1 INTRODUCTION

Tachycardia and increased ventricular contractility are fundamental compensatory mechanisms in conditions of cardiovascular stress such as sepsis and septic shock. During the progression of septic shock, overwhelming inflammation leads to vasodilation and capillary leakage, which alter CO by preload reduction, and a massive sympathetic activation is usually elicited in the physiological attempt to maintain vital perfusion. Thus, tachycardia and increased contractility, usually observed in septic patients, are classically considered as the main mechanisms in order to compensate a fall in preload [30], [168].

Current clinical guidelines for resuscitation of septic shock patients recommend the administration of fluids in order to restore the circulating blood volume and vasopressors in order to maintain a MAP higher than 65 mmHg; ScvO2 is monitored to be higher than 70% together with an antibiotic therapy to counteract the underlying source of infection [1], [33], [34]. In addition to these therapies, inotropes are administered to increase CO, if its value is still under a physiological threshold. As a result, it's common to have supranormal values of both HR and CO persisting even after correction of hypovolemia and hypotension, but the current clinical practice doesn't consider this condition deserving appropriate intervention.

To date only few studies have evaluated tachycardia as a mortality factor in patients suffering from septic shock [5], [74], [169], but they all found that high HR is an independent risk factor for increased mortality, similarly to many other clinical pathologies [170]–[174].

The underlying mechanism of such persistent tachycardia could be an altered chronotropic response, due to an impairment of the SNS and, in particular, of SNS mediated organs interactions [175]. In fact, it is now well understood that some septic shock patients may suffer from a

protracted and overshooting stimulation of the SNS, which may exceed in time and scope the beneficial short-term compensatory effect, leading to several adverse effects, such as prolonged tachycardia [5], [68], [176], [177] (see also §1.2). The duration of the positive effect of sympathetic stimulation depends on organ vulnerability to adrenergic overstimulation, for instance the heart, which is abundant of β adrenergic receptors, has to be considered as the main target of this sympathetic overstimulation [68]. Moreover, beside the physiological endogenous release of chatecholamines, the high doses of exogenous chatecholamines due to aggressive therapy with dobutamine or norepinephrine, further increase HR and the risk of cardiac failure [70]. For these reasons, in septic shock, tachycardia persisting after adequate volume resuscitation indicates an altered chronotropic response and can be considered as an early manifestation of myocardial dysfunction [175].

At present, most of scientific research into cardiac impairment in septic shock and multiorgan dysfunction syndrome (MODS) focuses on myocardial depression due to bacterial toxins and inflammatory mediators [178], [179]. However, recent studies show that a crucial issue in sepsis is not only the myocardial depression, but also the impaired regulation of cardiac function due to alterations of cardiovascular ANS.

Clinically, autonomic dysfunction can be quantified by HRV and BRS [6], [79], [84], [180], [181]. Several previous studies demonstrated that a reduction in HRV is one of the best predictors of death in critically ill patients [181]; among these, patients with septic shock and elevated HR have been shown to have also a reduced HRV, indicating an extreme attenuation of the vagal tone [84].

In this study, we focused on the autonomic control of cardiac contractility and HR during a controlled experiment of septic shock and resuscitation. In particular, we aimed 1) to propose a model able to separately characterize the static and dynamic control of ventricular contractility mediated by the autonomic arterial baroreflex and by the force-frequency autoregulation and 2) to determine how these control mechanisms are altered during septic shock or recovered after resuscitation. Both of these mechanisms may occur simultaneously and importantly contribute to the modulation of CO. For example, in

response to a fall in ABP, a baroreflex-mediated sympathoexcitation is supposed to occur, which will increase the inotropic state. At the same time the concurrent tachycardia could also increase the inotropic state independently via the force-frequency relation (or Bowditch effect) [182]. To our knowledge, no previous study has evaluated the static and dynamic control of ventricular contractility in septic shock condition.

3.2 MATERIALS AND METHODS

3.2.1 Study design and experimental procedure

We performed a controlled experimental study on a large animal model of septic shock induced by a polymicrobial peritonitis on adult swine in the Experimental Laboratory of Intensive Care (LA1230336), at the Université Libre de Bruxelles. The local animal ethics committee (Comité Ethique du Bien-Être Animal) approved the present study (protocol 641 N) and we followed the EU Directive 2010/63/EU for animal experiments and the ARRIVE guidelines for animal research.

Six pigs of both sex (age 4 to 6 months, weight 43.8 ± 3.9 kg expressed as mean ± SD) were obtained from a local farm (BE 400108–48). Animals were fasted for 18 h prior to the start of the experiment with free access to water. The animals were sedated with an intramuscular injection of 1.5 mg/kg midazolam (Dormicum; Roche, Belgium) and 5 mg/kg azaperone (Stresnil, Eli Lilly Benelux, Belgium) in the neck. After transportation to the operating room, a 14 G peripheral venous line (14G, Surflo IV Catheter, Terumo Medical Company, Belgium) was placed into a vein of the ear to provide vascular access and a 4,5 Fr arterial catheter (Leader- Cath, Vygon, France) was placed in the left common femoral artery for invasive arterial pressure monitoring and blood samples collection. The animals underwent endotracheal intubation following induction of anesthesia with the intravenous injection of 3 µg/kg sufentanil (Sufenta Forte, Janssen-Cilag, Belgium), 1 mg/kg propofol (Propovet, Zoetis, Belgium) and 0.5 mg/kg of rocuronium (Esmeron, Organon, The Netherlands). A central venous access for drugs infusion was obtained via a four lumen central venous line inserted inside the left femoral vein (Edwards LifeSciences, California, USA). General

anesthesia and analgesia were achieved using continuous inhalation of sevoflurane 1,8 to 2,5 % MAC (Sevorane, Abbott, Belgium) and continuous infusion of sufentanil 1 to 4 µg/kg/h (adapted according to the response to painful stimulations, as a nasal septum pinching), in association with rocuronium continuous infusion to avoid shivering. Mechanical ventilation was performed in volume-controlled mode (Primus, Draëger, Germany) with tidal volumes of 8 mL/kg and a PEEP set at 5 cmH2O. Arterial and venous blood gases were analyzed live in the operating room (Cobas b-123, Roche, Switzerland) and the respiratory rate was adjusted to maintain $PaCO_2$ values between 35 and 49 mmHg - aiming an arterial pH between 7,35 and 7,45 - while Fi_{O2} was set at a value of 0.30 at baseline and adjusted to keep PaO_2 > 90 mmHg. Fluid maintenance was provided by a balanced crystalloid (Plasmalyte, Baxter, Belgium) perfusion at 3 to 5 ml/kg/h, aiming a normal volemia at baseline, according to an arterial pulse pressure variation (PPV) ≤ 12% (and a negative fluid challenge in case of doubts, i.e. PPV remaining in the grey zone). Central temperature was maintained between 38,5 and 39,5°C with a warming blanket and hypoglycemia avoided by a continuous 10% glucose solution infusion (1 to 2 ml/kg/h). After a supra-pubic mini-laparotomy, a 14 Fr Foley catheter (Beiersdorf AG, Germany) was introduced into the bladder to monitor urine output and two large bore abdominal drains were placed in each side of the abdominal cavity for later introduction of the feces. A 7 Fr introducer was inserted into the right external jugular vein and a pulmonary artery catheter (CCO; Edwards LifeSciences, California, USA) was advanced in a pulmonary artery for continuous CO, right heart pressures and SvO_2 monitoring. Both femoral artery pressure and pulmonary artery pressure signals were continuously displayed (SC9000, Siemens, Germany) and exported to an A/D recording station (Notocord Hem, Notocord, France), similarly to electrocardiogram (ECG) and CO signals.

After instrumentation, the animals were allowed to rest for approximately 2 hours after which the first baseline measurements and blood samples were taken (baseline, T1). Sepsis was induced by the intraperitoneal instillation, via the two abdominal drains, of 3 g/kg of autologous feces collected in the cage, filtered and diluted in 300 ml of

glucose 10%. During septic shock onset, fluid maintenance was reduced to 1 ml/kg/h until the MAP has decreased below 50 mmHg. Thereafter, fluid maintenance perfusion was moderately increased to keep the animal alive for one more hour of severe hypotension (MAP goal between 45 and 50 mmHg), in order to consolidate peripheral hypoperfusion and MOF. At the end of this period, a second time point T2 was defined as reference for septic shock condition. Immediately after a series of hemodynamic measurements and blood samples, a full fluid resuscitation was initiated with both the same rate of the balanced crystalloid perfusion and an additional colloid perfusion (Geloplasma, Fresenius Kabi, France), aiming to reach a PPV <12%. After 120 min of hemodynamic stabilization, defined by a stable MAP and no further increase in CO, additional hemodynamic measurements and blood samples were taken again (T3, end of first resuscitation). Finally, a vasopressor therapy was administered, with a continuous infusion of norepinephrine at a fixed dose of 0.3 μg/kg/min for one hour, after which the last series of hemodynamic measurements and blood samples were taken again (T4, end of full resuscitation). Animal were then euthanized with a potassium chloride injection and an overdose of thiopental.

Two out of six animals were immediately fully resuscitated with vasopressors because of the severity of the distributive shock, therefore the measurements at time point T3 are missing. For this reason, in the following we refer only to baseline (T1), shock (T2) and to fully resuscitation (T4) period.

The overall timeline of the experiment is also reported in Figure 3.1 for the sake of clarity.

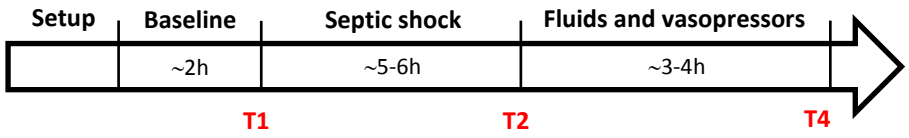

Figure 3.1. Timeline of the experimental protocol. Each phase, and its relative duration, is marked. Time points are highlighted in red.

At each time point, arterial blood samples were collected and EDTA-plasma isolated for laboratory analyses after immediate refrigerated centrifugation. In addition, hemoglobin concentration, blood lactate and electrolyte concentrations were measured (Cobas b-123, Roche, Switzerland). Further laboratory blood analyses were performed, such as cardiac troponin.

3.2.2 Hemodynamic data acquisition and preprocessing

Aortic BP and left ventricular pressure (LVP) were continuously recorded during the experiment. At each time point stationary segments of 15-minute length on average were selected. Time series of aortic SAP, DAP and MAP were obtained from aortic BP waveform using standard algorithms [152], [153], as already detailed in §2.2.3.

The time series of HP was obtained from the aortic BP waveform by computing the time difference between consecutive onsets of aortic BP beats and considered as a surrogate of the RR-intervals time series. The maximum of positive time derivative of LVP (dP/dt max) was derived on a beat-to-beat basis from LVP recording and it was taken as an indirect measure of ventricular contractility. An adaptive filter was then applied to the time series in order to remove outliers and irregularities [154] and each time series was finally resampled at 2 Hz by means of zero-order hold techniques. The pre-processed time series were then subdivided into 3-minute 50% overlapping windows and each window was detrended using a high-order polynomial function; stationarity was further verified using the Kwiatkowski–Phillips– Schmidt–Shin (KPSS) statistical test.

All the indices obtained from each 3-minute window were averaged and considered for successive statistical comparisons.

3.2.3 Ventricular contractility model

The black-box approach to mathematical modeling is a well-established and validated tool in the field of cardiovascular modelling [159], [183]. The black-box model implemented to study the control of ventricular contractility by the arterial baroreflex mechanism and the force-frequency autoregulation is depicted in Figure 3.2. We assumed

these mechanisms to be linear and time invariant, similarly to other literature works [184].

The transfer function ABP→dP/dt max represents the arterial baroreflex control of ventricular contractility, the transfer function HP→dP/dt max represents the control of the contractility by the force-frequency autoregulation. The perturbing noise source $N_{dP/dt\ max}$ represents the residual variability in dP/dt max not described by these two mechanisms, including estimation errors, noise or other mechanisms such as the cardiopulmonary baroreflex.

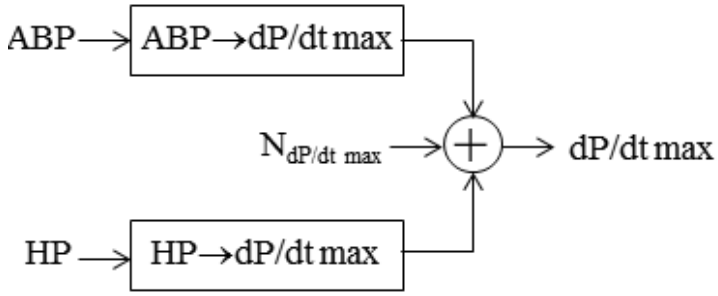

Figure 3.2. Block diagram for identification of the transfer functions relating fluctuations in aortic blood pressure (ABP) to dP/dt max (ABP→dP/dt max) and fluctuations in heart period (HP), surrogate of the heart rate variability to dP/dt max (HP→dP/dt max) from spontaneous beat-to-beat ABP, HP and dP/dt max variability. $N_{dP/dt\ max}$ represents the residual variability in dP/dt max not accounted for by the ABP and HP fluctuations.

The mathematical representation of the block diagram consists in the following autoregressive exogenous dual-input model:

$$\frac{dP}{dt}\max(n) = \sum_{i=1}^{p} a_i * \frac{dP}{dt}\max(n-i) + \sum_{i=s1}^{q} b_i * ABP(n-i) + \sum_{i=s2}^{r} c_i * HP(n-i) + W_{\frac{dP}{dt}\max} \quad (3.1)$$

where *n* is the discrete time, the coefficients a_i, b_i and c_i are the unknown parameters to be estimated, $W_{\frac{dP}{dt}\max}$ is the uncorrelated noise input of the model, *p*, *q*, and *r* define the model orders. Each term in the

model represents the beat-to-beat fluctuations of dP/dt max, mean aortic pressure (ABP) and heart period (HP). The sampling time interval is 0.5 sec.

Granger causality among the cardiovascular time series was verified before computation of the model [185]. The optimal model orders *p*, *q*, *r* were determined by minimizing the minimum description length (MDL) criterion on the overall population and were fixed to be equal to 2, *s1* and *s2*, respectively, where *s1*, *s2* are the input delays. The input delays were optimized at each time point T1, T2, T4 in order to obtain an impulse response and gain value which are in agreement with the physiology for each pig for both the transfer functions, i.e. with the first peak as negative. From our recursive analyses, the optimal set of values is the following: *s1*=3, *s2*=3 at baseline (T1), *s1*=2, *s2*=3 after development of septic shock (T2) and *s1*=6, *s2*=3 after full resuscitation with fluids and vasopressors (T4). Therefore, the model becomes the following at each time point:

$$T1: \frac{dP}{dt}\max(n) = \sum_{i=1}^{2} a_i * \frac{dP}{dt}\max(n-i) + \sum_{i=3}^{3} b_i * ABP(n-i) + \sum_{i=3}^{3} c_i * HP(n-i) + W_{\frac{dP}{dt}\max}(n) \tag{3.2}$$

$$T2: \frac{dP}{dt}\max(n) = \sum_{i=1}^{2} a_i * \frac{dP}{dt}\max(n-i) + \sum_{i=2}^{2} b_i * ABP(n-i) + \sum_{i=3}^{3} c_i * HP(n-i) + W_{\frac{dP}{dt}\max}(n) \tag{3.3}$$

$$T4: \frac{dP}{dt}\max(n) = \sum_{i=1}^{2} a_i * \frac{dP}{dt}\max(n-i) + \sum_{i=6}^{6} b_i * ABP(n-i) + \sum_{i=3}^{3} c_i * HP(n-i) + W_{\frac{dP}{dt}\max}(n) \tag{3.4}$$

Once the model orders were fixed, the parameters a_i, b_i and c_i were estimated from the stationary zero-mean fluctuations time series of ABP, HP and dP/dt max by using the standard least-square minimization procedure. Figure 3.3 shows an example of the impulse response of the baroreflex transfer function with a physiological shape: a fast negative peak followed by a smaller subsequent overshoot. The negative early

phase is consistent with the autonomic-mediated baroreflex dynamics, representing the rapid decrease in ventricular contractility that follows an increase in ABP. The area of the impulse response is expected to be negative according to this physiological interpretation, which means a negative static gain value of the baroreflex transfer function. Thus, dP/dt max would decrease (increase) in the steady state in response to a step increase (decrease) in mean ABP, as a result of the arterial baroreflex, in case of a stable HP.

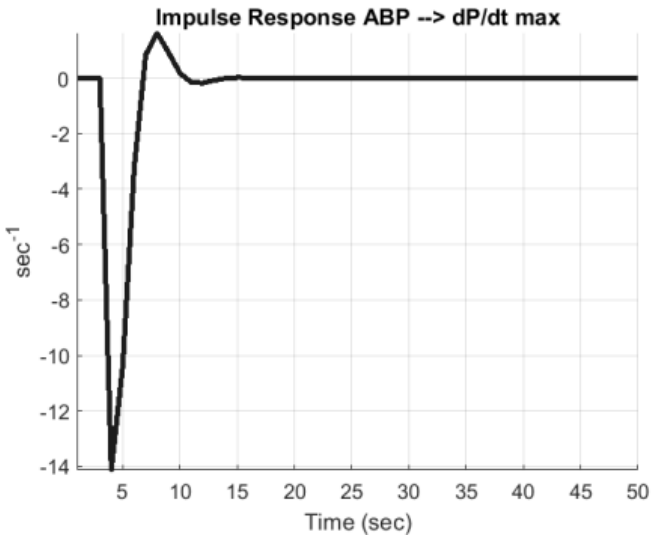

Figure 3.3. Impulse response of the baroreflex transfer function ABP→dP/dt max at baseline.

The dynamic of the force-frequency transfer function is similar: the impulse response shows a fast negative peak followed by a smaller subsequent overshoot. The static gain is expected to be negative, such as dP/dt max would decrease (increase) in the steady state in response to a step increase (decrease) in HP, due to the force-frequency autoregulation supposing any change in the mean ABP.

The model was assumed to be linear, thus a complete characterization of the dynamics of the arterial baroreflex and the force-frequency relation mechanisms were obtained from their respective impulse responses. In particular, we computed the following parameters for each transfer function:

- *Rise time* (t_{rise}), the time it takes for the step response to rise from 10% to 90% of the steady-state response.
- *Settling time* (t_{set}), the time it takes for the error $|y(t) - y_{final}|$ between the step response y(t) and the steady-state response y_{final} to fall within 2% of y_{final}.
- *Peak time* (t_{peak}), the time at which the peak of the step response occurs.
- *Static gain* (G), final value of the step response y_{final}, i.e. the area of the impulse response.

3.2.4 Cardiac baroreflex sensitivity analysis

The cardiac baroreflex, i.e. the ANS control of oscillations in the RR-intervals or HP induced by oscillations in SAP, was estimated via the bivariate model. Details about the bivariate model were previously described in §2.2.4. The parameter of interest is the feedback gain, which quantifies the cardiac BRS. Granger causality from SAP to HP was verified before computation of the gain [185]. The order of the model was optimized based on the Akaike Information Criterion, ranging from 5 to 15.

3.2.5 Heart rate variability and complexity analysis

We assessed HRV by means of linear and non-linear methods [81]. In particular, we computed the root mean square of successive differences between adjacent HP (RMSSD), the standard deviation of successive differences between adjacent HP (SDSD), and the SD of the overall HP time series. Moreover, we calculated the quadratic sample entropy (QSE), in order to investigate the non-linear characteristics of HRV as described in [186], and for this purpose the template length *m* was fixed at 1 and the tolerance *r* was computed as 20% of the SD of the time series.

3.2.6 Clinical data

Clinical variables collected at each time point were the following: CO (L min^{-1}), SV (mL), temperature T (°C), urine output UO (mL), arterial

pH, lactate (mmol L^{-1}), SvO2 (%), hematocrit Ht (%), base excess BE (mEq L^{-1}), cardiac troponin cTnT (ng/mL).

3.2.7 Statistical analysis

Friedman test was performed to detect differences in time points across multiple test attempts. In case of significant Friedman test p-value, i.e. < 0.05, we used then the Wilcoxon signed-rank test to assess significant changes among time points within the septic shock group of animals. Significance was considered with a p-value < 0.05.

The dynamic of the two mechanisms (ABP→dP/dt max and HP→dP/dt max) was compared in terms of rise time, settling time and peak time expressed in seconds. The values of the dynamic indices were compared by means of Mann-Whitney U test.

Parameters values are reported as median (25^{th}, 75^{th} percentile).

3.3 RESULTS

Figure 3.4 shows the median values (25^{th},75^{th} percentile) of MAP, HP and dP/dt max time series at each time point of the experiment.

Table 3.1 provides the median values (25^{th},75^{th} percentile) of the clinical, laboratory and hemodynamic variables. Septic shock induced a drop in MAP, CO and SV with a compensatory increase in HR (i.e. a fall in HP) and ventricular contractility, as expected. The rise in lactate values at T2 together with the decrease in BE, pH and SvO_2 denotes the typical anaerobic cellular metabolism of shock condition. Interestingly, at T4, after the resuscitation with fluids and vasopressors, even if MAP returned to values similar to baseline (except for one pig) all the other clinical variables highlight that the physiological condition of baseline was not completely restored, in particular, HR and dP/dt max further increased with respect to the shock condition (Figure 3.4).

Cardiac BRS feedback gain [mmHg ms^{-1}] showed a monotonic decreasing trend from T1 to T4: at T1 BRS was 2.4 (1.7,5.3), at T2 decreased to 0.6 (0.3,3.3) and at T4 reached values equal to 0.3 (0.2,0.4).

Table 3.2 shows the HRV indices. HRV was reduced at T2 and tended to further decrease at T4 (even if not significantly).

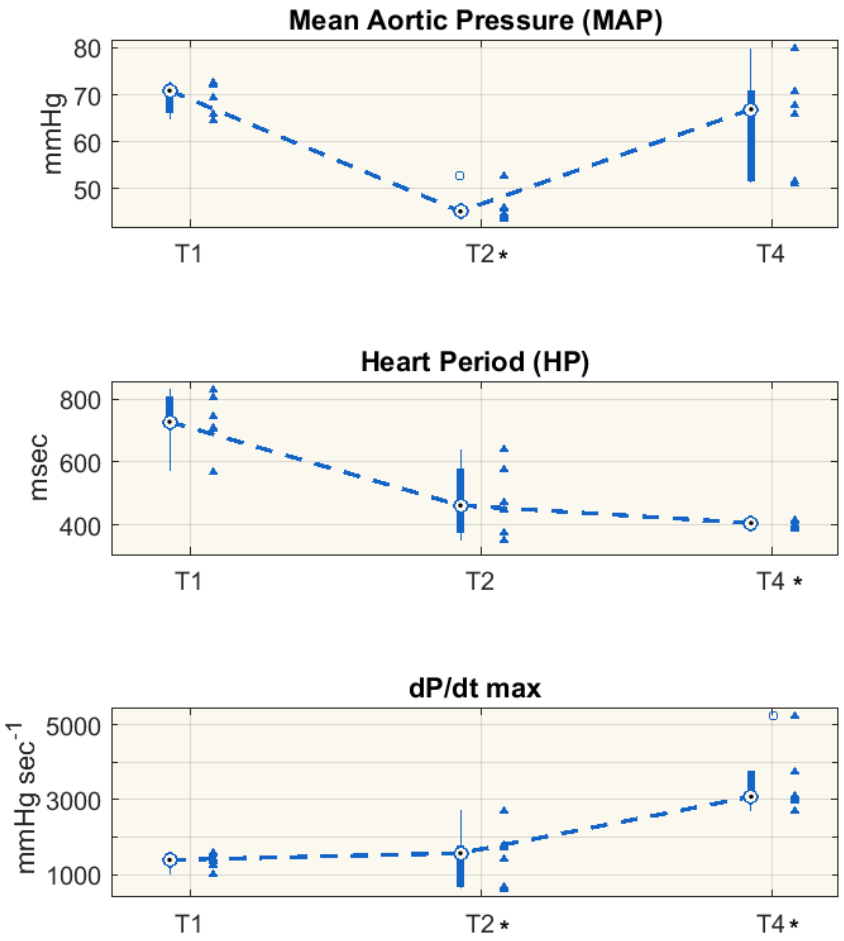

Figure 3.4. Distributions (median, 25th,75th percentile) of the values at each time point of the following variables: mean aortic pressure (MAP), heart period (HP) and maximum of positive time derivative of left ventricular pressure (dP/dt max). The values relating each pig are marked by blue triangles. T1 = baseline, T2 = after development of septic shock, T4 = after resuscitation with fluids and vasopressors. Wilcoxon signed test: *p-value<0.05 with respect to T1 (Friedman test p-value<0.05).

Table 3.1. Clinical and laboratory data at each time point of the experiment. Values are reported as median (25^{th},75^{th} percentile).

	T1	T2	T4
HR [bpm]	82.5 (74.3,84.8)	129.9 (105.6,159.7)	147.6 (144,150.6) *
SAP [mmHg]	84.8 (79.3,86.6)	64.8 (61.2,69.2)	92.7 (91.8,99.5) §§
DAP [mmHg]	54.7 (51.1,57.5)	33.5 (31.3,35.5) *	43.7 (30.8,44.8)
PAP [mmHg]	20 (16,21)	21 (19,29)	26 (23,33) *
RAP [mmHg]	6.5 (5,8)	6 (4.8,6.5)	8 (6,10)
CO [L min^{-1}]	4.9 (4.3,5.4)	3 (2.5,4.3)	9.4 (8,9.9) §
Lact [mmol L^{-1}]	0.9 (0.9,0.9)	1.7 (1.3,2.1)	2.45 (1.9,3.1) **
SV [mL]	65 (57,66)	23 (20.3,36.5)	64.5 (56,69) §
Ht [%]	27 (25,28.4)	33.5 (28,37)	25.6 (24,30)
BE [mol L^{-1}]	8.9 (8.4,11)	5.9 (5.2,5.9) *	5.7 (4.6,7) *
pH	7.48 (7.47,7.49)	7.42 (7.41,7.45)	7.44 (7.41,7.45)
UO [L]	1 (0.53,1.1)	0.1 (0.06,0.45)	0.22 (0.2,0.24)
SvO2 [%]	65 (61,69)	59.5 (53,68)	75 (68,78)
T [°C]	38.9 (38.8,39.1)	38.4 (37.7,39.2)	38.8 (38.2,39.1)
cTnT [ng l^{-1}]	11 (8,15)	21 (17,25)	35 (26,48) **

HR=heart rate, SAP=systolic aortic pressure, DAP=diastolic aortic pressure, PAP=pulmonary arterial pressure, RAP=right atrial pressure, CO=cardiac output, Lact=lactate, SV=stroke volume, Ht=hematocrit, BE=base excess, UO=urine output, SvO2=mixed venous oxygen saturation, T=temperature, cTnT=cardiac troponin. T1 = baseline, T2 = after development of septic shock, T4 = after resuscitation with fluids and vasopressors. Wilcoxon signed test: *p<0.05, **p<0.01 with respect to T1, §p-value<0.05, §§p-value<0.01 with respect to T2 (Friedman test p-value<0.05).

Table 3.2. HRV and HR complexity indices evaluated at each time point. Values are reported as median (25^{th},75^{th} percentile)

	T1	T2	T4
RMSSD [ms]	66 (55.1,91.4)	37.2 (11.5,93.6)	18.6 (12.6,20.3)
SDSD [ms]	3.5 (2.9,4.8)	2 (0.6,4.9)	1 (0.7,1.1)
SD [ms]	6.9 (4.6,8.4)	3.4 (1.4,5.4)	1.4 (0.8,1.8)
QSE	1.8 (1.7,2.2)	1.3 (0.4,2.1)	0.8 (0.4,0.9)

RMSSD=root mean square of successive difference, SDSD=standard deviation of successive difference, SD=standard deviation, QSE=quadratic sample entropy. T1=baseline, T2=after development of septic shock, T4=after resuscitation with fluids and vasopressors.

Figure 3.5 illustrates the average distribution of the impulse and step responses.

Table 3.3 reports the static and dynamic indices which characterize the two transfer functions ABP→dP/dt max and HP→dP/dt max. The gains of both the transfer functions are negative in agreement with the physiology. We observed an increasing trend in static gains of both the transfer functions from T1 to T4 and a delayed response in the contractility control mechanism mediated by baroreflex (ABP→dP/dt max) at T4, after resuscitation. This suggests that after resuscitation the autonomic-mediated baroreflex mechanism is slower in modulating ventricular contractility in response to a change in BP. A delayed response hints a depressed vagal activity during the resuscitation and this is in line with the results obtained from HRV and BRS analyses previously reported.

Table 3.3. Static and dynamic characteristics of ABP→dP/dt max and HP→dP/dt max transfer functions. Values are reported as median (25^{th},75^{th} percentile).

		T1	**T2**	**T4**
$\mathbf{t_{rise}}$ [s]	ABP→dP/dt	0.69 (0.65,0.75)	0.77 (0.64,0.95)	0.6 (0.49,0.67)
	HP→dP/dt	0.65 (0.65,0.67)	0.77 (0.62,0.95)	0.6 (0.48,0.75)
$\mathbf{t_{set}}$ [s]	ABP→dP/dt	5.9 (5,6.3)	5.8 (5.4,6.2)	6.2 (5.3,7.7)
	HP→dP/dt	5.9 (5.1,6.3)	6.4 (5.9,7.2)	4.3 (3.8,5)
$\mathbf{t_{peak}}$ [s]	ABP→dP/dt	2.5 (2.5,2.6)	2.2 (2,2.5)	3.8 (3.7,3.9) §
	HP→dP/dt	2.5 (2.5,2.5)	2.7 (2.5,3) $^{\#}$	2.3 (2.2,2.5) $^{\#\#}$
G	ABP→dP/dt	-5.8 (-7.5,-3)	-13.8 (-16.5,-12)	-21.9 (-30.5,-6)
	HP→dP/dt	-1.2 (-1.9,-0.5)	-13.2 (-43.8,-0.7)	-13.7 (-22.9,-7.8)

t_{rise}=rise time, t_{set}=settling time, t_{peak}=peak time, G=static gain [s^{-1} or mmHg/s ms^{-1}]. T1=baseline, T2=after development of septic shock, T4=after resuscitation with fluids and vasopressors. Wilcoxon signed test: §p-value<0.05 with respect to T2 (Friedman test p-value<0.05). Mann-Whitney U test: $^{\#}$p-value<0.05, $^{\#\#}$p-value<0.01 between ABP→dP/dt max and HP→dP/dt max transfer functions.

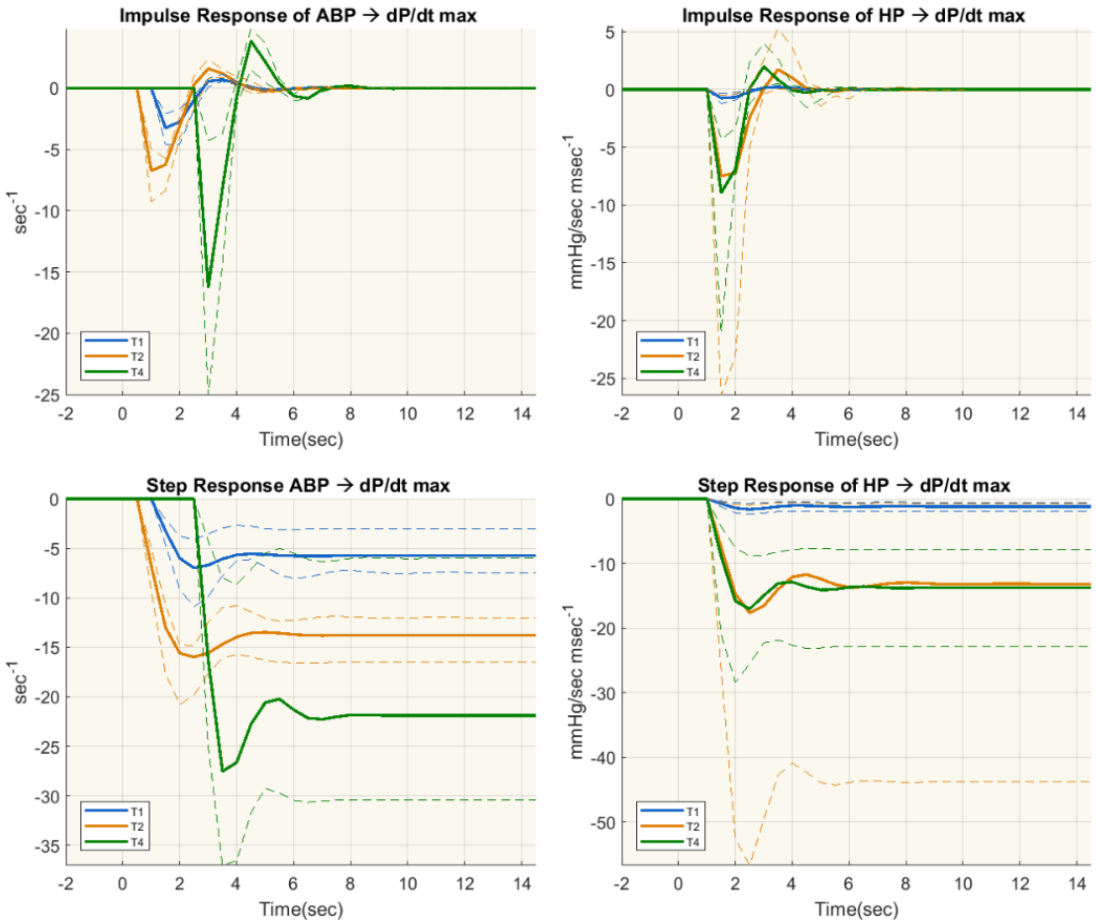

Figure 3.5. Median values (25^{th},75^{th} percentile) of ABP→dP/dt max and HP→dP/dt max impulse responses and step responses computed at each time point of the experiment. T1 = baseline, T2 = after development of septic shock, T4 = after resuscitation with fluids and vasopressors**.**

3.4 DISCUSSION

To the best of our knowledge, this is the first study that examines the static and dynamic control of ventricular contractility by the arterial baroreflex and force-frequency relation during a controlled experiment of septic shock and resuscitation. By means of a mathematical analysis of spontaneous beat-to-beat variability of the hemodynamic variables, we were able to separately characterize the contribution of the two mechanisms controlling ventricular contractility.

The main findings of this study consist in i) the feasibility of the proposed model; ii) the alteration of left ventricular contractility control mechanisms during shock which persists after resuscitation; iii) the emerging evidence that heart contractility should be considered a new therapeutic target for sepsis resuscitation.

The mechanisms under investigation significantly and independently control contractility with times of response in the order of seconds. Our results showed that the autonomic baroreflex mechanism was predominant at baseline (gain value of ABP→dP/dt max transfer function is higher than the gain of HP→dP/dt max transfer function, Table 3.3). Indeed, as Figure 3.4 shows, the impulse response of force-frequency mechanism is pretty negligible. After septic shock has fully developed (T2), both the mechanisms appeared amplified: both gain values were higher, in particular, the force-frequency related gain value increased to become comparable to the baroreflex related one. Interestingly, after resuscitation (T4) the physiological control of contractility was not restored and both the mechanisms maintained a gain value similar to shock condition or even increased.

These results, together with the clinical and laboratory variables, the HRV and the cardiac BRS analysis, highlighted that after resuscitation, i.e. after restoration of circulating volume and BP, the ANS control is still altered and this affects the heart and its functionality. An inefficient heart is prone to long term consequences such as cardiomyopathy or heart failure, which are very common in septic patients.

3.4.1 Hemodynamic changes during septic shock (T2)

Septic shock induced a severe hypotension and hypoperfusion as documented by the clinical and laboratory variables reported in Table 3.1. Moreover, an extensive hypovolemia occurred due to massive capillary leakage: the hematocrit increased, due to the higher density of the circulating blood, and CO was dramatically reduced due to low SV. Compensatory mechanisms, like tachycardia, were activated to maintain CO despite a fall in preload. Urine output decreased near to 0 mL, showing a certain degree of kidneys failure. It is interesting to observe that although HR rose more than 50% from baseline, only a slight increase occurred in ventricular contractility (dP/dt max, Figure 3.4). We hypothesized that this could be influenced by reduction in the end diastolic volume. Indeed, it is known that the index dP/dt max decreases per se, if the end diastolic volume decreases, as it is the case in shock condition.

The hemodynamics findings, such as the HRV and HR complexity analysis and the study of the cardiac BRS, suggest a shock-induced autonomic dysfunction by “uncoupling” the continuous physiological communication among vital organs through ANS signalling. In this context, the notion of complexity, as calculated by means of entropy estimates, refers to the regularity/irregularity of the time series, i.e. the degree to which template patterns repeat themselves: repeated patterns imply regularity and lead to reduced values of entropy. A loss of HR complexity, which means a higher regularity of the HR, is considered a feature of impaired adaptation of the ANS to physiological stress in several cardiovascular pathologies [187]–[189]. Accordingly, our results show that the autonomic modulation of HR in response to acute stress induced by septic shock was severely impaired (Table 3.2), in agreement with literature [82], [84]. Moreover, the cardiac baroreflex is severely depressed during shock; thus, variations in BP were not followed by a physiological adaptation of the HR in order to maintain homeostasis. Our hypothesis, as already proposed in [190], is that the elevated HR prevents any change in HRV and, thus, any adaptation to BPV.

The ventricular contractility model proposed in this work revealed that both mechanisms, i.e. the baroreflex and the force-frequency

autoregulation, increased their extent during shock condition. These results indicate that the combine effect of these mechanisms can significantly increase the level of contractility and thereby importantly aid in maintaining homeostasis during critical conditions such as septic shock. Although a notable rise in the gains was found, minor changes were reported in the dynamic characteristics of the transfer functions (Table 3.3). Only a slight delay in the peak time was observed in the step response of the force-frequency autoregulation related mechanism, and, oppositely, a slight acceleration in the peak time in the step response of the baroreflex mechanism (Table 3.3).

3.4.2 Hemodynamic changes after resuscitation (T4)

Clinical guidelines recommend resuscitation with intravascular fluid administration to counteract hypotension and tachycardia [33], [34]. However, as already reported in the literature, septic patients often have a persistent tachycardia even after a recovered hypovolemia [5], [68], [176], [177]. In this study, the animals showed a persistently elevated HR and contractility at T4, confirming what already observed in other septic populations. The reason for this phenomenon has been hypothesized to be an altered chronotropic response, probably related to an impairment of the SNS with disturbances in ANS signalling. Our data fully support this hypothesis. HRV and baroreflex functionality were still suppressed after resuscitation, indicating an ongoing dysfunctionality in autonomic control of BP and HR.

HRV is assumed to be mostly dependent on the parasympathetic outflow to the heart. Moreover, the recovery of baroreflex functionality has also been associated to an increased vagal activity in several pathologies [180], therefore, we could hint a vagal driver of an effective resuscitation [84]. An extensive literature is even pointing at the central role of the vagus nerve as therapeutic target: direct vagus nerve stimulation has been proved to be beneficial in several diseases, including sepsis and shock [58]–[60], [63], [64], [66] (see also §1.4.4).

As regard the contractility model, the results suggest that the contractility was maintained elevated by both the baroreflex and the force-frequency relation, as the gain values of the related transfer

functions were still similar to shock condition. Moreover, the dynamic characteristics showed a delayed response of the baroreflex mechanism at T4. The peak time increased whereas the one related to the force-frequency mechanism decreased, so to be significantly different (Table 3.3). This suggests that after resuscitation the autonomic-mediated baroreflex mechanism is slower in modulating ventricular contractility in response to a change in BP. A delayed response hints a depressed vagal activity during the resuscitation and this is in line with the results obtained from HRV and BRS analyses previously reported. The persistent increase of the gain related to the force-frequency relation after resuscitation could be also attributed to the overwhelming sympathoexcitation. In fact, it has been demonstrated that this mechanism is influenced by adrenergic stimulation and, in particular, the excitation of β adrenergic receptors has been shown to produce an important enhancement of the force-frequency influence on myocardial contractility [191].

Finally, also the clinical variables support the hypothesis of a stress condition unresolved by the resuscitation. Lactate, BE and urine output did not recover to baseline values, hinting a persistent alteration in either tissue perfusion and kidneys functionality even after resuscitation. Interestingly, the SV returned to physiological values (~60 mL) comparable to the baseline ones, but HR and CO were almost doubled (Table 3.1). We can hypothesize that this result indicates a situation of ventricular-arterial uncoupling [192], i.e. a condition where the left ventricle is not able to provide an adequate SV with the lowest possible energetic consumption. Cardiac troponin raised further at T4, suggesting an ongoing cardiomyocytes stress and supporting the idea of a condition deserving clinical attention.

3.4.3 Ventricular contractility indexes

We employed dP/dt max as an index of ventricular contractility because it is relatively independent from afterload compared to other indices which are, on the contrary, influenced by cardiac loading [193], [194]. However, the preload level can influence the value of dP/dt max, as it is known that the lower is the end diastolic volume (EDV) the lower

will be the value of dP/dt max, and vice versa [193]. For this reason, we verified that the large increase in dP/dt max found at T4 was really due to the altered physiological condition and not artificially generated by the fluid expansion following the resuscitation. Thus, we compared the index with the one normalized with respect to the value of EDV, at baseline and after resuscitation. The results showed a significant increase in the preload-adjusted index dP/dt max / EDV from T1 to T4, confirming the validity of our findings: T1, 10.7 (9.2,12.9) mmHg/s mL^{-1}; T4, 28.9 (26,36.4) mmHg/s mL^{-1}. We were not able to perform a similar verification during shock condition since the left ventricular volume measure was not available. However, in this case, the effect of a reduction in preload would have had the opposite influence over dP/dt max, leading to a possible underestimation of its value.

Although the maximal ventricular elastance (E_{max}) is generally recognized as the most specific index of ventricular contractility [184], E_{max} presents several computational limitations mainly due to the recording of the left ventricular volume, which requires frequent calibrations, e.g. at each time a variation in hematocrit occurs, as its measure depends on impedance properties of blood. The advantage of using dP/dt max instead, lies in its computational ease, since it is derived from LVP only. However, comparing the step responses at baseline of our transfer functions with those of the transfer functions illustrated in [184], such as ABP→ E_{max} and HR→ E_{max}, it can be noticed a similarity in the behavior; only the time scale is different (longer transients for ABP, HR→ E_{max} transfer functions) which is probably due to the computational difference of the two indices, i.e. dP/dt max has faster inter-beat changes than elastance.

3.4.4 Model assumptions

We characterized the dynamic ventricular contractility control via the arterial baroreflex and the force-frequency relation through the ABP→dP/dt max and HP→dP/dt max transfer functions, respectively. Thus, we assumed these mechanisms to be linear and time invariant. This hypothesis appeared to be reasonable in the context of this study as the

spontaneous hemodynamic fluctuations analyzed were quite small and obtained at rest, similarly to other literature works [184].

Furthermore, the following additional assumptions were made in order to build the dual-input autoregressive exogenous input model as represented by Equation 3.1. First, the transfer functions can be defined by their pole-zero representation, which has been proven to be successful in representing various physiological control systems as reported in [195]. Second, the terms *s1* and *s2* in the model equation were forced to be higher than zero in order to enforce causality, and also higher than one based on the convention used to describe the temporal relationships among the time series: given dP/dt max(*n*) as the peak of time derivative of the current LVP, HP(*n*) designated the difference between onset(*n*+1) and onset(*n*) on the ABP beat generated by the n^{th} cardiac contraction and MAP(*n*) was the average ABP value within HP(*n*). From this assumptions, it derived that MAP(*n*) cannot influence dP/dt max(*n*) as the physiological causal relationship has the opposite direction; the same applies also for the relation between HP(*n*) and dP/dt max(*n*).

3.4.5 Limitations of the study

The main limitation of this study consists in the small sample size, which may be the source of a high variability in the results so to affect the statistical tests. However, we think that the results, which showed evident trends, can still be considered important, even if not statistically significant.

Finally, the unavailability of direct measures of autonomic outflow, such as the CSNA, did not permit other than a speculation about the autonomic activity at the cardiac level during shock and resuscitation.

3.5 CONCLUSIONS AND REMARKS

The proposed analyses gave important insights into the autonomic regulation of ventricular contractility and HR during a protocol of septic shock and resuscitation. Besides clinical variables and standard hemodynamic indices as BRS and HRV, an advanced parametrical model has been proposed to study the static and dynamic properties of two

main mechanisms of ventricular contractility control: the autonomic-mediated arterial baroreflex and the force-frequency autoregulation mechanism.

The results highlighted a condition of cardiovascular inefficiency triggered by septic shock, which was not resolved after resuscitation. The ANS plays a key role to restore the lost homeostasis, as previously described in chapter 2, and, when its functionality is not successfully recovered, the resuscitation is not effective in restoring the ANS control mechanisms. Moreover, this work is in line with previous studies which observed a persistently elevated cardiac frequency and contractility after resuscitation in septic patients, and explained this phenomenon with an impairment of the ANS [175]. Our results further suggested that this persistent sympathoexcitation could be related to a dysfunction of both the arterial baroreflex and the force-frequency mechanisms.

We are aware that the proposed model requires the recordings of LVP, which is not commonly available in clinical setting; however, the cardiac dysfunctions are an import burden in septic patients and the possibility to have indexes which quantify and assess the heart contractility control in association to standard measures, like BP or echo indices, is worthy in the evaluation of different pharmacological therapies. For example, recent research studies are investigating the benefit of ivabradine administration, which is supposed to reduce the HR without affecting the cardiac contractility [175]. Our model can be used in preclinical studies in order to compare different pharmacological strategies.

Moreover, as detailed in §1.4.4, innovative technologies in the field of bioelectronic medicine are growing and they are advocated as the future medicine for the vast majority of diseases. These techniques are based on the direct stimulation of the ANS, in particular of the vagus nerve and clinical trials are ongoing [58]. Our model could be used to evaluate the efficacy of such treatment on left ventricular contractility and relating control mechanisms.

In conclusion, the proposed models could be valuable tools in assessing the autonomic control mechanisms of ventricular contractility and HR during shock and resuscitation, and they could be useful to

evaluate the effectiveness of treatments, combined with standard clinical measures. In addition, our results support the emerging evidence that heart contractility should be considered a new therapeutic target for sepsis resuscitation.

Moreover, the results here presented question other mechanisms which could be involved. Indeed, sepsis induces changes in the hemodynamics and mechanical properties of the vascular system, and these could contribute to the observed alteration of cardiac functionality. As reported in Table 3.1, the resuscitation maneuvers were able to restore the normal volemia and the SV returned to values similar to baseline condition; however, both HR and CO almost doubled compared to baseline, hinting an inefficient condition of cardiovascular coupling.

In the next chapter the same experimental model was used to investigate the hemodynamic changes on the arterial tree induced by septic shock. The peripheral vascular decoupling induced by septic shock has been already reported in the literature [196], such as an inversion of the usual physiological amplification of PP from central to peripheral arterial sites. This results in an increase of stiffness of aortic walls during shock state, and, if persistent after resuscitation, this condition could contribute to the cardiac functionality disarray.

4 VASCULAR DECOUPLING IN SEPTIC SHOCK: THE COMBINED ROLE OF AUTONOMIC NERVOUS SYSTEM, ARTERIAL STIFFNESS AND PERIPHERAL VASCULAR TONE

In this study we examined the hemodynamic changes along the arterial tree and their relation with autonomic functions in the same experimental protocol described in the previous chapter.

The objective is to investigate if the phenomenon of vascular decoupling occurs in polymicrobial septic shock, similarly to what reported by other researchers in endotoxin shock model [196], and to verify if the resuscitation maneuvers are able to restore a physiological condition of the arterial tree. Changes in vascular properties, e.g. arterial stiffening, are strictly related to autonomic functions, as already reported in a wide range of diseases, and markers of vascular functionality could be helpful as novel therapeutic targets to guide the administration of fluids and vasopressors. For example, a recent study, pointed out the detrimental effects of long-term administration of noradrenaline in septic shock patients, as it increases arterial wave reflections, thus leading to a further increase in left ventricular workload and to a worsening in left ventricular performance [197].

Our results show how septic shock induced a severe vascular disarray, decoupling the usual pressure wave propagation from central to peripheral sites; this phenomenon appeared as an inversion of the physiological PP amplification, with a higher PP in the central aorta than in the peripheral arteries during shock. At the same time, septic shock also induced a dysfunction of the ANS, which was found to be significantly correlated with the vascular derangements. This compromised condition was not resolved by administration of fluids and noradrenaline; thus, a persistent vascular and autonomic dysfunction were reported also in the resuscitated animals, as also previously described.

Details about rationale, methodology, results and conclusions of the study are described in the following paragraphs. These parts are material of a manuscript, which has been recently submitted to a peer-reviewed scientific journal in the field. A final remark on the main findings is reported at the end of the chapter.

4.1 INTRODUCTION

Acute inflammation and sepsis are known to impair endothelial functions, leading to an imbalance of vasodilatory and vasoconstrictive mechanisms, which may lead to multi organ failure [198]. Much research has focused on the analysis of the mechanisms underlying vasodilation of resistance vessels, but a major role is also played by mechanical properties of compliant arteries, especially the aorta. Elastic arteries are able to accommodate the ejected stroke volume and they allow to ensure the optimal flow conditions in the periphery, including during diastole. A reduction in the elastic properties (i.e. arterial stiffness) of the large arteries, and in particular of the aorta, leads to increased left ventricular afterload (increased myocardial oxygen demand), reduced coronary perfusion (decreased myocardial oxygen delivery) and mechanical fatigue of the arterial walls [199].

Sepsis and septic shock were proved to significantly increase the stiffness of the large arteries. One single injection of LPS in a nonlethal endotoxin shock model in rabbit has been shown to induce a transient but prolonged dysfunction of the endothelium-mediated vascular relaxation in the aorta [200]. Additional experimental observations in a swine model of endotoxin shock showed how sepsis differentially alters central and peripheral vasomotor tone, decoupling the usual pressure wave propagation from central to peripheral sites, with a stiffening of the central vasculature compartment (i.e. the aorta) and, on the opposite, an increase in compliance of the peripheral compartment [196]. A recent clinical observational study on arterial elastic properties has shown an increase in aortic stiffness, with respect to the general population, in a cohort of septic shock patients when pulse wave velocity (PWV, an

indirect measure of arterial stiffness in large arteries) was measured at the time of admission [201].

At the same time, the sympathetic autonomic nervous system (ANS) actively contributes, mainly through the release of noradrenaline, to the modulation of arterial smooth muscle tone, thereby it interacts with local endothelial mechanisms and it may affect the arterial mechanical properties. A relationship between muscle sympathetic nerve activity -an invasive measure of the peripheral ANS- and PWV was observed in healthy individuals, regardless of any other cofounding factors [202]. Moreover, an association between alterations in vascular properties (arterial stiffness), and dysfunction of the ANS has been widely documented in several cohorts of patients affected by different chronic pathologies, including diabetic patients [203]–[205], hypertensive patients [206], or heart failure patients [207].

Current clinical guidelines are mainly concerned to recover septic shock patients from hypotension and hypovolemia and the mean value of arterial pressure is one of the key therapy targets [33]; however, no further recommendation is addressed about the more subtle changes occurring in the arterial pressure waveform, which can give insight on peripheral vascular tone, backward pressure waves and large arteries compliance, and suffer from significant modifications. Interestingly a recent clinical observational study pointed out the effects of noradrenaline administration in septic shock patients, highlighting that high doses of noradrenaline increase arterial characteristic impedance, pulse wave velocity and reflection phenomena, while reducing aortic compliance [197].

Given these premises, the main objectives of this study are 1) to measure the vascular system properties, at central and peripheral levels, in an experimental model of polymicrobial septic shock with standard resuscitation, i.e. fluids and vasopressors administration; 2) to assess the effectiveness of these therapies at vascular level and to extend the previous observations made on endotoxin shock models [196]. Finally, we want to verify if these alterations of the vascular system properties in septic shock are accompanied by altered ANS regulatory mechanisms of arterial pressure.

4.2 METHODS

4.2.1 Experimental protocol

Six pigs of both sex (age 4 to 6 months, weight 43.8 ± 3.9 kg expressed as mean ± SD) were studied at time points: baseline (T1), after development of septic shock induced by fecal peritonitis (T2), and after full fluid resuscitation to reach a PPV <12% followed by a vasopressor therapy with continuous infusion of norepinephrine at a fixed dose of 0.3 µg/kg/min (T4). For details about instrumentation and experimental procedure refer to the previous chapter (§3.2.1).

4.2.2 Hemodynamic data acquisition and preprocessing

ABP was continuously recorded during the experiment at three different sites: in the ascending aorta (trough an internal carotid artery puncture), in the common femoral artery and in the radial artery. Aortic and femoral arterial pressures were measured using high-fidelity pressure transducers on solid tip catheters (respectively: 5F pressure catheter, Transonic System Europe, The Netherlands and SPR-350S Mikro-Tip®, Millar, USA). Radial arterial pressure was measured using a fluid filled catheter (3F Leader Cath, Vygon, France) and an external pressure transducer (TrueWave®, Edwards, Belgium); potential damping or resonance artefacts being excluded by careful cares and regular fast flush tests (so called Gardner's test). Each arterial pressure signal was exported to and A/D recording station (Notocord Hem, Notocord, France), similarly to the surface ECG signal, with a high temporal resolution (100 to 500 Hz).

At each time point stationary segments of about 15-minute length were selected. Time series of SAP, DAP, MAP and PP, computed as the difference between SAP and DAP within the same ABP pulse, were obtained from ABP waveforms using standard algorithms [152], [153]. The time series of HP was obtained from the aortic ABP waveform by computing the time difference between two consecutive onsets of ABP beats and considered as a surrogate of the RR-intervals time series. An adaptive filter was then applied to the time series in order to remove outliers and irregularities [154] and each time series was finally

resampled at 2 Hz by means of a zero-order hold technique. The pre-processed time series were then subdivided into 3-minute 50% overlapping windows and each window was detrended using a high-order polynomial function. In order to guarantee stationarity, the Kwiatkowski–Phillips– Schmidt–Shin (KPSS) statistical test was performed.

Except for the time constant τ described in the following, the indices considered for successive statistical comparisons are the average of the ones obtained from each 3-minute window.

Continuous cardiac output CO (L min^{-1}) monitoring was performed using a pulmonary artery catheter (CCO; Edwards LifeSciences, California, USA), from which were computed the continuous stroke volume SV (mL).

4.2.3 The Windkessel time constant τ

According to the 2-element Windkessel model reported in Figure 4.1, the time constant τ of the arterial tree can be computed by the following equation

$$\tau = TPR * AC \tag{4.1}$$

where TPR is the total peripheral resistance, and AC is the total arterial compliance [208].

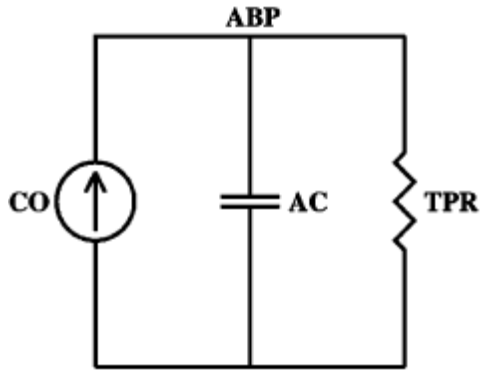

Figure 4.1. The 2-element Windkessel model of arterial tree. CO=cardiac output, ABP=arterial blood pressure, TPR=total peripheral resistance, AC=arterial compliance

This relationship is true on a beat-to beat basis if we consider the ABP waveform measured centrally where the cumulative effects of wave

reflections are attenuated [209]; in this case, ABP should decay like a pure exponential during each diastolic interval with a time constant τ. However, this relationship is also consistent if we consider a time scale sufficiently long such that the wavelengths of the propagating waves are much larger that the dimension of the arterial tree. At such time scales, the arterial tree acts as a single blood reservoir, and the Windkessel model is therefore valid. So, for example, if pulsatile activity abruptly ceased, then peripheral ABP may eventually decay like a pure exponential as soon as the faster wave reflections died out [210].

Based on this concept, we computed the time constant τ on long time intervals (6-minute windows) of the measured ABP waveforms by adopting the method proposed by Mukkamala and co-authors [210]. In particular, the technique is specifically implemented in three mathematical steps (see also [210]).

First, a cardiac contractions signal *x(t)* is created, which consists in an impulse train where each impulse is placed at the time instant of the onset of ABP upstroke and has an area equal to the ensuing PP.

Then, the relationship between the cardiac contractions signal *x(t)* and the ABP waveform named *y(t)* is characterized by estimating an impulse response function *h(t)*, which is found by minimizing in the least squares sense the convolution with *x(t)* in order to best fit *y(t)*. By mathematical definition, the estimated *h(t)* represents the ABP response to a single cardiac contraction (normalized approximately by the average PP). The impulse response function is specifically estimated according to the following autoregressive exogenous input equation:

$$y(t) = \sum_{k=1}^{m} a_k y(t-k) + \sum_{k=1}^{n} b_k x(t-k) + e(t) \qquad (4.2)$$

where *e(t)* is the unmeasured residual error, $\{a_k, b_k\}$ are unknown parameters, and *m* and *n* limit the number of these parameters (model order). For a fixed model order, the parameters are estimated from *x(t)* and *y(t)* in closed-form through the least-squares minimization of the residual error (i.e., linear least squares estimation). The model order is determined by minimizing the minimum description length criterion,

which penalizes for unnecessary parameters. With the estimated parameters $\{\hat{a}_k, \hat{b}_k\}$, *h(t)* is computed by definition as the response to an impulse, as follows:

$$h(t) = \sum_{k=1}^{m} \hat{a}_k h(t-k) + \sum_{k=1}^{n} \hat{b}_k \delta(t-k) \tag{4.3}$$

where $\delta(t)$ is the unit impulse function.

Next, the Windkessel time constant τ is determined over a selected interval, after the time of its maximum value based on the following exponential equation:

$$h(t) = Ae^{-\frac{t}{\tau}} + w(t) \tag{4.4}$$

The parameters A and τ are estimated through the least squares minimization of the unmeasured residual error *w(t)*. This optimization problem is solved via linear least squares estimation by log transforming *h(t)*.

The time interval for the determination of the time constant τ is updated at each time point based on the exponential decay of *h(t)*. An example of the obtained impulse responses at two different time points is reported in Figure 4.2; at T2 the faster response implies an earlier time window, as highlighted in red.

The time constant τ was determined on each 6-minute 50% overlapping window, then averaged for the successive statistical analysis.

The total arterial compliance AC was independently estimated with further two different methods: i) as the ratio between continuous SV and aortic PP ($AC = \frac{SV}{PP}$); ii) as proposed in [211], i.e. by using the following equation

$$AC = \frac{SV}{K(P_s^* - P_d)} \tag{4.5}$$

where $K = \frac{A_s + A_d}{A_d}$ represents the ratio between the total area under aortic pressure and the diastolic area, i.e. the area between the dicrotic notch and the end of diastole; P_s^* is the aortic pressure at the occurrence time of the dicrotic notch, and P_d is the aortic pressure at the end of diastole.

The total peripheral resistance TPR was computed as the ratio between mean aortic pressure and continuous CO, i.e. $TPR = \frac{MAP}{CO}$, according to the Windkessel model (Figure 4.1).

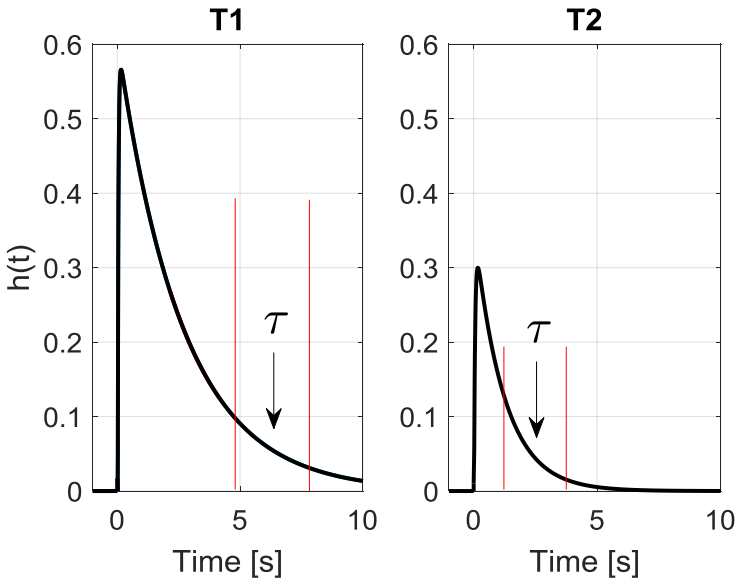

Figure 4.2. Example of two impulse responses *h(t)* computed at time point T1 and T2 for one pig. Highlighted in red the time windows selected for the computation of the time constant τ.

4.2.4 Cardiac BRS analysis

The cardiac baroreflex, i.e. the ANS control of oscillations in the RR-intervals or HP induced by oscillations in SAP, was estimated via the bivariate model method on the aortic ABP (see §3.2.4). The parameters of interest are the feedback gain (FB), which quantifies the autonomic-

mediated cardiac baroreflex, and the feedforward gain (FF) or runoff effect, which explains the oscillations in blood pressure generated by oscillations in HR, due to the mechanical coupling between the two systems. Granger causality from SAP to HP and vice versa was verified before computation of the gains [185]. The order of the model was optimized based on the Akaike Information Criterion, ranging from 5 to 15.

4.2.5 HRV analysis and spectral analysis

HRV analysis, as an indicator of the ANS control on HR, was performed on HP time series extracted from the aortic ABP signal (see §3.2.5). Such analyses have been described in the previous chapter.

Spectral indices obtained from power spectra of DAP included the normalized low frequency power (LF%), which represents the relative power in the low frequency (LF, 0.04–0.15 Hz) band and it is computed as the LF power divided by the total power without the very low frequency component (VLF, 0-0.04 Hz). LF oscillations in DAP values are meant to be mainly associated to the sympathetic ANS control of peripheral resistance.

4.2.6 Clinical data

We reported here again the clinical variables such as CO (L min^{-1}), SV (mL), urine output UO (mL), lactate (mmol L^{-1}), and mixed venous oxygen saturation SvO_2(%). Other variables were already reported in the previous chapter (see §3.2.6).

4.2.7 Statistical analysis

Friedman test was performed to detect differences among the three time points across multiple test attempts. In case of significant Friedman test p-value, i.e. < 0.05, we used then the Wilcoxon signed-rank test to assess significant changes among time points within the septic shock group of animals.

The values of aortic, radial and femoral BP components (SAP, DAP, MAP, PP) were compared at each time point among the different sites (radial and femoral) taking the aorta as a reference by means of Mann-

Whitney U test. Parameters values are reported as median (25^{th}, 75^{th} percentile).

In order to test for significant associations between changes in vascular characteristics and ANS activity, correlations analyses were performed by using the Spearman correlation test.

Significance was considered with a p-value < 0.05.

4.3 RESULTS

Table 4.1 provides the median values (25^{th},75^{th} percentile) of the hemodynamic parameters, the clinical variables, the estimated total arterial compliance AC and the total peripheral resistance TPR. Figure 4.3 shows the distribution of PP values computed from aortic, radial and femoral ABP signal at each time point.

At baseline (T1), PP was progressively higher from aortic to the more distal arterial sampling sites, reflecting the physiological peripheral PP amplification. The large increase in peripheral PP (~15 mmHg) was primarily due to a higher value in SAP, since DAP values resulted similar at all sampling sites; MAP was not significantly different at the three sites. HR, CO, SV and SvO2 have values within the physiological range. The arterial compliance AC was near the physiological value of 2 mL/mmHg [212].

After development of septic shock (T2), aortic PP slightly increased with no significant changes, whereas both femoral and radial PP progressively decreased and became slightly lower than aortic PP (Figure 4.3). The decrease in peripheral PP was due primarily to a larger decrease in SAP values in the peripheral sites as DAP values tended to decrease to a similar amount in all sites. HR increased as compensatory mechanism to sustain a condition of reduced venous return, CO and SV (Table 4.1). The rise in lactate values and the moderate decrease in SvO_2 denote the potential anaerobic cellular metabolism and heterogeneous microcirculatory alterations typical of shock. AC was more than halved in all pigs, hinting a certain degree of arterial stiffness.

After full resuscitation with fluids and vasopressors (T4), the overall cardiovascular condition seemed to be restored as MAP increased to be

>60 mmHg, SV returned to values similar to baseline, and all the main hemodynamic indices, e.g. CO, SAP, DAP, showed a positive trend, i.e. the differences between the values at T4 and T2 were positive (Table 4.1). However, the arterial pressure variables at different sites did not return to the baseline values, in particular, the physiological PP amplification was not restored as in aorta PP values did not return to be significantly lower than in peripheral arteries. These differences in PP were driven by SAP, as DAP changed similarly at all sites. The SV returned to values comparable to baseline, but CO was almost doubled, as expected in the compensatory hyperdynamic phase of a resuscitated distributive shock, mainly due to a sustained tachycardia. This inefficient cardiac condition, despite a full resuscitation and the $\beta 1$ adrenergic stimulation provided by the noradrenaline administration, indicates that the left ventricle was not able to provide a higher and adequate SV with the lowest possible energetic consumption, as already reported previously (§3.5). The values of AC were larger than in non-resuscitated shock but still lower than baseline. These results suggest that the large increase in central PP observed after resuscitation may be mostly due to the reduction in arterial compliance rather than an actual increase in heart efficiency as SV was only slightly increased compared to baseline (Table 4.1, Figure 4.3 and Figure 4.4).

It is important to recall that a decrease in arterial blood pressure could alter arterial compliance, independently on the sepsis condition, due to the volume-pressure relationship [213]. However, systemic hypotension, typical of circulatory shock state, would underfill the central vessels increasing arterial compliance. So the persisting decrease in AC in our experiment cannot be explained by the decrease in the arterial pressure. The same reasoning can also apply to the change in compliance observed from T2 to T4, where both AC and central blood pressure increased.

The estimated TPR showed a significant decrease at T4 with respect to un-resuscitated shock condition (T2) (Figure 4.4).

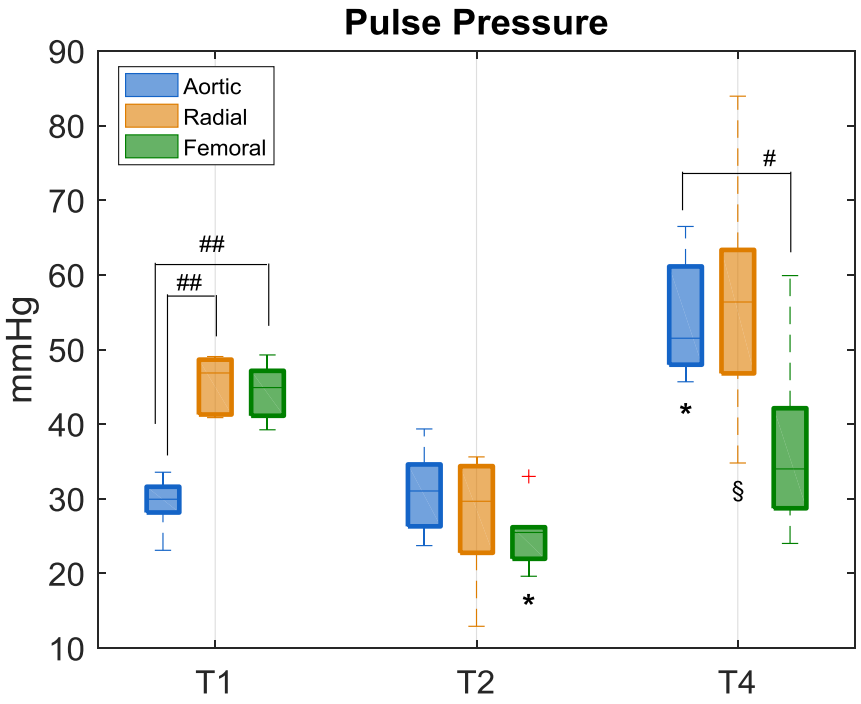

Figure 4.3. Distribution (median (25^{th},75^{th} percentile)) of PP values computed from aortic (blue), radial (orange) and femoral (green) ABP waveforms at each time point of the experiment. T1 = baseline, T2 = after development of septic shock, T4 = after resuscitation with fluids and vasopressors. Wilcoxon signed test: *p-value<0.05 with respect to T1, §p-value<0.05 with respect to T2 (Friedman test p-value<0.05). Mann-Whitney U test: #p-value<0.05 between the specified variables.

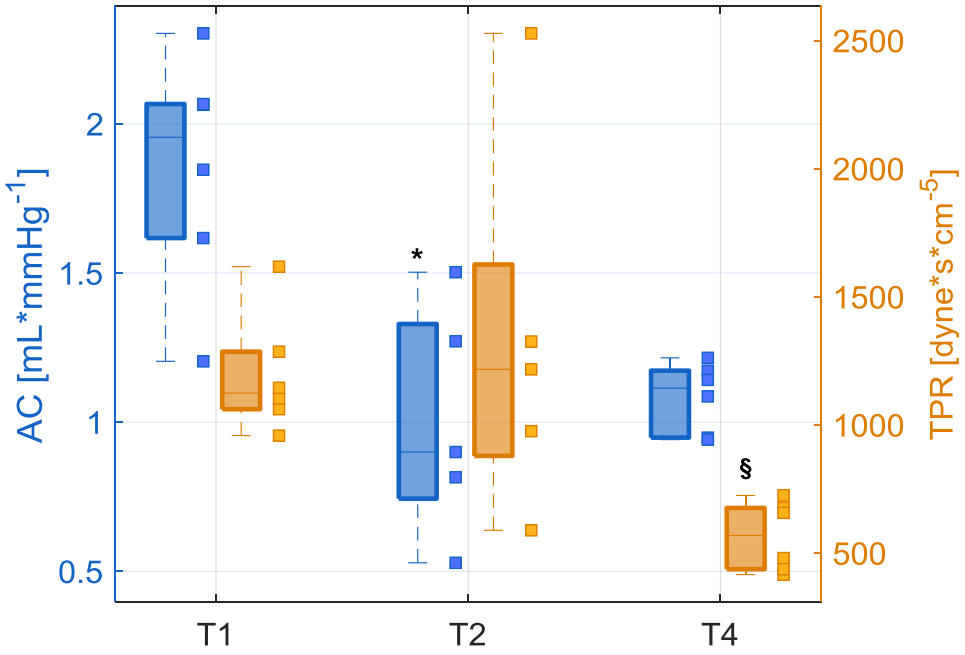

Figure 4.4. Distribution (median (25^{th},75^{th} percentile)) of total arterial compliance (AC) and total peripheral resistance (TPR) at each time point of the experiment. The values relating each pig are marked by the little squares. T1 = baseline, T2 = after development of septic shock, T4 = after resuscitation with fluids and noradrenaline. Wilcoxon signed test: *p-value<0.05 with respect to T1, §p-value<0.05 with respect to T2 (Friedman test p-value<0.05).

Table 4.1. Values of hemodynamic parameters, clinical data, estimated arterial compliance (AC) and total peripheral resistance (TPR) at each time point of the experiment. Values are reported as median (25^{th},75^{th} percentile).

	T1	T2	T4	Delta T2-T1	Delta T4-T1	Delta T4-T2
Aortic						
PP	29.9 (28.2,31.6)	31.1 (26.3,34.6)	51.5 (48,61.1) *	3.1 (-1.6,4.5)	22.8 (19.8,32.2)	19.7 (18.9,27.1)
SAP	84.1 (79.3,86.3)	65.6 (62.1,69.5)	94.9 (92.3,100.3) §§	-18.3 (-20.9,-16.3)	9.3 (6.2,14)	26 (24.3,32.3)
DAP	55.2 (50.5,56.6)	35.1 (33.6,35.8) *	45 (30,48.7)	-18.5 (-24.4,-15)	-11.9 (-18.3,-9)	7.5 (-1.2,12.9)
MAP	70.1 (65.9,71.8)	45.3 (45.2,45.5) *	67.6 (53.2,70.5)	-23.3 (-26.5,-18.9)	-3.5 (-12.9,0.6)	18.8 (7.8,23.9)
Femoral						
PP	44.9 (41.1,47.1) ##	25.5 (22,26.2) *	34 (28.7,42.1) #	-19.2 (-24.3,-14.1)	-12.5 (-19.9,2.9)	7.4 (2.6,17)
SAP	102.4 (95.1,105.3) ##	62.2 (55.5,63.5) **	82.4 (70.3,92.5)	-39.6 (-41.8,-36.5)	-22.5 (-30.4,-8)	14.9 (9.2,31.5)
DAP	57.5 (51.2,58.5)	36.6 (35.8,38.4) **	44.2 (40.7,49.7)	-19.8 (-21.1,-16.6)	-10.7 (-11.6,-8.5)	8.3 (4.8,14.4)
MAP	74.9 (68.7,75.8)	47.9 (45.4,49.8) **	64.4 (54.8,66.9)	-25.4 (-28,-23.3)	-9.1 (-16.4,-5.2)	14.9 (6.9,24.4)
Radial						
PP	46.8 (41.3,48.6) ##	29.7 (22.8,34.4)	56.4 (46.8,63.3) §	-14.4 (-24.6,-13.8)	8.1 (-1.8,22.5)	27.8 (11.2,36.5)
SAP	100.6 (96.5,101.7) ##	64 (60.8,68.6) *	97.4 (80.6,110.9)	-36.7 (-40.2,-34.1)	-5.5 (-15.9,11.2)	32 (12,47.3)
DAP	54.6 (51.5,55.3)	36.3 (33,38) **	41 (35,47.6)	-17.7 (-22,-14.9)	-13.7 (-14.1,-11.3)	4.4 (2.6,10.8)
MAP	71.6 (66.9,73)	45.6 (44.9,46.1) **	62.3 (52.8,68.9)	-25.4 (-27.8,-21.2)	-9.4 (-12.9,-4.3)	16 (6.7,23.5)

	T1	T2	T4	Delta T2-T1	Delta T4-T1	Delta T4-T2
HR	82.1 (76.3,85.3)	131 (103,158.8)	146 (144,150) *	43.4 (23.9,78.4)	66.9 (64.9,68.8)	12 (-13.5,44)
CO	5 (4.5,5.3)	2.97 (2.4,4.3)	9.5 (8.2,10.2) §	-1.9 (-2.2,-0.8)	4.9 (2.8,6.3)	5.2 (4.4,6.3)
SV	59 (52,60.5)	23.3 (20.1,36.8)	65.5 (57.6,69.6) §	-29.5 (-35,-21.7)	7.3 (-1.9,16.8)	34.3 (17,45)
UO	1 (0.53,1.1)	0.1 (0.06,0.45)	0.22 (0.2,0.24)	-0.7 (-0.98,-0.2)	-0.8 (-0.9,-0.6)	0.1 (0,0.2)
Lact	0.9 (0.9,0.9)	1.7 (1.3,2.1)	2.45 (1.9,3.1) **	0.8 (0.4,1.2)	1.55 (1,2.2)	0.45 (0.1,1)
SvO2	65 (61,69)	59.5 (53,68)	75 (68,78)	-6.5 (-11,1)	9 (3,15)	14.5 (-1,23)
AC (SV/PP)	1.98 (1.8,2.14)	0.88 (0.7,1.05)$^{n=5}$ *	1.17 (1.05,1.34)	-1.19 (-1.36,-0.85) $^{n=5}$	-0.78 (-0.99,-0.48)	0.38 (-0.03,0.57) $^{n=5}$
AC (area)	1.96 (1.62,2.07)	0.9 (0.74,1.33)$^{n=5}$ *	1.11 (0.95,1.17)	-0.79 (-1.29,-0.54) $^{n=5}$	-0.8 (-1.12,-0.4)	0.19 (-0.3,0.35) $^{n=5}$
TPR	1124 (1061,1286)	1217 (879,1626) $^{n=5}$	568 (437,676) §	155 (-161,363) $^{n=5}$	-572 (-870,-420)	-540 (-918,-447) $^{n=5}$

PP=pulse pressure [mmHg], MAP=mean pressure [mmHg], SAP=systolic pressure [mmHg], DAP=diastolic pressure [mmHg], HR=heart rate [bpm], CO=cardiac output [L/min], SV=stroke volume [mL], UO=urine output [L], Lact=lactate [mmol/L], SvO2=mixed venous oxygen saturation [%], AC=total arterial compliance [mL/mmHg], TPR=total peripheral resistance [dyne*s*cm^{-5}]. T1 = baseline, T2 = after development of septic shock, T4 = after resuscitation with fluids and noradrenaline. Wilcoxon signed test: *p<0.05, **p<0.01 with respect to T1, §p-value<0.05, §§p-value<0.01 with respect to T2 (Friedman test p-value<0.05). Mann-Whitney U test: $^{\#}$p<0.05, $^{\#\#}$p<0.01 with respect to aortic pressure at the specified time point.

Figure 4.5 shows the trend of the time constants τ computed at the three arterial sites (i.e. aorta, femoral and radial artery) at each time point of the experiment. All the time constants were characterized by a monotonically decreasing trend from T1 to T4; statistical significance was reached at T4 with respect to T1 only in aortic and radial τ. The time constant τ is a global characteristic of the arterial tree, reflecting both the contribution of total arterial compliance and total peripheral resistance of the overall arterial circulation. However, one could notice a slightly different trend in the values of τ computed at aortic level and in the periphery; this may be partly explained by a different balance between elastic and resistive components present at central and peripheral arterial sites, with a predominance of decreased elastic properties in the aorta and a predominance of loss of peripheral resistance in femoral and radial arteries. Moreover, the trend of both AC and TPR (Figure 4.4 and Table 4.1) reinforces this assumption as in shock (T2) the main driver of the decreased time constant seems to be the reduction in arterial compliance, whereas at T4 a dramatic loss of resistance further contributes to the low values of τ.

Table 4.2 reports the values of the normalized low frequency power (LF%) of DAP series. The index showed a monotonically decreasing trend from T1 to T4, hinting a persistent dysregulation of peripheral resistance by the ANS.

Table 4.3 shows the results obtained from BRS analysis and HRV analysis. The FB gain, i.e. the autonomic-mediated baroreflex mechanism, was decreased during shock (T2) and was even further depressed after resuscitation (T4). A similar trend was observed also for the HRV indices (see also HRV results in the previous chapter). On the opposite, an increasing trend was observed for the FF gain, or runoff effect, which was higher compared to baseline both in shock and after resuscitation.

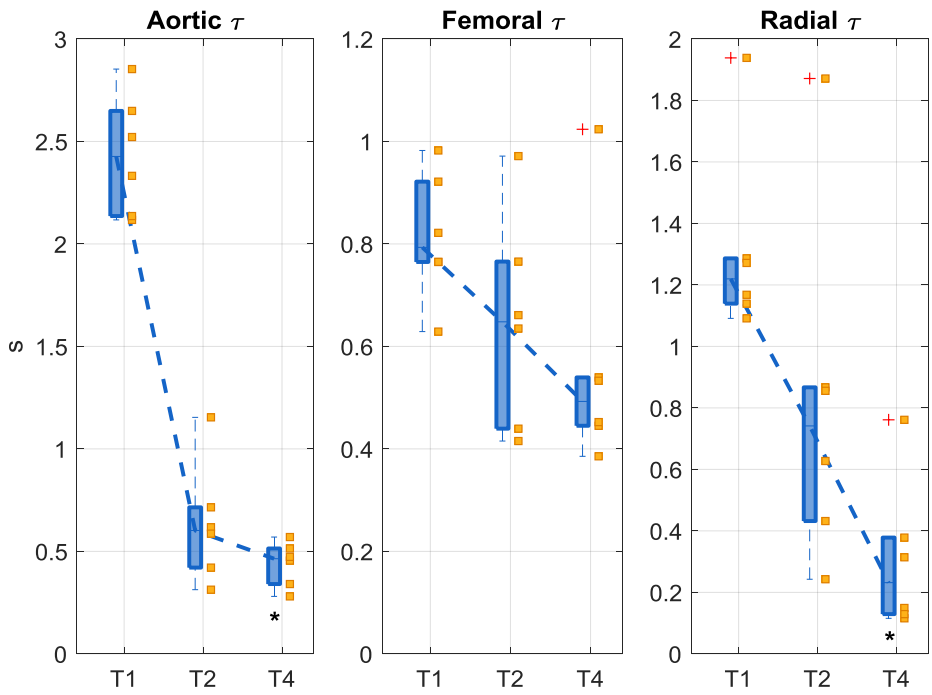

Figure 4.5. Distributions (median (25^{th},75^{th} percentile)) of the time constant τ values computed in aorta, femoral and radial artery at each time point of the experiment. The values relating each pig are marked by the little squares. T1 = baseline, T2 = after development of septic shock, T4 = after resuscitation with fluids and noradrenaline. Wilcoxon signed test: *p-value<0.05 with respect to T1 (Friedman test p-value<0.05).

Results of the correlation analysis are reported in Figure 4.6 for BRS and aortic τ (similar results, not shown, were found for the other variables). A strong positive correlation between the two indices was observed during the overall time course of the experiment. In particular, we noticed that the values at baseline were characterized both by an aortic τ higher than 2 s, representing the typical physiological value for humans [212], and by a BRS higher than 1 ms/mmHg. After shock and resuscitation the values can be grouped in an area characterized by much lower values of both BRS and τ. This further highlights that after resuscitation either the vascular mechanics and the autonomic control did not return to the baseline condition.

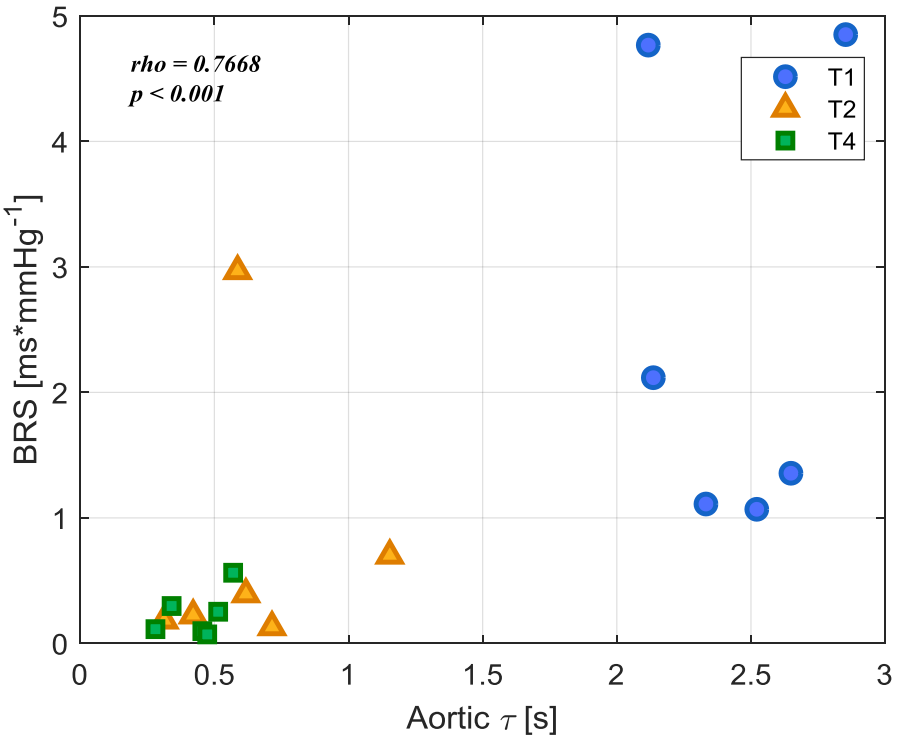

Figure 4.6. Correlation between characteristic time constant τ computed in aorta and baroreflex sensitivity (BRS) throughout the overall experiment. Baseline values (T1) are represented as blue circles, shock values (T2) as orange triangles, and values after full resuscitation with fluids and noradrenaline (T4) are reported as green small squares. In the upper left corner the correlation coefficient and the p-value are reported.

Table 4.2. LF normalized power computed for diastolic blood pressure components at each time point. Values are reported as median (25^{th},75^{th} percentile)

	T1	T2	T4	Delta T2-T1	Delta T4-T1	Delta T4-T2
DAP LF% [%]						
Aortic	7.9 (6.5,10.4)	2.2 (0.5,4) *	1.3 (1,2.9)	-5.5 (-8.9,-3.8)	-5 (-9.5,-3.5)	0.4 (-0.6,0.9)
Radial	9.3 (6.4,10.9)	2.3 (0.8,3.6) *	1.6 (1.3,3.8)	-7.6 (-7.9,-3.9)	-5.5 (-9,-3.4)	0.6 (-1.2,1.3)
Femoral	8.9 (8.2,11.9)	1.6 (0.7,3.9) *	1.7 (1.1,2.8)	-7.3 (-10.6,-5.1)	-6.2 (-10.7,-5.4)	0.6 (-0.2,1.5)

DAP=diastolic arterial pressures, LF%=normalized low frequency power. T1=baseline, T2=after development of septic shock, T4=after resuscitation with fluids and vasopressors. Wilcoxon signed test: *p<0.05 with respect to T1 (Friedman test p-value<0.05).

Table 4.3. Baroreflex feedback and feedforward gains and HRV indices computed at each time point. Values are reported as median (25^{th},75^{th} percentile)

	T1	T2	T4	Delta T2-T1	Delta T4-T1	Delta T4-T2
BRS						
FB [ms/mmHg]	1.7 (1.1,4.8)	0.3 (0.2,0.7)	0.2 (0.1,0.3) *	-1.4 (-4.5,-0.7)	-1.6 (-4.5,-1)	-0.2 (-0.4,0.1)
FF [mmHg/ms]	0.02 (0.02,0.08)	0.1 (0.04,0.3)	0.2 (0.19,0.21)	0.05 (0,0.3)	0.1 (0.08,0.2)	0.06 (-0.1,0.2)
HRV						
RMSSD [ms]	70.2 (49.9,86.6)	31.3 (25.9,56.8)	25.6 (22.5,29.4) *	-33.7 (-54,-15.9)	-45.1 (-60,-20.5)	-6.8 (-27.4,-3.6)
SDSD [ms]	3.7 (2.6,4.5)	1.6 (1.4,3)	1.3 (1.2,1.5) *	-1.8 (-2.8,-0.8)	-2.4 (-3.1,-1)	-0.4 (-1.4,-0.2)
SD [ms]	4.9 (3.5,7.6)	1.9 (1.6,4)	1.4 (1.2,1.8) *	-2.7 (-5.8,-0.5)	-3.5 (-6.2,-2.1)	-0.6 (-2.2,-0.4)

BRS=baroreflex sensitivity, FB=feedback gain, FF=feedforward gain, HRV=heart rate variability, RMSSD=root mean square of successive differences, SDSD=standard deviation of successive differences, SD=standard deviation. T1=baseline, T2=after development of septic shock, T4=after resuscitation with fluids and vasopressors. Wilcoxon signed test: *p<0.05 with respect to T1 (Friedman test p-value<0.05).

4.4 DISCUSSION

In this study a characterization of the arterial tree changes in mechanical properties, both at central and peripheral arterial sites, was investigated in a population of six pigs undergoing a fecal peritonitis-induced septic shock, including full resuscitation with fluids and noradrenaline. The interaction between the ANS dysfunction and these vascular changes were also assessed.

Our study confirms that septic shock induces a decoupling of the arterial pulse wave propagation from central to peripheral sites, without resolution after full resuscitation with fluids and noradrenaline. The ANS related indices, i.e. indices of BRS, BP spectral analysis and HRV, as previously described, showed that septic shock produces signs of autonomic dysfunction and these are associated to changes in vascular properties both at central and peripheral arterial sites.

4.4.1 Vascular changes during shock and following resuscitation

An inversion of the physiological PP amplitude propagation from central to peripheral arteries was observed after the development of septic shock, as already reported in a recent study of endotoxin shock [196]. Of interest, in this study we also observed a persistent vascular decoupling condition hours after a full resuscitation with fluids and noradrenaline (Table 4.1 and Figure 4.3). This finding, together with the trend of AC, TPR and the Windkessel time constants, hints an altered behaviour of the vascular compartments induced by septic shock.

The origin of this PP reversal could be related to the different tissue composition of central and peripheral arterial walls and, thus, to a different response to inflammatory vasoactive substances, such as nitric oxide (NO).

In large compliant arteries, local endogenous NO generation has been demonstrated to contribute to the regulation of large-artery stiffness at basal condition [214], and it was observed that acute systemic inflammation impairs the ability of the arterial endothelium to produce endogenous vasodilators in response to agonist and physical stimuli [215]. Reduced endogenous NO availability contributes to endothelial

dysfunction during sepsis and acute inflammation, and it may lead to a functional stiffening of the large arteries [216].

In peripheral resistance vessels, NO is recognized to be crucial in the modulation of vasomotor tone, and a growing body of evidence indicates that NO plays an important role in the hyporesponsiveness of resistance vessels to vasoactive agents [217]. The cellular source of NO generation within the microvasculature during sepsis is not completely clear, but there is some evidence to indicate that vascular smooth muscle, mostly present in peripheral vessels, may be the source [217]. In septic shock patients plasma concentrations of NO metabolites are markedly increased, and it was found that the raised production of NO is the result of the increased expression of the inducible form of nitric oxide synthase (iNOS), probably stimulated by the inflammatory cytokines [30]. This induces a persistent vasodilation despite high plasma concentrations of catecholamines [218].

The differences in structural components between large complaint and peripheral resistance vessels could partly explain the different endothelial response to inflammation: large-artery stiffening is caused by a reduction in NO bioavailability following inflammation and sepsis, and, conversely, the persistent peripheral vasodilation is caused by the large increase in NO at peripheral level occurring in sepsis and shock.

Although NO bioavailability was not measured in our experiment, in a previous work [219] we observed a decrease of the global arginine bioavailability, i.e. the ratio arginine/(ornithine+citrulline), in the same septic shock animals. As arginine is the precursor for the biosynthesis of NO, our data are consistent with the hypothesis of an increase in NO production supporting the previously outlined mechanisms.

Another important factor that can contribute to PP reversal and peripheral vascular decoupling observed in these animals may be a modification in the reflection of pressure waves. In [199] the authors show that acute systemic inflammation in healthy individuals increases arterial stiffness, assessed by PWV, and decreases wave reflection, estimated by means of the augmentation index on central aortic pressure waveform. The authors explained this phenomenon by the predominant

mechanism of peripheral vasodilation. In our study the estimated TPR and AC decrease in agreement with this work.

Finally, the increased capillary permeability secondary to endothelial dysfunction, may also contribute to the general vascular derangements observed in these animals. High capillary leakage may lead to changes in extracellular matrix composition and this, in turn, could have and influence on the contraction and relaxation properties of the vessels.

The vascular dysfunction induced by septic shock could also help in explaining the increase in FF gain, which measures the direct influence of heart interval duration on SAP, not mediated by autonomic control, but instead by a perturbation mechanism based on the Starling law and diastolic runoff (Table 4.3). The decreased arterial compliance inevitably limits the Windkessel effect of the large arteries [208], and, as a consequence, the blood flow could arrive at peripheral districts with a higher pulsatility; this, combined with the vasoplegic state typical of septic shock, could result in amplified oscillations in ABP induced by an elevated HR.

4.4.2 Autonomic dysfunction and association with vascular changes

The autonomic indices of cardiovascular regulation such as BRS, HRV and LF normalized power of DAP, all showed a depressed value in shock which persisted, or even worsened, after full resuscitation (Table 4.2 and Table 4.3). Thus, a condition of autonomic dysfunction, not resolved by the resuscitation maneuvers, is clearly depicted in these animals.

The reduced baroreflex FB gain and the depressed HRV, together with the elevated HR, may be interpreted as due to a depressed vagal activity at the heart level. HRV is assumed to be mostly dependent on the parasympathetic outflow to the heart, and an increase in baroreflex gain in a recovery phase has also been associated with an increased vagal activity [180]. These observations suggest a vagal driver for an effective resuscitation, and this hypothesis is in line with the recent literature on vagus nerve stimulation as an innovative therapeutic strategy, which has

been proven to be beneficial in several diseases, including septic shock [58]–[60], [63], [64], [66] (see also §1.4.4). However, we cannot rule out that the observed reduction in cardiac BRS was also due to a reduced sensitivity of the aortic baroreceptors caused by the increased stiffness of the vessel [220]. Moreover, also the elevated HR could be responsible of the reduced BRS, namely a very high HR prevented any change in HR oscillations in response to changes in arterial pressure values.

The depressed LF% power of DAP hints a dysfunction of the sympathetic nervous system also in the periphery. Changes in LF oscillations of arterial pressure can be related to changes in the outflow of the sympathetic nervous system, and spectral analysis of arterial pressure has been proven to be a powerful tool for identification of the different cardiovascular control mechanisms that regulate arterial pressure [77], [164]. From this perspective, the reduction of LF power can be interpreted as a withdrawal or a saturation of sympathetic activity, or an inability of adrenergic receptors to respond to further stimuli.

There are many recent indirect and direct evidences which suggest a very complex link between sympathetic activity and vascular function. Evidence from experimental studies indicates that the sympathetic ANS is influenced, both at central and peripheral level, by the most relevant factors regulating vascular function [221]. For example, neuronal NOS (nNOS) is constitutively expressed in neuronal cells of both peripheral and central nervous system, and in this latter it acts as a sympathoinhibitory substance [222]. Moreover, a relationship between arterial stiffness and sympathetic nerve traffic was also demonstrated, with the majority of studies reporting a positive correlation between sympathetic withdrawal and increased large artery elasticity [204], [205], [207], [221], [223]. In our study we can also confirm this association, as our analyses highlighted a significant positive correlation between a decrease in arterial compliance, i.e. increase in large artery stiffness, and a depressed vagal activity, as shown by the suppressed BRS, HRV and LF% index.

The relationship between autonomic and vascular dysfunction could be causal in both directions, that is autonomic impairment generates dysfunction in vascular tone or vice versa, or noncausal, that is

the two conditions may develop in parallel as inflammatory responses. Sympatho-excitatory maneuvers were found to impair endothelial function and to increase arterial stiffness, and markers of vascular dysfunction were shown to be inversely related to sympathetic discharge [221]. On the other hand, large artery stiffness can interfere with autonomic regulation by impairing arterial baroreceptors, as already discussed above. All these observations support a causality mechanism.

Another causal mechanism could be the increase in HR, as a sympathetic-mediated increase in HR per se results in stiffening of the central arteries [204]. Finally, arterial elasticity could modify autonomic activity through a change in cardiac afterload. An increase in arterial stiffness leads to an increased afterload, such that the heart needs a greater effort to eject the same SV, with a consequent sympathetic overstimulation to increase HR and ventricular contractility; if prolonged, this condition could lead to heart failure, considering also that an increased HR results in a shorter time for diastole. Moreover, an increased stiffness lead to an increase in PWV along the arterial tree with the consequence that the reflected waves arrive at the heart earlier in systole further increasing the cardiac afterload. This phenomenon could partly explain the persistent tachycardia and the elevated contractility observed in these animal population [224], and generally reported in septic shock patients [74], [175], [177], [178].

In conclusion, although this study did not demonstrate a direct causal relationship between vascular dysfunction and ANS activity, our observations clearly proved that both the vascular and the autonomic system are impaired in septic shock and they mutually influence each other. This compromised condition is not resolved by standard therapy, i.e. fluids and noradrenaline administration, and it can be macroscopically appreciated from the changes in morphology of blood pressure waveforms. Few cardiac beats of aortic pressure waveform are shown in Figure 4.7 for all the animals at each time point of the experiment. Interestingly, the shape of the aortic waveform mostly at T4 resembles the typical pressure waveform of a high cardiovascular risk subject, such as aged people as reported in [225], although these changes were developed only in a few hours of experiment.

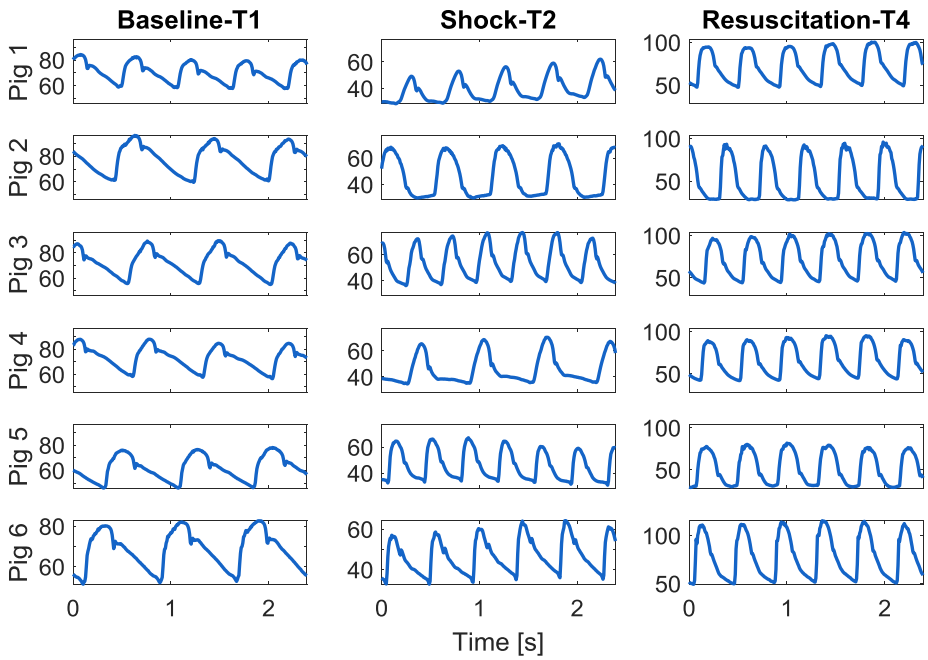

Figure 4.7. Example of few beats extracted from aortic waveform at each time point of the experiment for each animal.

4.4.3 Limitations of the study and future developments

The main limitation of this study consists in the small sample size, which may be the source of a high variability in the results so to affect the statistical tests. However, the results showed evident trends, that can still be considered important, even if not statistically significant.

Moreover, direct measures of peripheral arteries compliance and autonomic outflow were not available and this did not permit other than speculation about possible associations. Also, the lack of an invasive local flow measurement at some of the arterial pressure measurement sites impeded us to compute the local characteristic impedance, but it was the price to pay in order to keep a closed chest, closed pericardium model, with intact cardiopulmonary and interventricular interactions and less tissue damages.

Finally, it could be worthy to integrate these analyses with molecular mechanism involved by shock and resuscitation, since vasoactive agents released during sepsis, such as NO, have a directly

influence on the hemodynamic response to shock, and the responsible mechanisms still remain to be elucidated.

4.5 CONCLUSION AND REMARKS

The present study proposed an insightful analysis of the changes of vascular properties occurring during a protocol of polymicrobial septic shock and full resuscitation, and suggested a possible relationship with autonomic dysfunction typical of septic shock. In fact, a significant positive correlation between an increase in arterial stiffness, as shown by AC values, and a depressed vagal activity, as shown by BRS and HRV values, has been reported together with a decreased TPR and time constant τ and an impaired sympathetic activity at the peripheral sites. Interestingly, this abnormal condition generated by septic shock was not resolved after administration of fluids and noradrenaline. Although global hemodynamic markers were restored by the resuscitation maneuvers, such as MAP, HR, CO and SV, as shown in Table 4.1, we observed that indices of cardiovascular functions mediated by the ANS were still impaired, suggesting that the homeostatic condition of baseline was not completely recovered, as already reported in our previous studies [190], [224], [226], and in the previous chapters (chapter 2 and 3).

The proposed indices of autonomic functionality could represent a useful end-point to guide the resuscitation strategy, in combination with standard hemodynamic and clinical markers as prescribed by the current guidelines for septic shock management [33]. Moreover, our study suggests that measures of vascular properties should be taken into account in further studies in order to have a more comprehensive picture of the effectiveness of the therapy. The computation of time constant τ is a powerful approach to investigate the vascular behaviour in terms of total compliance and resistance, and it could be easily computed by the recorded pressure waveform.

Moreover, the present results highlight the importance of considering the heart and the circulation as coupled systems and they should be evaluated jointly and not as independent systems. We want to underline that this should be taken into account in the clinical guidelines

in order to optimize therapies for a better management of shock patients. For example, increased vascular stiffness caused by septic shock can directly affect the heart workload and clinicians should consider this when administering vasopressors; a prolonged and over-needed vasopressors support, while having a beneficial effect on arterial resistance and mean pressure, it could have a global detrimental effect on the heart performances, further increasing the cardiac afterload [197].

Finally, a more effective vasopressor therapy should take into account the different characteristics between central and peripheral arteries and further studies should be performed to understand the right balance in order to restore not only mean arterial pressure but also a physiological condition of cardiovascular interactions arterial pressure control.

5 BLOOD PRESSURE VARIABILITY AND HEART FUNCTIONALITY IN A PROTOCOL OF SEVERE HEMORRHAGIC SHOCK AND RESUSCITATION

In this study we investigated the hemodynamic changes and the autonomic mechanisms elicited by shock in an animal model of severe hemorrhagic shock and resuscitation. This work was partly presented at the 41st Annual Conference on Shock[1] and it was published as journal paper on Journal of Applied Physiology[2].

It is known that the SNS is crucial during bleeding to activate all the compensatory mechanisms in order to prevent collapse, and there is evidence that this response is lost at the point of hemodynamic decompensation, leading to irreversible shock.

Our purpose was to study the response of the ANS to hemorrhagic shock and standard resuscitation, testing the hypothesis that autonomic indices may be helpful in understanding the effectiveness of the resuscitation maneuvers. Moreover, we were interested in verifying if there are common hemodynamic patterns in septic and hemorrhagic shock, and, for this purpose, we applied similar analyses and we studied the trend of related autonomic indices.

The animals were studied at different time points: at baseline, after the massive blood withdrawal, after different resuscitation maneuvers were administered (i.e. after fluids and vasopressors infusion and after the shed blood infusion).

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[1] M. CARRARA, G. Babini, G. Baselli, G. Ristagno, M. Ferrario, "*The role of autonomic nervous sytem control of blood pressure during a protocol of severe hemorrhagic shock and resuscitation".* SHOCK, Vol. 49, No. 6S, pp. 87-88, 2018

[2] M. CARRARA, G. Babini, G. Baselli, G. Ristagno, R. Pastorelli, L. Brunelli, M. Ferrario, "*Blood pressure variability, heart functionality, and left ventricular tissue alterations in a protocol of severe hemorrhagic shock and resuscitation".* J Appl Physiol 125: 1011-1020, 2018

Our results highlight that after the first resuscitation mean BP reached the target value, but cardiovascular indexes were not fully restored, hinting at a partial recovery of the autonomic mechanisms. Only after shed blood was reinfused, all the indices returned to the baseline level.

Moreover, in this study we assessed the effects of severe hemorrhage on heart functionality by integrating the hemodynamic findings with measures of plasma high-sensitivity cardiac troponin T and metabolite concentrations in left ventricular tissue. The left ventricular metabolic profile and the values of cardiac troponin T confirmed the acute stress condition sensed by the cardiomyocytes.

Details about rationale, methodology, results and conclusions of the study are described in the following paragraphs. They are based on the related journal paper. A final remark on the main findings is reported at the end of the chapter.

The description of the metabolomics analyses and results are reported in Appendix A.

5.1 INTRODUCTION

Hemorrhage and unresolved hemorrhagic shock still represent the leading cause of mortality after trauma in both civilian and military settings, with the majority of deaths occurring because of the inability to control bleeding and to effectively resuscitate hemorrhage patients [35], [36].

Therefore, much effort has been spent on investigation of the fundamental underlying pathophysiology of hemorrhagic shock in order to develop innovative approaches or to discover new biological parameters capable of detecting the severity of blood loss, controlling bleeding, and guiding resuscitation.

During progressive hemorrhage, physiological compensatory mechanisms are usually elicited in trying to maintain homeostasis. Indeed, the cardiovascular system activates physiological responses with the aim of maintaining cerebral oxygenation and blood supply to central organs; for example, neuroendocrine-mediated modifications of

peripheral vascular resistance cause a redistribution of fluids that leads to non-uniform regional blood loss. Other compensatory mechanisms consist of an increase in HR and myocardial contractility to increase CO [105].

The role of the SNS has been demonstrated to be crucial during bleeding to prevent collapse through reflex tachycardia and peripheral vasoconstriction [112]–[114], [124], [227], [228] (see §1.5.2 for details). Schiller et al. [105] described a dynamic coupling between ABP and sympathetic outflow oscillations during progressive central hypovolemia. A decrease in ABP quickly initiates an increase in traffic of sympathetic nerve impulses by decreasing inhibitory afferent activity to the NST; the subsequent arterial vasoconstriction results in increased vascular tone and compensatory elevation in ABP, activating a baroreflex-mediated feedback reduction in sympathetic outflow. This baroreflex-mediated phenomenon of oscillatory coupling between BP and sympathetic activity represents an important compensatory mechanism during hypovolemia, and there is evidence that it may be lost at the point of hemodynamic decompensation [112]. These dynamics underscore the physiological importance of measuring oscillations of BP rather than relying on an average trend. Analysis of BPV could help in understanding the extent of ANS activation in response to volume depletion, the severity of hemorrhage, whether the patient is approaching shock, and, at the same time, whether he is likely to recover, i.e., when the resuscitation is effective.

Standards of resuscitation for prehospital hemorrhagic trauma patients include administration of fluids to stabilize BP and vascular volume before blood transfusion and surgical repair. However, after the replacement of blood loss the consequent restoration of CO and ABP does not accurately reflect the effectiveness of treatment [229], [230]. In fact, overzealous resuscitation with crystalloids dilutes oxygen-carrying capacity and clotting factor concentrations, thus exacerbating coagulopathy, inflammation, and hypoxia [9], [126].

For this reason, resuscitation science has tried to identify other surrogates able to assess cardiocirculatory status and tissue perfusion so

as to better depict shock severity and response to treatment (e.g., lactate, SvO_2, base deficit, and oxygen debt) [39], [231].

In this study, we analyzed the cardiovascular signals recorded during a protocol of severe hemorrhage and resuscitation with the aim of characterizing the compensatory response to hypovolemia and fluid repletion in terms of autonomic-mediated changes in BPV and HRV.

5.2 METHODS

5.2.1 Study design and experimental procedure

Nine male pigs (36 ± 2 kg) received anesthesia by intramuscular injection of ketamine (20 mg/kg), completed by ear vein injection of propofol (2 mg/kg) and sufentanil (0.3 μg/kg) and then maintained over the whole experiment with continuous intravenous administration of propofol (1–3 $mg \cdot kg^{-1} \cdot h^{-1}$), midazolam (2–4 $mg \cdot kg^{-1} \cdot h^{-1}$), and sufentanil (0.3 $\mu g \cdot kg^{-1} \cdot h^{-1}$). Animals were mechanically ventilated with a tidal volume of 10 ml/kg^{-1} and Fi_{O2} of 0.21 during baseline and 0.3 during and after the shock phase; respiratory frequency was adjusted to maintain the end-tidal P_{CO2} values in the range 35–40 mmHg. A 7-Fr catheter was placed in the descending aorta from the left femoral artery for blood sample collection. Another Millar Mikro- Tip pressure catheter was inserted in the right femoral artery for continuous monitoring of ABP. Continuous acquisition of CVP in the right atrium, pulmonary arterial pressure, and CO through thermodilution technique was obtained by means of an endovascular pentalumen 7-Fr catheter placed in the pulmonary artery from the right femoral vein. Continuous monitoring of LVP and left ventricular volume was achieved by means of a Millar Mikro-Tip pressure catheter inserted in the left ventricular cavity from the right carotid artery. Finally, a 14-Fr catheter was placed in the abdominal artery from the left femoral artery for blood withdrawal during the induction of shock, and a 14-Fr catheter was inserted in the left femoral vein for the successive blood reinfusion. The study was reviewed and approved by the Institutional Review Board and the governmental institution (Italian Ministry of Health).

After baseline measurements, animals were randomized to one of two study groups: 1) hemorrhagic shock and resuscitation (n = 6) and 2) sham-treated control (n = 3). Bleeding was induced by withdrawal of blood from the left femoral artery with a peristaltic pump at a rate of 20 ml/min over an interval of 60 min, until MAP reached values of 40 ± 5 mmHg. The hypoxic state with the consequent metabolic alteration was confirmed by serial blood lactate measurements. After 2 h of the shock condition, animals were resuscitated with a two-step procedure. Initially, fluid (normal saline) and vasopressor (norepinephrine) were administered to restore MAP of at least 60 mmHg and a PPV < 12%. Then, shed blood was reinfused with the aid of a peristaltic pump over an interval of 30 min. After 1 h of observation, animals were euthanized by intravenous injection of Tanax (1 ml/10 kg) and heart biopsies from the left ventricle were taken for histological, biochemical, and omics analyses.

For sham-treated animals the preparation was identical, but they did not undergo bleeding, fluid resuscitation, or blood reinfusion. One of six hemorrhagic shock animals was excluded because of elevated BP at baseline (MAP of 130 mmHg).

Animals were studied at four relevant time points: at baseline (T1), after the development of hemorrhagic shock (T2), after fluid and vasopressor resuscitation (T3), and after blood reinfusion (T4). The overall timeline of the experiment is also reported in Figure 5.1 for the sake of clarity.

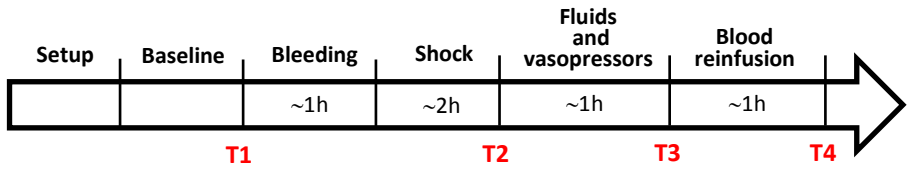

Figure 5.1. Timeline of the experimental protocol. Each phase, and its relative duration, is marked. Time points are highlighted in red.

At each time point a bolus of phenylephrine (3 µg/kg) and a bolus of epinephrine (10 µg) were intravenously administered to elicit the response of the ANS. Arterial and venous blood samples were collected

for blood gas analysis and laboratory analyses (i-STAT System; Abbott Laboratories, Princeton, NJ). Plasma high sensitive cardiac troponin T (hs-cTnT) was measured in a central laboratory by electrochemiluminescence immunoassay using commercial reagents (Elecsys 2010; Roche Diagnostics).

5.2.2 Clinical data

Clinical variables collected at each time point were the following: CO (l/min), temperature (°C), urine output (ml), arterial pH, lactate (mmol/l), $PaCO_2$ (mmHg), PaO_2 (mmHg), base excess (mEq/l), hs-cTnT (ng/ml), oxygen saturation (%), hematocrit (%), and left ventricular EF (%).

5.2.3 Hemodynamic analyses

Signal processing

ABP, ECG, LVP, and right atrial pressure (RAP) were continuously recorded during the experiment. At each time point stationary segments of 7-minute length on average were selected. RR intervals (RRI) and HR time series were extracted from the ECG waveform by means of the ECG Analysis Module available for LabChart software; time series of SAP, DAP and MAP were obtained from the ABP waveform with specific algorithms [152], [153]. Beat-to-beat RAP time series consist of the mean values of RAP within each cardiac cycle; dP/dtmax was derived on a beat-to-beat basis from the LVP recording, and it was taken as an indirect measure of heart contractility. Temporal relationships were maintained among the time series: given R(i) as the R peak of the current beat, RRI(i) designated the difference between R(i + 1) and R(i), SAP(i) follows R(i) and is followed by DAP(i), $dP/dt_{max}(i)$ is the slope of the upstroke right after R(i), and RAP(i) is the averaged RAP values within RRI(i). Finally, an adaptive filter was applied to the data to remove outliers and irregularities [154]. Each segment was subdivided into 3-minute 50% overlapping windows. Each time series was resampled at 2 Hz by means of zero-order hold techniques, and then it was detrended with a high-order polynomial function to guarantee stationarity, further verified with the Dickey-Fuller and Kwiatkowski-Phillips-Schmidt-Shin statistical tests.

All the indexes obtained for these series were averaged and considered for successive statistical comparisons.

Time and frequency indices

We computed the mean value of SAP, DAP, MAP, RRI, dP/dtmax, and RAP series. Spectral indexes obtained from power spectra included LF power, total power, which represents the total area under the spectrum and is a measure of the overall variability of the series, and LF%, which represents the relative power in the LF band.

Cardiac baroreflex sensitivity analysis

We adopted the bivariate model method [156] which was extensively detailed in §2.2.4. Briefly, the two parameters of interest are the feedback gain, which represents the baroreflex mediated by the ANS, i.e., changes in RR induced by oscillations in SAP and mediated by the ANS, and the feedforward gain, which denotes the mechanical influence of RRI on SAP through the heart and vasculature, also called the runoff effect. As the current RRI(i) cannot immediately affect SAP(i), a one-beat delay was considered for the feedforward relationship. Granger causality from SAP to RRI and vice versa was verified before computation of the gains [185], [232]. The order of the model was optimized based on the Akaike information criterion, ranging from 5 to 15.

Heart rate complexity analysis

We assessed HRV by means of linear and nonlinear methods. In particular, we computed the following indices, as previously reported: RMSSD, SDSD, and SD [81]. Moreover, we calculated the QSE to investigate the nonlinear characteristics of HRV [186]. The template length m was fixed at 1, and the tolerance r was computed as 20% of the SD of the time series, similarly to our previous study (see chapter 3).

Model-based system identification of DAP variability

To better investigate the different mechanisms of peripheral resistance control, we implemented a multi-input single-output causal black-box model for the prediction and spectral decomposition of DAP beat-to-beat fluctuations from variability signals of SAP, RAP, and RRI, as already illustrated in detail in §2.2.4. Assuming that DAP variability is a surrogate measure of peripheral vascular resistance [157], [158], the

current DAP value is assumed to be influenced by arterial and cardiopulmonary baroreflex control and by the mechanical coupling between the heart and the circulatory system, the so-called runoff effect. To disentangle these mechanisms and to highlight their different contributions in DAP variability, we modelled such mechanisms as follows:

$$DAP(i) = \sum_{j=s1}^{n} h_{sap}(j) * SAP(i-j) + \sum_{j=s2}^{n} h_{rap}(j) * RAP(i-j) + h_{rr}(1) * RR(i) + e(i) =$$
$$= DAP_{/sap} + DAP_{/rap} + DAP_{/rr} + DAP_{/noise} \tag{5.1}$$

We limited the effect of the mechanical runoff to a single gain parameter $h_{rr}(1)$, for physiological reasons. Granger causality among the cardiovascular time series was verified before computation of the model [185]. The optimal model order *n* was assessed with the "ARMA Parameter Reduction algorithm," as proposed in [233], starting from a maximal order value n equal to 12 and delays [s1, s2] equal to zero. Model parameters were determined by a least-squares minimization procedure. The black-box input-output relationships in Equation 5.1 can be assumed to be representative of the following mechanisms of DAP variability control:

- SAP→DAP ($DAP_{/sap}$) represents the black-box model for the arterial baroreflex-mediated sympathetic control of vasomotor tone
- RAP→DAP ($DAP_{/rap}$) represents the black-box model of vasomotor tone control related to the cardiopulmonary baroreflex; we assumed that RAP oscillations are representative of pressure oscillations sensed by cardiopulmonary baroreceptros.
- RR→DAP ($DAP_{/rr}$) represents the mechanical effect of diastolic runoff

The residual error ($DAP_{/noise}$) includes all the remaining sources of variability not measured.

To quantify the amount of DAP variability explained by the arterial baroreflex control or the mechanical runoff effect, a spectral decomposition was performed. In particular, we performed the spectral analysis of the model component series ($DAP_{/sap}$, $DAP_{/rap}$, $DAP_{/rr}$) and then

we assessed the ratio between LF power of $DAP_{/sap}$, $DAP_{/rap}$ or $DAP_{/rr}$ components over total LF power of DAP (LF $DAP_{/sap}$/LF DAP, LF $DAP_{/rap}$/LF DAP or LF $DAP_{/rr}$/LF DAP, respectively).

Analysis of response to phenylephrine and epinephrine

Changes in HR with respect to changes in MAP were analyzed during phenylephrine injection to quantify the ANS-mediated response to the stimulus [234]. Phenylephrine acts directly at the vessel level as a selective α1-adrenergic receptor agonist. In physiological conditions, a bolus of phenylephrine is expected to cause a rise in MAP accompanied by a decrease in HR mediated by the baroreflex autonomic mechanism. Changes in dP/dt_{max} during the injection of epinephrine were analyzed to assess the ANS-mediated response of heart contractility.

5.2.4 Statistical analyses

We adopted the Mann-Whitney U-test, also known as the Wilcoxon rank sum test, to verify significant differences in the index values between the two groups (shock and sham-treated) separately at each time point, whereas we used the Friedman test to assess significant changes among time points within the same group of animals. Significance was considered at p-value < 0.05.

5.3 RESULTS

Bleeding caused a significant decrease in MAP, CO, and filling pressures and a concomitant significant increase in HR, heart contractility (dP/dt_{max}) and lactate compared with sham-treated animals (Figure 5.2 and Table 5.1). After resuscitation with fluids and norepinephrine, MAP recovered to the target of 60 mmHg in all animals except one, but HR and heart contractility remained significantly higher than baseline values. Only after resuscitation with shed blood did all the indexes return to baseline values (Figure 5.2).

The high level of lactate in shock pigs confirmed the severity of the hypovolemic condition. However, the preserved EF suggested that the severity of shock was not sufficient to induce a concurrent acute heart failure.

Table 5.2 reports the HRV indexes. Variability of RRI was dramatically reduced at T2 and did not recover at T3, after fluid and vasopressor administration.

Table 5.3 shows that LF power of BP components (SAP, DAP, MAP) decreased during shock (T2) and remained lower with respect to baseline after fluid and vasopressor resuscitation (T3). After blood reinfusion (T4) the values returned similar to baseline and higher than T3. A similar trend was observed for LF power of HR and dP/dt_{max}, even if less marked. Baroreflex feedback gain was reduced at T2 and T3 (significantly at T2) and recovered at T4; the opposite trend was observed for feedforward gain.

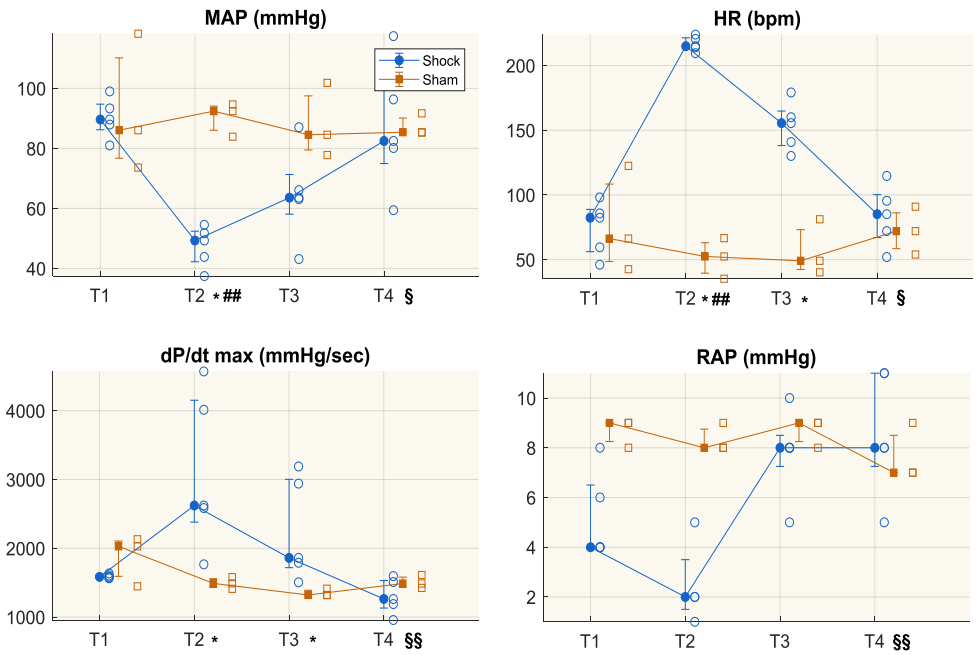

Figure 5.2. Distributions (median, (25^{th},75^{th} percentile)) of the averaged value at each time point of the following variables: mean arterial pressure (MAP), heart rate (HR), maximum of 1^{st} derivative of left ventricular pressure over time (dP/dt_{max}), and right atrial pressure (RAP) for both populations of hemorrhagic shock and sham-treated animals. Open symbols indicate values relating to each shock- or sham-treated pig. T1, baseline; T2, after development of shock; T3, after fluid and vasopressor resuscitation; T4, after blood reinfusion; bpm, beats per minute. *$P < 0.05$ shock- vs. sham-treated animals (Mann-Whitney U-test); $^{\#\#}P < 0.01$ vs. T1; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. T2 (Friedman test, only for shock-treated pigs).

Table 5.1. Clinical and laboratory data for hemorrhagic shock and sham animals at each time point. Values are reported as median (25^{th},75^{th}) percentile

	T1	T2	T3	T4
Hematocrit [%]				
Shock	23 (22.75,29.5)	21 (20,23.75)	10 (10,11) ##	20 (17.75,25.5)
Sham	26 (25.3,27.5)	25 (23.5,28)	26 (23.8,26) *	25 (24.3,25)
CO [L/min]				
Shock	3.6 (2.4,3.8)	1.5 (1.5,1.8)	5 (4,5.5) §§	2.6 (2.1,3.9)
Sham	3.5 (3,4.5)	3.3 (3.2,3.4)*	3.2 (3.1,3.6)	3.6 (3.5,3.8)
EF [%]				
Shock	59.9 (57.8,66.6)	72.5 (68.3,76.9)	64 (62.1,68.9)	62 (52.5,69.4)
Sham	60.5 (56.5,60.9)	60.9 (60.2,66.9)	64.4 (57.8,68.2)	57.1 (54.2,61.4)
Lactate [mmol/L]				
Shock	1.8 (1.5,2.2)	8.5 (7.9,8.8) #	5.9 (5.1,5.9) n=4	2.1 (1.6,2.3) §
Sham	1.2 (0.9,2.5)	0.9 (0.8,1.7) *	0.8 (0.6,1.3)	0.8 (0.7,1.1) *
hs-cTnT [ng/L]				
Shock	13.9 (9.4,33.1)	145.4 (124.3,589.6) ##	105.2 (86.6,508.5) #	61.9 (28.9,89.8)
Sham	3 (3,12.5)	4.9 (3.5,14) *	3.9 (3.2,40.4) *	14.7 (9.6,15.1) *
Urine output [mL]				
Shock	130 (107.5, 205) n=3	7.5 (0,20) n=4	260 (170,285) n=4	220 (125,342)
Sham	180 (97.5,270)	60 (85.5,97.5)	70 (47.5,85)	60 (60,75)
Temperature [°C]				
Shock	37.9 (37.6,38.2)	38.3 (38,39)	36.5 (35.8,36.9)	36.1 (35.5,36.7) §§
Sham	37.5 (36.8,37.7)	37 (36.3,37.4)	37.2 (36.3,37.4)	37.3 (36.3,37.5)
pH				
Shock	7.5 (7.49,7.51)	7.35 (7.3,7.39)	7.35 (7.23,7.37) ##	7.34 (7.33,7.43)
Sham	7.5 (7.45,7.5)	7.5 (7.49,7.5) *	7.51 (7.47,7.51) *	7.47 (7.43,7.52) n=2
$PaCO_2$ [mmHg]				
Shock	36.2 (34.4,39.4)	39.7 (35.8,40.6)	39.3 (38.1,46.5)	47.2 (41.6,48.7) #
Sham	40.6 (37.5,40.9)	39.6 (39.6,42,2)	41.4 (39.6,41.7)	41.7 (37.8,45.6) n=2
PaO_2 [mmHg]				
Shock	97 (88.8,126.3)	146 (142.8,157.5)	164 (148.5,166) #	131 (109.8,155.5)
Sham	89 (80.8,95)	146 (119,153.5)	133 (112.8,151)	124.5 (110,139)

CO=cardiac output, hs-cTnT=plasma high sensitive cardiac troponin T, $PaCO_2$=partial CO_2 pressure, PaO_2=partial O_2 pressure. T1 = baseline, T2 = after development of shock, T3. = after fluids and vasopressors resuscitation, T4 = after blood reinfusion.
Comparisons between shock and sham: *p-value<0.05 (Mann-Whitney U-test)
Comparisons between time points: #p-value<0.05 ##p-value<0.01 with respect to T1, §p-value<0.05 §§p-value<0.01 with respect to T2 (Friedman test)

Table 5.2. HRV and HR complexity indices for hemorrhagic shock pigs evaluated at each time point. Values ad reported as median (25^{th},75^{th}) percentile

	T1	T2	T3	T4
RMSSD	62.4 (37.8,302.1)	9 (6.4,16.6) #	20.7 (16.8,22.5)	58.3 (34.5,145.1) §
SDSD	3.3 (2,15.8)	0.5 (0.3,0.8) #	1.1 (0.9,1.2)	2.3 (1.8,7.6) §
SD	17.9 (5.4,34.4)	1.4 (1.1,2.7) #	2.8 (2.6,4.5)	14.1 (6.2,15.6) §
QSE	2.4 (1.9,3.5)	-0.1 (-0.4,0.8) #	0.9 (0.4,1.1)	2.3 (1.8,3.1) §

RMSSD=root mean square of successive difference [ms], SDSD=standard deviation of successive difference [ms], SD=standard deviation [ms], QSE=quadratic sample entropy, T1=baseline, T2=after development of shock, T3=after fluids and vasopressors resuscitation, T4=after blood reinfusion.
Comparisons between time points: #p-value<0.05 with respect to T1, §p-value<0.05 with respect to T2 (Friedman test)

Table 5.3. Frequency indices and baroreflex gains for hemorrhagic shock pigs evaluated at each time point. Values are reported as median (25^{th},75^{th}) percentile

	T1	T2	T3	T4
SAP				
LF power [a.u.]	105.3 (39.9,165.3)	18 (12.2,29.6)	12.5 (6.3,35.8) #	78.9 (48.8,102.1)
LF% power [%]	22.2 (7.2,39.4)	4.8 (2.4,6.2)	4.1 (3.3,12.8)	18 (14.5,21.7)
DAP				
LF power [a.u.]	155.2 (93,234.7)	37.9 (24.7,46.7) #	39.9 (33.5,71.3)	196.3 (110.6,248.3) §
LF% power [%]	38.1 (20.5,48.4)	11.4 (5.6,11.7)	11.6 (7.1,20.8)	53.6 (32.6,60.7) §
MAP				
LF power [a.u.]	80.3 (63.7,123.5)	25.2 (18.1,41.2)	24.8 (9.6,47.6)	119.1 (99.1,174.8) §°
LF% power [%]	16.6 (13.5,29.8)	8.7 (4.2,10.4)	7.4 (5.9,13.1)	29.3 (26.4,43.3) §
RRI				
LF power [a.u.]	171.5 (161.7,233)	97.3 (73.2,130.5)	125.8 (75,142)	116.3 (104.6,172.7)
LF% power [%]	42.8 (33.4,57.6)	32.2 (24.9,42.6)	31.1 (21.6,36.6)	30 (25.2,41.2)
dP/dt max				
LF power [a.u.]	147.8 (123.7,214.6)	75 (50.1,118.7)	104 (45.6,133.8)	152.1 (111.3,202.1)
LF% power [%]	31.6 (25.8,44.9)	21.3 (14.1,29.7)	22.7 (7.1,40.9)	30.1 (22.6,56.7)
BRS				
FB [ms/mmHg]	3.2 (1.1,11.4)	0.08 (0.06,0.2) ##	0.2 (0.1,0.6)	1.1 (0.8,5.7) §
FF [mmHg/ms]	0.05 (0.03,0.1)	0.3 (0.2,1.6)	0.4 (0.2,0.5)	0.03 (0.02,0.1) §

SAP=systolic arterial pressure, DAP=diastolic arterial pressure, MAP=mean arterial pressure, RRI=RR-intervals, BRS=baroreflex sensitivity, FB=feedback gain, FF=feedforward gain, T1=baseline, T2=after development of shock, T3=after fluids and vasopressor resuscitation, T4=after blood reinfusion. Comparisons between time points: #p-value<0.05 ##p-value<0.01 with respect to T1, §p-value<0.05 with respect to T2, °p-value<0.05 with respect to T3 (Friedman test)

The ANS response to the administration of phenylephrine in shock pigs was quantified by considering the amplitude of the change in MAP and HR during the administration of the bolus. Similarly, the response to epinephrine was analyzed by considering the magnitude of the increase in dP/dt_{max} during bolus injection. Both MAP and HR variations in response to phenylephrine and dP/dt_{max} variation in response to epinephrine were greatly reduced with respect to baseline at the end of the shock period (Table 5.4).

Table 5.4. Delta values (i.e. difference between the final value and the starting value) of MAP, HR and dP/dt max during the administration of phenylephrine or adrenaline, respectively, for shock animals at each time point. Values ad reported as median (25^{th},75^{th}) percentile

	T1	T2	T3	T4
		Responses to phenylephrine administration		
ΔMAP	33.5 (27.7,41.7)	10.6 (6.1,13) [#]	27.5 (16,31.5)	27 (19.4,29.5)
ΔHR	-13.4 (-27,-9.1)	0.08 (-5.4,1.9) [#]	-5.1 (-10.5,-0.6)	-10.1 (-12.5,-8.2)
		Responses to adrenaline administration		
ΔdP/dt max	3401.2 (2678.2,3552.7)	1602.7 (946.1,1986.5)[#]	2188.6 (1983.2,3082.3)	2349.2 (1826.8,2515.6)

MAP=mean arterial pressure, HR=heart rate, T1=baseline, T2=after development of shock, T3=after fluids and vasopressors resuscitation, T4=after blood reinfusion. Comparisons between time points: [#]p-value<0.05 with respect to T1, [§]p-value<0.05 with respect to T2 (Friedman test)

As regards the DAP model, the portion of DAP variability mediated by the arterial baroreflex mechanism (LF $DAP_{/sap}$/LF DAP) was predominant with respect to the other mechanisms in each phase of the experiment (Figure 5.3). A significant increase in LF $DAP_{/sap}$/LF DAP was observed in shock with respect to baseline. Furthermore, the mechanical influence of the heart on the circulatory system (LF $DAP_{/rr}$/LF DAP) and the portion of DAP variability regulated by the cardiopulmonary baroreflex (LF $DAP_{/rap}$ /LF DAP) presented a u-shaped trend: they decreased during the shock period, remained lower than baseline after resuscitation, and recovered to baseline values after blood reinfusion.

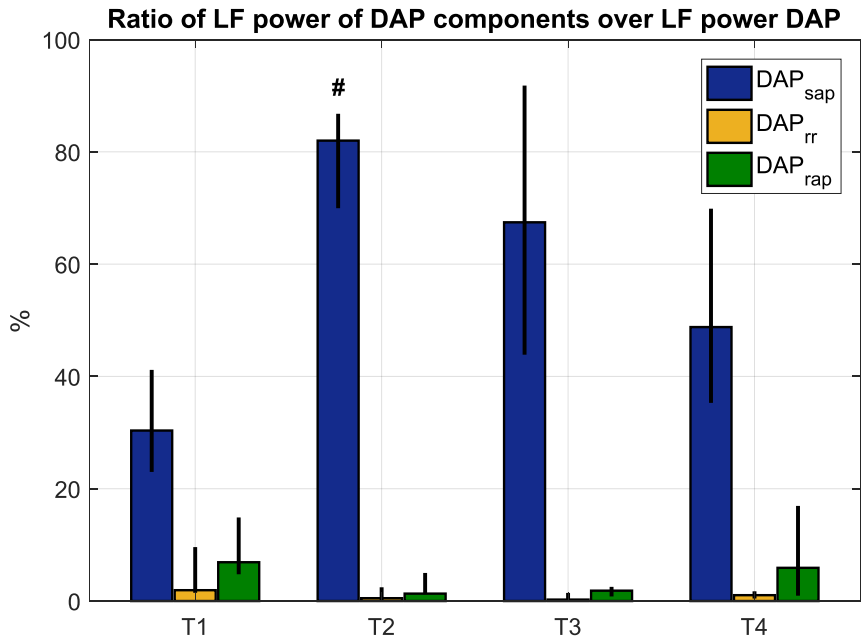

Figure 5.3. Ratio between low-frequency (LF) absolute power of each predicted component and LF absolute power of diastolic arterial pressure (DAP) at each time point for hemorrhagic shock animals. Column height is median value for the population; black bars indicate values of 25th and 75th percentiles. T1, baseline; T2, after development of shock; T3, after fluid and vasopressor resuscitation; T4, after blood reinfusion; SAP, systolic arterial pressure; RR, R-R interval. $^{\#}P < 0.05$ vs. T1 (Friedman test).

5.4 DISCUSSIONS

5.4.1 Cardiovascular indices and models

The trends of vital signs and lactate confirmed the hypovolemic shock associated with a hyperdynamic cardiovascular response (Figure 5.2 and Table 5.1).

Changes in LF oscillations of BP can be related to changes in the outflow of the SNS, and spectral analysis of BP has been proven to be a powerful tool for identification of the different cardiovascular control mechanisms that regulate BP [77], [164]. From this perspective, the reduction of LF power we observed during shock (Table 5.3) can be interpreted as a withdrawal or a saturation of sympathetic activity. Convertino et al. [235] and Cooke et al. [111] measured MSNA during increasing negative pressure in a LBNP protocol, supporting the hypothesis that sympathetic withdrawal may represent a fundamental mechanism for the development of circulatory shock. Our results are thus in line with this hypothesis, i.e., acute bleeding and hemorrhagic shock may cause a cardiovascular collapse characterized by a depressed peripheral sympathetic outflow.

Many hypotheses have been formulated in the past years, trying to explain this phenomenon. Some literature supports the concept that an "empty heart" might contribute to cardiac receptor stimulation, resulting in the activation of cardiac vagal afferents and subsequent sympathetic depression [105], [109], [122]. Koyama et al. [236] proposed that prolonged brain ischemia could be the triggering cause of sympathetic outflow depression documented in hypovolemic shock. Another possible mechanism that may contribute to the loss of sympathetic peripheral outflow is the resetting of baroreflexes, which leads to a loss of synchrony between ABP and MSNA, suggesting an impairment of arterial baroreflex control over sympathetic vasomotor activity [114], [118], [119], [237].

The available data and the results of our study do not fully support one of the above mechanisms. However, the hypothesis of prolonged brain ischemia-mediated sympathetic depression can be excluded since the shock condition was not prolonged for long and all animals recovered

after blood reinfusion. Interestingly, after resuscitation with fluid and vasopressor (T3) the level of LF power did not recover to baseline value despite the animals reaching the target MAP values, and CO was higher than baseline (Table 5.1). Therefore, the persisting depressed LF oscillations of BP cannot be attributed to vagal reflexes activated by an unloading of cardiopulmonary baroreceptors.

As regards the cardiac BRS we observed a significant decrease of sensitivity during shock (BRS feedback gain) that persisted even after the resuscitation maneuvers at T3 (Table 5.3). A dynamic reduction in BRS with decreased central blood volume has already been reported [228], [238]–[243], and since it is known to reflect baroreflex-mediated cardiac vagal withdrawal [228] it could be explained as a compensatory mechanism that leads to a greater tachycardia reserve [124].

Interestingly, the opposite trend was observed for the feedforward gain (BRS feedforward gain), or runoff effect, which was higher compared with baseline both in shock (T2) and after fluid and vasopressor resuscitation (T3). This may be due to a markedly high HR.

The black-box model of DAP variability, taken as a surrogate of peripheral vascular resistance, highlighted interesting changes in peripheral control mechanisms induced by severe blood loss. During shock the cardiac baroreflex-mediated DAP oscillations ($DAP_{/sap}$) significantly increased with respect to baseline, passing from ~30% to 80% in all animals. The other components ($DAP_{/rap}$, $DAP_{/rr}$) decreased at T2 and T3 with respect to baseline (Figure 5.3). These results highlighted that the large reduction in peripheral vascular resistance can be explained again by the high HR, which cannot sustain BP changes.

The results of phenylephrine and epinephrine maneuvers confirmed a reduced sympathetic modulation of HR and BP as previously reported. During shock there was a reduced responsiveness to the drugs, mostly in the HR: indeed, the variation during phenylephrine administration was negligible (Table 5.4).

Analysis of HRV revealed that hemorrhagic shock induced a decrease in HRV and HR complexity. A loss of HR complexity, which means a higher regularity of the HR, is considered a feature of impaired adaptation of the ANS to physiological stress in several cardiovascular

pathologies [187]–[189]. Accordingly, our results show that the autonomic modulation of HR in response to acute stress induced by hemorrhage and shock was severely impaired (Table 5.2), as already reported in other studies [140]. Interestingly, this condition is not relieved after administration of fluids and vasopressors but only after reinfusion of shed blood.

HRV is assumed to be mostly dependent on the parasympathetic outflow to the heart; moreover, the recovery of baroreflex functionality, which followed the same trend as HRV during the experiment, has also been associated with an increased vagal activity in several pathologies [180]. Thus we could suggest a vagal driver of effective resuscitation [92]. Furthermore, an extensive literature has recently pointed to the central role of the vagus nerve as therapeutic target: direct vagus nerve stimulation has been proven to be beneficial in several diseases, including hemorrhage and shock [60], [63].

The lack of a recovery in the ANS control after resuscitation with fluid and vasopressor could be explained by the fact that the massive fluid infusion produced a reduction in the arterial blood oxygen content and, consequently, in the delivery of oxygen to peripheral tissues. Indeed, the hematocrit was significantly reduced and blood lactate still high at T3 (Table 5.1), supporting the notion of an ongoing mismatch between whole body oxygen consumption and oxygen delivery. Furthermore, the time window between T2 and T3 may be too short to obtain a complete resolution of the oxygen debt. Finally, the volume expansion offered by fluid infusion is limited because of redistribution of fluids in the interstitium and excretion in urine that usually occur soon after the beginning of fluid resuscitation [244].

After the transfusion of the shed blood we observed a complete restoration of HRV and BPV, lactate clearance, and hemodynamics to near-baseline values. Probably the increase in hematocrit and arterial oxygen content was effective in restoring tissue oxygenation. Another hypothesis, based on the study of hemodilution physiology, is that the restoration of systemic and microvascular conditions after haemorrhage followed by hypovolemic shock depends mostly on blood rheological properties rather than on maintenance of oxygen carrying capacity. The

rationale for this hypothesis originates from experimental studies in hemorrhagic shock, showing that a threshold of blood/plasma viscosity is required to maintain microvascular perfusion and, in particular, functional capillary density [245].

5.4.2 Cardiac functionality

The shock protocol produced a clear decrease in CO and MAP and a hypoxic condition as supported by a lactate increase above 8 mmol/l. Left ventricular EF, however, was preserved, with an increased cardiac contractility during shock. After fluid resuscitation (T3) the EF returned to baseline value, but only after blood reinfusion (T4) both CO and dP/dt_{max} did recover to values similar to baseline.

These results suggest that the shock insult did not impair cardiac contractility. The main reason for this result could be the short period of shock before initiation of treatments. Indeed, in our study shock was maintained up to 2 hours after confirmation of lactate increase, while in previous experimental studies of hemorrhagic shock the onset of myocardial depression was usually observed between 2 and 5 hours after insult [246], [247]. In our study, shock was not sufficiently prolonged to significantly impair cardiac contractility and to induce acute heart failure.

Nevertheless, plasma hs-cTnT increased significantly in shocked animals, remained high after fluid resuscitation, and finally returned to baseline values after blood reinfusion. The severity and the kinetics of troponin release do not support the hypothesis of an acute coronary syndrome [248], [249]; indeed, the peak plasma concentration observed in shock was only modestly increased. Probably such an increase in hs-cTnT is the result of a general ischemic sufferance of cardiomyocytes. The most plausible hypothesis may consist in the mismatch between oxygen delivery and oxygen consumption, leading to a type II myocardial ischemia. In fact, during hemorrhage and resuscitation with fluids, several factors may determine decrease in myocardial oxygen delivery (hypotension, low CO, reduced diastolic time, reduced hematocrit following fluid resuscitation) and increase in myocardial oxygen consumption (extreme tachycardia, circulating catecholamines, both endogenous and administered as support, i.e., norepinephrine) [250].

Another possible explanation of the increase in hs-cTnT could be the mechanical stress on the left ventricular wall in the condition of elevated myocardial contractility and low filling volume, which causes sarcomere disruption in a highly susceptible spot (i.e., “zonal lesion”) [251]. Unfortunately, we did not investigate the presence of ischemic lesions in histological samples of the left ventricle, so we cannot confirm this hypothesis.

5.4.3 Limitations of the study

The main limitation of this study is the small sample size. Moreover, the short time between T2 (shock), T3 (resuscitation), and T4 (blood reinfusion) could have attenuated the effects of shock in the physiological and metabolic responses. Finally, the unavailability of direct measures of autonomic outflow, such as MSNA or CSNA, did not permit more than speculation about the compensatory mechanisms that are activated or suppressed during shock and resuscitation.

5.5 CONCLUSIONS AND REMARKS

The results of this study suggest that BPV, HRV, and baroreflex trends can be valuable tools to evaluate the severity of hemorrhagic shock and they may represent a more useful end point for resuscitation, in combination with standard measurements, such as mean values and biological measurements. In fact, they can add important insights into the recovery of the autonomic mechanisms of BP and HR regulation, which are fundamental for the recovery of organ dysfunction in shocked subjects. In this study, for example, resuscitation with fluid and vasopressor was effective in restoring circulating volume, CO, and mean BP, but indexes of autonomic functionality revealed that ANS control was not fully recovered. Interestingly, the high concentrations of cardiac troponin after resuscitation are signs of a stress condition induced by shock not resolved by the resuscitation maneuvers.

To conclude, the trends of autonomic indices obtained in hemorrhagic shock animals show similarities to those observed in septic

shock, highlighting how the ANS is similarly elicited and how it plays an important role in the recovery.

6 DISCUSSION AND CONCLUSIONS

The main purpose of this PhD thesis was to investigate the changes in short-term autonomic-mediated mechanisms of cardiovascular regulation induced by shock and by resuscitation therapies. The hypothesis is that the ANS, which is known to be the principal mediator of short-term compensatory mechanisms in response to shock development (see §1.5), may also be determinant in the recovery from shock, for an efficient restoration of a physiological homeostatic cardiovascular condition.

Nowadays, current clinical guidelines for management of shock patients do not include the monitoring of ANS activity, and they are based on the merely evaluation of global clinical and hemodynamic indices, e.g. mean BP, oxygen saturation, and lactate (§1.2.3, §1.3.3). However, recent studies and clinical trials highlighted serious limitations of the proposed management protocols (§1.6), and, although improved, the optimal therapy has not been found yet.

In this dissertation, we demonstrated the feasibility and the usefulness of the proposed indices in monitoring the subject's status during shock and resuscitation in different populations, including septic shock patients, septic shock pigs and hemorrhagic shock pigs.

Standard and advanced cardiovascular indices were proposed, together with new mathematical models to disentangle the contribution of different mechanisms in cardiovascular control.

To the best of our knowledge, no previous scientific work has extensively focused on the short term cardiovascular assessment in shock patients during the resuscitation phase. Many researchers developed algorithms for the prediction of shock development in critical ill populations, in the attempt to prevent such syndrome [252], [253]. Moreover, the majority of studies about shock resuscitation are mostly clinical trials, dedicated to compare well defined therapeutic strategies and standard drugs administration [3], [4] or to investigate new drugs and their impact on long-term outcomes [5], or they are focused on the

cellular pathways involved in the disease, with the aim of finding novel therapeutic targets [254], [255].

For these reasons, the results of this work represent a novel approach in the field, and could contribute to define new therapy targets and resuscitation protocols of shock patients tailored to each individual response to the therapy.

The next paragraphs summarize the main achievements and the results which were extensively outlined in the previous chapters, the limits of the study and the possible clinical impact. At the end of this chapter future possible directions are proposed and illustrated.

6.1 MAIN FINDINGS

Both septic and hemorrhagic shock studies showed how the standard resuscitation maneuvers were able to restore global hemodynamic variables, such as mean BP and circulating volumes, but this was not accompanied by a recover of a physiological condition, as highlighted by the cardiovascular indices and models. The shock subject may be considered resuscitated according to clinical guidelines, but the complementary information derived from autonomic cardiovascular indices reveals a persistent autonomic disarray, that, if not appropriately treated, could lead to development of MOF, cardiovascular collapse and death. Thus, clinical protocols should give importance to the trends of these cardiovascular indices, in order to make a step further in the optimization and personalization of the therapy and to reduce the long term effects, e.g. septic cardiomyopathy or left ventricular dysfunction, in survivors.

As reported in our septic shock patients population (chapter 2), the assessment of patients' severity is very complex since the inter-subject variability observed in the response to therapy can be very large. Although all patients reached the MAP target of 65 mmHg, our analyses highlighted important differences in terms of ANS control. In particular, only those patients who responded positively to the therapy, i.e. who improved their organ function according to SOFA score, were able to improve their autonomic control of BP, expressed in terms of LF

oscillations of BP components (SAP, DAP and MAP series). Moreover, these patients were found to have received a lower dosage of vasopressors during the first two days of ICU stay, and displayed a lower fluid balance with respect to non-responders. Sedation dosage was comparable between the two groups. The positive effect of the therapy can be viewed from the increase in mean BP among these patients despite a lower vasopressor dosage; on the opposite, the patients who did not respond to the therapy were not able to further increase their mean BP, which was just maintained above the targeted threshold, i.e. 65 mmHg, by means of a higher exposure to fluids and vasopressors. Overall, these results suggest how a restoration of ANS function is necessary for a restoration of organ function and a resolution of shock, and they indicate that the introduction of autonomic assessment into clinical practice may help in guiding the therapy for shock patients' resuscitation.

The worth of the present dissertation is represented by the availability of experimental animal data, that complete and integrate the preliminary observations of the clinical study. Animal experiments were designed to mimic the clinical situation although on a different time scale as the observation window of the subject's response is very different (i.e. a couple of days for humans, some hours for animals). However, the hemodynamic findings showed common patterns which all confirmed the initial hypothesis of the work.

The controlled condition of the experimental setup allowed to eliminate possible confounding factors, which may weaken the results of the clinical study. For example, an important factor to be considered is the level of sedation, since it is known that sedative drugs interact with the ANS by suppressing the autonomic cardiovascular regulation. In critical care patients the level of sedation is typically high, although in our patients the dosage of sedation drugs was comparable between responder and non-responder, so that we may exclude a large influence of sedation on the observed trends of the autonomic indices.

In the following, the hemodynamic findings concerning the control mechanisms of BP and HR will be argued in details, both in septic and in

hemorrhagic shock, and differences or similarities between the two types of shock will be outlined.

6.1.1 BPV control mechanisms in septic and hemorrhagic shock

A dysfunction in the autonomic control of BP was observed after shock, confirming what already found in other literature works (see §1.5.1, §1.5.2), moreover, such dysfunction was not resolved by resuscitation. In particular, the LF power of BPV and the cardiac BRS gain were suppressed in the experimental protocols of septic shock (Table 4.2 and Table 4.3) and hemorrhagic shock (Table 5.3).

A depressed variability of BP in the LF range (0.04-0.15 Hz) suggests an impaired sympathetic modulation of the vascular tone [164]. However, if this malfunction occurs at the central level or if it involves the afferent or the efferent branch of the system is still an open issue; moreover, if it is the result of a withdrawal or a saturation of the sympathetic activity, is not known, or if it may be due to an inability of adrenergic receptors to respond to further stimuli. We cannot respond to this question on the base of our available data, but our analyses confirm that the baroreflex mechanism is surely involved, both in septic and in hemorrhagic shock. An impairment of baroreflex could be due to different mechanisms such as i) a decrease in sensitivity of the baroreceptors, ii) a shifted set point at lower BP values with an impaired efferent sympathetic activity, or iii) a reduced responsiveness of the target organ.

As regard the hypothesis of a shifted set point of the baroreflex mechanism, our results seem not to support this mechanism, given that a depressed sensitivity with respect to baseline condition was reported both in the hypotensive condition of shock and in the resuscitated state, when BP is much higher than in shock. Recent studies have demonstrated an impairment of the baroreflex almost immediately after the induction of sepsis and in absence of BP changes [87], hinting a direct negative effect of the toxin on BRS. This could also be the case in hemorrhagic shock, as a secondary effect of blood volume depletion. In fact, acute hypoperfusion could lead to acute bowel ischemia with the resultant release of toxins and bacteria into the bloodstream. We can thus

speculate that tissue hypoxia can cause the release of numerous inflammatory cytokines which further promote vasodilation and hypoperfusion. This may also be supported by the fact that a complete recover of the hemorrhagic shock animals occurred after blood reinfusion, when the physiological oxygen content in the blood was restored.

The contribution of toxins and bacteria and of the consequent inflammation on baroreflex impairment, could involve the baroreflex mechanism at multiple levels. Acute inflammation is known to change the elasticity of large vessels and we demonstrated that the arterial compliance is decreased in septic shock and after resuscitation (Figure 4.4 in Chapter 4); a reduction in arterial distensibility implies a decrease in baroreceptors sensitivity, thus leading to a reduction in baroreflex gain. Moreover, the production of NO and ROS have been demonstrated to have a direct negative effect on BRS, although the exact mechanism is not fully elucidated yet; a sympathoinhibitory effect of NO in the CNS has been suggested [256], together with a possible inhibitory effect on sympathetic responses via pre- and postsynaptic mechanisms [257] (see also §4.4.2).

Finally, the hypothesis of an impairment of the vasomotor center in the brainstem is not supported by our results, although indirectly. In fact, other branches of the baroreflex were not suppressed. For example, the baroreflex control of peripheral resistance, as modeled by Equation 5.1 in §5.2.3, was significantly enhanced during hemorrhagic shock compared to baseline and remained elevated also after resuscitation; also the baroreflex control of ventricular contractility was increased both after development of septic shock and after resuscitation (chapter 3). Given these findings, we could hypothesize that it is unlikely that a central downregulation of baroreflex has occurred in these animals, at least for the short period of observation after the insult. Furthermore, also an extensive literature reported a different outflow of the sympathetic branch depending on the anatomical district, and many of these results are in line with our findings. An increase in renal sympathetic nerve activity (RSNA), not mediated by the baroreflex, was observed in septic sheep, together with a concomitant increase in cardiac sympathetic

nervel activity (CSNA), mediated by the baroreflex, and a depressed BRS for the HR [98]. Similar findings were reported in anesthetized rats after infusion of LPS [99], whereas a blunted muscle sympathetic nerve activity (MSNA) mediated by the baroreflex was described in a population of healthy human volunteers after injection of endotoxin [97].

Together our results may suggest that the efferent sympathetic outflow is not suppressed during septic shock, and the impaired autonomic control of BP may be the effect of a reduced responsiveness or a downregulation of α adrenoreceptors of the vessels [258]. This is consistent with much literature, that proves the autonomic imbalance in septic shock is often due to an excessive and prolonged sympathetic activation, or to an inappropriate regulation of the parasympathetic branch of the ANS [67], [68]. Indeed, the increased autonomic-mediated baroreflex control of ventricular contractility in the septic pigs, as discussed in the following paragraph, confirm this evidence.

In hemorrhagic shock animals, although they shared with the septic animals common trends of LF power of BP oscillations and cardiac BRS, both depressed after shock and resuscitation, it could be reasonable to affirm that the underlying cause of impaired BP autonomic control may be different. Indeed, the interaction between the ANS and the inflammatory and immune systems plays a crucial role in septic shock, but only a marginal role in hemorrhagic shock, where the volume loss is the main trigger of cardiovascular collapse. This hypothesis is supported by the opposite results obtained from the DAP variability decomposition model in the two groups of animals. In hemorrhagic shock pigs (§5.2.3) the baroreflex control of peripheral resistance, represented by the portion of DAP LF oscillations mediated by SAP oscillations ($DAP_{/sap}$), was significantly increased after shock and remained elevated also after fluids and vasopressors administration, whereas the LF oscillations driven by the mechanical effect of HR runoff followed the opposite trend and they were diminished (Figure 5.3). This may suggest that the responsiveness of the vessels' adrenergic receptors was not compromised during hemorrhagic shock. To confirm this hypothesis, the pharmacological stimulation by phenylephrine showed that the major contribution to

baroreflex depression was to be accounted for the heart and not for the vessels response to the sympathomimetic drug (Table 5.4).

An opposite result is emerged from the computation of DAP variability decomposition (Equation 2.4) in septic shock animal, and these additional results are included in the following (Figure 6.1). In this case, we used the time series of aortic SAP, which is the pressure really sensed by the aortic baroreceptors, the femoral DAP, which is a valid surrogate of peripheral vascular resistance, and the RR-intervals recovered from the ECG waveform. The results highlighted a decreased influence of the baroreflex-mediated control of peripheral resistance and, on the opposite, an increased role of the mechanical runoff. We may explain this result by a reduced responsiveness of peripheral adrenergic receptors, which is induced by sepsis, but not present in severe hemorrhage. The reason for this difference may lay in the overwhelming inflammation, typical of septic shock, which is known to induce a persistent vasodilation through the secretion of inflammatory vasoactive substances, such as NO; this latter was also directly proved to be implicated in the hyporesponsiveness of septic shock patients, as discussed in details in chapter 4. The role of inflammation in hemorrhagic shock is, instead, only secondary and the short observational period after the insult may be insufficient for an extended inflammatory response.

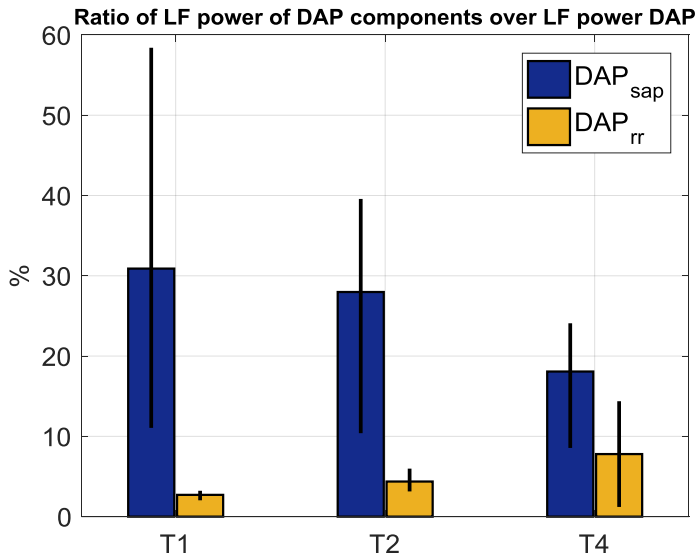

Figure 6.1. Ratio between low frequency (LF) absolute power of each predicted component and LF absolute power of diastolic arterial pressure (DAP) at each time point for septic shock animals. Column height is median value for the population; black bars indicate values of 25th and 75th percentiles. T1, baseline; T2, after development of septic shock; T3, after fluids and vasopressors resuscitation; SAP=systolic arterial pressure extracted from the aortic blood pressure waveform; DAP=diastolic arterial pressure extracted from the femoral blood pressure waveform; RR=RR-intervals extracted from the ECG waveform

Finally, other mechanisms involved in BP control could have a role in the suppression of BPV observed in shock subjects. BP and organ perfusion are known to be controlled by a variety of cardiovascular control systems, including feedback systems, such as the baroreceptor reflex, hormonal systems, e.g. the renin-angiotensin system, and local mechanisms, e.g. the myogenic vascular response and the endothelial NO system. The response time at which these mechanisms operate differs considerably, for example BP control by hormonal systems is slower than BP control via the baroreflex. Thus, cardiovascular control mechanisms elicit specific patterns of BPV and power spectral analysis of BPV can be used to assess their different contribution.

As regard the meaning of the LF oscillations, it's necessary to detail some issues in comparisons with other animal studies. The LF band is known to reflect the sympathetic modulation of vascular tone, both in

rats and in humans [77]. However, myogenic vascular function has been found to affect VLF and LF BPV in humans, but only VLF BPV in rats. Thus, in humans, LF BPV may not exclusively reflect sympathetic modulation of vascular tone. Similarly, in rats, LF BPV does not exclusively reflect sympathetic modulation of vascular tone, because, the endothelial NO system also affects LF BPV [77].

There is no such detailed characterization of the frequency range in which cardiovascular mechanisms regulate BPV in pigs; thus, we cannot be sure that the LF power of BPV only reflects sympathetic modulation of vascular tone, and other mechanisms could be involved and deserve further investigation.

As regard the hormonal systems and the local mechanisms, although slower in vascular tone modulation, they may play a role in the response. In particular, there is increasing evidence that the endothelial NO system, which is induced by inflammatory cytokines, plays an important role in the hypo-responsiveness of resistance vessels to vasoactive agents, generating a persistent vasodilation despite vasopressors administration, as detailed in §4.4.1. The non-responder group of patients in our clinical cohort showed a depressed LF power of BPV persisting during the first two days of ICU stay, despite an increasing administration of vasopressors (§2.3.2). Although an impaired sympathetic modulation is likely involved, we may also hypothesize the influence of the local NO mechanism in the overall response of these patients. This mechanism may also partly explain the monotonic decrease of the arterial tree characteristic time constant τ and the total peripheral resistance values of septic shock pigs population (§4.3).

Finally, it has been demonstrated that catecholamines, which are abundant in the circulation of shock subjects, enhance myogenic vascular responsiveness in rat small arteries [259].

Overall, our results demonstrated an impaired control of BP induced by shock, confirming the literature results, and they highlighted for the first time that a dysfunction of BP control mechanisms persists despite successful resuscitation, as targeted by the current resuscitation protocols. Although other mechanisms may be involved, the sympathetic modulation of BP resulted highly impaired both in septic and in

hemorrhagic shock, and for this reason we speculate that its monitoring through a real-time computation of BPV power spectrum analysis and of BRS, could be helpful in defining new therapy target tailored to the patient response.

6.1.2 HRV control mechanisms in septic and hemorrhagic shock

A dysfunction of the HR autonomic control was observed in shock as well, in line with the literature (§1.5.1, §1.5.2), and it was not resolved by resuscitation. In particular, HRV indices and the cardiac BRS gain, as already reported above, were highly suppressed both in septic (Table 3.2 and Table 4.3) and in hemorrhagic shock (Table 5.3).

A blunted cardiac baroreflex and HRV are signs typically associated to a predominance of sympathetic activity at the expense of vagal outflow. This uncontrolled sympathetic activation has already been reported in literature and it is known to be involved in the autonomic dysfunction typical of shock (§1.5). However, the exact underlying mechanism is still under investigation, and if it must be ascribed to a sympathetic overstimulation or to an inappropriate regulation of the parasympathetic branch is still unclear.

One possible mechanism explaining the suppressed HRV could be the saturation of the sinus node, i.e. a very high sympathetic drive would make the sinus node less capable of maintaining a rhythmic modulation. We can also speculate that, similarly in heart failure patients, high sympathetic drive may lead to saturation of LF oscillatory systems [102]. Moreover, β-adrenoreceptor downregulation could also contribute, or excessive concentrations of circulating catecholamines may compromise central autonomic controls [91], [97]. Finally, an impairment of the baroreflex mechanism is likely implicated, as already discussed in the previous paragraph.

In septic shock pigs we observed a depressed gain of the cardiac baroreflex (§3.3) in contrast to an increased influence of the baroreflex-mediated mechanism of ventricular contractility regulation (Figure 3.5). As previously discussed, an impairment of the cardiovascular center in the brainstem is unlikely, otherwise we could not explain why the sympathetic drive is enhanced in some physiological compartments and

suppressed in others. Moreover, also the hypothesis of a downregulation of cardiac β-adrenoreceptors is not supported by our findings, as cardiac contractility and HR resulted enhanced.

As a new hypothesis, we think that the depressed BRS could be explained by a saturation of HR, i.e. the elevated HR, which even exceeds 200 bpm in hemorrhagic shock pigs, prevents any change in HRV in response to a change in BP; thus, the two systems act as they were uncoupled and this further contribute to a loss of regulation.

Moreover, the runoff effect, i.e. the mechanical effect of oscillations in HR which induces oscillations in BP, was depressed in hemorrhagic shock and it was still negligible also after resuscitation ($DAP_{/rr}$ component of DAP variability decomposition model as in Figure 5.3). This result is a further evidence that the HR has a minor influence in BPV modulation and it could be the effect of an excessively high HR.

The $DAP_{/rr}$ component computed in the septic shock animals showed an increasing trend, opposite to the u-shape found in the hemorrhagic shock animals (Figure 6.1 above). We don't have an exhaustive explanation for this result, but many factors could contribute. For example, the HR did not increase to a level as high as the HR in hemorrhagic shock pigs, and, maybe, a margin of HRV was still available. Another hypothesis could be related to the dramatic reduction of peripheral resistance occurring in septic shock: a more dilated vessel is more prone to oscillate in response to an ejected SV. Furthermore, we should consider that a stiffening of the aortic blood vessel occurs, and this inevitably limits the windkessel effect of the large arteries; as a consequence, the blood outflow could arrive at peripheral districts with a higher pulsatility, and could induce higher oscillations.

Finally, the peak time of the transfer function representing the baroreflex-mediated control of ventricular contractility is delayed significantly after resuscitation (Table 3.3), which means that, after resuscitation, the autonomic-mediated baroreflex mechanism is slower in modulating ventricular contractility in response to a change in BP. This could suggest an inappropriate regulation of the vagal branch of the ANS, mostly after resuscitation, resulting in a withdrawal of vagal outflow, as a delayed response hints a depressed vagal activity.

A recovery of HRV and baroreflex functionality is usually associated to an increased vagal activity and our results could pave the way for new therapy strategies, such as the direct stimulation of the vagus nerve, which new findings proved to have beneficial effects in shock patients. There are evidences that the ANS reflexively regulates the inflammatory response in real time inhibiting the pro-inflammatory cytokines cascade through the cholinergic anti-inflammatory pathway (§1.4.4); also, a direct stimulation of the vagus nerve has been proven to reduce blood loss and duration of hemorrhage, and to reduce coagulopathy in hemorrhagic shock (§1.4.4). Our results indicate that the beneficial effects of vagal stimulation could also extend to the heart and its regulation. A lowering of the HR through an increased vagal outflow may reestablish a level of variability into a more physiological range and this may also be reflected into a more efficient BP regulation. Moreover, a lower HR implicates a higher time for diastole and, thus, a better oxygenation of the heart tissue.

Altogether these results strengthen the evidence that the heart and its variability should be considered as an additional therapeutic target in shock management. The ability of the cardiovascular system to maintain a high level of variability is a sign of health. Thus, a restored variability should be considered as important as the return to a physiological mean value, and indices, such as HRV and BRS, or models based on hemodynamic time series, such as the proposed ventricular contractility model, could be used to test new drugs or therapy targets.

Finally, we want to stress the importance of considering the heart and the circulation as coupled systems and we want to underline that current clinical guidelines should evaluate them jointly and not as independent systems in order to achieve a better optimization of the therapy. New drugs and therapies should also be studied with this perspective and their efficacy should be assessed on the overall cardiovascular system. For example, esmolol, a selective β1-blocker, has recently gained a lot of attention for its anti-inflammatory properties at vascular level [258]; moreover, preliminary analyses of our group on septic shock animals treated with ivabradine, a pacemaker I_f current inhibitor, revealed beneficial effects of the treatment on the circulation,

despite the drug was mainly administered to control HR. Preliminary results are reported in Appendix B.

6.2 CLINICAL IMPACT OF THE STUDY

Shock resuscitation protocols, although continuously revisited and updated in the last decades, present evident limitations, which explain the elevated mortality still associated with this syndrome. Novel therapy targets are needed in order to optimize drugs administration and tailor the treatments to each single patient, based on the response to therapy.

In this context, the clinical interest of the present thesis concerns the idea of a novel hemodynamic monitoring for shock patients, which can be functional and not symptomatic only. We studied a variety of mathematical indices and models representative of the short-term autonomic regulation of cardiovascular state, and we demonstrated that they add information on patient's response to therapy to standard clinical and hemodynamic indices (e.g. mean values), that are the cornerstones of current clinical guidelines. Thus, they may represent new possible non-invasive tools to guide shock resuscitation protocols in critical care.

The indices proposed are based on mathematical analyses of physiological waveforms which are routinely acquired in ICU patients, such as the ABP and the ECG. Thus, no other external or additional data is necessary and their real-time integration into ICU bedside monitors could be straightforward.

Moreover, new drugs and treatments in the field of shock are currently under intense investigation in critical care, and the proposed indices and models could be useful in order to verify the efficacy of the therapy and its impact on long-term outcomes, both in clinical trials and in preclinical researches. For example, the effect of esmolol or ivabradine administration on ventricular contractility control could be studied in animal experiments by means of the proposed ventricular contractility model, or their effect on vascular properties and vascular tone modulation could be assessed by computing the time constant of the arterial tree and the described variability indices.

Finally, the interest in monitoring and regulating the ANS activity is rapidly growing and its implications into clinical practice is more and more recognized. Innovative technologies in the field of bioelectronics medicine are advocated as the future medicine for a vast majority of diseases, and they are based on the direct stimulation of the ANS, in particular of the vagus nerve. The clinical interest concerning these techniques is enormous and clinical trials as well as animal experiments and in-vitro researches are ongoing. Our indices may be used to compare the effectiveness of different nerve stimulations on the overall cardiovascular regulation, as well as the effectiveness of a combination of different therapies.

6.3 LIMITATIONS AND FUTURE DEVELOPMENTS

Even if the results of the current work are very promising, some limitations must be discussed. As already underlined, an important limit of the study is represented by the small sample size, mostly of the experimental studies. This may be the source of a high variability in the results and this could affect the statistical tests. Moreover, septic shock patients analysed in chapter 2 were recruited in a single centre, and, although homogeneous, there could be some bias related to clinical routines of the hospital.

The most important limit of the experimental protocols consists in the limited time window; thereby, the duration between one time point and the other does not properly reflect what happens in clinical settings and only the short-term effects of shock and resuscitation protocols can be investigated. This inevitably results in the impossibility to directly compare the temporal trends of the variables obtained from the experimental protocols and the clinical study. However, common patterns in the hemodynamic variables were observed which allow to draw similar conclusions, as previously discussed.

Another limitation of the present works is the unavailability of direct measures of autonomic outflow which limited our conclusions to speculation about autonomic activity at the heart level and in the periphery. Although a comparison with published researches reinforces

our findings, we may suggest to include the direct microneurographic measurements of sympathetic activity in future studies.

Therefore, further investigations are needed to better elucidate the role of the ANS during shock and resuscitation, through direct and indirect measurements. Moreover, we suggest to include, in the study of sympathetic and vagal outflow, other physiological districts, not only the heart or the peripheral circulation, as it is known that, during shock, SNS activity is differentially modulated across the human body with, for example, a depressed sympathetic activity at the level of muscle vascular bed and an enhanced sympathetic activity at cardiac level. A complete characterization of the changes in autonomic activity at multiple organs and their correlation with development of MOF in shock could represent an intriguing field of research, paving the way for novel therapeutic targets in critical care. Among all the organs which have been already widely studied during shock development, such as the kidneys, recent evidences on the inflammatory reflex, as detailed in §1.4.4, revealed the dominant role of organs such the spleen or the liver in modulating cytokines production in response to vagal stimulation both in septic shock and in acute bleeding. Therefore, we suggest that they could carry important new knowledge for a global picture of the human body physiology, both in physiological and in pathological conditions.

Some analyses are ongoing in our group, concerning the data collected from a pig experimental protocol of septic shock performed at the Inselspital of Bern, which includes recordings of HR, CO, ABP, pulmonary arterial pressure, central, renal, portal and liver venous pressure as well as measurements of blood flow in the carotid, in the liver and in the kidneys. Standard laboratory exams and measurements of oxygenation and fluids or drugs administration are also included. Moreover, measurements of intracranial pressure and brain oxygenation will also be available, opening to the possibility of studying brain dysfunctions, such as cognitive impairment, which are important burdens of survived shock patients. This dataset will allow us to have a more comprehensive understanding of the local changes occurring during septic shock and resuscitation at target organs, advancing the knowledge of single organs functions into a more integrated and networked

knowledge of the human body. Network physiology is a new filed of research, which considers the human body as an integrated network, where physiological systems and organs, each with its own regulatory mechanism, continuously interact to coordinate their functions; this approach is of inspiration and will also be exploited.

Finally, the use of the indices proposed in this thesis can be exploited to test the efficacy of new drugs on the regulation of the target organ and on the overall control of cardiovascular homeostasis. Current studies are ongoing in our group, focusing, in particular, on the beneficial effect of two cardioselective drugs in septic shock, ivabradine and esmolol. While esmolol is a β1-selective blocker, ivabradine selectively inhibits only the pacemaker ionic current, leading to a reduction of HR without affecting intracardiac conduction, contractility or ventricular repolarization. Both the drugs have been already studied in the context of septic shock and MOF, demonstrating their beneficial effect on cardiac chronotropic dysfunction. However, it would be interesting to investigate their influence also on peripheral circulation, as a secondary mechanical effect of a reduced HR. Moreover, recent studies showed that a possible mechanism explaining the beneficial effect of esmolol on cardiac function is an indirect effect on vagal nerve activity [6]. The interest in the study of the ANS and ANS-mediated mechanisms is rapidly growing among clinicians, given its direct involvement in innovative therapeutic options.

The findings of this thesis demonstrate the fundamental role of the ANS in the recovery from septic and hemorrhagic shock, contributing to the development of a new concept of patients monitoring and care.

Appendix

A. METABOLOMICS ANALYSES OF LEFT VENTRICULAR TISSUE SAMPLE IN HEMORRHAGIC SHOCK PROTOCOL

These analyses are part of the study on the hemorrhagic shock animal experiment published in Journal of Applied Physiology (refer to chapter 5).

A.1 INTRODUCTION

Hemorrhage is known to induce a sort of myocardial injury [260], and the reduction in heart size with central hypovolemia stimulates the release of other vasoactive and volume regulatory hormones, such as arginine vasopressin and renin. Vasoactive hormonal responses to hemorrhage in animals may also be species dependent; for example, Thrasher [261] confirmed that arterial baroreflexes control vasopressin but not renin release during graded hypotension in the dog. For these reasons, we assessed the effects of severe haemorrhage on heart functionality and cardiac tissue by integrating the hemodynamic findings with metabolite concentrations in left ventricular tissue.

A.2 METHODS

Metabolic profile was obtained from tissue samples of the left ventricle for both shock- and sham-treated animals. Sample tissue preparation was performed as reported in [262]. Briefly, frozen tissue samples were disintegrated with a Mikro-Dismembrator S at 3,000 revolutions/ min for 40 s. The powder obtained was resuspended in ice-cold MeOH (3 µl/mg tissue) and homogenized for 1 min. Homogenized samples were subsequently centrifuged for 15 min at 10,000 g, and supernatants were stored at -80°C. Thirty microliters of each supernatant was used for targeted metabolomics analysis. A targeted quantitative approach using a combined direct flow injection and liquid chromatography tandem mass spectrometry (MS/MS) assay (AbsoluteIDQ 180 kit; Biocrates, Innsbruck, Austria) was applied to metabolomics analysis.

The assay quantifies 188 metabolites from five analyte groups: acylcarnitines, amino acids, biogenic amines, hexoses (sum of hexoses), phosphatidylcholines, and sphingomyelins. The method combines derivatization and extraction of analytes with the selective MS detection using multiple reaction monitoring pairs. Samples were analyzed with an LC/MS (Triple quad 5500; AB Sciex) method (for analysis of amino acids and biogenic amines) followed by flow injection analysis-MS (analysis of lipids, acylcarnitines, and hexose). Methodological details and data preprocessing have been extensively reported in previous articles [254], [255].

A.3 RESULTS

All the measured metabolites are not shown in this appendix but they are reported in Supplemental Table S1 available online at the Journal website. Only 11 metabolites significantly changed concentrations between sham-treated and hemorrhagic shock pigs in left ventricular tissue (Table A.1.). Left ventricular tissue of HS animals was mainly characterized by enhanced levels of long-chain phosphatidylcholine (PC) species and reduced concentrations of a few amino acids compared with left ventricular tissue from sham-treated animals.

A.4 DISCUSSION AND CONCLUSIONS

Only a small subset of the measured metabolites was differently abundant in left ventricular tissue between hemorrhagic shock and sham treatment. A disturbance in the composition of myocardial membrane PC species occurred. Indeed, shocked left ventricular tissue presented a marked increase in PC species, containing long-chain polyunsaturated fatty acids, such as PCaaC34:4, PCaaC36:4, and PCaaC36:5, with further elongation/desaturation products. We can speculate that since long-chain polyunsaturated fatty acids reduce T cell activation and dampen inflammation [263], their enhanced presence would help relieve serious inflammatory conditions. Concomitantly, raised plasmalogen levels such as PCasC38:0 and PCec42:3, which act as endogenous antioxidants [264], may protect cardiomyocytes during hypoxia in shocked left ventricle. Interestingly, plasmalogens, highly predominant in the sarcolemma and

sarcoplasmic reticulum of myocardial cells [265], play an important role in regulating myocardial electrical excitability [266] and sodium/calcium exchange in cardiac sarcolemmal preparations [267].

Together these findings converge toward a plausible adaptation of the left ventricle metabolic asset to cope with the insult. Such change in the metabolic asset might also be viewed as a key mechanism for either membrane structural rearrangement or damage to cardiomyocytes [268] and might directly or indirectly contribute to the production of electrophysiological abnormalities and arrhythmogenesis.

Moreover, shocked left ventricular tissue presented a dysregulated arginine metabolism, characterized by reduced arginine and ornithine levels, thus suggesting a possible imbalance of NO formation. In heart, alterations of the arginine-NO pathway have been reported in chronic heart failure [269]. NO synthesis requires the presence of arginine inside the cells of responsive tissues. Although some cell types can synthesize arginine from ornithine or citrulline, cardiac myocytes cannot produce it, and thus cardiac muscle must import this amino acid from the circulation. Depletion of arginine leads to NO synthase uncoupling. Combination of $O_2^{\cdot -}$ with NO from enzymatic or nonenzymatic sources will result in the production of peroxynitrite (ONOO−), an oxidizing agent associated with cell damage, decreased myocardial contractility, and congestive heart failure [270].

The dysregulated metabolism in the left ventricular tissue after resuscitation is a sign of a stress condition induced by shock not resolved by the resuscitation maneuvers.

Table A.1. Concentration values (μM) of the metabolites significantly different in the left ventricular tissue between sham and hemorrhagic shock animals. Values are reported as median (25^{th},75^{th}) percentile

	Shock	**Sham**
PC aa C34:4	0.7 (0.4,0.8)	0.3 (0.2,0.3)
PC aa C36:4	11.9 (7.8,17.9)	4.8 (3.2,5.2)
PC aa C36:5	2.6 (1.7,3.4)	0.9 (0.7,1.1)
PC aa C38:4	1.8 (1.2,2.3)	0.7 (0.4,0.8)
PC aa C38:6	1.8 (1.2,2.3)	0.65 (0.57,0.66)
PC ae C38:0	0.3 (0.2,0.4)	0.13 (0.1,0.13)
PC ae C42:3	0.08 (0.04,0.09)	0.029 (0.028,0.03)
Arginine	11.7 (6.8,14.3)	26.3 (24.4,28.2)
Isoleucine	6.5 (5.9,9.1)	11.9 (11.7,13.8)
Ornithine	2.5 (1.8,3.04)	4.4 (4.2,5.3)
SM C22:3	0.1 (0.09,0.14)	0.07 (0.05,0.07)

PC aa Cxx:x=Phosphatidylcholine diacyl C xx:x, SM C22:3=Sphingomyelin C 22:3

B. IVABRADINE-TREATED VERSUS SHAM-TREATED SEPTIC SHOCK PIGS: A CARDIAC EFFICIENCY ANALYSIS

B.1 INTRODUCTION

Tachycardia persisting despite fluids and vasopressors resuscitation is a hallmark of septic shock patients [175], and it is an independent risk factor for increased mortality [74]. The consequences of an elevated HR are multifactorial and include an impaired diastolic function with less time available for the heart to fill, myocardial ischaemia, tachyarrhythmia, and finally cardiac failure. The source of this persevering tachycardia has been demonstrated to be a protracted adrenergic stress at cardiac level, which exceeds in time and scope the beneficial short-term compensatory effects [177].

Based on the underlying mechanisms of non-compensatory tachycardia in septic shock, i.e. a high sympathetic stress, β-blockers, in particular esmolol, theoretically are the treatment of choice, because they can enable HR control and limit the adverse events related to sympathetic overstimulation [175]. However, β-blockers do bind not only to the β1-adrenoceptors of the sinoatrial node cells but also to β-adrenoceptors of many other cells, and, therefore, they do have not only negative chronotropic but also negative inotropic effects on the heart and they may interact in an unwanted mode with α-adrenoceptor stimulation on the coronary circulation [271]. Furthermore, they also have a BP-lowering effects, and therefore, in many patients, they cannot be given in the recommended dose.

Ivabradine specifically inhibits the pacemaker current (funny current, I_f) of the sinoatrial node cells, resulting in therapeutic HR lowering without any negative inotropic and BP-lowering effect [271].

The beneficial effects of ivabradine have been tested in different clinical trials and preclinical experiments, but no concordant results have been achieved.

While an improvement of clinical outcomes have been found in heart failure patients treated with ivabradine compared to placebo group in a large randomized clinical trial [272], no evident results have still been achieved in septic shock and MOF patients. In particular, despite a significant decrease in HR produced by the drug, no associated beneficial effects were observed in BP, lactatemia, vascular responsiveness to vasopressors and circulating levels of pro/anti-inflammatory cytokines in a rat experimental model of septic shock [273], and no differences were found in hemodynamics, disease severity, vasopressor use, mortality, and adverse events between ivabradine group versus placebo group in patients with MODS [274].

The objective of this work was to study the effect of ivabradine administration in a population of septic shock pigs compared to a sham group of pigs undergoing standard resuscitation. In particular, we compared the two groups in terms of cardiac efficiency, as assessed through the Buckberg index, and in terms of standard hemodynamic indices.

B.2 MATERIALS AND METHODS

B.2.1 Experimental procedure

We performed a controlled experimental study on a large animal model of septic shock induced by a polymicrobial peritonitis on adult swine in the Experimental Laboratory of Intensive Care (LA1230336), at the Université Libre de Bruxelles. The local animal ethics committee approved the present study.

Eight pigs of both sex (weight between 39 Kg and 52 Kg) were studied at time points: baseline (T1), after development of septic shock induced by fecal peritonitis (T2), after full fluids resuscitation to reach a PPV <12% (T3), after ivabradine administration at incremental doses until HR ≤ 90 bpm in the treated group or standard fluids in the control group (T4), and after noradrenaline infusion (T5). If the treated animal was not stable after ivabradine administration and returned to a hypotensive condition, additional fluids were infused to resuscitate the animal and then ivabradine was given again and time point T4 was taken at this point.

The eight septic shock pigs were divided into three sham control and five treated with ivabradine.

For details about instrumentation and experimental procedure refer to §3.2.1. At each time point, arterial blood samples were collected for laboratory analyses.

B.2.2 Signal processing

Aortic BP and LVP were continuously recorded during the experiment. At each time point stationary segments of about 15-minute length on average were selected. Time series of SAP, DAP, MAP, PP and HP were obtained from aortic BP waveform using standard algorithms [152], [153] (details in §2.2.3). An adaptive filter was then applied to the time series in order to remove outliers and irregularities [154] and each time series was finally resampled at 2 Hz by means of zero-order hold techniques. The pre-processed time series were then subdivided into 3-min 50% overlapping windows, and all the indices obtained from each 3-min window were averaged and considered for successive statistical comparisons.

B.2.3 Buckberg index

The Buckberg index is a measure of cardiac efficiency and it is defined as the ratio between the oxygen supply to the myocardium, the so called diastolic pressure time integral (DPTI) computed as the area between diastolic aortic pressure and LVP, and the oxygen demand by the myocardium, the so called systolic pressure time integral (SPTI) calculated as the area under systolic LVP curve. For the sake of clarity, the computation of the index is represented in Figure B.1.

The index was computed on a beat-to-beat basis and then averaged.

B.2.4 Statistical analysis

We adopted the Mann–Whitney U test, or Wilcoxon rank-sum test, to verify significant differences in the indices values between the two groups (treated versus sham animals) separately at each time point, whereas we used the Friedman test to detect differences in time points across multiple test attempts. In case of significant Friedman test p-value, i.e. < 0.05, we used then the Wilcoxon signed-rank test to assess

significant changes among time points within the same group of animals. Significance was considered with a p-value < 0.05.

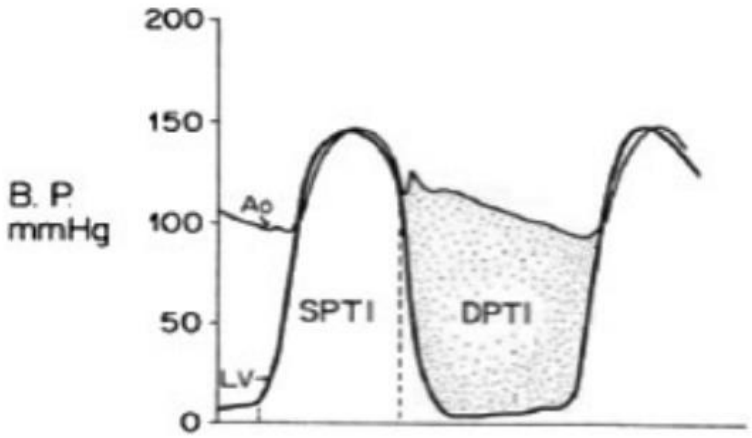

Figure B.1. The measurement of diastolic (DPTI) and systolic (SPTI) pressure time integrals. BP=blood pressure, Ao=aortic, LV=left ventricle. Reproduced from Hoffman and Buckberg [275]. The Buckberg index is computed as $\frac{DPTI}{SPTI} = \frac{O_2\ Supply}{O_2\ Demand}$

B.3 RESULTS

Table B.1 shows the values of clinical variables at each time point of the experiment in both groups of control and ivabradine-treated animals.

Figure B.2 reports the values distribution of the Buckberg index for both populations of pigs at each time point.

Figures B.3 and B.4 display the values distribution of hemodynamic variables such as HR (figure B.3) and BP components (figure B.4) for both groups of pigs during the experiment.

The Buckberg index indicates that a condition of cardiac efficiency (index equal or higher than one) was present only at baseline (T1) in all the animals. The development of septic shock triggered a state of cardiac inefficiency in both treated and sham animals, which persisted also following resuscitation, despite the administration of ivabradine significantly lowered HR in treated pigs.

Table B.1. Clinical data for sham control and ivabradine-treated group of animals at each time point. Values are reported as median (25^{th},75^{th}) percentile

		Ivabradine group	Control group
Lactate [mmol/L]	T1	0.9 (0.9,0.9)	0.9 (0.9,0.9)
	T2	NaN	NaN
	T3	1.2 (0.98,1.65)$^{n=3}$	0.9 (0.9,1.13)
	T4	1.35 (0.9,2.25)$^{n=4}$	1.1 (0.95,1.25)
	T5	1.9 (1.45,2.95)$^{n=3}$	1.8 (1.35,2.03)
CO [L/min]	T1	3.9 (3.5,4.4)	3.5 (3.5,4.5)
	T2	2.4 (2,2.96)	3.5 (3.5,3.5)$^{n=1}$
	T3	4.9 (4.2,6.8)$^{\#}$	5.4 (4.5,6.4)
	T4	4.9 (4,5.4)	4.7 (4.3,6.2)
	T5	6.4 (5.9,6.8)$^{\#\#}$	6.8 (6.5,8.1)
SV [mL]	T1	49.4 (48.3,55.7)	57.6 (54.1,58.7)
	T2	17.3 (15.9,19.9)	30.2 (30.2,30.2)$^{n=1}$
	T3	39.9 (38.1,44)	47.9 (42.8,58.2)
	T4	53.2 (47.3,59.1)$^{\#}$	41.6 (41.2,57.8)
	T5	63.1 (58.7,66.2)$^{\#\#}$	57.3 (56.4,58.2)
SVO2 [%]	T1	63 (61,64)	64 (55.8,72.3)
	T2	NaN	NaN
	T3	64 (63.3,64.8)$^{n=3}$	74 (68,77)
	T4	65 (58.5,66)	70 (64.8,73)
	T5	70 (65.5,71.5)$^{n=4}$	73 (72.3,74.5)

CO=cardiac output, SV=stroke volume, SVO2=mixed venous oxygen saturation $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus T2 (Friedman test)

From Table B.1 we observed a significant increase in SV both at T4 and T5 with respect to shock condition (T2) only in the treated animals; moreover, despite not significant, the values reached in the treated population are higher than the values of the sham group both at T4 and T5. The increase in SV in treated pigs was driven by the HR, given that the values of CO were comparable in the two populations at T4 and T5.

Regarding the values of BP, Figure B.4 highlights in treated animals a significant reduction of DAP and MAP following the administration of ivabradine (T4) compared to baseline (T1), so to become significantly different with respect to sham pigs. Interestingly, the values of PP became significantly higher in treated animals compared to sham animals both at T4 and T5, and significantly higher than baseline and shock values.

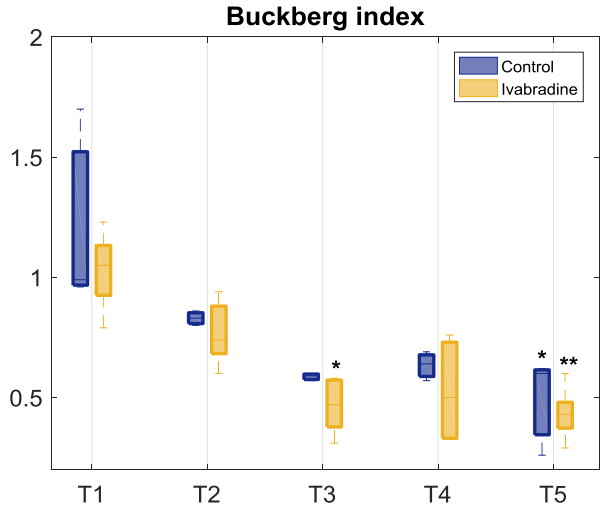

Figure B.2. Distribution (median (25^{th},75^{th} percentile)) of the Buckberg index for both groups of animals at each time point of the experiment. Control animals are represented in blue, ivabradine-treated animals are coloured in orange T1=baseline, T2=septic shock, T3=after fluids resuscitation, T4=after ivabradine administration (for treated animals), T5=after noradrenaline administration. Wilcoxon signed test: *p-value<0.05, **p-value<0.01 with respect to T1 (Friedman test p-value<0.05).

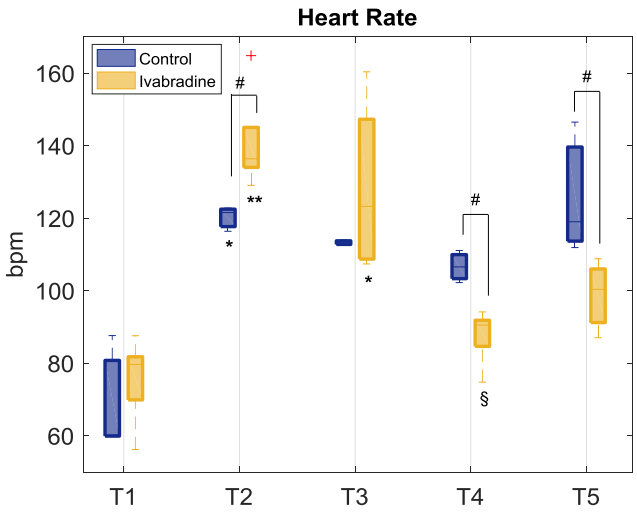

Figure B.3. Distribution (median (25^{th},75^{th} percentile)) of HR values for both groups of animals at each time point of the experiment. Control animals are represented in blue, ivabradine-treated animals are coloured in orange T1=baseline, T2=septic shock, T3=after fluids resuscitation, T4=after ivabradine administration (for treated animals), T5=after noradrenaline administration. Wilcoxon signed test: *p-value<0.05, **p-value<0.01 with respect to T1, §p-value<0.05 with respect to T2 (Friedman test p-value<0.05). Mann-Whitney U test: $^{\#}$p-value<0.05 between the specified variables

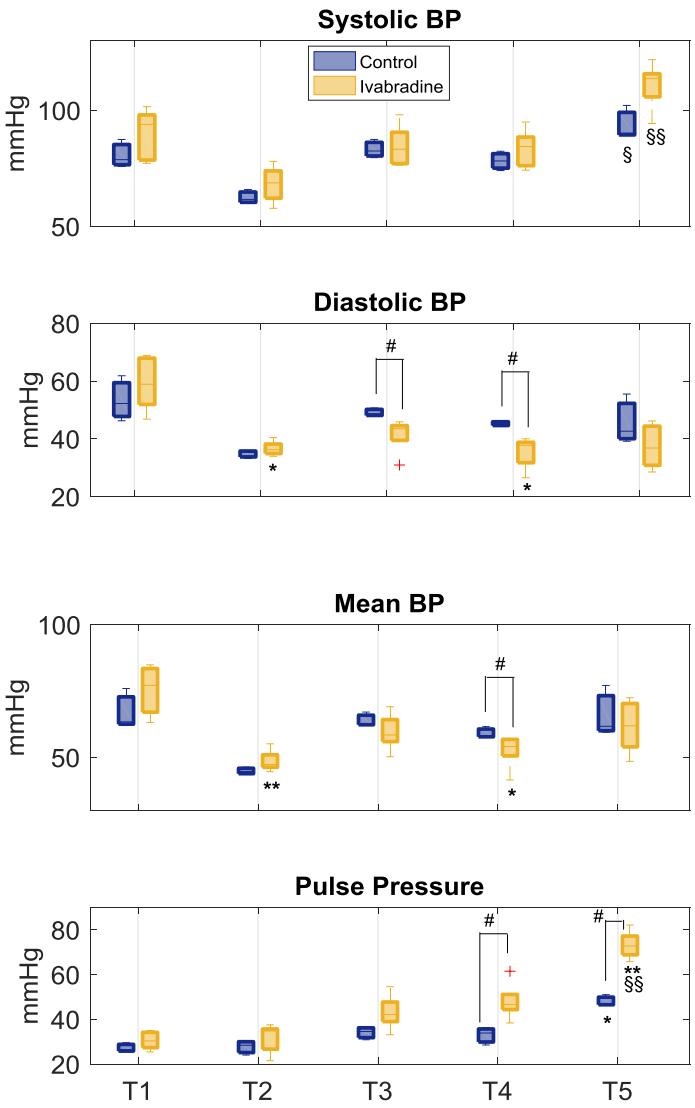

Figure B.4. Distribution (median (25^{th},75^{th} percentile)) of systolic, diastolic, mean aortic blood pressure (BP) and pulse pressure for both groups of animals at each time point of the experiment. Control animals are represented in blue, ivabradine-treated animals are coloured in orange T1=baseline, T2=septic shock, T3=after fluids resuscitation, T4=after ivabradine administration (for treated animals), T5=after noradrenaline administration. Wilcoxon signed test: *p-value<0.05, **p-value<0.01 with respect to T1, §p-value<0.05 with respect to T2 (Friedman test p-value<0.05). Mann-Whitney U test: $^{\#}$p-value<0.05 between the specified variables

B.4 DISCUSSION AND CONCLUSIONS

In this study we aimed at analyzing the difference in terms of cardiac efficiency, as assessed by the Buckberg index, in two populations of ivabradine-treated and sham septic shock pigs. The Buckberg index did not result significantly different between the two groups of animals and it was lower than baseline both during shock and after resuscitations in both populations. Thus, the administration of ivabradine drug did not ameliorate cardiac efficiency during septic shock in terms of the computed Buckberg index. However, the drug was effective in lowering the HR, as shown in figure B.2, and coherently with the literature.

The significant changes in hemodynamic variables such as PP and diastolic BP highlighted that the drug could also have an effect on the circulation; in fact, we observed an increase in aortic PP and a decrease in diastolic aortic BP in the treated group after the administration of ivabradine (time points T4 and T5). The reduced aortic BP indicates a decreased cardiac afterload which could lead to an increase in SV and a consequent elevation of PP, as observed in our treated animals. Moreover, the longer time for cardiac filling, due to the reduced HR, could also explain the increased SV.

These results shed light on the possible multiple beneficial effects of ivabradine administration in septic shock, which are not cardiac only but they also involve the circulation, such as reducing the HR, decreasing the cardiac afterload and increasing the SV. In these terms, we can affirm that the administration of ivabradine improved the cardiac efficiency of the treated animals.

The available data of this preliminary study did not allow to investigate the mechanisms responsible of the observed cardiocirculatory changes following ivabradine administration, but they underlined the importance of investigating the cardiocirculatory system as a whole in future studies.

C. LIST OF PUBLICATIONS

The complete list of published journal papers and conference proceeding contributions of the PhD candidate is here reported:

ISI Journal Papers

- CARRARA M, Herpain A, Baselli G, Ferrario M, "A mathematical model of dP/dt max for the evaluation of the dynamic control of heart contractility in septic shock", *IEEE Trans Biomed Eng.* 2019 Mar 12. doi: 10.1109/TBME.2019.2894333.

- CARRARA M, Babini G, Baselli G, Ristagno G, Pastorelli R, Brunelli L, Ferrario, M, "Blood pressure variability, heart functionality, and left ventricular tissue alterations in a protocol of severe hemorrhagic shock and resuscitation", *J Appl Physiol* (1985). 2018 Oct 1;125(4):1011-1020. doi: 10.1152/japplphysiol.00348.2018.

- CARRARA M, Bollen Pinto B, Baselli G, Bendjelid K, Ferrario M, "Baroreflex Sensitivity and Blood Pressure Variability can Help in Understanding the Different Response to Therapy During Acute Phase of Septic Shock", *Shock* 2018 Jul;50(1):78-86. doi: 10.1097/SHK.0000000000001046.

- CARRARA M, Baselli G, Ferrario M, "Mortality Prediction Model of Septic Shock Patients Based on Routinely Recorded Data", *Comput Math Methods Med.* 2015;2015:761435. doi: 10.1155/2015/761435.

- CARRARA M, Carozzi L, Moss TJ, de Pasquale M, Cerutti S, Lake DE, Moorman JR, Ferrario M, "Classification of cardiac rhythm using heart rate dynamical measures: validation in MIT-BIH databases", *J Electrocardiol.* 2015; Nov-Dec; 48(6):943-6. doi: 10.1016/j.jelectrocard.2015.08.002.

- CARRARA M, Carozzi L, Moss TJ, de Pasquale M, Cerutti S, Ferrario M, Lake DE, Moorman JR, "Heart rate dynamics distinguish among atrial fibrillation, normal sinus rhythm and sinus rhythm with frequent ectopy", Phy*siol Meas.*2015 Sep;36(9):1873-88. doi: 10.1088/0967-3334/36/9/1873.

Conference Proceedings

1 page

- CARRARA M, Aletti F, Baselli G, Ferrario M, "Mortality risk stratification in Septic Shock Patients: a data mining approach", *International Conference on Biomedical and Health Informatics* (BHI 2016)

- CARRARA M, Baselli G, Ferrario M, "Mortality prediction model of septic shock patients based on routinely recorded data", *14th International Conference on Complex Acute Illness* (ICCAI 2015)

- CARRARA M, Bollen Pinto B, Bendjelid K, Baselli G, Ferrario M, "Autonomic nervous system indices in the progression of organ failure in early septic shock patients", *15th International Conference on Complex Acute Illness* (ICCAI 2016)

- CARRARA M, Bollen Pinto B, Bendjelid K, Baselli G, Ferrario M, "Association between autonomic imbalance and recovery of organ function in early septic shock", *38th Annual International Conference of the IEEE Engineering in Medicine and Biology Society* (EMBC 2016)

- CARRARA M, Bollen Pinto B, Bendjelid K, Baselli G, Ferrario M, "The role of fluid accumulation in early septic shock: differences in ANS-driven response to therapy", *16th International Conference on Complex Acute Illness* (ICCAI 2017)

2 pages

- CARRARA M, Baselli G, Ferrario M, "Multiscale modelling of mortality risk in a septic shock population", *V congresso del Gruppo Nazionale di Bioingegneria* (GNB 2016)

4 pages

- CARRARA M, Baselli G, Ferrario M, "Mortality prediction in septic shock patients: towards new personalized models in critical care", *37th Annual International Conference of the IEEE Engineering in Medicine and Biology Society* (EMBC 2015).

- CARRARA M, Babini G, Baselli G, Ristagno G, Ferrario M, "Evaluation of the autonomic response during severe haemorrhage and resuscitation in anaesthetized pigs", *6th National Congress of Bioengineering* (GNB 2018)

Abstracts

- C CARRARA M, Bollen Pinto B, Bendjelid K, Baselli G, Ferrario M, "Different response to therapy in septic shock: an autonomic perspective", *40th Annual Conference on Shock* (SHOCK 2017)

- CARRARA M, Babini G, Luciani A, De Giorgio D, Staszewsky L, Baselli G, Ristagno G, Ferrario M, "A porcine model of severe haemorragic shock: evaluation of autonomic nervous system in blood pressure control", *17th Congress of European Shock Society* (ESS 2017)

- CARRARA M, Babini G, Baselli G, Ristagno G, Ferrario M, "The role of autonomic nervous system control of blood pressure during a protocol of severe haemorrhagic shock and resuscitation", *41st Annual Conference on Shock* (SHOCK 2018)

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