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NEW INSIGHT IN SEPSIS CAPILLARY LEAK SYNDROME:

α1AMPK, FROM THE COMPREHENSION OF KEY MOLECULAR MECHANISMS TO THE EXPLORATION OF A NEW THERAPEUTIC APPROACH

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AC	Actin cytoskeleton
ADP	Adenosine diphosphate
AICAr	5-Aminoimidazole-4-carboxamide ribonucleotide
AKI	Acute kidney injury
ARDS	Acute respiratory distress syndrome
AJs	Adherens junctions
Ang	Angiopoietin
AMP	Adenosine monophosphate
AMPc	3', 5'-cyclic adenosine monophosphate
AMPK	AMP-activated protein kinase
APC	Activated protein C
CaMKK-ß	Calcium/calmodulin-dependent protein kinase-ß
CDC42	Cell division control protein 42 homolog
Cox	Cyclooxygenase
Cx43	Connexin 43
CRP	C-reactive protein
DIC	Disseminated intravascular coagulation
EC	Endothelial cell
ECPR	Endothelial cell protein C receptor
eEF2	Eukaryotic elongation factor 2
eNOS	Endothelial nitric oxide synthase
FA	Focal adhesion
F-actin	Filamentous actin
G-actin	Globulous actin
GEF	Guanine exchange factor

GIV	G-alpha interacting vesicle associated protein
HCAEC	Human coronary arterial endothelial cells
HMEC	Human dermal microvascular endothelial cell
HMGB-1	High mobility group box 1
HSP27	Heat shock protein 27
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule 1
ICU	Intensive care unit
IEJs	Inter-endothelial junctions
IL	Interleukin
iNOS	Inducible NO synthase
INR	PT-international normalised ratio
LKB1	Liver kinase B1
LPS	Lipopolysaccharide
p38 MAPK	p38 mitogen-activated protein kinase
PAMPs	Pathogen associated molecular patterns
PRRs	Pattern recognition receptors
MAPK	Mitogen-activated protein kinase
MAPKAPK	Mitogen-activated protein kinase -activated protein kinase
MDCK	Madin-Darby canine kidney epithelial cells
MOF	Multi-organ failure
N-Cad	N-cadherin
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NFkB	Nuclear factor kappa B
NO	Nitric oxide
NOX	NAD(P)H oxidase
PAF	Platelet activating factor
PAI1	Plasminogen activator inhibitor, type 1
PAMPs	Pathogen-associated molecular patterns

PCT	Procalcitonin
PGI2	Prostacyclin
PRR	Pattern-recognition receptors
qSOFA	Quick sequential organ failure assessment
Rac1	Ras-related C3 botulinium toxin substrate 1
RhoA	Ras homolog gene family member A
ROCK	RhoA associated kinase
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
S1P	Sphingosine-1 phosphate
SEM	Standard error of the mean
Ser	Serine
SGLT2	Sodium-glucose cotransporter type 2
SGLT2i	Sodium-glucose cotransporter type 2 inhibitor
SIC	Sepsis-induced coagulopathy
siRNA	Small interfering RNA
SIRS	Systemic inflammatory response syndrome
sFLT-1	Soluble fms-like tyrosin kinase 1
SGLT	Sodium glucose cotransporter
SGLTi	Sodium glucose cotransporter inhibitor
SMC	Smooth muscle cell
SOFA	Sequential organ failure assessment
SPMs	Specialised proresolving mediators
TAB	Transforming growth factor a-activated kinase 1 binding proteins
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TLRs	Toll like receptors
TJs	Tight junctions
TNFα	Tumour necrosis factor α
Tyr	Tyrosine

VE-Cad	VE-cadherin
VEGF	Vascular endothelial growth factor
WHO	World health organisation
ZO-1	Zonula occludens-1

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INTRODUCTION

1 SEPSIS

1.1 STORY AND CONCEPT

Sepsis definition has continuously been reviewed in accordance with the characterisation of the medical history of the disease. The concept of sepsis was defined for the first time by Egyptians, with the Ebers Papyrus (1500 BCE) describing a disease developing from the intestine whose manifestations included suppuration and fever. The word *sepsis* then takes its origins in Greeks as denoting the rotting of flesh [1], before being precisely defined for the first time by Hippocrates (460-370 BCE) as the process of death and decay associated with illness, by opposition to what was called *Pepsis;* a decay that results in well-being, as do the fermentation of grapes to produce wine [2]. The concept of sepsis as a response of the host has emerged from different investigators of the 19th century, including Pfeiffer, Coley and Carswell [3].

The need to improve sepsis syndrome criteria and to develop new mediator-targeted therapies led the American College of Chest Physicians and the Society of Critical Care Medicine to the organisation of the first Sepsis Definitions Conference in 1991 [4]. This led to the emergence of the concept of systemic inflammatory response syndrome (SIRS), recognising that sepsis clinical syndrome could be observed in non-infectious diseases.

The second Sepsis Definitions Conference occurred in 2001, and proposed an update of SIRS criteria, as well as a template for a novel stratification system for sepsis – the predisposition, insult, response, organ dysfunction (PIRO) model [5]. Besides the prognostic aspects, the PIRO model laid the groundwork for a more individualised treatment which considers the characteristics of the patient to predict the potential response to specific therapies.

More recently, the Sepsis-3 consensus took place in 2016, and was conducted by a task force whose approach aimed to increase the focus on organ dysfunction, as well as to emphasise biological concepts that remain incompletely understood, such as genetic influences and cellular abnormalities. This led to the last version of sepsis definition: "*a life-threatening organ dysfunction caused by a deregulated host response to infection"* [6]. Thus, Sepsis-3 criteria, define sepsis as the presence of an infection combined with an acute change of 2 or more points in Sequential Organ Failure Assessment (SOFA) score (an objective tool for stratifying the risks of organ dysfunction, whose variables are presented in Figure 1). The declaration further defines septic shock as "*a subset of sepsis in which profound circulatory failure, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone*"[7]. Another major advance of the sepsis-3 consensus relies on the development and validation of the quick SOFA score (qSOFA, see variables Figure 1), a simple risk stratification tool constituted by three clinical criteria to help clinicians identify infected patients at risk of sepsis, disregarding the SIRS criteria. Finally, the consensus also allowed the raising of interesting points:

(1) The primacy of the nonhomeostatic host response
(2) The previous excessive focus on inflammation
(3) The determining role of organ dysfunctions for the prognosis
(4) The involvement of the fundamental changes in cellular metabolism and function in the evolution towards septic shock
(5) The paradoxal combination of both hyperinflammation and immunosuppression

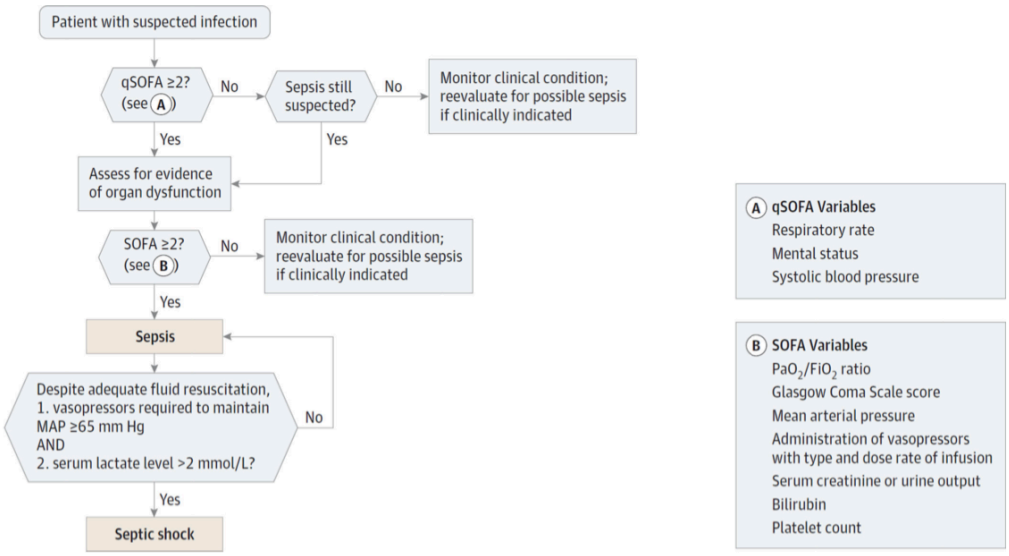

Figure 1 - Operationalization of clinical criteria identifying patients with sepsis and septic shock. SOFA indicates Sequential Organ Failure Assessment score; qSOFA, quick SOFA; MAP, mean arterial pressure. Taken from [6].

Although these considerations lead to a better characterisation of the disease, sepsis remains considered as a complex syndrome based on an uncertain pathobiology. At the bedside, Sepsis-3 updates are not generally accepted, and divide clinicians' opinions. The particular qSOFA measure as a substitute for SIRS criteria is highly debated, and despite being accepted to be marginally superior in predicting the high risk category of septic patients [7, 8], allows judgement for the identification of suspected sepsis [9-12].

The discussion on sepsis characterisation remains active in the critical care community, attesting that the definition process remains incomplete and will undoubtedly be refined with the further understanding of sepsis pathophysiology.

1.2 EPIDEMIOLOGICAL CONSIDERATIONS

Sepsis is a major public health concern [13]. It is considered as a leading cause of mortality and critical illness worldwide [13, 14], and has been recognised as a global health priority by the World Health Organization (WHO) in 2017. The true burden of the disease has long been difficult to determine with precision, particularly due to the lack of international epidemiological studies and the variability in defining and applying the diagnostic criteria. The first comprehensive global report of sepsis epidemiology, published in January 2020, reported 48,9 million cases of sepsis and 11.0 million sepsis-related deaths in 2017 worldwide [15]. Although these data must be considered with analytical constraints [16], they appear to be twice as thought previously, and make sepsis accountable for 19,7% of all global deaths, representing 20 deaths every minute.

The evolution of sepsis incidence during the past decades is discussed. Although most studies report a clear increase [17-20], some describe a relatively stable evolution [21], or even a decreasing incidence [15]. These discrepancies may potentially be explained by reporting bias caused by unclear sepsis definitions [22], changes in diagnostic codes or reimbursement-favourable coding [23], evolution of the recognition of the disease [6], or even extrapolation of the sepsis data sources from high to low income countries [16]. In the next decades, the aging of population – and associated comorbidity burdens – and growing number of campaigns increasing awareness and screening of the condition may certainly represent factors of significant increase in the incidence and mortality of sepsis [24, 25].

Sepsis incidence is now recognised to be affected by several factors. Age influences the onset of sepsis as well as its related mortality (see figure 2), with two major peaks of incidence occurring in early childhood and among adults older than 65 years [15]. Gender also seems to have an influence, as females present higher age-standardised sepsis incidence than men, while males are reported to present higher age-standardised

sepsis-related mortality. Location is another influencing factor, as countries with low socio-demographic Index (SDI) are described to be much more affected. This relationship is even stronger for mortality than for incidence. Independently, race may represent another influence, not only due to disparities in access to healthcare but also because of influences of genetic backgrounds [26, 27].

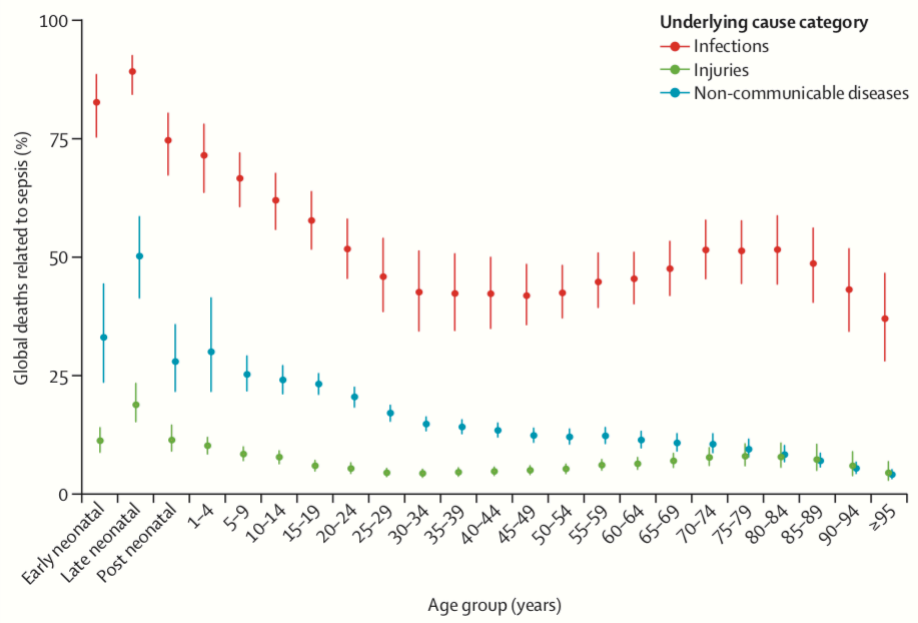

Figure 2 – Percentage of all sepsis-related death in each underlying cause category, by age group and for both sexes, in 2017. Bars represent 95% uncertainty intervals. Taken from [15].

At the world level, the first underlying cause of sepsis during the past two decades was diarrhoea, while the most common underlying cause of sepsis-related death was lower respiratory infections [15].

Despite the fact that notable progresses in the understanding of the disease and its pathophysiology have improved overall sepsis survival, long term sepsis mortality

remains important, with rates reaching 10% to 40%, depending on the severity of the disease [13, 18, 20]. The last epidemiological analysis of sepsis mortality reported a decrease of 52,8% from 1990 to 2017 [15]. This evolution had previously been demonstrated by several studies, although sometimes reported in lesser proportions [28, 29]. Anyway, the prominent contribution of sepsis in hospital mortality remains well recognised, and concerns between a third and a half of all in-hospital deaths in northern countries [19, 30-32]. This is particularly explained by the lack of specific treatment, since sepsis management still rely on supportive therapies. Indeed, none of the multiple therapeutic clinical trials in sepsis has yet yielded to an FDA approval [33].

In addition to high mortality, sepsis dramatically affects the quality of life of survivors [34-36]. As detailed in the end of the chapter (see section 1.4.3), several cognitive and functional disabilities are at the origin of a shift from acute care hospitals to chronic care facilities, and the emergence of a “post-sepsis syndrome”[37].

Finally, sepsis burden has major financial costs. These do not only include the hospitalisation care, but also the chronic use of healthcare resources after discharge and readmissions, both resulting from the multiple complications of sepsis [32, 38]. Sepsis has even been recognised as the costliest condition, far ahead of congestive heart failure and acute myocardial infarction [32].

Globally, sepsis epidemiological data must be interpreted with caution. Several factors - such as subjective clinical definitions, variable training of the staff in recognising sepsis, heterogeneous presentations, or increasing tendency to support sepsis awareness - induce major biases that may favour the inclusion of less severely ill patients in the number of sepsis case over time, and lead to a perceived increase of incidence and decrease of mortality [39]. Nevertheless, sepsis remains a quality-of-life and cost-burden healthcare issue making clinicians, policy makers, and patients agree on the urgency of considering the need for better prevention and management [15]. An easy

way to start may be to overcome the low public awareness [40] deplored by the Sepsis-3 Consensus [6].

1.3 PATHOPHYSIOLOGY

Sepsis syndrome is highly complex, and is still not fully understood [25, 41, 42]. The main sequence of its pathophysiology - infection, immune response, micro and macro-vascular failure, and organ dysfunction - is now generally accepted, and will structure this chapter. However, the precise mechanisms involved are fraught with uncertainties, and remain an active area of scientific research. In recent years, increasing importance has been attributed to metabolic and neurophysiological aspects of the processes underlying cellular bioenergetics alteration and the failure of epithelial and endothelial barriers [43]. Of note, fluid balance management and septic capillary leak syndrome occupy an important part of the sepsis research field [42, 44, 45].

1.3.1 SOURCE OF INFECTION

Sepsis may be triggered by virtually any infecting organism. The infection from which it arises can be classified as community- or hospital-acquired. At the world level, the most common sepsis aetiology during the last two decades was diarrhoeal disease and lower respiratory infections [15]. In addition, noncommunicable diseases are arising, with one third of sepsis cases and nearly half of all sepsis-related deaths due to underlying injury or chronic disease in 2017 [15].

In high-income countries (HICs), bacterial infections are responsible for the large majority of sepsis cases [46], while causative microorganisms are not identified in a large proportion of patients [30, 46]. The primary organisms identified in positive blood cultures of septic patients include *Esherichia coli*, *Staphylococcus aureus*, *Streptococcus A*, *B*, or *pneumonia*, and *Enterobacteriaceae faecalis* [46-48].

In Low- and middle-income countries (LMICs), sepsis origins include greater proportions of mycobacterial, viral and parasite infections, such as rickettsiosis, malaria, or dengue [49-51].

1.3.2 IMMUNE RESPONSE

The loss of loco-regional quarantine of pathogens exposes microbial components to the immune system and triggers an immune cascade (see Figure 3). Initially aimed at clearing pathogens, repairing injured tissue and resolving infection, the dissemination of inflammatory response can lead to its deregulation. This operates as a double-edged sword, and constitutes a determinant process in the onset of sepsis. For didactic reasons, the immune response will be theoretically dissociated in 3 phases - immune storm, early damage, resolution -, although they obviously present overlapping sequences.

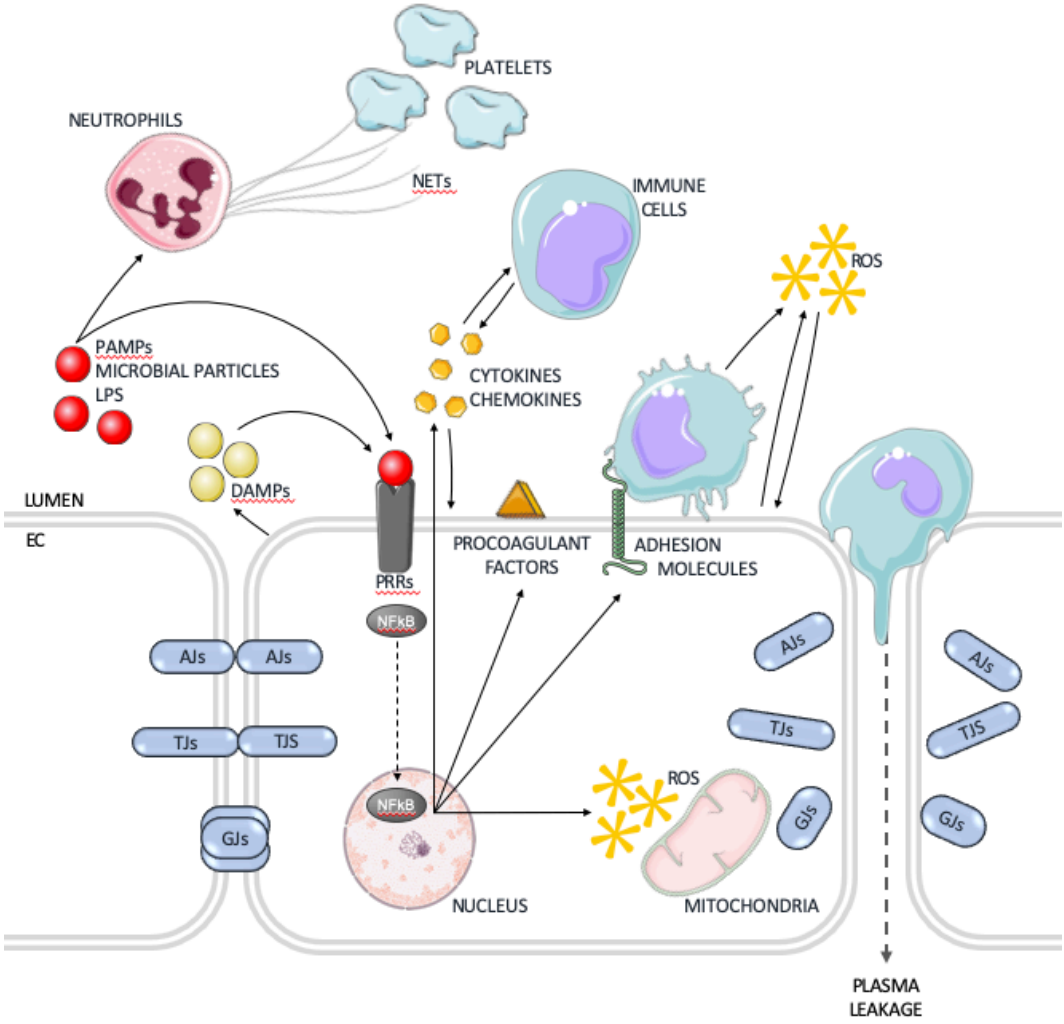

Figure 3 – Major determinants of the immune cascade in sepsis. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) components activate both immune and endothelial cells. They are recognised by pattern-recognition receptors (PRRs) and induce activation of transcription factors such as NFkB, leading to pro-inflammatory mediators production, which is self-perpetuating. Cytokines participate to immune and endothelial cells activation. Activated neutrophils produce neutrophil extracellular traps (NETs), which participate to platelets and immune cascade activation. Chemokines recruit immune cells from circulating blood and bone marrow. Endothelial adhesion molecules bind immune cells, support their activation and diapedesis. Extracellular reactive oxygen species (ROS) are produced by different cell types including immune and endothelial cells,

and further augment the inflammatory response. Pro-coagulant factors participate to platelet recruitment and activation, trapping the different actors and also exacerbating inflammatory processes.

1.3.2.1 IMMUNE STORM

The acute phase of sepsis is characterised by a high activation of the innate immune system, including macrophages, monocytes, granulocytes, natural killer and dendritic cells. Its activation is triggered by sensing the invading microorganisms through detecting highly conserved microbial exogenous signals called pathogen-associated molecular patterns (PAMPs), as well as endogenous production of damage-associated molecular patterns (DAMPs). This occurs via pattern-recognition receptors (PRRs), such as toll-like receptors (TLR), C-type lectin receptors (CLRs), or NOD-like receptors (NLRs) [52]. PRRs are expressed by different cell types (cells from the immune system, as well as endothelial cells or myocytes), in which their binding to PAMPs and DAMPs transduces a signal activating pro-inflammatory transduction factors such as nuclear factor kB (NFkB). NFkB activation results in a major release of cytokines. The main role of these low molecular weight glycoproteins is to promote defence mechanisms against microbial agents, such as the stimulation of innate and adaptive immune systems, endothelial cell-leukocyte adhesion, complement or clotting cascades, tissue repair, and the resolution of the inflammatory response. Because of various host or pathogen characteristics that are still not fully understood, this immune reaction may become improperly regulated, leading to exorbitant cytokine release (often referred to as a “cytokine storm”) and excessive inflammatory response [53].This cytokine storm encompasses the “early” phase of pro-inflammatory cytokines (i.e. tumor necrosis factor α (TNFα), interleukin (IL) 1B, IL17) which stimulate the “later” pro-inflammatory cytokines (i.e. IL6, IL8), and anti-inflammatory cytokines (i.e. IL4, IL10). Traditionally, this storm has been considered as a two-phase process, where the initial overwhelming pro-inflammatory response gives way to immunosuppression and immune anergy, often

resulting in secondary infections [54]. More recent considerations, some of which are based on genomic analysis of tissue samples from septic patients [55], propose pro- and anti-inflammatory phases to overlap, but varying in intensity according to various factors belonging both to the host – genetics, comorbidities – and the pathogen – type, virulence and burden [56-61]. Nevertheless, the maintenance of the homeostatic balance between pro- and anti-inflammatory pathways is of major importance, since imbalance between these processes is now recognised as a driving force in the pathophysiology of sepsis and its complications.

1.3.2.2 EARLY DAMAGE

Imbalanced pro- and anti-inflammatory responses modulate the physiological functions of virtually all populations of blood cells, causing a wide range of biological damage. Together with leukocytes and platelets, activated endothelial cells perpetuate immune, coagulation, and oxidative disorders. Despite their initial role consisting in containing and eliminating the pathogen, these mechanisms accelerate a vicious cycle of self-reinforcing damaging defence response. Specifically, activated endothelial cells increase the expression of cytokines, adhesion molecules, chemokines, and procoagulant factors [62]. Endothelial dysfunction is also typically characterized by loss of barrier function and disturbed vasoregulation. Innate immune cells present increased apoptosis, cytokine secretion, and decreased cytotoxic functions [57]. Among them, activated neutrophils release networks of DNA extracellular fibers, called neutrophils extracellular traps (NETs), which increase procoagulant state and are correlated with sepsis severity and organ dysfunction [63]. Adaptive immune cells also present reduced lifespan and activation state, generally resulting in decreased lymphocytes count and immune anergy, as reviewed by Hotchkiss *et al* [57]. Interestingly, endothelial barrier dysfunction has been incriminated in the decrease of circulating T-cells [41].

Altogether, these processes dramatically affect the structure and function of the vascular system, primarily affecting the capillary bed. Further details concerning the mechanisms involved are discussed in the section *1.3.3*, dedicated to the description of sepsis microvascular dysfunction.

1.3.2.3 TOWARDS RESOLUTION

Some patients spontaneously recover from this acute phase – which may then be considered as a physiological response to infection. Others (often showing extreme ages or comorbidities) progress to chronic immune dysfunction. In these patients, the immune system's ability to clear infections or repair injured tissues is altered, eventually promoting microvascular alterations [64]. Immune cell anergy, excessive anti-inflammatory cytokines, altered autophagy, and decreased efferocytosis (the phagocytic clearance of dying cells), are important mechanisms in this process [57, 65, 66]. Specialised pro-resolving mediators (SPMs), which include lipoxins and resolvins, also seem to play important roles in orchestrating the resolution of tissue inflammation [67]. SPMs are produced from essential fatty acids in a lipoxygenase (LOX)-dependant manner in various cell types. Their actions paradoxically allow to dampen inflammation while improving clearance of bacterial infection, promoting adequate host response and restoring tissue homeostasis after infection. This occurs notably by limiting neutrophils infiltration and enhancing the uptake of apoptotic cells by macrophages. Interestingly, exogenous SPMs administration could lower the dose of antibiotics required for the clearance of bacterial infections [68, 69], and even improve the survival of septic mice [70].

A better understanding of septic immune response remains a major challenge. Although this has never been objectified by solid study designs, it is strongly believed that genetic variability of the host is one of the major factors affecting the function of the immune system during sepsis, and that the impact of genetic variation on the sepsis

immunopathogenesis can influence predisposition to the disease, as well as its prognosis [41]. Genome-wide association (GWA) studies will help to validate these allegations.

1.3.3 MICROVASCULAR DYSFUNCTION

By disseminating throughout the body via the blood compartment, the dysregulated response to infection dramatically affects the microvascular system. Appropriate microvascular response to infection implies, as described in the first century, calor (heat), rubor (redness), dolor (pain) and tumor (swelling). These external signs reflect vascular hyperpermeability, changes in blood flow and coagulation, and recruitment of immune cells, all physiological and appropriate processes aiming to fight and contain the invading pathogen [71]. In sepsis, these processes disseminate, spread damage signals throughout the body and become deregulated. The endothelium thereby not only fails in executing its intrinsic functions, but also actively amplifies key processes of sepsis pathophysiology. The resulting microcirculatory dysfunction is characterized by (i) loss of barrier function, (ii) a procoagulant state, and (iii) vasomotor tone disorders [72], all contributing to impaired perfusion of small blood vessels (see Figure 4). These alterations together accentuate tissue hypoxia, directly impacting organ functions and outcome of septic patients [71, 73-75]. Supporting this notion, Rivers *et al* reported in the early 20th century that prompt optimization of blood flow had a beneficial impact on sepsis survival [76]. It should be noted that although organ-specificities undeniably influence endothelial function, the present chapter will discuss general endothelial concepts, with particular emphasis on permeability aspects.

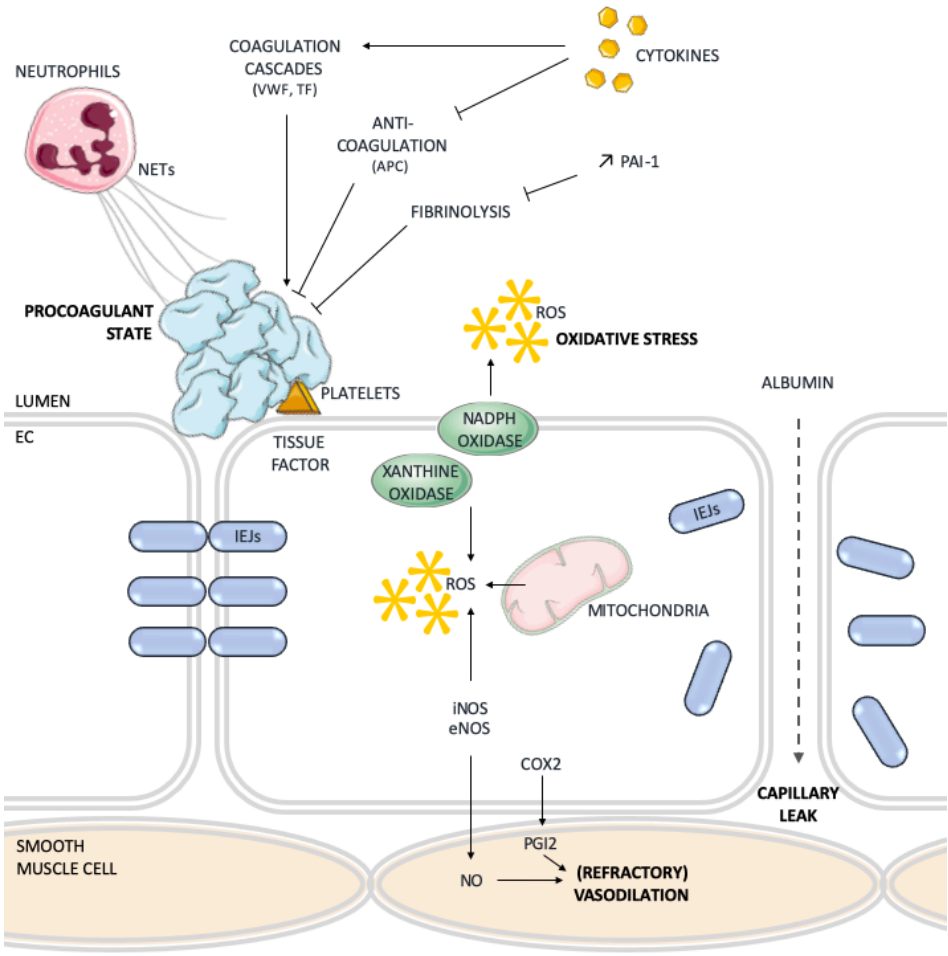

Figure 4 – Main determinants of microvascular dysfunction characterising sepsis. Endothelial cells (ECs), neutrophils and platelets are activated in response to the immune cascade described in Figure 3. Specifically, ECs undergo multiple phenotypic and functional changes altering endothelial functions. Stress signals transduction destabilise interendothelial junctions (IEJs), resulting in loss of endothelial barrier function. Increased expression of tissue factors (TF) and von Willebrand factor (VWF) activate coagulation cascade and platelets. This phenomenon is exacerbated by impaired anticoagulation pathways - notably due to activated protein C (APC) inhibition and reduced fibrinolysis resulting from increased plasminogen activator inhibitor (PAI-1) expression. Abnormal activation of nitric oxide synthases (iNOS and eNOS) and cyclooxygenase 2 (COX2) induce formation of high amounts of nitric oxide (NO) and prostacyclin (PGI2), resulting in hyporeactive vasodilation. Large amounts of ROS are generated

by NADPH oxidase, xanthine oxidase, eNOS and iNOS uncoupling, and mitochondrial dysfunction. These exceed compensating anti-oxidant mechanisms, which leads to oxidative stress.

1.3.3.1 THE MAJOR ISSUE OF VASCULAR HYPERPERMEABILITY

Vascular leakage, often referred to as capillary leak syndrome, is an integral component of the sepsis response [72, 77, 78]. More importantly, it has been recognised as an independent prognostic factor for sepsis outcome [45, 79] (see Figure 5). In physiological conditions, the endothelium acts as a semi-permeable membrane controlling the transfer of fluids, macromolecules, solutes and gases between the blood and the interstitial space. In response to the septic storm, the endothelial barrier is disrupted and allows the leakage of plasma and macromolecules in the underlying tissues. This vascular hyperpermeability results in the widespread accumulation of protein-rich fluid – which self-aggravates by an osmotic mechanism - in interstitial spaces, called oedema. Albumin, the main determinant of plasmatic oncotic pressure [80], is a decisive actor in this process. In addition to its dramatic hypovolaemic consequences, vascular leakage impairs microvascular blood flow. Oedema formation enhances the distance required for oxygen diffusion, increases interstitial pressure and compresses capillaries, resulting in altered oxygen delivery in peripheral tissues [78]. As described in section *1.4.2.1*, this process is exacerbated by the therapeutic fluid resuscitation administered in order to support arterial pressure and peripheral perfusion [81]. The subsequent damages directly affect organ functions and sepsis outcome [45, 82], by different processes:

(1) In the heart, myocardial oedema appears as a major contributor of the septic cardiomyopathy, notably by affecting the diastolic function due to increased myocardial stiffness [83-85].
(2) In the lung, vascular hyperpermeability combines with epithelial barrier dysfunction to flood the alveoli, inducing perfusion-ventilation mismatch,

arterial hypoxaemia, and altering lung compliance [86]. The resulting acute respiratory distress syndrome (ARDS) requires mechanical ventilation, itself inducing further lung injury, and increasing systemic inflammation [87].

(3) In the nervous system, the compromised blood-brain barrier fosters encephalopathy because of perivascular oedema, passage of immune cells, toxins and cytokines into the brain, and oxidative stress [88].

(4) In the liver, vascular hyperpermeability is largely characterised by heterogeneity of the microcirculatory blood flow, ultimately resulting in hepatocellular injury and dysfunction, with consequences including cholestasis - itself favouring bacterial translocation and impairment of the transport and processing of enteric pathogen lipids, further stimulating systemic inflammation - and coagulopathy [89, 90].

(5) In the gut, loss of vascular barrier function combines with epithelial hyperpermeability to enter a vicious cycle of bacterial translocation, gut injury by luminal content including activated pancreatic enzymes leading to auto digestion, and worsening of local and systemic inflammation [91, 92].

(6) In the kidney, oedema formation in the parenchyma associated with hypotension and hypovolaemia are proposed as determinant factors in the onset of acute kidney injury [93, 94].

(7) In the immune system, endothelial barrier breakdown has been associated with increased loss of lymphocytes, contributing to immune dysfunction, and higher susceptibility to opportunist infections [41].

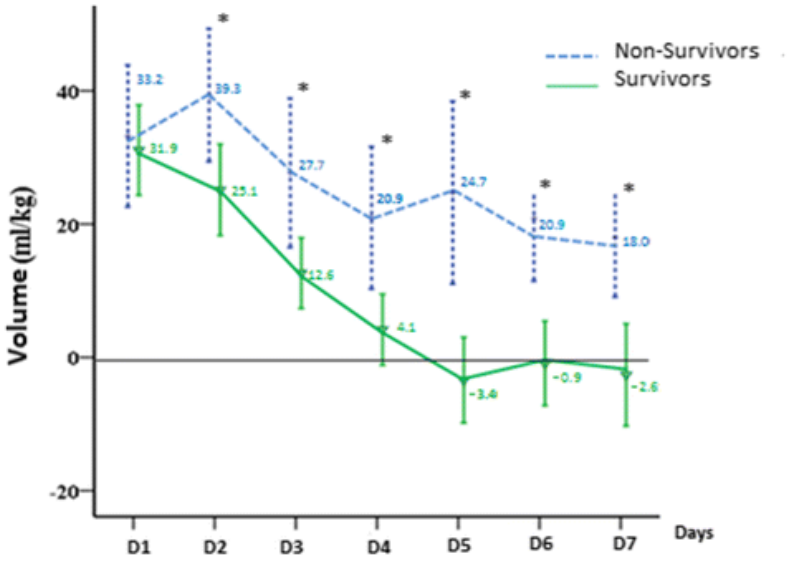

Figure 5 – Mean fluid balance (ml/kg) in survivors and non-survivors over the 7 days after sepsis onset. *Statistically significant difference at the $p<0.05$ level between survivors and non-survivors. Taken from [79].

Accordingly, a growing body of evidence highlights the beneficial effects of enhancing the stability of the endothelial barrier on sepsis outcome [62, 83, 95-99]. This makes the protection against sepsis capillary leak syndrome an expanding area of research.

Detailed molecular mechanisms controlling the endothelial barrier function are described in Chapter 2 of this introduction.

1.3.3.2 OTHER FACTORS OF MICROVASCULAR DYSFUNCTION

Coagulation, vasomotor tone, and oxidative stress disorders are also involved in microvascular dysfunction during sepsis and will be concisely mentioned here (for review, see [100, 101]).

a. Coagulation

Coagulation disorders almost invariably arise during sepsis, and are largely due to endothelial dysfunction. Excessive activation of the coagulation cascade, inhibition of natural anticoagulant pathways, and decreased endogenous fibrinolysis all converge towards a prothrombotic state. Mechanistically, Tissue Factor (TF), von Willebrand Factor (vWF), Activated Protein C (APC), or Plasminogen Activator Inhibitor type 1 (PAI-1), are key proteins whose production by the endothelial cell or exposure at cell surface are deregulated during sepsis [82, 101]. The subsequent coagulopathy may vary from infra clinical signs to diffuse microthrombosis or Disseminated Intravascular Coagulation (DIC), and plays a critical role in the progression of the disease by aggravating the microvascular alterations.

b. Vasomotor Tone

Vasomotor tone deregulation is another important feature of sepsis associated microvascular dysfunction, almost invariably characterised by vasodilation and decreased vascular smooth muscle reactivity [102]. Excessive Nitric oxide (NO) and peroxinitrites formation, increased prostacyclins synthesis, and deficiency of vasopressin are the main mechanisms involved in this dysregulation [102, 103]. The resulting loss of systemic vascular resistance importantly alters capillary blood flow and tissue perfusion, promoting shock. As already mentioned, the hypotension associated with this process is primarily managed by intravascular volemisation.

c. Oxidative Stress

Defined as an imbalance between reactive oxygen/nitrogen species (ROS/RNS) production and antioxidative defences, oxidative stress is mainly triggered by inflammatory storm and hypoxia during sepsis [104]. The production of ROS/RNS by

immune and endothelial cells is increased by mechanisms involving mitochondrial dysfunction, NO synthases (eNOS and iNOS) uncoupling, and activation of xanthine oxidase and nicotinamide dinucleotide phosphate (NADPH) oxidase. Excessive ROS/RNS have consequences on both the endothelium and the surrounding environment, causing cellular injuries (DNA and proteins oxidation, lipids peroxidation,…), mitochondrial dysfunction, or even apoptosis. These damages, by generating ROS/RNS themselves, pursue the self-sustaining lesion process that characterises sepsis. It is particularly the case with mitochondrial function, which is directly affected by ROS/RNS and whose impairment is a major mechanism of ROS/RNS production [105]. Of note, the involvement of mitochondrial dysfunction in the pathogenesis of sepsis-induced organ failure is increasingly recognised [106, 107].

In conclusion, coagulation, vasomotor tone, and oxidative stress disorders all combine with vascular barrier breakdown to alter microcirculatory flow and organ perfusion, which results in organ dysfunction. At the heart of the concept of sepsis, injured tissues contribute to the cycle of injury by releasing stress mediators, further damaging endothelial cells and the microcirculation.

1.3.4 ORGAN FAILURE

Organ failure is a continuous process that critically complicates sepsis pathophysiology. All organs can be concerned, and present damages ranging from hardly detectable to completely irreversible. The important crosstalk between the systems favours the evolution towards multiorgan failure (MOF), a critical condition dramatically affecting sepsis outcome [108-110].

The specific underlying mechanisms leading to MOF remain incompletely elucidated, but certainly involve a combination of haemodynamic and metabolic dysfunctions (see Figure 6).

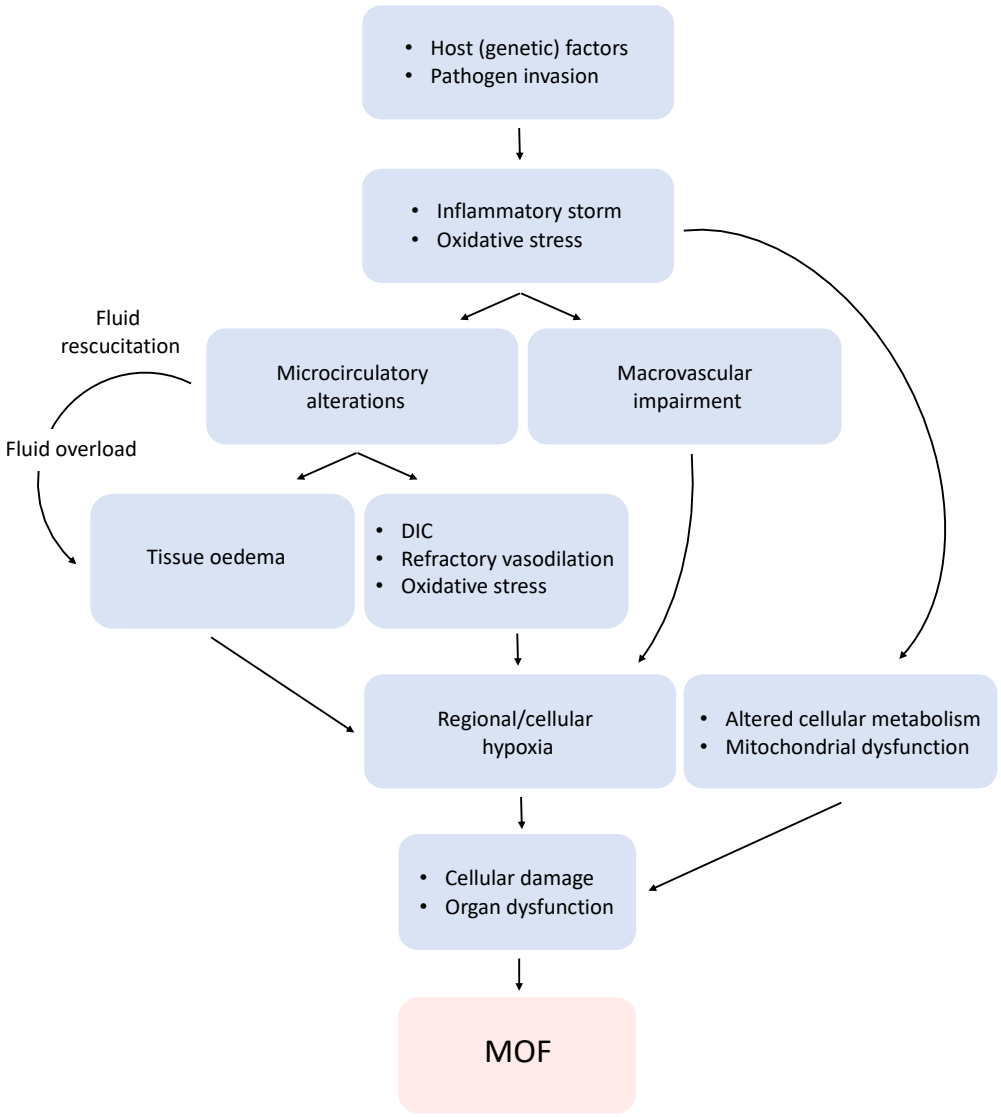

Figure 6 – Elements of organ failure pathophysiology in sepsis injury. Multiple factors can combine to induce cellular damage and subsequent (multi)organ failure in sepsis. Altered micro-perfusion, caused by microvascular alterations and aggravated by macrovascular failure, causes cellular hypoxia in peripheric tissues. Furthermore, cellular damage might also be a consequence of impaired cellular metabolism and mitochondrial dysfunction, although the specific contributions of these mechanisms need to be clarified. Due to major crosstalk and inter-organ dependence, organ dysfunction may rapidly affect other systems, and favour evolution towards multiorgan failure (MOF). DIC, disseminated intravascular coagulation.

First, it should be noted that cardiovascular impairment, even more than any other organ dysfunction, directly contributes to MOF. Indeed, commonly characterised by hypovolaemia and reduced vascular tone and ultimately coupled with myocardial depression, septic macrovascular alterations directly result in arterial hypotension, and altered tissue perfusion.

On the other hand, septic patients can develop MOF in the presence of normal systemic hemodynamic and blood oxygenation parameters, highlighting a critical role of the microcirculation in this process [54, 111]. Endothelial dysfunction and the subsequent disruption of microcirculatory homeostasis are indeed considered as major triggers of MOF [62, 71, 111]. Decreased capillary density and alterations of capillary perfusion, which typically characterise sepsis [75], result in functional shunting and ultimately tissue hypoxia, a predominant factor in organ dysfunction [112].

Finally, the hypothesis of “cytopathic hypoxia” has been increasingly considered in the past decades. This theory suggests that the compromised cellular bioenergetics is due to a disability of cellular oxygen utilisation rather than oxygen delivery [104, 106, 113, 114]. Among the arguments supporting this idea, clinical trials reported that sepsis non-survivors presented lower mitochondrial respiratory transcripts abundance, lower muscle ATP content, and normal or even higher muscular oxygen tension than survivors [115-117]. These data, however, probably vary depending on temporal and organ dependent factors [118].

Globally, sepsis induced organ dysfunction might be considered as a result of a cellular hibernation response, initially implemented in order to prevent ATP depletion and allow recovery [106]. Mitochondrial genesis seems to represent a factor of major importance in this paradigm, favouring recovery of mitochondrial respiration process [119], improvement of organ function, and eventual survival [115]. This theory finally suggests that the ability of a septic patient to survive MOF may depend on the ability of

its cells and mitochondria to activate the recovery mode at the most convenient time [115], beyond receiving adequate oxygen supply.

1.4 MANAGEMENT AND OUTCOMES

The clinical and biological patterns of sepsis greatly vary depending on the affected individuals. Age, underlying comorbidities, concurrent injuries and medications, and site of infection, greatly influence the signs and symptoms that are part of the host response [25, 58, 120]. This heterogeneous onset of the pathology renders sepsis condition difficult to recognise and diagnose (see Figure 7). Though, the early nature of diagnostic and support is now established as essential for effective management [121]. Sepsis therefore remains a major medical challenge despite the increasing improvement in the quality of care and the important efforts provided to better characterise the pathology.

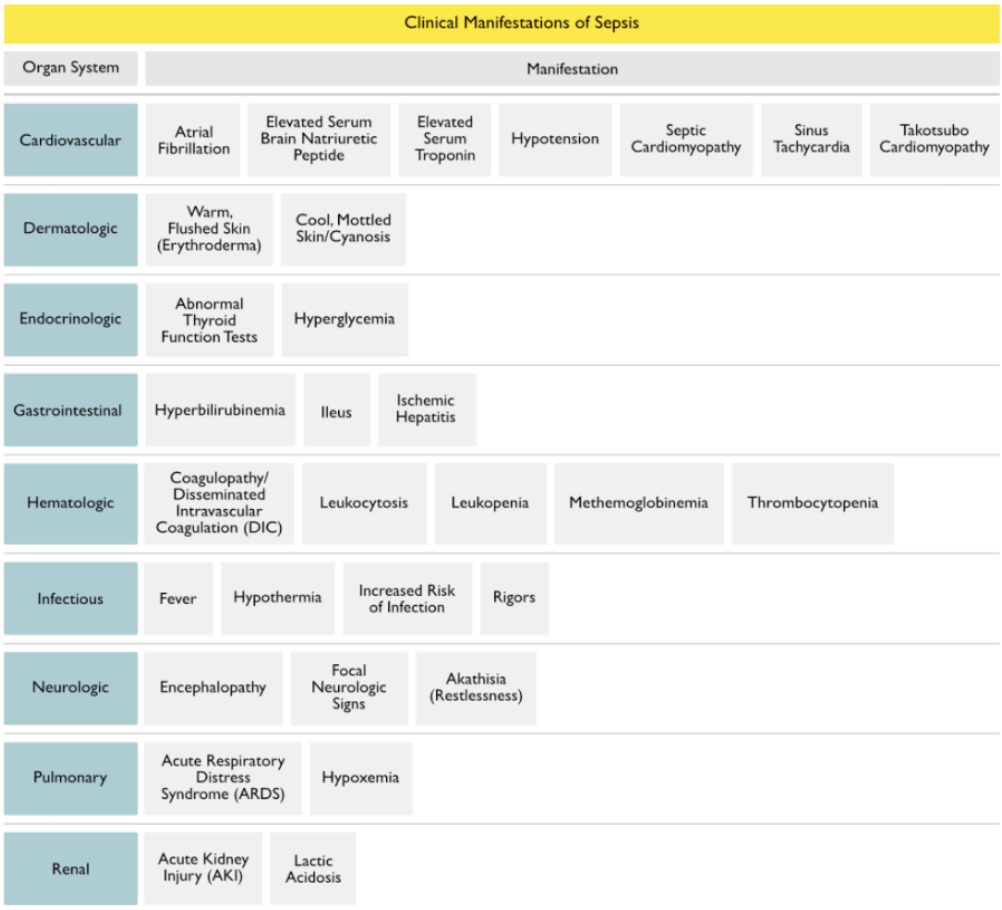

Figure 7 – Clinical manifestations of sepsis. Taken from https://www.mdnxs.com/topics-2/pulmonary-and-critical-care/sepsis.

1.4.1 DIAGNOSTIC TECHNIQUES

Gold standard diagnostic tests for sepsis are lacking [6, 122]. Sepsis diagnosis relies on a constellation of clinical signs suggesting organ dysfunction in a patient with declared or suspected infection. The evocative clinical presentation, which depends on several factors as detailed above, generally include general malaise and unspecific signs, such as fever (or hypothermia), tachycardia, tachypnoea, or altered mental status. Arterial hypotension, mottled skin, increased capillary refilled time, oliguria, or impaired gas

exchanges are indications of impaired organ perfusion. In order to standardise organ failure identification and facilitate diagnosis, the task force of the “sepsis-3 consensus” proposed the use of the SOFA score to help characterising the extent of organ failure in septic patients [6]. The SOFA score is not necessary for the management of sepsis patients, but is particularly recognised for its prognostic value; an increase of two points or more is associated with a significant increase of the mortality [109, 110].

Given the unspecific nature of the clinical picture, laboratory tests are useful to complement the clinical evaluation.

1.4.1.1 ROUTINE

The medical evaluation is mainly based on laboratory and radiological tests, which aim to confirm the infection, identify the source, and assess organ function.

Blood cultures, which represent the demonstrable evidence of systemic infection, should be collected promptly - ideally prior to the administration of antibiotics. The blood test should also include complete formula, C-reactive protein (CRP), coagulation, liver enzymes, and urinary function. Furthermore, serum lactate is a crucial marker of disease severity, considered as an indicator of inadequate tissue perfusion and recognised as a clinical criteria for septic shock, with important prognostic value and potential interest in guiding resuscitation strategy [121, 123]. Procalcitonin (PCT), although lacking in standardised immunoassays [124], is another biomarker of interest, especially in limiting the duration of antibiotherapy [125, 126]. Finally, prothrombin time and platelet count are important markers for DIC diagnosis, which is correlated with poor sepsis outcome [127].

An additional assessment is necessary to identify the source of infection, to put any samples in culture, and to allow targeted anti-infectious measures. These can include urine collection, chest radiography, abdominal ultrasound, and lumbar puncture.

1.4.1.1 NOVEL BIOMARKERS

Many new septic biomarkers are currently being investigated, with the aim of improving diagnostic criteria, better evaluating prognosis, and, eventually, helping to monitor therapeutic response. Some of them raise hope for potential clinical application. Among them, Soluble CD14 subtype (sCD14-ST), also known as presepsin, is associated with bacterial phagocytosis and may promise improvement in the differentiation of bacterial sepsis from other inflammatory conditions, as well as in the differentiation between sepsis severity groups [128]. Other proposed molecules include soluble urokinase plasminogen activator receptor (suPAR, pro-inflammatory activation of the immune system) [129], syndecan-1 and heparan sulphate (glycocalyx component) [130], neutrophil CD64 (nCD64) [131] or Interleukin-6 (IL-6, a pro- and anti-inflammatory cytokine)[132]. Interestingly, several molecules involved in vascular permeability regulation have also been highlighted, mainly for their potential prognostic values. Of those, angiopoietin 1 and 2 (Ang1 and Ang2) [82, 133], vascular endothelial growth factor (VEGF) [82], soluble vascular endothelial growth factor receptor 2 (sVEGFR2) [129], soluble fms-like tyrosine kinase 1 (sFLT-1) [82], heparin binding protein (HBP) [134], endothelial cell–specific molecule 1 (endocan), and thrombomodulin [135] seem to generate considerable interest.

Investigations are ongoing and continue to identify new potential biomarkers each year. The methodology, however, sometimes seems questionable since rarely based on standardised measurement methods, reproducible diagnostic criteria, and large repeated studies. The development of a more rigorous, standardised methodology should help to provide valuable, clinically relevant implications [136]. Moreover, it seems unlikely that one single molecule will provide information helping all diagnosis, prognosis, and therapeutic guidance. Of major interest, technologies allowing the measurement of multiple markers are under development, sometimes considering

results at the bedside, without laboratory requirements [137]. This would represent a major advance in the personalisation of patient monitoring, and eventually therapeutic management.

1.4.2 MEDICAL SUPPORT

Sepsis is a medical emergency. Proper identification and screening facilitate prompt intervention, which is of major importance for improving the outcome of septic patients [121, 138, 139]. Mechanistic insights have so far failed in the development of specific drug treatments [33]. Actual management of the septic patient thereby relies on infectious control and supportive therapies, which aim to relieve the organs in the fulfilment of their functions, allowing the body to overcome the acute phase, and recover.

1.4.2.1 MANAGEMENT OF THE SEPTIC PATIENT

A driving characteristic of the management of septic patients is that they require prompt, detailed initial assessment and ongoing re-evaluation of their therapeutic response [121]. The "1-hour bundle" protocol defined in 2018 by the Surviving Sepsis Campaign (SSC) - a collaboration of the Society of Critical Care Medicine (SCCM) and the European Society of Intensive Care Medicine (ESICM) committed to reducing mortality and morbidity from sepsis and septic shock worldwide - recommends, within the first hour following the recognition of the disease, to measure lactate level, obtain blood cultures, dispense broad spectrum antibiotics, administer intravenous fluid, and if required, apply vasopressors. Generally, timely intervention should also include complete clinical evaluation, control of the source of infection (such as removing an infected device or draining an abscess) and, eventually, supportive therapies [140]. The detailed guidelines of sepsis medical management can be found in the latest recommendation reviews [121, 140].

Special attention will, however, be given to the fluid resuscitation strategies, which have recently been highly debated. As previously discussed, vascular leakage (and subsequent decreased effective circulating blood volume) and vasodilation both reduce blood pressure and venous return, impairing tissue perfusion and altering organ function. Fluid resuscitation aims to restore intravascular volume, improve organ perfusion, and support their function. Particularly following the study of Rivers *et al.* [76], early fluid resuscitation was introduced in the international guidelines in 2013 [141]. Further data demonstrated that delay in fluid administration is associated with enhanced inflammatory response and excessive mitochondrial dysfunction [142, 143]. Nonetheless, a growing body of evidence raises concerns about the harm caused by fluid challenge, showing worsened outcome in response to excessive fluid administration [144-146]. Indeed, in a sepsis situation of vascular barrier breakdown, fluid resuscitation accentuates capillary leak, liquid accumulation in the interstitial space and tissue flooding. Endothelial glycocalyx degradation seems to play a significant role in the amplification of this process [147]. This phenomenon can be globally appreciated with the cumulative fluid balance of the patient, whose positivity has been recognised as a poor prognostic factor [79, 81, 148-150]. These considerations highlight the decisive contribution of capillary leak in sepsis pathophysiology, and the limits this process places on therapeutic management of the septic patient – complications associated with insufficient and excessive fluid administration in sepsis are summarised in Figure 8.

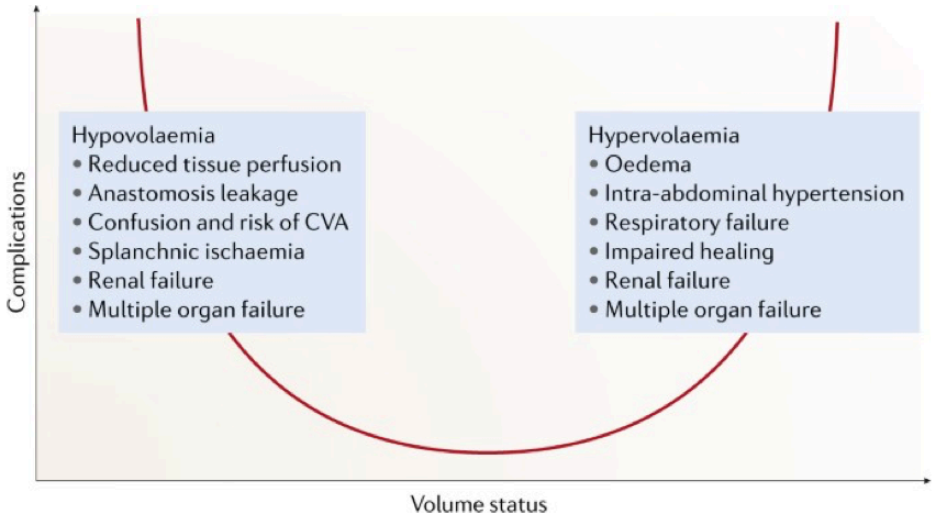

Figure 8 - The issue of fluid resuscitation during sepsis. Insufficient blood volume repletion can increase the incidence of sepsis complications mostly due to reduced tissue perfusion, whereas inadequate fluid administration is also associated with further complications, mostly due to tissue oedema. CVA, cerebrovascular accident. Taken from [107].

With this in mind, it is actually recommended to manage fluid resuscitation in 4 consecutive phases, encompassing rescue of fluid boluses, eventual weighted additional fluid administration, and then stabilisation and de-escalation of the fluid balance, in order to trigger aggressive fluid removal strategies [140]. Anyhow, fluid administration should be realised cautiously, and as much as possible guided by lactate [151] and dynamic measures testing response to resuscitation and hemodynamic tolerance [152]. Further development of microcirculation assessment at the bedside could reliably identify patients who particularly respond to fluid resuscitation [153]. Finally, the composition of the fluid administered also seems to have its importance [154]. Although colloids have greater intravascular expanding properties, studies failed to demonstrate their beneficial effects and cost effectiveness in the management of septic patients

[154-156]. Among crystalloids, balanced solutions should be preferred than normal saline in critically ill patients [157], although evidence seems insufficient to justify systematic use of balanced solutions. Two ongoing randomised control trials (RCTs) may provide stronger data to guide recommendations in the coming months [158, 159].

1.4.2.2 FUTURE THERAPIES

Despite considerable advances in the understanding of sepsis pathophysiology, no translation into specific treatments has yet been successful. Consistent research has been made on therapies focusing on limiting inflammation, stimulating the immune function, or controlling coagulation, but the underlying promise of a universal sepsis therapy remains illusive. The deep complexity of the mechanisms involved in the pathophysiology, the heterogeneity of the study population, the impact on survival as a reference for primary measure of efficacy, and the inadequate research models represent all factors that may explain the failure of this process [33]. Nevertheless, some ongoing perspectives may be of potential interest to improve sepsis management. Of those, intravenous vitamin C administration seems to represent a promising option. Several clinical trials have indeed been recently completed, and appear to show that high dose intravenous vitamin C (HDIVC, 50 to 200mg/kg/hour) may attenuate oxidative stress and inflammation, improve vasopressor synthesis, enhance immune cell function, improve endovascular function, induce epigenetic immunologic modifications, and show promising effects on mortality, without significant sides effects [160-162]. Other randomised controlled trials of HDIVC are under way and should provide additional data in the coming months. Blood purification techniques are another promising option, although based on less evidence. The techniques need to be better defined and optimised, but there is some evidence to support this attractive concept [163, 164]. Of note, several studies have pointed vascular permeability regulation as a potential

approach to improve sepsis outcome [45, 83, 95-99]. However, no therapeutic proposal specifically targeting vascular leakage has ever been brought to clinical trial.

Globally, the acquired knowledge seems to indicate that considering upstream, "pleiotropic" strategies (targeting transcription factors, mirco-RNAs, broad regulatory proteins,…) rather than focusing on single proteins may represent stronger approaches in the research of sepsis therapy. Furthermore, focusing on how to better individualise management strategies and adapt them to the molecular and biochemical profiles of each patient should represent another beneficial approach [25, 41].

1.4.3 OUTCOME

Sepsis death distribution has for long presented a biphasic pattern, characterised by a first peak in the early days - associated with inadequate resuscitation resulting in cardiovascular failure and multiorgan dysfunction - and a second one at several weeks - as the consequence of persistent organ failure, immune dysfunction and their complications [165]. This has evolved to a distribution that is rather considered as triphasic; characterised by a lesser amplitude of the first two peaks, and the emergence of a third one after 60-90 days, that keeps increasing after the next three years (see Figure 9) [36, 166]. The latter is suggested as a consequence of improved ICU care, prolonging the survival of the elderly or comorbid patients despite their chronic critically ill disease characterised by physiologic, immune, and metabolic alterations [36, 167].

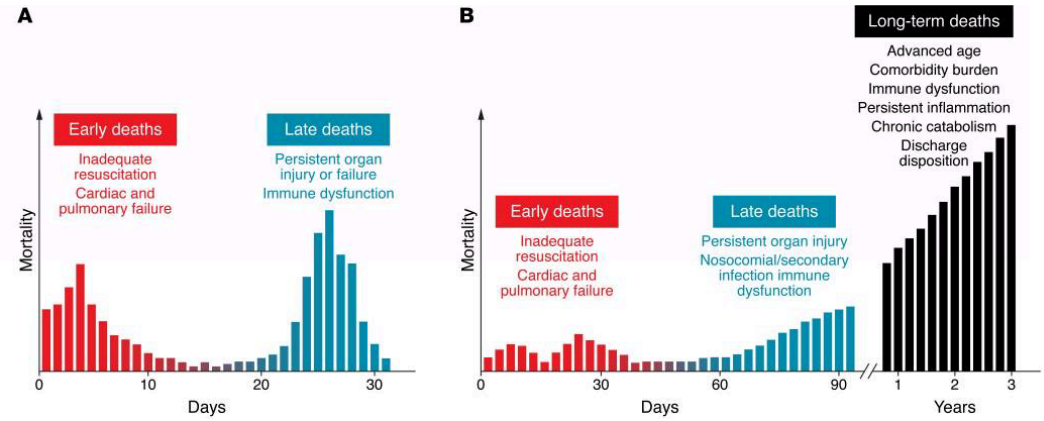

Figure 9 – Historical and current sepsis mortality distributions. (A) Historically, sepsis deaths have occurred in a biphasic distribution, with an initial early peak at several days attributed to inadequate resuscitation, resulting in cardiac and pulmonary failure, and a late peak at several weeks due to persistent organ injury and failure. Considering later trends in sepsis outcomes, the elderly population, and mounting long-term mortality, a trimodal distribution may be more indicative of the current sepsis-associated death distribution. (B) Current trimodal distribution of sepsis mortality. The two early peaks in mortality still exist, although with much lower magnitude than in the past. The third peak occurs approximately 60 to 90 days after the acute phase and continues to increase with time. This delay in sepsis mortality is thought to be a consequence of the more sophisticated ICU care that keeps the elderly and comorbid patients alive longer, in spite of ongoing immune, physiologic, and biochemical aberrations. Taken from [168].

On the other hand, sepsis survivors rarely present prompt recovery; return to normal organ function takes time. Patients surviving MOF develop a state of “chronic critical illness”, which can last several weeks [43, 169]. Thereby, compared to non-sepsis hospitalised patients, a significant proportion of sepsis survivors exhibit some kind of cognitive and functional disability, sometimes dramatically affecting their quality of life and requiring readmission in acute-care hospitals [35, 170]. This has been described as the “post-sepsis syndrome” [34, 37].

Improving the characterisation of molecular pathophysiological mechanisms, identifying the precise mechanisms triggering the maladaptive character of the host response, and possibly developing strategies to target them are key questions whose understanding would help improve sepsis management, outcomes, and societal burden.

2 THE ENDOTHELIAL BARRIER FUNCTION IN SEPSIS INJURY

The endothelium is the cell monolayer that lines the intima of blood vessels. In healthy condition, it forms a highly dynamic semi-permeable membrane between the intravascular compartment and the interstitium. Physiologically, transendothelial permeability can be enhanced by several mediators aimed at providing macromolecules and leukocytes within the underlying tissues to control eventual pathogen invasion. During sepsis, stress mediators drive homeostasis towards endothelial barrier breakdown, inducing the formation of intercellular gaps, uncontrolled flux of proteinaceous fluid into the interstitium, and subsequent impairment of microcirculatory perfusion resulting in multiorgan failure and death [45, 79, 82]. The present chapter will focus on the molecular mechanisms involved in the regulation of endothelial barrier function, and how they are affected by sepsis condition. These involve two main actors; interendothelial junctions (IEJs), and the actin cytoskeleton (AC), which directly anchors IEJs inside the plasma membrane [171](see Figure 10).

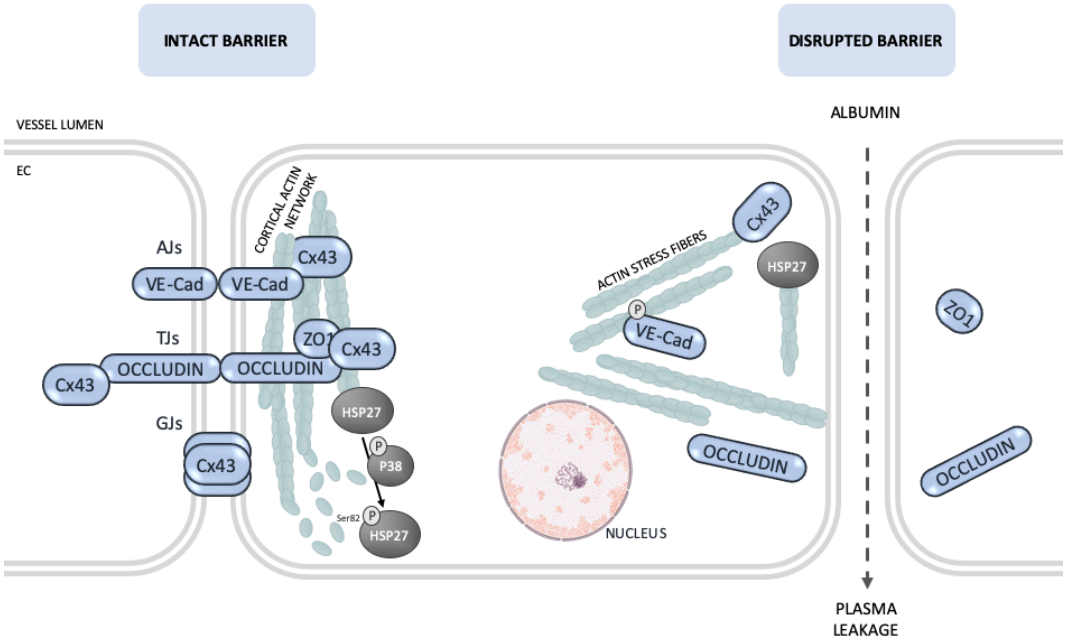

Figure 10 – Major actors composing intact and disrupted endothelial barrier. Under resting conditions (left part of the panel), microvascular endothelial barrier function is precisely regulated by tight junctions (TJs) whose anchoring is allowed by adherens junctions (AJs). Both TJs and AJs are anchored to the cortical actin network via several adaptor proteins, including ZO-1 and Cx43. In response to stress signals including inflammation (right part of the panel), signalling pathways disturb actin dynamics, and switch the balance from stabilising cortical actin to contractile actin stress fibers, which exert pulling forces and result in IEJs disruption, internalisation, and/or degradation.

2.1 INTERENDOTHELIAL JUNCTIONS

IEJs seal the endothelial cells to one another, in order to form a contiguous monolayer controlling the passage of components across the endothelial barrier in a size-selective manner [172, 173]. Endothelial permeability directly depends on the presence and organisation of IEJs at the plasma membrane, while the deregulation of these processes

is associated with loss of endothelial barrier function [171]. IEJs include three main types of protein complexes: adherens junctions (AJs), tight junctions (TJs), and gap junctions (GJs). Both AJs and Tjs directly contribute to the regulation of endothelial permeability and will represent the main focus of this chapter, while GJs consist of channels allowing the passage of small molecules for cell-cell communication [174]. Nevertheless, they all are involved in important crosstalks, and a multitude of protein interactions allowing subtle dynamic regulation of their expression, organisation, and stability. It should be mentionned that the composition and architecture of IEJs vary greatly along the vascular tree and the vascular beds, which requires to apprehend the theory with caution, and adapt it to the cellular models concerned. Importantly, TJs are more developed in small arterioles, whereas AJs are predominant in capillaries and post-capillary venules – mainly concerned by the capillary leak [171, 175].

2.1.1 ADHERENS JUNCTIONS

Adherens junctions (AJs) supervise major endothelial functions including cellular migration, immune cell trafficking, angiogenesis, and most importantly, endothelial permeability [176, 177]. Indeed, AJs are considered as the primary regulator of endothelial barrier function, acting as mechanical elements allowing strength adhesive bounds between ECs, on the basis of which TJs can be applied to refine the sealing of the intercellular contacts [171, 177]. Accordingly, AJs assembly is reported to be required for the the mechanical maintenance of TJs [174, 178], and for the expression of the TJs component Claudin-5 [179].

Vascular endothelial cadherin (VE-Cad) is the major component of AJs, described as the vital junctional protein in the regulation of endothelial permeability [180-182]. Indeed, the only loss of VE-Cad integrity is recognised as being the critical mechanism in intercellular gaps and tissue oedema formation [45, 176, 183], as shown in pulmonary microvessels submitted to calcium switch in the presence of VE-Cad antibodies [184]. Of

note, VE-Cad deletion in mice leads to embryonic mortality [185]. Other important AJs components are α-, β-, and p120-catenin adhesion complexes [186], themselves interacting with a variety of other actors such as Vinculin, actinin, afadin, N-WASP, or Arp2/3 [171, 176]. These proteins regulate AJs complexes stability, in particular by connecting them to the actin cytoskeleton, thereby modulating their mechanical anchorage in the plasma membrane [171]. Thanks to their close interaction with the actin cytoskeleton, AJs undergo various assembly, disassembly, and remodelling regulations in response to chemical and mechanical stimuli occurring in both physiological and pathological conditions [187]. These dynamic processes are crucial to fulfil AJs functions, and involve a large number of regulating mechanisms.

VE-Cad is composed of five ectodomains, a transmembrane domain, and a cytoplasmic tail [188]. The kinetics of its continuous assembly-disassembly sequence is strongly influenced by its homophilic dimerisation through *trans*- and *cis*-interactions [188]. *Trans*-interactions involve adhesive bounds with VE-Cad molecules from surrounding ECs, while cis-interactions concern lateral clustering of VE-Cad from the same EC which increases the strength of adhesive bounds [171]. On the other hand, the turnover of VE-Cad at the plasma membrane, depending on exchanges between junctional and cytoplasmic pools, is tightly regulated by VE-Cad interactions with catenin proteins and the actin cytoskeleton [189, 190]. These processes are dramatically disturbed by stress and inflammatory mediators, inducing various consequences.

First, loss of VE-Cad integrity. The main VE-Cad disruption sequence may be summarised as follows: formation of actin stress fibers - tyrosine phosphorylation of VE-Cad by kinases such as Src or Pyk2 - subsequent dissociation from P120-catenin - endocytosis/internalisation - eventual lysosomal degradation [182, 191]. This turnover is importantly regulated by VE-Cad post-traductional modifications such as phosphorylation, oxidation, and acetylation (for review, see [171]). Specifically, VE-Cad

phosphorylation on Tyr658 has been described as a key mechanism leading to VE-Cad internalisation in response to inflammatory mediators such as thrombin or lipopolysaccharide (LPS) [192], although this phosphorylation had previously been reported to be insufficient to trigger VE-Cad internalisation on its own [193].

Second, disturbed VE-Cad supra-molecular organisation. Stress mediators are associated with a shift from linear towards interrupted VE-Cad patterns within the plasma membrane (see Figure 11). Linear VE-Cad patterns are associated with peripheral junctions-associated actin filaments (JAAFs), mostly observed in confluent ECs, and known to stabilise the VE-Cad clusters [189, 194]. Interrupted VE-Cad patterns – often observed in the presence of intercellular gaps – are characterised by the presence of VE-Cad segments typically aligned either perpendicularly or diagonally to the longitudinal axis of the IEJs [177]. This VE-Cad organisation, typically associated with actin stress fibers, mainly occurs under inflammatory stimuli or during migration, and impairs the endothelial barrier function [195, 196].

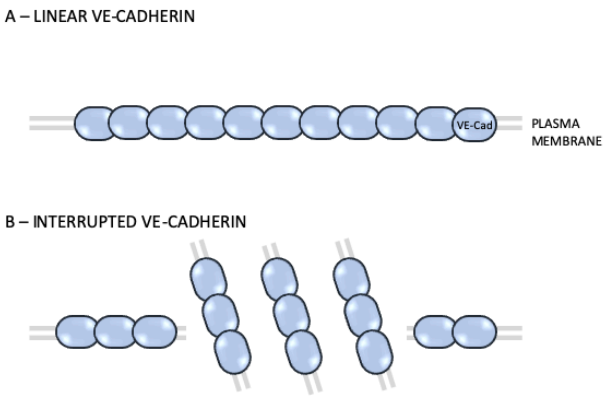

Figure 11 – VE-Cadherin (VE-Cad) patterns of interest in endothelial barrier function. (A) Linear VE-Cad patterns colocalise with cortical actin network, and are mostly described in confluent endothelial cells (ECs) *in vitro*, or quiescent ECs *in vivo*. These patterns are known to stabilise VE-Cad clusters and maintain

endothelial barrier integrity. (B) Interrupted VE-Cad patterns are connected with actin stress fibers, and described in sub-confluent ECs, under inflammatory conditions, or in migrating ECs. Their main functions are to promote paracellular permeability or cell migration. Adapted from [177].

Third, decreased VE-Cad expression. Actually, it remains unclear whether inflammatory mediators downregulate VE-Cad expression or not, since both outcomes are described. Chan *et al.* have specifically reviewed the impact of LPS on these mechanisms [182]. Mechanistically, increased MLC phosphorylation [197, 198], increased caveolae-mediated endocytosis [199], increased Ang2/Ras pathway [200], or inhibition of sirt3/AMPK pathway [200] have been reported to down regulate VE-Cad expression in response to LPS. On the other hand, LPS has also been described to induce VE-Cad internalisation without affecting its total expression. The mechanisms reported involve increased MLC phosphorylation (here specifically due to activated ROCK and inhibited Rac in response to reduced cyclic adenosine monophosphate (cAMP) levels) [201] and Src homology region 2-containing phosphatase (SHP2) oxidation [202]. In this latter sequence, VE-Cad is proposed to be recycled back to the membrane thanks to the recruitment of pro-recycling Rab5a GTPase [182]. Experimental conditions such as cell type or conditions of LPS incubation (duration, concentration, serotype), may activate one pathway rather than another, and explain the discordant reports on expression of VE-Cad in response to LPS challenge.

Finally, VE-Cad extracellular domain can be cleaved by inflammatory mediators such as metalloproteinases (ADAMs) or neutrophil elastase, and subsequently released as a soluble portion of VE-Cad (sVE) into the circulation [203, 204]. This raised the question of using sVE as a biomarker in pathologies associated with endothelial dysfunction, but clinical studies are lacking to draw any conclusion [205]. In the particular context of sepsis, studies showed conflicting results, with sVE being both reported to decrease [206, 207] or correlate with the severity of the disease [203, 208, 209] in septic patients.

Also of interest, it may be noted that N-Cadherin is another cadherin present in human endothelial cells [210], in which it mediates the recruitment and interaction with vascular smooth muscle cells and pericytes [211], and seems to contribute to the assembly of TJs [212].

2.1.2 TIGHT JUNCTIONS

Tight junctions (TJs) also play a major role in maintaining vascular barrier homeostasis. Their specific function is to seal the intercellular space, in order to selectively regulate the diffusion of fluids, ions, and small plasma proteins throughout the endothelium. Thereby, TJs allow fine control of endothelial permeability, as is required in specific vascular beds such as the blood–brain barrier [213, 214]. In addition, their signal transduction abilities allow TJs to contribute to the regulation of various cellular functions including proliferation, differentiation and apoptosis [215].

TJs preferentially localise in the apical part of the lateral EC membranes. Their transmembrane components include claudins (isoforms 1-24), occludin, tricellulin and junctional adhesion molecules (JAMs, isoforms A-C). Their cytoplasmic components are Zonula Occludens (ZO, isforms 1-3), which connect transmembrane TJs components to the actin cytoskeleton. These multiple proteins and isoforms allow a multitude of combinations for the constitution of tissue-specific TJs, in order to provide specific functions depending on the cell type and tissue concerned. ZO-1, claudin-5, and occludin are by far the best known TJs proteins in ECs.

ZO proteins are recognised to play a particular role in regulating endothelial barrier function. Specifically, ZO-1 or ZO-2 invalidation are embryonic lethal in mice [216], and lead to significant increase in paracellular permeability in human brain microvascular endothelial cells [217, 218]. ZOs contain important PDZ domains, capable of binding TJs transmembrane components as well as catenins in AJs and connexins in GJs.

Accordingly, ZO-1 has been described as a determinant modulator of VE-Cad stability [219]. On the other hand, ZO proteins are linked to actin filaments. Thereby, they act as scaffolds to anchor IEJs in the plasma membrane [220-222].

Stress mediators can modulate TJs architecture through multiple signalling pathways affecting expression, post-traductional modifications, or localisation of proteins [223]. Specifically, TJs proteins phosphorylation, which involves a wide variety of kinases, is generally accepted as an acute response to extracellular stimuli leading to TJs assembly or disassembly [220, 224]. The signalling pathways currently described to regulate TJs architecture involve the protein kinase C, PKA, PKG, Rho, mitogen activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and Wnt/β-catenin pathways [220].

TJs appear as important actors of endothelial permeability during sepsis. Studies have reported that endothelial barrier disruption was associated with lower protein contents of ZO-1 and occludin in this context [225, 226]. Moreover, although provided by two modest studies (one of them considering ZO-1 as a marker of the intestinal dysfunction rather than the vascular one), recent evidence seem to support the consideration of plasmatic ZO-1 levels as a predictor of sepsis severity and mortality [227, 228].

Of interest, more detailed considerations of TJs structure, assessment, signalling and functions can be found in the recent extensive review of Cong *et al.* [220].

2.1.3 IEJs REGULATING PROTEINS

IEJs are highly dynamic structures involved in a multitude of protein interactions to support adequate intercellular sealing in response to both intra- and extracellular stimuli. Among others, Cx43 has been identified as a key protein involved in their regulation through its non-canonical regulatory functions.

2.1.3.1 Cx43

Connexins (Cxs) are transmembrane hydrophilic proteins forming hexamers - called connexons - which associate between adjacent cells to build intercellular pores, called gap junctions (GJs) [229]. These allow cell-cell flux of small molecules and solutes, required for signal transduction within a confluent cell monolayer [230]. Cx37, Cx40 and Cx43 are the predominant isoforms in ECs. Specifically, ECs from capillaries predominantly express Cx43 and, to a lesser extent, Cx37 [229, 231, 232]. Of note, the differential features characterising each Cx have been related to the size and variability of their intracellular C-terminal loops.

The C-terminal loop of Cx43 constitutes a platform for diverse protein-protein interactions. This confers several non-canonical functions to Cx43, which acts as a scaffold protein to regulate the dynamics of other proteins at the cell membrane or to regulate gene transcription through transcription factors hijacking [229, 231, 233]. In this regard, the C-terminal tail of Cx43 was demonstrated to stabilise actin filaments to guide protein trafficking in cardiomyocytes [234]. It was also shown in neuronal cell models that both genetic invalidation and transient knockdown of Cx43 deeply affect the cellular transcriptome, modulating the expression of genes involved in various cellular functions including cell adhesion and migration, cytoskeleton dynamics, cell signalling, proliferation and differentiation [235-237]. Supporting the importance of these non-canonical functions, much of the Cx43 expressed in ECs exposed to disturbed blood flow is not located in sites of intercellular contacts [238]. Finally, the C-terminal tail of Cx43 contains many serine and tyrosine residues that are substrates for protein kinase A (PKA) [239], B (PKB)[240], C (PKC) [241], and ERK [241], and whose phosphorylation modulates its localisation and turnover.

Cx43 is importantly involved in IEJs assembly and disassembly [242], which supports its role in endothelial permeability regulation. Indeed, Ajs, Tjs, and cytoskeleton proteins

have been identified to interact with Cx43 C-terminal tail. Among junctional proteins, Cx43 directly interacts with β-catenin [241], ZO-1 [243-246], ad N-Cad [247]. Reciprocal regulation of GJs and AJs assembly has been prominently studied in the context of neuroectoderm, where Cx43 was shown to colocalise and coprecipitate with N-Cad [248-250]. Among cytoskeleton proteins, Cx43 has interestingly been shown to modulate actin dynamics by activating the p38/HSP27 pathway [251], which will be introduced in the section *2.2.2.1* of this chapter.

However, it remains controversial whether Cx43 expression and function are favourable or detrimental to endothelial barrier integrity [242]. Indeed, several studies indicate a detrimental role of Cx43 in the challenged endothelial barrier. Cx43 inhibition was accordingly shown to prevent from endothelial hyperpermeability in experimental models of acid- [252] and endotoxin-induced lung injury [253]. In the latter, the effect was interestingly associated with an up regulation of VE-Cad expression. In another study, a Cx43-overexpressing lentivirus was shown to induce the degradation of ZO-1 and claudin-5 in septic rats [254]. The same team had previously identified the involvement of the Rock-1/MLC20 pathway in Cx43 pro-permeability effects in a similar model of septic rats [255]. On the other hand, Cx43 down regulation or inhibition was shown to enhance endothelial permeability in rat retinal endothelial cells submitted to high glucose [256], in rat lungs and porcine BBB ECs [257], and *in vivo* in the blood-labyrinth-barrier in mouse cochlea [258]. Moreover, Cx43 up regulation was shown to counteract endothelial hyperpermeability in response to high glucose [258]. Although very contradictory, these data seem to favour a rather deleterious role of Cx43 in the regulation of the endothelial barrier upon sepsis. As recently reviewed, one may argue that this effect could involve a Cx43-mediated up regulation of inflammatory pathways through enhanced leukocyte recruitment [259].

2.1.3.2 OTHER IEJS REGULATING PROTEINS

As previously discussed, the dynamic regulation of IEJs involves plenty of proteins (for review, see [220, 260]). Among them, small GTPases from the Rho family are of major importance and will be detailed in the next chapter since primarily acting by modulating actin dynamics. In addition, growth factors (such as vascular endothelial growth factor receptor 2 (VEGFR2) or transforming growth factor (TGF)-ßRII/I), kinases (such as Src, PKA, PKC, PKG, PI3K or MAPKs) and phosphatases (such as VE-Cad phosphatase (VE-PTP), Src homology phosphatase-2 (SHP-2) or density-enhanced phosphatase-1 (DEP-1)) allow fine regulation, and make IEJs a functional hub controlling not only intercellular adhesion but also cellular signalling pathways [220, 260-262].

2.2 ACTIN CYTOSKELETON

The cytoplasmic part of IEJs complexes is directly anchored to the actin cytoskeleton via their scaffolding components (i.e. α-catenin, ZO-1 or Cx43). Particularly, the tethering of the actin cytoskeleton to AJs appears to be the driving force for VE-Cad stabilisation by reducing lateral mobility of cadherin molecules in the plasma membrane [260, 263]. On the other hand, actin cytoskeleton reorganisation and contraction importantly trigger endothelial barrier destabilisation and vascular leakage [264]. This confers a crucial role for actin dynamics in regulating the stability of IEJs and subsequent intercellular permeability [265].

2.2.1 ACTIN FILAMENTS

ECs mainly express non-muscle ß-actin, which appears as monomers (globular, G-actin) able to form microfilaments (F-actin) through polymerisation. Positive and negative ends have been conventionally assigned to F-actin, where G-actin associates or dissociates, respectively. In ECs, actin filaments are found in distinct but interrelated subcellular structures among which circumferential actin filaments and actin stress

fibers play pivotal roles in endothelial barrier regulation (see Figure 12). Circumferential actin filaments are predominant in basal conditions, associated with continuous VE-Cad pattern, and known to support IEJs anchorage in the plasma membrane [266]. Response to stress mediators results in actin reorganisation, as characterised by reduced cortical actin network and formation of stress fibers spanning throughout the EC [267-269]. The contractile features of actin stress fibers confer them critical power in cellular and junctions architecture remodelling. Indeed, initially aimed at supporting leukocyte transendothelial migration, stress fibers are known to promote IEJs disorganisation, as well as to increase centripetal tension favouring cells border retraction, internalisation of AJs and TJs, and IC gap formation [270]. Specifically, stress fibers formation is usually accompanied by remodelling of AJs, resulting in interrupted VE-Cad pattern [271]. In these processes, actin cytoskeleton remodelling is modulated by rapid turnover of the filaments which involve the regulation of many actin binding proteins and regulating pathways, as detailed below [266, 272].

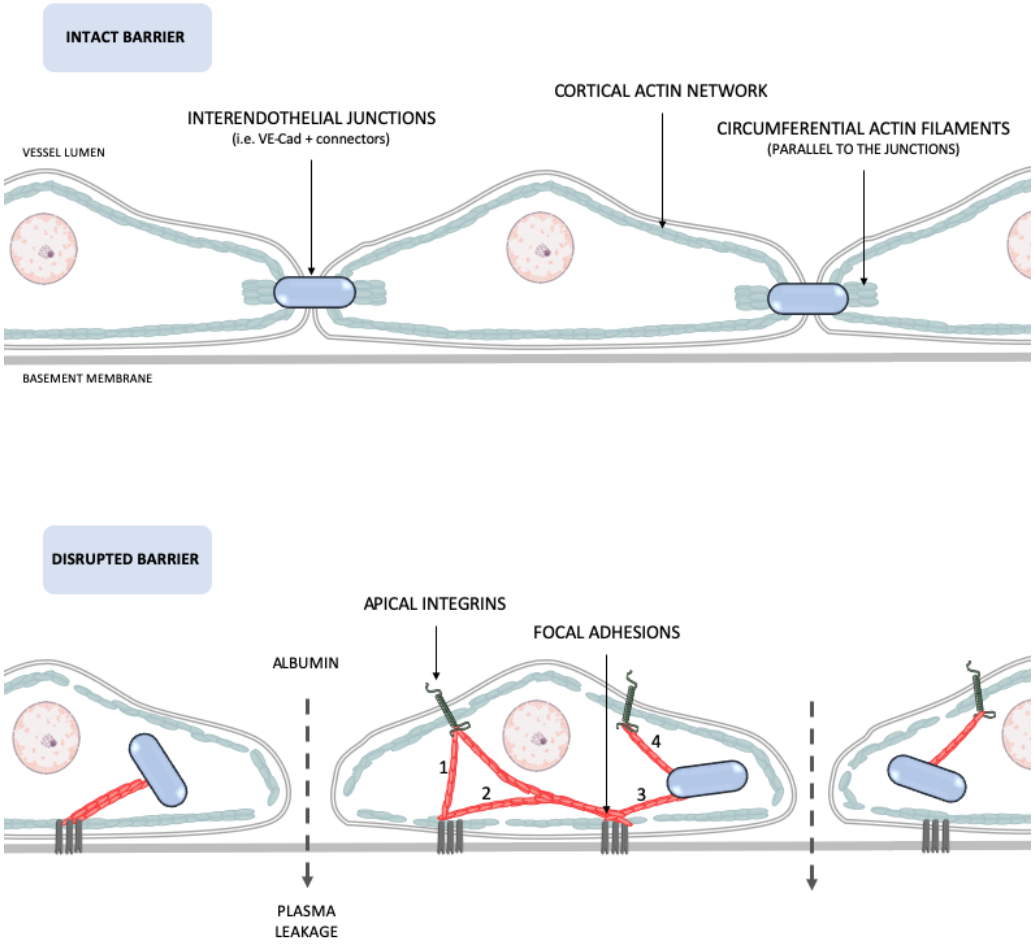

Figure 12 - Endothelial actin organisation. Potential appearance of actin filaments in endothelial cells. Confluent and resting endothelium (upper panel), is characterised by pronounced circumferential actin filaments, and no or few stress fibers. Subconfluent or activated endothelium (lower panel) generally presents pronounced stress fibers, and no or less circumferential actin filaments. Actin stress fibers can span between focal adhesion (FA) and apical membrane (1), FAs (2), interendothelial junctions (IEJs) and FA (3), or IEJs and apical membrane (4). Adapted from [266, 268].

2.2.2 KEY ACTIN REGULATING PROTEINS

The organisation of actin filaments involves numerous signalling pathways, notably ensuring fine loco-temporal regulation of intercellular contacts. This chapter will focus on two of them, known to be majorly involved in endothelial barrier function; the p38 MAPK/HSP27 pathway and the small GTPases from the Rho family. More details on the regulation of actin dynamics in ECs during sepsis can be found in the following review [264].

2.2.2.1 P38 MAPK/HSP27 PATHWAY

Mitogen activated protein kinases (MAPKs) are serine/threonine protein kinases activated by diverse extracellular stimuli, leading to the activation of numerous signalling pathways and transcriptional factors to promote fundamental cellular processes such as proliferation, differentiation, or apoptosis. Among them, the p38 MAPK (often referred to as p38) is well known for its effects on actin dynamics and subsequent modification of endothelial barrier function. It has been known for several years that p38 can be activated through three distinct pathways [273]: (1) via the MAPK-activated protein kinases (MAPKAPK) cascades, involving MAPKAPK 3 and 6 but also small GTP-binding proteins of the Rho family such as Rac1 and cdc42, (2) through its interaction with the transforming growth factor α-activated kinase 1 binding proteins (TAB) 1, 2, or 3, [274], (3) via autophosphorylation, which is facilitated by the tyrosine kinase Zap70 [274-276]. However, the impact of p38 activation on the endothelial barrier function is debatable, and its modulation by these different activation pathways remains to be elucidated. Obviously, extensive evidence shows a detrimental role of p38 activation in endothelial permeability [277-281]. Several studies, however, highlight contradictory protective effects [282, 283]. These involve the phosphorylation of the heat shock protein of 27 kda (HSP27*)*.

As a heat shock protein, HSP27 is a multifunctional protein aimed at increasing resistance of tissues to stress and injuries. HSP27 is constitutively expressed in numerous cell types, including ECs, and is up regulated by variety of stimuli including heat shock, oxidative stress, and exposition to cytokines or LPS [284]. Under physiological conditions, HSP27 tends to associate in dynamic multimeric structures of varying sizes, and is importantly characterised by a molecular chaperone function. HSP27 contains three phosphorylable serine sites (ser15, ser78 or ser82) targeted by several kinases. MAPKAPK2, and at a lesser extent MAPKAPK3 are by far the most concerned, and are both direct substrates of p38 MAPK [277, 284]. Other kinases that can phosphorylate human HSP27 include MAPKAPK5, PKC, and PKD [284]. In response to stress stimuli, HSP27 phosphorylation results in conformational changes, dissociated oligomers, and loss of chaperone activity [285]. Of note, HSP27(ser82) appears as the most relevant phosphorylation site in these dynamics. Indeed, ser15 phosphorylation seems to induce only small impact on HSP27 conformation and function, while ser78 was reported to be superfluous, mostly because inconstant in the animal kingdom [284, 286]. More importantly, HSP27 ser82 phosphorylation is recognised as an important modulator of endothelial barrier function.

HSP27 chaperone function underlies its pivotal involvement in the control of actin organisation [287, 288]. Indeed, unphosphorylated HSP27 binds the positive end of actin filaments, thereby preventing their polymerisation. In response to stress stimuli, the loss of its chaperone function releases HSP27 from actin, and allows the polymerisation of the filaments [289]. The characterisation of the functional impacts of HSP27 phosphorylation, however, is limited to a modest number of experimental models reporting conflicting evidence.

For example, HSP27 ser82 phosphorylation protects against endothelial hyperpermeability induced by burn serum challenge [282], hypoxia [290-292] and

anthrax lethal toxins [283]. Moreover, HSP27 phosphorylation was recently associated with lower efficacy of vascular disrupting agents in HUVECS submitted to hypoxia or energy depletion [302]. HSP27 was also shown to be down regulated in HUVECs submitted to LPS [293]. In contrary, pertussis toxin-induced endothelial permeability is temporally linked to HSP27 phosphorylation [277, 278]; and LPS-induced endothelial barrier dysfunction was shown to correlate with HSP27 phosphorylation *in vitro* and *in vivo* [294, 295]. The reason for these discrepancies is not clear but could involve crucial spatio-temporal factors. Moreover, it has been suggested that activation of the p38 MAPK/HSP27 pathway by LPS might be an adaptive response promoting IEJs supportive actin fibers and subsequent cell adhesion [290].

Beyond endothelial barrier function, HSP27 phosphorylation has been associated with vascular protective effects through reducing apoptosis under oxidative stress [296], TNFα stimulation [297], and high glucose [298]. These effects were shown to protect against atherosclerosis [299], and are supported by a study showing decreased HSP27 ser82 phosphorylation in endothelial cells from human diseased coronary arteries [300].

2.2.2.2 RHO FAMILY OF GTPASES

GTPases of the Ras homology (Rho) family are small signalling G proteins crucially involved in the maintenance and disease of blood vessels, particularly by involving the regulation of the actin cytoskeleton. Among the 21 Rho GTPases identified in mammals, RhoA, Rac1, cdc42 have been the most widely studied in endothelial permeability processes. They act as molecular switches carrying out their functions by hydrolysing GTP to GDP, and are regulated by RhoGTPase Activating Proteins (GAPs, which stimulate the hydrolysis of GTP), Rho Guanine-nucleotide Exchange Factors (GEFs, which promote the exchange of GDP for GTP), and Rho Guanosine-nucleotide Dissociation Inhibitors (GDIs, which inhibit their reactivation) [301]. These proteins are importantly recruited towards the cytoplasmic tail of VE-Cad, where they can define the local organisation of

actin fibers [176]. Importantly, timing and (sub)cellular localisation factors majorly influence the function of activated Rho GTPases, which can all have antagonist effects along with the spatio-temporal context and the nature of the stimuli underlying their activation. Altogether, these regulatory processes provide finely tuned signalling balances among Rho GTPases to maintain -or impair- IEJs stability.

RhoA, Rac1 and Cdc42 can all participate to support [189, 302, 303], and disturb [304-306] endothelial barrier function [307, 308]. In order to remain concise, this section will be limited to an overview of their major permeability functions (for review, see [309, 310]).

RhoA was first described to contribute to endothelial barrier breakdown [305, 306]. Indeed, hyperpermeability stimuli are often associated with RhoA activation, acting as a positive regulator of actin stress fibers formation and contractility through the activation of downstream effectors such as ROCK or mDia [311, 312]. This, typically occurs in response to sepsis [264, 311], oxidative stress [313], or inflammation [312]. However, data also reported the necessity of a certain level of RhoA activity to support the re-formation of intercellular contacts [314] and to prevent from vascular leakage during leukocyte diapedesis [315]. RhoA activation was even suggested to mediate barrier protection by S1P [316].

In contrast, Rac1 is primarily described to enhance basal endothelial barrier function and promote recovery after disruption [310]. These effects notably involve down regulation of RhoA activity [317, 318], and important crosstalk with VE-Cad. Indeed, VE-Cad dimerisation and engagement at forming AJs are known to promote Rac1 activation [308, 319], while Rac1 was demonstrated to be required for VE-Cad homophilic binding, and maintenance of endothelial barrier function in physiologic conditions [320]. Rac1 has also interestingly been shown to regulate HSP27 phosphorylation in human platelets [321, 322]. Furthermore, Rac1 activation has been found to protect the endothelial

barrier against inflammatory stimuli both *in vitro* and *in vivo* [201, 319, 323, 324]. Rac1 triggers barrier restoration through activating effector molecules, such as Arp2/3 complex, IQGAP and cortactin [325]. In this context, cAMP, Ang1 and S1P are critical signals mediating Rac1 activation, notably by activating specific GEFs (which involve Trio, Tiam1 or vav2)[260] [326]. Of note, some evidence indicate that Rac1 activation can also cause disassembly of VE-Cad and disruption of the endothelial barrier, notably in response to VEGF [304].

Beyond its role in the maintenance of basal barrier integrity, Cdc42 is typically activated in sites of junctional complex formation, and suggested to regulate the re-establishment of AJs after IEJs disruption, thereby playing a particular role in barrier recovery after damage [309, 327].

It appears clear that the activities of Rac1, RhoA and Cdc42 must be finely balanced for a proper control of endothelial permeability. In the context of sepsis, attempting to restore this balance by inhibiting RhoA or supporting Rac1 activities have been suggested as strategies of interest to promote endothelial barrier integrity [264, 319, 328].

2.3 MAIN TRIGGERS OF ENDOTHELIAL BARRIER MODULATION DURING SEPSIS

The septic storm induces endothelial barrier disruption through various mechanisms. Beyond inflammatory mediators and oxidative stress, several permeability regulating agents are typically known to regulate endothelial barrier function in sepsis capillary leak syndrome (see Figure 13).

Current evidence report a pivotal role for the Ang1/Ang2 balance in vascular permeability during sepsis. Ang1 and Ang2 both bind Tie2, an endothelial tyrosine kinase receptor on which they exert antagonistic effects. Ang1, the agonist, inhibits vascular

leakage. Ang2, on the other hand, increases endothelial permeability by antagonising Ang1/Tie2 interaction, and by inducing VE-Cad internalisation [329]. In response to inflammatory mediators, ECs increase Ang2 expression, resulting in disrupted Ang1/Ang2 equilibrium and increased vascular permeability. Fundamentally, data reports that endothelial specific Ang-2 overexpression in mice leads to the development of sepsis-like haemodynamic changes, an effect that can be abrogated by injecting mice with Ang1-encoding adenovirus [330]. Clinically, circulating Ang2 levels appeared to be up regulated in septic patients, and have emerged as a predictor of sepsis outcome [82, 331]. The possibility of Ang1 infusion as a therapeutic approach is, however, limited by side effects of pulmonary hypertension [332].

Sphingosine-1-phosphate (S1P) reinforces the endothelial barrier by stimulating its endothelial G protein coupled receptor 1 (S1PR1), leading to cytoskeleton reorganisation and adherens junctions assembly [62]. As previously mentioned, S1P is known to promote the activation of Rac1 (which is coupled to S1P1), and support VE-Cad and β-catenin expression in AJs [333]. Finally, S1P also protects ECs from the outside by inhibiting metalloproteinases and reducing glycocalyx shedding [334, 335]. Decreased serum S1P concentrations have been reported in sepsis patients and were correlated with the severity of the disease [336]. Several studies have even highlighted potential benefits of targeting S1P to improve sepsis outcome [337-339].

The vascular Endothelial Growth Factor (VEGF), was identified and characterised as a vascular permeability factor before being reported to promote proliferation, migration, and survival of endothelial cells, thereby dramatically contributing to angiogenesis [340, 341]. Mechanistically, VEGF is known to promote endothelial permeability by inducing VE-Cad [304], occludin and ZO-1 internalisation [342], notably by inducing hyper-activation of eNOS and subsequent protein nitrosylation. On the other hand, it has been reported that VEGF could have a protective role in endothelial barrier function, notably

by activating Rac1 [199]. Interestingly, various studies have reported elevated VEGF plasma levels in septic patients, which could be correlated with the severity of sepsis injury [343-345]. However, anti-VEGF treatments seem to show conflicted results, and arouse moderate enthusiasm [346-348].

Finally, it would be incorrect to close this chapter without naming Glycocalyx as an essential player in microvascular function and endothelial barrier integrity during sepsis (for review, see [349, 350]). However, while glycocalyx disruption is generally accepted to exacerbate the organism-wide septic insult, it is questioned whether restoring glycocalyx integrity *per se* would improve sepsis survival [100].

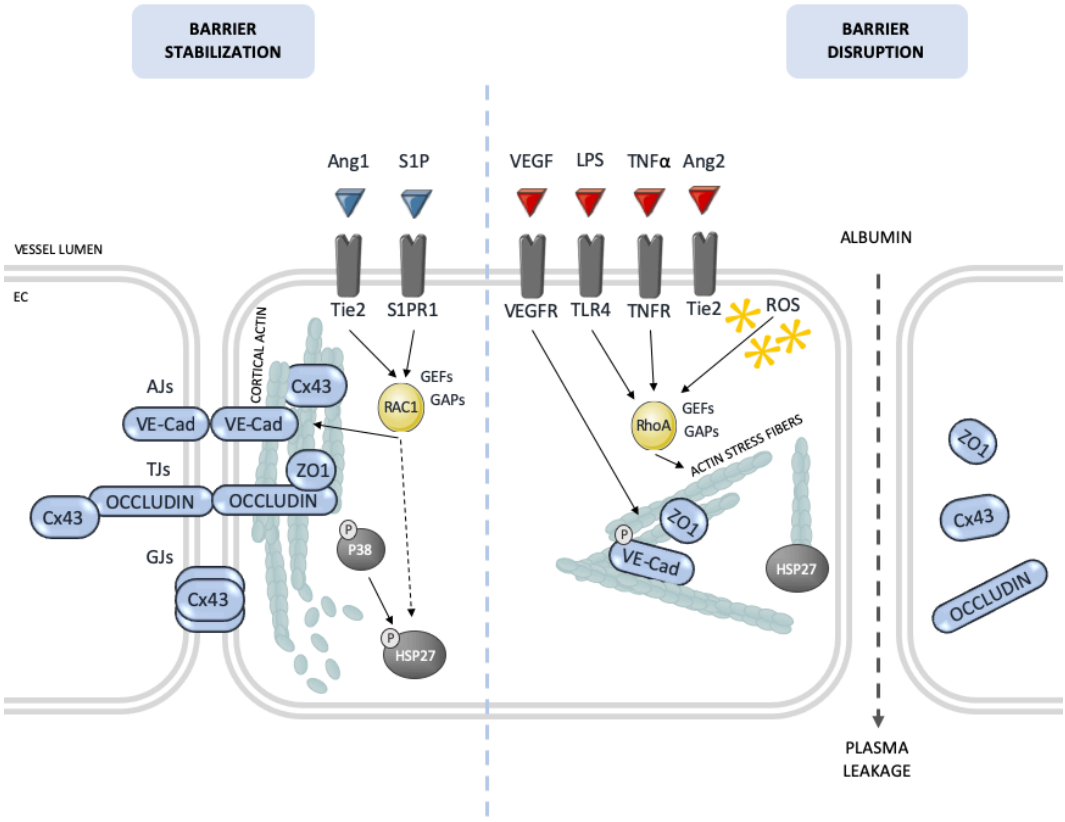

Figure 13 - Schematic, simplified signalling pathways regulating endothelial barrier during sepsis. Several different effectors can activate receptor-mediated actin remodelling, and subsequent interendothelial junctions (IEJs) organisation. These processes are importantly regulated by the p38 MAPK /HSP27 pathway and small Rho GTPases - such as Rac1 and RhoA. Rho GTPases are also regulated via guanine nucleotide exchange factors (GEFs) and GTPase-activated proteins (GAPs). Rac1 is the central signalling molecule which stabilises junctions and is a pivotal target of extracellular signalling molecules including sphingosine-1-phosphate (S1P) and angiopoietin-1 (Ang1) to enhance barrier function. HSP27(Ser82) phosphorylation, downstream of p38, allows actin polymerisation. In response to pro-inflammatory mediators, the pathways mediating endothelial barrier disruption importantly involve RhoA activation, which results in stress fibers formation that exerts pulling forces and destabilises IEJs. Tie2, tyrosine kinase with immunoglobulin and EGF homology domain 2; S1PR1, sphingosine-1-phosphate receptor 1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; LPS, lipopolysaccharide; TLR4, toll like receptor 4; TNFα, tumour necrosis factor α; TNFR, tumour necrosis factor receptor; Ang2, angiopoietin 2; ROS, reactive oxygen species; VE-Cad, VE-Cadherin; ZO-1, zonula-occludens-1; Cx43, connexin 43.

3 THE AMP-ACTIVATED PROTEIN KINASE

3.1 AMPK GENERALITIES

The AMP-activated protein kinase (AMPK) has been identified in 1987 as an enzyme responsible for the inhibition of fatty acids and cholesterol synthesis in rat liver [351]. Several years later, it was described as a metabolic master ubiquitously present in the body, playing a pivotal role in energy homeostasis by orchestrating integrated responses to energy deprivation.

Apart from these metabolic considerations, AMPK has then been shown to confer a plethora of tissue-specific cellular regulatory functions [352]. This chapter will focus on the endothelial aspects of AMPK.

3.1.1 STRUCTURE

AMPK is a highly conserved eukaryotic serine/threonine kinase consisting of one catalytic subunit (α) and 2 regulatory subunits (β and γ). Each subunit exists as multiple isoforms (α1/α2, β1/β2, γ1/γ2/γ3), giving 12 possible combinations of the holoenzyme [353].

The catalytic α subunit contains the protein kinase domain and a threonine residue (Thr172 on α1 and Thr174 on α2, referred to as Thr172), whose phosphorylation by upstream kinases is required for AMPK activation [354]. The regulatory β subunit acts as a scaffold for α and γ subunits and contains myristoylation sites that can modulate AMPK cellular localisation [355]. Finally, the γ subunit contains tandem repeat sequences which bind adenine nucleotides and are involved in allosteric AMPK activation [353].

Subunits isoforms decisively influence AMPK activity and function and confer special characteristics to AMPK heterotrimeric complexes, the expression of which varies considerably among the cell types.

3.1.2 BIOCHEMICAL MECHANISMS OF ACTIVATION

Whatever the stimulus, AMPK activation requires phosphorylation on Thr172 by an upstream kinase [356]. Importantly, the susceptibility of AMPK to be phosphorylated on Thr172 depends on a conformational change induced by the binding of AMP to the γ subunit [357], itself associated with a modest increase of AMPK activity [358].

Three distinct AMPK upstream kinases have been described (see Figure 14). First, the liver protein kinase B1 (LKB1) [357], whose activity on Thr172 is highly potentialized by the AMP-dependent conformational change of AMPK, and seems specific of the α2 isoform [359]. Second, the calcium/calmodulin-dependent protein kinase β (CaMKKβ) [360], which is activated by changes in intracellular calcium (Ca2+) concentrations,

independently of the energy status of the cell [361]. AMPK activation by CaMKKβ occurs in cell types which mainly express α1 isoform, including endothelial cells [362]. Finally, the transforming growth factor-β activated kinase 1 (TAK1) has been reported as an AMPK upstream kinase [363], at a lesser extent than the first two. Of interest, TLRs have recently been shown to stimulate this TAK1-AMPK axis in murine macrophages infected by *Salmonella thyphimurium* [364].

Finally, AMPK regulation may also involve additional phosphorylation sites (i.e. Ser485/491 and Thr479 of the α subunit), the intervention of phosphatases, and other post-translational modifications such as acetylation [365] or oxidation [366].

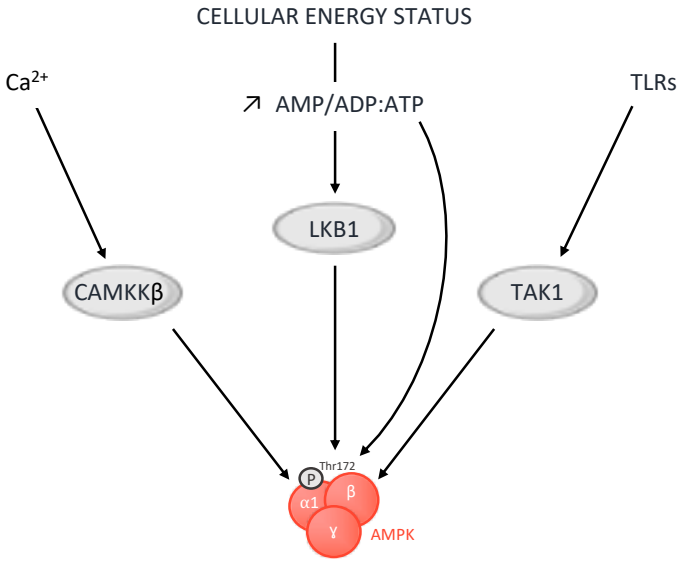

Figure 14 - Biochemical mechanisms of AMPK activation. AMP-activated protein kinase (AMPK) is activated by the upstream kinases LKB1, associated with the canonical pathway (triggered by an increased cellular AMP/ATP ratio) or CAMKKβ and TAK1, associated with the non-canonical pathways (triggered by an increased intracellular Ca^{2+} concentration or toll-like receptors (TLRs) activation). CAMKKβ, calcium/calmodulin-dependent kinase kinase β; LKB1, liver kinase B1; TAK1, transforming growth factor-ß-activated kinase 1. Adapted from [367].

3.2 AMPK AS A PHARMACOLOGICAL TARGET

3.2.1 AMPK ACTIVITY MODULATION IN BASIC RESEARCH

Since the 2000s, important research has been developed with the aim of developing pharmacological AMPK activators. Several molecules have been developed to activate or inhibit AMPK, always aiming to increase specificity and reduce side effects. The current challenge is to develop synthetic molecules or identify existing molecules capable of targeting AMPK in specific tissues or isoforms to refine treatments and avoid off-target effects.

The first promising molecule was the pro-drug 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAr), which is converted into the AMP analogue ZMP after cellular uptake. AICAr activates AMPK in all cell types expressing adenosine transporters and adenosine kinase activity, both being essential for AICAr conversion into ZMP [368]. However, the specificity of AICAR has been questionned [369], in particular through the use of murine models invalidated for AMPK at the hepatic level. Indeed, at high doses, AICAR is capable of affecting certain cellular functions and inhibiting mitochondrial respiration independently of AMPK [370]. In addition, AICAR has been shown to also activate AMP-dependent kinases other than AMPK [371].

Lately, direct allosteric effectors which bind the β subunit have been developed to activate AMPK. The A-769662 compound was presented in 2006 as the first direct AMPK activator [372]. Since then, several other molecules binding to the same site as A-769662 and showing higher selectivity have been synthesised [373]. Of those, the 991 compound is a powerful AMPK activator, described as five to ten times more effective than A-769662 [374].

Several AMPK inhibitors have also been described in the literature. Among them, the Compound C has for long been the most widely used, but was reported to inhibit a

number of other kinases and the results of studies using this AMPK inhibitor should be analysed with caution [375]. Recently, the SBI-0206965, initially thought to be an inhibitor of ULK-1, has been highlighted to directly inhibit AMPK by binding its catalytic α subunit [376]. SBI-0206965 appeared to be forty times more effective than compound C, with greater specificity, and is currently considered as the benchmark AMPK inhibitor.

3.2.2 AMPK ACTIVATION IN CLINICAL PRACTICE

Several clinically-usable molecules, most of which are prescribed as antidiabetic agents, have been identified to activate AMPK. Actually, as recently reported by Lu *et al.*, several anti-diabetic agents exert beneficial roles in the cardiovascular system through AMPK-dependent signalling pathways [377].

It was in 2000 that metformin was shown to exert its antidiabetic role by inhibiting complex 1 of the mitochondrial respiratory chain, which induces a reduction in mitochondrial respiration and therefore in ATP production [378]. In 2001, a study published a major result demonstrating the activation of AMPK by metformin in rat *in vitro* and *in vivo* models [379]. Since then, several studies have supported that the cardiovascular protective effects of metformin are mediated by the activation of AMPK, as interestingly reviewed by Lu *et al.* [377]. On the other hand, mitochondrial inhibition is associated with higher lactate production, making the administration of metformin controversial in certain pathological conditions at risk of lactic acidosis, such as sepsis [380, 381].

Many other molecules, often known as anti-diabetic agents or used in traditional Asian medicine, can also activate AMPK. Among these, statins, polyphenols (berberine, resveratrol), GLP-1 analogues (Liraglutide), thiazolidinediones, arctigenin, galegin or phloretin can be mentioned. However, the use of these activators appears to be limited,

in particular because of their low specificity and the importance of their off-target effects.

Of greater interest, SGLT2 inhibitors (SGLT2i), also known as gliflozins, are new oral antidiabetic agents with AMPK activation properties. Their inhibitory effect on SGLT2 in the proximal tubule decreases renal tubular thresholds for glycosuria and increases glucose urinary excretion, thereby preventing from hyperglycaemia. Four gliflozins have currently been approved by FDA; canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin. The clinical trials conducted for the cardiovascular risk assessment of these molecules surprisingly highlighted a dramatic cardiovascular protection, which can be considered as a class effect [382-386]. The molecular mechanisms underlying this protection remain largely unknown and are the subject of wide investigation in basic and translational research. In this context, an increasing number of studies are describing activating effects of SGLT2i on AMPK. Initially, canagliflozin but not empa- or dapagliflozin was described to activate AMPK at clinically relevant concentrations in human embryonic kidney cells (HEK293) and in mouse liver [387]. Since then, further evidence has reported an impact of empagliflozin and dapagliflozin on AMPK (Thr172) phosphorylation in murine total heart samples [388, 389], and isolated cardiofibroblasts [390]. Empagliflozin was even shown to promote AMPK activation in several endothelial models [391-393], and this effect was suggested to depend on elevation of AMP/ATP ratio [391]. However, the most described SGLT2i to activate AMPK at low concentrations remains Canagliflozin.

3.2.3 CANAGLIFLOZIN, A NEW BREATH FOR (ENDOTHELIAL) AMPK ACTIVATION?

The SGLT2i canagliflozin was approved by the FDA in 2016 and has been commonly prescribed as glucose lowering agent in diabetes since then. As other SGLT2i, canagliflozin treatment has been associated with impressive beneficial cardiovascular effects. Clinical trials conducted over the past five years have notably reported

significant protection against heart-failure and renal injury in both diabetic and non-diabetic patients [385, 386, 394]. The mechanisms underlying these protective effects cannot be fully explained by glucose lowering properties and remain poorly understood, despite arousing massive interest in the scientific community. In this context, AMPK as emerged as a downstream target of canagliflozin in several cell types – such as HEK293 [387], mouse macrophages [395, 396], human prostate and lung cancer cells [397], HUVECS and HAECs [396] – and tissues – such as mice liver [387, 395], kidney [398], and subcutaneous adipose tissue [399].

Hawley *et al.* elegantly demonstrated the mechanisms by which clinically relevant concentrations of canagliflozin, but not empagliflozin or dapagliflozin, activate AMPK in HEK293 and in primary mouse hepatocytes [387]. They highlighted increased cellular ADP/ATP ratios (used as a surrogate for AMP/ATP), loss of AMPK phosphorylation in cells expressing the AMP/ADP-insensitive R531G mutant of AMPK-γ2, reduced cellular oxygen uptake, and reduced oxygen uptake in permeabilised mouse hepatocytes provided with substrates that feed into complex I, altogether suggesting inhibition of complex I of mitochondria and subsequent increase of AMP/ATP ratio to induce AMPK activation in response to canagliflozin.

Whether AMPK activation by canagliflozin also depends on the inhibition of SGLT2 remains uncertain, particularly under pathological conditions. SGLT2 has long been thought to be expressed primarily in the kidney, and to a lesser extent in pancreatic alpha cells. However, its expression has been recently highlighted in HUVECs [396, 400-402], human aortic endothelial cells [396], and in murine aortic endothelium [392], although the detection of protein and mRNA contents is sometimes discordant. More obviously, SGLT2 endothelial expression was described to increase in response to high glucose in endothelial cells from murine aorta [392] and porcine coronary arteries [403]. Anyway, canagliflozin has been shown to reduce glucose uptake independently of SGLT2

expression in HEK-293, while this effect was associated with modest AMPK activation, and suggest to poorly participate to canagliflozin impact on AMPK activity in this cell type [387].

It should be noted that canagliflozin administration in clinical practice is associated with a nearly twofold increased risk of lower limb amputations [382, 404], which preferentially concerns elderly patients with baseline cardiovascular disease. Some evidence is starting to help understand the causes of this side effect, which do not appear to be mediated by inadequate glycaemic control and has not been reported with other SGLT2 inhibitors [405]. First, Mancini *et al.* reported that canagliflozin treatment was associated with modest reduction of healthy cells viability, suggested to be due to reduce glucose uptake and subsequent compromised ATP synthesis [396]. More recently, Behnammanesh *et al.* confirmed that 10 µM canagliflozin concentration had a robust cytostatic impact on HUVECs and HAECs, and identified the blockade of cyclin A expression to be partly responsible for this effect [406]. They also showed that higher canagliflozin concentrations (50µM) also impaired HUVECs migration, and importantly reduced tube formation. Moreover, a study reported that canagliflozin could reduce intra-tumour vascularisation in a xenograft model of liver cancer [401]. Meanwhile, since the risk of amputation remains low (4 more amputations in 1000 person-years with canagliflozin than with a GLP-1 agonist), and has been associated with age-related susceptibilities and comorbidities, it is proposed to be contextualised and put in the risk-benefits balance for patient-doctor decision-making before prescription [404].

Beyond these considerations, a growing body of evidence suggests the involvement of AMPK in the clinical benefits of canagliflozin treatment. Indeed, several studies have recently pointed out several overlapping mechanisms between cardiovascular protection by AMPK and canagliflozin. Accordingly, AMPK activation by canagliflozin has been shown to tamper inflammation [389, 396], inhibit NADPH oxidase [407, 408],

regulate nitric oxide production [389, 409], and enhance autophagy [410]. This field of research is currently booming and should allow a better understanding of the mechanisms involved in the near future.

The recent extensive evidence that canagliflozin activates AMPK in a large number of cell types and experimental conditions raises outstanding questions. Among these, the evaluation of new therapeutic indications for canagliflozin in pathological contexts associated with vascular damage is of major interest. More fundamentally, considering AMPK as a pivotal mediator of cardiovascular protection by canagliflozin, and possibly other SGLT2i, might allow a big step in unravelling this enigmatic protection.

3.3 AMPK IN THE ENDOTHELIAL CELL

AMPK has a critical role in regulating vascular function (see Figure 15). The mechanisms involved rely on the regulation of various signalling and gene expression pathways, which go far beyond cellular metabolic status (for review, see [411]). Of note, α1AMPK isoform is predominantly responsible for total AMPK activity in ECs [97, 411-413].

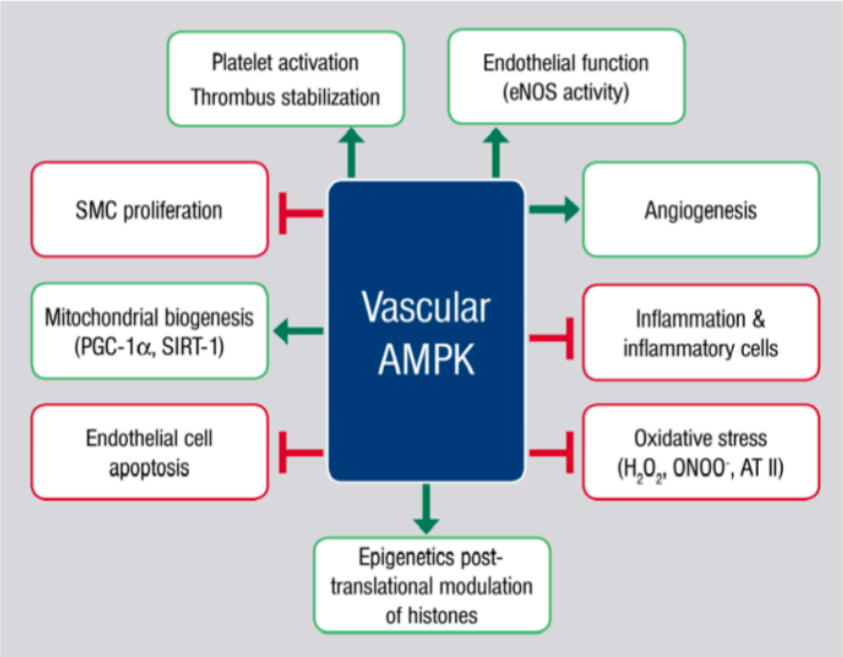

Figure 15 – Physiological functions of vascular AMPK. Activation of vascular adenosine monophosphate-activated protein kinase (AMPK) leads to endothelial nitric oxide synthase (eNOS) phosphorylation and reduction of oxidative stress by controlling notably mitochondrial biogenesis and mitochondrial gene expression. Furthermore, inflammatory processes are regulated by AMPK. In this context, AMPK is able to promote angiogenesis, reduce EC apoptosis and inhibit smooth muscle cell (SMC) proliferation. ATII, angiotensin II; H_2O_2, hydrogen peroxide; $ONOO^-$, peroxynitrites anion; PGC-1α, peroxisome proliferative-activated receptor gamma coactivator 1-α; SIRT-1, sirtuin-1. Taken from [414]

3.3.1 AS A METABOLIC MASTER

As extensively described in other mammalian cells, but to a lesser extent than in higher metabolic tissues, AMPK activation is known to reduce energy consumption and enhance energy production in ECs [414-416]. Indeed, it has been validated that AMPK activation by the pharmacological compound AICAr was associated with increased fatty oxidation in HUVECs [417]. AMPK was also found to normalise impaired fatty acid

oxidation and insulin signalling in response to high glucose [418]. Furthermore, a crucial involvement of α1AMPK in glycolysis regulation has been recently reported in mouse aortic ECs and HUVECs [419]. Finally, increasing evidence supports that endothelial AMPK increases mitochondrial biogenesis [420, 421] and prevents from mitochondrial fission [422].

3.3.2 AS A CELLULAR MANAGER

Endothelial AMPK is described as a metabolic sensor detecting and mediating cellular adaptations to several vascular disruptive stimuli, such as hypoxia, low glucose or shear stress. Its activation, which can also be mediated by CamKKβ in response to vasoactive molecules including thrombin, VEGF or S1P, promotes anti-inflammatory, anti-oxidative, anti-contractile, and anti-atherogenic defences in blood vessels [411].

One of the important downstream effects of endothelial AMPK is its anti-inflammatory action. It involves the reduction of NFkB [423-425], Jun N-terminal kinase (JNK), and Janus kinase-STAT signalling (JAK-STAT) [426] activities. These major organisers of the inflammatory response are known to be stimulated by TNFα, IL-1β and IL-6, three key mediators of sepsis inflammatory storm [411].

Endothelial AMPK is also widely demonstrated to prevent ROS production by different mechanisms [411]. First, AMPK reduces ROS formation by inhibiting NAD(P)H oxidase (Nox) [427, 428] and eNOS uncoupling [429]. AMPK also promotes mitochondrial biogenesis and reduces endoplasmic reticulum stress [430]. Finally, AMPK increases the levels of the antioxidant defence enzymes such as superoxide dismutase-2, catalase, and thioredoxin [431, 432].

Endothelial AMPK is also a great provider of NO, a critical regulator of vascular functions with pro-relaxing, anti-inflammatory, and antiaggregant properties [433]. This is allowed by several mechanisms, including endothelial NO synthase (eNOS) phosphorylation or

enhancement of tetrahydrobiopterin levels, preventing the generation of superoxide by eNOS instead of NO [434].

Angiogenesis is another crucial ATP consuming function whose regulation involves endothelial AMPK. Indeed, stimulators such as hypoxia, VEGF or adiponectin induce EC migration and angiogenesis by mechanisms requiring AMPK [435-439]. Furthermore, AMPK activation itself stimulates VEGF expression and signalling [436].

AMPK also seems to be involved in other endothelial cell functions including cell cycle [440], autophagy [441], or mechanotransduction [442], although these mechanisms have been predominantly described in other cell types.

Finally, during the last ten years, the role of AMPK in vascular barrier function has been described in several experimental models and will be the subject of the next chapter.

3.3.3 AS A GUARDIAN OF PARACELLULAR PERMEABILITY

While AMPK is unanimously recognised as a crucial player in the regulation of epithelial barrier function, its homologous role in the endothelium has been more recently investigated and is still being characterised.

3.3.3.1 FROM EPITHELIAL TO ENDOTHELIAL PERMEABILITY

The role of AMPK in the regulation of intercellular junctions has first been investigated in epithelial models, in which junctions disruption had been associated with ATP depletion [443]. Thereby, the first demonstration of junctional protein regulation by AMPK activation was made in 2006, by identifying that AMPK was required for TJs (and more precisely ZO-1) reassembly after epithelial barrier breakdown by calcium switch [444]. This study also highlighted that AMPK activation by AICAr improved epithelial barrier function. Since then, Epithelial AMPK has been reported to regulate TJs proteins

and subsequent epithelial barrier function in epithelial Madin-Darby canine kidney (MDCK) cells [445], in human airway epithelial cells (H441 cells) [446], in hepatectomised rat livers [447], and in human intestinal epithelial cells (Caco-2) [448]. Furthermore, reciprocal association between IEJs and AMPK has been described, since it was shown that AMPK is activated by AJs mediated mechanotransduction signals, through an LKB1 dependent mechanism [47, 449]. Finally, of major interest, epithelial barrier reinforcement by AMPK activation has been associated with potential therapeutic consequences, such as protecting against LPS-induced lung injury [212], inhibiting respiratory infection by reinforcing airway epithelial barrier [450], alleviating inflammatory bowel disease [451], reducing ischemic renal tubular injury [452], and ameliorating recovery from acute ischemic stroke by reducing blood brain barrier disruption [453].

The overall molecular composition of the epithelial and endothelial barriers only exhibits few differences. Of those, the replacement of epithelial AJs protein Epithelial Cadherin (E-Cadherin) by vascular endothelial-cadherin (VE-Cadherin) and the presence of N-Cadherin in the endothelium are the most important. One would therefore expect that the mechanisms by which AMPK regulates epithelial barrier are also involved in the endothelium.

3.3.3.2 KEY AMPK TARGETS

Further argument supporting the role of AMPK in endothelial permeability is the demonstration, most of the time in other cell types, that AMPK can regulate proteins playing key roles in the endothelial barrier.

Some of these targets are part of the intercellular junctions as such; Cx43, a key scaffold protein of both AJs and TJs in the endothelium [235, 242, 245], has appeared to be down regulated in the absence of AMPK and up regulated by AMPK activation in

cardiomyocytes [454, 455], and cardiac fibroblasts [456, 457]. The mechanisms involved in this regulation remain unclear. ZO-1 has been identified to be regulated by AMPK in Caco-2 cells submitted to calcium switch [448]. Afadin has previously been described to be regulated by AMPK [445] and to promote Cadherins assembly [458] in epithelial cells. The TJs components Claudin-1 and -4 were also shown to be direct AMPK targets in SV40 immortalised rat submandibular acinar cell lines and epithelial Eph4 cells, respectively [51, 71]. In pulmonary microvascular endothelial cells, N-Cadherin has been shown to mediate AMPK reinforcement of the TJs [212, 413].

Other AMPK targets can modulate IEJs stability, notably by modulating the actin cytoskeleton organisation. Of note, the high amounts of ATP required for the reworking of actin filaments have been proposed as the reason of AMPK recruitment in these processes [47]. Several actin regulating proteins have been shown to be regulated by AMPK. The small GTPase RhoA was described to be directly inhibited by AMPK in vascular smooth muscle cells [459], while Regulatory Myosin Light Chains (MLCs), MLC Kinase (MLCK), Cofilin, and Vasodilator-Stimulated Phosphoprotein (VASP) where shown to be regulated by AMPK in human platelets [460], epithelial MDCK [461, 462] and U2OS [463], rat vascular smooth muscle cells [464] and mouse mesenteric arteries [465]. Of interest, p38 MAPK, the main upstream kinase of the actin binding protein HSP27, has also been reported to be regulated by AMPK in HUVECs [466-468], in human immune cells [469], and in the ischemic heart [469, 470]. Moreover, it was recently demonstrated in HUVECs that AMPK activation was associated with HSP27 phosphorylation and protective actin filaments remodelling during hypoxia and energy depletion [302]. Finally, G-alpha interacting vesicle associated protein (GIV) phosphorylation by AMPK has been shown to enhance AJs stability by promoting the linking of AJs to the actin cytoskeleton [471].

From a broader point of view, AMPK may also modulate endothelial barrier function by regulating agonists affecting cell permeability. Specifically, a role for S1P-AMPK relationship has been recently described to stabilise the endothelial barrier in Human microvascular endothelial cells (HMECs) [472]. Longer ago, endothelial AMPK was shown to attenuate Ang2 expression in response to shear stress via a FoxO1a dependent mechanism [473]. AMPK would also be involved in a crosstalk with VEGF. Indeed, AMPK has been shown to increase VEGF expression by a mechanism depending on p38 MAPK in myoblasts [436] and in endothelial cells [474], while being reported to be activated by VEGF in several endothelial models [435, 437, 475]. Finally, the ability of AMPK to improve mitochondrial function, enhance autophagy [441], or tamper ROS and inflammatory cytokines production [396, 425], known as factors disturbing endothelial barrier regulation, are also logically associated with beneficial impacts on endothelial barrier function [391, 476-480].

3.3.3.3 CURRENT FUNCTIONAL EVIDENCE

The role of AMPK as an endothelial barrier regulator has emerged over the past decade through being described in a growing number of studies, most of them based on models of sepsis. Initially, Creighton *et al.* have reported in 2011 that α1AMPK was required for IEJs integrity, and that its activation by AMP-mimetic AICAr allowed endothelial barrier repair [413]. This work was realised on human pulmonary microvascular endothelial cells (HPMECs) and *ex vivo* mouse lungs challenged with thapsigargin, used to mimic inflammatory Ca^{2+} signalling [413]. Later, the same team worked on HPMECs and mice submitted to LPS and highlighted a protective effect of AMPK activation by metformin on endothelial barrier function, both *in vitro* and *in vivo* [97]. This study also reported the primary role of the α1 isoform in this function. The same year, similar results obtained with murine models of α1AMPK and α2AMPK invalidations were interestingly published, demonstrating the protective role of AICAr, and the requirement of basal

αAMPK (α1 at a greater extent than α2) to control lung oedema during LPS challenge [481]. Accordingly, our own group also highlighted that the mortality of mice exposed to sublethal doses of lipopolysaccharide (LPS) was dramatically increased by α1AMPK invalidation, and was associated with enhanced vascular permeability [83]. In this study, Castanares-Zapatero *et al.* also reported that AMPK activation by AICAr attenuates LPS-induced myocardial oedema formation in wild-type, but not in α1AMPK KO mice. These effects were completed with *in vitro* experiments, highlighting a role for endothelial AMPK in the regulation of ZO-1 in tight junctions. Since then, others confirmed the endothelial barrier protection conferred by AICAr in human brain [477] and lung microvascular endothelial cells, and by the A769662 compound in a C57BL/6 murine model of cecal ligation and puncture [482]. Nevertheless, among all the activators tested in these different studies, only metformin is currently used in clinical practice, while its administration during sepsis remains controversial because of the potential evolution towards lactic acidosis [380].

Very interestingly, the SGLT2 inhibitor empagliflozin was recently reported to protect the myocardial microvascular barrier by AMPK dependent mechanisms in a model of diabetes [391]. Experiments were performed on cardiac microvascular cells (CMECs) isolated from mice submitted to streptozotocin and subsequently treated with empagliflozin for several weeks. Results show that empagliflozin treatment preserves VE-Cad expression and actin organisation, by preventing mitochondrial fission.

An additional recent study claims that the exercise hormone Irisin can also protect against endothelial hyperpermeability in HMECs and mice submitted to LPS [478]. The protective mechanisms involve AMPK and are associated with an activation of Rac1/Cdc42, an improvement of mitochondrial function, and the preservation of VE-Cad integrity. The authors also demonstrate that serum Irisin levels are decreased and inversely correlated with the severity of the disease and the mortality of ARDS patients.

Based on these results, they propose that exogenous irisin administration might present therapeutic perspectives in pathological contexts associated with endothelial barrier breakdown.

This expanding field of research suggests great therapeutic potential for AMPK activation in microvascular leakage-associated diseases. Strong evidence, however, is still needed to support the development of this therapeutic strategy.

AIMS OF THE WORK

Sepsis accounts for one-fifth of total deaths worldwide [15], and has been identified as a global health priority by the World Health Organization. Despite major advances in the understanding of the pathophysiology, major uncertainties remain and underlie the failure in identifying specific therapeutic approaches. In a context where microvascular failure is generally accepted to play a pivotal role in the progression of the disease, capillary leak considerably affects organ function but has never been the subject of therapeutic proposals going beyond clinical trials. Taking these considerations into account, Castanares-Zapatero *et al.* highlighted α1AMPK as a major protector in sepsis pathophysiology, and suggested endothelial barrier reinforcement to underlie its protective action [83].

In this context, we first aimed to identify molecular mechanisms by which α1AMPK might regulate endothelial permeability in human microvascular endothelial cells submitted to sepsis-like conditions. Based on both the state of the art and the preliminary results obtained by Castanares-Zapatero *et al.*, we have articulated this work around two main points. On the one hand, we evaluated the effects of α1AMPK on the regulation of key proteins composing interendothelial junctional complexes. On the other hand, we further characterised the role of α1AMPK in the regulation endothelial actin dynamics.

Second, we sought to delineate the role of endothelial α1AMPK in the pathophysiology of sepsis. We investigated whether the endothelial specific deletion of α1AMPK would reproduce the phenotype observed in total knockout animals in terms of vascular permeability and survival.

Third, knowing that several AMPK activators are commonly prescribed in clinical practice, we considered their potential use as a therapeutic support to prevent sepsis capillary leak syndrome. Known for its cardiovascular protective properties and recently described to activate AMPK in endothelial cells at clinically relevant concentrations, the SGLT2i canagliflozin has been considered with special attention.

RESULTS

CHAPTER I – MECHANISTIC CONSIDERATIONS

This first chapter dedicated to our work was published in the International Journal of Molecular Science in 2020. The aim of the article was to identify molecular mechanisms underlying α1AMPK protection of the endothelial barrier, by focusing on the two main actors regulating paracellular permeability - interendothelial junctions and actin cytoskeleton.

In this article, we reported that:

1) α1AMPK is required to maintain basal expression levels of VE-Cad, ZO-1 and Cx43.

2) The loss of scaffolding Cx43 is likely to mediate the impact of α1AMPK invalidation on VE-Cad and ZO-1 expression.

3) AMPK activation protects the integrity of VE-Cad, ZO-1 and Cx43 against LPS challenge, thereby improving endothelial barrier function.

4) AMPK activation reinforces cortical action network by mechanisms involving the p38 MAPK/HSP27 pathway, independently of Rac1 activation.

PDF article can be found in annex 2.

α1AMP-Activated Protein Kinase Protects Against Lipopolysaccharide-Induced Endothelial Barrier Disruption via Junctional Reinforcement and Activation of the p38 MAPK/HSP27 Pathway

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Abstract

Vascular hyperpermeability is a determinant factor in the pathophysiology of sepsis, and AMP-activated protein kinase (AMPK) is known to play a role in maintaining endothelial barrier function in this condition. Thus, we investigated the underlying molecular mechanisms of this protective effect. α1AMPK expression and/or activity was modulated in human dermal microvascular endothelial cells using either α1AMPK-targeting small interfering RNA or the direct pharmacological AMPK activator 991, prior to lipopolysaccharide (LPS) treatment. Western blotting was used to analyse the expression and/or phosphorylation of proteins that compose cellular junctions (zonula occludens-1 [ZO-1], vascular endothelial cadherin [VE-Cad], connexin 43 [Cx43]) or that regulate actin cytoskeleton (p38 MAPK; heat shock protein 27 [HSP27]). Functional endothelial permeability was assessed by *in vitro* Transwell assays, and quantification of cellular junctions in the plasma membrane was assessed by immunofluorescence. Actin cytoskeleton remodelling was evaluated through actin fluorescent staining. We consequently demonstrate that α1AMPK deficiency is associated with reduced expression of CX43, ZO-1, and VE-Cad, and that the drastic loss of CX43 is likely responsible for the subsequent decreased expression and localisation of ZO-1 and VE-Cad in the plasma membrane. Moreover, α1AMPK activation by 991 protects against LPS-induced endothelial barrier disruption by reinforcing cortical actin cytoskeleton. This is due to a mechanism that involves the phosphorylation of p38 MAPK and HSP27, which is nonetheless independent of the small GTPase Rac1. This results in a drastic decrease of LPS-induced hyperpermeability. We conclude that α1AMPK activators that are suitable for clinical use may provide a specific therapeutic intervention that limits sepsis-induced vascular leakage.

1. Introduction

Sepsis, a major global health problem [14, 19, 483], is defined as a deregulated host response to an infection [6]. Its evolution towards multi-organ failure (MOF), which is known as a crucial predictor of survival [110], directly results from microcirculatory dysfunction [74, 82, 107]. The latter is highly dependent on the regulation of vascular extravasation [72], and the maintenance of intravascular volume remains one of the most important challenges for the clinical support of septic patients.

Dysregulation of transendothelial paracellular permeability is the main determinant of sepsis-induced vascular extravasation. This results from the disruption of inter-endothelial junctions (IEJs) and the disorganisation of actin cytoskeleton [171]. IEJs include adherens junctions (AJs) and tight junctions (TJs), which are both composed of multiprotein complexes. Some of their components, such as vascular endothelial cadherin (VE-Cad) and zonula occludens-1 (ZO-1), form part of the IEJ structure *per se* and thus constitute critical modulators of paracellular permeability [171]. Specifically, VE-Cad is recognised as the most important gatekeeper of the endothelial barrier [180, 191, 484, 485]. Other constituents play major roles in the organisation of protein–protein complexes in plasma membranes. They include connexin 43 (Cx43), which can directly and indirectly affect TJ and AJ assembly [235, 242, 245]. Actually, Cx43 signalling microdomains can influence a wide range of junctional- and cytoskeleton-associated proteins [233, 486] through mechanisms that involve direct protein–protein interactions and transcriptional regulation [233, 243, 247]. The complexes of IEJs are anchored to the actin cytoskeleton, which contributes to defining the IEJs' architecture [264, 272]. Actin filaments can exert antagonist effects on IEJs integrity, depending on their polymerisation activity, localisation, and contractility. These actin bundles can be categorised into two main types: cortical actin fibers that support the IEJs' anchorage to the plasma membrane, which are predominant at the basal state, and contractile

transcellular filaments called stress fibers that promote IEJs disassembly and internalisation by exerting pulling forces. Stress fibers are mostly formed in response to pro-inflammatory agents. Their contraction contributes to the onset of intercellular-gap formation [264, 272, 487]. The key mediators involved in actin-dynamic regulation include the small heat shock protein 27 kDa (HSP27), which plays a critical role in the regulation of actin polymerisation [282, 290]. Unphosphorylated HSP27 behaves like a filamentous actin (F-actin) cap-binding protein by inhibiting actin polymerisation. Phosphorylation of several distinct serine residues (Ser15, Ser78, and Ser82) relieves its capping activity and promotes actin microfilament formation. HSP27 is phosphorylated by mitogen-activated protein kinase-activated protein kinases (MAPKAPK) 2 and 3, which are both direct substrates of p38 MAPK α and β [277]. The role of HSP27 phosphorylation in the regulation of endothelial permeability remains controversial [277, 282, 290, 294, 488]. In addition, small Rho GTPases are crucial orchestrators of actin organisation and contractility [272, 309]. RhoA promotes stress fibre contraction, while Rac1 reinforces IEJs' anchorage to the plasma membrane by promoting the development of a cortical actin network [189, 194]. Altogether, junctional and cytoskeleton proteins regulate paracellular flow through tight control of the opening-closure sequences of the intercellular space. In pro-inflammatory conditions, paracellular permeability is enhanced because macromolecules and leukocytes are recruited within underlying tissues to regulate pathogen invasion. Extreme sepsis conditions drive homeostasis towards endothelial dysfunction, formation of intercellular gaps, subsequent massive plasma leakage and impaired oxygen diffusion into peripheral tissues, causing MOF. Targeting mediators that contribute to the vascular barrier integrity might therefore improve support for septic patients.

AMP-activated protein kinase (AMPK) is a key metabolic master that has broad effects on cellular functions, including organisation of IEJs [212, 448, 489] and actin cytoskeleton [460-465]. Its main endothelial isoform, α1AMPK, has been reported to

regulate paracellular permeability [97, 413, 490-492] and, more specifically, sepsis-induced vascular leakage [83, 97, 413, 481, 482]. Our group previously demonstrated that the mortality of mice exposed to sublethal doses of lipopolysaccharide (LPS, from *Escherichia coli* O55:B5) was dramatically increased by α1AMPK invalidation. This was also shown to be associated with enhanced vascular permeability [83]. Moreover, we showed that AMPK activation by the AMP-mimetic 5-aminoimidazole-4-carboxamide ribonucleotide (AICAr) in wild-type mice can attenuate LPS-induced tissue oedema formation. Other direct [83, 212, 481, 482] and indirect [97, 480, 493] AMPK activators have already demonstrated their capacity to protect the vascular barrier in in-vivo models of sepsis, and some are even associated with anti-inflammatory effects [480, 482, 493] and improved survival [482]. Among these activators, only metformin is currently used in clinical practice. However, its administration during sepsis remains controversial because of the potential evolution towards lactic acidosis [380]. Considering the urgency to improve medical support for septic patients [494], special attention should be paid to identifying new molecule targeted therapies that are clinically applicable to the particular context of sepsis.

In this study, we sought to further elucidate mechanisms through which AMPK regulates junctional assembly and permeability in LPS treated microvascular endothelial cells. Specifically, we examined the effect of the specific and direct pan-AMPK activator 991 on IEJs and cytoskeleton organisation. We report for the first time that AMPK plays a critical role in the maintenance and assembly of IEJs by regulating Cx43 expression, HSP27 phosphorylation and subsequent actin cytoskeleton remodelling.

2. Results

2.1. α1AMPK Preserves IEJs Integrity

2.1.1. α1AMPK Deficiency is Associated with Down regulation of VE-Cad, ZO-1, and Cx43 Expression

We first investigated whether α1AMPK regulates VE-Cad, ZO-1, and Cx43 expression in unstimulated and LPS-treated HMECs. Cells transfected with AMPKα1-targeting or scrambled control small interfering RNAs (siRNAs) were either treated or not treated with the best-available AMPK activator 991 (1 µM, 1h), prior to LPS stimulation (50 µg/mL, 6h) (Figure 1a). While AICAr can have off targets, the 991 compound has been recently described and is a direct and more specific AMPK activator [374]. Its effect is mainly allosteric, inducing a change in AMPK conformation and subsequent stimulation. Unlike LPS, treatment by 991 activates AMPK in HMECs, as reflected by AMPK phosphorylation on Thr172 and acetyl-CoA carboxylase (ACC) phosphorylation on Ser79. α2AMPK is not expressed in HMECs (data not shown). Transfection of α1AMPK-targeting siRNA abrogates α1AMPK expression and 991-mediated ACC phosphorylation (Figure 1b and 1c). Western blot analysis also reveals that VE-Cad, ZO-1, and Cx43 protein expression are significantly down regulated in α1AMPK-depleted cells, although they are not affected by 991 or LPS treatment (Figure 1b and 1c). Considering the importance of Cx43 in the formation and scaffolding of IEJs between endothelial cells, we subsequently utilised a specific siRNA-targeting Cx43 to characterise the impact of its deletion on VE-Cad and ZO-1 expression. The protein level of Cx43 is significantly lower in cells transfected with the siRNA anti-Cx43, compared to scramble-transfected cells (Figure 2a). Cx43 deficiency is associated with significantly reduced expression of VE-Cad and ZO-1 (Figure 2b and 2c). Accordingly, fluorescence analysis confirms the drastic loss of VE-Cad and ZO-1 signals at the edges of Cx43-silenced cells (Figure 2d and 2e). These results suggest that α1AMPK plays a critical role in the regulation of Cx43 expression

and subsequent maintenance and assembly of TJs and AJs, independently of LPS treatment.

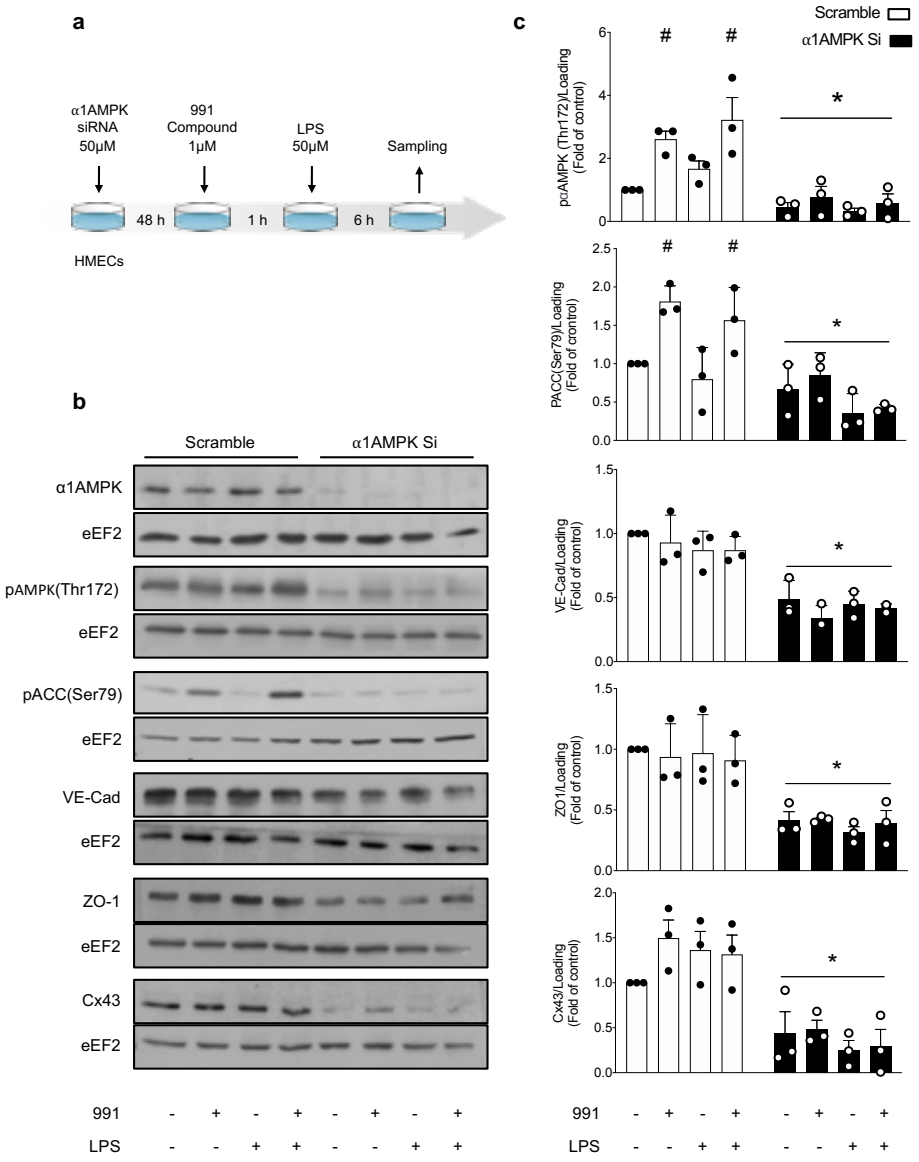

Figure 1. Basal α1AMPK regulates VE-Cad, ZO-1, and Cx43 expression. (**a**) Schematic representation of the experimental design, in which HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50nM) for 48 h. Then, they were treated with 991 (1μM) or dimethyl sulfoxide (DMSO) for one

hour and subsequently exposed or not exposed to LPS (*Escherichia coli* O55:B5, 50µg/mL) for six hours. (**b, c**) HMECs were treated according to the protocol detailed in (**a**). Cell lysates were submitted to Western blot analysis and probed with α1AMPK, phospho-AMPK (Thr172), phospho-ACC (Ser79), VE-Cad, ZO-1, and Cx43 antibodies (Abs). Anti-eukaryotic elongation factor 2 (eEF2) was used as a loading control. Representative western blots (**b**) and quantifications (**c**) are shown. Data are expressed as mean ± standard deviation (SD) (three biological replicates for each condition). $^{\#}p < 0.05$ is relative to corresponding untreated HMECs and $^{*}p < 0.05$ is relative to cells transfected with the scrambled siRNA. The data underwent two-way analysis of variance (ANOVA).

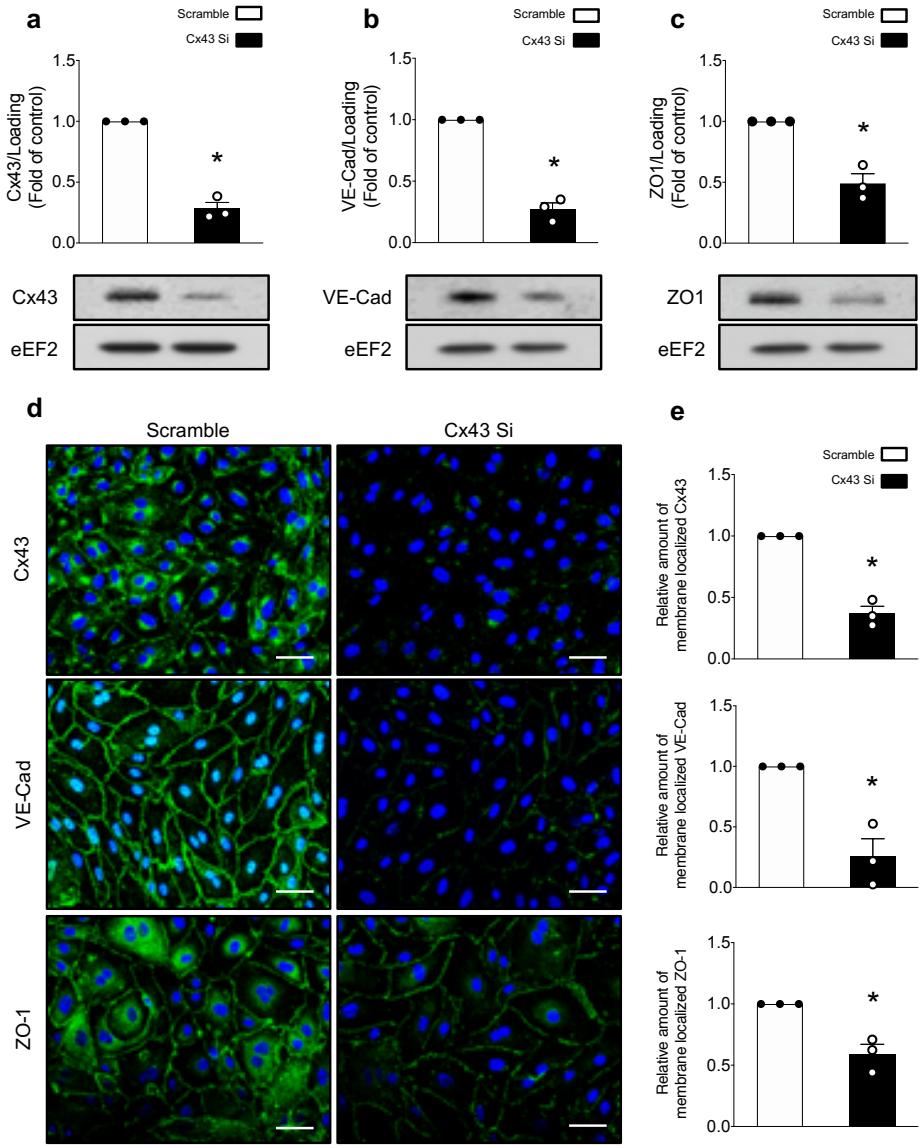

Figure 2. Cx43 deficiency is associated with decreased VE-Cad and ZO-1 expression. (**a**–**e**) HMECs were transfected with control non-targeting siRNA or Cx43 siRNA (50nM) for 48 h. (**a–c**) Cell lysates were submitted to Western blot analysis and probed with Cx43 (**a**), VE-Cad (**b**), and ZO-1 (**c**) Abs. eEF2 was used as a loading control. Representative Western blots (top panel) and quantifications (bottom panel) are shown. (**d–e**) Cx43, VE-Cad, and ZO-1 immunostainings. Nuclei are stained with DAPI. Scale bar, 50µm. Representative images (**d**) and quantifications (**e**) are shown. Data are expressed as mean ± SD (three biological replicates for each condition). *$p < 0.05$ is relative to cells transfected with the scrambled siRNA. Analyses were performed using Student's *t*-test.

2.1.2. AMPK Activation Prevents LPS-Induced Disruption of IEJs and Endothelial Barrier Dysfunction

LPS drastically impairs the integrity of VE-Cad (Figure 3a and 3b), ZO-1 (Figure 3c and 3d), and Cx43 (Figure 3e and 3f). Indeed, the linear shape of cell–cell junctions, which is observed in basal conditions, appears jagged and disconnected after LPS treatment. Accordingly, membrane staining quantification for VE-Cad, ZO-1, and Cx43 is significantly lower in LPS-treated cells, compared to control cells (Figure 3b, 3d, 3f). These changes are associated with the formation of intercellular gaps, which indicates endothelial barrier disruption. As we show that LPS does not affect VE-Cad, ZO-1, and Cx43 total protein expression (Figure 1), the decreased membrane staining of IEJs is likely due to their mechanical disruption and internalisation. Next, we determined the effects of the small-molecule AMPK activator 991. The 991 compound does not affect IEJs organisation in unstimulated HMECs. However, it reinforces Cx43, VE-Cad, and ZO-1 anchorage to the plasma membrane upon LPS stimulation and also prevents the appearance of intercellular gaps. This protective effect is lost in α1AMPK-depleted cells, indicating an AMPK-dependent action (Figure 3 a–f). Of note, the absence of α1AMPK induces a drastic decrease of VE-Cad, ZO-1, and Cx43 signals at the periphery of cells, regardless of the conditions, which confirms our previous Western blot data (Figure 1b and 1c). Finally, the involvement of the AMPK pathway in endothelial barrier integrity was demonstrated by measuring the clearance of HRP-coupled streptavidin through the HMEC monolayer. As expected, 991 treatment significantly reduces LPS-induced hyperpermeability (Figure 3g). To inactivate AMPK, we used the pan-AMPK inhibitor SBI0206965. AMPK inactivation significantly impairs endothelial barrier function and completely abrogates 991-mediated protection (Figure 3g).

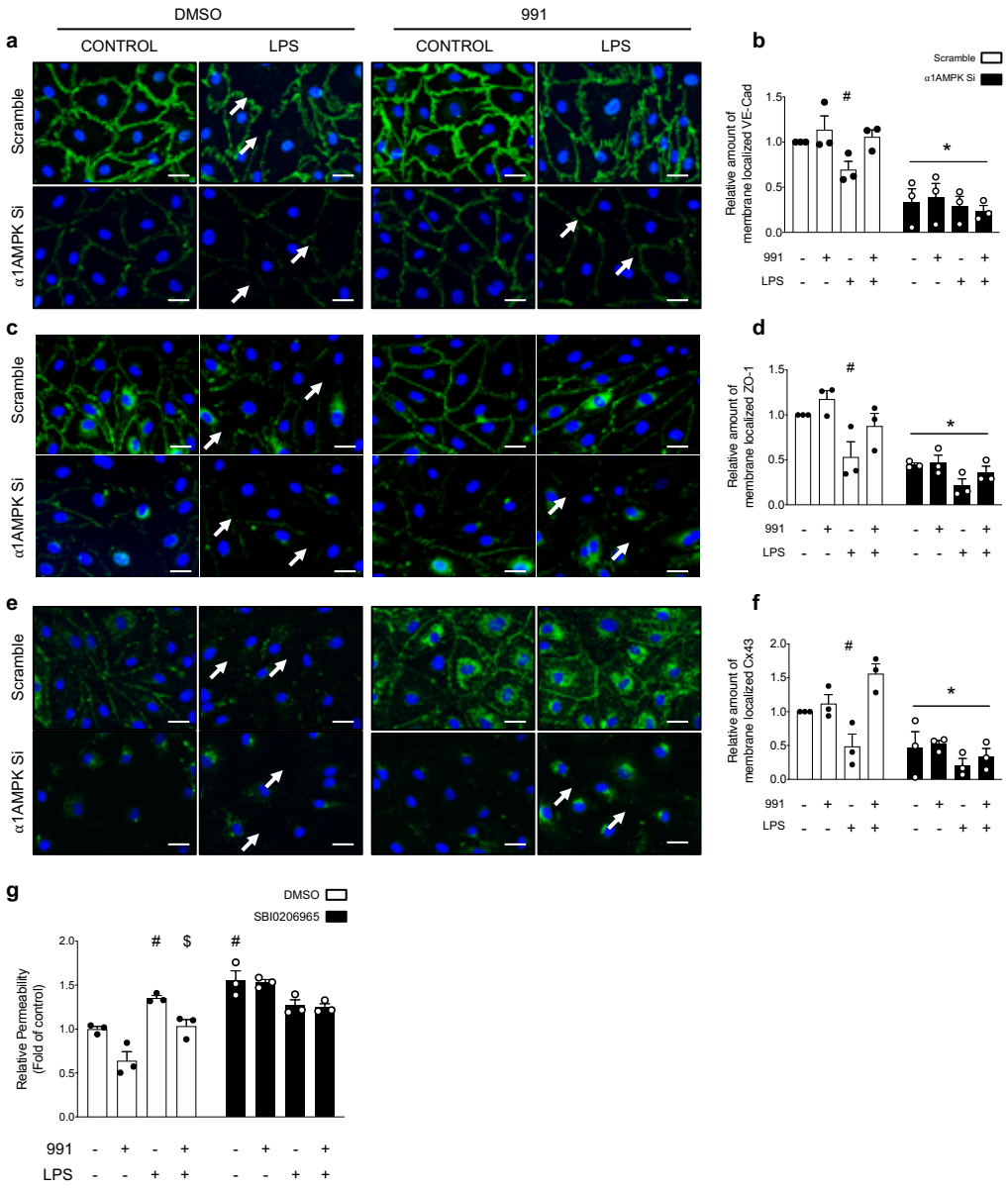

Figure 3. The AMPK Activation Preserves IEJs Organisation and Endothelial Barrier Function in HMECs Under LPS Challenge. (a-f) HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50nM) for 48 h. Then, HMECs were treated with dimethyl sulfoxide (DMSO) or 991 (1µM) for one hour, after which they were either exposed or not exposed to LPS (from *Escherichia coli* O55:B5, 50µg/mL) for six hours. Immunostainings and membrane-staining quantification of VE-Cad (**a, b**), ZO-1 (**c, d**), and Cx43 (**e, f**) are shown. Intercellular gaps are indicated by white arrows. Nuclei are stained with DAPI. Scale bar,

50µm. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}p < 0.05$ is relative to corresponding untreated HMECs and $*p < 0.05$ is relative to cells transfected with scrambled siRNA. The data underwent two-way ANOVA. **(g)** Endothelial permeability in response to AMPK activation, LPS challenge, and AMPK inhibitor SBI0206965. HMECs were grown on Transwell inserts for 72 h and treated or not treated with SBI0206965 (10µM) for 15 min before undergoing 991 and LPS treatments. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}p < .05$ is relative to respective non-treated HMECs and $^{\$}p < .05$ is relative to LPS-only treated HMECs. The data underwent two-way ANOVA.

2.2. α1AMPK Induces Actin Cytoskeleton Remodelling

2.2.1. AMPK Activation Modulates Actin Organisation and Polymerisation

Since junctional complexes are proposed to associate with the actin cytoskeleton, considered as a key component of cell permeability, we then investigated the impact of AMPK activation on LPS-induced F-actin morphological changes. In resting HMECs, F-actin is mainly organised in peripheric beams within the cytoplasm (Figure 4a). After LPS stimulation, peripherally located F-actin is substituted by centralised and irregular stress fibers running across the cytoplasm (Figure 4a and 4b). This effect is even more pronounced in the absence of α1AMPK, despite a significant decrease in the fluorescent signal that corresponds to F-actin (Figure 4c). AMPK activation by 991 strongly reinforces peripheral actin bands, both in unstimulated cells and in cells treated with LPS. This effect is reduced in the absence of α1AMPK (Figure 4a–c).

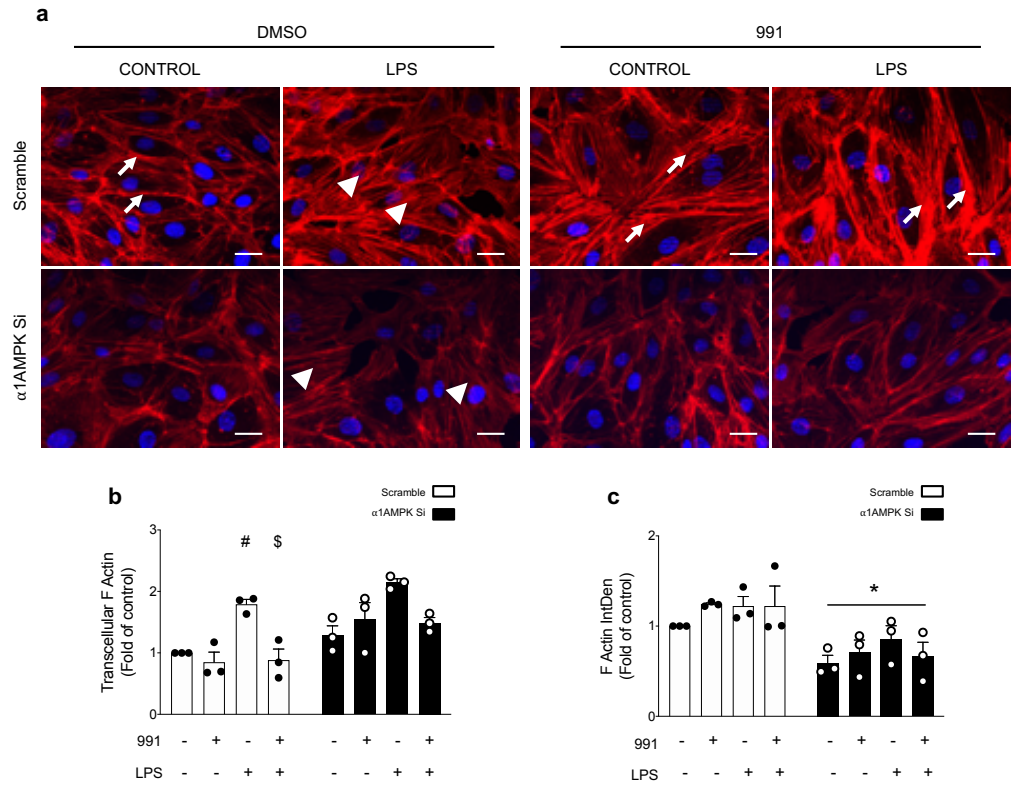

Figure 4. AMPK Activation Reinforces the Cortical Actin Network and Counters Stress-Fiber Formation During LPS Challenge. HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50nM) for 48 h and treated with dimethyl sulfoxide (DMSO) or 991 (1µM) for one hour. They were then exposed to LPS (from *Escherichia coli* O55:B5, 50µg/mL) for six hours. After fixation, HMECs were stained with Alexa Fluor 568 Phalloidin. (**a**) Representative fluorescence microscopy images of F-actin. Arrows indicate peripheric actin; arrowheads indicate centralised stress fibers. (**b**) Quantification of transcytoplasmic actin filaments. (**c**) Quantification of global F-actin amounts, which were assessed by integrated density measurement. Nuclei are stained with DAPI. Scale bar, 25 µm. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}p < 0.05$ is relative to corresponding untreated HMECs, $^{\$}p < 0.05$ is relative to corresponding LPS-treated HMECs, and $^{*}p < 0.05$ is relative to cells transfected by scrambled siRNA. The data underwent two-way ANOVA.

2.2.2. α1AMPK Regulates Actin Polymerisation via Activation of the p38 MAPK/HSP27 Pathway

Due to its pivotal role in actin polymerisation, we investigated whether HSP27 was involved in the protective action of α1AMPK against LPS-induced endothelial barrier impairment. The total expression and phosphorylation sate of HSP27(Ser82) and p38 MAPK(Thr180/Tyr182) were measured in the presence or absence of 991, before LPS stimulation. Results show that 991 and LPS increase p38 MAPK phosphorylation and can individually and additionally enhance HSP27 phosphorylation (Figure 5a and 5b). Unlike LPS, the effect of 991 on HSP27 phosphorylation is attenuated in α1AMPK-depleted cells (Figure 5a and 5b). It clearly depends on p38 MAPK activation since preincubation with the selective p38 MAPK inhibitor SB203580 significantly reduces signal intensity, regardless of the conditions (Figure 5c and 5d). Surprisingly, treatment with SB203580 increases MAPK p38 phosphorylation, possibly because of feedback activation induced by the inhibition of p38 MAPK activity (Figure 5c) [495]. Next, we examined the effect of direct inhibition of p38 MAPK on the actin cytoskeleton. SB203580 globally reduces F-actin content and impairs the well-organised peripheral actin network of unstimulated cells, which acts in favour of the formation of stress fibers across the cytoplasm (Figure 6a–c). More interestingly, p38 MAPK inactivation prevents 991-induced enrichment of thick actin bundles around the cell periphery of LPS-treated cells (Figure 6a–c). Altogether, these results indicate that α1AMPK activation modifies the cytoskeleton response to LPS injury by potentiating the p38 MAPK/HSP27 pathway. Accordingly, while basal permeability is barely affected by p38 MAPK inhibition, SB203580 completely prevents the protective effect of 991 (Figure 6d). Since p38 MAPK has been reported as a downstream target of Rac1, a small GTPase intimately involved in regulating cortical actin structures and endothelial barrier function [496], Rac1

activation was measured in the presence or absence of 991, before LPS stimulation. G-LISA analysis shows that LPS and 991 can individually enhance Rac1 activity, while there is no additional effect of the combined treatments (Figure 7a). Preincubation with NSC23766 inhibits Rac1 activity (Figure 7a) but does not significantly change 991—or LPS-induced HSP27 phosphorylation (Figure 7b), indicating that the activation of the p38 MAPK/HSP27 axis is independent of Rac1 in both conditions.

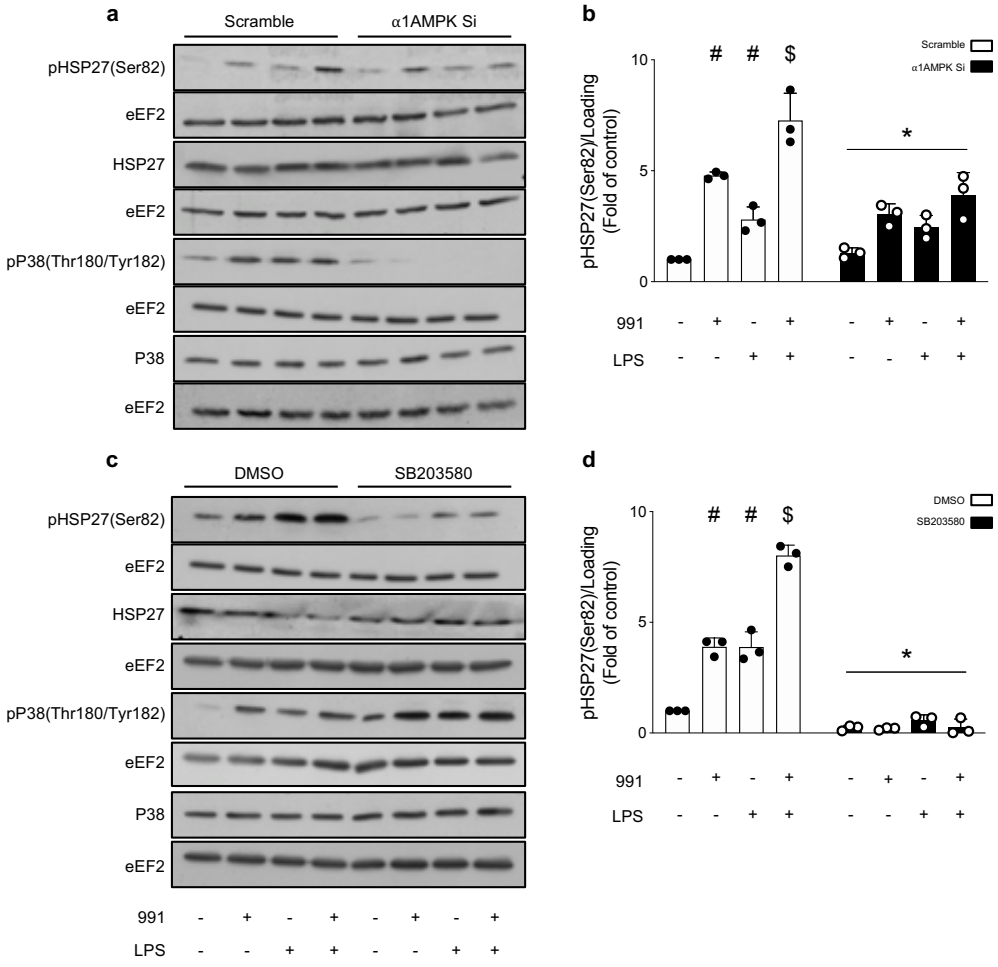

Figure 5. AMPK Activation Leads to Increased HSP27 Phosphorylation, via a p38 MAPK-Dependent Way. (**a–b**) HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50nM) for 48 h and

then treated with dimethyl sulfoxide (DMSO) or 991 (1µM) for one hour. They were then exposed to LPS (from *Escherichia coli* O55:B5, 50µg/mL) for six hours. (**c–d**) HMECs were preincubated with DMSO or with the p38 MAPK inhibitor SB203580 (10µM) for 15 min. Then, they were treated with 991 (1µM) or DMSO for one hour and subsequently exposed or not exposed to LPS (from *Escherichia coli* O55:B5, 50µg/mL) for six hours. (**a–d**) Cell lysates were submitted to western blot analysis and probed with total HSP27, total p38 MAPK, phospho-HSP27 and phospho-p38 MAPK Abs. eEF2 was used as a loading control. Representative Western blots (**a, c**) and quantification of HSP27 (Ser82) phosphorylation (**b, d**) are shown. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}p < 0.05$ is relative to corresponding non-treated HMECs, $^{\$}p < 0.05$ is relative to corresponding LPS-treated HMECs, and $*p < 0.05$ is relative to cells transfected with non-targeting siRNA or that were treated with the vehicle. The data underwent two-way ANOVA.

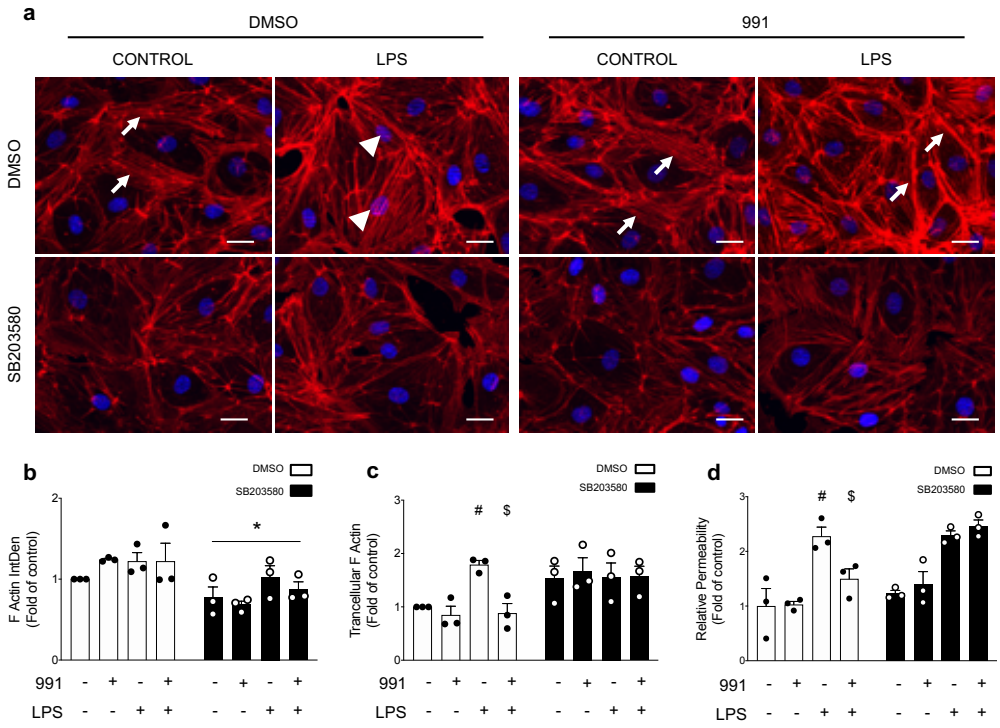

Figure 6. AMPK Activation Regulates Actin Organization and Cell Permeability via the p38 MAPK/HSP27 Pathway. (**a–c**) HMECs were fixed and stained with Alexa Fluor 568 Phalloidin. (**a**) Representative

fluorescence microscopy images of F-actin. Arrows indicate peripheric actin ; arrowheads indicate centralised stress fibers. (**b**) Quantification of area occupied by transcytoplasmic actin filaments. (**c**) Quantification of global F-actin amounts, which were assessed by integrated density measurement. Nuclei are stained with DAPI. Scale bar, 25 µm. Data are expressed as means ± SD (three independent experiments for each condition). *$p < 0.05$ is relative to cells treated with DMSO. The data underwent two-way ANOVA. **(d)** Endothelial permeability in response to AMPK activation, LPS challenge, and p38 MAPK inhibitor SB203580. HMECs were grown on Transwell inserts for 72 h and treated or not treated with SB203580 (10µM) for 15 min before undergoing 991 and LPS treatments. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}p < 0.05$ is relative to respective non-treated HMECs and $^{\$}p < 0.05$ is relative to LPS-only treated HMECs. The data underwent two-way ANOVA.

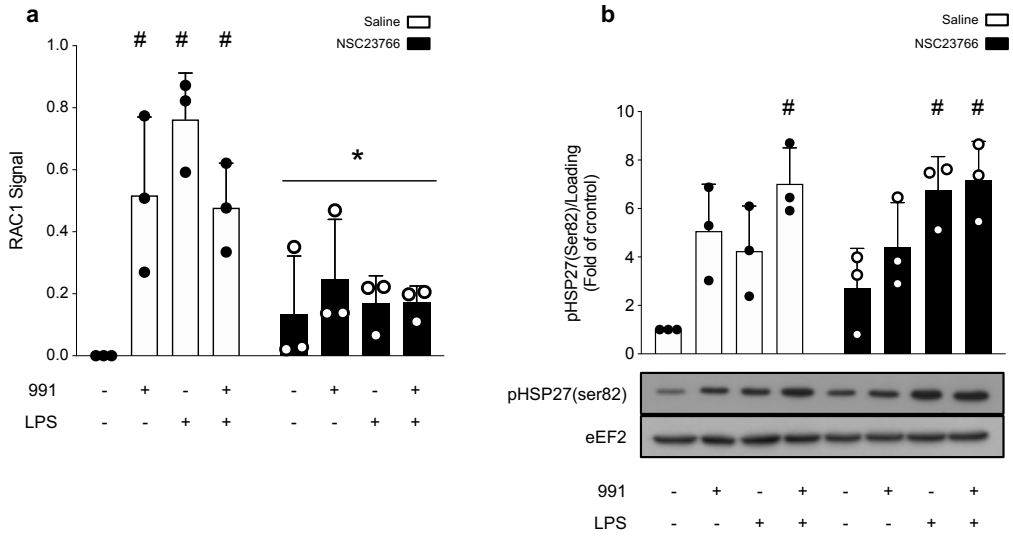

Figure 7. AMPK Regulates Actin Organization Independently of Rac1. (**a, b**) HMECs were treated with saline or the Rac1 inhibitor NSC23766 (100µM) for 15 min before adding dimethyl sulfoxide (DMSO) or 991 (1µM) for one hour. Then, they were exposed to LPS (from *Escherichia coli* O55:B5, 50µg/mL) for six hours. (**a**) Lysates were engaged in G-LISA assays to precisely monitor Rac1 activity. Quantification represents the differential signal, compared to basal, in fold of control. (**b**) Representative Western blots and quantification of HSP27 (Ser82) phosphorylation. eEF2 was used as a loading control. Data are

expressed as means ± SD (three biological replicates for each condition). $^{\#}p < 0.05$ is relative to corresponding non-treated HMECs. The data underwent two-way ANOVA.

3. Discussion

In this study, we first demonstrate that α1AMPK is essential in maintaining the proper expression and architecture of IEJs in basal conditions. Second, we identify the p38 MAPK/HSP27 pathway and actin cytoskeleton as central mediators of the protective effect of activated α1AMPK on the endothelial barrier. Experiments were performed using HMECs, which constitutes a highly relevant model for the assessment of vascular leakage mechanisms that mainly occur at the level of capillaries and post capillary venules. The α1AMPK isoform plays a predominant role in these cells since its specific invalidation using a siRNA strategy almost completely abrogates the 991 effect on ACC phosphorylation, which is the bona fide substrate of AMPK. Several studies, including ours, have reported the beneficial role of AMPK in endothelial barrier regulation during sepsis [83, 97, 413, 481, 482]. Here, we confirm this protective action in microvascular endothelial cells that are exposed to LPS and highlight the involvement of new molecular mechanisms.

Our previous work has already reported the protective role of basal α1AMPK in the maintenance of endothelial barrier function, through the preservation of ZO-1 integrity in TJs [83]. Here, we demonstrate that, in addition to ZO-1, VE-Cad is also distorted and down regulated in the absence of α1AMPK. Our data further support the emerging concept that Cx43 can contribute to the basal expression and organisation of ZO-1 and VE-Cad. Cx43, which is a protein normally associated with gap junctional communication, has recognisable scaffolding functions that contribute to various biological processes [233, 235]. The AMPK-Cx43 pathway has been highlighted in cardiomyocytes [454] and in cardiac fibroblasts [456] in which it plays a role in the

fibrotic response commonly seen with a variety of cardiac pathologies [457]. The link between AMPK and Cx43 has never been demonstrated in the endothelium. We show for the first time that AMPK deficiency is associated to a drastic loss of Cx43 in endothelial cells, which leads to the subsequent disruption of tight and adherens junctions. The fact that the 991 compound has no impact on Cx43 expression supports the notion that non-catalytic functions of AMPK are rather involved in this regulation. These non-catalytic functions may include the scaffolding of protein complexes, the competition for protein interactions, allosteric effects on other enzymes or subcellular targeting. Among others, ERK1 and ERK2 were shown to influence their substrates not only by phosphorylation, but also by direct protein-protein interactions, independently of kinase activity [497]. We believe that this type of regulation likely explains how AMPK may influence Cx43 expression.

While the direct interaction of Cx43 with ZO-1 has been extensively demonstrated [243, 245, 246], the mechanism is more speculative for VE-Cad. The interaction between Cx43 and VE-Cad likely involves other partners, such as N-cadherin (N-Cad), p 120, and α- and ß-catenin, all of them being known to bind Cx43 and promote VE-Cad stability [191, 212, 241, 247, 498, 499]. In addition to its scaffolding action, Cx43 is also described as a modulator of gene transcription, by the interaction of its carboxy tail with transcription factors driving its translocation to the nucleus and binding to gene promoters [233, 500]. However, these potential mechanisms do not exclude Cx43-independent transcriptional regulation of ZO-1 and VE-Cad by α1AMPK. Indeed, AMPK can directly (via the phosphorylation of transcriptional regulators) or indirectly (via the induction of sirtuins) regulate gene transcription [501]. Post-transcriptional (changes in micro-RNAs expression) and post-translational (acetylation or nitrosylation) mechanisms might also be involved [502-505]. Future experimental work is necessary to delineate the molecular regulation of VE-Cad, ZO-1, and Cx43 by AMPK.

Our present work emphasises the critical role of actin cytoskeleton in the α1AMPK dependent protection of IEJ integrity in LPS-treated endothelial cells. Our observations are supported by data showing that, in epithelial and endothelial cells, activated AMPK can reinforce the interaction between cadherin-catenin complexes and actin cytoskeleton via both regulation of N-Cad [212, 498] and phosphorylation of G-alpha interacting vesicle associated protein (GIV) [471, 506]. Other protective mechanisms have been highlighted. In Caco-2 cells submitted to a calcium switch [444, 448, 489, 507], α1AMPK activation is associated with a change in the expression level of VE-Cad, ZO-1, or Cx43 [448, 490], whereas our present data show that the expression of these proteins is not changed in 991-treated HMECs. In other cellular models, activated α1AMPK may affect the integrity of TJs and AJs by phosphorylating some of their components. For example, claudin-1 and -4 have been described as direct AMPK substrates in epithelial Eph4 cells and SV40 immortalised rat submandibular acinar cell lines, respectively [492, 508]. Afadin is another AMPK target involved in reinforcing TJs in Madin-Darby Canine Kidney (MDCK) cells, via its binding to ZO-1 [445].

We and others have previously demonstrated that α1AMPK regulates the organisation of actin cytoskeleton in different cell types [460-465]. Interestingly, HSP27 plays an important role in actin dynamics, through its phosphorylation downstream of p38 MAPK [277, 282, 290]. Very interestingly, a link between Cx43 and HSP27 was previously demonstrated in HeLa cells [251]. In this model, Cx43 regulates actin dynamics and migration by binding p21-activated protein kinase 1 (PAK1), which then activates the downstream p38 MAPK/HSP27 pathway. Our data show that HSP27 phosphorylation protects the endothelial barrier function. However, the role of HSP27 in the regulation of endothelial permeability remains controversial. For example, HSP27 phosphorylation protects against hypoxia- or burn serum-induced permeability of endothelial cells [277, 282, 290, 294, 488]. In contrary, pertussis toxin-induced endothelial permeability is temporally linked to p38 MAPK activation and phosphorylation of HSP27 [277, 278]; and

LPS-induced endothelial barrier dysfunction correlates with HSP27 phosphorylation *in vitro* (our own data) and *in vivo* [294, 295]. The reason for these discrepancies is not clear. It has been suggested that activation of the p38 MAPK/HSP27 pathway by LPS might be an adaptive response promoting centralised stress fibers and subsequent cell adhesion in order to counteract contractility which is rather regulated by the RhoA pathway and downstream myosin light chain phosphorylation [290].

Given that p38 MAPK is a downstream effector of AMPK in endothelial cells [466], we postulated that it could be involved in the protective action of 991 on cell cytoskeleton and permeability. Subsequently, we asserted that HSP27 phosphorylation was significantly increased in 991-treated HMECs. While LPS is associated with the formation of centralised stress fibers, 991 increases cortical actin structures, suggesting the likely involvement of two different p38 MAPK activation pathways. Typically, p38 MAPK is activated through small GTPases, such as Rac1, and the downstream canonical three-tiered kinase cascade [273, 509]. Rac1 is activated by LPS and 991 to the same extent, but, surprisingly, we observed that its inhibition had no significant impact on LPS- or 991-induced HSP27 phosphorylation. This suggests the involvement of other non-canonical mechanisms of p38 MAPK activation. Accordingly, it was recently shown that the p38α isoform in HMECs can be autoactivated through its interaction with the transforming growth factor α-activated kinase 1 binding proteins (TAB) 1, 2, or 3, which is independent of the MAPK cascades [274]. Notably, the direct activation of p38 MAPK by AMPK, via the scaffold protein TAB1, has been previously described in apoptotic lymphocytes and the ischemic heart [469, 470], strengthening the relevance of this mechanism in 991-treated HMECs. Finally, a different non-canonical pathway for p38 MAPK activation can be mediated through autophosphorylation, which is facilitated by the tyrosine kinase Zap70 [274-276]. The role of these non-canonical mechanisms of p38 MAPK in the regulation of endothelial barrier disruption remains poorly understood and warrants further exploration.

In summary, our data show that α1AMPK is required to maintain the integrity of TJs and AJs in microvascular endothelial cells. Moreover, α1AMPK activation leads to the phosphorylation of HSP27 via a non-canonical p38 MAPK dependent mechanism, which results in the reinforcement of the submembrane actin network during LPS challenge, contributing to consolidation of IEJs in the plasma membrane and intercellular tethering (Figure 8). Therefore, α1AMPK activation might have therapeutic potential to alleviate conditions in which microvascular barrier formation is dramatically compromised, such as sepsis [510].

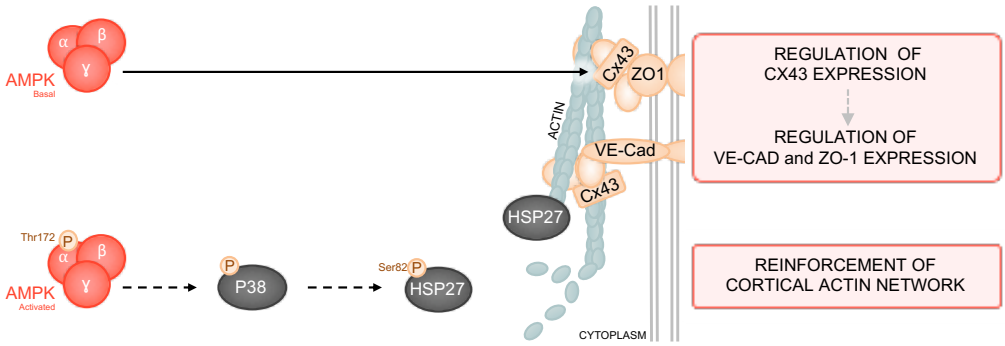

Figure 8. Molecular mechanisms involved in endothelial barrier protection by α1AMPK. α1AMPK is essential in maintaining the proper expression and architecture of IEJs in basal conditions. It regulates Cx43 expression which, via its scaffolding functions, may contribute to ZO-1 and VE-Cad organisation at plasma membrane. On the other hand, the specific and direct pan-AMPK activator 991 protects HMECs against LPS-induced hyperpermeability. The mechanism involves activation of the p38 MAPK/HSP27 pathway and subsequent cortical actin polymerisation, which reinforces IEJs anchorage at the plasma membrane and subsequent intercellular tethering. Abbreviations: AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (P38), Heat shock protein 27 (HSP27), VE-cadherin (VE-Cad), Zonula occludens-1 (ZO-1), Connexin 43 (Cx43).

4. Materials and Methods

4.1. Reagents and Antibodies

The 991 compound was kindly provided by Benoit Viollet (Institut Cochin, DR2 Inserm, Paris, France). SBI0206965 (#SML1540), SB203580 (#S8307), NSC23766 (#SML0952), dimethyl sulfoxide (DMSO, #485519) and LPS from *Escherichia coli* O55:B5 (50μg/mL, #L2880) were from Sigma-Aldrich. We also used a halt protease and phosphatase inhibitor cocktail (#78442; Thermo Fisher Scientific), Lipofectamine RNAiMAX reagent (#13778-150; Invitrogen), Opti-MEM (#31985070; Gibco), siRNA negative control (#AM4635; Ambion), siRNA PRKAA1 (#AM51334; Ambion), siRNA GAJ1 (#4392422; Ambion), BM chemiluminescence blotting system (#11500694001; Roche), 12mm μ-dish culture inserts (634-1577P; VWR), Alexa Fluor 488 Deoxyribonuclease I (DNAse I) (#D12371; Invitrogen), Alexa Fluor 568 Phalloidin (#A12380; Invitrogen), Transwell inserts (#3413; Corning), M200 medium (#M200500; Gibco), HRP-coupled streptavidin (#15:1000, DY998; R&D Systems), TMB substrate (#T040; Sigma-Aldrich), H_2SO_4 (#30743; Sigma-Aldrich), and RAC1 G-LISA kit (#BK128; Cytoskeleton, Inc, Denver, CO). The antibodies used were α1AMPK (#MA5-15815; Thermo Fisher Scientific), phospho-AMPK Thr172 (#2535; Cell Signaling Technology), phospho-ACC S79 (#3661; Cell Signaling Technology), Cx43 (#3512; Cell Signaling Technology), VE-Cad (#36-1900; Thermo Fisher Scientific), ZO-1 (#339100; Thermo Fisher Scientific), p38 MAPK (#9212; Cell Signaling Technology), phospho-p38 MAPK T180/Y182 (#9211; Cell Signaling Technology), heat shock protein of 27 kDa (HSP-27) (#2402; Cell Signaling Technology), phospho-HSP27 S82 (#44534; Life Technologies), eEF2 (#PA5-17794; Thermo Fisher Scientific), secondary horseradish peroxidase (HRP)-conjugated antibodies (#A0545; Sigma-Aldrich or#554002; BD Biosciences), and Alexa Fluor-coupled secondary antibodies (#A21202 or #A21206; Invitrogen).

4.2. Cell Culture and Treatments

HMECs were purchased from Promocell (C-12225) and cultured according to the manufacturer's recommendations, using Endothelial Cells Growth Medium MV (C-22020; Promocell) containing 1% penicillin-streptomycin, at 37°C and 5% CO_2 in a humidified incubator. Cells were sub-cultured when reaching 80% confluence and used until subculture number 7. To proceed with the experiments, cells were seeded at a density of $10^4/cm^2$ and cultured for 48–72 h, until total confluence was reached. Medium deprivation was performed for two hours prior to treatment or experimentation.

4.3. siRNA Transfections

For endothelial α1AMPK and Cx43 silencing, HMECs were seeded the day before transfection with heparin-free Endothelial Cells Growth Medium MV2 (C-22022; Promocell) to reach 60% confluence within 24 h. Reverse transfection was then performed for 48 h with either a control non-targeting siRNA construct (50nM), a siRNA specifically targeting PRKAA1 (50nM), or GAJ1 (50nM). This was performed using a lipofectamine RNAimax transfecting reagent, which adhered to the manufacturer's instructions.

4.4. Western Blotting

Western blot analysis was performed on HMEC lysis, as previously described [511]. Protein content was measured using the Bradford method, which used bovine serum albumin (BSA) as a reference. Proteins (15μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electro blotted. Membranes were then probed with the primary antibody overnight at 4°C, in order to assess protein phosphorylation or total expression level. Primary antibodies included α1AMPK

(1:1000), phospho-AMPK Thr172 (1:1000), phospho-ACC (1:5000), Cx43 (1:1000), VE-Cad (4:1000), ZO-1 (2:1000), p38 MAPK (1/1000), phospho-p38 MAPK T180/Y182 (1/1000), HSP27 (1:1000), phospho-HSP27 S82 (1:1000), and eEF2 (1:1000). Membranes were then incubated with appropriate secondary HRP-conjugated antibodies (1:20000, #A0545 or 1:10000, #554002; both Sigma-Aldrich) for one hour at room temperature. A chemiluminescence blotting system was used for detection. Quantification was executed by ImageJ software and normalised with eEF2 as a loading control on the same gel. Each experiment was repeated three times.

4.5. Immunofluorescence Microscopy

HMECs were seeded on non-coated glass coverslips at 20×10^3 cells/cm^2, 72 h before treatment. After treatment, cells were fixed in 4% paraformaldehyde, permeabilised with 0.3% triton X-100 for ten minutes, and blocked with 10% BSA for 45 min. For intercellular junction studies, cells were stained as previously described [83], using the following primary antibodies: Cx43 (1:100), VE-Cad (1:25), or ZO-1 (1:50). Cells were then incubated with the appropriate Alexa Fluor-coupled secondary antibody (1:1000, #A21202 or #A21206). For cytoskeleton studies, HMECs were incubated with Alexa Fluor 568 Phalloidin (150nM) for 15 min at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Stainings were visualised under a Zeiss Imager Z1 microscope that was equipped with an ApoTome device. Pictures were acquired with a 20× objective. Each experiment was repeated three times.

4.6. Image Analysis

Quantitative image analysis was performed on uncompressed images (native format: zvi) with Fiji 1.52n on MacOS (10.14.5). The intercellular junctions evidenced by ZO-1 or VE-Cad stainings were automatically delimited by a fixed-value threshold method. Stained area was quantified, and the mean signal intensity was calculated within this

selection. Stained membrane segments were subsequently detected using the Analyse Particles and Skeletonize tools, and automatically counted. Due to the irregular distribution of the Cx43 signal, all cell membranes were manually defined as regions of interest. The Cx43 positive area and mean signal intensity were then quantified within these regions.

To analyse cytoskeleton organisation, F-actin and nuclei were sequentially detected using threshold methods in the red and blue channels, respectively. Stained area and integrated density were measured for F-actin. The area fraction of F-actin that was colocalised within nucleus areas was subsequently calculated and defined as stress fibers. The mean signal intensity was also measured.

For normalisation purposes, the nuclei in all images were automatically counted using a threshold method and the Analyse Particles tool.

4.7. Endothelial Permeability Assessment

For the endothelial permeability assay, HMECs (10^5 cells/well) were seeded on gelatin-coated Transwell inserts of 24-well plates, in 250µL complete with Endothelial Cells Growth Medium MV. They were then incubated for 72 h at 37°C and with 5% CO^2. Cells were incubated in free M200 medium for two hours before stimulation. Cells were then incubated with the different compounds, as indicated in the figure legends. After treatment, the medium from the upper chamber was replaced by 300µL of M200, containing HRP-coupled streptavidin. The medium of the lower chamber was collected after ten minutes of incubation at 37°C, and every condition was aliquoted in triplicate. The TMB substrate was added for ten minutes, and an Elisa reader was used to stop the reaction with 2N H_2SO_4 before acquiring 450 nm absorption. Resultant absorption intensity values were normalised over the vehicle control condition. Each experiment was repeated three times.

4.8. Small GTPases Activity Assay

Rac1 activity was measured in protein lysates from HMECs using the commercially available G-LISA assay kit, according to the manufacturer's instructions. Each experiment was repeated three times.

4.9. Statistical Analysis

Statistical analyses were conducted using SPSS v.25 software (IBM Corp., Armonk, New York) and graphs build with GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). All tests were two-sided, with significance set at the 0.05 probability level. Data were expressed as mean ± standard deviation. Means were compared using an unpaired student *t*-test. One—or two-way analysis of variance with *f* test were used to compare more than two groups. The Bonferroni correction was used for multiple comparisons.

Author Contributions

Conceptualisation: M.A., D.C.-Z., C.B. and S.H.; Formal analysis: J.D.P., C.D., G.C. and C.B.; Funding acquisition: D.C.-Z, L.B., C.B. and S.H.; Investigation: M.A., J.D.P. and C.D.; Methodology: G,C,, Caroline Bouzin and Rozenn Quarck; Supervision, Diego Castanares-Zapatero and Sandrine Horman; Writing —original draft: M.A. and S.H.; Writing—review & editing: D.C.-Z, R.Q., L.B., C.B. and S.H.

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Conflicts of Interest

The authors declare no conflicts of interest.

CHAPTER II – POTENTIAL THERAPEUTIC IMPLICATIONS

After delineating molecular mechanisms mediating endothelial barrier protection by α1AMPK, we asked whether clinically usable AMPK activators could be considered as a new therapeutic approach to protect against sepsis capillary leak syndrome.

This second chapter is dedicated to our work recently submitted in the Journal of Experimental Medicine. The aim of the article was to describe the protection conferred by clinically relevant concentrations of canagliflozin on sepsis-induced vascular barrier disruption, and to confirm the involvement of the α1AMPK dependent pathways previously identified in this effect.

In this article, we report that:

1) Canagliflozin activates the α1AMPK/p38 MAPK/HSP27 pathway in HMECs.

2) AMPK activation by canagliflozin protects the integrity VE-Cad and reinforces endothelial barrier function in HMECs submitted to LPS injury.

3) AMPK activation by canagliflozin preserves VE-Cad integrity in HMECs challenged with human septic plasma.

4) Canagliflozin protects against capillary leak syndrome in septic mice, by mechanisms depending on endothelial α1AMPK.

Canagliflozin protects against sepsis capillary leak syndrome by activating endothelial α1AMPK

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Abstract

Sepsis capillary leak syndrome (SCLS) is an independent prognostic factor for poor sepsis outcome. We previously demonstrated that α1AMP-activated protein kinase (α1AMPK) prevents sepsis-induced vascular hyperpermeability by mechanisms involving VE-cadherin (VE-Cad) stabilization and activation of p38MAPK/HSP27 pathway. Canagliflozin, a SGLT2 inhibitor, has recently been proven to activate AMPK in endothelial cells. Therefore, we hypothesized that canagliflozin could be of therapeutic potential in patients suffering from SCLS. We herein report that canagliflozin, used at clinically-relevant concentrations, counteracts lipopolysaccharide (LPS)-induced vascular hyperpermeability and albumin leakage in wild-type, but not in endothelial-specific α1AMPK-knockout mice. *In vitro*, canagliflozin was demonstrated to activate α1AMPK/p38MAPK/HSP27 pathway and to preserve VE-Cad's integrity in human endothelial cells exposed to human septic plasma. In conclusion, our data demonstrate that canagliflozin protects against SCLS via an α1AMPK-dependent pathway, and lead us to consider novel therapeutic perspectives in SCLS.

1 Introduction

Sepsis is a major health concern worldwide [15], and is defined as a syndrome of dysregulated host response to infection causing life-threatening organ dysfunction [6]. Despite significant advances in the understanding of the disease, the therapeutic management of septic patients primarily relies on supportive care and mortality rates remain unacceptably high, around 40% [13]. Sepsis capillary leak syndrome (SCLS), mainly caused by vascular hyperpermeability, is a critical process in sepsis pathophysiology and has been demonstrated to be an independent prognostic factor of survival [79]. Moreover, growing evidence supports that maintenance of vascular barrier integrity improves sepsis outcome [62, 83, 95-99]. However, no therapeutic proposal that targets SCLS has so far reached the clinical trial stage.

SCLS is caused by vascular barrier disruption. Under healthy conditions, endothelial cells are sealed to one another by inter-endothelial junctions (IEJs) that effectively control the passage of molecules in a size-selective manner. Vascular endothelial cadherin (VE-Cad), the major component of adherens junctions (AJs), is a protein essentially involved in this regulation [180, 181]. Its stability depends on the actin cytoskeleton [264], whose polymerization is notably regulated by the phosphorylation of heat-shock protein of 27kDa (HSP27), downstream of the p38 MAP kinase (p38MAPK)[277]. Upon sepsis, stress mediators trigger signaling cascades that induce actin cytoskeleton contraction, AJs disruption, and loss of endothelial barrier function [45, 176, 183]. This event is characterized by the formation of intercellular gaps, leading to plasma leaking through the endothelium and resulting in widespread edema [45]. Albumin, the main determinant of plasmatic oncotic pressure [80], notably drives this process through osmotic forces. In addition to dramatically reducing circulating blood volume, capillary leaking directly compromises the microcirculation [75]. First, the fluid accumulating in the interstitial space mechanically compresses capillaries and, thus, impairs

microvascular blood flow. Second, the perivascular fluid enhances the distance required for oxygen diffusion. Impaired tissue perfusion and oxygenation processes progressively induce organ failure and ultimately affect patient survival [71, 74].

The catalytic subunit of AMP-activated protein kinase (AMPK) is primarily expressed under its α1-isoform within the microvascular endothelium; there, it acts as a major regulator of the actin cytoskeleton and IEJs [212, 461, 512]. Our team and others have previously demonstrated the pivotal role of α1AMPK in the maintenance of endothelial barrier function, specifically in SCLS [83, 413, 513]. In mechanistic terms, we demonstrated that endothelial barrier protection by α1AMPK was mediated by p38MAPK/HSP27-dependent enhancement of VE-Cad stability [512]. Nevertheless, none of the AMPK activators used in the different studies can be safely employed *in vivo* or securely administered to septic patients [83, 97, 493, 513]. In this context, a particularly interesting therapeutic strategy would be to identify AMPK activators that are clinically-usable and protect against sepsis-induced vascular leakage.

Canagliflozin, an inhibitor of sodium-glucose co-transporter 2 (SGLT2i), is currently prescribed as oral glucose-lowering agent to patients with diabetes. Independently of modulating glucose transport, clinically-relevant canagliflozin concentrations also activate AMPK in different cell types, including human endothelial cells [387]. Interestingly, in addition to increasing renal glucose excretion, strong evidence supports that canagliflozin exerts significant cardiovascular protective effects, whose exact mechanisms are still poorly understood [382, 385, 514]. On account of its effect on AMPK activity, we hypothesized that canagliflozin may constitute a new therapeutic option to target SCLS.

In the current study, we have evaluated *in vivo* the potential benefits of canagliflozin-induced AMPK activation on SCLS. Using a murine model of specific and conditional endothelial α1AMPK deletion, we have demonstrated that canagliflozin protects against

vascular leakage by mechanisms that are dependent upon endothelial α1AMPK. This protection involves both activation of p38MAPK/HSP27 pathway and preservation of VE-Cad integrity. By validating these results in endothelial cells submitted to human plasma collected from septic shock patients, we have laid the groundwork for further clinical investigations.

2 Results

2.1 Generation and validation of conditional e-AMPK KO mice

To better delineate the role played by endothelial α1AMPK in protecting against SCLS, we first generated a mouse model in which endothelial α1AMPK was conditionally and specifically deleted in the endothelium. With this aim in mind, mice expressing the tamoxifen-responsive conditional Cre-ERT2 fusion protein under the Cdh5 promoter ($Cdh5Cre^{+/-}$) control were crossed with mice expressing LoxP-flanked PRKAA1 gene ($\alpha1AMPK^{fl/fl}$). The $Cdh5Cre^{-/-}/\alpha1AMPK^{fl/fl}$ (e-AMPK WT) littermates were employed as controls. To validate the efficacy and specificity of tamoxifen-induced α1AMPK deletion, α1AMPK gene expression was measured using TaqMan qPCR on endothelial cells that were immunoprecipitated from lung tissues. Results demonstrate 60% α1AMPK depletion in endothelial cells that were isolated from e-AMPK KO, in comparison with e-AMPK WT mice (Fig. 1 A). Notably, this invalidation was not observed in immunoprecipitated supernatant that contained non-endothelial cells (Fig. 1 B).

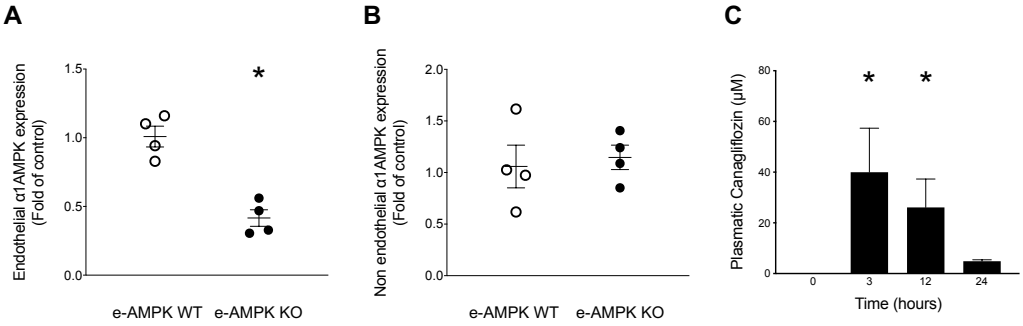

Figure 1. Generation and validation of the experimental model. e-AMPK WT/KO mice were intraperitoneally administered tamoxifen (500µg/mice) for five consecutive days in order to induce α1AMPK invalidation specifically in the endothelium. 3 weeks after the last Tamoxifen injection, mice were sacrificed and endothelial cells were immunoprecipitated from lung tissue with dynabeads. α1AMPK gene expression was detected by TaqMan qPCR on both isolated endothelial cells and supernatants. (**A**) Validation of endothelial α1AMPK deletion's extent in e-AMPK KO. α1AMPK expression detected by TaqMan qPCR in immunoprecipitated endothelial cells. (**B**) Validation of endothelial α1AMPK deletion's specificity. α1AMPK expression detected by TaqMan qPCR on lysates of lung tissue depleted with endothelial cells. (**C**) Time course of Canagliflozin plasma levels in mice treated by oral gavage, 100mg/kg, during indicated time. The data are mean ± SEM, n=3 to 5/group. The data were analyzed using one-way ANOVA test.

2.2 Canagliflozin protects against SCLS via endothelial α1AMPK-dependent mechanisms

We next investigated whether canagliflozin treatment could prevent SCLS in septic mice. Canagliflozin was administered by oral gavage at a dose of 100mg/Kg, thereby reaching the clinically relevant plasma concentrations of ∼10µM throughout the 24-hour experiment duration, as validated by liquid chromatography-mass spectrometry (Fig. 1 C)[515]. Sepsis was induced by intraperitoneal injections of sublethal doses of lipopolysaccharide O55:B5 (LPS), an endotoxin produced by *Escherichia coli* (*E. coli*). Our

experimental setup is summarized in Fig. 2 A. Capillary leak was monitored using Evans Blue Dye (EBD), as previously reported [83]. As expected, EBD detection on myocardial sections of e-AMPK WT mice submitted to LPS was indicative of relevant capillary leakage. These data were reinforced by measuring plasmatic albumin levels, which clearly revealed that LPS treatment was associated with reduced albuminemia, probably due to albumin leakage. Importantly, canagliflozin administration drastically reduced LPS-induced myocardial edema and maintained albumin plasma levels, which is possibly indicative of reinforced vascular barrier function (Fig. 2 B, D and F). Likewise, this outcome may also be explained by renal protection, given that several clinical studies have recently reported a decreased albuminuria in SGLT2i-treated patients [382, 516]. Finally, similar experiments performed in e-AMPK KO mice demonstrated that canagliflozin protection was abrogated in the absence of endothelial α1AMPK. Indeed, sepsis-induced myocardial edema and albumin leakage persisted despite canagliflozin treatment in e-AMPK KO animals (Fig. 2 C, E and G). Taken all results together, these data demonstrate that canagliflozin is indeed able to protect septic mice against SCLS, based on an endothelial α1AMPK-dependent mechanism.

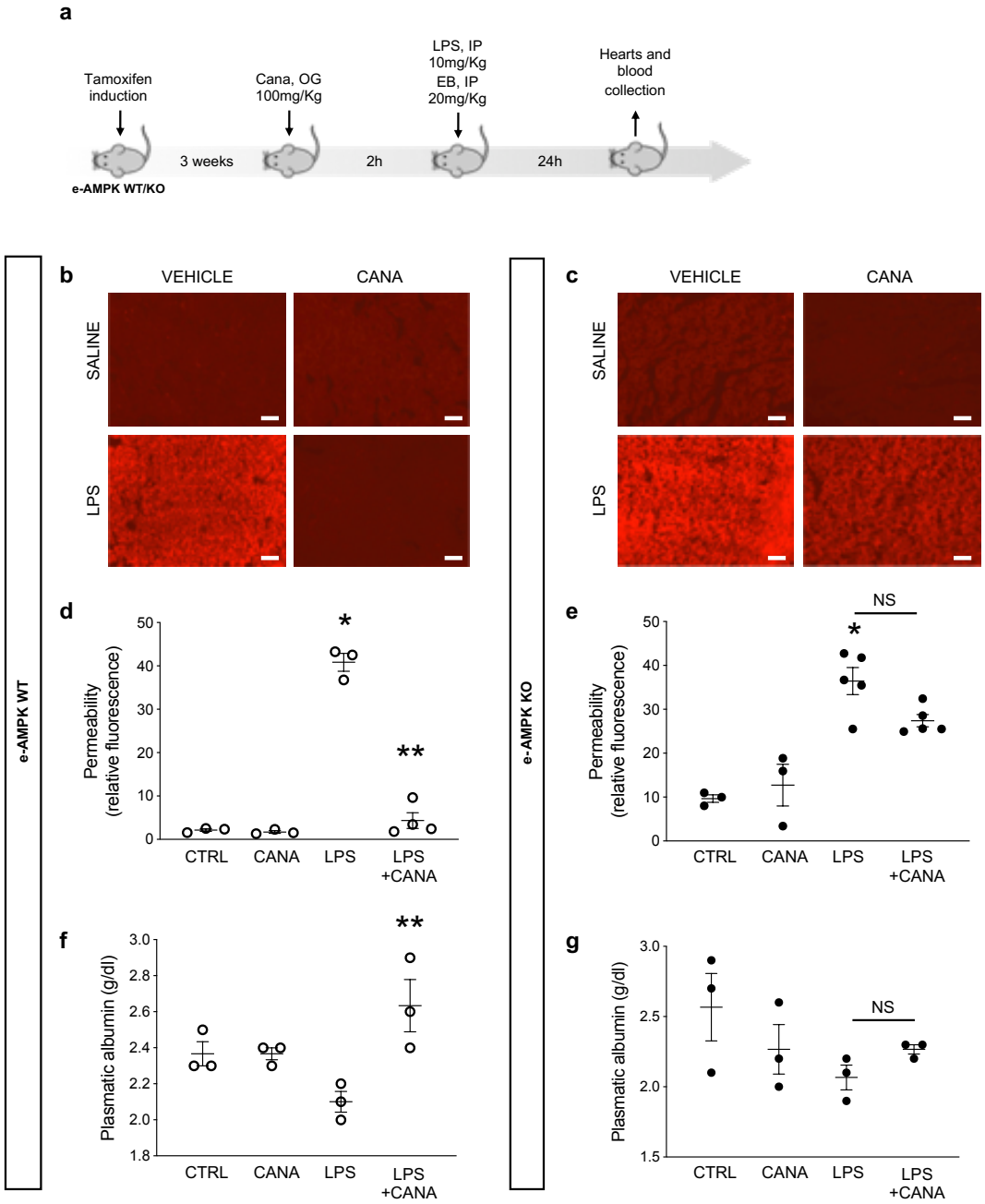

Figure 2. Canagliflozin protects against Lipopolysaccharide (LPS) induced capillary leak syndrome via endothelial α1AMPK dependent mechanisms. (A) Schematic representation of the experimental protocol for *in vivo* permeability assessment. e-AMPK WT/KO mice were intraperitoneally administered tamoxifen

(500µg/mice) for five consecutive days in order to induce α1AMPK invalidation specifically in the endothelium. 3 weeks after the last Tamoxifen injection, mice were treated with Canagliflozin (Cana) (100mg/Kg) by oral gavage, two hours before being submitted to LPS treatment (sublethal doses, 10mg/kg) by intraperitoneal (IP) injection. Evans Blue Dye (EBD) was administered by IP simultaneously with LPS. (B-E) Cardiac vascular permeability was assessed via EBD fluorescence quantification on myocardial sections of hearts sampled 24 hours after injection of LPS or saline vehicle. Representative images (B,C) and quantifications (D,E) are shown. Scale bar, 200µm. (F,G) Plasmatic albumin levels were measured on blood samples collected 24 hours after injection of LPS or saline vehicle. The data are mean ± SEM, n=3 to 5/group. *$p < 0.05$ is relative to control saline group, **$p < 0.05$ is relative to LPS treated group. NS= nonsignificant. The data underwent two-way ANOVA.

2.3 Canagliflozin activates α1AMPK/p38 MAPK/HSP27 pathway in HMECs

To better understand the molecular mechanisms underlying canagliflozin protection of endothelial barrier function, we assessed the impact of canagliflozin treatment on AMPK activation and its downstream p38MAPK/HSP27 pathway in human endothelial cells. The p38MAPK/HSP27 pathway is known to mediate AMPK-dependent stabilization of inter-endothelial junctions by reorganizing the actin cytoskeleton [277, 282, 512]. Indeed, HSP27 is an actin-capping protein that inhibits actin polymerization by binding the microfilaments positive ends. HSP27 phosphorylation downstream of the p38MAPK releases HSP27 from actin, thereby enabling further filament polymerization in order to reinforce IEJ anchorage. Since SCLS primarily occurs at the level of capillaries and post-capillary venules, experiments were performed on human endothelial cells derived from the microcirculation (HMECs). Results indicate that canagliflozin dose-dependently activates AMPK, as assessed via phosphorylation of AMPK (Thr172) and its bona fide substrate acetyl-CoA carboxylase (ACC) (Ser79) (Fig. 3 A-D). Furthermore, canagliflozin significantly increases phosphorylation of both p38 MAPK (Thr180/Tyr182) and HSP27 (Ser82), and this in a dose-dependent manner (Fig. 3 E-H). Incubation of HMECs with 3µM canagliflozin for increasing time periods also resulted in a significant and sustained

AMPK activation, as represented by both AMPK (Thr172) and ACC (Ser79) phosphorylation (Fig. S1 A-D). Taken together, these results demonstrate that clinically relevant canagliflozin concentrations do indeed activate the α1AMPK/p38MAPK/HSP27 pathway in HMECs, thereby potentially reinforcing inter-endothelial junctions by modulating actin cytoskeleton organization.

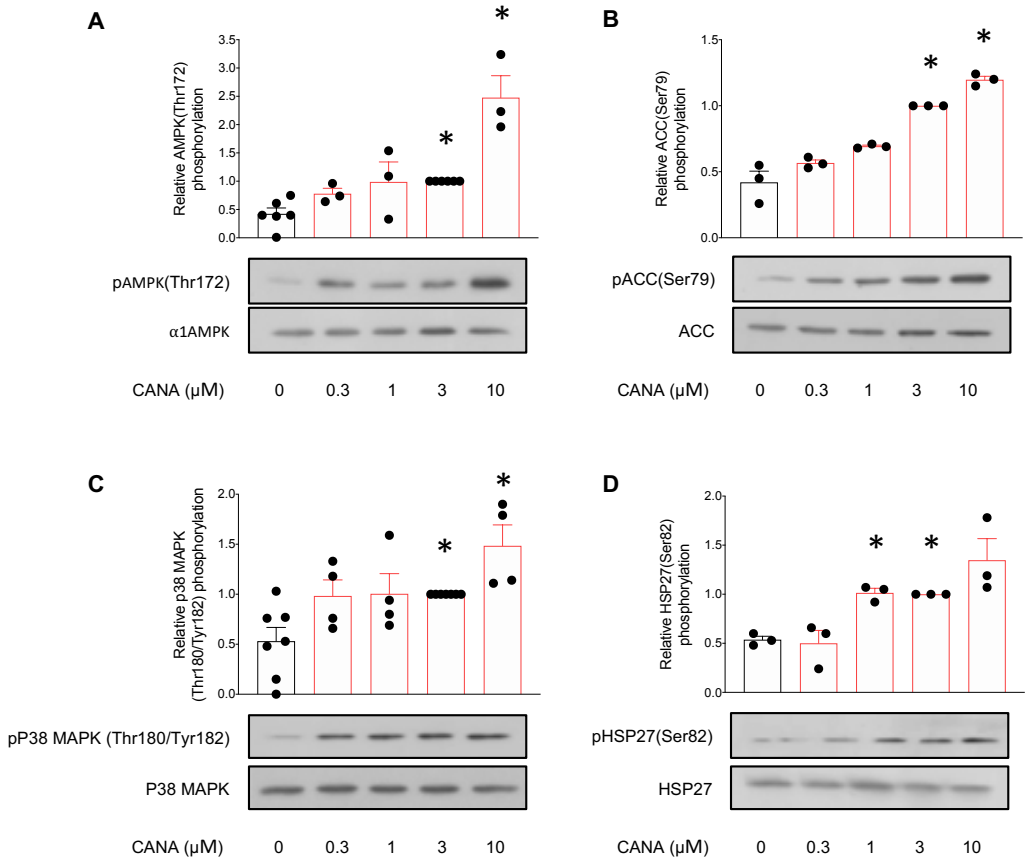

Figure 3. Canagliflozin activates the α1AMPK/p38MAPK/HSP27 pathway in HMECs. Human microvascular endothelial cells (HMECs) were treated with Canagliflozin (Cana) for the indicated concentrations during one hour. Cell lysates were submitted to Western Blot analysis and probed with total and phosphorylated (**A**) α1AMPK (Thr172), (**B**) ACC (Ser79), (**C**) p38 MAPK (Thr180/Tyr182) and (**D**) HSP27(Ser82) antibodies. Representative western blots and quantification are shown. Data are fold of the

3µM condition, and expressed as mean ± SEM (three to six biological replicates for each condition). *p < 0.05 is relative to untreated HMECs. The data underwent one-way ANOVA.

2.4 Canagliflozin-induced AMPK activation protects VE-Cad organization and endothelial barrier function against LPS injury

Next, we investigated the impact of canagliflozin on VE-Cad, *i.e.,* the junctional protein known to be the gatekeeper of endothelial barrier function [180]. Therefore, immunostainings were performed on HMECs, either depleted or not in α1AMPK, before being treated with canagliflozin (3µM) and LPS (50µg/mL) (Fig. 4 A). Fig. 4B illustrates that the continuous peripheral staining of VE-Cad under basal conditions, appears to be disorganized in response to LPS treatment. This is associated with the formation of intercellular gaps. In contrast, canagliflozin likely strengthens VE-Cad anchorage within the plasma membrane, preserving its organization and preventing the formation of intercellular gaps in response to LPS. The response to canagliflozin treatment is abrogated in α1AMPK-deficient cells, as confirmed by VE-Cad signal quantifications (Fig. 4 B and C). Finally, the impact of canagliflozin-induced AMPK activation on the endothelial barrier function was evaluated *in vitro* by measuring the clearance of HRP-coupled streptavidin through the HMECs monolayer (Fig. 4 D). Because cellular transfection affects by itself the barrier integrity, we employed the pan-AMPK inhibitor SBI0206965 to abrogate AMPK activation. As expected, LPS treatment increases endothelial permeability, whereas canagliflozin interestingly protects against LPS-induced endothelial barrier disruption. The SBI0206965 compound, when given alone, significantly impairs endothelial barrier function, whereas this agent completely abolishes canagliflozin-induced protection. Of interest, these results are remarkably supported by our previous data that mechanistically demonstrated the essential role of α1AMPK in both maintaining expression and architecture of IEJs under basal conditions,

and mediating the protective effect of 991 compound, its best pharmacological activator, in preventing IEJs disruption caused by LPS insult [512].

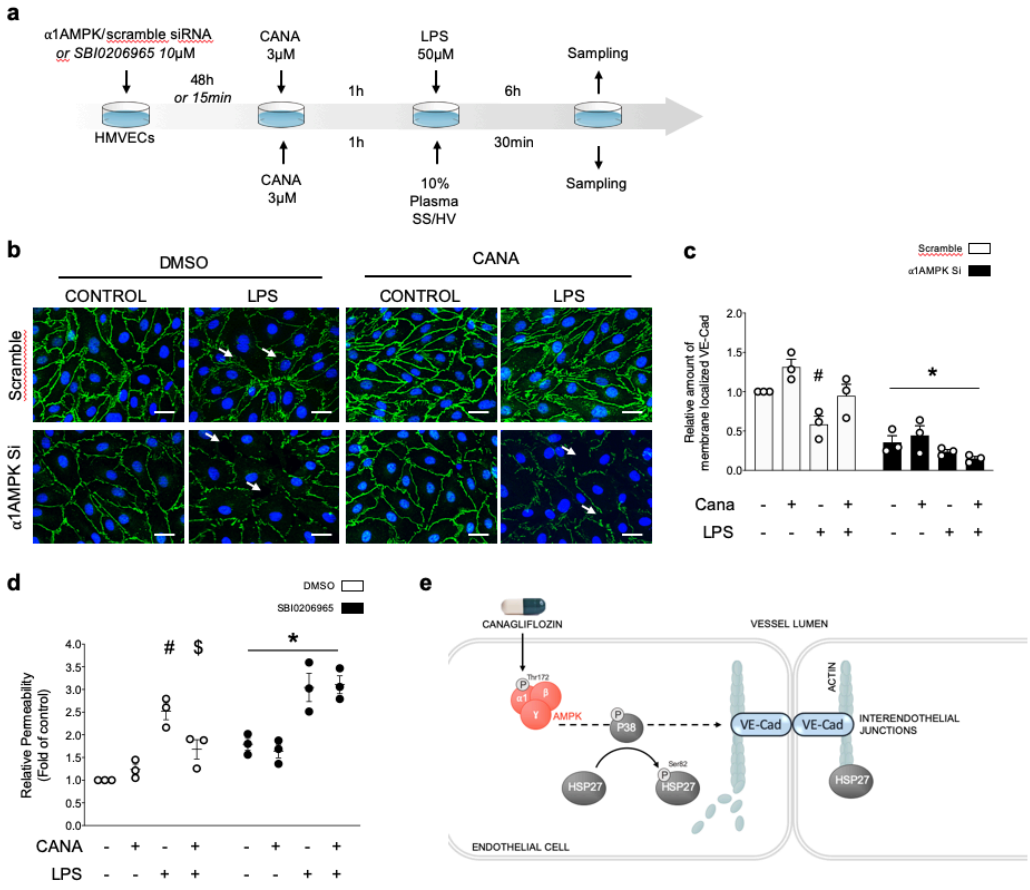

Figure 4. AMPK activation by Canagliflozin protects VE-Cad organization and endothelial barrier function against LPS injury. (**A**) Schematic representation of the experimental protocol. For (B, C), Human microvascular endothelial cells (HMECs) were transfected with scramble or α1AMPK targeting siRNA (50nM) for 48h, then treated with Canagliflozin (Cana) (3μM) or dimethyl sulfoxide (DMSO) for one hour, before adding Lipopolysaccharide (LPS) or vehicle (50μg/mL) for 6 hours. For (D), SBI compound was incubated for 15 minutes, then Canagliflozin (3μM) or DMSO were incubated for one hour, before adding

LPS (50µg/mL) for 6 hours. (**B**, **C**) VE-Cad Immunostainings performed on HMECs treated according to the protocol detailed in (A). Representative images (B) and quantifications (C) are shown. Intercellular gaps are indicated by white arrows. Nuclei are stained with DAPI. Scale bar, 50µm. (**D**) Endothelial permeability in response to Canagliflozin, LPS challenge, and AMPK Inhibitor SBI0206965. HMECs were grown on gelatin-coated Transwell inserts for 72h and treated according to the protocol detailed in (A). (**E**) Proposed molecular mechanisms involved in SCLS protection by α1AMPK. Data are expressed as mean ± SEM (three biological replicates for each condition). $^{\#}p < 0.05$ is relative to respective non-treated HMECs, $^{\$}p < 0.05$ is relative to LPS-only treated HMECs, and $*p < 0.05$ is relative to cells treated with DMSO. The data underwent two-way ANOVA.

2.5 Canagliflozin-induced AMPK activation protects VE-Cad integrity in HMECs challenged with human septic plasma

Finally, in order to reinforce the translational perspectives of our work, the effects of canagliflozin were evaluated on HMECs incubated with human plasma collected from either control healthy volunteers (HV) or septic shock patients (SS) (Fig. 5 A, B and Fig. S2). Immunostainings show that both HV and SS plasma affect VE-Cad architecture, with SS plasma inducing higher VE-Cad disruption, as represented by discontinuous jagged signals and intercellular gaps formation. Of major interest, canagliflozin importantly preserved VE-Cad integrity and linear organization, while slightly enhancing its membrane expression in HMECs exposed to both HV and SS plasma. On the other hand, α1AMPK depletion was associated with reduced, disrupted VE-Cad signal, and drastic decrease of canagliflozin protective effects. These, however, also seem to involve AMPK-independent mechanisms, since their abrogation appears inconstant in AMPK depleted cells.

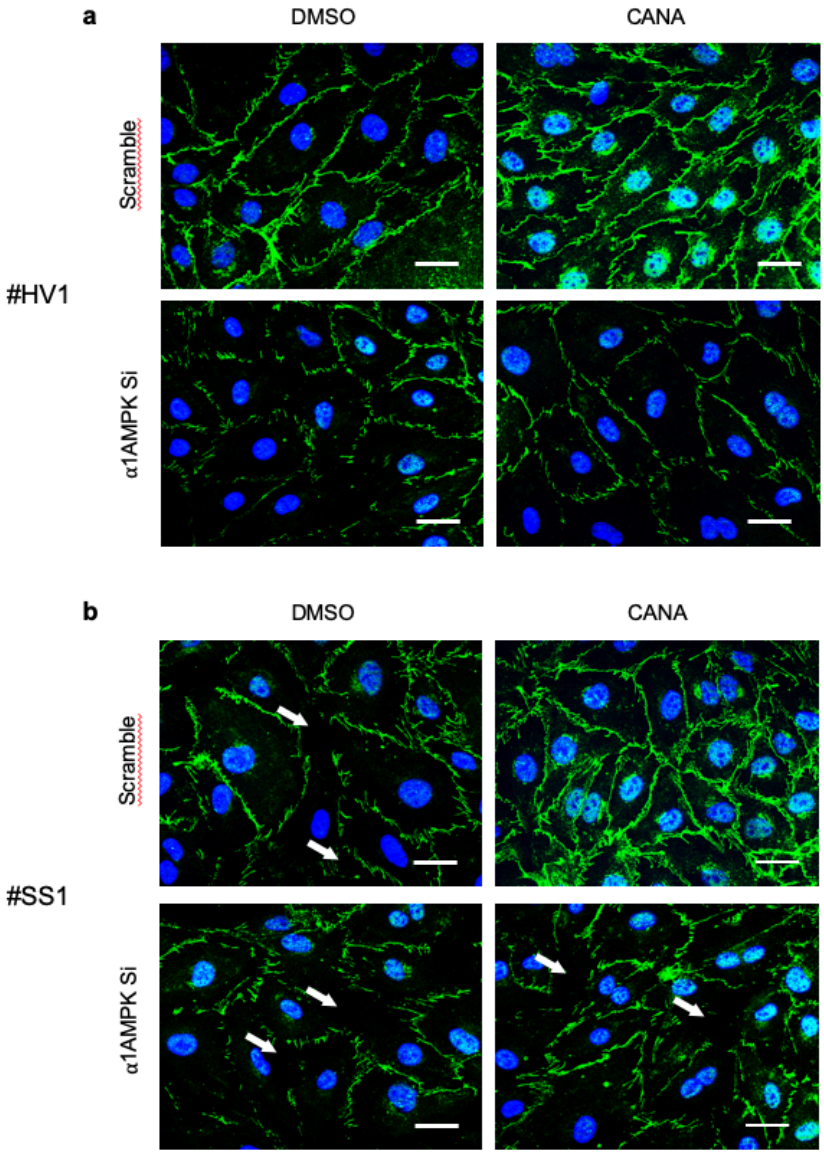

Figure 5. AMPK activation by Canagliflozin protects VE-Cad integrity in HMECs challenged with human healthy and septic plasma. VE-Cad immunostainings performed on Human microvascular endothelial cells (HMECs). HMECs were transfected with scramble or α1AMPK targeting siRNA (50nM) for 48h, before being treated with Canagliflozin (3µM) or dimethyl sulfoxide (DMSO) for one hour, then incubated with 10% plasma of healthy volunteers (HV) or septic shock patients (SS) for 30 minutes. Typical examples of pictures are shown in this figure. This experiment has been repeated with 4 different donors for each

group (see supplemental, Fig. S2). Intercellular gaps are indicated by white arrows. Nuclei are stained with DAPI. Scale bar, 50µm.

3 Discussion and conclusions

Our work highlights canagliflozin's protective effects on sepsis-induced vascular hyperpermeability, and demonstrates that endothelial α1AMPK and its downstream p38MAPK/HSP27/VE-Cad regulatory pathway are involved in this protection. During the past decade, canagliflozin, along with other SGLT2i, have emerged as antidiabetic drugs that exhibit remarkable cardiovascular protection [382-386], which is not fully explained by their blood glucose-lowering properties [517-519]. Extensive clinical studies are currently conducted to further characterize this protective action. Emerging hypotheses notably suggest that glucosuria and natriuresis, decreased inflammation, or reduced oxidative stress may all contribute to improve cardiovascular function [519]. Interestingly, the diuretic effects of SGLT2i have been shown to selectively reduce interstitial edema with minimal depletion of circulating blood volume [520-522]. Beyond postulating that endothelial barrier integrity possibly plays a significant role in this particular SGLT2i feature, such integrity appears particularly relevant in the SCLS setting.

Here, we have demonstrated that canagliflozin-induced microvascular protection depends, at least to some extent, on endothelial α1AMPK activation. While the SGLT2-induced AMPK activation is attracting growing interest, the hypothesis that this kinase mediates SGLT2's cardiovascular protective effect is still incompletely explored. Tampering inflammation [388, 389, 396], reducing oxidative stress [392, 393, 407], regulating nitric oxide production [389, 398, 409], or preventing energy depletion [389, 391, 523] are overlapping cardiovascular protective mechanisms of SGLT2i and AMPK. Our data combined with recent findings supporting an empagliflozin-induced AMPK-dependent protection of microvascular barrier function [391] enable us to postulate

that the endothelial barrier regulation as induced by AMPK activation also represents a key mechanism contributing to the SGLT2i–related cardiovascular protection.

One limitation of our study is that in our model, canagliflozin treatment was administered before LPS challenge. This protocol does, thus, not reflect the clinical reality of sepsis. The canagliflozin impact should further be evaluated and figured as a treatment of declared sepsis. A recent study demonstrating improved survival of septic mice subsequently treated with SGLT2i supports that promising results may reasonably be expected [524]. This perspective, however, raises several issues and questions. It should, first, be determined whether other SGLT2is could exert a similar protective mechanism. Indeed, although canagliflozin was initially described to activate AMPK more robustly compared to other SGLT2i [387], both empagliflozin and dapagliflozin were subsequently reported to activate AMPK *in vivo* in both mice total heart samples [388, 389] and cardiac fibroblasts [388]. Moreover, empagliflozin was proven particularly beneficial for microvascular barrier function [391] and against sepsis injury [389, 524]. Therefore, we believe that vascular barrier protection would not be restricted to canagliflozin. Second, SGLT2i administration could be further optimized in order to avoid *per os* formulations for intensive care unit (ICU) settings. In this respect, it is worth mentioning that the feasibility of intravenous canagliflozin administration has been demonstrated recently [409]. Finally, owing to the heterogeneity of clinical sepsis presentations and based on the increasing relevance attached to genetic variants concerning host septic responses, it is unlikely that all septic patients would benefit to the same extent from receiving SGLT2i inhibitors. Dynamic protocols reflecting the integrity of the microcirculation – *i.e.,* orthogonal polarization spectral imaging [525] – could be useful for early identifying patients that are most likely to respond to this new therapeutic approach.

Given the urgent need for therapies targeting SCLS [45], along with the emerging cardiovascular protective role of SGLT2i, we strongly believe that these aforementioned findings will likely help better link these two research fields and ultimately provide a promising therapeutic approach for SCLS. It must additionally be mentioned that SGLT2i have been recently approved in other indications than diabetes such as heart failure with reduced ejection fraction, extending their clinical applications and daily uses.

4 Materials and Methods

4.1 Materials and reagents

Tamoxifen (#T5648), LPS O55:B5 (#L2880), SBI0206965 (#SML1540), TMB substrate (#T040), Evans Blue (#E2129), dimethyl sulfoxide (DMSO, #485519) and Transwell inserts (#3413) were from Sigma-Aldrich (Overijse, Belgium). Cells-to-CT 1-Step TaqMan kit (#A25603), PRKAA1 FAM probe (#Mm01296696_m1), RPL32 VIC probe (Mm02528467_m1), siRNA negative control (#AM4635), siRNA PRKAA1 (#AM51334), and Lipofectamine RNAiMAX reagent (#13778-150) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). We also used Canagliflozin (#HY-10451, Medchem Express, Monmouth Junction, NJ, USA), and HRP-coupled streptavidin (#15:1000, DY998; R&D Systems, Minneapolis, MN, USA). The antibodies employed were α1AMPK (#MA5-15815; Thermo Fisher Scientific), phospho-AMPK Thr172 (#2535; Cell Signaling Technology, Danvers, MA, USA), phospho-ACC S79 (#3661; Cell Signaling Technology), p38 MAPK (#9212; Cell Signaling Technology), phospho-p38 MAPK T180/Y182 (#9211; Cell Signaling Technology), heat shock protein of 27 kDa (HSP-27) (#2402; Cell Signaling Technology), phospho-HSP27 S82 (#44534; Life Technologies, Thermo Fisher Scientific), eEF2 (#PA5-17794; Thermo Fisher Scientific), secondary horseradish peroxidase (HRP)-conjugated antibodies (#A0545; Sigma-Aldrich or #554002; BD Biosciences, San Jose, CA, USA), and Alexa Fluor-coupled secondary antibodies (#A21206; Invitrogen).

4.2 Mice and breeding

Animal handling and experimental procedures were approved by local authorities at UCLouvain (Comité d'éthique facultaire pour l'expérimentation animale, 2016/UCL/MD/027) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication, revised 2011). All animals were housed with a 12-hour/12-hour light/dark cycle, with the dark cycle occurring from 6.00 p.m. to 6.00 a.m. Mice were observed daily with free access to water and standard chow. C57BL/6J males (age 8-12wk) were purchased from the Janvier labs (Le Genest Saint Isle, France). C57BL/6J Cdh5-iCreERT2 mice [526] were kindly provided by Ralf Adams, and crossed with mice carrying a floxed allele of PRKAA1 gene ($PRKAA1^{fl}/^{fl}$, #014141, the Jackson Laboratory). Cdh5-iCreERT2 +/- $PRKAA1^{fl}/^{fl}$ mice were administered tamoxifen (500μg, intraperitoneally) for five consecutive days at 8 weeks, and used for experiment three weeks after the last tamoxifen injection. Endothelial α1AMPK invalidation was confirmed with Taqman PCR technology on endothelial cells isolated from lung tissues. Data were analyzed with the 2 (-Delta Delta C(T)) method [527], and expressed as fold of controls. The animals were maintained under a 12:12-hour light-dark cycle with free access to food and water.

4.3 In vivo model of endotoxemia, cardiac permeability assessment, and plasmatic measurements

Canagliflozin was suspended in saline solution containing 0.5% carboxymethylcellulose and 0,025% Tween-20 and administered by oral gavage (100mg/Kg, 10μL/g), as described previously [387]. Endotoxemia was induced by intraperitoneal (IP) injections of either LPS (10mg/Kg) or saline vehicle. For myocardial permeability studies, Evans Blue Dye (EBD) was administered by IP injections (20mg/Kg) and used to quantify albumin extravasation, as described previously [83]. For heart sampling, animals were euthanized with IP injections of pentobarbital (300mg/Kg) following 24 hours. Vascular

leakage, corresponding to the dye amount within the extravascular compartment, was quantified using image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD), as the relative fluorescence (594nm) surface on frozen sections (6-um thick). For blood collection, mice were bled under ketamine and xylazine anesthesia from the retro-orbital plexus. Plasma was obtained by centrifugation at 3000g for 15 minutes, followed by 14800g for 3 minutes. Albumin was measured by colorimetric method, using FUJI Dry-Chem NX500 biochemical system. For plasma quantification, canagliflozin was analyzed by HPLC-MS/MS system consisting in a Xevo TQ-S mass spectrometer (Waters) coupled to an Acquity UPLC Class H system (Waters). Dapagliflozin was employed as internal standard. The chromatographic separation was performed using a Kinetex C18 HPLC column. Multiple reaction monitoring analysis was performed following electrospray ionization in positive mode.

4.4 Human plasma sampling

The study was approved in 2018 by the institutional ethics committee (V1 04/12/2018), and its conduct complied with good clinical practice guidelines. All participants provided written informed consent. Patients with septic shock admitted at *Cliniques universitaires Saint-Luc*, Brussels, were included in the analysis. Septic shock was defined as a sepsis with vasopressor therapy needed to elevate mean arterial blood pressure (MAP) ≥65mmHg, and lactate >2mmol/L, despite adequate fluid resuscitation of 30mL/kg of intravenous crystalloid within 6 hours. Patients on therapeutic oral or parenteral anticoagulation therapy (including heparins, fondaparinux, vitamin K antagonist, or novel oral anticoagulants), with previous history of thrombocytopenia (<100 000 platelets/mm3), recent (less than 1 month) chemotherapy, cirrhosis (Child Pugh > A), or recent (less than 48 hours) major surgery, and those patients with active inflammatory disease, hemophilia, or other coagulopathy were excluded from the analysis. The control group comprised healthy volunteers. For the experimental group, blood samples

were obtained in the ICU using the routinely inserted central venous catheter, within 48 hours f septic shock diagnosis. For the control group, blood samples were collected by venous puncture. Plasma-rich-platelet (PRP) was obtained after centrifugation at 800*g* for 5 seconds, followed by centrifugation at 100*g* for 5 minutes. Next, platelets were pelleted by centrifugation at 400*g* for 10 minutes. Apyrase and Integrilin were added to limit platelet activation during the preparation.

4.5 Cell culture and treatments

Human Microvascular Endothelial Cells (HMECs) were purchased from Lonza (#cc-2543) and cultured according to the manufacturer's recommendations, using EGM-2 MV microvascular endothelial cell growth medium (#cc-3202, Lonza, Verviers, Belgium) containing 1% penicillin-streptomycin, at 37°C and 5% CO_2 in a humidified incubator. The cells were subcultured when reaching 80% confluence and used until subculture number 7. Medium deprivation was performed for two hours prior to treatment or experimentation.

4.6 Western blotting

Protein content was measured by means of the Bradford method, using bovine serum albumin (BSA) as reference. Proteins (15µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then electroblotted. Membranes were probed with the primary antibody overnight at 4 °C in adequate dilution; α1AMPK (1:1000), phospho-AMPK Thr172, (1:1000), phospho-ACC (1:5000), p38MAPK (1:1000), phospho-p38 MAPK T180/Y182 (1:1000), HSP27 (1:1000), phospho-HSP27 S82 (1:1000), and eEF2 (1:1000). Bound antibodies were detected by means of chemiluminescence. Loading was controlled with anti-eukaryotic elongation factor 2. Quantification was assessed using Image J software. Each experiment was repeated at least three times.

4.7 In vitro transwell assay

For the endothelial permeability assay, HMECs (10^5cells/well) were seeded on gelatin-coated Transwell inserts of 24-well plates, in 250µL complete with Endothelial Cells Growth Medium MV. They were then incubated for 72 hours at 37 °C and with 5% CO2. The cells were incubated in free M200 medium for two hours before stimulation. The cells were then incubated with the different compounds, as indicated in the figure legends. After treatment, the upper chamber medium was replaced by 300µL of M200, containing HRP-coupled streptavidin. The lower chamber medium was collected after 10-minute incubation at 37°C, and every condition was aliquoted in triplicate. The TMB substrate was added for 10 minutes, and 2N H_2SO_4 was applied to stop the reaction before acquiring 450nm absorption in an Elisa reader. Resultant absorption intensity values were normalized over the vehicle control condition. Each experiment was repeated three times.

4.8 In vitro immunofluorescence staining and image analysis

HMECs were seeded on non-coated glass coverslips at a density of $20x10^3$cells/cm^2, 72 hours before treatment. After treatment, cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% triton X-100 for 10 minutes, and then blocked with 10% BSA for 45 minutes. Cells were then stained as previously described [83], using VE-Cad primary antibodies (1:25) and Alexa Fluor-coupled secondary antibodies (1:1000). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). Stainings were visualized under a Zeiss Imager Z1 microscope that was equipped with an ApoTome device. Pictures were acquired using an x20 objective. Each experiment was repeated three times.

Quantitative image analysis was performed on uncompressed images (native format: zvi) with Fiji 1.52n on MacOS (10.14.5). The intercellular junctions, evidenced by VE-Cad

staining, were automatically delimited using a fixed-value threshold method. The stained area was quantified, and the mean signal intensity was calculated with this section. Stained membrane segments were subsequently detected using the Analyze Particles ad Skeletonize tools, and automatically counted. For normalization purposes, all images' nuclei were automatically counted using a threshold method and the analyze particles tool.

4.9 Statistical analyses

The sample size was not pre-determined based on statistical analysis, and it was chosen according to previous publications. Statistical analyses were conducted using SPSS v.25 Software (IBM Corp., Armonk, NY, USA), and graphs were build using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). All tests were two-sided, with statistical significance set at the 0.05 probability level. Data were expressed as mean ± standard deviation. Means were compared using unpaired Student's *t*-test or a one-way or two-way analysis of variance, as appropriate. The Bonferroni correction was applied for multiple comparisons.

Author contributions

Conceptualization: M.A., D.C.-Z., S.H. and C.B.; Formal analysis: M.D, G.M., J.M., S.D., and T.M.; Funding acquisition: D.C.-Z, L.B., S.H., and C.B.; Investigation: M.A., A.G., J.D.P., S.B., and M.R; Methodology: G.M. and T.M.; Supervision: D.C.-Z., S.H. and C.B.; Writing—original draft: M.A., S.H., and C.B.; Writing—review & editing: D.C.-Z, L.B., S.H., and C.B.

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Abbreviations

ACC	Acetyl-CoA carboxylase
AJs	Adherens junctions
AMPK	AMP-activated protein kinase
eEF2	Eukaryotic elongation factor 2
HMEC	Human dermal microvascular endothelial cell
HSP27	Heat shock protein 27
IEJs	Inter-endothelial junctions
LPS	Lipopolysaccharide
p38 MAPK	p38 mitogen-activated protein kinase
SCLS	Sepsis capillary leak syndrome
SGLT2i	Sodium-glucose co-transporter 2 inhibitor
siRNA	Small interfering RNA
VE-Cad	VE-cadherin

Suplemental data

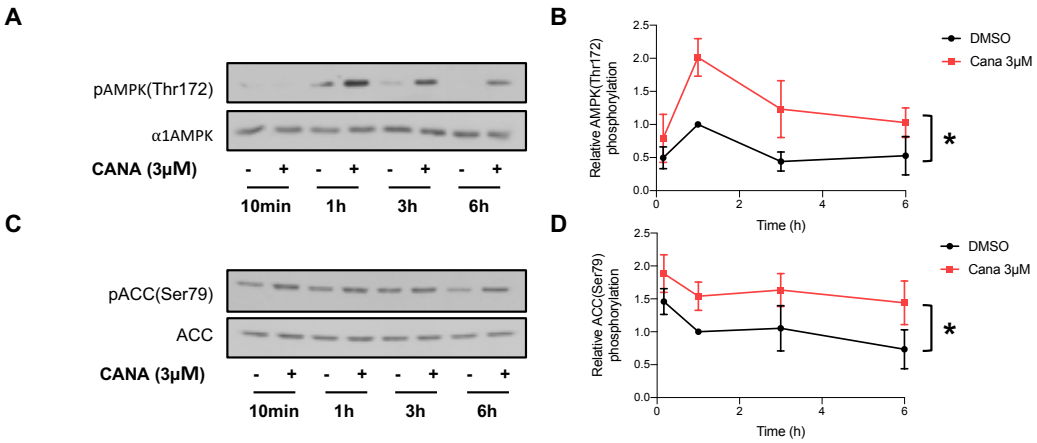

Figure S1. Canagliflozin activates the α1AMPK/p38MAPK/HSP27 pathway in HMECs. Human microvascular endothelial cells (HMECs) were treated with Canagliflozin (Cana) for the indicated time. Cell lysates were submitted to Western Blot analysis and probed with total and phosphorylated α1AMPK (Thr172) and ACC (Ser79) antibodies. Representative western blots (A,C) and quantifications (B,D) are shown. Data are fold of the 1 hour control condition, and expressed as mean ± SEM (three to six biological replicates for each condition). $^{*}p < 0.05$ is relative to HMECs treated with DMSO. The data underwent one-way ANOVA.

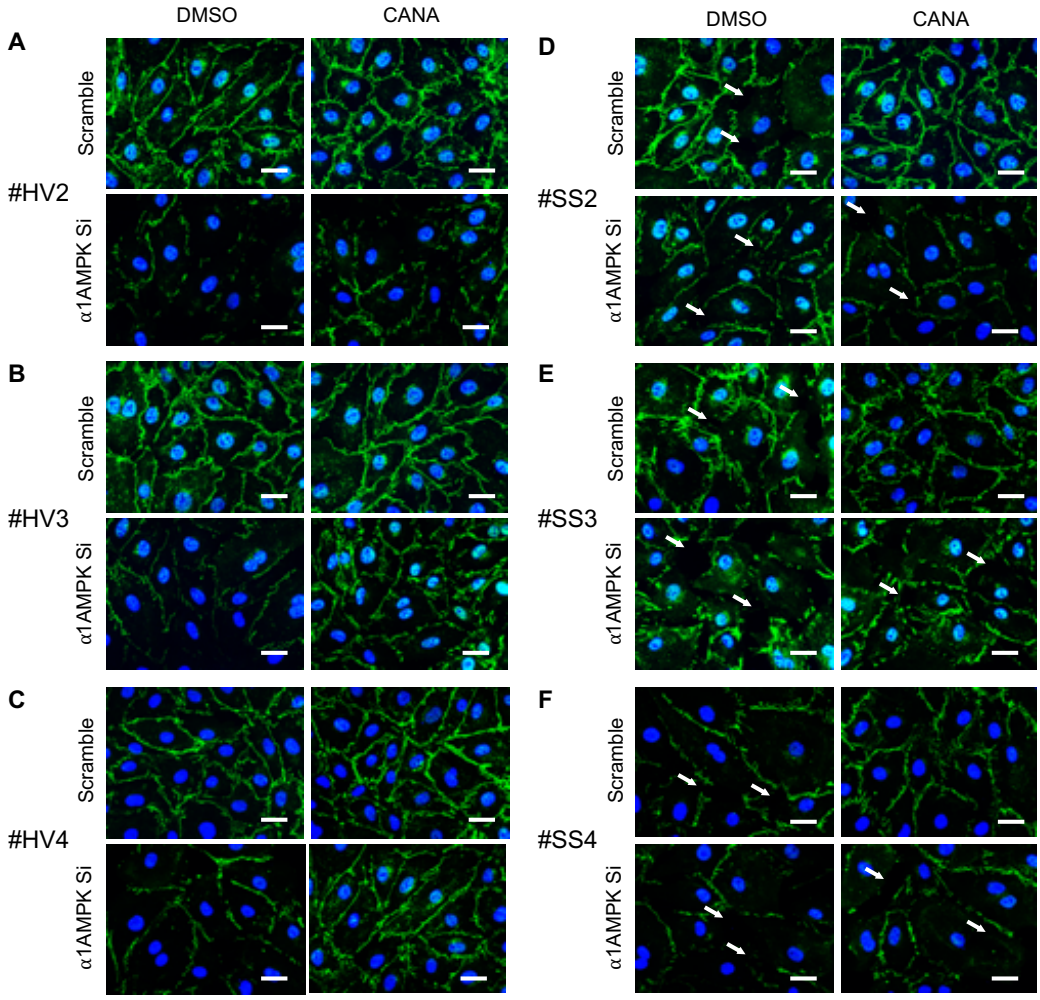

Figure S2. AMPK activation by Canagliflozin protects VE-Cad integrity in HMECs challenged with human control and septic plasma. VE-Cad immunostainings performed on Human microvascular endothelial cells (HMECs). HMECs were transfected with scramble or α1AMPK targeting siRNA (50nM) for 48 hours, before being treated with Canagliflozin (Cana) (3µM) or DMSO for 1 hour, then incubated with 10% plasma of (**A**-**C**) healthy volunteers (HV) or (**D**-**F**) septic shock patients (SS) for 30 minutes. Intercellular gaps are indicated by white arrows. Nuclei are stained with DAPI. Scale bar, 50µm.

CHAPTER III – MAJOR UNPUBLISHED FINFINGS

This chapter covers results of interest that were not included in publications. These describe mechanistic elements about the mechanisms of IEJs regulation by AMPK, impact of endothelial α1AMPK on survival of septic mice, as well as further results allowing to understand the impact of SGLT2i on endothelial function and tolerance to sepsis. These may be considered as preliminary results for future directions of the project, and are discussed in the section *General discussion and perspectives*.

1 AMPK INHIBITION IMPAIRS IEJS ORGANIZATION WITHOUT AFFECTING THEIR EXPRESSION

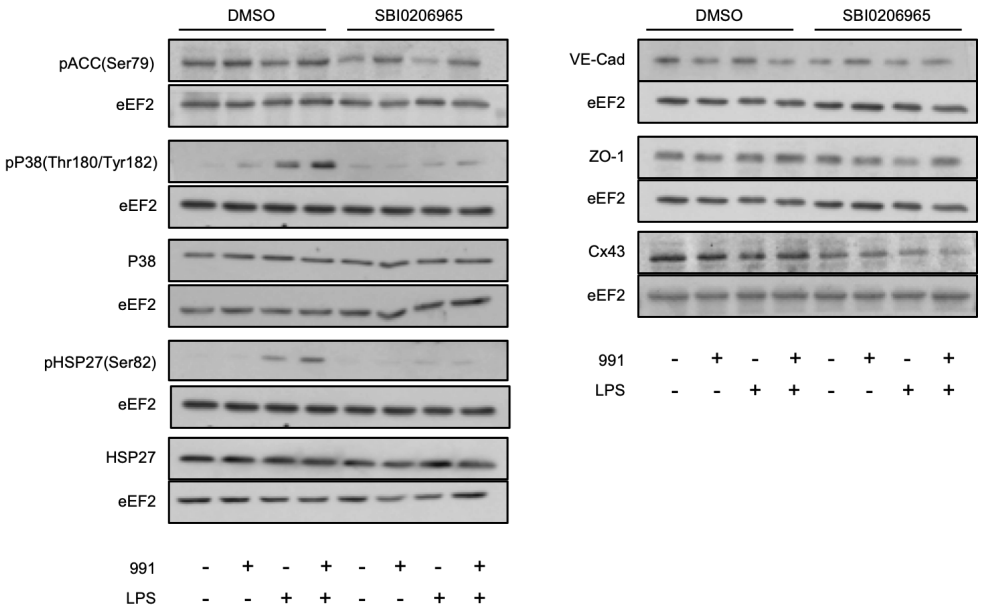

Figure I – Effect of AMPK inhibition on the P38/HSP27 pathway and on the expression of key junction targets in HMECs submitted to LPS challenge. Human microvascular endothelial cells (HMECs) were treated with the SBI compound or DMSO for 15 minutes, before adding canagliflozin (3µM) or DMSO for one hour, then lipopolysaccharide (LPS) for 6 hours. Cell lysates were submitted to Western Blot analysis

and probed with phosphorylated ACC (Ser79), phosphorylated and total P38 and HSP27, Ve-Cad, ZO-1, and Cx43 antibodies. Anti-eukaryotic elongation factor 2 (eEF2) was used as a loading control.

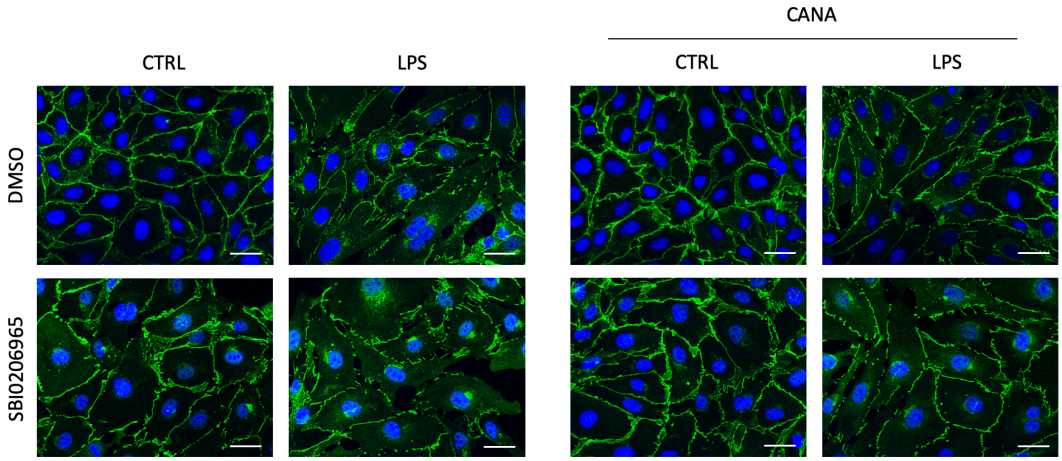

Figure II – Effect of AMPK inhibition on VE-Cad integrity in HMECs submitted to LPS challenge. (A,B) VE-Cadherin (VE-Cad) immunostainings performed on Human microvascular endothelial cells (HMECs) treated with SBI compound or DMSO for 15 minutes, before adding Canagliflozin (3µM) or DMSO for one hour, then lipopolysaccharide (LPS) for 6 hours. Representative images (A) and quantifications (B) are shown. Intercellular gaps are indicated by white arrows. Nuclei are stained with DAPI. Scale bar, 50µm.

2 ENDOTHELIAL A1AMPK DELETION DOES NOT AFFECT SURVIVAL OF SEPTIC MICE

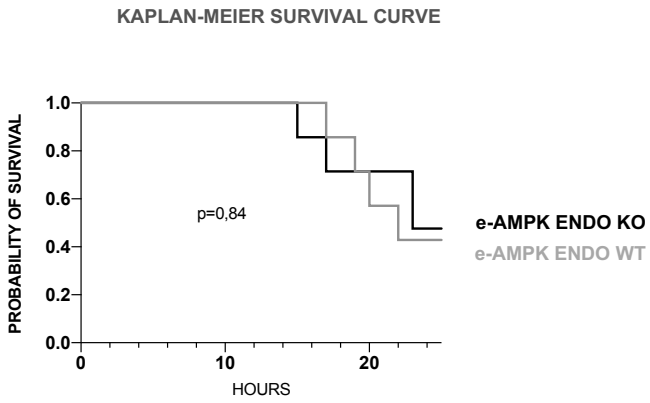

Figure III – Impact of endothelial α1AMPK deletion on survival of septic mice. Cdh5Cre$^{+/-}$ x PRKAA1fl/fl (e-AMPK WT/KO) mice were intraperitoneally administered tamoxifen (500µg/mice) for five consecutive days in order to induce α1AMPK invalidation specifically in the endothelium. 3 weeks after the last Tamoxifen injection, mice were submitted to LPS treatment (lethal dose, 80mg/kg) by intraperitoneal injection. n=6/group.

3 SGLT2 IS WEAKLY EXPRESSED IN HMECS

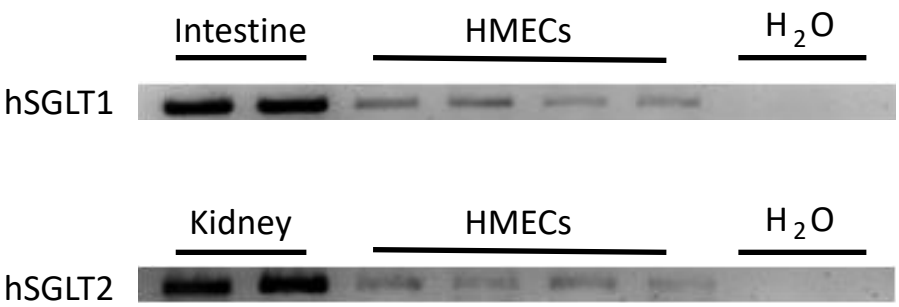

Figure IV – Expression of SGLTs in HMECs. ARN was extracted from human microvascular endothelial cells (HMECs) lysates, then submitted to reverse transcription and polymerase chain reaction. Human intestine and human kidney tissues were used as positive controls for SGLT1 and SGLT2, respectively. hSGLT, human sodium glucose cotransporter primer.

4 OTHER GLIFLOZINS ACTIVATE AMPK IN HMECS

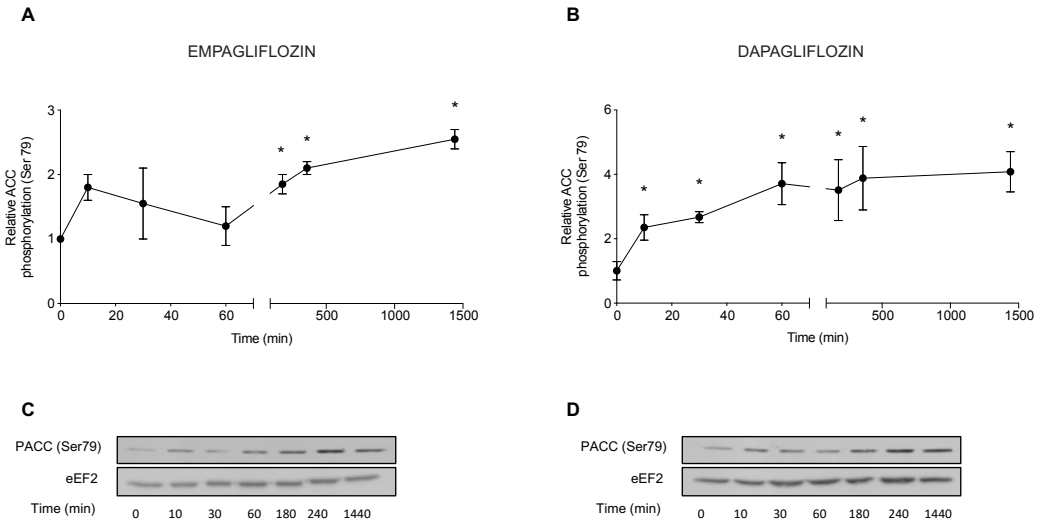

Figure V – Empagliflozin and dapagliflozin activate the α1AMPK in HMECs. (A, C) Human microvascular endothelial cells (HMECs) were treated with 10 µM empagliflozin for the indicated timings. Cell lysates were submitted to Western Blot analysis and probed with phosphorylated ACC (Ser79) antibody. Anti-eukaryotic elongation factor 2 (eEF2) was used as a loading control. (B, D) Same protocol was followed with 10 µM dapagliflozin. Representative western blots (C, D) and quantifications (A, B) are shown. Data are expressed as mean +- SEM (three biological replicates for each condition). *$p < 0.05$ is relative to untreated HMECs. The data underwent one-way ANOVA.

5 CANAGLIFLOZIN EXERTS DOSE DEPENDENT EFFECTS ON IEJS

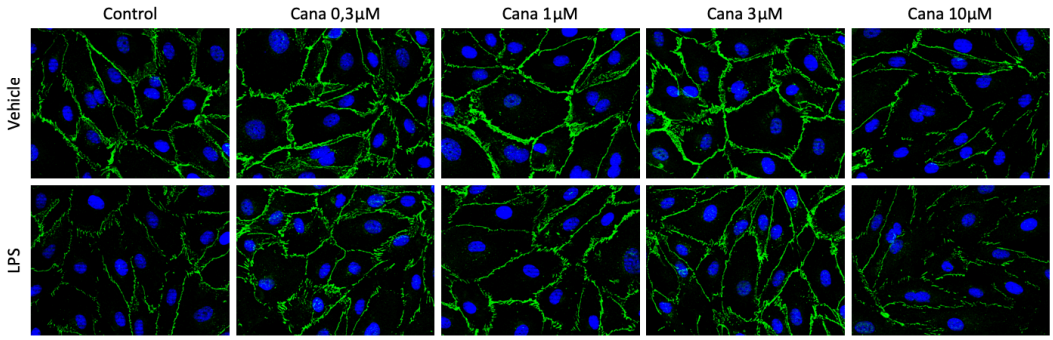

Figure VI – Dose-dependent effects of canagliflozin on VE-Cad integrity in HMECs submitted to LPS challenge. VE-Cadherin (VE-Cad) immunostainings performed on Human microvascular endothelial cells (HMECs) treated with canagliflozin (at the indicated concentrations) or equal amounts of DMSO for one hour, before adding lipopolysaccharide (LPS) for 6 hours. Nuclei are stained with DAPI. Scale bar, 50µm.

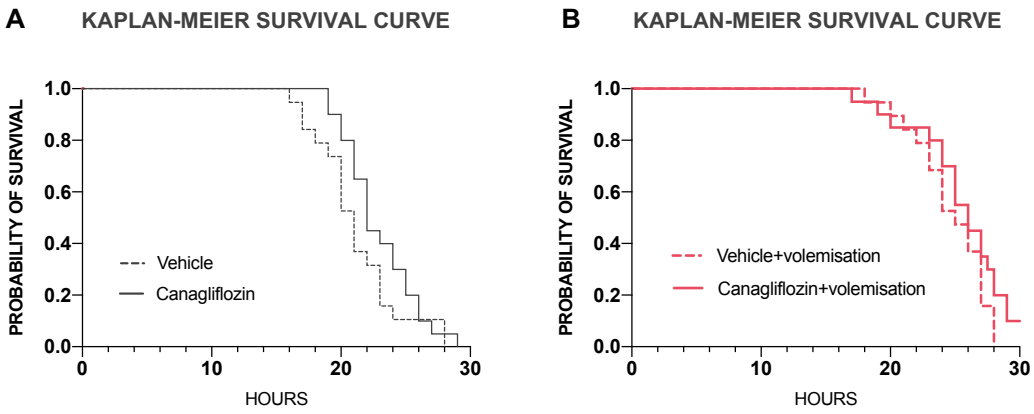

Figure VII – Effect of canagliflozin on survival of septic mice. (A) C57BL/6 were treated with canagliflozin (100mg/kg) by oral gavage two hours before receiving LPS injection (dose lethal 50, varying with LPS batches) by intraperitoneal injection. (B) C57BL/6 mice were treated according to protocol described in (A), in addition to receiving fluid resuscitation. Fluid resuscitation was achieved by subcutaneous injections of saline (50ml/kg), concomitantly to LPS administration. N=18 to 20/group.

GENERAL DISCUSSION AND PERSPECTIVES

For decades, clinical and fundamentalist researchers have searched for evidence to reduce the burden of sepsis. As the supportive management of septic patients continues to improve, no therapeutic approaches have yet been approved to help minimising sepsis injury. Most of the options proposed have focused on inflammatory and coagulation mechanisms. Vascular permeability factors, although also recognised as being at the centre of the pathophysiology, have not yet been targeted by significant therapeutic proposals. Aware of the complexity of the ongoing processes, our work had the main objective of placing the vascular barrier function at the centre of the debate.

A growing body of evidence from our team and others supports the protective role of α1AMPK against sepsis injury. In addition, the protective effects of AMPK activation on endothelial barrier function are increasingly described. In this context, we went further in the mechanisms underlying the role of α1AMPK in endothelial barrier protection during sepsis. Interestingly, we highlighted protective effects of the SGLT2i canagliflozin, an oral antidiabetic drug recently described to activate AMPK, on sepsis-induced capillary leak. These results raise several questions and give way to a large possibility for future investigations.

1 QUESTIONING THE EXPERIMENTAL MODEL OF SEPSIS

Lipopolysaccharide (LPS) is an endotoxin - a component of the cell membrane of gram-negative bacteria - considered as one of the most prominent instigators of the inflammatory cascade in sepsis (with comparable substances composing gram-positive organisms, yeasts, or viruses) [528]. Since found in the plasma of septic patients, recognised to induce an intense inflammatory response, and to reproduce hemodynamic and metabolic derangements of clinical sepsis, endotoxins could be considered as a robust model of sepsis. However, the pathophysiological mechanisms induced by their administration in mice appear to differ from those reported in human sepsis [529-531]. First, the remarkably lower LPS sensitivity of mice compared to

humans imposes the use of disproportionate LPS doses in experimental procedures. Indeed, the dose leading to death in approximately half of mice (LD50 dose) is reported as about 1000 fold to 10 000 fold greater than the LPS dose required to induce human sepsis shock [532]. Murine expression of circulating factors capable of suppressing cytokines release in response to TLR agonists has been suggested to explain this discrepancy [533]. Second, a systematic analysis of murine and human genomic responses to conditions activating the innate immune system demonstrated minimal correlation of leukocyte gene expression changes in humans and mice submitted to endotoxemia [534]. It should be noted, however, that this study only involved one strain of mice, which received a dose of LPS 250 times greater than that of the healthy human volunteers unrolled. Third, the temporal kinetics of cytokines release is generally reported to be far more transient in endotoxemic mice than in septic humans [535].

Other models of sepsis have been developed to try reproducing the complexity of human sepsis. The Cecal Ligation and Puncture (CLP), a model of bacterial peritonitis, has aroused great interest since characterised by poly microbial infection [536] and reproducing a number of key features observed in human peritonitis [537]. Several deficiencies still remain, and weigh the benefits provided by this model. Majorly, the lack of interlaboratory standardisation due to differences in experimental procedures [538] or host enteric bacteria involved in bacteraemia are pointed out [539]. In addition, inconstancy in sepsis associated organ failure, notably depending on the age of mice induced by CLP, were reported [540]. Finally, the fact that proper therapeutic support of CLP treated mice (including antibiotics, resuscitation, surgical removal of abscess and necrotic tissues) results in 100% survival with no visible sequelae [541] demonstrates major differences with the pathophysiology of human sepsis. Recently, Alverdy *et al.* suggested several measures to enhance the relevance of murine CLP sepsis model. These include modification of the diet, fluid and antibiotics administrations, and working on mice of older age, with underlying condition, or “humanised” microbiome [539]. It is

suggested that incorporating these elements into the CLP model may allow to put the emphasis on the complications associated with the septic reaction, rather than on the infectious event initiating the pathological process.

Various other approaches were proposed, such as injections of bacteria, intraperitoneal injection of human stool batches, intranasal/intratracheal administration of endotoxins or bacteria, colon ascendens stent peritonitis, or acute pancreatitis by chemical injection. Yet, none of them appears as the ideal sepsis model [542], and the choice of one over another should rather be guided by research questions. Of note, humanised mouse models are now available. These consist in mice that can be added with genetic variants by transplantation of stem cells from different donors, which allow specific aspects of the human septic response to be examined [543, 544].

Beyond these model-dependent considerations, it appears essential to consider the major differences separating murine and human (patho)physiologies, especially in response to a septic insult (see Figure 16) [545]. Accordingly, several promising therapeutic approaches that were effective in animal studies failed to show any benefits in human clinical trials [546]. These limitations add to the animal welfare arguments to support - more than ever - refinement, reduction and replacement of animal models. Translational research is thereby encouraged for reliable exploration of the complexity of human sepsis. In this respect, experiments incubating human cellular models with human septic plasma are increasingly described in the literature [163, 547]. Our latest results are based on such a model, and could be further developed in the future directions of the project. Evaluating the effects of septic plasma on endothelial permeability in the presence of SGLT2 inhibitors, both in pre- and post-treatments, could help to define potential clinical perspectives of our results.

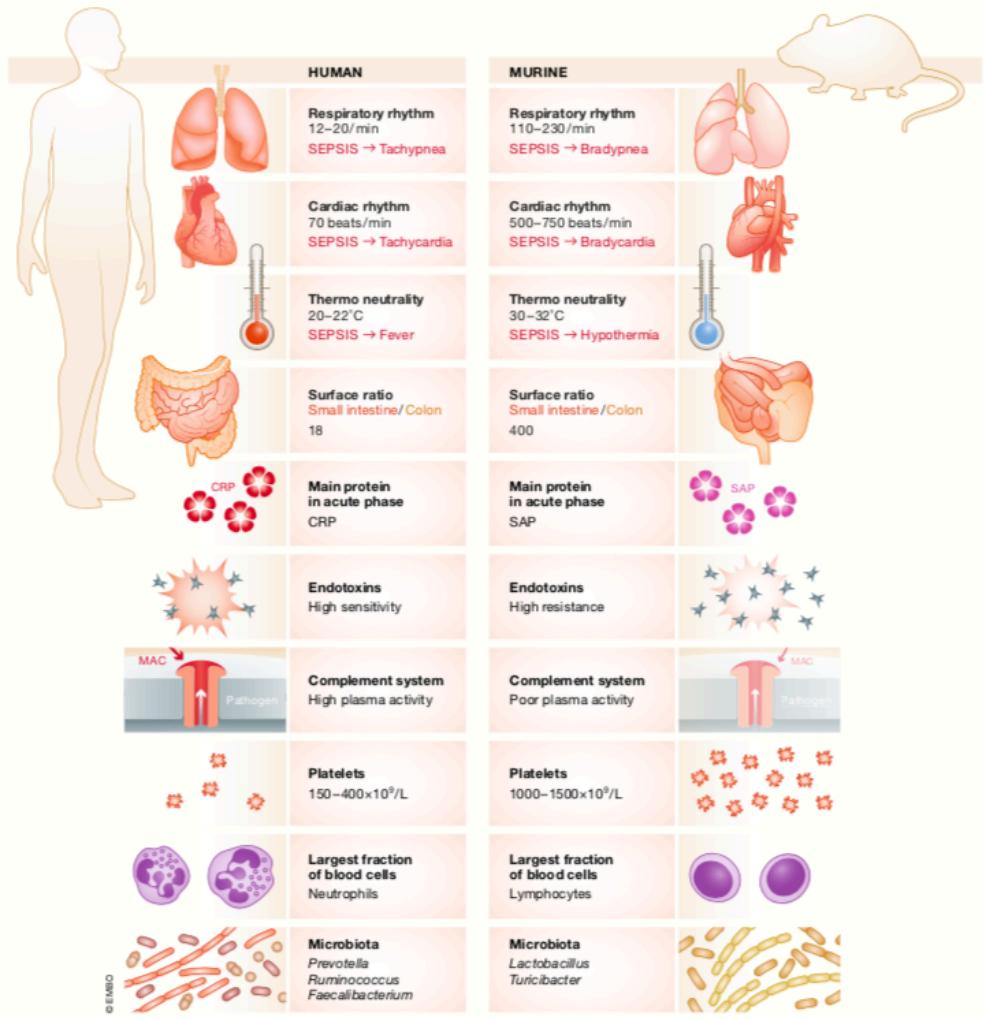

Figure 16 – Key differences in murine and human physiology that affect the response to sepsis. CRP, C-reactive protein; MAC, membrane attack complex; SAP, serum amyloid protein). Taken from [545].

2 AMPK AND THE ENDOTHELIAL BARRIER, POTENTIAL SUBORDINATE PATHWAYS

In the past decades, α1AMPK has emerged as a major actor in the context of paracellular permeability. Each of its functions of cell shape, polarity, or metabolism regulator seem

to be involved in these processes. First, AMPK activation by mechano-sensing AJs has been shown to provide the energy supply required for ATP-consuming actin reorganisation and stabilisation of IEJs [47, 443, 449]. Second, α1AMPK has been identified to regulate actin cytoskeleton remodelling through regulating different actin binding proteins. Our work highlights the involvement of the p38 MAPK/HSP27 pathway in this process. Finally, α1AMPK has appeared as a direct regulator of IEJs proteins. Here, we characterise the impact of AMPK activation on the maintenance of IEJs organisation. We also highlight the control of IEJs proteins expression by AMPK, and propose Cx43 to be a crucial mediator of this regulation.

Both activated and basal α1AMPK appear to be involved in these different processes. First, AMPK activation appears as a pivotal event in the regulation of actin dynamics, and subsequent IEJs organisation. We indeed show that AMPK activation by 991 protects IEJs integrity, while its inhibition by the SBI0206965 compound induces their disruption. Supporting our results, it has been shown in epithelial and endothelial cells that activated AMPK can reinforce the interaction between cadherin-catenin complexes and actin cytoskeleton via both regulation of N-Cad [212, 498] and phosphorylation of G-alpha interacting vesicle associated protein (GIV) [471, 506]. In other cellular models, activated α1AMPK also directly affected the integrity of TJs and AJs by phosphorylating some of their components. For example, afadin, claudin-1 and -4 have been described as direct AMPK substrates in epithelial cells [445, 492, 508]. Second, we demonstrated that basal α1AMPK is required for proper IEJs proteins expression and integrity, which are poorly impacted by 991 compound or SBI0206965. This suggests the involvement of kinase-independent functions of AMPK in this regulation. It is actually noteworthy that, although primarily considered for its kinase activity, AMPK can programme its biological responses through involving non-catalytic functions. These may include the scaffolding of protein complexes, the competition for protein interactions, allosteric effects on other enzymes or subcellular targeting. Among others, ERK1 and ERK2 were shown to

influence their substrates not only by phosphorylation, but also by direct protein-protein interactions, independently of kinase activity [497]. It could reasonably be assumed that AMPK is involved in such type of regulation. The fact that the AMPK inhibitor SBI0206965 does not reproduce the effects of α1AMPK deletion on reducing IEJs expression, although it dramatically affects their organisation (see Results section, Chapter III.1) supports this hypothesis.

Considering the variety of mechanisms through which α1AMPK appears to regulate paracellular permeability, it could be questioned whether α1AMPK directly modulates the different targets highlighted, or if intermediate processes should be considered. Accordingly, several mechanisms could be hypothesised to mediate AMPK catalytic and non-catalytic regulations of permeability actors (see Figure 17).

2.1 GENE TRANSCRIPTION

First, α1AMPK could regulate endothelial barrier effectors by modulating gene transcription, either directly - via the phosphorylation of transcriptional regulators -, or indirectly - via the induction of sirtuins - [501]. Accordingly, AMPK has been highlighted to exert anti-inflammatory effects by phosphorylating the transcriptional coactivator p300 (also known to regulate ß-catenin [548]) [549] and the KLF2-associated HDAC5, in human ECs respectively submitted to TNFα and LPS. Furthermore, endothelial AMPK was also reported to regulate antioxidant defence by activating the transcription factor FOXO3a [550]. Even more interestingly, AMPK has been shown to enhance epithelial barrier function by up regulating CDX2, a transcription factor known to modulate TJs proteins [551].

2.2 POST-TRANSCRIPTIONAL MODIFICATIONS

Second, α1AMPK could regulate endothelial barrier effectors via post-transcriptional mechanisms, such as modulation of micro-RNAs (miRNAs, or miRs) expression. miRs are small single-stranded non-coding RNAs (~22 nucleotides) which regulate gene expression by affecting specific mRNA stability and/or protein translation. In 2017, approximately 2500 miRs were identified in the human genome, and they were shown to modulate the activity of 30-50% of proteins (for review, see [552]). Interestingly, miR regulation appears as a new emerging regulatory mechanism of AMPK, while several of miRs are shown to modulate endothelial barrier function (for review, see [553]). Even more interestingly, results of our laboratory (Dufeys *et al.*, under revision) and others revealed an α1AMPK dependent regulation of miR-125b [554], which seems to be of particular interest in the context of sepsis. Indeed, miR-125b has recently been shown to be up regulated in the plasma of septic patients [555, 556]. In this context, it was associated with higher inflammatory response, and correlated poor sepsis outcome, suggesting its potential use as a prognosis marker. Mechanistically, miR-125b was shown to inhibit VE-cadherin translation in HUVECs submitted to VEGF [557] and to repress the expression of Cx43 in human cardiac fibroblasts (Dufeys *et al.*, under revision). Taken together, these arguments suggest a potential involvement of miRs, and more specifically miR-125b, in endothelial barrier protection by AMPK during sepsis injury.

2.3 POST-TRANSLATIONAL MODIFICATIONS

Finally, α1AMPK may regulate endothelial barrier effectors through indirectly modulating other post-translational modifications than phosphorylation. Indeed, processes such as acetylation - introduction of acetyl functional group - or nitrosylation/nitration - covalent incorporation of a NO/NO_2 molecule - have been

highlighted to modulate the interactions of IEJs proteins, particularly in AJs [171]. Supporting the hypothesis of acetylation, AMPK has been shown to increase the expression of Sirtuin1 (Sirt1), a deacetylase, in lung tissues of rats submitted to LPS from two distinct studies [503, 504]. This was associated with reduced NFκB activity and improved epithelial barrier function in both of them. More recently, canagliflozin was also shown to promote the AMPK-Sirt1 pathway in adipocytes [399]. It is interesting to note that acetylation of AJs proteins, such as ß-catenin, was reported to play a role in the stability of these junctional complexes, while the invalidation of deacetylase HDAC6 has shown protective effects on pulmonary oedema in endotoxemic mice [558]. On the other hand, AMPK is known to modulate NO production and biodisponibility [412], which directly impacts nitration and nitrosylation of proteins. S-nitrosylation is described as an emergent regulatory mechanism of microvascular permeability [505], while nitration appears as an important factor modulating the activity and function of Rac1 [559] and RhoA [560]. Finally, O-glcNAcylation - addition of N-acetylglucosamine on serine or threonine residues of proteins - is affected by AMPK activation in different cell types, including endothelial cells [561, 562]. The involvement of O-glcNAcylation in endothelial barrier regulation has not yet been investigated but may be suspected of having its importance, if only because of the competition it induces on protein phosphorylation sites.

While we can postulate that α1AMPK modulates p38 MAPK via its kinase functions - although we do not know whether this involves direct phosphorylation or intermediate kinases -, future experimental work is needed to delineate the molecular regulation of Cx43, VE-Cad, and ZO-1 by α1AMPK, which may potentially involve several of the above-mentioned mechanisms at a time.

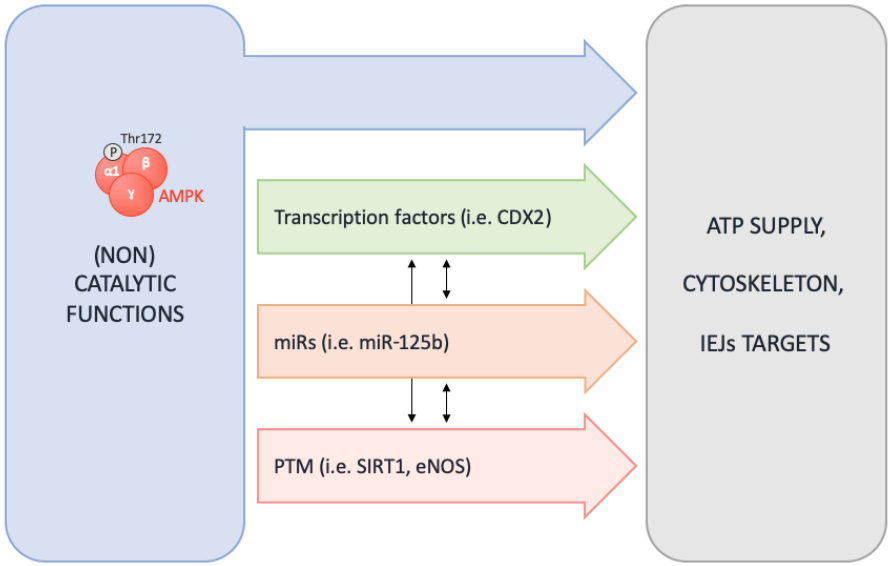

Figure 17 - Suggested mechanisms involved in the regulation of endothelial permeability by α1AMPK. α1AMPK, via its catalytic and non-catalytic functions, modulates endothelial ATP supply, cytoskeleton and interendothelial junctions (IEJs) targets. This regulation may occur directly, or involve transcriptional, post-transcriptional, or post-translational modifications.

3 AMPK IN SEPSIS, BEYOND VASCULAR PERMEABILITY

AMPK appears as a pivotal actor in sepsis injury. Castanares-Zapatero *et al.* demonstrated that α1AMPK total invalidation (α1AMPK total KO) in mice dramatically affected their tolerance to sub-lethal doses of LPS in terms of vascular permeability, coagulation, vasomotor tone and more importantly, survival [83].

In the present project, we highlight that the specific invalidation of endothelial α1AMPK interestingly reproduces the increased basal vascular permeability observed in total KO animals. We also highlight that the protective effect of AMPK activation on vascular

leakage is greatly - but not totally - mediated by endothelial α1AMPK. However, mortality experiments did not show any impact of endothelial α1AMPK deletion on sepsis survival (see Results section, Chapter III.2). This strongly suggests that α1AMPK acts in other cell types to confer protective effects against sepsis injury.

Thus, reflecting the complexity that governs the pathophysiology of sepsis, AMPK activation seems to modulate the septic response by controlling cellular processes which go beyond endothelial permeability. If the protective nature of this complex regulation is increasingly recognised, the characterisation of the mechanisms involved is only emerging. Major hypotheses are presented here.

3.1 INFLAMMATION

First, AMPK is now generally accepted to have anti-inflammatory actions [563], some of which are described in the vasculature (for review, see [414]). AMPK activation has been shown to suppress multiple pro-inflammatory signalling cascades, including NFκB, JNK, and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways, and reduce cytokines production in ECs [396, 426, 549]. Such effects have been confirmed in sepsis models, which demonstrated that AMPK activation is associated with attenuated local and systemic inflammation, mostly assessed by measuring cytokines production and adhesion molecules expression both *in vitro* and *in vivo* [503, 564-566]. Interestingly, our team previously demonstrated that compared to the WT, α1AMPK total KO mice submitted to LPS presented enhanced inflammatory response in the heart, as reflected by gene expression of IL1ß, TNF-α, IL6, CXCL-1, and CXCL-2 cytokines (Castanares-Zapatero *et al.*, unpublished data). Plasma levels of these cytokines however indicated no significant impact of α1AMPK deletion on systemic inflammation during sepsis [83]. In addition, specific invalidation of α1AMPK in myeloid cells (through LysM Cre recombinase system) did not reproduce the increased vascular

permeability or excess in mortality observed in α1AMPK total KO animals, suggesting that the absence of AMPK in myeloid cells was not accountable for the total α1AMPK KO phenotype. Thereby, it can be postulated that AMPK exerts beneficial anti-inflammatory actions during sepsis, although these are not sufficient to explain its protection against sepsis injury.

3.2 COAGULATION

Second, our laboratory is working on elucidating the role of α1AMPK in counteracting the pro-coagulant state associated with sepsis. Initially, it was shown that total α1AMPK deletion was associated with increased thrombin generation in septic mice (Castanares-Zapatero *et al.*, unpublished data). A PhD thesis (conducted by Julien De Poortere) currently aims to identify the specific contributions of AMPK in platelets, ECs and neutrophils in the regulation of sepsis pro-coagulant state. Mechanistically, our laboratory previously demonstrated that AMPK modulates platelets activity by phosphorylating Acetyl Carboxylase (ACC). This was shown to affect platelets phospholipids contents, thereby supporting thromboxane A2 (TXA2) generation, dense granules release, and thrombus formation [567]. Interestingly, this pathway was shown to be activated in platelets collected from septic shock patients (Dechamps *et al.*, unpublished data). Investigating the impact of the AMPK/ACC pathway on platelets metabolic and inflammatory functions during sepsis is the object of a second ongoing PhD thesis in our laboratory (conducted by Mélanie Dechamps).

3.3 VASOMOTOR TONE

A third mechanism potentially underlying AMPK protection during sepsis is the regulation of vasomotor tone. Indeed, preliminary results indicated that α1AMPK total KO mice present a prolonged drop in blood pressure in response to LPS as compared to WT animals (Castanares-Zapatero *et al.*, unpublished data). The mechanisms underlying

this effect have not yet been investigated. Considering that AMPK inhibits NFκB in ECs [568], we may hypothesise that an up regulation of this pathway would lead to the induction of iNOS and cyclooxygenase (COX-2) expression, and result in increased production of NO and prostaglandins – two major vasodilators in sepsis. Beyond the characterisation of these mechanistic considerations, reproducing telemetry analyses on septic α1AMPK endo KO mice would allow to characterise the contribution of endothelial AMPK in the regulation of vasomotor tone during sepsis. In addition, evaluating the impact of AMPK activation on this parameter would be of major clinical interest.

3.4 OXIDATIVE STRESS

Based on its ability to prevent the formation of ROS and the depletion in anti-oxidants, AMPK may also temper sepsis reaction by reducing oxidative stress. Indeed, AMPK has been described to contribute to several pathways regulating vascular redox balance (for review, see [414]). Among others, it was demonstrated that AMPK directly activates the Nuclear factor erythroid-2 related factor 2 (Nrf-2), a transcription factor promoting the expression of antioxidant defence proteins [569]. Moreover, our team and others highlighted an AMPK mediated inhibition of NOX-2 in different cell types, including ECs [408, 570]. The role of endothelial AMPK in redox balance regulation seems to be of particular importance in the vasculature, since the specific deletion of endothelial α1AMPK (Cdh5Cre x AMPK fl/fl) was shown to induce oxidative damage *in vivo* [571]. Interestingly, this was associated with impaired endothelial barrier function, and enhanced recruitment of inflammatory cells in the vascular wall. Similar observations were reported in the context of sepsis, in which AMPK was shown to preserve redox balance, notably by promoting mitochondrial biogenesis [421]. Accordingly, AMPK was recently shown to mediate protective effects of molecules such as Honokiol or Salidroside against oxidative stress in the endothelium under sepsis challenge [200,

572]. Given the broad impacts of ROS on vascular functions, it seems logical to suppose that the beneficial effects of AMPK on oxidative stress may participate in the reduction of self-sustaining loops of other mechanisms characterising sepsis pathophysiology.

3.5 ENERGY STATUS

Finally, AMPK also seems to mitigate sepsis injury by regulating cellular energetic status [573]. It was shown in myeloid cells from septic mice that AMPK invalidation leads to an increase in pyruvate kinase isozyme M2 (PKM2)-dependent aerobic glycolysis, associated with increased release of HMGB1 (a late mediator of systemic inflammation), lactate accumulation, and mortality [574]. In addition, AMPK is shown to protect against sepsis by promoting autophagy, a catabolic process allowing orderly degradation and recycling of cellular components, and playing essential role in cellular homeostasis [564]. These AMPK-mediated mechanisms seem to support the function of different organs during sepsis [482, 575, 576].

3.6 IMPACT ON SEPSIS OUTCOME

Taken together, AMPK appears to govern different pathways that cooperate to protect microvascular function during sepsis (see Figure 18). This importantly results in improved organ function, as supported by studies reporting the protective effects of AMPK activators (AICAr [564, 577, 578], A-769662 compound [513] and, beyond the problematic of lactic acidosis, metformin [579]) in fighting against MOF and improving sepsis outcome. AMPK seems to regulate the septic response by involving various pathophysiological processes in various cell types, which act in an intricate and synergistic manner. Therefore, if cell-type-specific mouse models of deletion allow further characterisation of its protective effects, it seems unlikely that any of them will reproduce the phenotype of the total knockout model in terms of survival to sepsis injury.

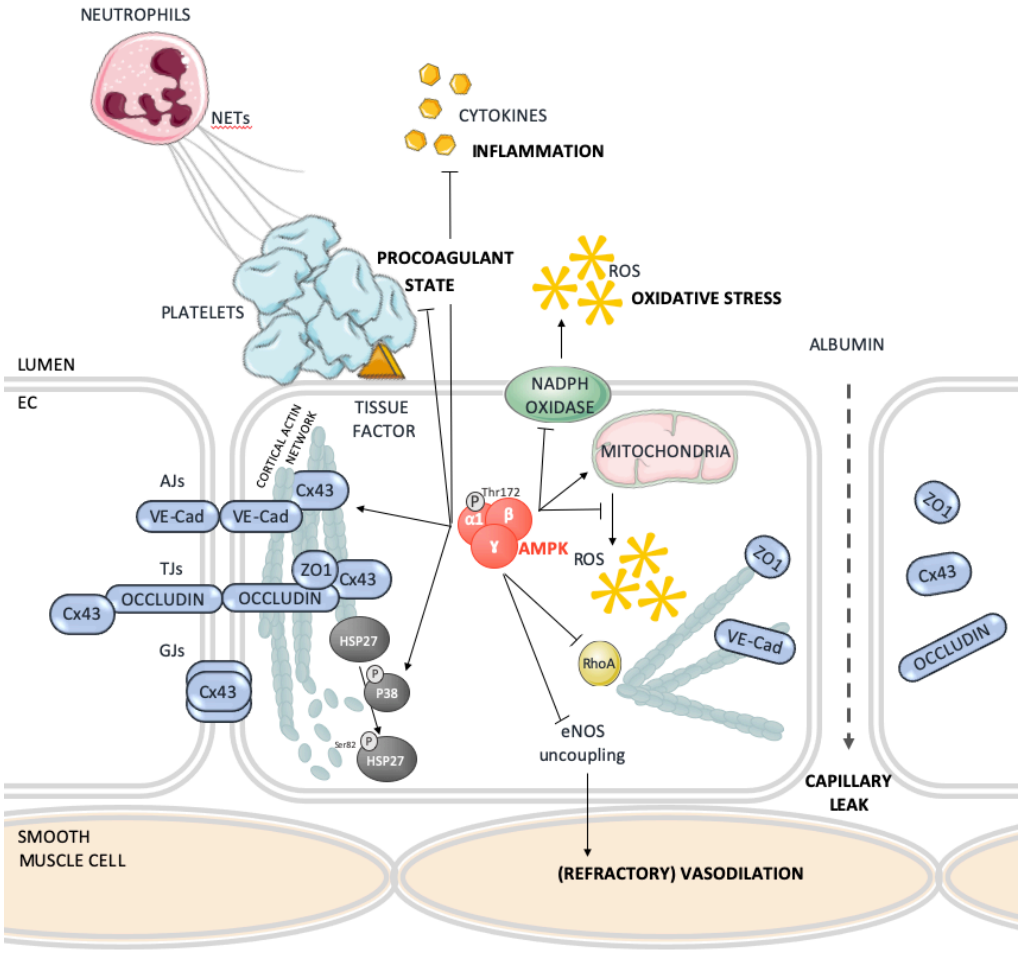

Figure 18 – Endothelial α1AMPK protects against sepsis-induced microvascular dysfunction, suggested mechanisms. Endothelial α1 isoform of AMP-activated protein Kinase protects the endothelial barrier function through mechanisms notably involving regulation of connexin 43 (Cx43) expression, p38 MAPK/HSP27 pathway, and RhoA activity. These effects are reinforced by AMPK protective effects against deregulated inflammation, pro-coagulant state, oxidative stress, and vasomotor tone.

4 SGLT2 INHIBITORS, AMPK AND SEPSIS, WHAT THE FUTURE HOLDS?

4.1 SGLT2 INHIBITORS IN CARDIOVASCULAR DISEASE

Sodium/Glucose cotransporter 2 (SGLT2) is mainly expressed in the apical membrane of epithelial cells in the S1 segment of the proximal renal tubule, where it is responsible for the reabsorption of 90% of filtered urinary glucose by ensuring the transport of glucose coupled with sodium with a stoichiometry of 1/1 (see Figure 19). In addition to the kidney, SGLT2 mRNA has been detected in human testes, cerebellum, pancreas, and cerebral arteries [580, 581]. Our PCR results highlight low but detectable SGLT2 expression in HMECs (see Results section, Chapter III.3). Interestingly, it has been shown in animal-isolated epithelial and endothelial cells that SGLT2 expression can be induced in pathological conditions, including exposition to high glucose or pro-inflammatory cytokines (such as IL-6 and TNF-α) [403, 582]. These evidences suggest a potential increase in endothelial SGLT2 expression during sepsis challenge commonly associated with hyperglycaemia and inflammation. Also of interest, glucose transport by SGLT-1 and -2 was reported to be associated with increased ROS production and oxidative stress in cardiomyocytes and renal epithelial cells [582, 583].

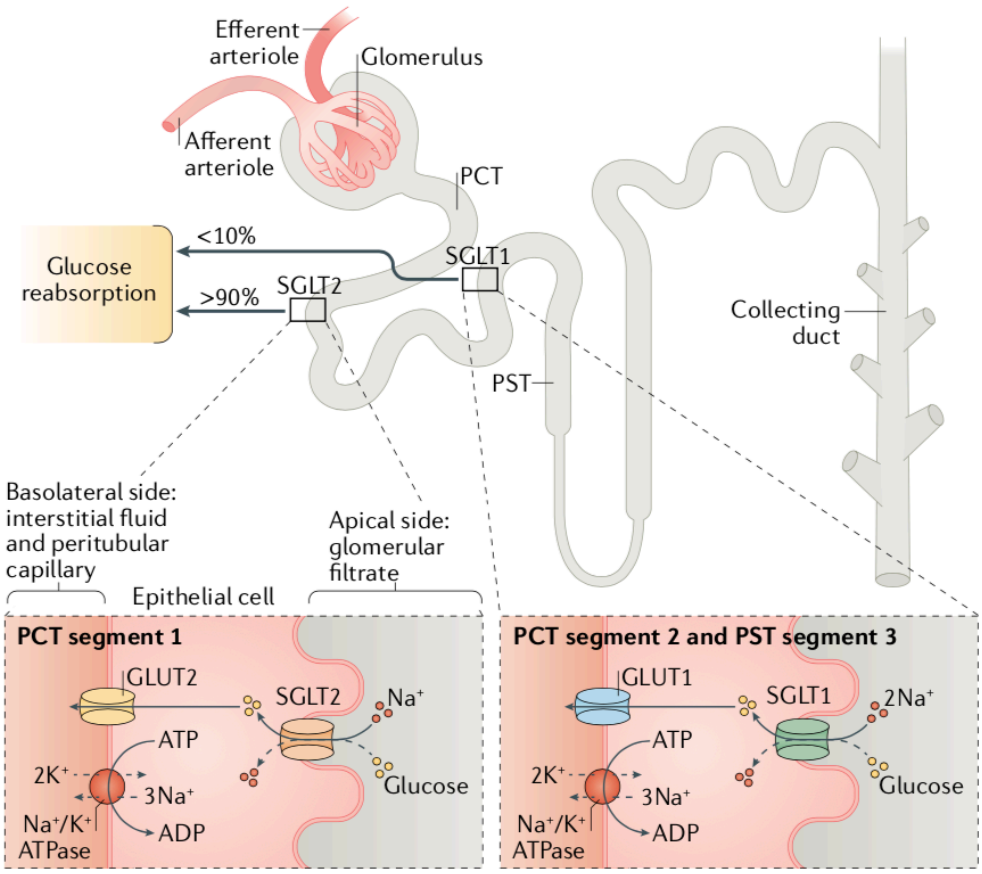

Figure 19 – Role of sodium glucose transporters (SGLTs) in glucose reabsorption in the kidney. Most (>90%) of the glucose is reabsorbed from the glomerular filtrate into the epithelial cells via SGLT2 in segment 1 of the proximal convoluted tubule (PCT). The remainder of the glucose (<10%) is reabsorbed via SGLT1 in PCT segment 2 and proximal straight tubule (PST) segment 3. The glucose then passes into interstitial fluid via the glucose transporter 2 (GLUT2) in segment 1 or via GLUT 1 in segments 2 and 3. Taken from [584].

SGLT2 inhibitors (SGLT2i) have attracted increasing attention in the past few years and are now commonly prescribed as oral anti-hyperglycaemic agents in type 2 diabetes. In the past 5 years, cana- [382], empa- [383], and dapagliflozin [384] were shown to

provide robust heart failure-related benefits in clinical trials evaluating cardiovascular outcomes in both diabetic and non-diabetic contexts [385, 585]. As reported by the notorious meta-analysis of Zelnicker *et al.*, SGLT2i appear to reduce the risk of cardiovascular death or hospitalisation for heart failure by 23% in type 2 diabetes, with similar benefits in patients with and without history of atherosclerotic cardiovascular disease or heart failure. While it becomes clear that this cardiovascular protection does not only depend on glucose lowering properties (see Figure 20), the precise mechanisms involved arouse great interest in the scientific world, but remain for the most part hypothetical and incompletely defined.

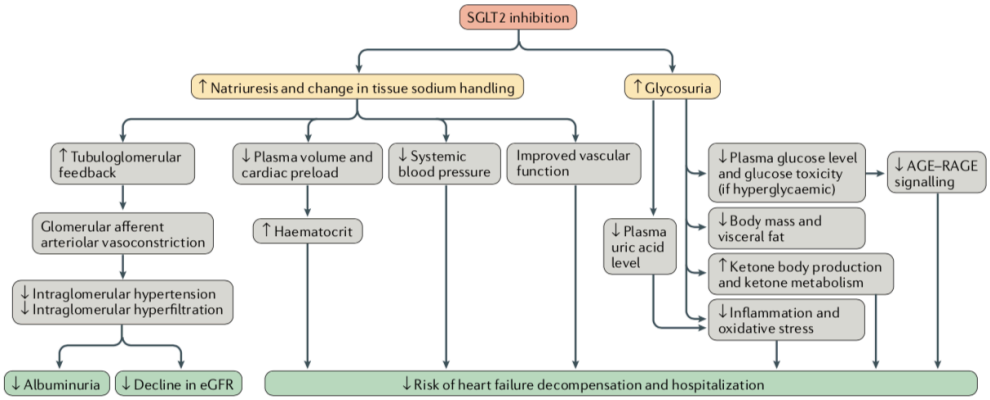

Figure 20 – Integrated effects of SGLT2 inhibitors likely to produce cardiovascular benefits. Sodium-Glucose cotransporter 2 (SGLT2) inhibitors increase natriuresis and glycosuria, resulting in various beneficial effects on the cardiovascular system. Together, these effects reduce the risk of heart failure decompensation and hospitalisation. AGE, advanced glycation end products; eGFR, estimated glomerular filtration rate; RAGE, receptors for advances glycation end products. Taken from [584].

Supporting the outcomes of human clinical trials, preclinical studies demonstrate beneficial class effects of SGLT2 inhibitors on endothelial function (for review, see

[586]). As reported by a meta-analysis comparing several classes of oral antidiabetic agents, only SGLT2 inhibitors significantly improve endothelial function, as assessed by flow mediated dilation [587]. Two main mechanisms have been proposed to mediate this protection. First, by reducing inflammation. Indeed, several teams have shown that all cana-, empa- and dapagliflozin markedly reduce expression of pro-inflammatory adhesion markers and cytokines such as IL-6, monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) in rodent models of diabetes, HFrEF, hypertension, or obesity [388, 586, 588-591]. These observations were supported by *in vitro* experiments performed on human models of endothelial cells [396, 403, 592]. Furthermore, it was proposed that anti-inflammatory effects of canagliflozin could be explained by inhibition of intracellular glucose metabolism and increased autophagy in immune cells [410]. Second, SGLT2i may protect endothelial function by preventing oxidative stress. *In vitro* experiments demonstrated improvement of eNOS function, and suppression of mitochondrial reactive oxygen species by SGLT2i in human ECs [391-393], while complementary mechanism of SGLT2i-mediated inhibition of NADPH oxidase activity was highlighted in other cell types [593]. Others suggested that improved redox balance by SGLT2i was explained by restored NO bioavailability rather than modulation of eNOS expression or signalling [402]. These effects may logically be supposed to be interdependent due to the major crosstalk existing between oxidative and inflammatory pathways.

For the first time, our results highlight the protective effects of canagliflozin on endothelial barrier function in HMECs submitted to LPS, and on myocardial oedema formation in septic mice. Although currently poorly reported in the literature, modest evidence seems to support a beneficial impact of SGLT2i on microvascular permeability regulation. Indeed, empagliflozin was shown to preserve microvascular barrier function of cardiac microvascular ECs (CMECs) isolated from diabetic mice [391]. Although not directly reported as vascular barrier protection, the team of Jason Dyck also highlighted

significant reduction of LPS-induced mesangial congestion in septic mice treated with empagliflozin [524]. *In vitro* experiments performed on human ECs further report restoration of glycocalyx integrity by empagliflozin [594]. Finally, Ipragliflozin was shown to protect blood-retinal barrier in the case report of a patient with steroid resistant diabetic macular oedema, which recovered after replacing metformin by Ipragliflozin treatment [595]. These results need, however, to be balanced with a study demonstrating no impact of empagliflozin on hyperpermeability in HUVECS challenged with TNFα [402].

Quite independently, a growing body of evidence indicates that all cana-, empa- and dapagliflozin activate AMPK in different cell types (see Introduction, section 3.3.2). This effect seems greater for canagliflozin, reported to activate AMPK at clinically relevant concentrations in multiple experimental models, through mechanisms which obviously involve the inhibition of mitochondrial respiratory chain [387, 396-399, 410, 596]. AMPK activation by empa- and dapagliflozin appears more subtle, since dependent on cell types, concentration, or timing of stimulation [387, 389, 390, 596, 597]. In HMECs, we show significant and sustained AMPK activation with 10uM concentration of both molecules (see Results section, Chapter III.4). While never reported for dapagliflozin yet, these observations are supported by other studies working with empagliflozin on ECs [391-393].

Although currently weakly associated, these two observations of cardiovascular protection and AMPK activation by SGLT2i may raise two major research hypotheses.

4.2 SGLT2I AS POTENTIAL THERAPEUTIC SUPPORT FOR SEPTIC PATIENTS

Given the central role played by the EC in the microvascular alterations that characterise sepsis pathophysiology, considering the emerging beneficial actions of SGLT2i on endothelial function in this pathological context appears of major interest. Beyond our

results highlighting endothelial barrier protection by canagliflozin, other benefits of SGLT2i have already been described in different models of sepsis. Canagliflozin was shown to reduce inflammatory response, lung congestion and lung inflammatory infiltration in mice submitted to LPS [410, 598], by mechanisms suggested to involve increased autophagy in immune cells [410]. *In vitro*, canagliflozin but not empa- or dapagliflozin reduced IL-6 expression in HACECs submitted to LPS [596]. Empagliflozin was also highlighted to confer major protective effects in LPS models of septic mice, notably by improving cardiac contractility, vasomotor tone, systemic inflammation, acute kidney injury, and even more importantly, survival [389, 524]. Finally, dapagliflozin may also be of interest, since reducing inflammasome in a model of cardiac fibroblasts isolated from diabetic mice and submitted to LPS challenge [388].

On the basis of these considerations, it remains to be determined which gliflozin would be the most interesting to consider the therapeutic perspectives of SGLT2i in sepsis. While further experiments are obviously required to compare the benefits provided by each molecule, particular attention should be paid to the dose-dependent paradoxical effects of canagliflozin. First questioned for the increased risk of distal amputation with which it is associated in elderly diabetic patients with history of vascular disease [404], this molecule showed surprising double edge sword effects in our experimental model (see Results section, Chapter III.5). Never described so far, we indeed observed that 0,3; 1; and 3 µM canagliflozin reinforced IEJs integrity in control and LPS-challenged HMECs. However, it appeared that 10 µM - a concentration often reached in the plasma of orally treated patients - canagliflozin significantly impaired the expression and organisation of VE-Cad, while affecting ECs proliferation and morphology. Supporting our results, canagliflozin as recently been highlighted to inhibit HUVECs proliferation, migration and angiogenesis at concentrations from 10 µM [406]. Of note, empagliflozin was shown to slightly affect HUVECs migration at the concentration of 50 µM - which is clinically poorly relevant -, while dapagliflozin did not reveal any toxicity. Beyond these effects on EC

proliferation cycle, inhibition of mitochondrial respiration by canagliflozin and, more recently described, empagliflozin, must also be considered with caution. Although never reported to be associated with lactic acidosis, similar effects of metformin have caused the systemic removal of this drug in septic patients due to a controversial syndrome of metformin-associated lactic acidosis (MALA). It is thereby primordial to delineate the cellular, tissue, and systemic implications of these effects before considering the administration of gliflozins in the context of sepsis.

In our experimental model, we could not show any significant impact of canagliflozin treatment on septic mice survival (see Results section, Chapter III.6). Based on what has been described in the recent literature, several points could be raised to interpret these results, and possibly optimise the characterisation of the impact of gliflozins on sepsis injury.

- Dose, mode, and timing of SGLT2i administration could be modified. In our model, canagliflozin is administered by oral gavage, 2 hours before LPS treatment, with a unique dose of 100mg/kg that induces plasma concentrations of ∼10µM throughout the 24-hour duration of the experiment. Considering intravenous administration [409] after sepsis onset [524], and targeting lower plasma concentrations could be of major clinical interest.
- If not questioning the model of sepsis (see discussion, section 1) LPS doses may also be adapted in order to modify the severity of the insult and eventually evaluate potential benefits on the different mortality peaks, which are proposed to rely on distinct pathophysiological mechanisms (see Introduction, section 1.4.3).
- Considering the introduction of comorbidities - working on models of diabetes, high-fat diet, arterial hypertension, or extreme periods of age - or medical support - systematic administration of antibiotics or sedatives, fluid

resuscitation, or invasive ventilation - could, in spite of increased experimentation costs, make it possible to get closer to concrete clinical contexts and improve the relevance of the results obtained.

- Finally, the consideration of mortality as an endpoint as recently been questioned, in a context where quality of life is strongly affected after sepsis and could be considered as a valuable therapeutic objective for predicted survivors [545].

Altogether, these elements should help point the way for potential therapeutic perspectives of SGLT2i in sepsis injury. In addition to the fundamental aspects, carrying out clinical studies evaluating the incidence and/or outcome of sepsis in patients chronically treated by SGLT2i as compared to the one receiving other oral antidiabetic therapies could also be of interest.

4.3 AMPK AS A PIVOTAL EFFECTOR OF SGLT2I

Our result support the hypothesis of AMPK as a mediator of SGLT2i cardiovascular protective properties. Surprisingly, this hypothesis has never been considered as such. Yet the similarity of the mechanisms of action of gliflozins and AMPK may deserve attention.

As recently reviewed by Lopaschuk *et al.*, the hypotheses currently proposed to underlie gliflozins cardiovascular protective effects concern a plethora of different mechanisms: (1) blood pressure lowering, (2) increasing diuresis/natriuresis, (3) improving cardiac energy metabolism, (4) preventing inflammation, (5) weight loss, (6) improving glucose control, (7) inhibiting the sympathetic nervous system, (8) preventing adverse cardiac remodelling (9) preventing ischaemia/reperfusion injury, (10) inhibiting the cardiac Na^+/H^+ exchanger, (11) inhibiting SGLT1, (12) reducing hyperuricemia, (13) increasing autophagy and lysosomal degradation, (14) decreasing epicardial fat mass, (15)

increasing erythropoietin (EPO) levels, (16) increasing circulating vascular progenitor cells, (17) decreasing oxidative stress, and (18) improving vascular function [519].

Among these mechanisms, several clearly appear to overlap the effects of AMPK on cardiovascular function. Indeed, AMPK is generally recognised as an upstream regulator of several pathways regulating cardiac energy metabolism, inflammation, glucose control, ischaemia/reperfusion injury, autophagy, oxidative stress, and vascular function.

Over the past two years, several studies have pointed out the capacity of AMPK to mediate the protective effects of gliflozins in inflammation [388, 389, 396, 410], energy repletion [389, 399], autophagy [410], oxidative stress [398] and mitochondrial fission [391, 399, 597]. Even more interestingly, the protection of cardiac microvascular barrier function by empagliflozin was shown to depend on AMPK [391].

It therefore seems surprising that the question of AMPK as a pivotal mediator of overall cardiovascular protection by gliflozins has not been raised as such. In a context where the scientific world is deploying major resources to deepen the mechanisms of action and the therapeutic implications of gliflozins, it seems that we can expect this question to be investigated in the near future.

5 IMPLICATIONS FOR THE SEPTIC CHILD

Sepsis is responsible for a substantial proportion of global childhood morbidity and mortality [599], making children represent a large proportion of sepsis victims (see Introduction section, Figure 2). This may be explained by immune immaturity that makes children prone to infectious episodes, while various paediatric physiological and anatomical factors increase their susceptibility to develop organ dysfunctions.

The paediatric specificities of sepsis pathophysiology - and the specific role of the endothelium in this setting - have been marginally investigated compared to adult sepsis [600]. Age specific differences in clinical picture and management requirements, however, exist (see Figure 21), and demand particular investigation to improve therapeutic approach and outcomes. If the precise description of these differences is beyond the scope of this chapter, it is convenient to emphasise on the major importance of capillary leak in the clinical presentation and therapeutic management of paediatric sepsis. Indeed, early fluid resuscitation is at the centre of the therapeutic management of the septic child. In fact, insufficient or delayed fluid administration have been associated with increased mortality [601]. Moreover, it is currently accepted that children proportionately require more fluid resuscitation than adults [602]. The deleterious effects of unnecessary volemisation should, however, not be underestimated and volume overload must be clinically tracked in critically ill children. Finally, Ang1 and 2, both major modulators of microvascular barrier function during sepsis (see introduction, section 2.3), have recently been reported as relevant biomarkers of sepsis severity in young children [603, 604].

	Paediatric sepsis	**Adult sepsis**
Etiology	Age dependent. Pneumonia, diarrhea, meningitis, and virus are most common.	Abdominal, respiratory and genitourinary sources are most common.
Clinical presentation	High systemic vascular resistances. Hypotension is a late and ominous sign of shock.	Low systemic vascular resistances. Hypotension.
Initial rescucitation	Aggressive fluids up to 60ml/kg in the first hour, as long as absence of clinical signs of fluid overload. Norepinephrine if poor effects of volemisation.	Guarded fluid rescucitation, 1-2L boluses, (titrated to SVV and CVP), early vasopressors.
Outcomes	Early intervention in the first hour reduces mortality by two-fold. Less overall mortality (10-20%).	Early intervention in the first 1-3 hours reduces mortality. Higher overall mortality (10-40%).

Figure 21 – Key differences between paediatric and adult sepsis. SVV, stroke volume variation; CVP, central venous pressure. Adapted from [602].

Focusing on fundamental aspects, it may be appropriate to raise the emerging evidence supporting the involvement of AMPK in paediatric sepsis, since AMPK has been reported to improve autophagy and reduce the inflammatory response in neonatal septic mice [605]. Of more modest interest, respiratory syncytial virus - the main causative agent of acute lower respiratory tract infections in children worldwide - has been shown to activate AMPK-dependent signalling pathways in host epithelial cells [606].

Finally, in order to consider the paediatric perspectives of this work, it should be noted that the use of SGLT2i is not yet recommended for children, since studies focusing on the effects of these drugs in paediatric populations are lacking. The safety of one single oral dose of dapagliflozin in type 1 diabetes adolescents was interestingly reported in 2017, and associated with decreased insulin need [607]. Since then, SGLT2i are proposed as a conceivable approach for combination therapy in the young diabetic population, although poor evidence has been brought. Of note, the possible increased risk of ketoacidosis may be a deterrent in this process. Beyond diabetes, a clinical trial (NCT03867487) is currently ongoing to evaluate the impact of empagliflozin on non-alcoholic fatty liver disease (NASH) in non-diabetic adolescents. After two years of follow-up, final data collection is expected for May 2021.

6 TALKING SEPSIS IN 2020, NOT WITHOUT A WORD ON SARS COV2

The severe acute respiratory syndrome coronavirus 2 (SARS-Cov2), responsible for de corona virus disease 2019 (COVID-19) pandemic, causes a wide range of clinical pictures. Symptomatic patients progressing to critical illness are primarily characterized by

pulmonary lesions. Recent studies, however, tend to highlight a major role of endothelial dysfunction in the progression of the disease (for review, see [608]). This endothelial dysfunction, associated with the deregulated responses it induces and the resulting organ deficiencies, leads COVID-19 patients to often meet sepsis criteria as defined by the sepsis-3 consensus [6].

Loss of anti-thrombotic and anti-inflammatory properties appear as major factors of endothelial dysfunction in COVID-19 patients, with thrombotic microvascular injury reported as a classical feature of pulmonary abnormalities [609, 610]. Furthermore, histological data have highlighted the role of capillary thrombosis in the pathogenesis of organ dysfunction during SARS-CoV-2 infection [611, 612]. Although it is not described as a central factor, endothelial barrier disruption is also reported to have its importance in microvascular dysfunction of COVID19 patients [613, 614]. Supporting this idea, plasma levels of Ang2 and IL-6, both recognized to impair endothelial barrier function [329, 615], were proposed as good predictive factors for severe Covid19 cases [616-618].

It worth mentioning that AMPK has been shown to modulate the expression and stability of Angiotensin-conversing enzyme 2 (ACE2), the main receptor mediating the entry of SARS-CoV-2 in host cells, in HUVECs [619]. This has led to the postulate that AMPK could play a role in COVID-19 disease [620], while the potential benefits of AMPK activation in protecting against acute lung injury have also been suggested [621]. These hypotheses are of particular interest in the context of our research project, but are currently based on weak evidence and remain to be explored.

Also of interest, SGLT2i have recently been considered as a potential therapeutic support for COVID-19 patients [622]. This suggestion, which is not yet supported by scientific evidence, relies on the cardiovascular protective properties of gliflozins, and on the cardiovascular / cardiometabolic comorbidities that classically characterize

severe COVID-19 cases. Anyway, it may be assumed that research is ongoing in this area, and that promising results may be expected.

These evidences together suggest potential COVID-19 implications of the present project. Beyond their still unclear cardiovascular properties, considering SGLT2i could indeed be of interest to support microvascular barrier function in COVID19 patients - if not in typical cases, in the ones complicating with bacterial surinfection. Experiments evaluating the effects of SGLT2i on HMECs incubated with human plasma collected from septic COVID19 patients are planned in the coming weeks, and should provide preliminary informations to guide this research hypothesis.

CONCLUSION

« An understanding of the function of the endothelial barrier, endothelial repair, and endothelial regeneration may one day lead to new therapies. For frustrated pharmaceutical companies, for clinicians, and most importantly for patients with sepsis, this day cannot come a moment too soon », Lee *et al*., NEJM, 2010 [45].

Vascular hyperpermeability is recognised as an independent prognostic factor for poor sepsis outcome, while there are currently no therapeutic options to mitigate this process. Our team has been working for years on the protective role of α1AMPK, known as a regulator of the endothelial barrier, in the pathophysiology of sepsis. Besides, SGLT2i - new oral antidiabetic agents - emerge for their cardiovascular protective properties, and are parallelly described to activate AMPK. In this work, we go further in understanding the role of AMPK in endothelial barrier regulation, and identify SGLT2i as a potential therapeutic option to attenuate sepsis capillary leak syndrome.

Although important work remains to be done before considering the outcome of this hypothesis, the previous approbation of gliflozins by the FDA represents a significant advantage in this process.

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Annex 1

Letter to the Editor, American Journal of Physiology-Heart and Circulatory Physiology, 2019

Role of AMP-activated protein kinase in sepsis-induced cardiovascular dysfunction

Marine Angé, Diego Castanares-Zapatero, Luc Bertrand, Sandrine Horman, and Christophe Beauloye

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LETTER TO THE EDITOR

Role of AMP-activated protein kinase in sepsis-induced cardiovascular dysfunction

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TO THE EDITOR: As founding members of a laboratory working on AMP-activated protein kinase (AMPK) for more than 20 yr, we have carried a lot of interest to the recent study regarding the effect of AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) on age-dependent cardiac function during experimental sepsis performed by Inata et al. (4) and highlighted by an Editorial Focus (7). We would like to comment on the proposed hypothesis taking into account data we previously published on the same issue (2).

In agreement with Inata et al.'s paper, we also think that AMPK activation has to be considered as a new potential therapeutic approach in sepsis-induced cardiovascular impairment. However, we believe that the protection conferred by AMPK more likely relies on an endothelial action (as endothelial cells mainly express the α_1-AMPK isoform) rather than on cardiomyocytes (which mainly express the α_2-AMPK isoform). This conclusion is based on data that we already published in *Critical Care Medicine* in 2013 (2). Our arguments are supported by several observations.

First, we demonstrated that α_1-AMPK knockout (KO) mice did not tolerate sublethal doses of LPS (10 mg/kg, *Esherichia coli* O55B5). Indeed, a dramatic increase in mortality has been observed in young mice (2 mo of age), associated with an increased cardiac vascular permeability leading to a massive myocardial edema. We identified α_1-AMPK as a key contributor for the control of intercellular endothelial junctions. The latter is a crucial determinant of vascular leakage, which constitutes one of the main features of septic cardiomyopathy (2). More importantly, we showed no significant phenotypic differences between α_2-AMPK KO and wild-type mice submitted to sepsis concerning mortality, left ventricular function, myocardial edema, and endothelial barrier integrity, ruling out a major role of α_2-AMPK and cardiomyocytes in sepsis-induced dysfunction.

Second, we and others have shown that AICAR is unable to activate AMPK in adult cardiomyocytes (6), suggesting that the protective effects of AICAR do not result from a direct action on cardiomyocytes. Accordingly, AICAR treatment barely affects cardiac AMPK activity (as assessed by Thr^{172} phosphorylation) in the study of Inata and colleagues (4).

Third, sepsis in young mice mostly affects left ventricular end-diastolic volume, a parameter that crucially depends on blood volemia, and microvascular dysfunction (4). The resulting decreased cardiac preload could explain the reduced ejection fraction, independently of myocardial injury. This is reinforced by the clear disconnection between myocardial injury and left ventricular function observed by Inata et al. (4). Moreover, AICAR treatment in septic young mice led to a preservation of left ventricular end-diastolic volume. One may postulate that AICAR improves endothelial function and lowers vascular leakage, leading to enhanced blood volemia, tissue perfusion, and oxygen diffusion. This is in line with our previous observation that AICAR prevents LPS-induced left ventricular edema (2).

In conclusion, AMPK protects against sepsis-induced cardiovascular impairment. The pathophysiological mechanisms involved, as well as the potential protection that AMPK activation could offer, need to be deepened. Nevertheless, on the basis of several arguments from others and us, we postulate that those mechanisms primarily involve the preservation of vascular integrity. Given that AICAR application in vivo remains challenging, new clinically usable AMPK activators like antidiabetic drugs (1, 3, 5) should be considered as a perspective to unravel the involvement of AMPK in septic cardiomyopathy.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.A. and C.B. drafted manuscript; M.A., D.C.-Z., L.B., S.H., and C.B. edited and revised manuscript; M.A., D.C.-Z., L.B., S.H., and C.B. approved final version of manuscript.

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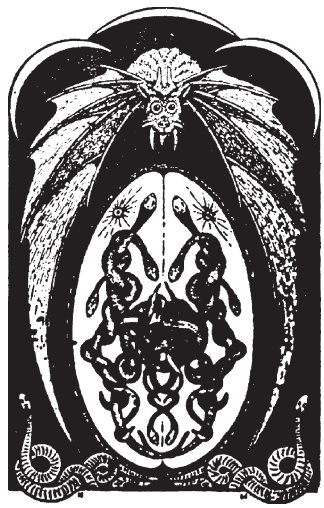

Annex 2

Article, International Journal of Molecular Sciences, 2020

α1AMP-Activated Protein Kinase Protects Against Lipopolysaccharide-Induced Endothelial Barrier Disruption via Junctional Reinforcement and Activation of the p38 MAPK/HSP27 Pathway

Marine Angé, Diego Castanares-Zapatero, Julien De Poortere, Cécile Dufeys, Guillaume E. Courtoy, Caroline Bouzin, Rozenn Quarck, Luc Bertrand, Christophe Beauloye, and Sandrine Horman

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MDPI

Article

α1AMP-Activated Protein Kinase Protects against Lipopolysaccharide-Induced Endothelial Barrier Disruption via Junctional Reinforcement and Activation of the p38 MAPK/HSP27 Pathway

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Abstract: Vascular hyperpermeability is a determinant factor in the pathophysiology of sepsis. While, AMP-activated protein kinase (AMPK) is known to play a role in maintaining endothelial barrier function in this condition. Therefore, we investigated the underlying molecular mechanisms of this protective effect. α1AMPK expression and/or activity was modulated in human dermal microvascular endothelial cells using either α1AMPK-targeting small interfering RNA or the direct pharmacological AMPK activator 991, prior to lipopolysaccharide (LPS) treatment. Western blotting was used to analyze the expression and/or phosphorylation of proteins that compose cellular junctions (zonula occludens-1 (ZO-1), vascular endothelial cadherin (VE-Cad), connexin 43 (Cx43)) or that regulate actin cytoskeleton (p38 MAPK; heat shock protein 27 (HSP27)). Functional endothelial permeability was assessed by in vitro Transwell assays, and quantification of cellular junctions in the plasma membrane was assessed by immunofluorescence. Actin cytoskeleton remodeling was evaluated through actin fluorescent staining. We consequently demonstrate that α1AMPK deficiency is associated with reduced expression of CX43, ZO-1, and VE-Cad, and that the drastic loss of CX43 is likely responsible for the subsequent decreased expression and localization of ZO-1 and VE-Cad in the plasma membrane. Moreover, α1AMPK activation by 991 protects against LPS-induced endothelial barrier disruption by reinforcing cortical actin cytoskeleton. This is due to a mechanism that involves the phosphorylation of p38 MAPK and HSP27, which is nonetheless independent of the small GTPase Rac1. This results in a drastic decrease of LPS-induced hyperpermeability. We conclude that α1AMPK activators that are suitable for clinical use may provide a specific therapeutic intervention that limits sepsis-induced vascular leakage.

Keywords: α1AMPK; sepsis; lipopolysaccharide; endothelial permeability; VE-cadherin; connexin 43; zonula occludens-1; heat shock protein 27; actin cytoskeleton

1. Introduction

Sepsis, a major global health problem [1–3], is defined as a dysregulated host response to an infection [4]. Its evolution toward multi-organ failure (MOF), which is known as a crucial predictor of survival [5], directly results from microcirculatory dysfunction [6–8]. The latter is highly dependent on the regulation of vascular extravasation [9], and the maintenance of intravascular volume remains one of the most important challenges for the clinical support of septic patients.

Dysregulation of transendothelial paracellular permeability is the main determinant of sepsis-induced vascular extravasation. This results from the disruption of inter-endothelial junctions (IEJs) and the disorganization of actin cytoskeleton [10]. IEJs include adherens junctions (AJs) and tight junctions (TJs), which are both composed of multiprotein complexes. Some of their components, such as vascular endothelial cadherin (VE-Cad) and zonula occludens-1 (ZO-1), form part of the IEJ structure per se, and thus, constitute critical modulators of paracellular permeability [10]. Specifically, VE-Cad is recognized as the most important gatekeeper of the endothelial barrier [11–14]. Other constituents play major roles in the organization of protein–protein complexes in plasma membranes. They include connexin 43 (Cx43), which can directly and indirectly affect TJ and AJ assembly [15–17]. Actually, Cx43 signaling microdomains can influence a wide range of junctional- and cytoskeleton-associated proteins [18,19] through mechanisms that involve direct protein–protein interactions and transcriptional regulation [19–21]. The complexes of IEJs are anchored to the actin cytoskeleton, which contribute to define the IEJs' architecture [22,23]. Actin filaments can exert antagonist effects on IEJs integrity, depending on their polymerization activity, localization, and contractility. These actin bundles can be categorized into two main types: cortical actin fibers that support the IEJs' anchorage to the plasma membrane, which are predominant at the basal state, and contractile transcellular filaments called stress fibers that promote IEJs disassembly and internalization by exerting pulling forces. Stress fibers are mostly formed in response to pro-inflammatory agents. Their contraction contributes to the onset of intercellular-gap formation [22–24]. The key mediators involved in actin-dynamic regulation, include the small heat shock protein 27 kDa (HSP27), which plays a critical a role in the regulation of actin polymerization [25,26]. Unphosphorylated HSP27 behaves like a filamentous actin (F-actin) cap-binding protein by inhibiting actin polymerization. Phosphorylation of several distinct serine residues (Ser15, Ser78, and Ser82) relieves its capping activity and promotes actin microfilament formation. HSP27 is phosphorylated by mitogen-activated protein kinase-activated protein kinases (MAPKAPK) 2 and 3, which are both direct substrates of p38 MAPK α and β [27]. The role of HSP27 phosphorylation in the regulation of endothelial permeability remains controversial [25–29]. In addition, small Rho GTPases are crucial orchestrators of actin organization and contractility [23,30]. RhoA promotes stress fibers contraction, while Rac1 reinforces IEJs' anchorage to the plasma membrane by promoting the development of a cortical actin network [31,32]. Altogether, junctional and cytoskeletal proteins regulate paracellular flow through tight control of the opening-closure sequences of the intercellular space. In pro-inflammatory conditions, paracellular permeability is enhanced because macromolecules and leucocytes are recruited within underlying tissues to regulate pathogen invasion. Extreme sepsis conditions drive homeostasis towards endothelial dysfunction, formation of intercellular gaps, subsequent massive plasma leakage and impaired oxygen diffusion into peripheral tissues, causing MOF. Targeting mediators that contribute to the vascular barrier integrity might therefore improve support for septic patients.

AMP-activated protein kinase (AMPK) is a key metabolic master that has broad effects on cellular functions, including organization of IEJs [33–35] and actin cytoskeleton [36–41]. Its main endothelial isoform, α1AMPK, has been reported to regulate paracellular permeability [42–46] and, more specifically, sepsis-induced vascular leakage [45–49]. Our group previously demonstrated that the mortality of mice exposed to sublethal doses of lipopolysaccharide (LPS) was dramatically increased by α1AMPK invalidation. This was also shown to be associated with enhanced vascular permeability [47]. Moreover, we showed that AMPK activation by the AMP-mimetic 5-aminoimidazole-4-carboxamide ribonucleotide (AICAr) in wild-type mice can attenuate LPS-induced tissue edema formation. Other

direct [33,47–49] and indirect [45,50,51] AMPK activators have already demonstrated their capacity to protect the vascular barrier in in vivo models of sepsis, and some are even associated with anti-inflammatory effects [49–51] and improved survival [49]. Among these activators, only metformin is currently used in clinical practice. However, its administration during sepsis remains controversial because of the potential evolution toward lactic acidosis [52]. By considering the urgency to improve medical support for septic patients [53], special attention should be paid to identifying new molecule targeted therapies that are clinically applicable to the particular context of sepsis.

In this study, we sought to further elucidate mechanisms through which AMPK regulates junctional assembly and permeability in LPS treated microvascular endothelial cells. Specifically, we examined the effect of the specific and direct pan-AMPK activator 991 on IEJs and cytoskeleton organization. We report for the first time that AMPK plays a critical role in the maintenance and assembly of IEJs by regulating Cx43 expression, HSP27 phosphorylation and subsequent actin cytoskeleton remodeling.

2. Results

2.1. α1. AMPK Preserves IEJs Integrity

2.1.1. α1. AMPK Deficiency is Associated with Downregulation of VE-Cad, ZO-1, and Cx43 Expression

We first investigated whether α1AMPK regulates VE-Cad, ZO-1, and Cx43 expression in unstimulated and LPS-treated HMECs. Cells transfected with AMPKα1-targeting or scrambled control small interfering RNAs (siRNAs) were either treated or not treated with the best-available AMPK activator 991 (1 μM, 1 h), prior to LPS stimulation (50 μM, 6 h) (Figure 1a). While, AICAr can have off-targets, the 991 compound has been recently described and is a direct and more specific AMPK activator [54]. Its effect is mainly allosteric, inducing a change in AMPK conformation and subsequent stimulation. Unlike LPS, treatment by 991 activates AMPK in HMECs, as reflected by AMPK phosphorylation on Thr172 and acetyl-CoA carboxylase (ACC) phosphorylation on Ser79. α2AMPK is not expressed in HMECs. Transfection of α1AMPK-targeting siRNA abrogates α1AMPK expression and 991-mediated ACC phosphorylation (Figure 1b,c). Western blot analysis also reveals that VE-Cad, ZO-1, and Cx43 protein expression are significantly downregulated in α1AMPK-depleted cells, although they are not affected by 991 or LPS treatment (Figure 1b,c). Considering the importance of Cx43 in the formation and scaffolding of IEJs between endothelial cells, we subsequently utilized a specific siRNA-targeting Cx43 to characterize the impact of its deletion on VE-Cad and ZO-1 expression. The protein level of Cx43 is significantly lower in cells transfected with the siRNA anti-Cx43, compared to scramble-transfected cells (Figure 2a). Cx43 deficiency is associated with significantly reduced expression of VE-Cad and ZO-1 (Figure 2b,c). Accordingly, fluorescence analysis confirms the drastic loss of VE-Cad and ZO-1 signals at the edges of Cx43-silenced cells (Figure 2d,e). These results suggest that α1AMPK plays a critical role in the regulation of Cx43 expression and subsequent maintenance and assembly of TJs and AJs, independently of LPS treatment.

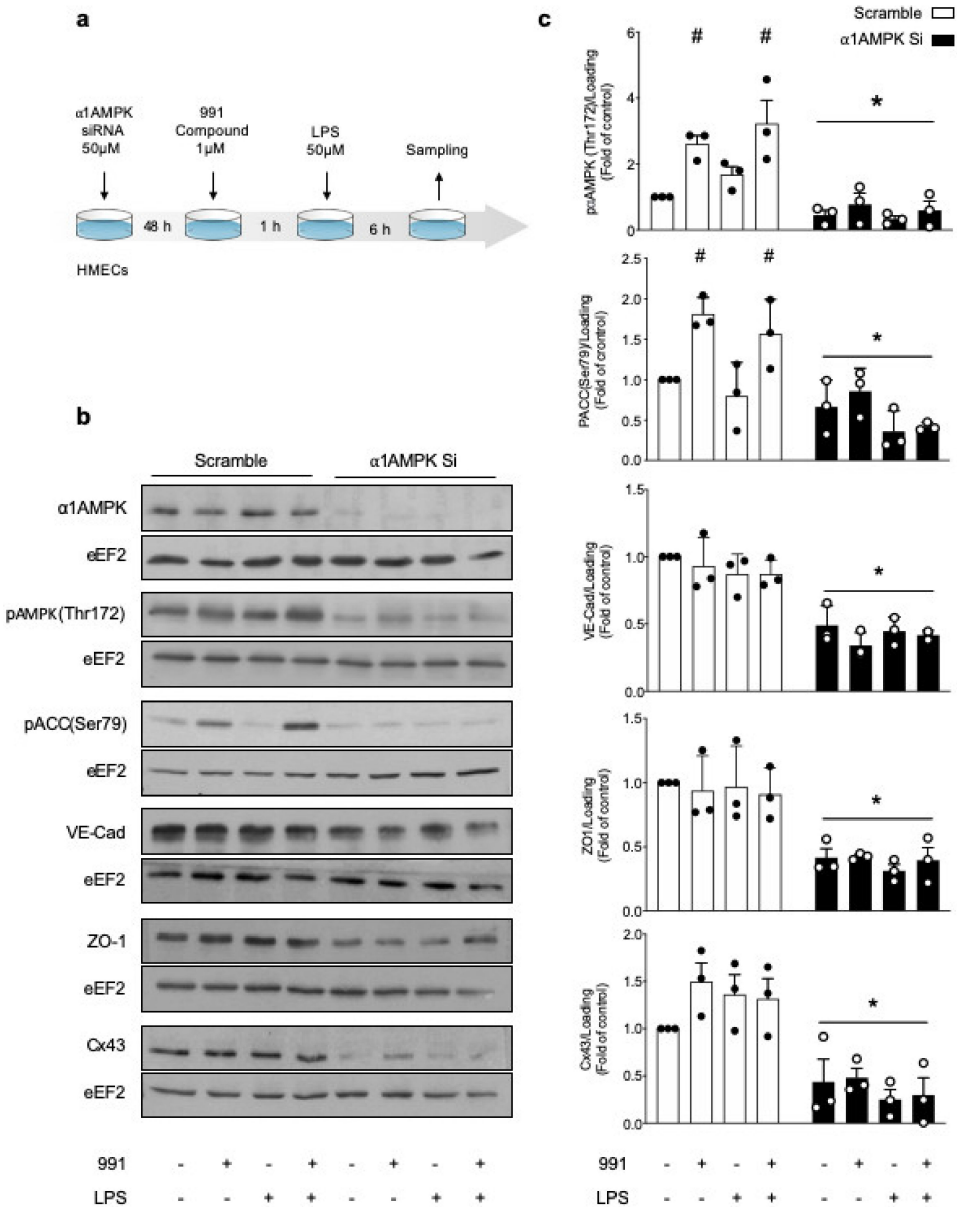

Figure 1. Basal α1AMPK regulates VE-Cad, ZO-1, and Cx43 expression. (**a**) Schematic representation of the experimental design, in which HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50 nM) for 48 h. Then, they were treated with 991 (1 μM) or DMSO for one hour and subsequently exposed or not exposed to lipopolysaccharide (LPS, 50 μM) for six hours; (**b**,**c**) Human dermal microvascular endothelial cells (HMECs) were treated according to the protocol detailed in (**a**). Cell lysates were submitted to Western blot analysis and probed with α1AMPK, phospho-AMPK (Thr172), phospho-ACC (Ser79), VE-Cad, ZO-1, and Cx43 antibodies (Abs). Anti-eukaryotic elongation factor 2 (eEF2) was used as a loading control. Representative western blots; (**b**) and quantifications (**c**) are shown. Data are expressed as mean ± standard deviation (SD) (three biological replicates for each condition). $^{\#}$ $p < 0.05$ is relative to corresponding untreated HMECs and * $p < 0.05$ is relative to cells transfected with the scrambled siRNA. The data underwent two-way analysis of variance (ANOVA).

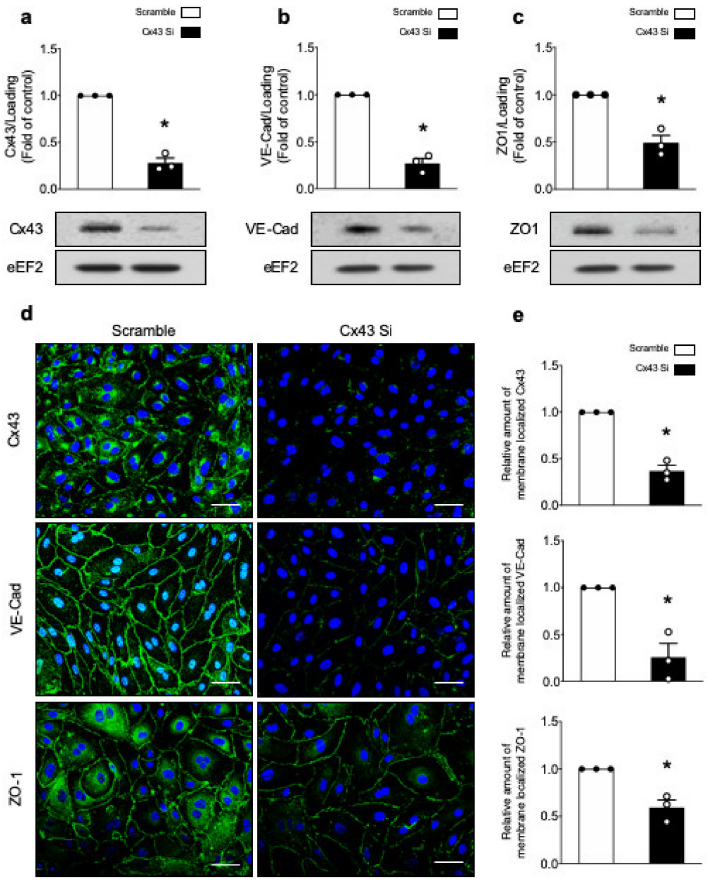

Figure 2. Cx43 deficiency is associated with decreased VE-Cad and ZO-1 expression. (**a**–**e**) HMECs were transfected with control non-targeting siRNA or Cx43 siRNA (50nM) for 48 h. (**a**–**c**) Cell lysates were submitted to Western blot analysis and probed with Cx43 (**a**), VE-Cad (**b**), and ZO-1 (**c**) Abs. eEF2 was used as a loading control. Representative Western blots (**top** panel) and quantifications (**bottom** panel) are shown; (**d**,**e**) Cx43, VE-Cad, and ZO-1 immunostainings. Nuclei are stained with DAPI. Scale bar, 50 µm. Representative images (**d**) and quantifications (**e**) are shown. Data are expressed as mean ± SD (three biological replicates for each condition). * $p < 0.05$ is relative to cells transfected with the scrambled siRNA. Analyses were performed using Student's *t*-test.

2.1.2. AMPK Activation Prevents LPS-Induced Disruption of IEJs and Endothelial Barrier Dysfunction

LPS drastically impairs the integrity of VE-Cad (Figure 3a,b), ZO-1 (Figure 3c,d), and Cx43 (Figure 3e,f). Indeed, the linear shape of cell–cell junctions, which is observed in basal conditions, appears jagged and disconnected after LPS treatment. Accordingly, membrane staining quantification for VE-Cad, ZO-1, and Cx43 is significantly lower in LPS-treated cells, compared to control cells (Figure 3b,d,f). These changes are associated with the formation of intercellular gaps, which indicates endothelial barrier disruption. As we show that LPS does not affect VE-Cad, ZO-1, and Cx43 total protein expression (Figure 1), the decreased membrane staining of IEJs is likely due to their mechanical disruption and internalization. Next, we determined the effects of the small-molecule AMPK activator 991. The 991 compound does not affect IEJs organization in unstimulated HMECs. However, it reinforces Cx43, VE-Cad, and ZO-1 anchorage to the plasma membrane upon LPS stimulation and also prevents the appearance of intercellular gaps. This protective effect is lost in α1AMPK-depleted cells, indicating an AMPK-dependent action (Figure 3a–f). Notably, the absence of α1AMPK induces a drastic decrease of VE-Cad, ZO-1, and Cx43 signals at the periphery of cells, regardless of the conditions, which confirms our previous Western blot data (Figure 1b,c). Finally, the involvement of AMPK pathway in endothelial barrier integrity was demonstrated by measuring the clearance of HRP-coupled streptavidin through the HMEC monolayer. As expected, 991 treatment significantly

reduces LPS-induced hyperpermeability (Figure 3g). To inactivate AMPK, we used the pan-AMPK inhibitor SBI0206965. AMPK inactivation significantly impairs endothelial barrier function and completely abrogates 991-mediated protection (Figure 3g).

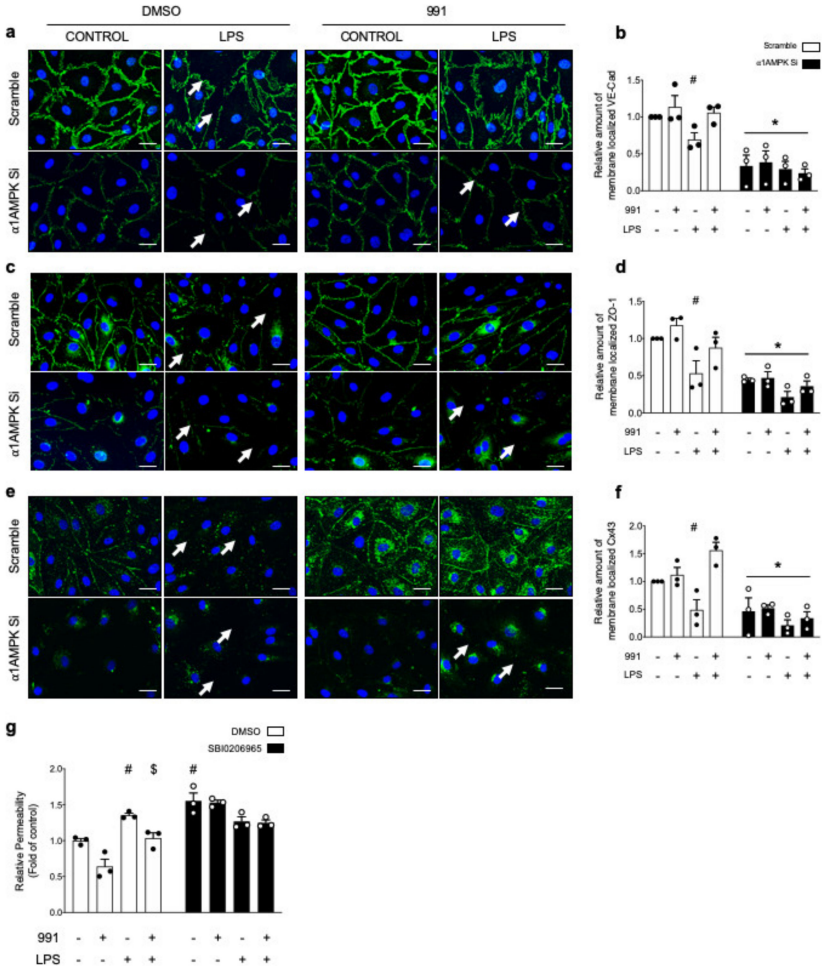

Figure 3. AMPK Activation Preserves IEJs Organization and Endothelial Barrier Function in HMECs Under LPS Challenge. (**a**–**f**) HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50 nM) for 48 h. Then, HMECs were treated with DMSO or 991 (1 μM) for one hour, after which they were, either exposed or not exposed to LPS (50 μM) for six hours. Immunostainings and membrane-staining quantification of VE-Cad (**a**,**b**), ZO-1 (**c**,**d**), and Cx43 (**e**,**f**) are shown. Intercellular gaps are indicated by white arrows. Nuclei are stained with DAPI. Scale bar, 50 μm. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}$ $p < 0.05$ is relative to corresponding untreated HMECs and * $p < 0.05$ is relative to cells transfected with scrambled siRNA. The data underwent two-way ANOVA; (**g**) Endothelial permeability in response to AMPK activation, LPS challenge, and AMPK inhibitor SBI0206965. HMECs were grown on Transwell inserts for 72 h and treated or not treated with SBI0206965 (10 μM) for 15 min before undergoing 991 and LPS treatments. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}$ $p < 0.05$ is relative to respective non-treated HMECs and $^{\$}$ $p < 0.05$ is relative to LPS-only treated HMECs. The data underwent two-way ANOVA.

2.2. *α1. AMPK Induces Actin Cytoskeleton Remodeling*

2.2.1. AMPK Activation Modulates Actin Organization and Polymerization

Given that junctional complexes are proposed to associate with the actin cytoskeleton, considered as a key component of cell permeability, we then investigated the impact of AMPK activation on LPS-induced F-actin morphological changes. In resting HMECs, F-actin is mainly organized in

peripherical beams within the cytoplasm (Figure 4a). After LPS stimulation, peripherally located F-actin is substituted by centralized and irregular stress fibers running across the cytoplasm (Figure 4a,b). This effect is even more pronounced in the absence of α1AMPK, despite a significant decrease in the fluorescent signal that corresponds to F-actin (Figure 4c). AMPK activation by 991 strongly reinforces peripheral actin bands, both in unstimulated cells and in cells treated with LPS. This effect is reduced in the absence of α1AMPK (Figure 4a–c).

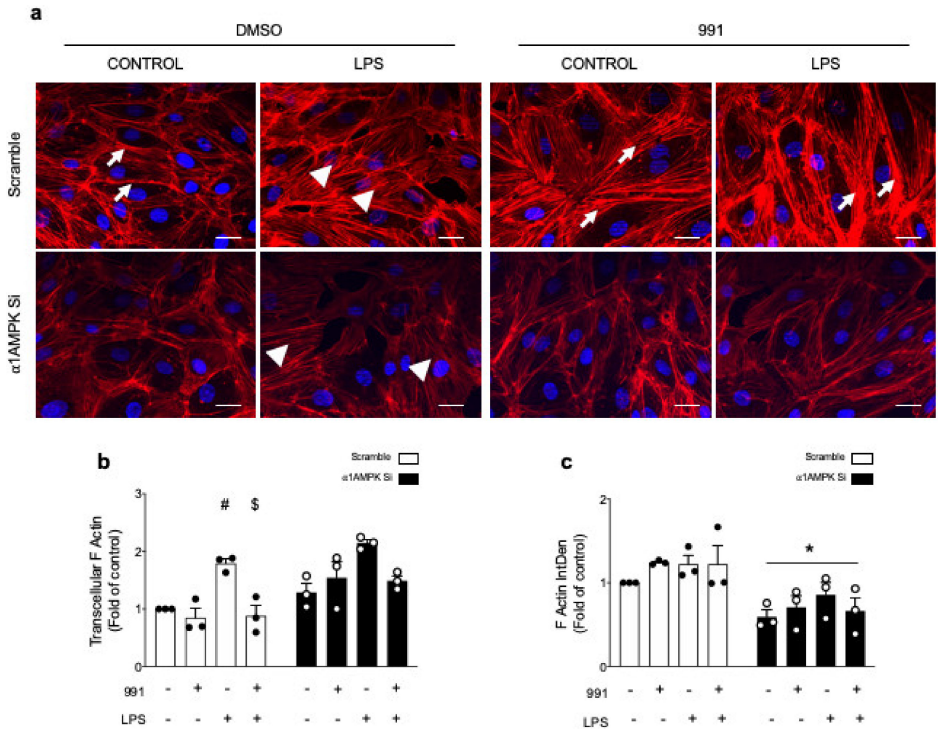

Figure 4. AMPK Activation Reinforces the Cortical Actin Network and Counters Stress-Fiber Formation During LPS Challenge. HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50 nM) for 48 h and treated with DMSO or 991 (1μM) for one hour. They were then exposed to LPS (50 μM) for six hours. After fixation, HMECs were stained with Alexa Fluor 568 Phalloidin. (**a**) Representative fluorescence microscopy images of F-actin. Arrows indicate peripherical actin; arrowheads indicate centralized stress fibers; (**b**) Quantification of transcytoplasmic actin filaments; (**c**) Quantification of global F-actin amounts, which were assessed by integrated density measurement. Nuclei are stained with DAPI. Scale bar, 25 μm. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}$ $p < 0.05$ is relative to corresponding untreated HMECs, $^{\$}$ $p < 0.05$ is relative to corresponding LPS-treated HMECs, and * $p < 0.05$ is relative to cells transfected by scrambled siRNA. The data underwent two-way ANOVA.

2.2.2. α1. AMPK Regulates Actin Polymerization via Activation of the p38 MAPK/HSP27 Pathway

Due to its pivotal role in actin polymerization, we investigated whether HSP27 was involved in the protective action of α1AMPK against LPS-induced endothelial barrier impairment. The total expression and phosphorylation sate of HSP27(Ser82) and p38 MAPK(Thr180/Tyr182) were measured in the presence or absence of 991, before LPS stimulation. The results show that 991 and LPS increase p38 MAPK phosphorylation, and can individually and additionally enhance HSP27 phosphorylation (Figure 5a,b). Unlike LPS, the effect of 991 on HSP27 phosphorylation is attenuated in α1AMPK-depleted cells (Figure 5a,b). It clearly depends on p38 MAPK activation since preincubation with the selective p38 MAPK inhibitor SB203580 significantly reduces signal intensity, regardless of the conditions (Figure 5c,d). Surprisingly, treatment with SB203580 increases MAPK p38 phosphorylation, possibly because of feedback activation induced by the inhibition of p38 MAPK activity (Figure 5c) [55]. Next, we examined the effect of direct inhibition of p38 MAPK on the actin cytoskeleton. SB203580 globally

reduces F-actin content and impairs the well-organized peripheral actin network of unstimulated cells, which acts in favor of the formation of stress fibers across the cytoplasm (Figure 6a–c). More interestingly, p38 MAPK inactivation prevents 991-induced enrichment of thick actin bundles around the cell periphery of LPS-treated cells (Figure 6a–c). Altogether, these results indicate that α1AMPK activation modifies the cytoskeletal response to LPS injury by potentiating the p38 MAPK/HSP27 pathway. Accordingly, while basal permeability is barely affected by p38 MAPK inhibition, SB203580 completely prevents the protective effect of 991 (Figure 6d). Since p38 MAPK has been reported as a downstream target of Rac1, a small GTPase intimately involved in regulating cortical actin structures and endothelial barrier function [56], Rac1 activation was measured in the presence or absence of 991, before LPS stimulation. G-LISA analysis shows that LPS and 991 can individually enhance Rac1 activity, while there is no additional effect of the combined treatments (Figure 7a). Pre-incubation with NSC23766 inhibits Rac1 activity (Figure 7a) but does not significantly change 991—or LPS-induced HSP27 phosphorylation (Figure 7b), indicating that the activation of the p38 MAPK/HSP27 axis is independent of Rac1 in both conditions.

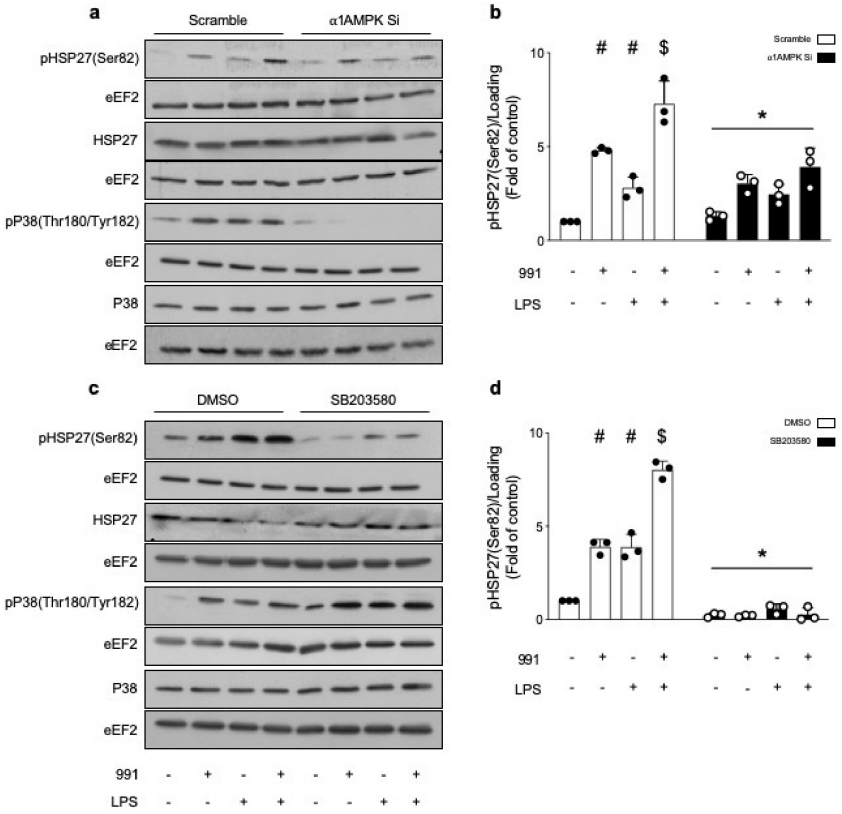

Figure 5. AMPK Activation Leads to Increased HSP27 Phosphorylation, via a p38 MAPK-Dependent Way. (**a**,**b**) HMECs were transfected with control non-targeting siRNA or α1AMPK siRNA (50 nM) for 48 h and then treated with DMSO or 991 (1 μM) for one hour. They were then exposed to LPS (50 μM) for six hours; (**c**,**d**) HMECs were preincubated with DMSO or with the p38 MAPK inhibitor SB203580 (10 μM) for 15 min. Then, they were treated with 991 (1 μM) or DMSO for one hour and subsequently, either exposed or not exposed to LPS (50 μM) for six hours. (**a**–**d**) Cell lysates were submitted to western blot analysis and probed with total HSP27, total p38 MAPK, phospho-HSP27 and phospho-p38 MAPK Abs. eEF2 was used as a loading control. Representative Western blots (**a**,**c**) and quantification of HSP27 (Ser82) phosphorylation (**b**,**d**) are shown. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}$ $p < 0.05$ is relative to corresponding non-treated HMECs, $^{\$}$ $p < 0.05$ is relative to corresponding LPS-treated HMECs, and * $p < 0.05$ is relative to cells transfected with non-targeting siRNA or that were treated with the vehicle. The data underwent two-way ANOVA.

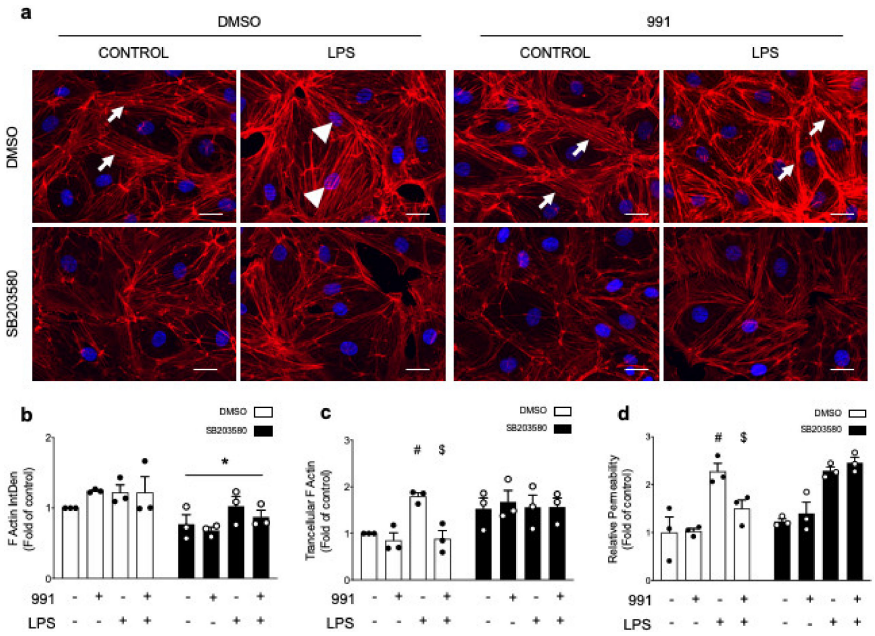

Figure 6. AMPK Activation Regulates Actin Organization and Cell Permeability via the p38 MAPK/HSP27 Pathway. (**a**–**c**) HMECs were fixed and stained with Alexa Fluor 568 Phalloidin. (**a**) Representative fluorescence microscopy images of F-actin. Arrows indicate peripherical actin; arrowheads indicate centralized stress fibers; (**b**) Quantification of area occupied by transcytoplasmic actin filaments; (**c**) Quantification of global F-actin amounts, which were assessed by integrated density measurement. Nuclei are stained with DAPI. Scale bar, 25 µm. Data are expressed as means ± SD (three independent experiments for each condition). * $p < 0.05$ is relative to cells treated with DMSO. The data underwent two-way ANOVA; (**d**) Endothelial permeability in response to AMPK activation, LPS challenge, and p38 MAPK inhibitor SB203580. HMECs were grown on Transwell inserts for 72 h and treated or not treated with SB203580 (10 µM) for 15 min before undergoing 991 and LPS treatments. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}$ $p < 0.05$ is relative to respective non-treated HMECs and $^{\$}$ $p < 0.05$ is relative to LPS-only treated HMECs. * $p < 0.05$ is relative to cells treated with the vehicle. The data underwent two-way ANOVA.

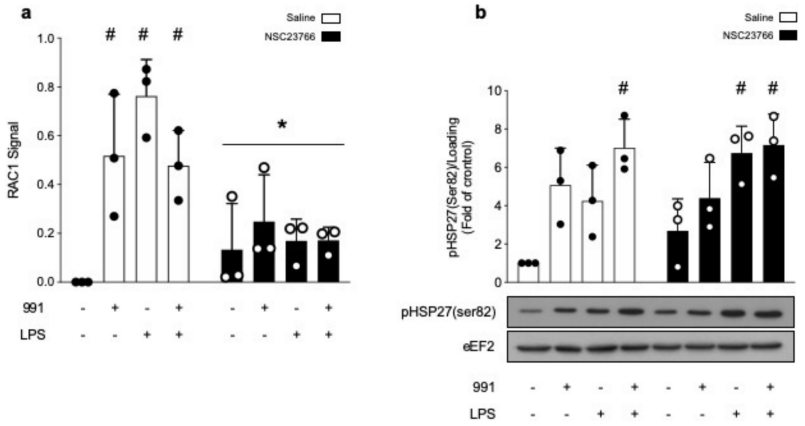

Figure 7. AMPK Regulates Actin Organization Independently of Rac1. (**a**,**b**) HMECs were treated with saline or the Rac1 inhibitor NSC23766 (100µM) for 15 min before adding DMSO or 991 (1µM) for one hour. Then, they were exposed to LPS (50µM) for six hours. (**a**) Lysates were engaged in G-LISA assays to precisely monitor Rac1 activity. Quantification represents the differential signal, compared to basal, in fold of control; (**b**) Representative Western blots and quantification of HSP27 (Ser82) phosphorylation. eEF2 was used as a loading control. Data are expressed as means ± SD (three biological replicates for each condition). $^{\#}$ $p < 0.05$ is relative to corresponding non-treated HMECs. * $p < 0.05$ is relative to cells treated with the vehicle. The data underwent two-way ANOVA.

3. Discussion

In this study, we first demonstrate that α1AMPK is essential in maintaining the proper expression and architecture of IEJs in basal conditions. Second, we identify the p38 MAPK/HSP27 pathway and actin cytoskeleton as central mediators of the protective effect of activated α1AMPK on the endothelial barrier. Experiments were performed using HMECs, which constitute a highly relevant model for the assessment of vascular leakage mechanisms that mainly occur at the level of capillaries and postcapillary venules. The α1AMPK isoform plays a predominant role in these cells since its specific invalidation using a siRNA strategy almost completely abrogates the 991 effect on ACC phosphorylation, which is the bona fide substrate of AMPK. Several studies, including ours, have reported the beneficial role of AMPK in endothelial barrier regulation during sepsis [45–49]. Here, we confirm this protective action in microvascular endothelial cells that are exposed to LPS and highlight the involvement of new molecular mechanisms.

Our previous work has reported on the protective role of basal α1AMPK in the maintenance of endothelial barrier function, through the preservation of ZO-1 integrity in TJs [47]. Here, we demonstrate that, in addition to ZO-1, VE-Cad is also distorted and downregulated in the absence of α1AMPK. Our data further support the emerging concept that Cx43 can contribute to the basal expression and organization of ZO-1 and VE-Cad. Cx43, which is a protein normally associated with gap junctional communication, has recognizable scaffolding functions that contribute to various biological processes [16,19]. The AMPK-Cx43 pathway has been highlighted in cardiomyocytes [57] and in cardiac fibroblasts [58] in which it plays a role in the fibrotic response commonly seen with a variety of cardiac pathologies [59]. The link between AMPK and Cx43 has never been demonstrated in the endothelium. We show for the first time that AMPK deficiency is associated with a drastic loss of Cx43 in endothelial cells, which leads to the subsequent disruption of tight and adherens junctions. The fact that the 991 compound has no impact on Cx43 expression supports the notion that non-catalytic functions of AMPK are rather involved in this regulation. These non-catalytic functions may include the scaffolding of protein complexes, the competition for protein interactions, allosteric effects on other enzymes, or subcellular targeting. Among others, ERK1 and ERK2 were shown to influence their substrates not only by phosphorylation, but also by direct protein-protein interactions, independently of kinase activity [60]. We believe that this type of regulation likely explains how AMPK may influence Cx43 expression.

While the direct interaction of Cx43 with ZO-1 has been extensively demonstrated [17,21,61], the mechanism is more speculative for VE-Cad. The interaction between Cx43 and VE-Cad likely involves other partners, such as N-cadherin (N-Cad), p120, and α- and ß-catenin, all of them being known to bind Cx43 and promote VE-Cad stability [11,20,33,62–64]. In addition to its scaffolding action, Cx43 is also described as a modulator of gene transcription, by the interaction of its carboxy tail with transcription factors driving its translocation to the nucleus and binding to gene promoters [19,65]. However, these potential mechanisms do not exclude Cx43-independent transcriptional regulation of ZO-1 and VE-Cad by α1AMPK. Indeed, AMPK can directly (via the phosphorylation of transcriptional regulators) or indirectly (via the induction of sirtuins) regulate gene transcription [66]. Post-transcriptional (changes in micro-RNAs expression) and post-translational (acetylation or nitrosylation) mechanisms might also be involved [67–70]. Future experimental work is necessary to delineate the molecular regulation of VE-Cad, ZO-1, and Cx43 by AMPK.

Our present work emphasizes the critical role of actin cytoskeleton in the α1AMPK dependent protection of IEJ integrity in LPS-treated endothelial cells. Our observations are supported by data showing that, in epithelial and endothelial cells, activated AMPK can reinforce the interaction between cadherin-catenin complexes and actin cytoskeleton via both regulation of N-Cad [33,63] and phosphorylation of G-alpha interacting vesicle associated protein (GIV) [71,72]. Other protective mechanisms have been highlighted. In Caco-2 cells submitted to a calcium switch [34,35,73,74], α1AMPK activation is associated with a change in the expression level of VE-Cad, ZO-1, or Cx43 [34,42], whereas our present data show that the expression of these proteins is not changed in 991-treated

HMECs. In other cellular models, activated α1AMPK may affect the integrity of TJs and AJs by phosphorylating some of their components. For example, claudin-1 and -4 have been described as direct AMPK substrates in epithelial Eph4 cells and SV40 immortalized rat submandibular acinar cell lines, respectively [44,75]. Afadin is another AMPK target involved in reinforcing TJs in Madin-Darby Canine Kidney (MDCK) cells, via its binding to ZO-1 [76].

We and others have previously demonstrated that α1AMPK regulates the organization of actin cytoskeleton in different cell types [36–41]. HSP27 plays an important role in actin dynamics, through its phosphorylation downstream of p38 MAPK [25–27]. Very interestingly, a link between Cx43 and HSP27 was previously demonstrated in HeLa cells [77]. In this model, Cx43 regulates actin dynamics and migration by binding p21-activated protein kinase 1 (PAK1), which then activates the downstream p38 MAPK/HSP27 pathway. Our data show that HSP27 phosphorylation protects the endothelial barrier function. However, the role of HSP27 in the regulation of endothelial permeability remains controversial. For example, HSP27 phosphorylation protects against hypoxia- or burn serum-induced permeability of endothelial cells [25–29]. On the contrary, pertussis toxin-induced endothelial permeability is temporally linked to p38 MAPK activation and phosphorylation of HSP27 [27,78]; and LPS-induced endothelial barrier dysfunction correlates with HSP27 phosphorylation in vitro (our own data) and in vivo [28,79]. The reason for these discrepancies is not clear. It has been suggested that activation of the p38 MAPK/HSP27 pathway by LPS might be an adaptive response, thereby promoting centralized stress fibers and subsequent cell adhesion, in order to counteract contractility. This is rather regulated by the RhoA pathway and downstream myosin light chain phosphorylation [25].

Given that p38 MAPK is a downstream effector of AMPK in endothelial cells [80], we postulated that it could be involved in the protective action of 991 on cell cytoskeleton and permeability. Subsequently, we asserted that HSP27 phosphorylation was significantly increased in 991-treated HMECs. While, LPS is associated with the formation of centralized stress fibers, 991 increases cortical actin structures, suggesting the likely involvement of two different p38 MAPK activation pathways. Typically, p38 MAPK is activated through small GTPases, such as Rac1, and the downstream canonical three-tiered kinase cascade [81,82]. Rac1 is activated by LPS and 991 to the same extent, but surprisingly, we observed that its inhibition had no significant impact on LPS- or 991-induced HSP27 phosphorylation. This suggests the involvement of other non-canonical mechanisms of p38 MAPK activation. Accordingly, it was recently shown that the p38 isoform in HMECs can be autoactivated through its interaction with the transforming growth factor α-activated kinase 1 binding proteins (TAB) 1, 2, or 3, which is independent of the MAPK cascades [83]. Notably, the direct activation of p38 MAPK by AMPK, via the scaffold protein TAB1, has been previously described in apoptotic lymphocytes and the ischemic heart [84,85], strengthening the relevance of this mechanism in 991-treated HMECs. Finally, a different non-canonical pathway for p38 MAPK activation can be mediated through autophosphorylation, which is facilitated by the tyrosine kinase Zap70 [83,86,87]. The role of these non-canonical mechanisms of p38 MAPK, in the regulation of endothelial barrier disruption, remains poorly understood and warrants further exploration.

In summary, our data show that α1AMPK is required to maintain the integrity of TJs and AJs in microvascular endothelial cells. Moreover, α1AMPK activation leads to the phosphorylation of HSP27 via a non-canonical p38 MAPK dependent mechanism, which results in the reinforcement of the submembrane actin network during LPS challenge, contributing to the consolidation of IEJs in the plasma membrane and intercellular tethering (Figure 8). Therefore, α1AMPK activation might have therapeutic potential to alleviate conditions in which microvascular barrier formation is dramatically compromised, such as sepsis [88].

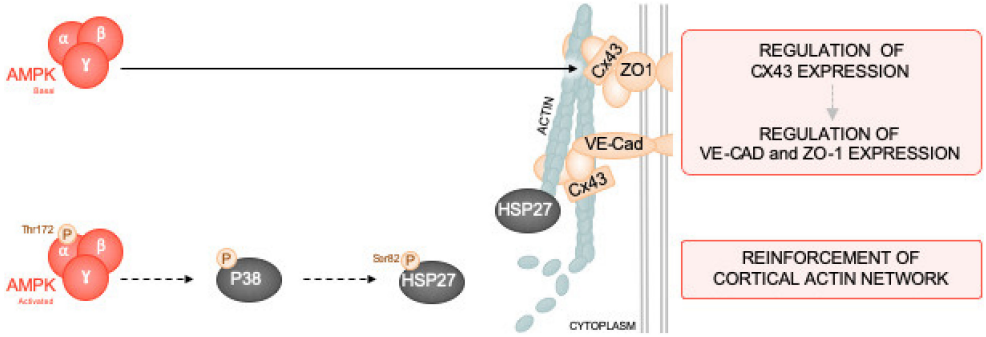

Figure 8. Molecular mechanisms involved in endothelial barrier protection by α1AMPK. α1AMPK is essential in maintaining the proper expression and architecture of IEJs in basal conditions. It regulates Cx43 expression, which through its scaffolding functions, may contribute to ZO-1 and VE-Cad organization at plasma membrane. On the other hand, the specific and direct pan-AMPK activator 991 protects HMECs against LPS-induced hyperpermeability. The mechanism involves activation of the p38 MAPK/HSP27 pathway and subsequent cortical actin polymerization, which reinforces IEJs anchorage at the plasma membrane and subsequent intercellular tethering. Abbreviations: AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (P38), Heat shock protein 27 (HSP27), VE-cadherin (VE-Cad), Zonula occludens-1 (ZO-1), Connexin 43 (Cx43).

4. Materials and Methods

4.1. Reagents and Antibodies

The 991 compound was kindly provided by Benoit Viollet (Institut Cochin, DR2 Inserm, Paris, France). SBI0206965 (#SML1540), SB203580 (#S8307), NSC23766 (#SML0952), and LPS O55:B5 (50 µg/mL, #L2880) were from Sigma-Aldrich (Overijse, Belgium). We also used a halt protease and phosphatase inhibitor cocktail (#78442; Thermo Fisher Scientific, Waltham, MA, USA), Lipofectamine RNAiMAX reagent (#13778-150; Invitrogen, Thermo Fisher Scientific), Opti-MEM (#31985070; Gibco, Thermo Fisher Scientific), siRNA negative control (#AM4635; Ambion, Thermo Fisher Scientific), siRNA PRKAA1 (#AM51334; Ambion), siRNA GAJ1 (#4392422; Ambion), BM chemiluminescence blotting system (#11500694001; Roche, Sigma-Aldrich), 12 mm µ-dish culture inserts (634-1577P; VWR), Alexa Fluor 488 Deoxyribonuclease I (DNAse I) (#D12371; Invitrogen), Alexa Fluor 568 Phalloidin (#A12380; Invitrogen), Transwell inserts (#3413; Corning, Sigma-Aldrich), M200 medium (#M200500; Gibco), HRP-coupled streptavidin (#15:1000, DY998; R&D Systems, Minneapolis, MN, USA), TMB substrate (#T040; Sigma-Aldrich), H_2SO_4 (#30743; Sigma-Aldrich), and RAC1 G-LISA kit (#BK128; Cytoskeleton, Inc., Denver, CO, USA). The antibodies used were α1AMPK (#MA5-15815; Thermo Fisher Scientific), phospho-AMPK Thr172 (#2535; Cell Signaling Technology, Danvers, MA, USA), phospho-ACC S79 (#3661; Cell Signaling Technology), Cx43 (#3512; Cell Signaling Technology), VE-Cad (#36-1900; Thermo Fisher Scientific), ZO-1 (#339100; Thermo Fisher Scientific), p38 MAPK (#9212; Cell Signaling Technology), phospho-p38 MAPK T180/Y182 (#9211; Cell Signaling Technology), heat shock protein of 27 kDa (HSP-27) (#2402; Cell Signaling Technology), phospho-HSP27 S82 (#44534; Life Technologies, Thermo Fisher Scientific), eEF2 (#PA5-17794; Thermo Fisher Scientific), secondary horseradish peroxidase (HRP)-conjugated antibodies (#A0545; Sigma-Aldrich or#554002; BD Biosciences, San Jose, CA, USA), and Alexa Fluor-coupled secondary antibodies (#A21202 or #A21206; Invitrogen).

4.2. Cell Culture and Treatments

HMECs were purchased from Promocell (C-12225) and cultured according to the manufacturer's recommendations, using Endothelial Cells Growth Medium MV (C-22020; Promocell, Heidelberg, Germany) containing 1% penicillin-streptomycin, at 37 °C and 5% CO_2 in a humidified incubator. The cells were sub-cultured when reaching 80% confluence and used until subculture number 7. To

proceed with the experiments, cells were seeded at a density of $10^4/cm^2$ and cultured for 48–72 h, until total confluence was reached. Medium deprivation was performed for two hours prior to treatment or experimentation.

4.3. siRNA Transfections

For endothelial α1AMPK and Cx43 silencing, HMECs were seeded the day before transfection with heparin-free Endothelial Cells Growth Medium MV2 (C-22022; Promocell) to reach 60% confluence within 24 h. Reverse transfection was then performed for 48 h with either a control non-targeting siRNA construct (50 nM), a siRNA specifically targeting PRKAA1 (50 nM), or GAJ1 (50 nM). This was performed using a lipofectamine RNAimax transfecting reagent, which adhered to the manufacturer's instructions.

4.4. Western Blotting

Western blot analysis was performed on HMEC lysis, as previously described [89]. Protein content was measured using the Bradford method, which used bovine serum albumin (BSA) as a reference. Proteins (15 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted. Membranes were then probed with the primary antibody overnight at 4 °C, in order to assess protein phosphorylation or total expression level. Primary antibodies included α1AMPK (1:1000), phospho-AMPK Thr172 (1:1000), phospho-ACC (1:5000), Cx43 (1:1000), VE-Cad (4:1000), ZO-1 (2:1000), p38 MAPK (1:1000), phospho-p38 MAPK T180/Y182 (1:1000), HSP27 (1:1000), phospho-HSP27 S82 (1:1000), and eEF2 (1:1000). Membranes were then incubated with appropriate secondary HRP-conjugated antibodies (1:20,000, #A0545 or 1:10,000, #554002; both Sigma-Aldrich) for one hour at room temperature. A chemiluminescence blotting system was used for detection. Quantification was executed by ImageJ software and normalized with eEF2 as a loading control on the same gel. Each experiment was repeated three times.

4.5. Immunofluorescence Microscopy

HMECs were seeded on non-coated glass coverslips at 20×10^3 cells/cm^2, 72 h before treatment. After treatment, cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% triton X-100 for ten minutes, and blocked with 10% BSA for 45 min. For intercellular junction studies, cells were stained as previously described [47], using the following primary antibodies: Cx43 (1:100), VE-Cad (1:25), or ZO-1 (1:50). Cells were then incubated with the appropriate Alexa Fluor-coupled secondary antibody (1:1000, #A21202 or #A21206). For cytoskeleton studies, HMECs were incubated with Alexa Fluor 568 Phalloidin (150 nM) for 15 min at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Stainings were visualized under a Zeiss Imager Z1 microscope that was equipped with an ApoTome device. Pictures were acquired with a 20× objective. Each experiment was repeated three times.

4.6. Image Analysis

Quantitative image analysis was performed on uncompressed images (native format: zvi) with Fiji 1.52n on MacOS (10.14.5). The intercellular junctions evidenced by ZO-1 or VE-Cad staining were automatically delimited by a fixed-value threshold method. The stained area was quantified, and the mean signal intensity was calculated within this selection. Stained membrane segments were subsequently detected using the Analyze Particles and Skeletonize tools, and automatically counted. Due to the irregular distribution of the Cx43 signal, all cell membranes were manually defined as regions of interest. The Cx43 positive area and mean signal intensity were then quantified within these regions.

To analyze cytoskeleton organization, F-actin and nuclei were sequentially detected using threshold methods in the red and blue channels, respectively. Stained area and integrated density were measured for F-actin. The area fraction of F-actin that was colocalized within nuclei areas was subsequently calculated and defined as stress fibers. The mean signal intensity was also measured.

For normalization purposes, the nuclei in all images were automatically counted using a threshold method and the Analyze Particles tool.

4.7. Endothelial Permeability Assessment

For the endothelial permeability assay, HMECs (10^5 cells/well) were seeded on gelatin-coated Transwell inserts of 24-well plates, in 250 µL complete with Endothelial Cells Growth Medium MV. They were then incubated for 72 h at 37 °C and with 5% CO_2. The cells were incubated in free M200 medium for two hours before stimulation. The cells were then incubated with the different compounds, as indicated in the figure legends. After treatment, the medium from the upper chamber was replaced by 300 µL of M200, containing HRP-coupled streptavidin. The medium of the lower chamber was collected after ten minutes of incubation at 37 °C, and every condition was aliquoted in triplicate. The TMB substrate was added for ten minutes, and an Elisa reader was used to stop the reaction with 2N H_2SO_4 before acquiring 450 nm absorption. Resultant absorption intensity values were normalized over the vehicle control condition. Each experiment was repeated three times.

4.8. Small GTPases Activity Assay

Rac1 activity was measured in protein lysates from HMECs using the commercially available G-LISA assay kit, according to the manufacturer's instructions. Each experiment was repeated three times.

4.9. Statistical Analysis

Statistical analyses were conducted using SPSS v.25 software (IBM Corp., Armonk, NY, USA) and graphs build with GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). All tests were two-sided, with significance set at the 0.05 probability level. Data were expressed as mean ± standard deviation. Means were compared using an unpaired Student's *t*-test. One- or two-way analysis of variance with *f* test was used in comparisons wtih more than two groups. The Bonferroni correction was used for multiple comparisons.

Author Contributions: Conceptualization: M.A., D.C.-Z., C.B. (Christophe Beauloye) and S.H.; formal analysis: J.D.P., C.D., G.E.C. and C.B. (Caroline Bouzin); funding acquisition, D.C.-Z., L.B., C.B. (Christophe Beauloye) and S.H.; investigation, M.A., J.D.P. and C.D.; methodology: G.E.C., C.B. (Caroline Bouzin) and R.Q.; Supervision, D.C.-Z. and S.H.; writing—original draft: M.A. and S.H.; writing—review and editing: D.C.-Z., R.Q., L.B., C.B. (Christophe Beauloye) and S.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACC	Acetyl-CoA carboxylase
AICAr	5-Aminoimidazole-4-carboxamide ribonucleotide
AJs	Adherens junctions
AMPK	AMP-activated protein kinase
Cx43	Connexin 43
eEF2	Eukaryotic elongation factor 2
GIV	G-alpha interacting vesicle associated protein
HMEC	Human dermal microvascular endothelial cell
HSP27	Heat shock protein 27
IEJs	Inter-endothelial junctions

LPS	Lipopolysaccharide
p38 MAPK	p38 mitogen-activated protein kinase
MAPKAPK	Mitogen-activated protein kinase -activated protein kinase
N-Cad	N-cadherin
siRNA	Small interfering RNA
TAB	Transforming growth factor a-activated kinase 1 binding proteins
TJs	Tight junctions
VE-Cad	VE-cadherin
ZO-1	Zonula occludens-1

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Annex 3

Review, European Journal of Physiology, 2020

Glucose transporters in cardiovascular system in health and disease

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INVITED REVIEW

Glucose transporters in cardiovascular system in health and disease

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Abstract

Glucose transporters are essential for the heart to sustain its function. Due to its nature as a high energy-consuming organ, the heart needs to catabolize a huge quantity of metabolic substrates. For optimized energy production, the healthy heart constantly switches between various metabolites in accordance with substrate availability and hormonal status. This metabolic flexibility is essential for the maintenance of cardiac function. Glucose is part of the main substrates catabolized by the heart and its use is fine-tuned via complex molecular mechanisms that include the regulation of the glucose transporters GLUTs, mainly GLUT4 and GLUT1. Besides GLUTs, glucose can also be transported by cotransporters of the sodium-glucose cotransporter (SGLT) (SLC5 gene) family, in which SGLT1 and SMIT1 were shown to be expressed in the heart. This SGLT-mediated uptake does not seem to be directly linked to energy production but is rather associated with intracellular signalling triggering important processes such as the production of reactive oxygen species. Glucose transport is markedly affected in cardiac diseases such as cardiac hypertrophy, diabetic cardiomyopathy and heart failure. These alterations are not only fingerprints of these diseases but are involved in their onset and progression. The present review will depict the importance of glucose transport in healthy and diseased heart, as well as proposed therapies targeting glucose transporters.

Keywords Cardiac metabolism · GLUT · SGLT · SMIT1 · Akt · AMPK · Glucotoxicity · Diabetic cardiomyopathy · Cardiac hypertrophy · Heart failure

Introduction

The heart is an astonishing organ that requires lots of energy to sustain its pumping function. Every single day, the left ventricle of a human heart ejects approximatively 8 tons of blood for 100,000 beatings. Such heavy work needs a tremendous mass of energy. Not less than 6 kg of ATP, the main cellular energy-providing molecule, is utilized by the heart in 24 h, corresponding to 20 times its own weight. This makes the heart the most energy-consuming organ (per unit weight) of the human body [70]. Due to the lack of a significant store of energy (the heart possesses a level of ATP for few seconds and metabolic store, such as glycogen, for several minutes only), the energy should be continuously provided by the catabolism of extracellular substrate, working on a "just-in-time" basis. The production of energy is subject to body needs, hormonal status and nutrient availability, equilibrating energy production to energetic demand. It is currently accepted that the fine-tuned energetic homeostasis is essential for the maintenance of optimal and healthy cardiac function. Inadequacy between energy production and needs, as well as alterations of metabolic regulatory mechanisms, are triggers of cardiac pathologies and heart failure [100, 110, 170]. Glucose is one of the main energy-providing substrates used by the heart. Its transport into the cardiomyocytes, the first step of its catabolism, is highly regulated and is mainly under the control of glucose transporters GLUTs (mainly GLUT4 in the adult heart).

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However, other glucose transporters, like the sodium-dependent glucose cotransporters (SGLTs), are immerging as important regulators of cardiac function. The goal of the present review is to summarize the importance of all these glucose transporters in both healthy and diseased heart.

Principles of cardiac metabolism of the healthy heart

The vast majority of the cardiac energy is produced from mitochondrial oxidation of substrates. Carbohydrates (glucose and its derived metabolites lactate and pyruvate) and fatty acids are the main substrates used by the heart [70]. Fatty acids are the major ATP-generating metabolites under fasting whereas glucose is mainly oxidized during postprandial state. Randle and colleagues were the first to describe this metabolic switch in 1963 [59, 106]. Fatty acid catabolism basically inhibits glucose uptake and oxidation. This intrinsic inhibition of glucose use by fatty acids was largely depicted and involved a mix of allosteric, phosphorylation-mediated and transcriptional regulations [17, 60]. However, whereas the molecular events involved in the inhibition of glucose oxidation (via a complex regulation of pyruvate dehydrogenase activity by allosteric regulation and phosphorylation) and of glycolysis (via citrate-mediated inhibition of 6-phosphophofructo-1-kinase) are clearly identified, the mechanism involved in the fatty acid-induced decrease in glucose uptake [106, 113] is still debated with the implication of insulin resistance [113] but also of acetylation processes [108]. After a meal, the increase in plasma glucose level provokes the secretion of insulin. Insulin is a potent inducer of glucose uptake, glycolysis and glucose oxidation; meanwhile, it blocks fatty acid utilization [17]. Apart from glucose and lipids, the heart, which is an omnivore organ, can also use alternative substrates such as ketone bodies and branched chain amino acids, counteracting the catabolism of main substrates [68, 70]. In relation to the present review, our research group recently characterized the inhibition of cardiac glucose uptake by ketone bodies and leucine [109]. In parallel, the inhibition of glycolysis by ketone esters was demonstrated in humans [29].

Regulation of glucose uptake in the healthy heart

Glucose is a hydrophilic molecule that cannot pass through the cell membrane, which is of lipophilic nature. Cardiac glucose transport is principally under the control of members of the glucose transporter GLUT family. GLUTs are uniporters allowing facilitated diffusion of glucose along its gradient of concentration. Fourteen members of GLUTs are existing but differ in terms of tissue-specific expression and in terms of substrate specificity and affinity [123]. In addition to GLUT1 and GLUT4, which are the predominant GLUT isoforms expressed in the heart and which centralize the majority of the research studies, the expression of GLUT3, GLUT8, GLUT10, GLUT11 and GLUT12 has been also reported in cardiac muscle in a small number of publications [123, 130]. In terms of mRNA level, GLUT8 represents the third most abundant GLUT isoform expressed in the adult rodent heart but is 25 times less expressed than GLUT4 [2]. It has been shown to be insulin sensitive and particularly expressed in the atrial tissue [83]. GLUT3, GLUT10 and GLUT12 are particularly expressed during cardiac development [62, 151, 169].

GLUT 1 is a ubiquitous isoform particularly expressed in the heart during embryonic and early postnatal phases [146]. GLUT1 resides in the sarcolemma and is mainly regulated at the transcriptional level by SP1 (as an activator) and SP3 (as repressor) transcription factors [123]. Its constitutive localization at the plasma membrane explains the high level of basal glucose uptake in embryonic and neonatal cardiomyocytes, where glucose metabolism remains the main source of ATP production. As a consequence, neonatal cardiomyocytes poorly respond to insulin with an increase in glucose transport of approximatively 60–80% (only under supra-physiological concentrations) [82, 152]. It has to be mentioned that immortalized cardiomyocyte-like cell lines, such as HL-1 [53, 120, 124] and H9C2 [6, 23], are also characterized by high GLUT1 expression and poor insulin responsiveness (around 50% increase) even if supra-physiological concentrations of insulin of 100 to 200 nM are used. More recently, it has been shown that the same applies to IPS-derived cardiomyocytes [18], proving that all these cell lines resemble more to neonatal than to adult cardiomyocytes. The importance of GLUT1 during development is highlighted by the fact that its genetic deletion leads to embryonic lethality [55].

Whereas GLUT1 expression is rapidly downregulated after birth, GLUT4 is upregulated to become the main glucose transporter expressed in the adult cardiomyocyte. In unstimulated conditions, GLUT4 mainly resides in specialized intracellular vesicles usually named GLUT4 storage vesicles (GSVs) (Fig. 1). Accordingly, basal glucose uptake is relatively low [16]. The stimulation of glucose transport is mediated by the translocation of GSVs into the sarcolemmal membrane by stimuli such as insulin [17]. The healthy heart is strongly sensitive to the insulin that promotes a rapid and potent stimulation of glucose uptake. A 3- to 8-fold increase in glucose transport is frequently found in adult cardiomyocytes incubated with physiological concentrations (around 3.10^{-9} M) of insulin [16, 121]. The first and main signalling event involved in the translocation of GSVs downstream insulin is driven by the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt) pathway [17]. The heterodimeric PI3K (of class Ia) is composed of a p110 catalytic and

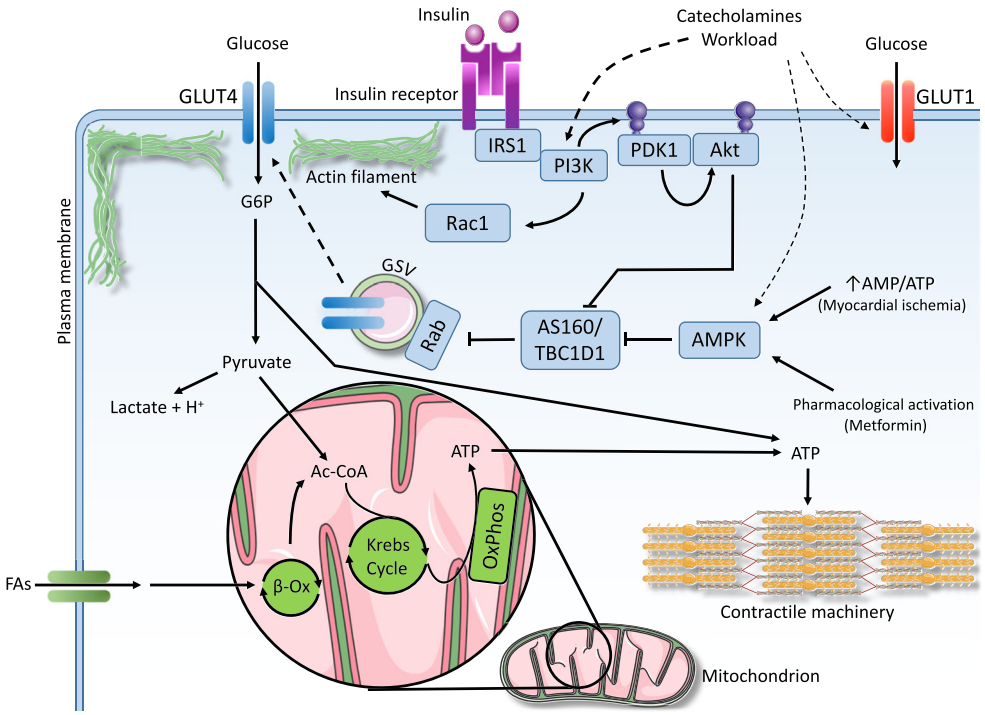

Fig. 1 Molecular mechanisms regulating glucose uptake in the adult cardiomyocyte. See the main text for details. Ac-CoA, acetyl coenzyme A; β-Ox, fatty acid β-oxidation; FAs, fatty acids; G6P, glucose 6-phosphate; OxPhos; oxidative phosphorylation system

a p85 regulatory subunit. This regulatory subunit contains SH2 domains that bind to the insulin receptor substrate 1 (IRS1) on tyrosine residues phosphorylated by the activated insulin receptor (IR) [57]. Once recruited and activated, PI3K catalyses the phosphorylation of the phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) at the 3 positions to produce phosphatidylinositol trisphosphate ($PI3,4,5P_3$) in the plasma membrane. This lipid promotes the recruitment and co-localization of the phosphoinositide-dependent kinase 1 (PDK1) and the protein kinase B (PKB)/Akt at the plasma membrane where PKB/Akt is phosphorylated and activated by PDK1 [17]. The pharmacological inhibition of PI3K by wortmannin is sufficient to block the glucose uptake stimulation generated by insulin [16, 36]. In the same way, the study of genetic mouse models deficient for PDK1 [91] or PKB/Akt (mainly PKBβ/Akt2 isoform) [32] nicely demonstrated the necessity of these elements for the insulin-mediated stimulation of cardiac glucose uptake. One of the numerous substrates of PKB/Akt is the Akt substrate of 160 kDa (AS160). AS160 is a GTPase-activating protein (GAP) of the small G protein Rab that is localized on GSVs. The phosphorylation of AS160 by PKB/Akt induces its inactivation and, in consequence, promotes Rab activation. AS160 is expressed and phosphorylated upon insulin incubation in adult cardiomyocytes [49]. But, whereas its role is essential in skeletal muscle [24, 147], a recent study showed that its deletion does not shrink glucose uptake in the heart [5]. A possible explanation of this apparent conflict is the existence of a AS160 paralog, called TBC1D1. Accordingly, the deletion of TBC1D1 was latterly shown to abrogate the insulin-mediated increase in cardiac glucose transport [13].

If TBC1D1 looks clearly mandatory, another pathway controlled by the small G protein Rac1 plays an important in the insulin-stimulated glucose uptake (Fig. 1). Rac1, via the p21-associated kinase (PAK), ensures the reorganization of cortical actin, allowing GSVs to reach plasma membrane [25, 61, 88, 99]. The molecular mechanism involving Rac1 has been mainly evaluated in skeletal muscle [99]. Rac1 is activated downstream PI3K and looks to parallel PKB/Akt, albeit these two pathways seem intrinsically coordinated [129]. The insulin-mediated Rac1 activation is of great influence on muscle glucose uptake stimulation [128, 129, 141]. Less is known concerning the role of Rac1 on glucose transport in cardiac muscle, which is actually under investigation in our research lab. Among others, our recent data (unpublished observation) revealed that the pharmacological inhibition of Rac1 seriously reduces the stimulation of glucose uptake upon insulin stimulation in primary cultures of adult cardiomyocytes (Fig. 2).

Cathecolamines and increased cardiac workload, being intimately linked to increased metabolism, are other inducers of GLUT (GLUT4 and GLUT1) translocation and glucose uptake [37, 107, 150, 168] (Fig. 1). The stimulation of contraction activates the AMP-activated protein kinase (AMPK) in isolated cardiomyocytes [79]. Similarly, AMPK activity of ex vivo perfused working heart is higher than that of hearts perfused using the unloaded Langendorff model [4]. In the same study, beta-adrenergic agonists were also shown to activate AMPK. Accordingly, it was proposed that AMPK (see also next chapter) is responsible for the stimulation of glucose transport upon increased workload and cathecolamines. However, an acute increase in workload (using the working

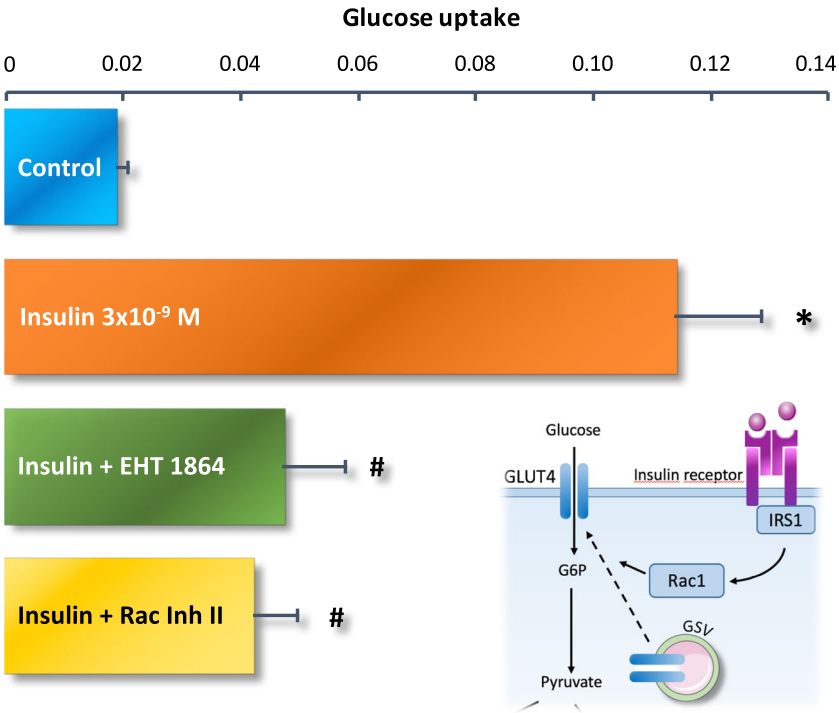

Fig. 2 Inhibition of the insulin-stimulated glucose uptake by Rac1 inhibitors in cultured adult cardiomyocytes. Adult cardiomyocytes were isolated from male Wistar rats and cultured as described [16]. Cultured cardiomyocytes were pre-treated with two different Rac inhibitors (EHT 1864, 10 μM or Rac inhibitor II, 50 μM) for 15 min before insulin treatment (3 nM, 30 min). Glucose uptake was measured using the detritiation rate of [2-^{3}H]glucose as described [16]. The values are in micromoles of glucose transported per milligram of protein per hour. They are the means ± SE of 3 to 9 independent experiments. *p value ≤ 0.05 vs. control cells; #p value ≤ 0.05 vs. insulin treatment

heart model) does not stimulate the AMPK pathway, whereas it efficiently increases glucose metabolism [15, 168]. The exact mechanism involved is not yet fully identified but PI3K looks, at least partially, implicated [15, 34]. Lastly, using the immortalized cardiomyoblast cell line HL-1 as well as cardiomyocytes from genetically deficient mice, it was shown that protein kinase D could be another pathway regulating glucose uptake under electrical stimulation [33].

The PI3K-dependent insulin signalling is fundamental for glucose uptake but also for other metabolic checkpoints and other cellular processes such as protein synthesis, cell growth and anti-apoptotic systems [17]. Subsequently, the genetic invalidation of PI3K, PDK1, PKB/Akt or TBC1D1 leads to obvious prejudicial phenotypes with cardiac disorders [13, 32, 91, 148]. More peculiar, cardiac-specific GLUT4 knockout mice have no deleterious impact on cardiac function under unstressed conditions [1]. These deficient mice are characterized by moderate and physiological hypertrophy with an unexpected increase in basal glucose uptake that could be explained by an upregulation of GLUT1.

Importance of cardiac glucose uptake in myocardial ischaemia

In absence of oxygen, such as during myocardial ischaemia, the anaerobic glycolysis becomes the sole ATP-producing pathway available for the cardiomyocyte. The lower ATP yield provided by glycolysis requires the stimulation of glucose metabolism including glucose uptake. Myocardial ischaemia is associated with the rapid increase in GLUT4 translocation and in glucose transport [89, 159] and is due to the activation of the AMPK [115] (Fig. 1). AMPK is known to be a fuel gauge that rapidly senses energy imbalance [58, 118, 126]. Metabolic stress occurring during myocardial ischaemia results in the increase in the intracellular AMP/ATP ratio. AMPK can bind AMP or ATP in a mutually exclusive manner. Binding of AMP induces AMPK activation via a combination of direct allosteric stimulation and phosphorylation by upstream kinases. The ischaemia-mediated activation of AMPK coordinates a metabolic response with the goal to maintain an appropriate ATP level for cell survival under this energetic stress condition. AMPK is composed of one catalytic (α) and two regulatory (β and γ) subunits. Several isoforms, encoded by different genes, exist for each subunit, two for α (α1 and α2) and β (β1 and β2) subunits and three for the γ subunit (γ1, γ2, γ3). Their expression differs from one organ to another [58]. The AMPKα2 is the most abundant catalytic subunit in cardiomyocytes, whereas AMPKα1 is predominant in endothelial and smooth muscle cells as well as in fibroblasts [14, 58, 94]. Inasmuch as the AMPK substrates seem to be indiscriminately phosphorylated by both isoforms, the respective role of AMPKα1 and AMPKα2 comes from this cell specificity of expression. Among other things, AMPK maintains cellular energy balance by switching on catabolic pathways [14, 58]. Comparably to insulin, AMPK stimulates glucose uptake by promoting the translocation of GLUT4. AS160 has been rapidly shown to be targeted by AMPK in muscle [72, 140] and later in cardiomyocytes [49, 104]. TBC1D1 was also similarly shown to be a substrate

of AMPK [144]. Future research remains necessary to evaluate the respective role of these two paralogs in the ischaemia-mediated increase in glucose uptake. Besides the regulation of GLUT4 translocation to the plasma membrane, it has been also shown that AMPK reduces the endocytotic recycling of GLUT4, contributing to the overall increase of its membrane content [157].

By stimulating glucose uptake, but also increasing anaerobic glycolysis [84], AMPK naturally protects the heart against distress induced by the ischemic episode (Fig. 3). Accordingly, AMPK knock-out or dominant-negative mouse models have impaired glucose handling during myocardial ischaemia accompanied by drastic ATP depletion, rapid and severe ischemic contracture, greater infarct size and delayed or poorer post-ischemic contractile recovery [22, 116, 155, 162]. The same applies to GLUT4 knock-out mouse hearts, which present altered glucose metabolism and accelerated ATP depletion during ischaemia and irreversible cardiac dysfunction after reperfusion [135]. Fascinatingly, the natural AMPK activation occurring during myocardial ischaemia is not maximal and the addition of a pharmacological activator triggers the overactivation of the protein kinase [21, 67, 84, 137]. Such acute pharmacological AMPK overstimulation during the ischemic episode increases glucose uptake and glycolytic rate, preserves mitochondrial function, reduces reactive oxygen species (ROS) production and apoptosis, ameliorates cell survival and finally promotes a better post-ischemic function recovery [21, 67, 137].

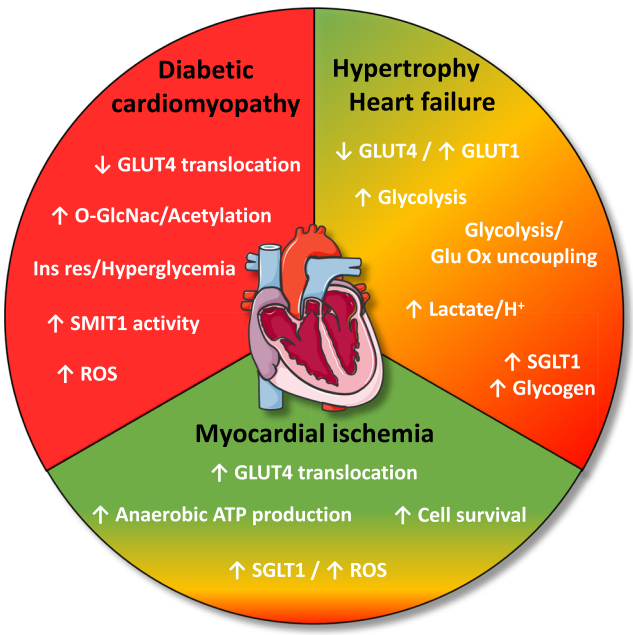

Fig. 3 Modifications of glucose transport in cardiac diseases. Green represents an adaptive response; red corresponds to the maladaptive response. See the main text for details. Glu Ox, glucose oxidation; Ins res; insulin resistance; O-GlcNAc, O-GlcNAcylation

AMPK remains activated and GLUT4 persists at the plasma membrane during a certain period upon reperfusion [54, 73, 159, 161]. However, their impact on glucose uptake looks different depending on the models used. When low-flow ischaemia is performed, a clear stimulation of glucose uptake can be shown at reperfusion [87, 159]. By contrast, no more stimulation can be observed after global no-flow ischaemia suggesting a possible inhibition of GLUT4 activity in the plasma membrane [161].

Metabolic reprogramming in hypertrophied heart and heart failure

The metabolic flexibility that characterized the healthy heart is critically altered in numerous chronic cardiac pathologies. Pathological hypertrophy and its frequent outcome heart failure are associated with a metabolic remodelling where glucose becomes the main and constant ATP-generating substrate [77, 100] (Fig. 3). This alteration is due to a complex reorganization of the gene expression profile that resembles the foetal pattern. Especially, GLUT1 expression is increased, whereas GLUT4 is downregulated [90, 97, 136]. This shift in favour of glucose is an adaptive response of the hypertrophic heart and is linked to the fact that glucose has a higher oxygen efficiency than fatty acids for energy production. Although protective during the first step of hypertrophic development, this seems to be deleterious at the long term. Indeed, a genetic model where the shift of substrate towards glucose is prevented (acetyl-CoA-carboxylase 2 cardio-specific deficient mice) is protected from pathological hypertrophy development [69]. Similarly, the acute overexpression of GLUT1 (mice with cardiomyocyte-specific inducible overexpression of GLUT1) does not preclude contractile dysfunction and amplifies hypertrophy development [98]. It has to be noted that, in contrast, the transgenic mice chronically overexpressing GLUT1 have adapted phenotype, have no basal myocardial dysfunction and are, even, protected from pathological hypertrophy [76]. A similar shift of expression from GLUT4 to GLUT1 occurs in post-infarction heart failure [114]. And, in line with the aforementioned data, the chronic pharmacological blockage of glucose transport does not aggravate and, even, ameliorates the cardiac contractile function 2 weeks after ischaemia/reperfusion [56]. The deleterious impact of a basal increase in glucose transport looks independent of the GLUT transporter isoform overexpressed. Indeed, Wende and coworkers recently characterized a mouse model of inducible and cardio-specific overexpression of GLUT4. These mice have elevated level of basal glucose uptake and suffer from myocardial dysfunction [149].

Several factors can explain the deleterious effect of the increased and uncontrolled glucose uptake and catabolism (Fig. 3). First, glucose can be rerouted to the hexosamine

biosynthesis pathway (HBP). A small proportion of glucose, less than 0.1% for the heart [95], enters into HBP to produce UDP-GlcNAC, the moiety for the particular post-translational modification called O-GlcNAcylation [26, 40, 81]. The hypertrophic and failing hearts are characterized by an elevated level of protein O-GlcNAcylation [48, 80]. The increased O-GlcNAcylation level is not solely a signature of the pathological hypertrophy but can be considered as a major trigger of its development. Indeed, inhibiting O-GlcNAcylation is sufficient to block the development of the disease [39, 48]. This was confirmed more recently by using the cardiac-specific overexpression or deletion of GFAT, the rate-limiting step of HBP [139]. Indeed, the deletion of GFAT reduces cardiac hypertrophy and cardiac dysfunction induced by pressure overload, whereas its overexpression aggravates the development of the disease. Of great medical interest, the metformin-mediated AMPK activation has been shown to inhibit protein O-GlcNAcylation and to prevent cardiac hypertrophy development in rodents [47, 48] (Fig. 3).

A second characteristic of the hypertrophic and failing hearts is the uncoupling of glucose oxidation and glycolysis. Indeed, the increase in glucose uptake is associated with accelerated glycolysis but reduced glucose oxidation [92, 165, 166]. Such uncoupling may result in the production of lactate and protons via the anaerobic glycolysis, reducing contractility and cardiac efficiency [78]. Accordingly, the stimulation of glucose oxidation looks protective against heart failure development [65].

Finally, glucose metabolic derivatives other than lactate have been recently pointed out as putative contributors to the progression of the disease. First, with the help of an in silico metabolic control analysis, Taegtmeyer and colleagues propose that glucose 6-phosphate, which is known to accumulate in hypertrophic hearts, is responsible for the stimulation of the mammalian target of rapamycin (mTOR) pathway involved in the increase in protein synthesis and, so, in biomass [64, 122]. Besides, Tian's lab showed that the increase in glucose consumption contributes to aspartate synthesis, which is a driver of nucleotide synthesis during cardiac hypertrophy [112].

Metabolic inflexibility in favour of fatty acids in the heart of type 2 diabetic patients

Diabetic cardiomyopathy is defined as a myocardial dysfunction that occurs in patients suffering from type 2 diabetes in the absence of hypertension and coronary artery disease [30, 110]. The alterations in cardiac metabolism are among the earliest events induced by diabetes in the heart, preceding myocardial dysfunctions. Among the metabolic events implicated, we can cite a metabolic inflexibility with the constant and almost exclusive use of fatty acids as a source of energy. In line, the diabetic heart does not use glucose anymore even if hyperglycaemia is a hallmark of the diabetic situation. Strikingly, glucose uptake is drastically decreased in both diabetic animal models [19, 20, 110] and in diabetic patients [28, 93] (Fig. 3). The decline in glucose uptake is mainly explained by the insulin-resistant state which is characteristic of the diabetic heart [17]. Here, it is important for the reader to discriminate the notion of systemic and local insulin resistance. Diabetes is characterized by a systemic insulin resistance where plasmatic glycaemia is poorly controlled. This systemic insulin resistance certainly contributes to the local cardiac insulin resistance [7]. However, local cardiac insulin resistance could appear rapidly and even before other systemic events in certain diabetic models [96]. This cardiac insulin resistance, which is characterized by an altered PI3K/PKB/Akt stimulation and a reduction of the translocation of GLUT4 to the plasma membrane, is crucial for the development of diabetic cardiomyopathy [7, 17, 20, 51, 110]. The fact that diabetic cardiomyopathy development is amplified in PI3K-deficient mice is an illustration of this concept [31]. In consequence, therapies normalizing cardiac insulin sensitivity in favour of glucose utilization were proposed. In line, PI3K-targeted gene therapy protects the diabetic heart from adverse remodelling [102]. Targeting AMPK to promote glucose use looks also promising [14, 58]. First, AMPK, by acting on glucose uptake independently of PI3K/PKB/Akt, can bypass insulin resistance. Second, as previously mentioned, AMPK inhibits protein O-GlcNAcylation which is another deleterious feature of diabetes [35, 47, 48, 81]. Third, AMPK activation by pharmacological compounds such as the antidiabetic drug metformin has been shown to improve cardiac insulin sensitivity by enhancing PKB/Akt signalling [16]. The insulin sensitization capacity of AMPK on PKB/Akt is explained by its counteracting effect on the insulin-negative feedback loop involving the mTOR/p70 ribosomal S6 protein kinase pathway, which is enhanced in a diabetic state [49]. However, an additional mechanism, which still needs to be identified, looks required to explain the enhancement of insulin response in terms of glucose transport [49]. The AMPK-mediated RAC activation, firstly identified in muscle cells [74], could be this missing link. AMPK-related therapy has been already successfully used in a rodent model of diabetes [42, 154].

It should be borne in mind that insulin sensitization and restoration of controlled metabolic flexibility must stay the objectives in diabetic cardiomyopathy treatment. It should be preferred to the simple and uncontrolled increase of glucose uptake. By contrast to that occurred in heart failure, chronic increased glucose uptake by GLUT1 overexpression favours cardiac dysfunction in diet-induced obesity although it reduces high fatty acid oxidation [156]. The increase of glucose delivery that happens in the inducible and cardio-specific overexpressed GLUT4 mouse model does not protect from diabetic

cardiomyopathy development [149]. Accordingly, the local insulin-resistant state could also be viewed as a protective mechanism against energy substrate overload observed in the diabetic state, limiting its cellular uptake and accumulation. This hypothesis has been recently nicely reviewed [131, 132]. It has to be noticed that several insulin-sensitizing agents (including those acting through AMPK) also block energy substrate overload-induced toxicity. As an example, we demonstrated that AMPK inhibits hyperglycaemia-induced ROS production by blocking the activation of the NADPH oxidase 2 (NOX2) [10].

Another feature of the diabetic heart is the increase in protein acetylation [44, 108]. Besides histones, mitochondrial proteins have been rapidly shown to be acetylated. The metabolic overfueling occurring in diabetes increases the production of mitochondrial acetyl-CoA, the substrate of the acetyl transferases responsible for this post-translational modification. The acetylation of mitochondrial proteins essentially activates fatty acid oxidation, whereas it blocks glucose oxidation [44]. We recently established that acetylation also negatively regulates cardiac glucose uptake [108, 109]. Metabolic overfueling of cardiomyocytes or perfused hearts rapidly downregulates GLUT4 translocation to the plasma membrane and this inhibition can be reverted by treatment with inhibitors of lysine acetyl transferases [109]. The precise identification of the acetylated protein involved in this effect still needs to be formally identified [108]. No matter what are the acetylation processes mainly involved in the development of cardiac metabolic inflexibility, it is clear that increasing protein acetylation exacerbates the development of the disease. For example, the mouse model deficient for the main mitochondrial lysine deacetylase Sirtuin 3 suffers from exaggerated diabetic cardiomyopathy [160]. Inversely, the induction of protein deacetylation via the stimulation of Sirtuin 3/1 protects the diabetic heart [9, 127].

Glucose transport through SGLTs in a normal and diseased heart

Beyond GLUT transporters, a second family of glucose transporters, namely SGLTs, has been described (Fig. 4). These transporters rely on the sodium gradient to bring sugar into the cells [153]. The SGLT family comprises seven isoforms including SGLT1, SGLT2, SGLT3, SGLT4, SGLT5, SGLT6 (SMIT2) and SMIT1 [153]. These isoforms differ regarding their substrate specificity, but they all transport glucose with different affinities, SGLT1 having the highest affinity for glucose (Km ~ 0.5 mM). SGLT1 was the first isoform that has been reported to be expressed in the heart [12] (Fig. 4). Under normal conditions, cardiac SGLT1 does not play a crucial role as far as SGLT1 knock-out mice exhibit a normal cardiac phenotype [143]. Moreover, although initial observations using Phlorizin suggested that SGLT1 could contribute to the overall glucose uptake under basal or insulin-stimulated state, we (personal communication) and others recently ruled out this hypothesis using a genetic model of SGLT1 downregulation or deletion [75]. Therefore, the role of SGLT1, if any, in normal conditions, remains to be determined. It is reinforced by the fact that an active secondary transport is not necessary to fuel the heart with glucose because of the highly favourable glucose gradient across the plasma membrane. One may wonder whether the transmembranal glucose gradient is not the driving force for ions transport. It has to be noticed that recent findings showed that SGLT1 protein is

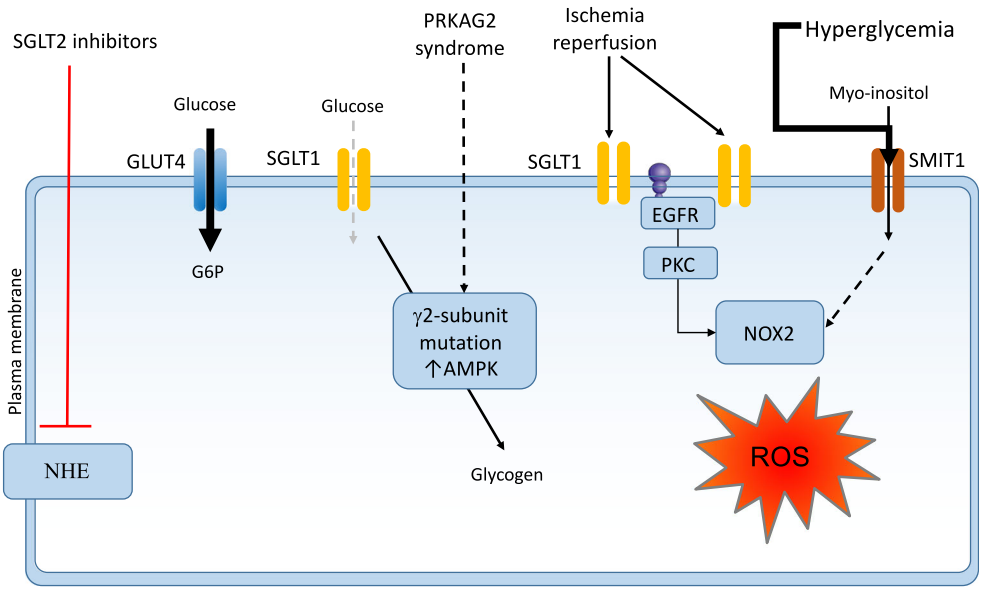

Fig. 4 Role of sodium-glucose cotransporters under normal and diseased conditions. Among SGLT isoforms, SGLT1 and SMIT1 are expressed in cardiomyocytes. See the main text for more details. EGFR, epidermal growth factor receptor; G6P, glucose 6-phosphate; NOX2, NADPH oxidase 2; NHE, cardiac Na^+/H^+ exchanger; PKC, protein kinase C

actually rather localized in the heart capillaries than in myocytes sarcolemma [145].

The role of cardiac SGLT1 appears clearer under pathological conditions (Figs. 3 and 4). It has been advocated in the pathophysiology of cardiomyopathy linked to the PRKAG2 gene (expressing AMPK regulatory subunit) mutation [105]. The "coupling" between SGLT1 and a chronic increase in AMPK activity mediates the increase in glycogen storage induced by the γ2 subunit mutation of AMPK. This metabolic hypertrophic phenotype could be blunted by SGLT1 knockdown, through a reduction in cardiac glycogen content and an improvement in cardiac function [105]. The absence of SGLT1 has been shown to counteract transaortic constriction-induced cardiac hypertrophy, resulting in well-preserved left ventricular function under pressure overload [85]. Finally, SGLT1 expression is increased following myocardial infarction and favours cardiac ischaemia-reperfusion injury. SGLT1 downregulation decreases infarct size, myocardial dysfunction and oxidative stress. The signalling pathway downstream of SGLT1 involves protein kinase C activation and subsequently p47phox phosphorylation, a subunit of NOX2, leading to increased oxidative stress [75]. Basically, glucose transport through SGLT1 activates signalling pathways in a diseased heart. However, conflicting results have been observed concerning the impact of pharmacological inhibition of SGLT1 during ischaemia reperfusion. KGA-2727, a selective SGLT1 inhibitor, reduces post-myocardial infarction adverse left ventricular remodelling in mice [119] while T-1095, a dual SGLT1-2 inhibitor, exacerbates cardiac dysfunction in the post-myocardial infarction setting (after ligation of the left anterior descending coronary artery) [27].

SGLT2 is a new therapeutic target in the treatment of type 2 diabetes and heart failure. Indeed, SGLT2 inhibitors, including canagliflozin, empagliflozin or dapagliflozin, reduce cardiovascular death and heart failure hospitalizations of type 2 diabetic patients either in a primary or secondary prevention context [164]. The protection conferred by SGLT2 inhibition has also been demonstrated in non-diabetic patients. Indeed, dapagliflozin reduces the risk of worsening heart failure or death from cardiovascular causes in patients with heart failure and reduced ejection fraction (HFrEF) regardless of the presence or absence of diabetes [86]. However, our group confirmed that SGLT2 is expressed neither in the cardiac tissue nor in isolated cardiomyocytes, excluding a direct action on SGLT2 inhibitors into the cardiomyocytes [143]. The cardiovascular protection conferred by SGLT2 inhibitors has to be attributed to an extracardiac action (i.e. diuretic or natriuretic action, increased ketone concentrations, …) or off-target effects. Notably, SGLT2 inhibitor treatment of isolated cardiomyocytes and perfused hearts exerts a direct cardiac effect by lowering myocardial Na^+ cytoplasmic concentration through impairment of cardiac Na^+/H^+ exchanger flux and could mitigate elevated Na^+ that is known to occur in heart failure and diabetes [8, 142]. Many other protective mechanisms have been argued and have been recently summarized [163]. However, the key element explaining the cardioprotective effect of SGLT2 either cardiac or extracardiac have to be better defined.

In addition to SGLT1, we have also demonstrated that SMIT1 is, at least equally, expressed in the heart tissue and in cardiomyocytes form mice, rats and humans [143] (Fig. 4). SMIT1 has a high affinity for myo-inositol and a poor affinity for glucose with Km above 50 mM. Myo-inositol is an important precursor of inositol phosphates and phospholipids that are central in the membrane and cell signalling. Once again, the role of SMIT1 at the basal state remains to be determined as far as no cardiac abnormalities could be detected in SMIT1 knock-out mice [143]. By contrast, under hyperglycemic conditions, our group formally demonstrated that SMIT1 could sense hyperglycaemia, triggering a signalling pathway leading to NOX2 activation and increased ROS production [143]. This could participate in the redox signalling in the hyperglycemic heart and contribute to the pathophysiology of diabetic cardiomyopathy (Fig. 3). No data exist concerning the role of SMIT1 in other cardiac diseases.

Glucose transport in non-myocyte cells

A high number of non-myocyte cells are present in the human and rodent heart, among which, endothelial cells (ECs) and cardiac fibroblasts (CFs) [11]. CFs are usually defined as spindle/stellate shape located between cardiomyocytes and near capillaries. They are the main producer of extracellular matrix proteins acting as scaffolding support for the myocardial tissue, and as a platform for para- and autocrine signals which participate in heart homeostasis [133]. Most cardiomyopathies involve CF activation, which refers to the transition from a resting phenotype to activated cells characterized by heightened proliferation, migration, contractility and extracellular matrix production, and contributing to fibrosis [43, 71]. The most widely used markers for identifying activated fibroblasts are α-smooth muscle actin (α-SMA) and collagen expression [43]. It is well established that adult CFs express more GLUT1 than GLUT4 [50] indicating that their glucose uptake is mostly insulin independent. Interestingly, neonatal CFs are insulin-sensitive and express GLUT4 [103]. In line, their metabolic substrate preferences are very different with neonatal CFs oxidizing glucose and lactate, while adult CFs primarily use fatty acids [111]. These differences may explain conflicting results regarding the impact of hyperglycaemia on CF activation. Indeed, while several studies in neonatal CFs have concluded that hyperglycemic conditions enhanced collagen synthesis and α-SMA expression [41, 125, 167], it was recently demonstrated that hyperglycaemia does not alter

glucose transport or activation status of healthy adult CFs [50]. This resistance to hyperglycaemia is an interesting characteristic of adult CFs that needs further investigation. Importantly, these in vitro studies suggest that fibrosis in diabetic cardiomyopathy does not result from direct effects of hyperglycaemia on CFs but likely involves other contributors such inflammatory factors and/or oxidative stress [117]. Nevertheless, it cannot be excluded that acute treatment with high-glucose concentration is not enough to promote fibrotic effects, whereas chronic exposure might stimulate CF activation.

ECs line the inner surface of the entire cardiovascular system, where they act as communicators between blood and the underlying tissue. Their capacity to transport glucose is highly dependent on their location along the vascular tree. GLUT1 is recognized as the most abundant isoform expressed in ECs composing coronary arteries and aorta [45, 158, 101]. Endothelial GLUT1 is expressed at the basolateral pole of cells and is characterized by better extrusion than intake properties, thereby passively diffusing glucose molecules from the cytosol to the adjacent tissue [46]. In addition, the expression of SGLT1 at the luminal pole of ECs, where it allows active glucose entry in the cytosol, has been described in several reports [134, 63, 52]. Recently, the presence of SGLT2 was also demonstrated in the mouse aortic endothelium [38]. The endothelial expression of these glucose transporters is affected in pathological conditions. Indeed, prolonged hyperglycaemia causes GLUT1 downregulation in ECs [3, 138]. More recently, lower GLUT1 expression was shown to be associated with an upregulation of SGLT1 and SGLT2 in porcine coronary artery rings and cultured ECs submitted to high glucose [66]. In addition, the authors highlighted that SGLT1/2 dual inhibition by empagliflozin significantly reduced the endothelial dysfunction induced by hyperglycaemia, supporting a key role of SGLT1/2 in EC glucotoxicity [66].

Conclusion

As we depicted here, the vision of cardiac glucose uptake cannot be reduced to the action of GLUT4 in the adult cardiomyocyte to load glycolysis and glucose oxidation for the production of ATP in the healthy heart. Behind this simplistic view, glucose uptake and its main regulators, GLUTs and SGLTs, are involved in numerous metabolic and signalling events not only in cardiomyocytes but also in other cardiac cell types. These events play important roles in the maintenance of cardiac function but also in the development of cardiovascular diseases. Novel therapies targeting these molecular mechanisms can be considered as very promising.

Authors' contributions J.A. and E.R. performed the RAC experiments. M.A. and J.C. collaborated in writing the chapter dedicated to non-myocyte cells. L.B., S.H. and C.B. wrote the manuscript with input from all authors.

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