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EFFECTS OF SERINE PROTEASES ON ENDOTHELIAL PERMEABILITY

PhD thesis

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LIST OF ABBREVIATIONS

AFM	atomic force microscopy
AJ	adherent junction
B2R	B2 bradykinin receptor
BK	bradykinin
BM	basal membrane
cAMP	cyclic adenosine monophosphate
DMR	dynamic mass redistribution
EC	endothelial cell
EnLaB	endothelial label-free biochip
GPCR	G-protein coupled receptor
HAE	hereditary angioedema
HMWK	high molecular weight kininogen
HUVEC	human umbilical vein endothelial cell
IL	interleukin
LPS	lipopolysaccharide
MASP	mannan-binding lectin-associated serine protease
MBL	mannan-binding lectin
ML-7	MLCK inhibitor
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
N-cadherin	neural cadherin
NF-κB	nuclear factor κB
OWLS	optical waveguide lightmode spectroscopy
p-	plasma derived
p38-MAPK	p38 mitogen-activated protein kinase
PAR	protease activated receptor
PECAM-1	platelet endothelial cell adhesion molecule 1
PKC	protein kinase C
PRM	pattern recognition molecule
QCM	quartz crystal microbalance

r-	recombinant
Rac1	Ras-related C3 botulinum toxin substrate 1
Rap1	Ras-related protein 1
rhoA	Ras Homology A
ROCK	Rho kinase
RWG	resonance waveguide grating
SGMI-1	*Schistocerca gregaria* protease inhibitor based MASP inhibitor-1
TAFI	thrombin activatable fibrinolysis inhibitor
TEER	transendothelial electrical resistance
TJ	tight junction
TNF-α	tumor necrosis factor α
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
Y-27632	ROCK inhibitor

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1. INTRODUCTION

The innermost layer of vessel walls is constituted by endothelial cells (ECs), which play key roles in several physiological processes, but most importantly, they control both the quality and the quantity of cells and molecules that can cross the endothelial barrier and get to other tissues by a process called permeability regulation. ECs are present everywhere in the body in large quantities and are affected by several diseases (e.g., atherosclerosis, edematous conditions, sepsis, cancer, etc.), moreover, because of their very special position, ECs are also immediately affected by blood cells and soluble molecules of blood plasma. Therefore, it is crucial to understand the mechanisms by which active plasma components (either naturally occurring or therapeutically administered) can modify their behavior.

Enzymes of hemostasis and innate immunity are indispensable for maintaining the integrity of the body. In the operation of these vital processes, soluble serine proteases play a crucial an indispensable role, activating each other in a cascade-like manner. The activation of plasma serine protease cascades can result in various outcomes, such as the formation or the resolution of blood clots, opsonization, damage and clearance of pathogens and apoptotic cells, as well as the regulation of inflammation. One of the best-known plasma serine proteases, thrombin has been long acknowledged to directly activate ECs and its background (the cleavage of EC surface protease-activated receptors (PARs) and subsequent cell signaling and cytoskeletal changes), as well as its consequence (an increased endothelial permeability) are well understood. However, this is not the case with other serine proteases of blood plasma, which are also in direct physical contact with ECs, as the EC activating potency of these proteases has not yet been investigated. Therefore, during my doctoral research, I have studied the effects of several plasma serine proteases on ECs using different approaches.

1.1 Endothelial cell characteristics

ECs are squamous cells of mesodermal origin, typically organized into a thin monolayer that constitutes the inner lining of our vessel walls. These cells are laying on a basal membrane (BM) synthesized by themselves, which serves as an adhesive substrate anchoring EC adhesion molecules. Adjacent ECs are also interconnected by different

adhesion molecules allowing them to form a barrier between blood plasma and the surrounding tissues.

Beyond this classical barrier function, ECs have a great variety of physiological roles – they produce vasoconstrictor and vasodilator molecules to regulate blood pressure, anticoagulants and procoagulants to maintain hemostasis and they also express inter- and intracellular structures to actively control transport processes.

1.1.1 Endothelial adhesion molecules

Endothelial adhesion molecules are transmembrane proteins with an intracellular part connected to the cytoskeletal elements via junction associated proteins and an extracellular part bound to the adhesion molecules of adjacent cells or to the underlying BM.

The connections between ECs and the basal membrane serve to anchor these cells. These interactions are concentrated in focal adhesion complexes, where the various types of integrin heterodimers bind laminin, collagen IV, heparan-sulfate, fibronectin and other proteoglycans of the BM [1, 2].

Adhesion molecules of cell-to-cell connections can also be organized into well-defined structures, such as the tight junctions (TJ) and the adherent junctions (AJ). TJs – as their name suggests - are responsible for the tight sealing of adjacent cells as they create a sealing ring, that separates the apical and the basolateral sides.

The intracellular part of TJs consist of claudins, occludin and JAM-A that are connected to the actin cytoskeleton via intracellular adapter proteins, such as zonula occludens 1 and 2 (ZO-1, ZO-2), cingulin and rab13 [3].

An important prerequisite for successful TJ formation is the existence of AJs. In contrast to TJs, the most important role of AJs is to hold the cells together rather than completely preventing paracellular transport. The intracellular side of AJs is made up of cadherins - mainly vascular endothelial (VE-) and neural (N-) cadherin - that are also connected to cytoskeletal actin with the mediation of cytoplasmic adapter proteins p120, β-catenin and plakoglobin.

ECs also express adhesion molecules that are independent of the multiprotein junctional complexes. One such molecule is platelet endothelial cell adhesion molecule 1 (PECAM-1), distributed homogenously on the cell surface [4].

1.1.2 Endothelial protease activated receptors

Like most cell types, ECs also express protease activated receptors (PARs), a special type of membrane receptors belonging to the most populous receptor family of the G-protein coupled receptors (GPCRs). Similarly to other GPCRs, PARs also show the classical, seven-transmembrane-domain structure, but they also have a very unique feature, a so-called "tethered", endogenous ligand, that is part of the receptor itself. As their name suggests, PAR activation is triggered by proteases, which cleave the peptide chain of the receptor, close to its N-terminal. The new N-terminal then acts as a tethered ligand by leaning back and binding to the second extracellular loop of the receptor, initiating conformational changes and signal transduction. Nowadays, the so-called biased signaling of PARs is a subject of intensive research. This signaling bias is common in the case of PARs and means that one particular type of these receptors can induce different cellular responses depending on where exactly it was cleaved by the activating protease, what other type of PARs it is paired to or what signaling molecules are surrounding it intracellularly [5].

Four types of PARs have been identified to date (PAR1-4) with different functions and tissue expression pattern, however, all four types were found to be expressed in ECs [6, 7]. PAR-1 is the most well-known among all PARs. Although cleaved by several proteases, the most important role of PAR-1 described to date is the mediation of the cellular effects of thrombin (that is why PAR-1 is also called thrombin receptor). By the cleavage of PAR-1, thrombin can induce platelet aggregation, vascular smooth muscle cell proliferation and EC activation, the latter leading to endothelial barrier disruption and increased vascular permeability [8].

The role of the other EC-expressed PARs in permeability is less well-characterized, although our group has previously shown that PAR-4 can be cleaved by mannan-binding lectin-associated serine protease 1 (MASP-1), which leads to intracellular Ca^{2+}

mobilization, nuclear translocation of nuclear factor κB (NF-κB) and activation of p38-mitogen-activated protein kinase (p38-MAPK) signaling [9].

1.1.3 Endothelial permeability – underlying molecular mechanisms and regulation

Endothelial permeability means the capacity of a vessel to allow for the passage of a wide range of circulating molecules. This transport can occur via two routes, through the ECs or between them; called transcellular and paracellular transport, respectively. To our current knowledge, the transcellular route is responsible for just a small fraction of the total mass transport and is significant primarily in special, highly restrictive endothelial barriers, like in the case of the microcapillaries of the blood-brain barrier. In most tissues, however, the endothelium can be considered as a size-selective molecular sieve, which allows for the free diffusion of particles ≤3 nm between ECs, while limits the transport of particles sized 3-11,5 nm (for example IgG) to the transcellular route via vesicle trafficking. However, the transport of particles bigger than 11,5 nm is not possible in the case of an intact endothelium [10].

Besides the concentration gradient, the intensity of paracellular transport also greatly depends on the stability of cell-to-cell junctions. Although a well-organized network of TJs can restrain even the passing of water molecules, for example in the case of brain microvasculature, the apical and basolateral space are not so strongly isolated in the vessels of most tissues (for example the umbilical vein). There are places of extreme permeability too, as in the case of the liver, where endothelial junctions are almost completely missing, creating a sinusoidal endothelium suitable for the extremely intensive transport processes [11].

Although confined within certain, tissue-specific limits, endothelial permeability is dynamically changing in response to different stimuli, therefore the magnitude of transport processes can quickly become many times higher than the basal state, even in the case of well-organized barriers.

Under resting conditions, when the surrounding tissues perform their physiologically assigned tasks, the main role of most endothelia is to create a barrier between the circulating bodily fluids and the tissues. However, there are several – either physiological or pathological – stimuli that can temporarily disrupt the endothelial barrier and increase permeability by loosening cell-to-cell junctions and simultaneously triggering EC

contraction. This way, ECs draw apart from each other, creating paracellular gaps and allowing a more intensive paracellular transport [10]. Although the outcome may seem quite simple, a very well-coordinated cooperation of several complex signaling pathways is needed to increase permeability as we intended to depict it in our recent review [12]. Most of the well-known permeability increasing agonists – such as histamine, bradykinin (BK), vascular endothelial growth factor (VEGF) or thrombin stimulate their corresponding receptor in the plasma membrane of ECs, which initiates intracellular signaling. Thrombin is special among the above listed permeability increasing agonists as it not only binds to its cell surface PARs, but – being a serine protease – activates them via proteolytic cleavage. Activation of PAR1 (the primary thrombin receptor on ECs) provokes conformational changes leading to the further activation of signal transducing molecules in the cytoplasm (for a schematic representation of thrombin induced permeability signaling, see **Fig. 1**). A key step of cell activation and early intracellular signaling is the biphasic elevation of intracellular free Ca^{2+} concentration [13]. Free cytosolic Ca^{2+} can then activate – among others – myosin light-chain kinase (MLCK) by binding to its associated regulatory molecule, calmodulin. Active MLCK phosphorylates the light chain of non-muscle type myosin at two sites (Thr18 and Ser19), which causes a conformational change in the myosin head, that increases its ATP-ase activity in the presence of actin, leading to enhanced actomyosin contractility [14]. However, newer results with a specific MLCK inhibitor (ML-7) indicate that MLCK does not play a major role in thrombin induced endothelial hyperpermeability [15].

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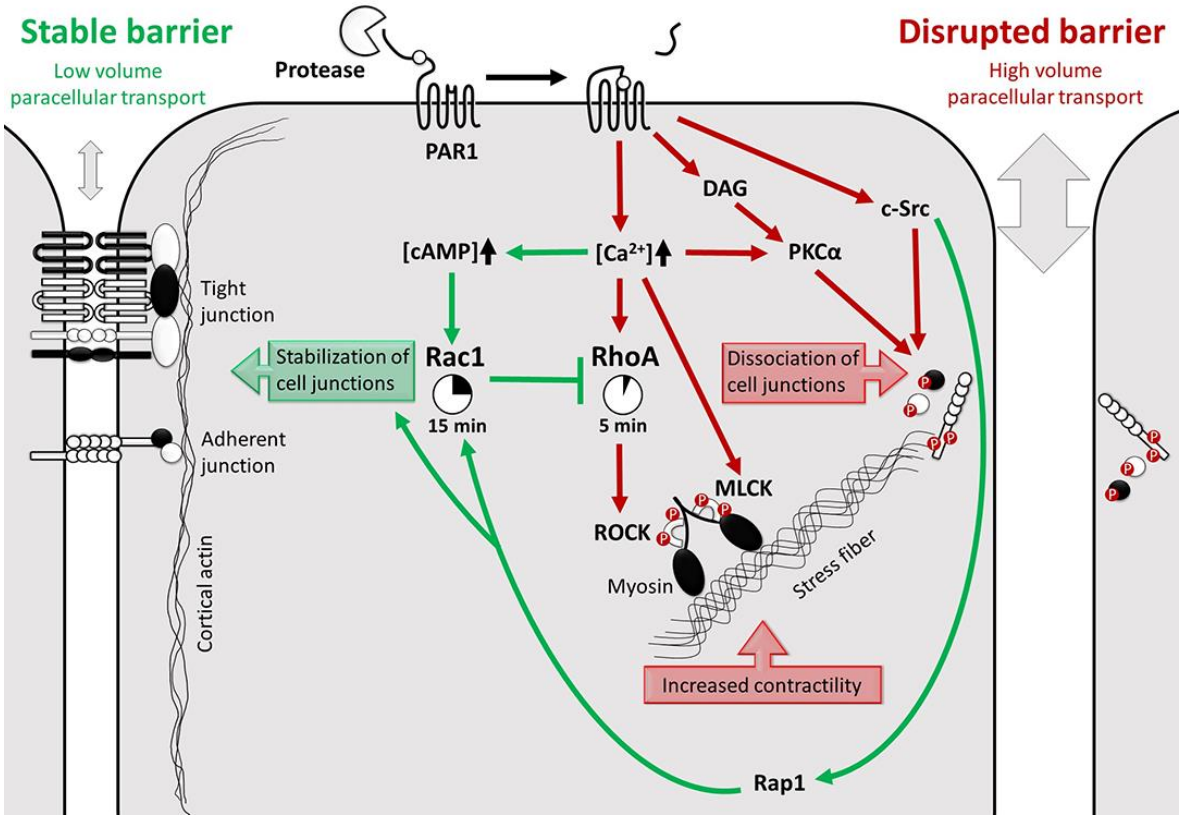

Figure 1. The schematic representation of the key steps of PAR-1 mediated endothelial permeability. Protease mediated cleavage causes the activation of cell surface PAR-1, initiating intracellular signaling in ECs. Key signaling events leading to barrier protection (stabilization of cell junction and the cortical actin network), are emphasized by green color, whereas barrier disruptive signaling leading to hyperpermeability (phosphorylation and dissociation of cell junction components, phosphorylation of myosin light chain and formation of actin stress fibers) are indicated by red color. (This figure is a modified version of Fig. 2 from our review [12].)

Besides MLC activation, EC contraction requires the remodeling of the entire cytoskeleton. The cortical actin ring is intended to stabilize the cell shape and keep the adhesion molecules in place under the plasma membrane. During the contractile response, this cortical actin structure reorganizes to form thick, myosin-associated filamental actin bundles crossing the cytoplasm, called stress fibers, which can function as a framework for myosin to exert centripetal force. One key step in this process is the activation of small GTP-ase Ras Homology A (RhoA), a process mediated by RhoGEF and Ca^{2+} dependent protein kinase C α (PKC-α) activation [3]. RhoA becomes activated within 5 minutes after PAR-1 activation [16] and helps maintain the phosphorylated state of MLC by activating Rho-kinase (ROCK) and at the same time, inhibiting MLC phosphatase (MLCP), thereby facilitating actomyosin interaction, stress fiber formation and actin polymerization [17]. Along the actin stress fibers, the centripetal force - exerted by

actomyosin contraction – pulls the cytoskeleton-associated adhesion molecules (including AJ protein VE-cadherin that is of key importance in cell-layer cohesion) towards the center of the cell, resulting in cell contraction and destabilization of homotypical junctions between adjacent cells [13]. In AJs, VE-cadherin and its associated adaptors are affected not only by the centripetal force, but in parallel, they also undergo phosphorylation by kinases (e.g. PKC-α and c-Src), which causes their dissociation and endocytosis [10].

Despite being a strong inducer of endothelial hyperpermeability, thrombin also initiates a negative-feedback mechanism to facilitate barrier restabilization [15, 16]. The main controller of junctional stabilization is the small G-protein Ras-related C3 botulinum toxin substrate1 (Rac1), which serves as a hub for different signaling pathways to inhibit RhoA-mediated barrier disruption [18]. Upon thrombin stimulation of endothelial cells, Rac1 becomes activated at 15 minutes, which can happen either by cyclic adenosine monophosphate (cAMP) signaling [15] or the Ras-related protein 1 (Rap1) axis [16]. The Rac1-mediated RhoA inhibition and junctional restabilization ensures a fast barrier recovery within 1 hour.

The pathological magnitude or duration of increased permeability plays an important role in the pathomechanism of several edematous conditions (e.g. sepsis or hereditary angioedema), therefore, the reliable, high-throughput and simple measurement of endothelial permeability is of great importance to understand these diseases.

1.2 Plasma serine proteases

Serine proteases are enzymes containing a serine amino acid residue as part of the so-called catalytic triad in their active site that mediates the endopeptidase activity of these enzymes. Besides the digestive system, serine proteases also have important roles in blood plasma, where they operate vital processes, such as hemostasis and innate immunity.

1.2.1 General characteristics of plasma cascade systems and their physiological roles

Soluble serine proteases circulate in the vessels mostly as inactive zymogens, but they can become instantly activated in a cascade-like manner when danger-associated stimuli

occur (such as vessel injury or pathogen invasion). These cascade systems cannot be separated neither spatially, nor functionally as their enzymes affect each other in several ways and share common regulators, therefore, growing evidence suggest that we should talk about a single, but very intricate network, however, due to historical and didactic reasons, the common practice is to discuss this enormous and diversified system decomposed into subsystems for the better understanding. According to their function, we usually distinguish four subsystems, such as i) the coagulation, ii) the fibrinolytic, iii) the kinin-kallikrein and iv) the complement system [19].

The coagulation cascade is meant to protect the body from losing its bodily fluids during vessel injuries and also to prevent the dissemination of invading microbes by the occlusion of small vessels. The fibrinolytic system is intended to break down the clots produced by the coagulation cascade in order to restore blood flow during tissue regeneration. The kinin-kallikrein system's main function is the production of bradykinin, a small peptide with strong proinflammatory and edematogenic effects [20]. Interestingly, bradykinin also mediates the perception of inflammatory pain by the acute excitation and electrical sensitization of nociceptor sensory neurons [21] serving as an adaptive mechanism for the individual to spare the inflamed tissues, moreover, it regulates neuroinflammation, neuroprotection and neuroregeneration [22]. The complement system is the humoral arm of innate immunity, responsible for the immediate, but non-specific elimination of pathogens upon entrance. It can be activated via three routes: the classical pathway is initiated by IgM or IgG containing immunocomplexes, the lectin pathway activates on pathogen-associated sugar motifs, while alternative pathway activation occurs mainly on pathogen-associated surfaces lacking sialic acid.

1.2.2 A promiscuous plasma serine protease: MASP-1

Mannan-binding lectin-associated serine protease 1 (MASP-1) is a key enzyme of the lectin pathway of complement, being also the most abundant among MASPs with a plasma concentration of 140 nM. As its name suggests, MASP-1 is associated to pattern recognition molecules (PRMs) of the lectin pathway – such as mannan-binding lectin (MBL), collectin-10, collectin-11, ficolin-1, ficolin-2, and ficolin-3 – together with the other MASPs and the non-catalytic regulatory molecules, MBL-associated protease of 19 kDA (MAp19) and MAp44.

The domain structure of MASP-1 is identical to that of the other serine proteases of the lectin pathway, namely MASP-2 and MASP-3; they contain 6 domains (CUB-1, EGF, CUB-2, CCP-1, CCP-2, SP), with the function-determining serine protease domain being on the C-terminal, while Ca^{2+}- and PRM-binding ability is limited to the three N-terminal domains [23]. As it is quite challenging to purify MASPs and some other plasma serine proteases from human plasma in sufficient quantity and quality, our collaborators have managed to express the recombinant (r) forms of MASP-1, MASP-2, MASP-3, C1s and C1r in E. coli and to effectively purify them by methods described earlier [24, 25]. These recombinant enzymes contain only the three C-terminal domains (CCP1, CCP2 and SP), but as the other three N-terminal domains have no role in the enzymatic activity (it is exclusively limited to the SP domain), the physiological function of the proteases can still be effectively studied using the recombinant forms. This is supported by *in vitro* experimental evidence: our earlier study showed that recombinant MASP-1 (rMASP-1) had the same effects on ECs as the plasma purified, full-length form [26].

Being the oldest discovered enzyme of the lectin pathway [27], MASP-1 has been studied for nearly three decades. After overcoming the experimental artefacts due to the impurity of early MASP-1 preparations, it soon became evident that MASP-1 cannot generate the C3 convertase itself (it is unable to cleave complement C4), although it is far from being just an auxiliary enzyme in lectin pathway activation. It is the exclusive physiological activator of MASP-2 and is also responsible for 60% of C2 cleavage [28]. MASP-1 activated MASP-2 becomes able to cleave C2 and also C4, thereby generating the lectin pathway C3 convertase [29, 30].

Having a wide substrate-binding cleft that can encompass parts of many types of molecules, the role of MASP-1 is not limited to just lectin pathway activation. Substrates for MASP-1 identified to date are fibrinogen, factor XIII, thrombin activatable fibrinolysis inhibitor (TAFI), high molecular weight kininogen (HMWK), prothrombin and PARs [30]. The aforementioned list suggests that beyond the complement system, MASP-1 can have important roles in the other three plasma cascades, and indeed, the effects of MASP-1 on blood coagulation and fibrinolysis has been an intensively researched field in the recent years [31-34].

Our group has been studying MASP-1 for over a decade now. My colleagues showed that MASP-1 can activate ECs *in vitro* by cleaving PARs [9, 26], which increased the

production of cytokines interleukin-6 and 8 (IL-6, IL-8) and lead to the effective recruitment and transmigration of neutrophil granulocytes [35]. We later found that this process is further facilitated by MASP-1 as it also up-regulated the cell surface expression of E-selectin, which increased leukocyte adherence to the MASP-1 activated endothelium [36]. Since neutrophil granulocytes are known as the first line of host cellular defense against bacteria and fungi, therefore, hypothetically, MASP-1 itself could prompt the effective local elimination of these pathogens supplementing the pathogen clearance function of down-stream complement activation. We also studied the effects of MASP-1 on the expression of inflammation related genes in ECs and found that MASP-1 indeed regulates the expression of several chemokines, inflammatory receptors, and other inflammatory factors, thereby inducing a shift in ECs towards a more proinflammatory phenotype as early as 2 hours after treatment [37]. In contrast to MASP-1, there were no known direct cellular effects of MASP-2 and MASP-3, the other two serine proteases of the lectin pathway.

As MASP-1 has no selective natural inhibitor, our partners have developed a high-affinity, high-specificity small molecule inhibitor, *Schistocerca gregaria* protease inhibitor based MASP inhibitor-1 (SGMI-1) for facilitating the investigations regarding the physiological functions of MASP-1. However, this new MASP-1 inhibitor has not been tested in cellular systems yet.

1.2.3 Hereditary angioedema as a link between C1-inhibitor, MASP-1 and endothelial permeability

C1-inhibitor is a plasma serine protease inhibitor belonging to the serpin superfamily, encoded by the *SERPING1* gene (localized on chromosome 11). Being one of the most heavily glycosylated human proteins, approximately one third of its total molecular weight (that is observed to be >100 kDa with SDS-PAGE) is made up of sugars. C1-inhibitor forms covalent complexes with its target serine proteases, disabling their enzymatic activities.

Being an effective inhibitor of many plasma serine proteases, C1-inhibitor is the most important inhibitor of the kinin-kallikrein system and also the early complement activation, although it keeps all four plasma cascade subsystems under control [19, 38-41].

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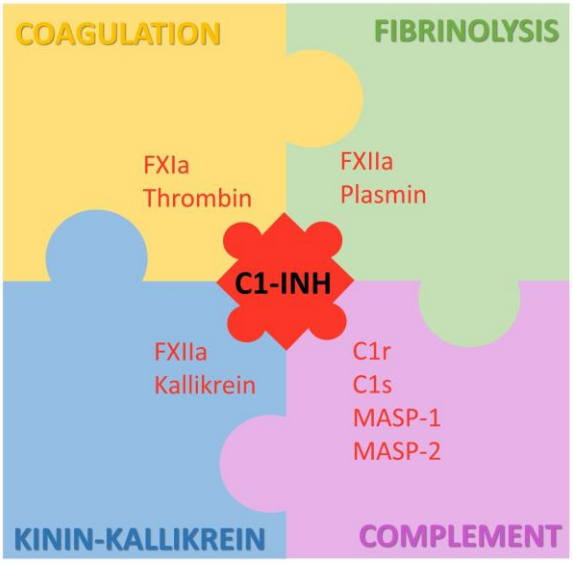

Figure 2. The common regulator of plasma cascade systems: C1-inhibitor controls several enzymatic processes in all four plasma cascade subsystems. The numerous proteases of blood plasma can be divided into four subsystems, such as i) the coagulation, ii) the fibrinolytic, iii) the kinin-kallikrein and iv) the complement system, although these subsystems are all heavily interconnected and constitute an enormous and diversified network. Those enzymes of the four subsystems that can be blocked by C1-inhibitor are listed (red).

The central role of C1-inhibitor can be best understood from the pathomechanism of hereditary angioedema (HAE), a rare but potentially life-threatening disease caused by the genetic deficiency of functional C1-inhibitor. The recurrent edematous attacks characterizing HAE can affect nearly any part of the body (with the interesting exception of the lungs) but can also cause the swelling of the larynx and lead to suffocation. These edematous attacks occur due to an instability in the plasma cascade systems caused by the lack of C1-inhibitor, which can be seen as a conductor of the plasma serine protease orchestra (see **Fig. 2**). According to the generally accepted explanation in the field, the direct cause of HAE attacks is the overproduction of small vasoactive peptide bradykinin, generated from HMWK mainly by kallikrein-mediated cleavage. Bradykinin binds to B2 bradykinin receptors (B2R) on ECs and activates them, causing hyperpermeability [42]. However, the permeability state of endothelium is determined by a delicate balance between the countless permeability increasing and decreasing effects, therefore, the potential pathogenetic role of other molecules should also be investigated in order to see the whole picture. Other potential pathogenetic factors in HAE can be searched among the target enzymes of C1-inhibitor (enzymes written in red on **Fig. 2**). MASP-1 - a serine protease naturally inhibited by C1-inhibitor - has an enzymatic activity resembling that

of thrombin, a well-known edematogenic agent. We have shown previously that MASP-1 can activate ECs [9] and my colleagues have also reported that ECs become activated during HAE attacks [42]. These findings lead us to ask the question whether MASP-1 or other target serine proteases of C1-inhibitor (for example FXII, kallikrein, plasmin, C1r, C1s and MASP-2) can directly increase endothelial permeability.

1.3 Techniques and challenges in studying cellular monolayers

In the current phase of our research, we wanted to test the effect of serine proteases on the endothelial monolayer *in vitro* to eliminate as many confounding factors as possible. It has long been a demand to somehow quantify the barrier properties of a cellular monolayer, but the realization can be quite challenging. The main requirements for such methods are reliability and data reproducibility, but it is also desirable that a platform offers high-throughput measurements and causes minimal interference with cellular functions. This last consideration divides the existing techniques to "label-using" and label-free ones, out of which the latter techniques typically require a high-tech instrumental background.

1.3.1 *In vitro* permeability tests using labelled molecules

One of the oldest described and still expansively used permeability test is the Transwell method [43]. The technique utilizes special porous inserts with defined pore-size that can be placed in the wells of cell-culture plates and are suitable for the creation of confluent cellular monolayers on their surface. The permeability of the cell layer can be measured by the injection of a labelled molecule (e.g., fluorescently labelled proteins, carbohydrates, or horse radish peroxidase) into the insert and monitor its concentration in the outer fluid space. A big advantage of the Transwell method is that it can measure true mass-transport, moreover, with the possibility of using different sized analytes it is suitable for testing the size-selectivity of the cellular monolayer. Despite being simple in its principles, implementation of a Transwell test can be quite challenging. A serious drawback is that it requires good manual skills from the experimenter, and still, one can easily scratch the cell layer while pipetting – an error that is hard to notice afterwards. Another critical point is to ensure equal hydrostatic pressure in- and outside the inserts when changing the media or moving the insert to another well, as small differences in the fluid levels can cause changes in the intensity of mass transport. For the above-mentioned

reasons, the method is not robust enough and it requires several parallel measurements to get reliable data. These extra measurements consume lots of cells and reagents, and the expensive inserts further raise the costs.

To overcome these problems, Dubrovskyi and colleagues have developed an easy-to-use, cost effective and high-throughput technique, called XPerT Permeability Assay [44], which we slightly modified (**Fig. 3**). Briefly, the assay principle is to use biotinylated gelatin for the coating of cell culture plates (gelatin coating is used routinely for the successful seeding of ECs on plastic surfaces as it mimics the basement membrane) and after the cell monolayer reaches confluency, permeability changing agonists are added and the newly formed paracellular gaps are stained with a streptavidin-conjugated fluorescent dye. Permeability change can be quantified using different approaches: the fluorescence intensity can be detected by a fluorescence microplate reader (as it is written in the original protocol) or individual wells can be photographed using a fluorescence microscope (with which we supplemented the detection in our study). Due to its simplicity and robustness, XPerT offers a very promising alternative to the Transwell method.

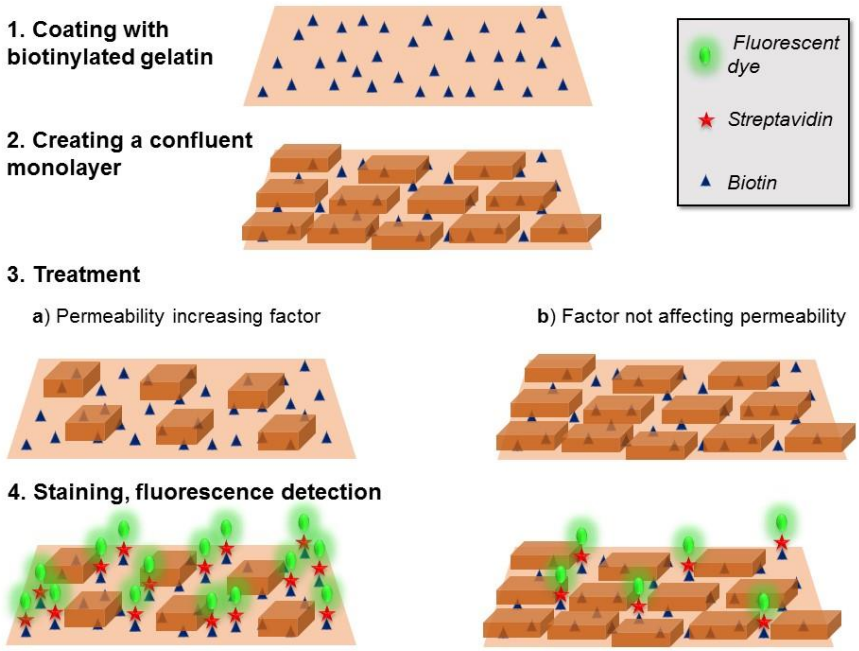

Figure 3. The principle of the modified version of the XPerT permeability assay. The surface of a multiwell cell culture plate is coated with biotinylated gelatin, then cells are seeded and cultured to reach full confluency. Cells are then treated with the compound to be tested and a streptavidin conjugated fluorescent dye is added to the wells to visualize the paracellular gaps.

Paracellular permeability of the monolayer can be quantified by the analysis of fluorescence microscopy images or data provided by a fluorescence microplate reader.

1.3.2 Label-free technologies for high-throughput screening of cellular monolayers

We can use label-free technologies and avoid the usage of indicator molecules of any kind to exclude the possibility that the labeling itself will interfere with cell signaling and modify the effect of the tested compound. In general, label-free technologies cannot directly measure the intensity of mass-transport through a cellular monolayer (as that would require the application of dyes/labelled molecules) and the detected signal should not be interpreted as evidence of permeability change. This, however, is not always a drawback if we use these methods for screening purposes, because in some of them – for example the later explained resonance waveguide grating (RWG) technology – the output signal is of exceptional biological complexity and can represent practically any kind of cellular events. Nowadays, as label-free efforts come to the fore, more and more techniques are being developed – out of which I will briefly describe some of the more important ones for this dissertation.

Transwell inserts can also be used in a technique called transendothelial electrical resistance (TEER) measurement. In this method, electrodes are placed both in the inner and the outer fluid space and thereby the electrical resistance of the monolayer can be measured. The electrical resistance is determined mostly by small ions (Na^+, Cl^-) passing between ECs. Its principle makes TEER almost impossible to use in the case of endothelia with looser adhesive bonds - for example human umbilical vein ECs (HUVECs) - as small ions can cross these barriers almost freely and the basal resistance can hardly be lowered. Also, as mentioned above, handling of Transwell inserts requires very good manual skills and the potential scratches done to the monolayer can easily go unnoticed, resulting in greater standard deviation of data.

The xCELLigence system is a label-free approach for the quantification of endothelial/epithelial barrier function in real-time [45]. In this method, cells are cultured on the surface of gold electrodes that measure the impedance (alternating current equivalent of resistance), which increases with better barrier functions (i.e. stronger cell-cell and cell-matrix adhesion) because surface attached cells act as an insulation between the gold electrodes, therefore, xCELLigence can be used in the case of endothelia with

looser adhesive bonds. Compared to TEER, xCELLigence is more robust and easier to use, even in high-throughput mode, simultaneously monitoring up to 384 wells.

Compared to xCELLigence, much more complex biological signals can be monitored real-time with the RWG-based optical biosensor technologies, which can *in situ* monitor refractive index changes in an evanescent electromagnetic field, generated in close proximity (~150 nm) of the surface of the sensor in 96- or 384-well microplates [46, 47]. The refractive index is affected by any kind of mass translocation in the sensing depth, therefore, the sensor output, which is an integrated signal of these mass translocations, is also called dynamic mass redistribution (DMR) [48]. This allows all types of cellular responses to be visualized with RWG based optical biosensor techniques. The technology was successfully used to monitor the kinetics of cell–surface and cell membrane receptor–ligand interactions [46, 49-51], binding affinity [52], cellular signaling [53, 54], cytotoxicity [55], nanoparticles [56] and the functional state of surface-adhered cells down to the single cell level [57]. A commercially available RWG based optical biosensor platform is the Epic BenchTop (BT) system seemed particularly suitable for our screening purposes, as it can monitor any type of a cellular response on the nanometer scale in a real-time and completely label-free manner. It is worth noting that because of the integrated nature of the DMR signal, the underlying molecular events cannot be identified exactly, and further, targeted tests are needed to investigate the background of the detected signal.

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2. OBJECTIVES

Due to their spatial position as the innermost layer of vessel walls, endothelial cells come into direct physical contact with the components of blood plasma, including plasma serine proteases. Thrombin, a well-described plasma serine protease can activate endothelial cells to increase endothelial permeability, however, information is very limited on the cellular effects of other plasma serine proteases. MASP-1, a key serine protease of complement lectin pathway activation shows enzymatic similarities with thrombin and is a natural target of C1-inhibitor. The observations that the genetic or acquired deficiency of C1-inhibitor underlies the pathomechanism of some life-threatening edematous conditions made it highly relevant to know whether MASP-1 is able to directly increase endothelial permeability. As C1-inhibitor controls the activity of numerous other plasma serine proteases yet uninvestigated in cellular systems, there was also a need for a high-throughput assay suitable for the detection of complex endothelial responses and thereby the screening of plasma serine proteases on endothelial monolayers. Therefore, we have set the following objectives for our studies:

- **Investigation of the potential permeability increasing effect of MASP-1 and the underlying molecular mechanisms**

- **Development of a label-free screening method for the real-time investigation of endothelial cell behavior**

- **Investigation of the effects of plasma serine proteases on the physical properties of endothelial monolayers using the newly developed label-free assay**

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3. RESULTS

All the following results were obtained using *in vitro* cultures of freshly isolated human umbilical vein endothelial cells (HUVECs) and the protocol was approved by the Semmelweis University Institutional Review Board (permission number: TUKEB 141-2015). As the scope of the dissertation does not allow for a separate Methods section, please see the methodological details in the relevant original articles [58, 59].

3.1 Effect of MASP-1 on endothelial permeability

The potential effect of MASP-1 on endothelial permeability would be of high relevance in edematous conditions characterized by C1-inhibitor deficiency. To clarify the role of MASP-1 we aimed to take a complex approach.

3.1.1 Effect of rMASP-1 treatment on the impedance of the endothelial monolayer

To test whether rMASP-1 has any effect on the barrier function of the endothelial monolayer, we performed real-time impedance measurements using the xCELLigence system (**Fig. 4**). Treating HUVECs with 2 μM rMASP-1 resulted in a prominent decrease of monolayer impedance, with an overall duration of approximately 1 hour and a maximal response occurring 15-20 min after rMASP-1 treatment. This effect of rMASP-1 was similar to that of the well-known permeability increasing agonist, thrombin.

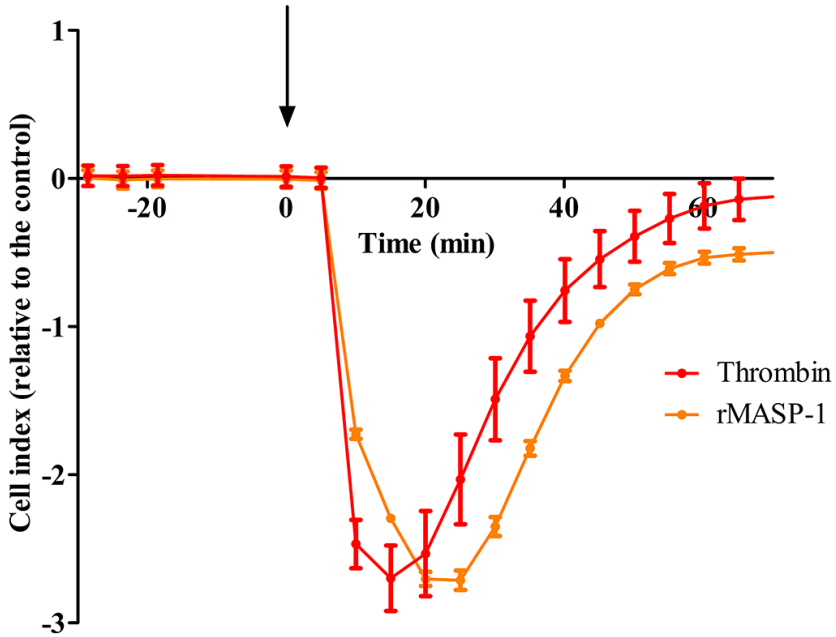

Figure 4. rMASP-1 treatment reversibly decreases the impedance of the endothelial monolayer. HUVECs were seeded onto 96-well E-plates (Roche, Hungary) mounted by golden microelectronic sensor arrays (pre-coated with 0.5% gelatin). After 3 days of cell culturing, confluent HUVEC monolayers were treated with 2 μM rMASP-1 or 100 nM thrombin or the culture medium only. Cells were kept in incubator at 37◦C and monitored every 1 min throughout the experiments. The following formula was used to determine cell index for each time point: (Rn—Rb)/15, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the medium alone. Therefore, values of cell index are dimensionless and directly proportional to the electrical permeability of the monolayer. Cell indices of rMASP-1 and thrombin treated cells were normalized with that of the control cells. The figure is a representative of three independent experiments. The arrow indicates the addition of treatment. Source of this figure is the candidate's own publication [58].

3.1.2 Effect of rMASP-1 treatment on endothelial permeability

Although the results of xCELLigence measurements suggested that rMASP-1 can increase

endothelial permeability, these results needed to be confirmed as the system does not measure true mass transport. For the permeability measurements, we used a modified version of the recently developed XPerT technique, a simple method suitable for the detection of paracellular transport. In these permeability tests, rMASP-1 treatment resulted in a fivefold increase in endothelial permeability, which was dose dependent and comparable to the effect of thrombin (**Fig. 5**). To assess the inhibitability of the rMASP-1 triggered hyperpermeability, we used its natural inhibitor C1-inhibitor and also the highly specific, artificial MASP-1 inhibitor, SGMI-1, which latter we found to be non-cytotoxic even if applied in high doses for 24 hours (data not shown). The permeability increasing effect of rMASP-1 was completely blocked by both C1-inhibitor and SGMI-1.

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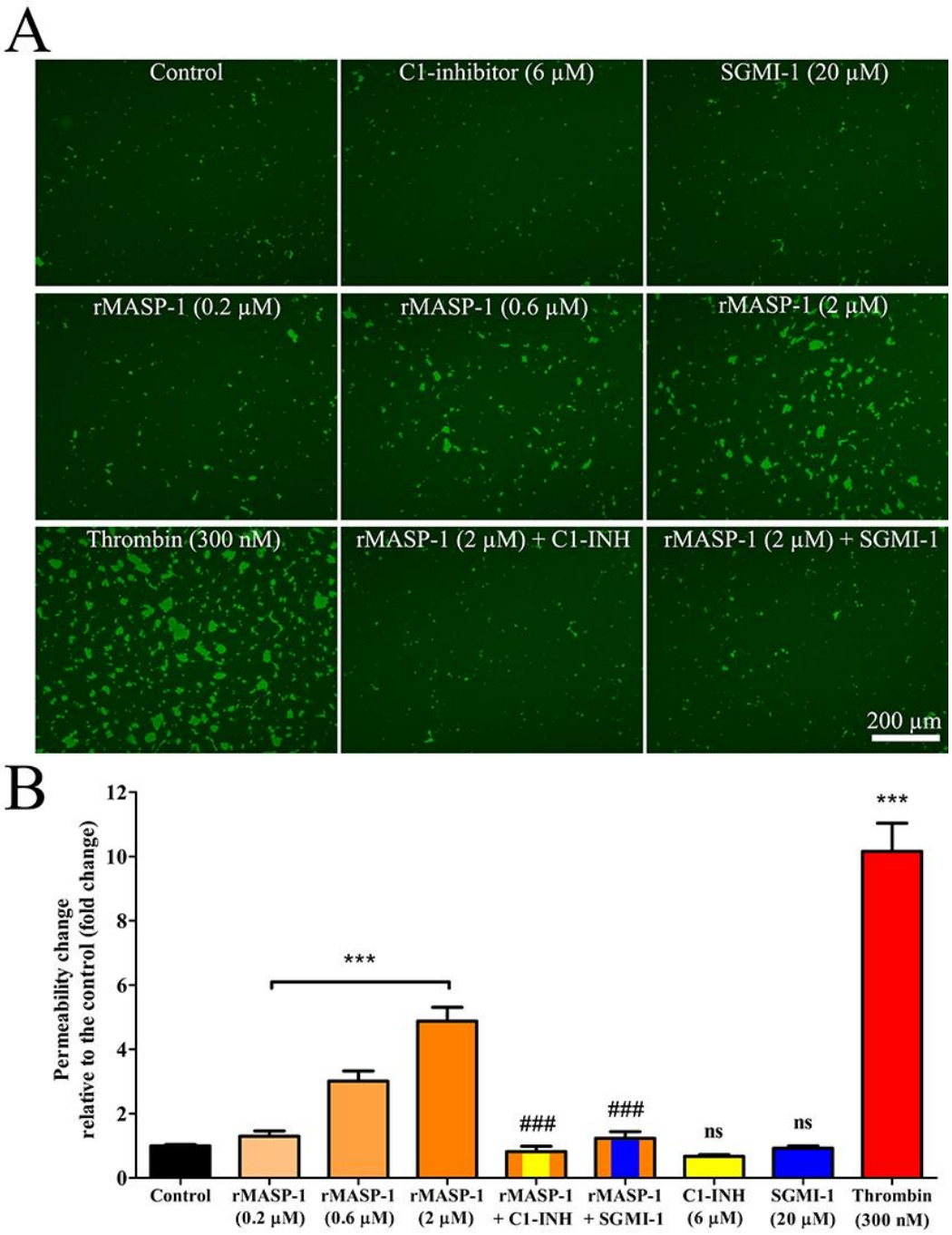

Figure 5. rMASP-1 treatment increases endothelial permeability in a dose dependent and inhibitable manner. Confluent monolayers of HUVECs were seeded onto biotinylated gelatin precoated 96-well plates. Cells were treated with various concentrations of rMASP-1 or a mixture of rMASP-1 and its inhibitors (SGMI-1 or C1-inhibitor) or with the culture medium alone (control) for 20 min. Streptavidin-Alexa488 was added to the wells, and after paraformaldehyde fixation, pictures were taken using fluorescence microscopy. A) Representative images of three independent experiments. B) Size of the stained area was determined on each image using the CellP software. Mean values of three independent experiments normalized to the controls are shown. ***$p < 0.005$, compared to the control, analyzed by one-way ANOVA and post-test for linear trend; ns, non-significant; ###$p < 0.005$, compared to the 2 µM rMASP-1 treated. Source of this figure is the candidate's own publication [58].

3.1.3 Signaling of the rMASP-1 induced permeability change

We have shown earlier that MASP-1 is able to cleave cell surface PAR-1, PAR-2 and PAR-4 on HUVECs. Since intracellular Ca^{2+} mobilization is a crucial step of the PAR-mediated permeability increase, we investigated the role of each PAR using PAR agonists and antagonists. Treating HUVECs with PAR-1 agonist resulted in a considerably large Ca^{2+} release, the PAR-4 agonist had only a mild effect, while the PAR-2 agonist failed to induce any response (**Fig. 6 A,B**). Accordingly, only the PAR-1 antagonist pretreatment could significantly dampen the rMASP-1 triggered calcium flux (**Fig. 6 C,D**).

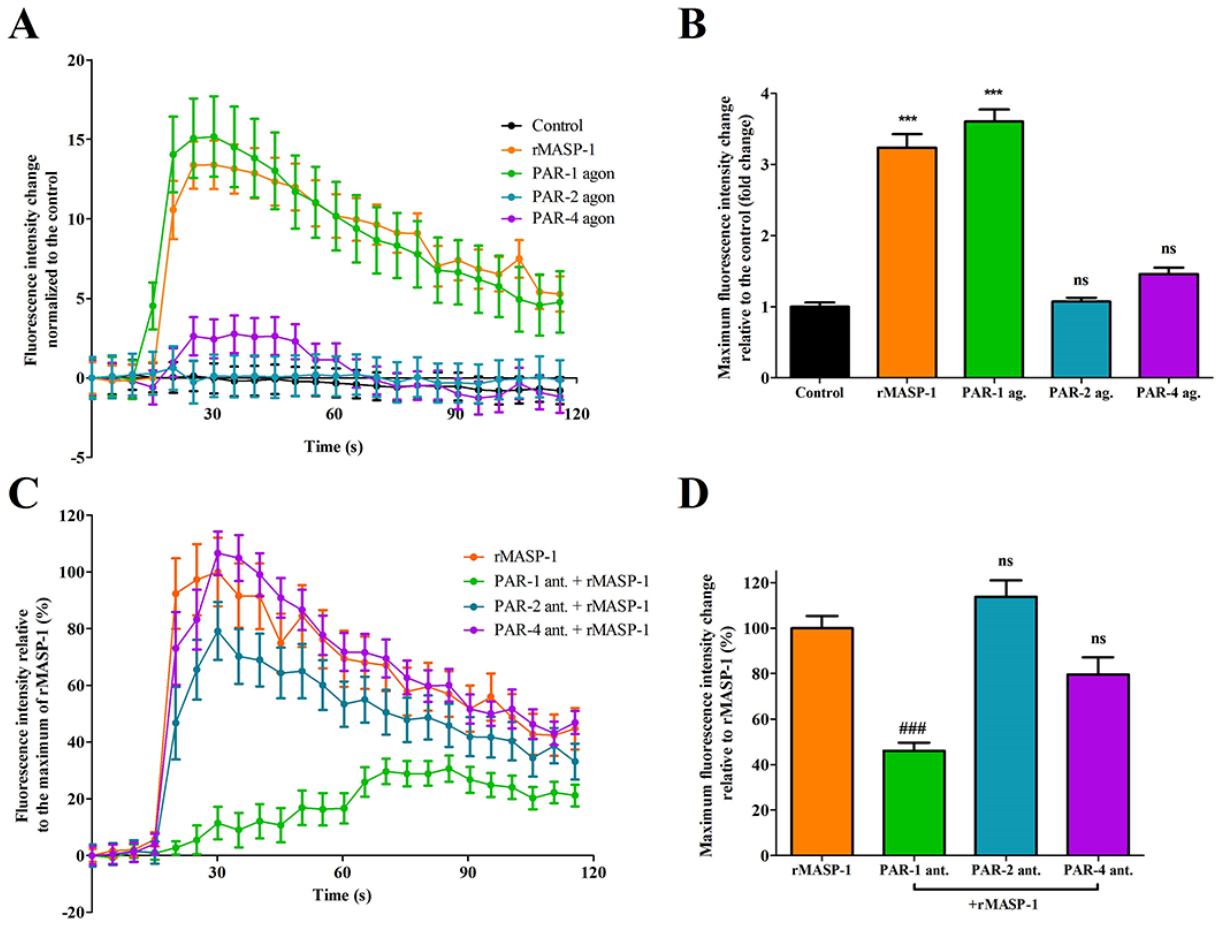

Figure 6. PAR-1 mediates the rMASP-1 triggered intracellular Ca^{2+} mobilization in HUVECs. Confluent layers of HUVECs were seeded onto 96-well plates and cultured for 1 day. Cells were loaded with a fluorescent calcium indicator (Fluo-4 AM). A,B) Sequential images were obtained every 5 s by fluorescence microscopy: three photos were taken to determine the baseline fluorescence, then cells were treated with 2 μM rMASP-1 or PAR-agonists (4 μM PAR-1 agonist, 0.4 μM PAR-2 agonist or 1 mM PAR-4 agonist) or the culture medium only (control). (C,D) Cells were pretreated with or without PAR antagonists (0.68 μM PAR-1 antagonist; 20 μM PAR-2 antagonist or 0.28 μM PAR-4 antagonist) for 10 min. Sequential images were obtained

every 5 s by fluorescence microscopy. Three photos were taken to determine the baseline fluorescence, then cells were treated with rMASP-1 or with culture medium alone (control) and the response was measured for 2 min. Images were then analyzed using the CellP software. A,C) data from a single, representative experiment, where fluorescence intensity values were background corrected and normalized to the control. B) Means of the maximum fluorescence intensity values normalized to that of the control are presented. Data from three independent experiments. D) Means of the maximum fluorescence intensity values are expressed as the percentage of rMASP-1 treatment (control: 0%). Data from three independent experiments. ***p < 0.005, compared to the control; ###p < 0.005 compared to the rMASP-1 treated; ns, non-significant. Source of this figure is the candidate's own publication [58].

Then, we tested the PAR-dependence of the permeability response induced by rMASP-1. The PAR-1 and the PAR-4 agonists could significantly increase endothelial permeability (to a similar extent as rMASP-1), while the PAR-2 agonist was found to be ineffective (**Fig. 7 A**). However, only the PAR-1 antagonist pretreatment could prevent the permeability response triggered by rMASP-1 **(Fig. 7 B**).

Next, we investigated the role of two major protein kinases of cytoskeletal remodeling – ROCK and MLCK – in the permeability increasing effect of MASP-1 (**Fig. 7 B**). HUVECs pretreated with the synthetic ROCK inhibitor Y-27632 showed even stronger barrier properties than the untreated cells, this inhibitor could entirely avert the effect of rMASP-1. ML-7 (inhibitor of MLCK), however, failed to prevent the rMASP-1 induced permeability.

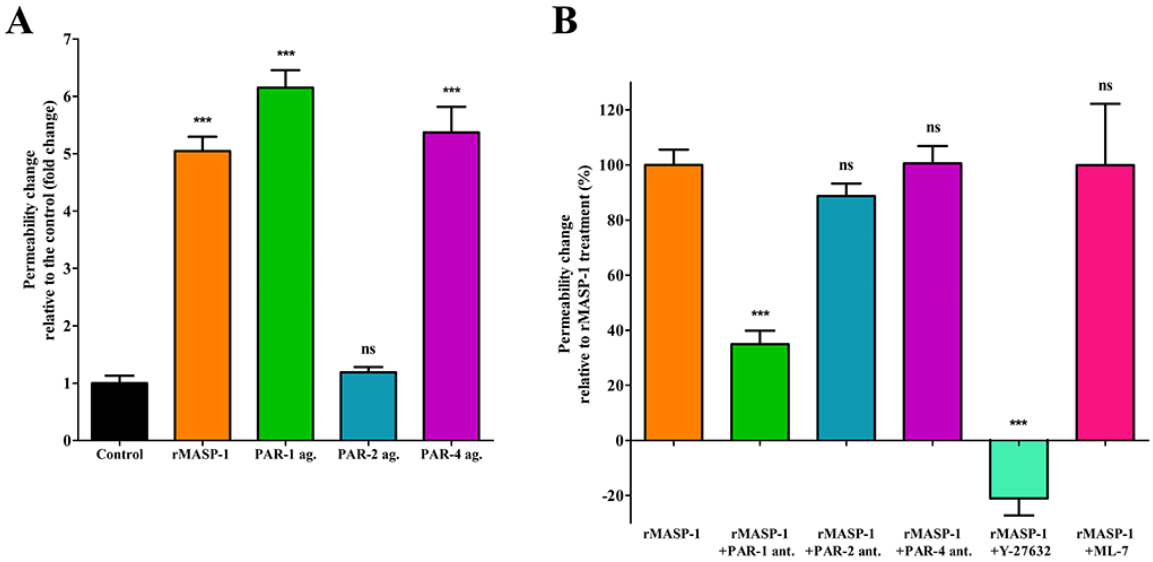

Figure 7. PAR-1 and ROCK plays a key role in the rMASP-1-induced endothelial permeability. Confluent layers of HUVECs were seeded onto 96-well plates pre-coated with biotinylated gelatin and were cultured for 2 days. Following cell treatment, streptavidin-Alexa488 was added to each well and after cell fixation, pictures were taken using fluorescence microscopy. Size of the stained area was determined on each image using the CellP software in three

independent experiments. A) Cells were treated with 2 μM rMASP-1, PAR-agonists or the culture medium alone for 20 min. Values are expressed as fold-change relative to the control. B) Cells were pretreated with or without PAR antagonists for 10 min then treated with 2 μM rMASP-1 for 20 min. Values are expressed as the percentage of rMASP-1 treatment (0% = cells treated only with the culture medium). ***$p < 0.005$, compared to the control; ###$p < 0.005$, compared to rMASP-1 treated; ns, non-significant. Source of this figure is the candidate's own publication [58].

3.1.4 Effects of rMASP-1 on the actin cytoskeleton of endothelial cells

We also examined the cytoskeletal changes underlying the rMASP-1-triggered permeability change. Similarly to thrombin, rMASP-1 treatment induced a strong MLC phosphorylation and cytoskeletal rearrangement, resulting in the formation of actin stress fibers (**Fig. 8**). All the above-mentioned effects of rMASP-1 could be inhibited by C1-inhibitor and SGMI-1. Among the PAR-antagonists, only the PAR-1 antagonist pretreatment could significantly block the effects of rMASP-1, while antagonists of PAR-2 and PAR-4 proved ineffective. Furthermore, ROCK inhibitor Y-27632 could completely prevent rMASP-1 induced cytoskeletal events – this pretreatment led to a total absence of diphosphorylated MLC and actin stress fibers. MLCK inhibitor ML-7, on the other hand, again failed to block the effect of rMASP-1.

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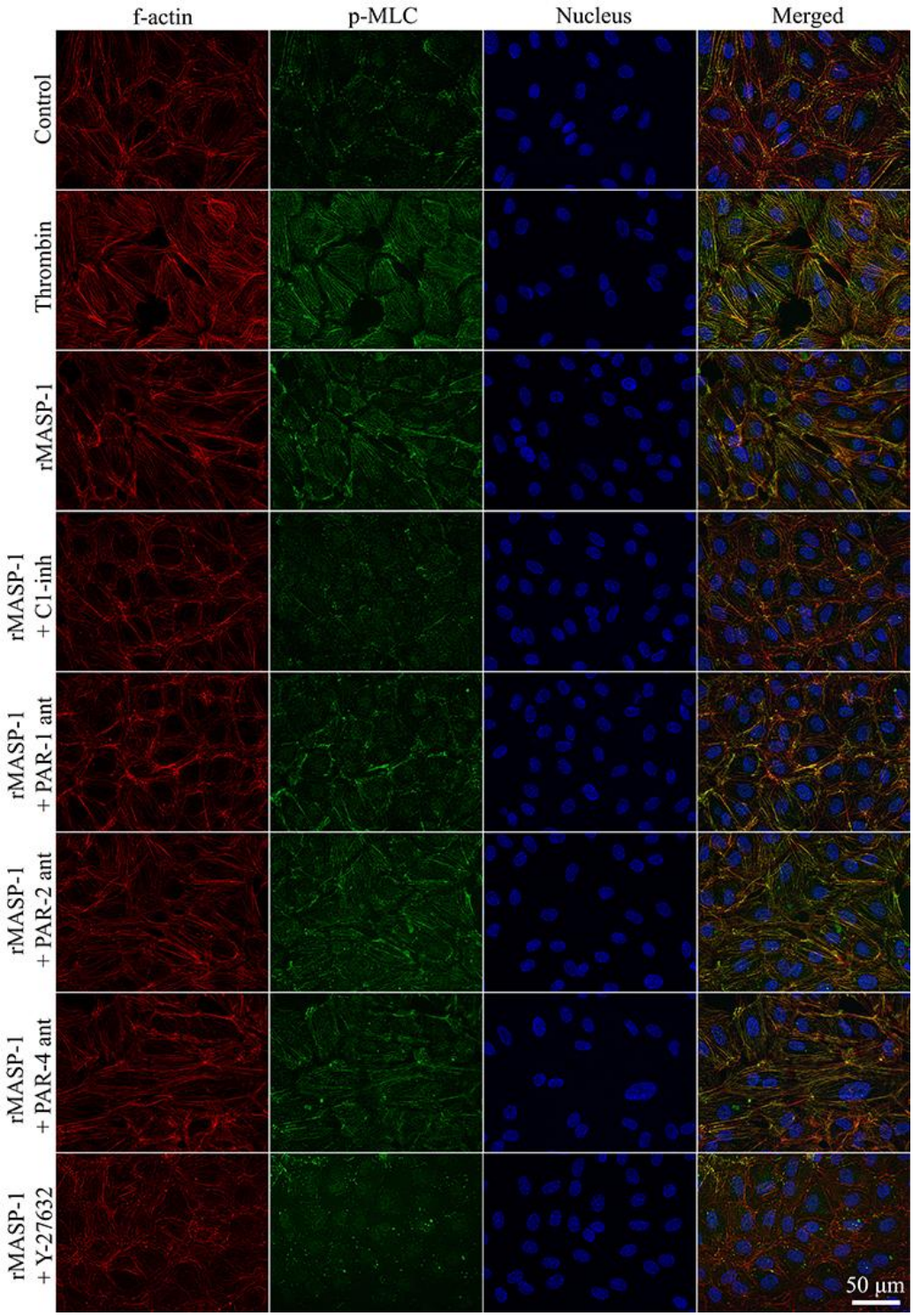

Figure 8. rMASP-1 treatment reorganizes the actin cytoskeleton of endothelial cells. Confluent layers of HUVECs were seeded onto 18-well ibidi™ slides and were cultured for 2 days. Cells were pretreated with or without PAR antagonists for 10 min or ROCK inhibitor (Y-27632) for 15 min, then treated with rMASP-1, thrombin or culture medium for 20 min. After fixation, cells were stained with anti-pMLC antibody. Filamentous actin cytoskeleton was stained with phalloidin-Alexa488, cell nuclei were labeled with Hoechst 33258 and pictures were taken

using a fluorescence microscope. Representative images from three independent experiments are shown. Source of this figure is the candidate's own publication [58].

3.1.5 Effects of rMASP-1 on the pattern of molecules important in endothelial cell adhesion

Next, we investigated the effect of rMASP-1 on the pattern of some important EC adhesion molecules. On **Fig. 9** a well-organized and uninterrupted network of adhesion molecules VE-cadherin and PECAM-1, and intracellular adaptor molecule ZO-1 can be seen around the untreated cells. This pattern was spectacularly disrupted by rMASP-1 treatment, resulting in the formation of paracellular gaps. The effect of rMASP-1 could also be completely blocked by C1-inhibitor.

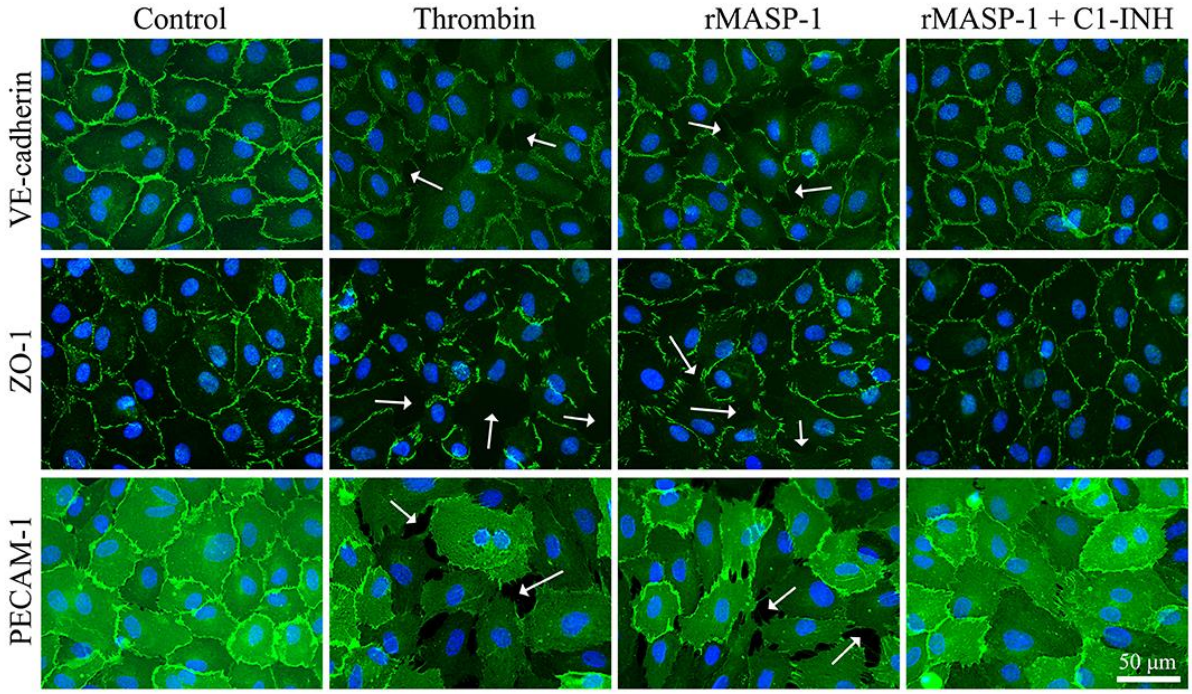

Figure 9. rMASP-1 treatment changes the pattern of endothelial cell adhesion molecules. Confluent layers of HUVECs were seeded onto 18-well ibidi™ slides and cultured for 2 days. Cells were either treated with thrombin, rMASP-1, a mixture of rMASP-1 and C1-inhibitor or with the culture medium alone (control) for 20 min. After fixation, cells were stained with anti-VE-cadherin, anti-ZO-1, or anti-PECAM-1 antibodies (green). Cell nuclei were labeled with Hoechst 33258 (blue) and images were taken using fluorescence microscopy. Paracellular gaps are indicated with white arrows. Source of this figure is the candidate's own publication [58].

3.1.6 Effects of rMASP-1 on the expression of permeability related genes

We also wanted to investigate whether rMASP-1 can influence the expression of permeability related genes in HUVECs as a prolonged effect, therefore, we analyzed the

data of our previously performed whole transcriptome microarray analysis. The 2-hour rMASP-1 treatment significantly changed the expression levels of 25 permeability-related genes. Out of these, we found 12 genes (9 up-regulated and 3 down-regulated) with direct experimental evidence verifying their role in endothelial permeability regulation (in the then-current literature) (**Table 1**). An interesting example is that rMASP-1 up-regulated the expression of B2R, the EC surface receptor for bradykinin, which is a very potent edematogenic peptide. Among the 25 MASP-1 regulated genes, we could also identify an additional set of 6 genes (3 up-regulated and 3 down-regulated) with indirect experimental evidence for their permeability-related roles (in the case of these genes, we found experimental evidence for their role in modulating the effects of known regulators of endothelial barrier functions). The remaining 7 genes (4 up-regulated and 3 down-regulated) lacked experimental evidence for their permeability-regulating effects but are suspected to affect barrier functions (a **Supplemental table** containing also the last two categories can be found at https://www.frontiersin.org/articles/10.3389/fimmu.2019.00991/full#supplementary-material). Furthermore, the effects of well-known permeability increasing agonists such as lipopolysaccharide (LPS), histamine, thrombin and tumor necrosis factor α (TNF-α) on the gene expression of HUVECs were also tested and we found that 17 (68%) of the permeability related genes regulated by MASP-1 were also modulated by one or more of the above-mentioned mediators, while 8 genes were exclusively regulated by rMASP-1 (**Table 1** and **Supplemental Table 1**). An interesting finding was that with a single exception, genes that were found to be regulated both by rMASP-1 and any of the above-mentioned permeability increasing agonists manifested expression level alterations in the same direction regardless of the agonist applied.

Table 1. rMASP-1 significantly changes the expression of permeability-related genes
Confluent layers of HUVECs from four individuals were cultured in 6 well plates and treated for 2 h with 0.6 μM rMASP-1. To compare the effects of rMASP-1 with other endothelial cell activators, 300 nM thrombin, 10 ng/mL TNFα, 100 ng/mL LPS, or 50 μM histamine treatment was applied. The table contains the median fold-change (FC) values of the 4 independent HUVECs. Possible permeability-related genes were filtered out from the set of genes significantly changed by rMASP-1 using REACTOME, KEGG, and GO databases. Experimental evidence for the permeability related function of these genes were verified according to the current literature. The table contains only those genes with direct experimental evidence to support their role in permeability regulation. The following terms and pathways were used to filter out possible permeability related genes from each database: GO—permeability; junction; actin; adhesion; camp; cgmp; rho; calcium. REACTOME—Cell junction organization; Extracellular matrix organization; Cell surface interactions at the vascular wall; Toll-like receptors cascades;

Complement cascade; Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways. KEGG—Cell adhesion molecules; Adherens junction; Focal adhesion; Tight junction; Gap junction; Complement and coagulation cascades; Toll-like receptor signaling pathway; NOD-like receptor signaling pathway; RIG-I-like receptor signaling pathway; Leukocyte transendothelial migration.
↑: Gene expression was up-regulated by the given activator;
↓: Gene expression was up-regulated by the given activator.
Source of this table is the candidate's own publication [58].

Median of fold change by rMASP-1	Gene symbol	Gene name by HUGO Gene Nomenclature Committee (HGNC)	Description of the permeability related function	Other activators			
				LPS	Histamine	Thrombin	TNF-α
Up-regulated							
4.09	F3	coagulation factor III, tissue factor	Important mediator of the endothelial hyperpermeability induced by TNF-α, which exerts its permeability increasing effect by the up-regulation of endothelial cell surface tissue factor [59].	↑	↑	↑	↑
3.38	FZD7	frizzled class receptor 7	AJ protein which colocalizes with VE-cadherin and has an important role in the stabilization of the endothelial barrier [60].				
3.35	RCAN1	regulator of calcineurin 1	Synthesized in response to histamine treatment and has a role in reducing endothelial barrier breakdown [61].	↑	↑	↑	↑
2.78	GHSR	growth hormone secretagogue receptor	Mediates the effect of ghrelin. Reduces the permeability increasing effect of LPS [62] and also prevents the increase in the blood-brain barrier permeability following traumatic brain injury [63].	↑			
2.54	FOXF1	forkhead box F1	Transcription factor important in the maintenance of endothelial barrier function [64].	↑	↑	↑	↑
2.32	TLR2	toll like receptor 2	Mediates postischemic permeability increase [65].	↑			
2.24	KITLG	KIT ligand	Binds to endothelial c-Kit receptor and increases endothelial permeability through the stimulation of VE-cadherin internalization [66, 67].	↑	↑		
2.17	BDKRB2	bradykinin receptor B2	Receptor for the well-known edematogenic factor bradykinin. Also, an important pharmacological target in HAE therapy [68].			↑	↑
2.03	TGFBR1	transforming growth factor beta receptor 1	TGF-β induces endothelial cell contraction and increases endothelial permeability through this receptor [69, 70].	↑	↑	↑	

Median of fold change by rMASP-1	Gene symbol	Gene name by HUGO Gene Nomenclature Committee (HGNC)	Description of the permeability related function	Other activators			
				LPS	Histamine	Thrombin	TNF-α
Down-regulated							
-2.90	EDNRB	endothelin receptor type B	Receptor for endothelin-1, which reduces the permeability increasing effect of bradykinin and ATP through this receptor [71].	↓	↓	↓	↓
-2.58	APLNR	apelin receptor	Mediator of the effects of apelin, which is important for the stabilization of the endothelial barrier of both blood and lymphatic vessels [72-74].				
-2.30	CCR3	C-C motif chemokine receptor 3	Eotaxin increases endothelial permeability through this receptor [75].	↓			

3.2 Investigation of the effects of plasma serine proteases on ECs using a label-free optical biosensor

Seeing the results with MASP-1, we were curious to know whether other plasma serine proteases could also directly affect the physical properties of the endothelium. Therefore, we aimed to develop an optical biosensor-based, high-throughput and label-free assay, suitable for the real-time monitoring of endothelial cell responses to screen a set of plasma serine proteases on endothelial monolayers.

3.2.1 Biosensor surface modification: optimization of gelatin coatings for cell seeding

Due to its ideal properties, we chose the commercially available Epic BT platform for the biosensor assay development. The surface of the biosensor was made biocompatible with gelatin coating, which mimics the basal membrane and facilitates EC adhesion. We tested two gelatin concentrations (0.2 and 5 mg/mL) during the coating experiments and our cooperation partners (Inna Székács, Boglárka Kovács, András Saftics and Róbert Horváth - Nanobiosensorics Momentum Group, Institute of Technical Physics and Materials Science, Centre for Energy Research, Budapest, Hungary) characterized the resulting gelatin layer with optical waveguide lightmode spectroscopy (OWLS), quartz crystal microbalance (QCM) and atomic force microscopy (AFM). According to these experiments, coating the biosensor surface with 0.2 mg/mL gelatin creates an ideal

substrate for EC adhesion as this concentration results in a ~16 nm thick, highly hydrated and homogenous layer, which is thin enough for the biosensor to "see through" while also has ideal viscoelastic properties providing a stable basis for ECs.

The preparation of the biological interface containing ECs and the principles of the endothelial label-free biochip (EnLaB) measurement is schematically explained in **Fig. 10**. Additional figures and detailed information on the coat optimization can be found in our open-access original paper at https://www.nature.com/articles/s41598-020-60158-4.

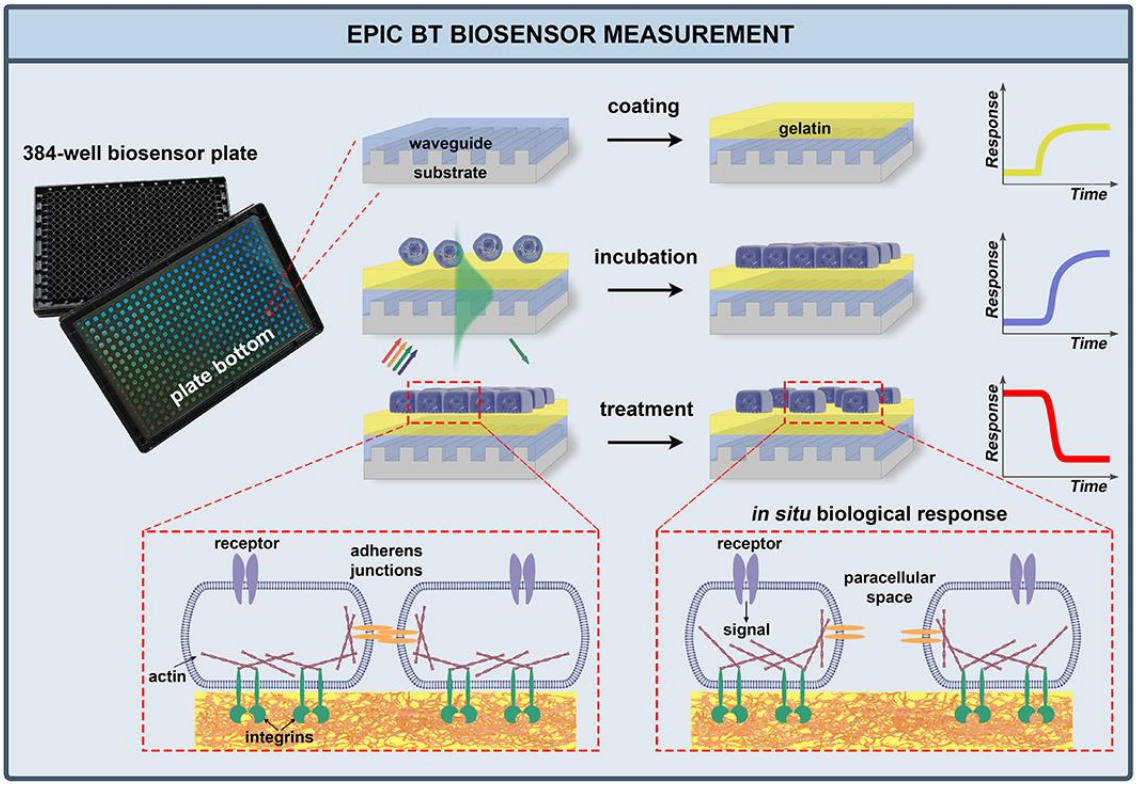

Figure 10. Schematic overview of the preparation of the biological interface containing ECs and the proposed endothelial label-free biochip (EnLaB) measurement setup. The figure illustrates the steps of the biosensor measurements and typically obtained biosensor responses (the detected shifts in the resonant wavelength): coating of the chip with gelatin, followed by cell attachment to the gelatin surface, and subsequent cell treatment using the studied molecular compounds (screening). The biological effects of the treatment are illustrated in the dashed boxes. Source of this figure is the candidate's own publication [59].

3.2.2 Optimization of the Epic BT-based EnLaB system for the label-free investigation of complex EC responses to plasma serine protease treatments

Confluent monolayers of HUVECs were successfully created on the surface of 0.2 mg/ml gelatin coated 384-well Epic biosensor microplates (cultured in a humidified incubator).

The wavelength shift curve was not affected by the addition of growth medium to the HUVEC monolayers (**Fig. 11 A**), but when trypsin treatment was applied and ECs completely detached from the surface, it resulted in a great negative wavelength shift of -1400 pm (**Fig. 11 B**). Thrombin – a serine protease that triggers paracellular gap formation, but not cell detachment – induced a more moderate, but still definite negative wavelength shift (**Fig. 11 C**).

The effect of thrombin was further investigated in 6 different concentrations in the range of 12.5 nM up to 400 nM and was found to be dose dependent ($p<0.001$, ANOVA, post-test for linear trend, **Fig. 11 D**). According to these results, EnLaB appeared to be suitable for testing the effects of other plasma serine proteases on ECs.

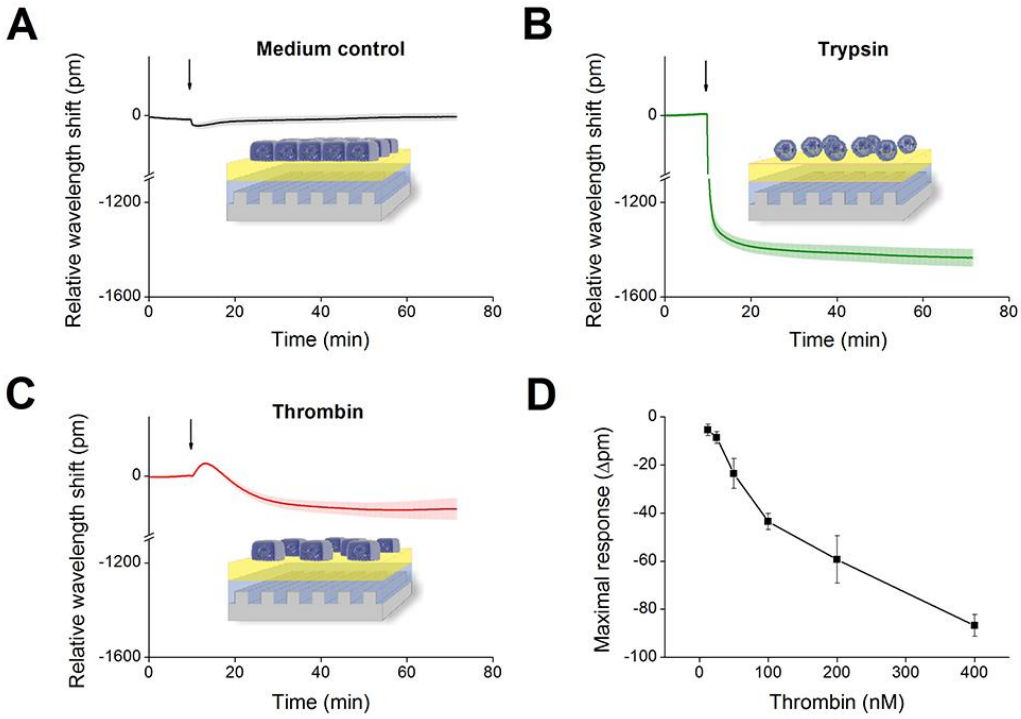

Figure 11. Fine-tuning of the label-free measurement of dynamic mass redistribution on confluent HUVEC monolayers. A 384 well biosensor microplate was precoated with 0.2 mg/ml gelatin, then wells were washed with PBS and confluent monolayers of HUVECs were created on the surface (24 h). The microplate was placed into the Epic BT reader, and after the stabilization of the baseline, cell treatments were added directly to the culture medium, and the wavelength shift was monitored for 60 min. A) The culture medium was added alone. B) 2 μM of trypsin was added. C) 300 nM of thrombin was added. D) Various concentrations of thrombin were added. The response curves were normalized to the medium control and the maximum values of the wavelength shift were determined. The figures show representative graphs of at least 3 independent measurements. Source of this figure is the candidate's own publication [59].

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3.2.3 Investigating the effects of plasma serine proteases on endothelial monolayers using the EnLaB system

After fine-tuning the system with thrombin, we screened other plasma serine proteases that can be interesting in HAE as targets of C1-inhibitor but have not yet been investigated thoroughly on endothelial monolayers, such as rMASP-2, kallikrein, rC1s, rC1r and FXII (**Fig. 12**). We also included MASP-3, a serine protease that cannot be blocked by C1-inhibitor. From the tested enzymes, rMASP-3, rC1s, and rFXII did not alter EC behavior, but rMASP-2, rC1r and kallikrein treatment resulted in characteristic wavelength shift curves significantly different from the medium control. A negative wavelength shift was induced by rMASP-2 and kallikrein, while rC1r had an opposite effect and caused a positive signal. The effect of all three proteases was dose dependent. Covalent complex formation between rMASP-2, kallikrein and their natural inhibitor, C1-inhibitor completely blocked the effects of these proteases. However, surprisingly, the rC1r retained its cellular effects when applied in complex with C1-inhibitor, although PAGE tests verified that the covalent complex was enzymatically inactive (data not shown).

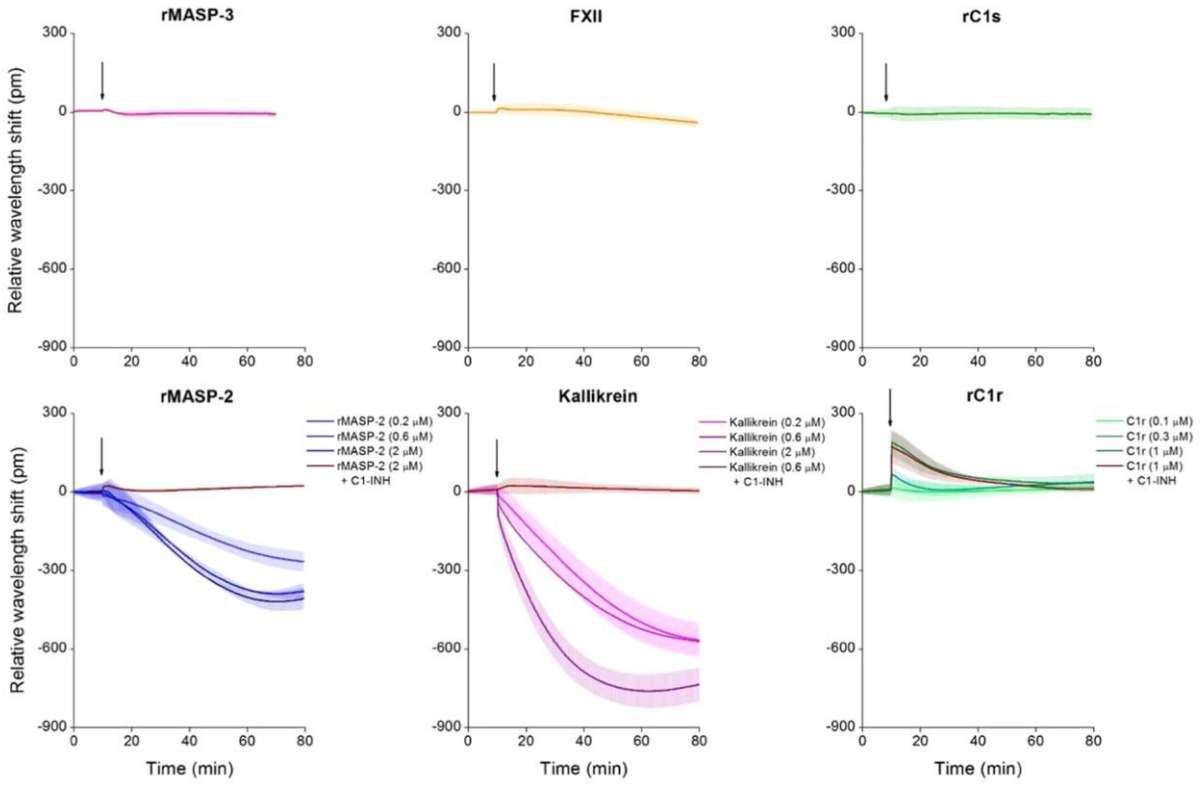

Figure 12. Effects of plasma serine proteases on the dynamic mass redistribution of confluent endothelial monolayers. A 384 well biosensor microplate was precoated with 0.2 mg/ml gelatin, then the wells were washed with PBS, and confluent monolayers of HUVECs were grown on the surface (24 h). The microplate was placed into the Epic BT reader, and after the stabilization of the baseline, serine proteases rMASP-3 (1 μM), FXII (2 μM), rC1s (1 μM), rMASP-2 (0.2, 0.6 or 2 μM) and kallikrein (0.2, 0.6 or 2 μM), rC1r (0.1, 0.3 or 1 μM) or culture medium alone was added. Proteases rMASP-2, kallikrein and rC1r were also applied in complex with their inhibitor, C1-INH (using a three-fold molar excess of C1-INH). Cell treatments were added directly to the culture medium, and the wavelength shift was monitored for 60 min. Wavelength shift curves were normalized to the medium control. The figures show representative graphs of at least 3 independent measurements. Source of this figure is the candidate's own publication [59].

3.2.4 Validation of the new hits found with EnLaB using well-established assays

To validate our results obtained using EnLaB, we performed intracellular Ca^{2+} mobilization assays and permeability tests. Treating HUVECs with rMASP-2 and kallikrein induced a Ca^{2+} response similarly to thrombin (which was used as a positive control). The proteases showed no such effects if applied in complex with C1-inhibitor. On the other hand, rC1r could not trigger the mobilization of intracellular Ca^{2+}. We chose rMASP-3 to represent the proteases that did not alter the wavelength shift curve in the Epic BT system, and consistently with those results, it failed to trigger a Ca^{2+} signal (for a visual representation of the results of Ca^{2+} measurements, see Figure 7 of our original paper at: https://www.nature.com/articles/s41598-020-60158-4). In the permeability tests, rMASP-2, rC1r and kallikrein were all able to increase paracellular permeability (**Fig. 13 B**) by the triggering of VE-cadherin disappearance from AJs leading to the disruption of EC-EC adhesion (**Fig. 13 A**). In accordance with the Epic BT results, rMASP-2 and kallikrein could be blocked by C1-inhibitor, while the effect of rC1r was again found to be insensitive to its natural inhibitor; moreover, the rC1r/C1-inhibitor complex induced an even greater monolayer permeability than rC1r alone. Another interesting observation was that rC1r (applied alone or in complex with C1-inhibitor) caused the total, homogenous loss of VE-cadherin from the surface of the cells, while the other proteases triggered the disappearance of this adhesion molecule only in the close proximity of the paracellular gaps. We repeated these experiments also with the plasma derived, full-length form of C1r (pC1r), which behaved similarly to the recombinant version in every respect (data not shown).

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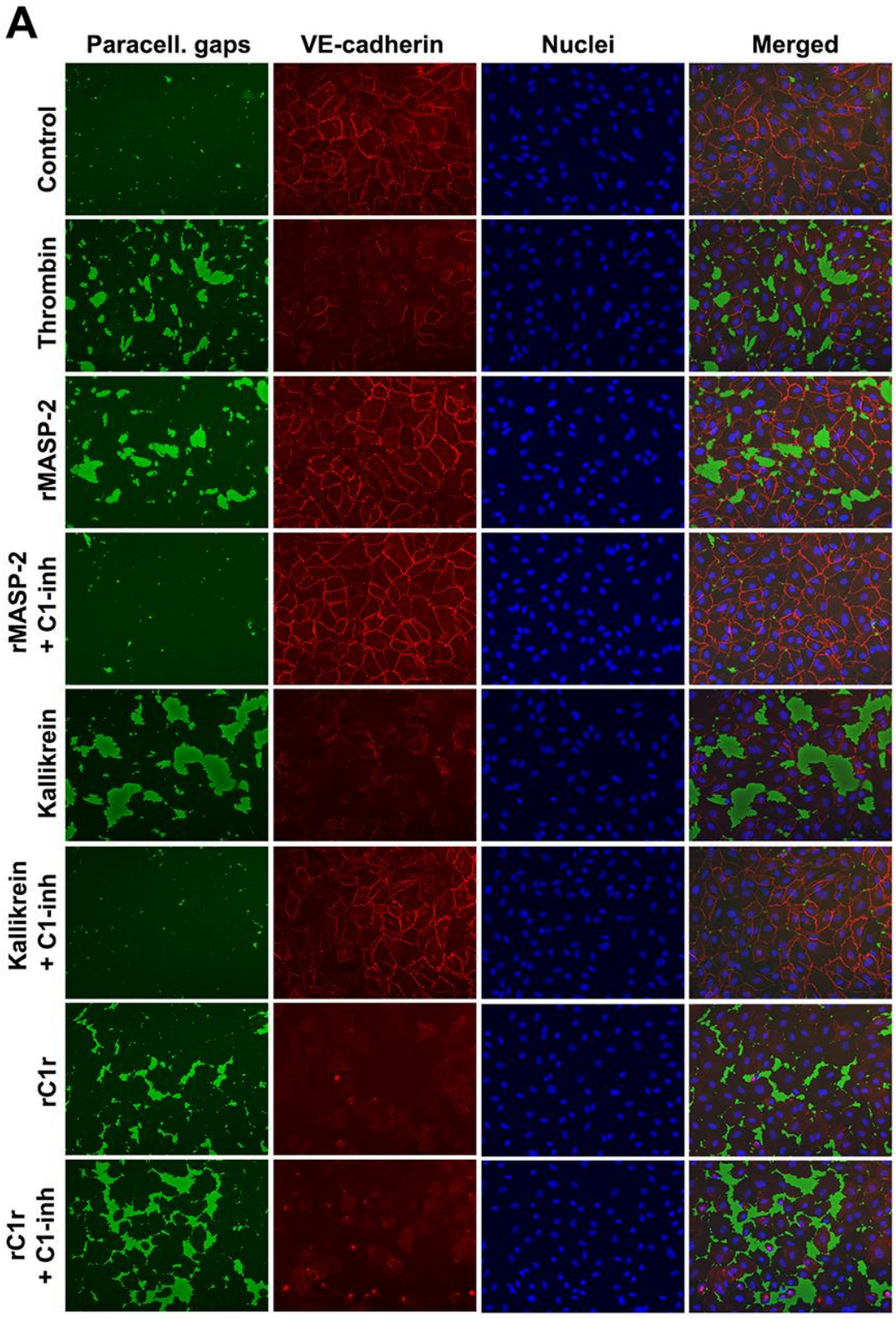

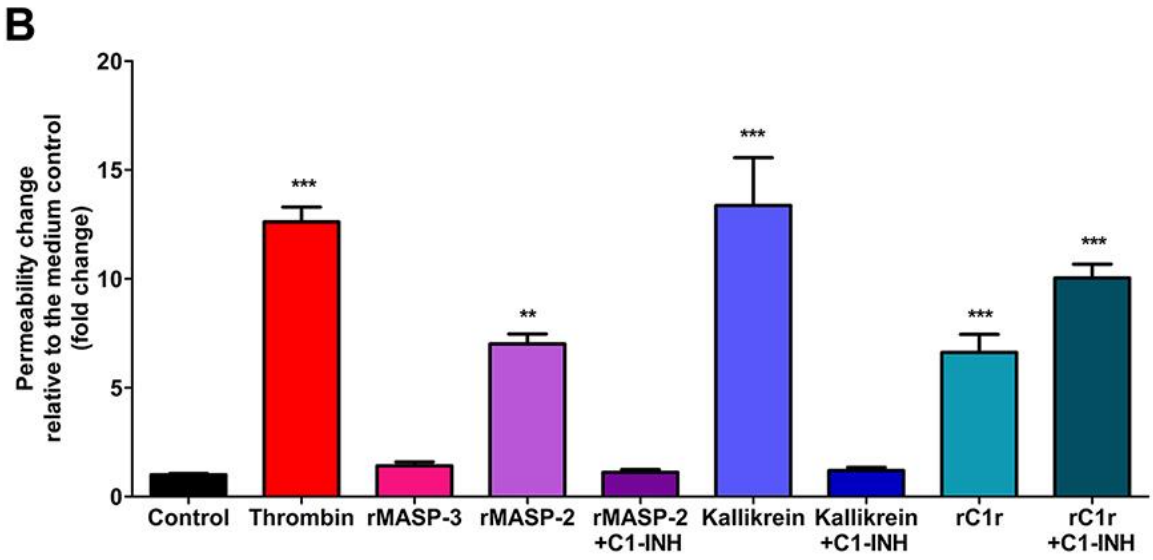

Figure 13. Effects of plasma serine proteases on VE-cadherin adhesion junctions and the permeability of HUVEC monolayers. A) A modified version of the XPerT technique was used for the permeability tests. Briefly, confluent layers of HUVECs were seeded onto biotinylated gelatin-precoated 96-well microplates and cultured for 2 days. Cells were treated with thrombin (300 nM), rMASP-3 (1 μM), rMASP-2 (2 μM), rMASP-2/C1-INH complex (2 μM/ 6 μM), kallikrein (2 μM), kallikrein/C1-INH complex (2 μM/ 6 μM), rC1r (1 μM), rC1r/C1-INH complex (1 μM/ 3 μM), or culture medium alone for 20 min and streptavidin-Alexa488 was added to each well for 2 min. After 2% paraformaldehyde fixation, VE-cadherin adhesion molecules were stained with anti-VE-cadherin antibody followed by an Alexa 568-conjugated seconder antibody. Cell nuclei were stained with Hoechst 33258. B) The total area of the streptavidin-Alexa 488 stained paracellular gaps was determined on each image using the CellP software. Fold-change data are the mean (+SEM) of three independent experiments calculated after normalization to the medium control.
**$p < 0{,}001$; ***$p < 0{,}0001$, relative to the control (One-way ANOVA with Tukey post-test).
Source of this figure is the candidate's own publication [59].

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4. DISCUSSION

Despite their direct physical contact, very little is known about the interactions between ECs and the circulating serine proteases of blood plasma. The one exception is thrombin, which is long known to activate ECs via cell surface PARs, leading to endothelial hyperpermeability. Therefore, we aimed to investigate the possible endothelial effects of other important serine proteases of the plasma cascades, concentrating mainly on the assessment of endothelial barrier properties. Among plasma serine proteases, we focused on those inhibited by C1-inhibitor as our laboratory has long been investigating the rare and potentially life threatening edematous disease, hereditary angioedema (HAE), a condition that develops in the absence of this serine protease inhibitor.

The first target serine protease of C1-inhibitor that came to the forefront of our attention was complement MASP-1. The question, whether MASP-1 can directly increase endothelial permeability arose from the facts that i) this protease shows structural and functional similarities with the well-known permeability increasing agonist thrombin, and ii) in the pathophysiology of HAE, deficiency of MASP-1's inhibitor, C1-inhibitor leads to edematous attacks, during which ECs become activated [42].

Using the xCELLigence system, which is an ideal platform for the real-time screening of potential permeability increasing agents [60] we observed that MASP-1 decreased the impedance of the endothelial monolayer, similarly to the well-known permeability increasing agonist thrombin. These results were confirmed at the level of macromolecular transport with XPerT permeability tests [44], where rMASP-1 could indeed increase endothelial permeability in a dose dependent manner and the effect could be blocked by its inhibitors (C1-inhibitor and SGMI-1). The contraction of ECs is achieved as the endpoint of an intricate, multistep intracellular signaling, which is initiated by the agonist-mediated activation of cell surface receptors (PAR-1 in the case of thrombin), shortly followed by a transient increase of intracellular [Ca^{2+}], a common sign of cell activation [61]. In previous studies of our group, it was shown that active MASP-1 can activate ECs, however, the catalytically inactive form is unable to initiate any cellular response. Therefore, the receptor of MASP-1 was expected to be among PARs, which was supported by the finding that MASP-1 can cleave synthetic, PAR mimicking substrates, most efficiently that of PAR-4 [9, 26]. PAR-4 was also found to be important in the inflammatory activation of HUVECs [9]. In the current work, we also tested the PAR-

dependence of the MASP-1 induced Ca^{2+}-signal. According to our current results, we identified PAR-1 as the main mediator of this process as only the PAR-1 antagonist pretreatment could prevent the effects of rMASP-1, although not completely. We observed a similar pattern regarding the PAR-dependence of the MASP-1 induced permeability: it also proved to be PAR-1 mediated. According to our earlier results with PAR-4 and the current observations with PAR-1 (especially that the MASP-1 triggered EC activation was only partly inhibited by the PAR-1 antagonist), it is plausible that besides the main player PAR-1, PAR-4, PAR heterodimers or other biased PAR-signaling could have an auxiliary role in the MASP-1 triggered permeability. Also, the differential contribution of PAR-1 and PAR-4 to the permeability and the inflammatory response, respectively, shows that MASP-1 may regulate EC phenotype in a complex way.

The formation of paracellular gaps in a monolayer is normally achieved via cell contraction, which requires the rearrangement of the cortical actin ring into stress fibers, along which centripetal force can be exerted by myosin activity. Cell contraction can only happen if MLC is in a phosphorylated state, which can be catalyzed by MLCK and ROCK [62]. In our experiments rMASP-1 had a similar effect on the cytoskeleton of ECs as thrombin: a prominent stress fiber formation and MLC phosphorylation was observed in both cases. The selective inhibitor of ROCK (Y-27632) could completely prevent the rMASP-1 induced cytoskeletal changes and permeability, while MLCK inhibitor ML-7 was ineffective. Similar findings were reported recently about thrombin as Y-27632 treatment completely prevented thrombin-induced MLC phosphorylation, stress fiber formation, and hyperpermeability in ECs, supporting the essential role of ROCK [15, 63], while ML-7 could not block the thrombin induced MLC phosphorylation, questioning the involvement of MLCK in the process [15]. Our findings about the indispensable role of ROCK in rMASP-1 induced endothelial hyperpermeability are also in agreement with the mechanism of action of this kinase as not only does it directly phosphorylate MLC but it can also inhibit MLC dephosphorylation which makes ROCK generally accepted as a key regulator of cytoskeletal changes [15, 64].

The moving apart of adjacent cells in a monolayer also requires the disruption of cell-to-cell junctions. We investigated the role of MASP-1 in this process by the visualization of some key molecules involved in EC adhesion. Treating HUVECs with rMASP-1 induced a marked disintegration of the network of VE-cadherin - a known indicator of endothelial

hyperpermeability [65] - and also lead to the disappearance of the TJ component ZO-1 from the junctional areas. The staining of PECAM-1 clearly demonstrated that similarly to thrombin, rMASP-1 greatly increases the size and density of paracellular gaps, which serves as a good visual explanation for the observed paracellular hyperpermeability.

Using whole transcriptome microarray analysis, we also investigated whether rMASP-1 can alter the expression of permeability-related genes and found that it can significantly change the level of 25 genes that can be linked to permeability regulation. The transcriptome analysis shed light to a possible interesting mechanism, namely rMASP-1 treatment was found to upregulate the expression of bradykinin receptor B2R, which mediates the edematogenic effect of bradykinin, known as a key factor in the development of HAE attacks [66]. By the upregulation of B2R, MASP-1 could possibly sensitize the endothelium towards bradykinin-induced hyperpermeability.

These results broadened our knowledge about the physiological effects of MASP-1 which can be summarized as follows (**Fig. 14**). Upon PRM binding to an activator surface (mostly pathogens of bacterial or fungal origin or damaged self-molecules), zymogen MASP-1 autoactivates. Active MASP-1 can then cleave its various soluble substrates in blood plasma – i.e., activates zymogen MASP-2 and C2 to continue lectin-pathway activation, also activates proteins of blood coagulation and fibrinolysis to regulate hemostasis, moreover it can possibly cleave HMWK to produce the edematogenic peptide bradykinin. Besides its soluble substrates, we have shown that MASP-1 can also cleave EC surface PARs, leading to an inflammatory activation of ECs, characterized by endothelial IL-6 and IL-8 cytokine production, and up-regulation of E-selectin expression. The EC-derived chemotactic cytokines then recruit neutrophil granulocytes to the endothelium, on which the surface-expressed E-selectin mediates their effective rolling. Our group has also shown earlier that the transmigration of neutrophils through the endothelial monolayer is facilitated by MASP-1, which can potentially contribute to the elimination of pathogens as the effector arm of innate immunity. In the current study, we demonstrated that this mechanism can possibly be augmented by the MASP-1-triggered endothelial hyperpermeability, which may allow the extravasation of soluble immune effector molecules (i.e., complement components, defensins and immunoglobulins) and thereby help in the fight against pathogens as the humoral arm of innate immunity. We also provided evidence that MASP-1 can possibly regulate

endothelial permeability by altering the expression level of permeability-related genes (for example B2R) as a prolonged effect.

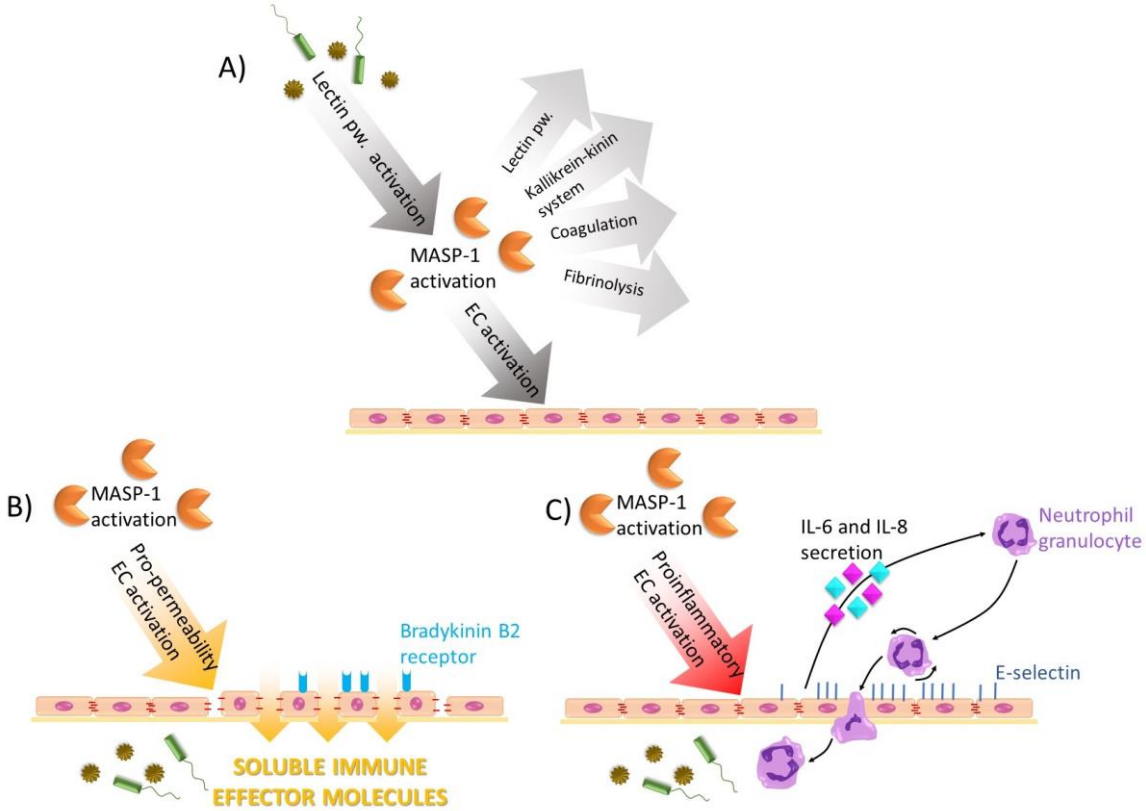

Figure 14. A visual summary of the effects of MASP-1 known to date. A) Upon bacterial or fungal infection, the lectin pathway of complement becomes activated and MASP-1 activation occurs. MASP-1 further activates its targets in the lectin pathway, the kinin-kallikrein system, the coagulation and the fibrinolytic cascades, moreover, it activates endothelial cells via PAR cleavage. B) MASP-1 induces a rapid permeability response in endothelial cells by which it can increase the extravasation of soluble immune effector molecules into the infected tissues. MASP-1 also changes the expression of permeability-related genes, for example it upregulates bradykinin B2 receptor expression and can thereby possibly sensitize endothelial cells towards other hyperpermeability stimuli. C) MASP-1 also induces a proinflammatory shift in the gene expression of endothelial cells and increases IL-6 and IL-8 secretion as well as E-selectin expression. These events together support neutrophil granulocyte recruitment, adherence and extravasation, facilitating the elimination of the invading pathogens.

Seeing the results with MASP-1 urged us to investigate the potential effects of other plasma components, especially serine proteases on the barrier properties of the endothelium. Given the great number of the molecules to be tested, this aim required a method that is suitable for high-throughput screening. We also wanted to exclude the risk that any kind of labeling alters the cellular effects of the tested compound, therefore we searched for a label-free solution. Among the label-free techniques, the RWG based Epic BT platform seemed to be the most ideal for our screening purposes as its principle of

operation offered the possibility of detecting any kind of cellular response with the real-time monitoring of dynamic mass redistribution and it is also capable of high-throughput screening [47, 48]. Therefore, we chose the Epic BT platform as a basis for our newly developed assay (EnLaB) for the label-free, high-throughput screening of plasma serine proteases and other soluble compounds on confluent endothelial monolayers.

In the process of assay development, the first issue was to make the biosensor surface compatible with HUVECs. On plastic surfaces this is routinely achieved by gelatin coating to mimic the basal membrane, however, its application on the biosensor surface should be carefully considered. The surface coating connects the endothelial monolayer with the optical transducer, therefore the optimization of surface coatings in evanescent field-based biosensor applications is a crucial step. The challenge is to create a film of gelatin on the biosensor surface thin enough to sense cellular DMR in the exponentially decaying evanescent optical field, while still providing full coverage and biological functionality (i.e. it should be robust enough to support EC attachment and growth). We optimized the process of gelatin coating using QCM, OWLS, AFM and RWG techniques and found that a gelatin coating applied in 0.2 mg/mL on the 384-well biosensor microplates creates a homogenous and highly hydrated layer thin enough for the biosensor to see through, while demonstrating ideal physical properties for the mechanical support of ECs. On this optimized surface coating we have successfully created a confluent and homogenous HUVEC monolayer, a widely accepted *in vitro* model of the inner vessel wall.

After the fine-tuning of cell-culturing, we worked out a simple and quick protocol for biosensor experiments using trypsin and thrombin – serine proteases that are known to affect the barrier properties of endothelial monolayers. These experiments proved that the new EnLaB method is an effective tool for the label-free screening of plasma serine proteases on ECs. Using EnLaB, we then tested the possible EC-related effects of a subset of plasma serine proteases that are natural targets of C1-inhibitor and thus are potentially interesting in HAE but are still underinvestigated on ECs. Among the tested serine proteases, rMASP-2, rC1r and kallikrein caused characteristic wavelength-shift curves fundamentally different from the untreated control. Effects of these proteases were further tested in well-established cell activation and permeability assays and their EC-related functions were verified.

MASP-2 has crucial roles in complement lectin pathway activation – in its absence, lectin pathway mediated complement C4 cleavage cannot take place, and the lectin pathway ceases before C3 convertase complex could form, therefore the effector functions of the complement system cannot be realized [67]. Despite its well-established importance in the complement system, to date, there are no direct cellular effects described for MASP-2, although it was found to be critically important in the pathomechanism of renal ischemia–reperfusion injury [68]. The characteristic wavelength shift curve observed with the EnLaB assay was found to be dose dependent and could be blocked if we applied rMASP-2 in complex with its natural inhibitor, C1-inhibitor. During the validation of the EnLaB results, the effects of rMASP-2 was further investigated using traditional assays. In agreement with the biosensor experiments, rMASP-2 treated HUVECs exhibited intracellular Ca^{2+} mobilization, a classical sign of GPCR-mediated EC activation. Furthermore, rMASP-2 treatment also induced endothelial hyperpermeability and markedly disintegrated the network of adhesion molecule VE-cadherin. The fact that all these effects could be blocked by C1-inhibitor suggests that the protease activity of MASP-2 is responsible for its cellular effects and its receptor is among PARs (similarly to MASP-1 [9] and thrombin [61, 69]), although further investigations with inactive mutant forms of MASP-2 are needed to confirm this hypothesis.

C1r has indispensable roles in the activation of the classical pathway of complement (similar to the roles MASP-1 has in lectin pathway activation), it autoactivates upon PRM (C1q) binding to activator surfaces (IgM or IgG containing immune complexes). Although the absence of C1r contributes to the development of the severe autoimmune condition systemic lupus erythematosus (SLE) [70], there are no known direct cellular effects of this protease. However, when we treated HUVEC monolayers with rC1r in the EnLaB assay, it resulted in a characteristic wavelength shift curve fundamentally different from the untreated control. The effect was found to be dose dependent, although, intriguingly, when we applied rC1r in complex with its only known natural inhibitor C1-inhibitor, it did not affect the response. Another interesting observation was that unlike all the other proteases tested, rC1r induced a positive wavelength shift in the EnLaB assay, similarly to the well-known permeability increasing small molecule histamine (data not shown). Due to the unique properties of the RWG based Epic BT platform – that the response curve can represent very complex cellular events and integrates all kinds

of mass redistribution happening in the evanescent field – it is currently impossible to identify the exact molecular mechanisms behind this effect. The validation experiments also provided interesting results as both rC1r and pC1r failed to elicit an intracellular Ca^{2+} mobilization in HUVECs (which was also unaffected if C1r was applied in complex with C1-inhibitor). On the contrary, rC1r and pC1r treatment resulted in a dramatic, homogenous loss of VE-cadherin from the junctional areas, and C1-inhibitor was not only unable to prevent this, but even intensified the effect. Consequently, rC1r (and also pC1r) significantly increased endothelial permeability, and this response was also greater when HUVECs were treated with C1r/C1-inhibitor complexes. Our results suggest that C1r disrupts the endothelial barrier independent of its protease activity, therefore we assume that the EC receptor of C1r cannot be among PARs.

Being the central protease of the kinin-kallikrein system, kallikrein is the main contributor to the generation of small proinflammatory and vasoactive peptide bradykinin, a well-known mediator of edema formation, especially important in the pathomechanism of HAE attacks [71]. Buillet et al. provided some experimental evidence suggesting the direct cellular effects of kallikrein, which was found to be able to increase endothelial permeability independent of bradykinin. The authors explained this effect by the kallikrein-mediated cleavage of VE-cadherin on ECs, but they did not investigate the phenomenon thoroughly [72]. In agreement with the above-mentioned results, we found that kallikrein indeed causes a characteristic negative wavelength shift curve, which effect was dose dependent and could be completely prevented if kallikrein was applied in complex with C1-inhibitor. Similarly to MASP-1, MASP-2 and thrombin, kallikrein was also able to induce intracellular Ca^{2+} mobilization in HUVECs, suggesting that this protease can activate intracellular signal transduction pathways in ECs. In our experiments, kallikrein treatment increased endothelial permeability by the disruption of VE-cadherin adhesion junctions and as these effects could also be completely blocked by C1-inhibitor, it can be suspected that the EC receptors of kallikrein are also possibly among PARs. Taken together our results with those of Buillet et al. and others suggest a triple effect of kallikrein on endothelial monolayers as it i) generates edematogenic factor bradykinin, ii) cleaves cell surface VE-cadherin on ECs, and iii) directly activates ECs possibly through PAR-cleavage and induce their active contraction.

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5. CONCLUSIONS

During my doctoral studies, I have contributed to the improvement of a permeability test and the development of an optical biosensor based cellular assay. With these novel approaches, we revealed hitherto unknown cellular functions of plasma serine proteases MASP-1, MASP-2, C1r and kallikrein. All these proteases were found to disrupt the endothelial barrier by the disintegration of adhesion junctions, which lead to an increased monolayer permeability. In the case of MASP-1, we have successfully identified some of the key players in the most important steps of the molecular mechanism of permeability induction. The directions are also clear for future studies, as the exact molecular events mediating the endothelial effects of MASP-2, C1r and kallikrein are waiting to be explored.

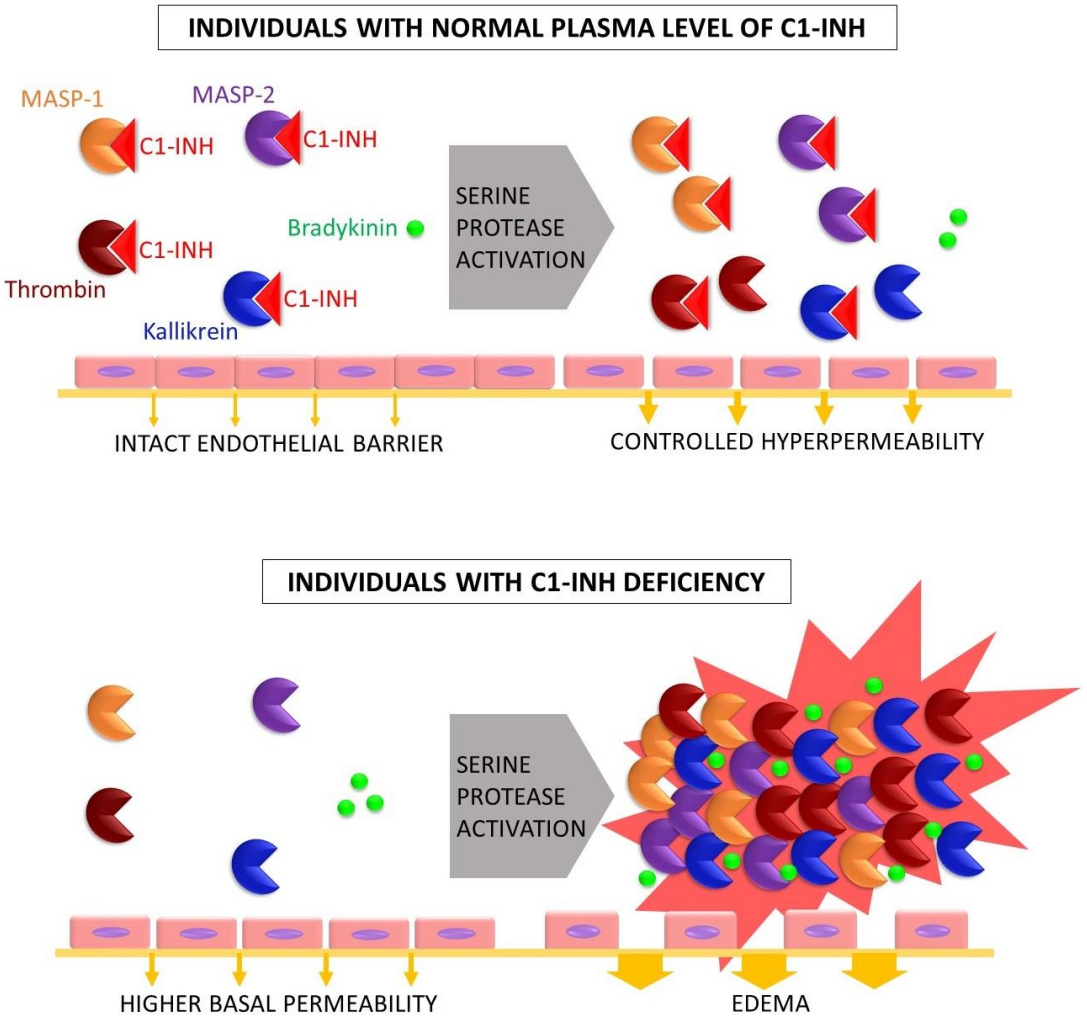

Figure 15. Hypothesis on the role of plasma serine proteases in the pathomechanism of hereditary angioedema. Based on our results we can make the following update to the hypothesis

of hereditary angioedema pathomechanism. In an individual with normal plasma levels of C1-inhibitor, plasma serine proteases and bradykinin production are under constant control, which supports an intact endothelial barrier. Upon infection, injury or other stimuli, serine protease activation occurs, but C1-inhibitor can still keep this enzyme activation and bradykinin production under control, therefore the hyperpermeability reaction is limited. On the contrary, individuals with C1-inhibitor deficiency are characterized by a higher basal enzyme activity, higher bradykinin levels and a higher basal permeability. If serine protease activation occurs in the affected individuals, endothelial permeability rises uncontrollably in the absence of C1-inhibitor, leading to edematous attacks.

Our findings also have pathophysiological implications. Normally, all the newly identified permeability increasing serine proteases are controlled by the same serpin, C1-inhibitor, therefore in HAE, where C1-inhibitor is deficient, these proteases may contribute to the onset, the worsening or the sustainment of edematous attacks (**Fig. 15**). Moreover, these serine proteases may also play a role in other life-threatening edematous conditions such as sepsis or ischemia-reperfusion injury and once their exact role is clarified, they can potentially become targets of future drug development. Finally, we have also successfully worked out a very promising, easy-to-use, label-free assay, EnLaB that can later be used for the high-throughput screening of other subsets of plasma components or even drug candidates on endothelial as well as epithelial monolayers.

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6. SUMMARY

Constituting the inner lining of vessel walls, ECs come into direct physical contact with the components of blood plasma, including plasma serine proteases. It is well-known that serine protease thrombin can activate ECs and increase endothelial permeability, however, very few other serine proteases have been tested on ECs in this respect. One important serine protease of the complement system is MASP-1, which plays a key role in the activation of the lectin pathway. Similarly to thrombin and many other plasma serine proteases, activity of MASP-1 is also controlled by C1-inhibitor. In certain conditions – such as HAE, sepsis or SARS-CoV-2 infection – deficiency of C1-inhibitor is accompanied by pathologic edema formation. As MASP-1 shows enzymatic similarities with thrombin and is a target of C1-inhibitor, it was important and clinically relevant to find out whether MASP-1 is also able to increase endothelial permeability.

Using several methods, we took a complex approach to investigate the effects of MASP-1 on the permeability of the endothelial monolayer and to reveal some of the key steps of the underlying intracellular signaling. We proved that MASP-1 can indeed significantly increase endothelial paracellular permeability by the rearrangement of the endothelial cytoskeleton and adhesion molecules and we identified PAR-1 as the main receptor and ROCK as an important kinase mediating this effect. The results with MASP-1 have encouraged us to search for other plasma serine proteases that could exert direct effects on the endothelium. For this purpose, we developed a new, optical biosensor based, high-throughput and label-free assay, which we found to be suitable for the real-time screening of plasma serine proteases on endothelial monolayers. Using this new approach validated with traditional assays, we identified additional target serine proteases of C1-inhibitor – MASP-2, C1r and kallikrein – as hyperpermeability agonists on endothelial cells.

The discovery of the permeability increasing effect of four plasma serine proteases – for three of which had no known direct cellular effect published before – suggests yet unknown mechanisms of innate immunity, i.e., that these enzymes could prompt ECs to contract and thereby to actively contribute to the enhancement of complement leakage to the infected tissues. Additionally, our research can also have pharmaceutical significance as our newly developed label-free method can be suitable for the simple and high sensitivity screening of drug candidates and other molecules on endothelial and epithelial monolayers.

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8. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

8.1 Publications on which the dissertation is based

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8.3 Publications independent of the subject of the dissertation

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