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ALTERED INTERACTIONS BETWEEN THE ENDOTHELIUM AND MYELOID CELLS AFFECT THE VASCULAR RESPONSE TO INJURY IN PATIENTS WITH CARDIOVASCULAR DISEASE

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ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACE	angiotensin-converting enzyme
ADP	Adenosine diphosphate
Akt	protein kinase B
ApoA1	apolipoprotein A1
ATP	adenosine triphosphate
BK	bradykinin
CCR2	C-C chemokine receptor type 2
CETP	Cholesteryl ester transfer protein
cGMP	cyclic guanosine monophosphate
CV	cardiovascular
CVD	cardiovascular diseases
CX3CR1	C-X-X-X-C chemokine receptor 1; fraktalkine receptor
CXCR4	C-X-C chemokine receptor type 4
cGMP	cyclic guanosine monophosphate
DPP-4	dipeptidyl peptidase 4
DNA	Deoxyribonucleic acid
EOC	early outgrowth cells
EC	endothelial cell
EPC	endothelial progenitor cell
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinases
GPCR	G-protein coupled receptor
GF	Growth factors
HDL	high density lipoprotein
ICAM-1	intercellular adhesion molecule 1
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	Janus kinase
JAM	junctional adhesion molecule
JNK	c-Jun N-terminal kinase
B1R	kinin B1 receptor
B2R	kinin B2 receptor
LDL	low density lipoprotein
LOX-1	lectin-like oxidized LDL receptor type 1
LFA-1	leukocyte functional antigen 1
MAPK	mitogen-activated protein kinase
miR	microRNA
mRNA	messenger RNA
NADPH	reduced Nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

NO	nitric oxide
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphoinositol-3-phosphate
PKA	protein kinase A
ROS	reactive oxygen species
RNA	ribonucleic acid
S1P	sphingosine-1-phosphate
SDF-1	Stromal cell-derived factor 1
SERCA	sarco/endoplasmic reticulum Ca2+-ATPase
SMV	shed microvesicles
SMC	smooth muscle cell
SR-B1	scavenger receptor B1
STAT	signal transducers and activators of transcription
SDMA	symetric dimethyl arginine
TF	tissue factor
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VLA-4	very late antigen 4
WHO	World Health Organization

3. Introduction

3.1.   Cardiovascular diseases and their epidemiological implications

The vascular system permeates the entire human body, delivering oxygen and nutrients to organs, removing metabolic products and providing a transport system for cells and biological mediators, thus facilitating communication between distant organs. Blood circulation is maintained by the heart, a chambered hollow muscle, which is directly connected to the vasculature. Reflecting those basic physiological observations, one can easily apreciate that dysfunctions of the cardiovascular system are potentially life-threatening. Indeed, cardiovascular diseases (CVD) constitute the main cause of death world wide. According to the 2008 survey of the World Health Organization (WHO), more than 30% of all cases of death were caused by CVD *(Mendis et al, 2011)*.

The term "cardiovascular diseases" is not sharply defined, summarizing diverse pathologies of the heart and of the vascular system. In the field of epidemiological statistics, for example, congenital malformations of the heart and vasculature are usually not included *(„International Statistical Classification of Diseases and Related Health Problems (ICD)" of the World Health Organization)*, while in applied medicine, those diseases are often included.

The largest part of CVD is caused by dysfunctions of the vasculature and organs due to hypertonus and/or atherosclerosis. Apart from non-modifyable risk factors (such as genetic predisposition, age and sex), cardiovascular mortality among the general population is enhanced to a high and still rising degree by lifestyle-associated cardiovascular risk factors, including smoking, physical inactivity and calorie uptake exceeding demand over long time periods *(Mendis et al, 2011)*. In fact, even in patients with an unfavourable genetic background, a healthy lifestyle can reduce the cardiovascular risk to a level below that of patients with a favourable genetic background, but unfavourable lifestyle *(Khera et al, 2016)*. This observation is in line with the concept that genetic dysfunctions gaining relevance only when challenged by lifestyle factors, which has been develped for various diseases wit multigenetic cause, including type 2 diabetes mellitus *(Fuchsberger et al, 2016)*. With the improvement of the economic situation for large parts of the world's population in countries such as India and China, associated to the adoption of high-calorie diets and the reduction of physical activity, a rise in the number of CVD cases is expected word-wide, leading to increasing economical as well as social costs incurred due to treatment costs and loss of productivity, but also to reduced quality of life.

A better understanding of the molecular and cellular processes contributing to the maintenance of vascular health, as well as cost-effective, life-style based means to protect and improve endothelial integrity, might thus aid the delopment of tools and strategies to combat the expected wave of CVD cases in the near and mid-future.

3.2. Physiological and pathological relevance of the vascular endothelium

The luminal side of all blood vessels is lined with a single layer of endothelial cells. This endothelial layer represents an interface between substances, vesicles and cells within the blood and the outer layers of the vessel wall as well as the surrounding tissue. Contradicting the historical understanding of the blood vessels as passive conduits, the blood vessel wall plays an active role in modulating the exchange of substances between blood and tissue. The vascular endothelium regulates the function of adjacent cells and provides a docking scaffold and interaction platform for circulating cells and extracellular vesicles.

The density, permeability and molecular and functional characteristics of the endothelium differ between various types of blood vessels *(reviewed in (Aird, 2007a; Aird, 2007b))*. Concomittantly, pathological disorders of the endothelium vary between the types of vessel afflicted. Atherosclerotic remodelling of the vascular wall is observed in the arteries, while thrombotic events occur in both, arterial and venous vessels albeit following distinct mechanisms, and retraction and dissociation of microvessels - responsible for impaired wound healing and organ perfusion in type 2 diabetes - affect capillaries and arterioles.

The functions of the vasculature include the reglation of vasotonus, and thereby blood pressure, the modulation of hemostasis, the regulation of transport processes between blood and underlying tissue, and the adaptation of the vascular layout to altered conditions, such as during organogenesis, during healing of acute injuries, or upon altered oxygen and nutrient requirements, as in exercising athletes.

Barrier function: The endothelium regulates the passage of molecules and cells between blood or lymph and the surrounding tissue. This regulation is effected by the establishment or degradation of inter-cellular junctions in response to stimuli received by the endothelial cell, thus creating and adjusting a size cut-off for molecules of certain size, or cells (*Zihni et al, 2016*). Tight junction molecules – such as occludin, claudins, JAMs and tricellulins – appear to play a major role in cardiovascular diseases (*Taddei et al, 2008*). Large molecules or cells preferentially use tricellular junctions, i.e. locations where three endothelial cells meet (*Krug, 2017; Krug et al, 2009*). Cells can furthermore use the "transcellular route", i.e. through channels within an endothelial cell, as opposed to the pericellular route between endothelial cells. Through both, the transcellular and the pericellular routes, leukocytes are actively aided and guided by the endothelial cell (*van Buul et al, 2007; van Buul et al, 2010b*). It appears that adhesion and the "guidance" of leukocytes through the endothelial layer utilize the same or at least co-regulated signalling mechanisms *(Krug, 2017; Taddei et al, 2008; van Buul et al, 2010b)*. The regulation of junctional proteins, and thereby endothelial barrier function, can be regulated by blood flow characteristics (velocity and pattern, such as laminar versus turbulent) and inflammatory and metabolic messengers (reactive oxygen species, cytokines, lipoproteins) (*Garcia-Polite et al, 2017; Kang et al, 2014; Magalhaes et al, 2016; Nourshargh & Alon, 2014; van Buul et al, 2010a; Weber et al, 2007*). Indeed, the partial loss of endothelial barrier function at sites of non-laminar blood flow, in combination with inflammatory/metabolic stimuli is considered to consist the first step in the development of atherosclerotic plaques (*Dhawan et al, 2010*).

Endothelial modulation of the vasotonus: Upon sensing physical (shear or strain) or chemical (e.g. kinins) cues endothelial cells act on the vasotonus by secreting relaxing as well as constricting factors affecting the underlying smooth muscle cell layer. A prominent vasorelaxing factor - termed endothelial-derived relaxing factor before its molecular identification - is the gas nitric oxide (NO). NO is a radical, stable for only few seconds, but it can pass cell membranes and thereby affect neighbouring smooth muscle cell. NO synthesized by endothelial cells diffuses into the adjacent smooth muscle cells, where it induces a rise in intracellular Ca^{2+} levels via the small messenger molecule cyclic guanosine monophosphate (cGMP), a product of the NO-sensitive guanylate cyclase. Subsequent phosphorylation events lead to the opening of Ca^{2+}-sensitive K^+ channels and myosin light chain phosphorylation (*Archer et al, 1994*). Ultimately, those signalling events modulate actin-myosin interaction and cause cellular

hyperpolarization, respectively, effecting the relaxation of the smooth muscle cell. Endothelial-derived vasorelaxing factors, including bradykinin, make use if this activation mechanism (*Archer et al, 1994*).

Re-adaptation of the vascular layout: During embryogenesis and postnatal growth, but also in the adult, the vascular system is subject to constant re-organization and adaptation. Growth processes, such as vasculogenesis (mainly in embryonic development) and angiogenesis (pre- and post-natal) as well as remodeling processes, such as arteriogenesis, and retraction of capillaries are regulated by the interplay between endothelial cells, extracellular matrix, paracrinally active cells and pericytes/smooth muscle cells, which stabilize the vascular wall and also affect endothelial cell behaviour (*Campagnolo et al, 2010; Form et al, 1986; Kodelja et al, 1997; Nicosia et al, 1993; Tattersall et al, 2016*). Depending on the patho-physiologic situation, remodelling by arteriogenesis, or growth processes like angiogenesis may posess larger importance. Therefore, individual molecular and cellular players affecting vascular layout vary in different pathological states (*Hoefer et al, 2013; Tuttle et al, 2002; Wahlberg, 2003*). In patients with established cardiovascular or peripheral vascular disease, but already before the manifestation of the disease, in states of increased cardiovascular risk, such as hypertension or diabetes, reduced capillarization and altered capillary layout have been observed in the heart and limbs, affecting wound healing and organ perfusion, but also the remodelling responses necessary to bypass occluded vessel sections (*de Groot et al, 2009; Lind & Lithell, 1993; Rakusan & Turek, 1986*). While dysfunction of endothelial cells - the effector cells of neo-vascularisation - had been extensively studied earlier, their control by cells with paracrine activity has moved to the focus of attention during the recent decades (*Al Haj Zen et al, 2010; Patel et al, 2013a*). Moreover, immature cells with endothelial differentiation potential are thought to be present also in the adult and might be involved in vascular growth or healing by homing to the site of injury and differentiating/maturing into the needed vascular cell type (endothelial, smooth muscle) in situ, albeit these events appear to be extremely rare (*Daniel et al, 2010; Purhonen et al, 2008*). The majority of bone marrow-derived cells homing to injured or newly forming vessels might indeed belong to the haematopoietic lineage, providing paracrine support and guidance (*Purhonen et al, 2008*). In addition to chemical cues, remodelling processes such as arteriogenesis, i.e. the increase in vessel diameter of arteries, are stimulated by mechanical variables, such as an increase in shear stress and radial wall stress (*Heil & Schaper, 2004; Prior et al, 2003*). In this context, a large increase of flow is necessary to induce an increase of vessel diameter, while decreased flow very reliably induces a decrease in vessel diameter (*Brownlee & Langille, 1991; Tuttle et al, 2001*). Flow also appears to be a major driving factor in microvascular organization, guiding migration patterns of endothelal cells (*Franco et al, 2015; Gebala et al, 2016*).

Hemostasis/Coagulation/Thrombosis: Quiescent endothelial cells do not activate intrinsic or extrinsic coagulation pathways, but instead exert potent anti-coagulative action via their expression of heparan sulfate and thrombomodulin (*(Dekker et al, 2006); reviewed in: (Martin et al, 2013)*). Moreover, via the secretion of prostacyclin and by the conversion of ADP into ATP, endothelial cells inhibit platelet activation (*reviewed in (Schafer & Bauersachs, 2008)*). However, upon injury or exposure to pro-inflammatory agents as well as to non-laminar or reduced shear stress, endothelial cells – as well as other cell types, such as monocytes or platelets - can assume a pro-coagulant state (*Wu et al, 1995*). A main role in the initiation of the extrinsic pathway of coagulation in those circumstances is occupied by the transmembrane receptor tissue factor (TF). Moreover, the removal of endothelial cells, or their detachment during endothelial erosion, allow collagen and TF within the vascular wall to contact plasma factors, which also activates the extrinsic coagulation cascade (*reviewed in: (Mackman et al, 2007)*).

3.3. Molecular and cellular regulators of endothelial function

While the endothelium impacts on other cell types in the vicinity, endothelial function in itself is regulated by a variety of biochemical and mechanical cues. Biochemical signalling factors can act over short (paracrine) or long

(endocrine) distances, depending on their stability and reactivity (affected by degrading enzymes and reaction partners), polarity and size (both of which affect their capacity to pass through biological membranes) and the local distribution of interaction partners, such as receptors, binding proteins or radicals. The mechanisms regulating endothelial function therefore resemble an intricate network of interconnected regulatory signalling pathways. In engineering terms, the tight integration of endothelial function into a network of feedback mechanisms allows for immediate reaction to acute stimuli, while at the same time conveying a measure of stability on a longer time scale despite recurring challenges. The same principles however, can result in a long-term dysregulation if individual variables and corrective mechanisms are only slightly off. In a molecular context, the efficiency of therapies and prevention strategies, such as statins or regular exercise training, relies on their targetting of multiple pathways, thereby affecting endothelial function via direct effects as well as via the modulation of circulating (inflammatory) factors *(reviewed in: (Golbidi & Laher, 2012; Jasinska et al, 2007))*. Disappointing shortcommings of recent therapy forms, such as the transplantation of autologous stem/progenitor cells might in part also be explained by the fact, that adverse milieu conditions remain insufficently treated, thereby hampering survival and behaviour of the transplanted cells at the target site.

3.3.1. Endocrine factors

The variety of circulating molecules identified to affect endothelial function encompasses proteins, lipids/lipoproteins and nucleic acids, especially short or long non-coding RNAs. Circulating factors can occur "naked", bound in complexes or contained within membranous vesicles, such as exosomes or shed microvesicles. Free or complexed circulating factors can bind to endothelial cell surface receptors or can be taken up via membrane transporters *(reviewed in: (Besler et al, 2012; Mause & Weber, 2010; Tedgui & Mallat, 2006))*. Factors transported within membrane vesicles can furthermore enter the endothelial cell upon endocytosis of the vesicle *(reviewed in: (Mathivanan et al, 2010; Mause & Weber, 2010))*.

Cytokines can act over short as well as long distances; therefore it is difficult to ascribe purely para- or endocrine function to them. Originally, the definition for a cytokine required it to be involved in immune response as well as inflammation. In endothelial cells, however, cytokines can induce inflammatory changes even in the absence of pathogens. Cytokines and their receptors include molecules with wide structural heterogeneity, such as the immunoglobulin and tumor necrosis factor receptor superfamilies and G-protein coupled receptors. In endothelial cells, cytokine exposure, via the transcription factor NF-κB, leads to the enhanced expression of cell adhesion molecules (ICAM-1 and VCAM-1) on the endothelial surface *(De Martin et al, 2000)*, facilitating the recruitment of leukocytes from the circulation, which in parallel get activated by the present cytokines, resulting in the release of reactive oxygen species, proteases and further paracrine factors (*as discussed below*). By direct effects on endothelial gene expression, as well as via the recruitment of leukocytes, cytokines can furthermore induce the dysregulation of junctional proteins and thereby mediate the loss of endothelial barrier function *(Edens & Parkos, 2000; Wojciak-Stothard et al, 1998)*. The resulting "seeping out" of plasma proteins from the blood stream into the sub-endothelial vascular layers is considered an initial event in the formation of atherosclerotic plaques *(reviewed in (Guyton et al, 1990))*. Chemokines are considered a sub-group of cytokines which are able to induce the directed migration – chemotaxis – of cells, such as leukocytes or endothelial cells. Chemokines posess some structural heterogeneity with regard to the number of amino acid residues interspacing their first two cysteine residues, thus forming four groups, CC, CXC, CX$_3$C and C chemokines. All chemokines interact with G-protein coupled receptors (GPCRs), which then transmit signalling via small G proteins unto kinase pathways, including PI3K, PKA and MAPK pathways [*reviewed in: (Curnock et al, 2002; Patel et al, 2013b)*]. Monocyte chemoattractant protein-1 and fractalkine, via their receptors CCR2 and CX3CR1 take a prominent role in the recruitment of distinct subtypes of monocytes/macrophages to the vascular wall (*Auffray et al, 2007*). Both recruitment mechanisms, as

well as the distinct monocyte subtypes, play a role in regenerative, as well as pathologic processes within the vascular wall (*Dawson et al, 1999; Saederup et al, 2008*).

Growth factors (GF) represent another heterogenous group of proteins affecting endothelial cell functions. Most GFs signal through specific tyrosine kinase receptors. Upon extracellular ligand binding, the receptor dimerizes, which allows autophosphorylation of intracellular tyrosine residues, followed by adaptor protein binding and a cascade of further phosphorylation events, e.g. initiation of the ERK1/2 MAPK and PI3K pathways, leading to gene expression changes, cytoskeletal alterations and generation of NO, finally affecting adhesive proterties, survival, proliferation and migration of the cell *(Fry et al, 1993; Jeltsch et al, 2013)*. Dysfunctions in kinases downstream to GF receptors, such as the JNK and the protein kinase B (Akt), as well as increased phosphatase activity are implicated in the development of endothelial dysfunction in states of increased cardiovascular risk, such as adipositas or type 2 diabetes *(Hirosumi et al, 2002)*.

Lipids and lipoproteins greatly affect endothelial homeostasis, as well as the inflammatory reaction which is so essential in the development of atherosclerosis *(Avraham-Davidi et al, 2013)*. While LDL is considered the "bad" cholesterol, a beneficial role in vascular biology is usually ascribed to HDL, since circulating levels of LDL are positively correlated and circulating levels of HDL are negatively correlated with CV risk *(Castelli et al, 1986; Gordon et al, 1981)*. However, a number of studies support the notion that the composition of the particle, as well as post-translational modifications, such as oxidation and carbamylation might carry the greater importance for the effects of HDL in vascular biology, than only quantity *(Schofield et al, 2013; Schwartz et al, 2012; Van Lenten et al, 1995)* *(Figure 1)*. In addition to lipoprotein particles, some of their lipid components, such as the lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid, can also circulate in the blood bound to plasma proteins *(Avraham-Davidi et al, 2013)*.

The various effects of LDL, native, as well as in its modified, i.e. oxidized and carbamylated, forms, on the development and progression of atherosclerosis have been well studied *(Badimon & Vilahur, 2012; Brown & Goldstein, 1983; Gouni-Berthold & Sachinidis, 2004)*. LDL - native or modified - signals through several receptors, including the LDL receptor, the lectin-like oxidized LDL receptor type 1 (LOX-1) and the macrophage scavenger receptor, thus mediating the generation of reactive oxygen species as well as pro-apoptotic and pro-inflammatory signalling of differential extent in various vascular cells, including endothelial and smooth muscle cells, but also monocytes and macrophages *(Allen et al, 1998; Apostolov et al, 2009; Frostegard et al, 1991; Shi et al, 2011; Yokode et al, 1988; Zhu et al, 1999)*. A number of enzymes can oxidize LDL components or regulate this process, including myeloperoxidase and lipoxygenase. Oxidized LDL induces foam cell development, but also acts as an autoantigen, inducing immune defense mechanisms *(Samson et al, 2012; Yokode et al, 1988)*. In addition, a role of carbamylation of LDL in atherosclerotic signaling has been identified *(Apostolov et al, 2009; Carracedo et al, 2011)*.

HDL can confer anti-inflammatory actions to endothelial as well as immune cells (*Bursill et al, 2010; Cockerill et al, 1999; Fleisher et al, 1983*). Apolipoprotein A1 (ApoA1), the typical Apolipoprotein of HDL, exhibits signalling as well as scaffold properties. By binding to the ABCA1 transporter, ApoA1 promotes the efflux of cholesterol from macrophages to the HDL particle, which then effects cholesterol transport back to the liver. Via other components, HDL can also bind further receptors expressed on endothelial cells, including the sphingosine-1-phosphate receptor 1/3 and the scavenger receptor BI, subsequently activating G-protein mediated pro-survival, -migration and -proliferation signaling cascades *(reviewed in (von Eckardstein & Rohrer, 2009))*. Furthermore, adenosine triphosphate-binding cassette transporters ABCA1 and ABCG1 are involved in HDL transcytosis through endothelial cells, the cholesterol uptake by macrophages, anti-oxidant and anti-inflammatory signalling and also

Figure 1: HDL and LDL effects on leukocyte and endothelial cell function. [*Kränkel, unpublished*]
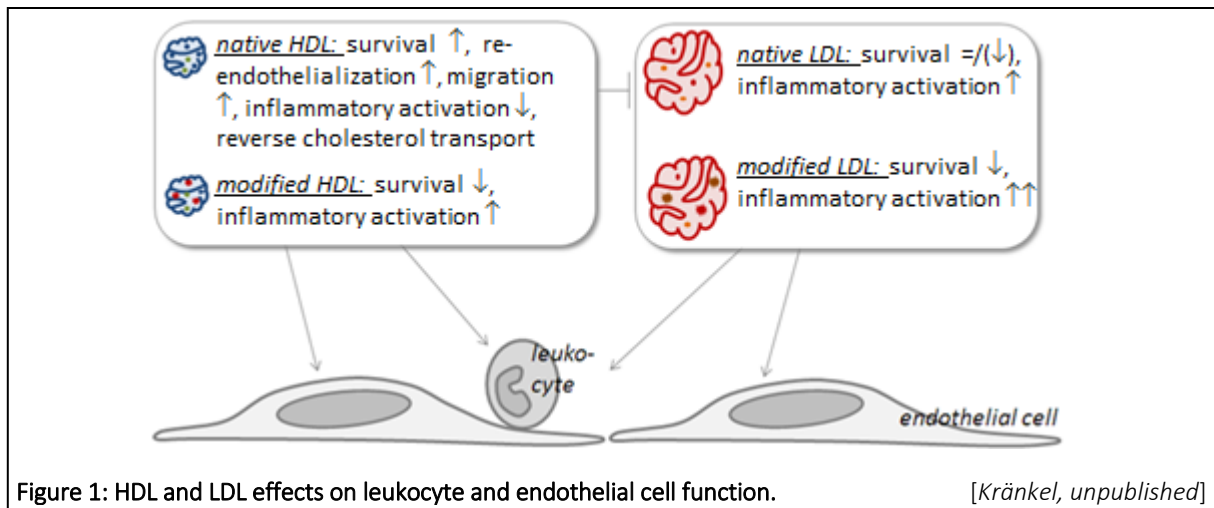
monocyte and neutrophil generation (*(Rohrer et al, 2009; Westerterp et al, 2013), reviewed in (Tall, 2008))*. ApoA1, and by extension HDL, are therefore accounted major anti-atherogenic molecules. However, this view of the functional properties of HDL has proven somewhat simplistic, as therapy strategies targeting only HDL quantity within the circulation, such as the application of Cholesteryl ester transfer protein (CETP) inhibitors, did not succeed in reducing cardiovascular risk *(Barter et al, 2007; Schwartz et al, 2012)*. It might therefore be that therapies targeting multiple biological pathways, such as statins or physical exercise, are necessary in order to improve HDL quality, i.e. composition and post-translational modification of its components, in addition to quantity. Within the circulation, ApoA1 can serve as a stabilizing factor, e.g. for protacyclin, and as a scaffold protein, e.g. for symetric dimethyl arginine (SDMA, an inhibitor of NO synthesis) and can thereby allow them to expand their active distance and repertoire of binding partners *(Speer et al, 2013; Yui et al, 1988)*. Therefore, cellular effects evoked by ApoA1 as well as HDL-mediated mechanisms might be affected in pathology due to an altered repertoire of associated proteins. Moreover, both of these mechanisms might affect endothelial cells differently than macrophages. To date, we are only beginning to survey the spectrum of proteins bound within the lipoprotein particles in physiological and pathological conditions. In parallel, seminal studies are under way to delineate the effects of lipoprotein modification in the different pathologies as well as with regard to its localization (flowing blood versus vascular wall, where it is much more exposed to the action of oxidating enzymes and radicals). Towards that end, there is evidence that HDL modifications within atherosclerotic plaques might differ from HDL quality within the circulation of the same patient *(Didonato et al, 2013)*. Several groups of drugs (such as statins) as well as lifestyle interventions (exercise training) are able to target cholesterol particles as well as inflammatory markers *(Adams et al, 2013; Ridker et al, 2008; Ridker et al, 2005)*. In fact, their pleiotropic effects, including the modification of cholesterol-associated lipoproteins and proteins, might be necessary to achieve a relevant reduction in cardiovascular risk, which cannot be achieved by the quantitative modulation of circulating levels of lipoproteins alone (Bruckert et al, 2010; Ridker et al, 2009). In a similar manner, exercise training, has recently been shown to improve HDL functional effects on endothelial cells *(Adams et al, 2013)*.

Extracellular Vesicles: The term "extracellular vesicles" describes a heterogenous population of membrane vesicles of cellular origin, found in virtually all fluids of the human body. Their size ranges from ca. 30nm to ca. 1μm. Vesicles shed from the cell membrane, measuring about 100nm to 1μm are usually referred to as microparticles or shed microvesicles (SMV). Instead, smaller vesicles (ca. 30nm to 100nm in size), derived from the intracellular multivesicular bodies, have been termed exosomes (ca. 50-100nm) or exosome-like vesicles (ca. 30-50nm). Extracellular vesicles in general can transport nucleic acids, lipids and proteins from the cell of origin to the target cell in an endocrine or paracrine fashion. The actual mechanism of signal transduction can vary from the activation of endothelial surface receptors by ligands exposed on the surface of the vesicle, via the transfer of proteins and

lipids by surface transporters to the transfer of the soluble and membrane fractions of the vesicle to the cell upon endocytosis *(Mause & Weber, 2010)*. Within the target cell, this leads to a change in signalling events ultimately modulating cellular function.

Currently the spectrum of effects on physiological and pathophysiological processes of exosomes and microvesicles is only partially understood. It has been observed that microvesicle release is enhanced upon activation of the cell of origin by certain stressors including tumor necrosis factor α (TNF-α) *((Jimenez et al, 2003), reviewed in: (Montoro-Garcia et al, 2011))*. One recently identified mechanism with high vascular relevance is the release of vesicles containing the microRNA-126 (miR-126) from apoptotic endothelial cells. In the adjacent (recipient) endothelial cells, the uptake of miR-126 containing vesicles leads to the upregulation of anti-apoptotic signalling, thus conveying a measure of protection to endothelial cells adjacent to injured endothelial areas *(Zernecke et al, 2009)*. On the other hand, microvesicles might also be involved in inducing thrombotic events and in maintaining a systemic state of endothelial dysfunction *(Angelillo-Scherrer, 2012; Boulanger et al, 2001; Densmore et al, 2006; Siljander et al, 1996)*. At the moment, investigations are still ongoing to determine the individual effects of microvesicles in various disease conditions or upon therapy.

While shed microvesicles (SMV) carry signature molecules of their cells of origin on their surface, thus allowing to quantify SMVs derived from platelets, leukocytes or endothelial cells within the blood, additional research is required to identify markers denoting the cell of origin for exosomes although the existence of several exosome populations is evidenced (*Koliha et al, 2016; Willms et al, 2016*). Moreover, their small size precludes their high-throughput quantification by commercially available flow cytometers, while following careful setup this is possible for SMVs (*Shah et al, 2008; van der Pol et al, 2014*). First studies investigating the potential of microvesicles for clinical diagnostics are therefore focussed on SMVs rather than on exosomes. Indeed, certain SMV species could be predictive for cardiovascular outcome in certain patient populations *(Amabile et al, 2012; Nozaki et al, 2010; Sinning et al, 2011; Werner et al, 2006)*. However, more studies are needed to better define extracellular vesicle species with a predictive value, as well as the relevance of those in the distinct pathologies. The high inter-individual variability in SMV numbers as well as not well standardized quantification protocols currently pose additional limitations to their diagnostic employment at the moment (*Ayers et al, 2015*). Of note, the markers used to define „endothelial" SMVs in particular differ between working groups. The presented data therefore need to be revised carefully, as many markers thought before to be specific for endothelial cells are also present on other cell types (e.g. CD31 on platelets).

Non-coding RNAs: Micro-RNAs (miRs) represent a class of short regulatory nucleic acid molecules. After transcription, miRs form a hairpin structure, which is then processed within the nucleus by an enzyme complex containing the nuclease Drosha. The pre-miR is then transferred into the cytoplasm via the transporter exportin 5. Following further pocessing, by an enzyme complex containing dicer, a short single-stranded mature miR is generated, which can interact with the target messenger RNA (mRNA), to which it is imperfectly complementary, within the RISC complex *(Kim, 2005)*. The mechanisms by which mRNA-to-protein translation is impeded comprise the blockade of translation, the facilitation of mRNA degradation as well as de-adenylation of the mRNA, which also results in its instability *(Winter et al, 2009)*. Interestingly, miRs do not only play a fundamental role in controlling protein synthesis within their cell of origin, but they can also be secreted and circulate within virtually all body fluids (*(Hunter et al, 2008; Valadi et al, 2007); reviewed in (Creemers et al, 2012))*. MiRs can thus affect protein content within a receiving cell far away from the cell of origin.
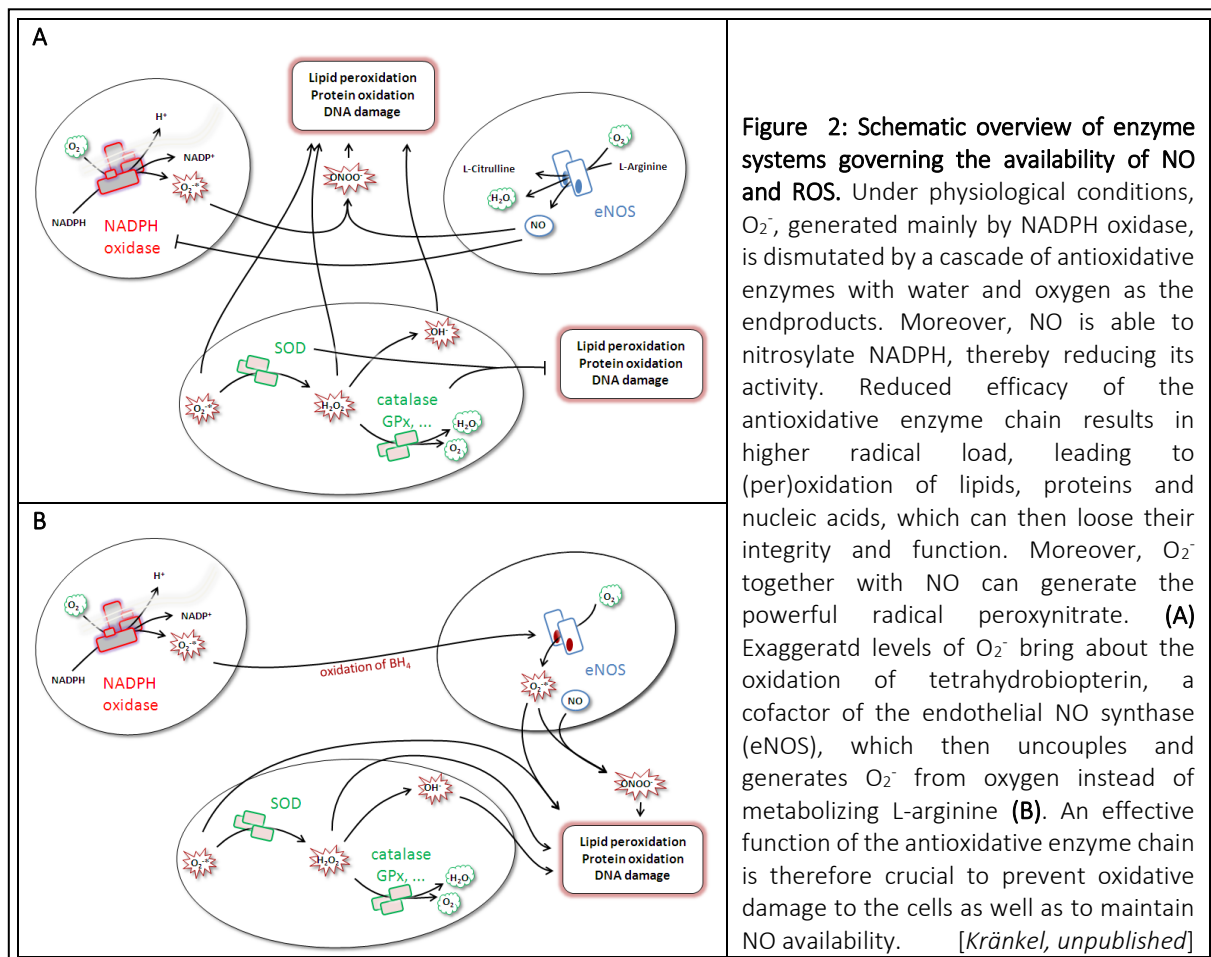
MiRs within the circulation are surprisingly stable, which has been attributed to both, their short size and hairpin structure, but also to their association with carrier proteins or their containment within extracellular vesicles *(Arroyo et al, 2011; Li et al, 2012; Turchinovich et al, 2011; Wang et al, 2010)*.
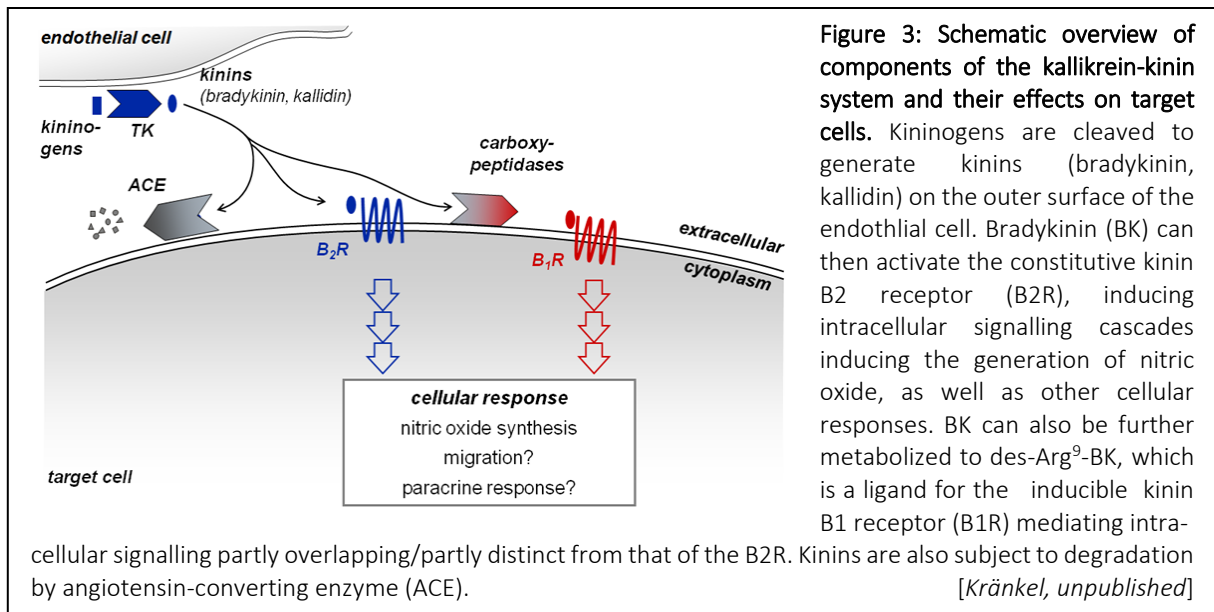
3.3.2. Paracrine Factors

Most of the above mentioned molecules and molecule complexes with endocrine activity also act in a paracrine manner if originated from cells in the vicinity to the endothelium (e.g. adjacent endothelial cells, plaque macrophages, vascular smooth muscle cells, adhering leukocytes). In addition, substances with short half-life, such as reactive oxygen species or peptides prone to fast degradation play a role in mediating endothelial function.

Nitric oxide and reactive oxygen species: The balance between NO and various reactive oxygen species (ROS) exerts a crucial impact on vascular function. The most prominent enzyme systems involved regulating the NO-ROS-balance are NADPH oxidase, which generates oxgen radicals during proteolytic cleavage of NADPH, the cascade of antixodative enzymes, dismutating O_2^- into water and oxygen, as well as the endothelial NO synthase (*Figure 2*). In cardiovascular diseases, impaired or inefficient function of the antioxidative enzyme chain, as well as increased activity of NADPH oxidase result in higher radical load which leads to oxidation of lipids, proteins and nucleic acids, thereby endangering their integrity and function, further curbing the inflammatory process and finally leading to cell death *(reviewed in (Del Maestro et al, 1980))*. Moreover, O_2^- has been shown to upregulate the generation of inflammatory cytokines, such as TNF-α *(Wang et al, 2000)*. Thus, increased oxidative stress does not only exert local effects, but - if unchecked - can induce a systemic inflammatory state.

The endothelial NO synthase (eNOS) differs from the inducible NO sythase (iNOS) involved in immune defense by its kinetics of NO generation as well as by its sensivity to Ca^{2+} levels. All NOS isoforms - endothelial, neuronal and inducible - metabolize L-arginine into L-citrulline under the consumption of NADPH, H^+ and O_2. NO is a second product of this reaction. In the endothelium, NO harbours potent anti-inflammatory and anti-atherogenic activities and is of paramount importance for the regulation of vascular tone and blood flow as well as haemostasis *(Harrison et al, 2006)*. In addition, it has been shown that NO can nitrosylate the p47[phox] subunit of NADPH oxidase, thereby

Figure 2: Schematic overview of enzyme systems governing the availability of NO and ROS. Under physiological conditions, O_2^-, generated mainly by NADPH oxidase, is dismutated by a cascade of antioxidative enzymes with water and oxygen as the endproducts. Moreover, NO is able to nitrosylate NADPH, thereby reducing its activity. Reduced efficacy of the antioxidative enzyme chain results in higher radical load, leading to (per)oxidation of lipids, proteins and nucleic acids, which can then loose their integrity and function. Moreover, O_2^- together with NO can generate the powerful radical peroxynitrite. **(A)** Exaggeratd levels of O_2^- bring about the oxidation of tetrahydrobiopterin, a cofactor of the endothelial NO synthase (eNOS), which then uncouples and generates O_2^- from oxygen instead of metabolizing L-arginine **(B)**. An effective function of the antioxidative enzyme chain is therefore crucial to prevent oxidative damage to the cells as well as to maintain NO availability.   [*Kränkel, unpublished*]

Figure 3: Schematic overview of components of the kallikrein-kinin system and their effects on target cells. Kininogens are cleaved to generate kinins (bradykinin, kallidin) on the outer surface of the endothlial cell. Bradykinin (BK) can then activate the constitutive kinin B2 receptor (B2R), inducing intracellular signalling cascades inducing the generation of nitric oxide, as well as other cellular responses. BK can also be further metabolized to des-Arg^9-BK, which is a ligand for the inducible kinin B1 receptor (B1R) mediating intracellular signalling partly overlapping/partly distinct from that of the B2R. Kinins are also subject to degradation by angiotensin-converting enzyme (ACE). [*Kränkel, unpublished*]

reducing NADPH activity *(Qian et al, 2012)*. However, in the presence of elevated O_2^- levels, NO and O_2^- can form peroxynitrite, a powerful oxidant itself *(Beckman et al, 1990; Darley-Usmar et al, 1992)*. Moreover, O_2^- can oxidize the eNOS co-factor BH$_4$, thus leading to uncoupling of eNOS from L-Arginine and to the generation of superoxide by eNOS *(Beckman & Koppenol, 1996)*. The efficient function of the antioxidant enzyme chain is therefore crucial for vascular NO levels, besides preventing excessive damage to DNA/RNA, lipids and proteins. However, radical oxygen species can also serve as signalling mediators, activating redox-sensitive pathways, including NF-kB, MAPK and PGC-1α signalling *(De Keulenaer et al, 2000; Kabe et al, 2005; Trachootham et al, 2008)*, thereby feeding back into the regulation of survival and stress defense pathways.

Beyond its role in regulating vascular tone, NO is also an endothelial survival factor and can modulate the recruitment of cells from the blood into the vessel wall, e.g. by modulating the expression of cell adhesion molecules at the luminal endothelial cell surface and by regulating the stability of endothelial cell junctions, thus acting on leukocyte adhesion as well as permitting their transmigration through the endothelial layer *(Hoffmann et al, 2003; Pfenniger et al, 2010; Predescu et al, 2005)*. Reduced NO availability is therefore widely considered to be causally involved in the endothelial dysfunction observed in patients with CVD *((Shimokawa & Vanhoutte, 1989); reviewed in: (Brunner et al, 2005; Fleming & Busse, 1999)*.

Besides reduced activation of eNOS due to altered kinase/phosphatase activity, endothelial cells of CVD patients also exhibit increased levels of ROS, partly due to enhanced NADPH oxidase activity and partly due to impaired dismutation *(Guzik et al, 2000)*. As discussed above, the result of those signalling dysregulations is an enhanced oxidation of proteins, lipids and DNA, impairing their function and integrity, thus inducing cell death. As has been demonstrated by own work (please see "original papers") and other groups, the reduced NADPH oxidase activity, improved antioxidative capacity, and restoration of eNOS activating phosphorylation greatly contribute to the rescue of endothelial function by regular exercise training *(Hambrecht et al, 2003)*.

Vasoactive peptides: Kinins: Another important signalling network with crucial importance for endothelial function and integrity is the kallikrein-kinin-network. Kinins are vasoactive peptides, generated from precursors, the kininogens, by kallikreins. Differen isoforms of kallikreins have been identified in plasma (plasma kallikrein) and in the vascularwall (tissue kallikrein). Both harbour different substrate specificity. While plasma kallikrein cleaves only low molecular weight kallikrein to bradykinin (BK), tissue kallikrein may use both, high and low molecular weight kallikrein to generate either kallidin - which can then be further metabolized to bradykinin by aminopeptidases - or high molecular weight kininogen to generate bradykinin. Both, BK and kallidin can activate the kinin B2 receptor

(B2R), which is constitutively expressed on the endothelium and several other cell types. However, carboxypeptidases can cleave both, BK and kallidin to generate substrates for the kinin B1 receptor (B1R), which is usually low expressed, but quickly upregulatd in inflammatory conditions. Finally, BK can be degraded into inactive peptides by the action of angiotensin-converting enzyme (ACE) (*Figure 3*). Both kinin receptors, B2R and B1R, share partially overlapping intracellular signalling cascades and use partially divergent signalling. B2R activation via G-proteins triggers several intracellular signalling cascades, including phospholipase C and PI3K/Akt-dependent activation of eNOS, which finally enable the release of vasorelaxants, such as NO, prostaglandins and prostacyclin *(reviewed in: (Leeb-Lundberg et al, 2005)).* Therefore, BK protection by inhibitors of ACE allows for a major part of the ACE-inhibitor mediated improvement of vasorelaxation and B2R activation is generally considered beneficial for vascular function. In contrast, the role of the B1R in vascular biology remains incompletely understood. There is indication that the B1R might result in deleterious effects in acute settings, such as myocardial infarction or acute inflammation *(Austinat et al, 2009; Xu et al, 2005; Yin et al, 2007)*, while in certain other settings, e.g. when the B2R is inactivated, it might take over the role of the B2R and exert protective actions *(Xu et al, 2013)*. One possible explanation can be derived from the the observation that the B1R can signal via the inducible NO synthase, thus generating NO with different kinetics as compared to eNOS *(Brovkovych et al, 2011)*. While this might not exert overt effects in "normal" quiescent settings, iNOS-derived additional NO generation might contribute to tissue damage in acute inflammation, where iNOS is upregulated and high levels of oxygen radicals are available to generate further effectors, such as peroxynitrite.

3.3.3. Paracrinally active cells involved in the vascular response to injury

Similar to other organs, the endothelial layer underlies a constant "surveying" by myeloid cells *(Auffray et al, 2007)*. Upon endothelial activation (e.g. by low or turbulent shear stress) or acute injury, those and further factors within the blood initiate the vascular response to injury, which can proceed in a time frame of months, potentially leading to the pathological expansion of the intimal layer and reduction of vessel lumen. In recent years, other vascular and perivascular resident cell types in addition to smooth muscle cells (SMCs), as well as the role of the vasa vasorum to deliver cells and mediators from the abluminal side have been better appreciated for their contribution to the vascular response to injury *(Krankel et al, 2013b)*.

Acute removal of endothelial cells exposes the underlying collagen of the internal elastic lamina, thus providing a surface for glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) activation and initiation of coagulation. Moreover, externalization of phosphatidylserine during apoptosis and other activation processes facilitates assembly of coagulation factors and thus initiation of coagulation *(Lambers et al, 1987)*. The involved platelets provide a „platform" for adhesion of other circulating cells types, but also modulate the survival, proliferation and adhesive properties of adjacent endothelial and other vascular cells *(Gros et al, 2014; Malczak & Buck, 1977; Tschoepe et al, 1993)*.

The other major blood-borne cell lineage modulating the vascular response to injury are represented by the myeloid cells *(De Ciuceis et al, 2005)*. In the narrow sense, those include monocytes of the "classical" fraction expressing high levels of CD14, but no or only low levels of CD16 (CD16^{hi}CD16^{-}) two fractions of "alternatively activated" or "regulatory" CD16^{+} monocytes, which differ in their level of CD14 expression (CD14^{hi}CD16^{+} and CD14^{lo}CD16^{+}), and their offspring, macrophages of the M1 or M2 varieties *[reviewed in (Das et al, 2015; Zawada et al, 2012)]*. In addition, the contribution of haematopoietic or angiogenic progenitor cells in this process is discussed *[reviewed in (Rafii et al, 2002)]*. During the early 2000s, a functional separation between CD133^{+}/CD34^{+} progenitor cells –then thought to give rise to endothelial cells *(Peichev et al, 2000)* - and monocytes/macrophages - paracrinally modulating endothelial cell function - was presumed in the cardiovascular field. However, it might well be that those different cells rather represent different states within a continuous spectrum, exhibiting a high functional flexibility. Especially the lack of in vivo verification of a major role of CD34^{+} progenitor cell-derived stable

endothelium *(Purhonen et al, 2008)* supports the notion that the main mechanism by which myeloid cells, as well as progenitor cells support re-endothelialization is paracrine *(Bautz et al, 2000; Ratajczak et al, 2013; Sahoo et al, 2011)*. In contrast, other "flavours" of the myeloid cell spectrum can enhance inflammatory endothelial activation, inducing the upregulation of cell adhesion molecules and a weakening of cell-cell-junctions *(Daniel & van Buul, 2013)*. One major emerging line of current research therefore adresses how haematopoietic/myeloid cells can be influenced towards a more endothelial supportive/-protective phenotype in patients with CVD.

Following the work of Kalka et al. *(Kalka et al, 2000)*, several working groups have adopted a culture technique by which a population of mononuclear cells from the peripheral blood (containing lymphocytes, monocytes and some contaminating granulocytes, platelets and erythrocytes) is allowed to adhere to fibronectin-coated culture dished in the presence of endothelial-specific growth factors over a duration of one week, while non-adhering cells are removed by washing and media changes. The adhering cells posess pro-angiogenic action and have the potential to improve re-endothelialization upon injury (*Kalka et al, 2000; Sorrentino et al, 2007*). However, in contrast to earlier descriptions, with the refinement of analytical methods it became clear that the majority of cells generated by this technique consist predominantly of myeloid cells, with only a small fraction constituting immature stem/progenitor cells *(Liu et al, 2009; Rehman et al, 2003; Yoder et al, 2007)*. This heterogenous cell population has therefore been termed "early outgrowth cells" (EOC) by some working working groups, as compared to "late outgrowth cells", obtained from the population which initially do not adhere (thereby allowing for a depletion of myeloid cells to some degree by an initial shot-term adhesion step), but after a culture period of ca. three weeks contain a more endothelial-like population *(Yoder et al, 2007)*. We and others have characterized EOC as being mainly composed of M2-like macrophages (*Jakob et al, 2012; Krankel et al, 2013a; Medina et al, 2011*). EOC/M2 macrophages represent an interesting working model for studying the mechanisms of support provided to endothelial cells by a heterogenous blood-derived cell population. Technically and ethically, EOC/M2 macrophages can easily be obtained from patients or healthy control subjects in amounts which allow antigenic, genetic and functional characterisation, thereby providing a good tool for translational research close to the pathologic subject of interest.

4. Research question / Aim of the work

It has been an aim of my work to better understand the bi-directional interactions between the vascular endothelium and blood-borne cells of myeloid lineage in the context of the vascular response to injury and its special case (*Mannarino & Pirro, 2008*) - the development of atherosclerotic plaques.

5. Results

The interactions between the endothelium and recruited myeloid cells in the context of the vascular response to injury comprise a number of interwoven bi-directional processes. Starting from analyses of the molecular dysfunctions of both partners - the endothelium and the circulating cell which is to be recruited - I have investigated alterations in the recruitment processes and in the effect which the altered spectrum of recruited cells, as well as their altered functions, have upon the endothelium and its capacity to compensate an injury. Towards this end, I have mainly worked with samples from patients with coronary artery disease or with high cardiovascular risk, such as those with diabetes mellitus.

5.1. Impaired functions of circulating cells

We have in the past provided evidence that in patients with high risk to suffer from a cardiovascular event, such as patients with diabetes mellitus individual subpopulations of circulating cells are compromized in their survival capability under stress *(Krankel et al, 2005; Madeddu et al, 2008)*, and are altered in their paracrine functions *(Barcelos et al, 2009; Jakob et al, 2012; Paneni et al, 2016)*, as well as in their survival and maturation/polarization potential *(Albiero et al, 2013; Fadini et al, 2013)*. The study summarized below stands exemplary to this, focusing on the PI3Kγ, which is relevant in angiogenic signalling.

5.1.1. Phosphoinositide 3-kinase γ gene knockout impairs postischemic neovascularization and endothelial progenitor cell functions.

Refers to: Madeddu P, Kraenkel N, Barcelos LS, Siragusa M, Campagnolo P, Oikawa A, Caporali A, Herman A, Azzolino O, Barberis L, Perino A, Damilano F, Emanueli C, Hirsch E. Phosphoinositide 3-kinase gamma gene knockout impairs postischemic neovascularization and endothelial progenitor cell functions. *Arterioscler Thromb Vasc Biol*. 2008 28(1):68-76. doi: https://doi.org/10.1161/ATVBAHA.107.145573

Beyond its role in vasorelaxation, nitric oxide is an important factor in wound healing, potentially affecting several adaptive processes, among them angiogenesis (*Konturek et al, 1993; Ziche et al, 1994*). One upstream element of the eNOS signalling pathway, the phosphoinositide 3-kinase (PI3K), couples incoming signals (via G protein coupled receptors or tyrosine kinase receptors) to phosphorylation of protein kinase B and subsequently to nitric oxide synthase. Four different classes of the PI3K have been described with partially divergent and partially ovelapping functions in vascular biology. While the members of the class IA, comprising α, β and δ isoforms of the catalytic subunit and being activated by tyrosine kinase receptors, the PI3K gamma (PI3Kγ) contains the gamma isoform of the catalytic subunit p110 and is a member of the class IB, activated by signaling from G protein-coupled receptors. PI3Kγ exerts its biological functions not only through its kinase activity, but also provides a scaffold for protein complex assembly (*Alloatti et al, 2004; Hirsch et al, 2000*). Thus, kinase-dead mutants have been developed in order to disect kinase-mediated functions of the PI3Kγ.

We used a PI3Kγ kinase-dead (PI3K$^{KD/KD}$) mutant mouse strain as well as a mouse strain with deletion of the PI3Kγ catalytic subunit (PI3K$^{-/-}$) and wild type controls with full PI3Kγ functional activity (WT) in order to specifically assess the role of the kinase activity of the PI3Kγ in "endothelial progenitor cells" and their capacity to induce and support ischemia-induced angiogenesis.

The PI3Kγ knockout mice exhibited a delayed neovascularization response upon induction of unilateral hindlimb ischaemia, characterized by reduced restoration of blood flow (assessed by laser Doppler measurement), and

number of immunohistochemically recognized capillaries and arterioles. In contrast, kinase-dead mutant mice exhibited a normal blood flow restoration. The subsequent analysis of functional characteristics of bone marrow-derived "endothelial progenitor cells" revealed functional impairments - outgrowth from bone marrow mononuclear cells, phosphorylation of protein kinase B, endothelial NO synthase and the transcription factor Foxo1, as well as migration toward SDF-1 and integration into endothelial networks - revealed a critical role of the PI3Kγ in all of the studied functions, with different requirement of the kinase function. In fact, cells from kinase-dead mice were not different from wild type cells with regard to their outgrowth potential (i.e. survival and maturation) as well as their migration towards SDF-1, but showed a reduced capacity to integrate into endothelial networks in vitro. In line with this, we observed differences in the distribution of leukocytes invading the ischaemic tissue: while in wild type mice, leukocytes are usually aligned with the newly forming vasculature, they were scattered across the tissue in samples PI3K$^{-/-}$ mice.

Our data indicate that the PI3Kγ plays an important role in the restoration of blood flow upon ischaemia, although only a part of the relevant cellular functions is mediated by the kinase activity and the scaffold function of the catalytic isoform might play a more crucial role than estimated before. Despite reduced activating phosphorylation of the protein kinase B at its residue Ser^{473}, eNOS phosphorylation was maintained in the kinase-dead cells, suggesting functional overlap from other kinases.

5.2.   Altered recruitment mechanisms

As previously shown, subsets of myeloid cells can support the endothelium in a paracrine manner (*Becher et al, 2014; Medina et al, 2011*). In contrast, other subsets aggravate endothelial dysfunction (*Bevilacqua et al, 1985*). The following studies therefore investigate mechanisms of selective leukocyte recruitment and their effects on the capacity of the endothelium to restore its integrity or form new capillaries.

5.2.1.   Role of kinin B2 receptor signaling in the recruitment of circulating progenitor cells with neovascularization potential.

Refers to: Kränkel N, Katare RG, Siragusa M, Barcelos LS, Campagnolo P, Mangialardi G, Fortunato O, Spinetti G, Tran N, Zacharowski K, Wojakowski W, Mroz I, Herman A, Manning Fox JE, MacDonald PE, Schanstra JP, Bascands JL, Ascione R, Angelini G, Emanueli C, Madeddu P. Role of kinin B2 receptor signaling in the recruitment of circulating progenitor cells with neovascularization potential. *Circ Res*. 2008;103(11):1335-43. doi: https://doi.org/10.1161/CIRCRESAHA.108.179952

The close local proximity of myeloid cells and endothelial cells in the vasculature, especially the "patrolling" alternative myeloid cell types (*Auffray et al, 2007*) theoretically allows for a functional relevance of messenger peptides, which usually are quickly degraded by peptidases, such as kinins, in the interaction between both cellular partners. In several projects I have elucidated this hypothesis and interrogated the individual role of the B1 and B2 kinin receptors *(Krankel et al, 2010; Krankel et al, 2008; Krankel et al, 2013a)* .

Initially, we investigated the role of kinin signalling in the recruitment of cells supporting the restoration of blood flow after induction of ischaemia and potential alteratins of this pathway in patients with coronary artery disease. We observed expression of the kinin B2 receptor on circulating CD133+ and CD34+ cells as well as cultured "early outgrowth cells" of healthy subjects, but only low expression of the B1 kinin receptor. The B2 receptor ligand bradykinin elicited a potent chemotactic response from "early outgrowth cells" in an ex vivo transwell migration assay. The involvement of kinin B2 receptor, PI3K and eNOS as signal mediators was verified.

When employed as a chemoattractant in a transwell assay, bradykinin was able to induce an enrichment of CD34+ and CD133+ cells in the migrating as compare to the non-migrating cell fraction. The cell fraction migrating towards BK showed more prominent pro-angiogenic capacity in an *in vitro* matrigel assay as well as when transplated into a mouse model of hindlimb ischaemia.

Circulating CD34+ and CD133+ cells from human patients with cardiovascular disease expressed less B2R and were less efficiently migrating towards bradykinin. In line, bone marrow cells from kinin B2 receptor-deficient mice promoted blood flow recovery less efficient than bone marrow cells from B2 receptor expressing mice. This was paralleled by a reduced homing of the B2 receptor deficient sca-1+ and cKit+flk1+ progenitor cells to the ischaemic tissue. Similar results were obtained with systemic B2 receptor inhibition when wild type cells were transplanted.

Thus, our results underline the role of the bradykinin B2 receptor for the recruitment of progenitor cells supporting the reconstitution of blood flow to an ischaemic tissue. The reduction of B2 receptor expression in patients with cardiovascular disease might limit the sensitivity of these pro-angiogenic cells towards the homing signal and thus represent a novel mechanism by which microangiopathy might be mediated in these patients.

5.2.2. Distinct kinin-induced functions are altered in circulating cells of young type 1 diabetic patients.

We aimed to understand whether kinin-mediated recruitment of circulating progenitor cells with endothelial-supportive functions is altered in young patients with well-controlled type 1 diabetes already before the clinical onset of cardiovascular disease.

Towards this end, we assessed the expression of the kinin B1 and B2 receptors on various leukocyte subsets in peripheral blood of young type 1 diabetes patients (age: 34.8 ± 1.6 years, HbA1C: 7.9 ± 0.2%) and non-diabetic controls. In addition, peripheral blood mononuclear cells were exposed to bradykinin as a chemotactic stimulus in a transwell migration assay and migrating cells were further tested for their generation of nitric oxide and superoxide as well as their capacity to support endothelial network formation in a matrigel assay. In addition, we assessed the effect of high, medium and low glucose levels during culture on the capacity of „early outgrowth cells" (EOC) to generate nitric oxide upon stimulation with bradykinin.

While in those well-controlled, young patients, no difference was obvious in the expression of the B1 or the B2 kinin receptor on the three known monocyte subtypes, or in CD34+ progenitor cell populations with or without co-expression of the CXCR4 and the VEGF receptor 2, if compared to a non-diabetic control group of similar age and sex distribution. Moreover, superoxide generation was comparable between EOC of T1D patients and healthy controls. In contrast, bradykinin-induced nitric oxide production in EOC and endothelial network formation supported by EOC were reduced in the T1D group. EOC obtained from healthy blood donors exhibited a low baseline migratory activity, with was significantly enhanced by the addition of bradykinin. When cultured for 7 days in intermediate glucose concentrations (10mM D-glucose), baseline migratory activity was slightly increased, with a further increase upon bradykinin administration which reached levels of the normal glucose group, but did not reach statistical significance. Under high glucose conditions (25mM), bradykinin did not elicit any significant increase of migratory activity beyond the baseline level.Bradykinin elicitedan increase of nitric oxide production in EOC cultured under low glucose (5mM) conditions, but had no effect in the intermediate glucose group and even induced a reduction in NO production in the high glucose group. At the same time, EOC cultured under high glucose conditions increased their superoxide production upon bradykinin administration, an effect that was not seen under normal or intermediate glucose concentrations.

Thus, our data underline the importance of stringend glucose control in patients with type 1 diabetes mellitus in order to delay the onset of cardiovascular disease, although this might not be easy in young, active patients. Kinin receptor expression and migration of various progenitor and monocyte subpopulations was not altered in our T1D patients. Thus, the observed glucose-induced dysfunctions might initially be rather with the signalling mechanisms downstream of the kinin receptors and might reduce nitric oxide availability and the associated pro-angiogenic actions of EOC first, only later on or at higher glucose concentrations followed by enhanced generation of superoxide. Once might therefore speculate that over time, kinin-sensitive cells are still recruited to the vascular wall or ischaemic tissues at need, but are less efficient in providing nitric oxide. Thus, no damage might at first provided by this mechanism, while it´s supportive effects decline. Only at later stages of diabetic vasculopathy or

cardiovascular disease, the previously described alterations occur, including the generation of superoxide, adding to the vascular damage.

5.2.3. Novel insights into the critical role of bradykinin and the kinin B2 receptor for vascular recruitment of circulating endothelial repair–promoting mononuclear cell subsets.

Refers to: Kränkel N, Kuschnerus K, Müller M, Speer T, Mocharla P, Madeddu P, Bader M, Lüscher TF, Landmesser U. Novel insights into the critical role of bradykinin and the kinin B2 receptor for vascular recruitment of circulating endothelial repair-promoting mononuclear cell subsets: alterations in patients with coronary disease. *Circulation*. 2013;127(5):594-603. doi: https://doi.org/10.1161/CIRCULATIONAHA.112.118117

The previous studies investigated kinin-mediated cell recruitment in an ischaemic environment. Yet, in macrovascular disease, the vascular wall suffers from recurring damage to endothelial cells in the absence of ischemia (induced by physical factors like blood flow conditions as well as bio-chemical factors). In addition, ballon inflation during percutaneous catheter intervention induces physical starin-induced damage to the arterial wall. We investigated the role of kinin-mediated cell recruitment in this non-ischaemic injury setting.

We observed that the kinin B2 receptor ligand bradykinin or inhibition of the angiotensin converting enzyme - a major kininase *in vivo* - by captopril supported the adhesion of $CD34^+CXCR4^+$ progenitor cells, but not of $CD14^+$ monocytes to human aortic endothelial cells *ex vivo*. The bradykinin-enhanced adhesion of progenitor cells was blocked by addition of the B2 receptor inhibitor icatibant. We were able to verify our observations in a mouse model of carotid injury, where systemic treatment with icatibant reduced CD34+CXCR4+ progenitor cell recruitment to an injures arterial wall segment and likewise it′s re-endothelialization, but not monocyte recruitment. In order to exclude systemic effects of the icatibant treatment on endothelial cells, we next transplanted bone marrow cells from B2R deficient mice or wild type controls into syngenic recipient animals after the induction of carotid artery endothelial injury. Similar to the pharmacologic approach, bone marrow cells from B2R-expressing mice were able to support re-endothelialization of the injured carotid artery segment, while the same cell fraction of the B2R-deficient mice was not able to support endothelial regeneration. In an ex vivo adhesion assay, the CXCR4+Tie2+ pro-angiogenic fraction of bone marrow cells exhibited a stronger dependence on the bradykinin signal than the CXCR4-Tie2- fraction. In an in vitro scratch assay of endothelial cell gap closure, the CXCR4+Tie2+ bone marrow cells exhibited a more prominant support of re-endothelialization than CXCR4-Tie2- bone marrow cells.

Early outgrowth cells of human patients with coronary artery disease (CAD) or apparently healthy controls of similar-age were then assessed for their capacity to adhere to aortic endothelial cells with or without bradykinin stimulation, and subsequently support re-endothelialization of an induced scratch in the endothelial cell layer. Similar to the murine model, bradykinin enhanced adhesion of early outgrowth cells and subsequent support of re-endothelialization. When early outgrowth cells from patients with CAD were used, this effect was lost, probably due to a downregulation of B2 receptor expression in the CAD patient cells. Overexpression of B2R in EOC of patients with CAD prior to transplantation into a mouse model of carotid artery injury rescued the impairment in kinin-mediated cell recruitment, and improved subsequent re-endothelialization.

The paper extends earlier findings of a downregulation of the kinin B2 receptor in patients with CAD and provides new evidence for the relevance of kinin-mediated recruitment of circulating cells supporting not only angiogenesis in ischaemic tissues, but also promoting the re-consitiution of an intact andothelial layer upon injury.

5.2.4. Short-term inhibition of DPP-4 enhances endothelial regeneration after acute arterial injury via enhanced recruitment of circulating progenitor cells.

Refers to: Brenner C, Kränkel N, Kühlenthal S, Israel L, Remm F, Fischer C, Herbach N, Speer T, Grabmaier U, Laskowski A, Gross L, Theiss H, Wanke R, Landmesser U, Franz WM. Short-term inhibition of DPP-4 enhances endothelial regeneration after acute arterial injury via enhanced recruitment of circulating progenitor cells. *Int J Cardiol*. 2014;177(1):266-75. https://doi.org/10.1016/j.ijcard.2014.09.016

Myeloid cell types from the circulation are recruited to injured and ischaemic tissue upon the release of a plethora of cytokines and peptides from the damaged tissue. In acute ischemia (myocardial infarction, limb ischemia), SDF-1 is considered a – if not "the" - major chemotactic factor. It has initially been considered to recruit specifically immature cells (stem/progenitor cells) which then mature to endothelial cells in situ (*Mohle et al, 1998*). We therefore investigated whether inhibition of the DPP-4 might accelerate the repair of injured vascular section. If so, this might be a novel approach for the prevention of thrombus formation after percutaneous catheter intervention.

Towards that end, we employed a mouse model of defined carotid artery injury, with subgroups treated with sitagliptin (via their diet), sitagliptin and the CXCR4 inhibitor AMD3100 (administered intraperitoneally daily), or none of these. Protection of SDF-1 integrity was assessed in murine plasma samples by mass spectrometry. Locally, SDF-1 concentrations were indeed elevated in the injured carotid segments as compared to the contralateral non-injured carotid section. Consistently, recruitment of systemically injected CXCR4+ BM-derived progenitor cells was higher in the injured than in the non-injured section of the *a. carotis*. In addition, treatment with sitagliptin further increased progenitor cell recruitment to the injured carotid section. This effect was prevented by co-administration of the CXCR4-inhibitor AMD3100. On a functional level, endothelial regeneration was more pronouced in the sitagliptin-treated animals as compared to untreated controls or sitagliptin/AMD3100 co-treated mice.

We therefore concluded that systemic treatment with sitagliptin is indeed able to accelerate vascular regeneration upon injury, via the protection of SDF-1/CXCR4 signalling. Further studies are, however, needed in order to investigate long-term effects as well as the relevance of this treatment approach in patients with cardiovacular disease, where other proteases and recruitment mechanisms might take over.

5.2.5. DPP-4 inhibition ameliorates atherosclerosis by priming monocytes into M2 macrophages.

Atherosclerosis might be viewed as a misguided or exaggerated vascular response to injury *(Mannarino & Pirro, 2008)*. In order to better understand the role of SDF-1/CXCR4-mediated cellular recruitment in the long-term setting of atherosclerosis, we therefore employed a mouse model of high-fat diet in ApoE-deficient mice.

We employed a murine model of atherosclerosis, consisting of a high-cholesterol diet for 3 months in mice lacking the ApoE gene (ApoE$^{-/-}$). Two subgroups of ApoE$^{-/-}$ mice received sitagliptin mixed in with the high-cholesterol chow, one of them together with the CXCR4 inhibitor AMD3100 infused via implanted osmotic pumps, in a similar manner as in the previous study (*Brenner et al, 2014*). A control group of ApoE$^{-/-}$ mice received normal diet during the same period.

ApoE$^{-/-}$ mice had developed lipid deposits along the aorta after the 3-month high cholesterol diet, mimicking human atherosclerotic lesions. The extent of lipid deposition was significantly reduced when sitagliptin was fed together with the high cholesterol chow. This might in part be due to reduced circulating LDL levels, as recently described for human patients with type 2 diabetes mellitus (*Shigematsu et al, 2014*). However, co-treatment with the CXCR4 inhibitor AMD3100 abolished the sitagliptin-mediated reduction in atherosclerotic lesion area, thus suggesting an effect mediated by cell recruited via the SDF-1/CXCR4 axis. In line with this, we observed higher recruitment rate of M2-like macrophages in the sitagliptin group as compared to the mice fed a high cholesterol diet only, as well as the group treated with AMD3100 in addition to sipagliptin. Instead, total macrophage number as well as M1-type macrophage numbers were consistently higher in all mice fed a high cholesterol diet as compared to the mice on standard chow. In addition, CXCR4 expression was consistently higher in M2-type macrophages than in M1-type macrophages without influence of the diet. Moreover, a negative correlation was established between the number of M2-type macrophages recruited to the aorta and the extent of aortic lipid depositions in the same animals. In vitro studies performed on human macrophages suppoertedhigher CXCR4 expression in M2-type macrophages as compared to M1-type macrophages. Moreover, culture of human primary monocytes in conditions supporting maturation into macrophages revealed that presence of sitagliptin shifted macrophage polarization towards the M2 phenotype.

Thus, our data support the notion that the M2 phenotype of macrophages might play an anti-atherosclerotic role, at least in the initial stages of atherosclerosis. Moreover, sitagliptin might play a two-fold role, supporting CXCR4-mediated recruitment of these cells into the vascular wall and favouring marcophage polarization towards an M2 instead of an M1 phenotype.

5.3. Development of ex vivo assays to study recruitment mechanisms

In translational research, one often has to bridge the gap between well-standardized molecular assays and the biochemically very complex situation in the human being from which samples are derived. It has therefore been one focus of my work to develop and improve ex vivo assays to enable phenotypical and functional characterization of cell populations which influence endothelial cell function.

In order to study selective recruitment processes across the endothelial barrier into the sub-endothelial tissue, we have advanced a previously established migration assay, where a population of cells is exposed to a chemotactic gradient. The cells are separated from the stimulus by a porous membrane with defined pore size (e.g. 3um), allowing the slow diffusion f the stimulus, as well as the active migration of the cell towards the neighborung chamber containing the stimulus, but preventing the passive "falling through" of cells from the upper to the lower chamber. The "classical" assay is highly artificial, as usually a pre-selected cell population is tested, thus ignoring cell-cell interactions which occurr in vivo, and furthermore a passive separation is performed by the membrane. Instead, the passage of leukocytes from the lumial side of the endothelial layer into sub-endothelial vascular positions is regulated by the edothelial cell layer itself in vivo. The novel assay therefore includes *(1)* a heterogenous cell population as starting material and *(2)* a separating endothelial cell layer grown to confluency on the upper side of the membrane *(Krankel et al, 2011).*

Cell-specific fluorescence labelling prior to the assay, combined with a flow cytometric analysis and bead-based quantification, now allows the study of complex interactions between the diverse cell types and the endothelium under the influence of pre-defined stimuli. The assay has enabled the better understanding of the dysfunction of the SDF-1 signalling pathway in patients with cardiovascular disease in subsequent studies *(Ascione et al, 2015).*
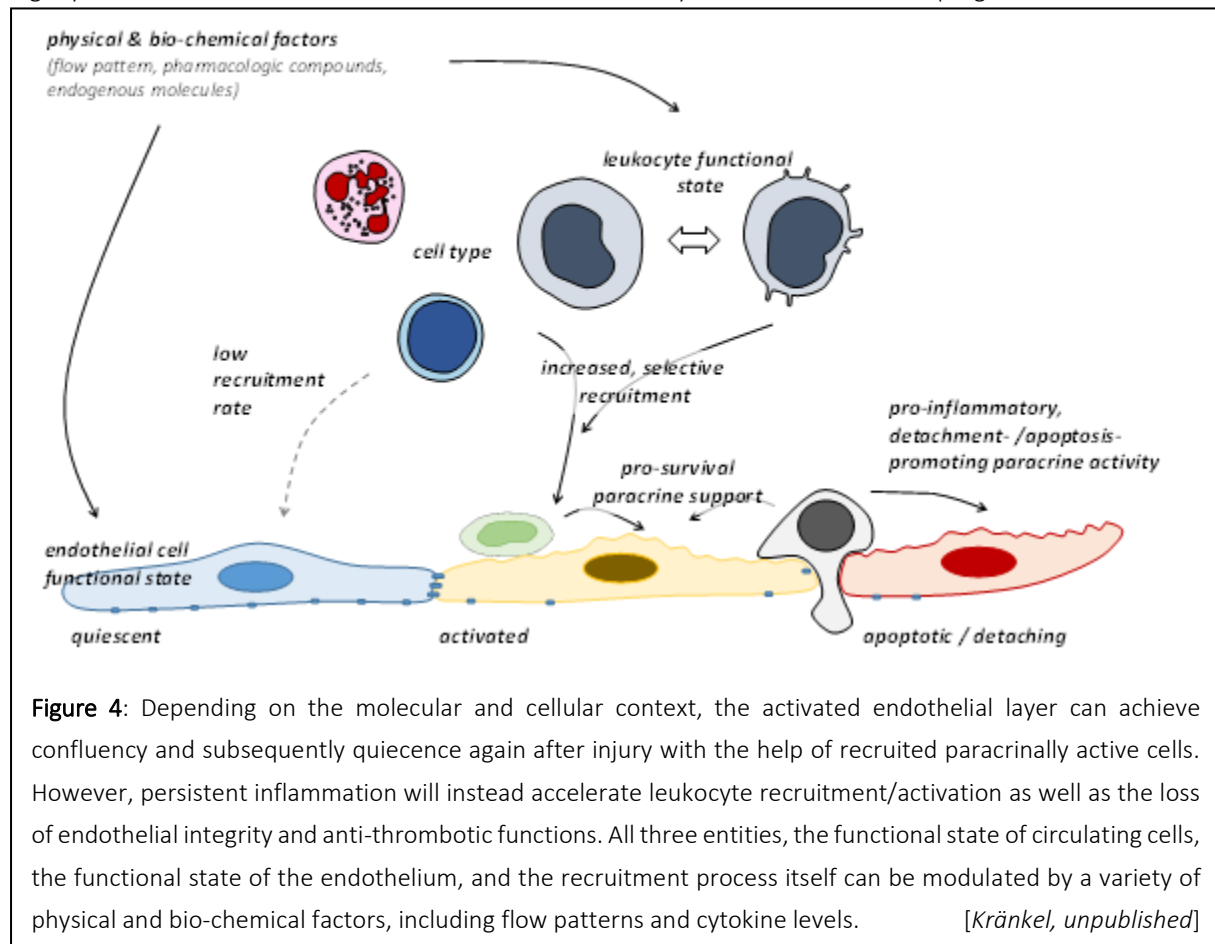
6. Discussion

The increased incidence of cardiovascular disease translates into an increased social and economical burden world-wide - impairing quality of life for the patients, increasing healthcare costs and reducing productivity. In order to limit further escalation in the mid- and long-term future, efforts are undertaken globally to better understand the molecular and cellular mechanisms underlying vascular diseases, develop cost-effective widely available forms of primary and secondary prevention and therapy, and implement them with the general population *(Kotseva et al, 2016)*. The work I have contributed to in the past and intend to continue and expand in the future targets all of those objectives.

On a methodological scale, I am working with translational aproaches, assessing primary human biological material using and adjusting functional cell biology assays, as well as molecular analysis techniques. Thus, observations from a broader patient basis can be transformed into pathophysiological hypotheses, which are tested experimentally before being verified in human patients.

6.1.   The vascular response to injury: A dance for two

During the recent decades the field has expanded its static view of the vasculature and the cellular and molecular components involved in regulating its physiological and pathophysiological processes. The concept has evolved that the vascular endothelium underlies constant re-arrangement processes, depending on a variety of cues, including physical (flow/shear, strain) and bio-chemical (cytokines/chemokines, growth factors, nitric oxide/reactive oxygen species, oligonucleotides/nucleic acids, multimolecular complexes). Specifically, the important role for various blood-borne cell types to modulate endothelial function has been (re-)discovered. Both partners, the endothelium and the recruited "accessory" cell affect each other: Enhanced endothelial activation, e.g. by non-laminar flow conditions or increased inflammatory status - leads to the upregulation of endothelial

Figure 4: Depending on the molecular and cellular context, the activated endothelial layer can achieve confluency and subsequently quiecence again after injury with the help of recruited paracrinally active cells. However, persistent inflammation will instead accelerate leukocyte recruitment/activation as well as the loss of endothelial integrity and anti-thrombotic functions. All three entities, the functional state of circulating cells, the functional state of the endothelium, and the recruitment process itself can be modulated by a variety of physical and bio-chemical factors, including flow patterns and cytokine levels.   [*Kränkel, unpublished*]

adhesion molecules, facilitating the recruitment of a number of circulating cells (*Figure 4*). On the other hand, adverse metabolic and inflammatory conditions affect functional response and/or differentiation/polarization of myeloid cells (references) as well as their adhesive state, thus modulating recruitment on the part of the circulating cell (*Figure 4*).

6.1.1. Functions of myeloid cells and their recruitment to the endothelium

EOC/M2 macrophages have been shown to release NO as well as a wide spectrum of growth factors and cytokines and this paracrine activity is altered in patients with CVD and/or diabetes *(Krankel et al, 2013a; Tan et al, 2010; Urbich et al, 2005; Yang et al, 2010)*. In order to provide efficient paracrine input to endothelial cells, recruitment of supporting paracrine cells needs to be efficient and specific to the target site. As is known from leukocyte adhesion studies, initial contact is made between selectins on the activated endothelial cell which bind to specific carbohydrate residues on leukocyte transmembrane glycoproteins, such as P-selectin glycoprotein ligand-1. Vice versa, leucocyte selectin (L-selectin) can also bind endothelial antigens, such as CD34. Those binding forces are still weak so that the leukocyte can still be affected by the flow forces of the blood, resulting in a rolling movement along the endothelial surface. Subsequently, integrins on the leukocyte get activated, resulting in a conformational change of the integrin molecules which allows interaction with endothelial cell adhesion molecules, such as ICAM-1 [*reviewed in: (Campbell & Humphries, 2011)*]. Interations between integrin and cell adhesion molecules, as well as junctional adhesion molecules also mediate the following passage of leukocytes from the luminal to the abluminal side of the endothelial barrier (Bradfield et al, 2007; Muller, 2014; Schnoor et al, 2015). While most studies generating this information have been performed with freshly isolated leukocytes, some studies have reported the involvement of the integrins CD18 (component of the LFA-1 complex) and CD49d (component of the VLA-4 complex) for EOC/M2 macrophage recruitment and retention within injured tissue *(Duan et al, 2006)*. Surface expression of involved molecules, as well as integrin conformational changes and clustering of membrane protein complexes involved in leukocyte adhesion and transmigration unterlie the regulation of intracellular signal cascades, which are modulated by cytokines (Avraamides et al, 2007; van Buul et al, 2010a).

A series of feedback loops therefore control the (selective) recruitment of cells from the circulation to the endothelial surface as well as their transmigration into and retention within the sub-endothelial layers of the vascular wall. After platelets, myeloid cells are among the first cell type to populate the vicinity of an endothelial lesion, likely supported by the former *(Spinetti et al, 2011)*. The paracrine and phagocytic activity of recruited myeloid cells, in turn, affect endothelial cell activation and further cell recruitment. In addition, their high functional flexibility in response to various stimuli as well as their high paracrine activity makes myeloid cells important amplifiers and organizers of the processes within the vascular wall – including endothelial integrity and function.

6.1.2. Molecular mechanisms of selective myeloid cell recruitment to the endothelium

Kinin signaling: One interest of my further work was therefore to identify mechanisms governing changes in the recruitment of EOC to a site of endothelial injury, or to an angiogenic focus, where they could support the function of the resident endothelial cells. During my early postdoctoral work, I started to investigate the role of the kallikrein-kinin-system for the recruitment of cells with pro-angiogenic action in the setting of ischaemia *(Krankel et al, 2010; Krankel et al, 2008)*. Bradykinin, a vasoactive peptide cleaved from high molecular weight kininogen by tissue kallikrein had before been known to act directly on endothelial cells via the kinin B2 receptor *(Leeb-Lundberg et al, 2005)*. Angiotensin converting enzyme (ACE) and other peptidases rapidly degrade bradykinin *in vivo*, which therefore only acts over short distances *(Erdos, 1990; Ura et al, 1987)*. In theory, however, it would still be possible to serve as a means of communcation between the endothelium and a cell rolling or crawling along

the endothelial surface, as has been described for M2-type macrophages *(Auffray et al, 2007)*. We therefore first investigated the expression of the B1 and the B2 kinin receptors, as well as their functional effect in various leukocytes and progenitor cells in patients with cardiovascular disease and diabetes *(Krankel et al, 2010; Krankel et al, 2008; Krankel et al, 2013a)*, findings which were also reported by other working groups *(Dabek et al, 2008)*. Subsequently, we characterized kinin-dependent recruitment of progenitor cells and monocytes/macrophages onto endothelial cells, respectively the injured vascular wall. Those studies verified in *in vitro*, as well as *in vivo* models the relevance of cells recruited to a site of injury in a kinin-dependent manner for the healing of the lesion and re-establishment of an intact endothelial layer *(Krankel et al, 2013a)*.

SDF-1/CXCR4 signalling: Our current understanding supports the notion of atherosclerosis as a "special case" of misguided or exaggerated vascular response to injury *(Mannarino & Pirro, 2008)*. In two further projects we therefore investigated the SDF-1/CXCR4 pathway – more precisely it´s protection by inhibitors of the dipeptidyl peptidase 4 (DPP-4) – in an acute model of vascular injury *(Brenner et al, 2014)* and in a murine model of atherosclerosis *(Brenner et al, 2015)*. While acutely DPP-4 inhibition improved the recruitment of circulating progenitor cells to the site of vascular injury and resulted in a faster healing response, in the long-term atherosclerosis model stem/progenitor cell recuitment was not beneficially modulated *(Brenner et al, 2015; Brenner et al, 2014)*. Instead, DPP-4 inhibition allowed for the enhanced recuitment of M2 macrophages to the vascular wall, which was associated with smaller atherosclerotic fatty depositions *(Brenner et al, 2015)*. Those observations underline the role of SDF-1/CXCR4 signalling not only in acute, but also in chronic regenerative settings, where distinct cell types might be addressed. Inhibition of the dipeptidyl peptidase-4 (DPP4), which degrades SDF-1, might therefore convey protection of this pathway and provide an additional field of application for the substance class of gliptins, currently used in type 2 diabetes for it´s capacity to lower glucagon and increase incretin levels and thereby improve peripheral glucose uptake.

Nitric oxide synthesis: NO synthesis is considered crucial for both, the endothelium as well as the myeloid cell. While NO can serve as a vasorelaxant as well as a survival factor for the endothelium *(Motterlini et al, 1996; Struck et al, 1995)*, it is also part of a pathogen defense mechanism and might injure surrounding tissue, especially if superoxide is available *(Kroncke et al, 1991; Phan et al, 1994)*. The three isoforms of NO synthase release NO at different kinetics and thus allow a certain fraction to react with superoxide to form peroxynitrite. The consequences of increased or reduced NO levels therefore need to be interpreted in context with timing and location as well as with superoxide scavenger and production status *(McQuaid et al, 1996)*. Consistently, we have aimed at developing therapy strategies improving endothelial nitric oxide synthesis and reducing the oxygen radical burden *(Adams et al, 2005; Hambrecht et al, 2003; Spescha et al, 2015)*.

We have investigated the role of the PI3-kinase γ, an isoform of the PI3-kinase which is G-protein coupled in contrast to the PI3-kinase isoforms α, β and δ *(Madeddu et al, 2008)*. In addition to its kinase activity, PI3Kγ also posesses a scaffolding function, allowing the activation of the phosphodiesterase 3 (PDE3), thereby affecting intracellular cAMP levels *(Bondeva et al, 1998; Patrucco et al, 2004)*. By studying a kinase-dead mutant of PI3Kγ we were able to discern the scafolding function from the kinase-mediated function of the enzyme. The activating phosphorylation of the protein kinase B - a crucial activator of the endothelial NO sythase - was reduced as expected in the kinase-dead mutant cells, together with reduced translocation of the transcription factor Foxo1, a downstream target of the protein kinase B. In contrast, eNOS phosphorylation and subsequently NO synthesis were not affected (Madeddu et al, 2008). Cells from PI3Kγ knockout mice showed both, reduced Foxo1 translocation, as well as reduced eNOS phosphorylation and NO synthesis *(Madeddu et al, 2008)*. Our observations associate the altered NO synthesis to the impaired capillarization response and delayed blood flow restoration of the ischaemic hind limb in the PI3Kγ knockout, but not the kinase-dead mice *(Madeddu et al, 2008)*. In this study,

we were not able to detect injected bone marrow-derived lin- cells or their decendants in the ischaeic hindlimb tissue, while a higher number of PI3Kγ deficient cells were detected in the spleen of recipient mice as compared to the circulation. Those findings indicate a more rapid degradation of PI3Kγ deficient cells, possibly because they are detected to be dysfunctional (*Madeddu et al, 2008*). A time course as well as the transplantation of cells at various differentiation stages would be necessary to derive better information about their homing to injured/ischaemic hindlimb.

The activity of eNOS is regulated on several levels *((Du et al, 2001; Kalinowski et al, 2016), reviewed in: (Govers & Rabelink, 2001))*, including phosphorylation and de-phsphorylation of the Ser^{1177} residue. While phosphorylation of this residue by the PKB is associated with increased NO synthesis, Ser^{1177} is dephosphorylated by the protein phosphatase 2a (PP2A). I have earlier investigated this later process within endothelial-supportive M2-like macrophages, at that time still referred to as „endothelial progenitor cells" *(Krankel et al, 2005)*. Taking together those findings with other reports, one might conclude that in diabetes a reduced release of NO by the endothelium as well as the recruited endothelial-supportive M2 macrophages together with a reduced homing capacity itself *(Sorrentino et al, 2007)* lead to an even lower provision of macrophage-derived NO and growth factors to the endothelium and/or sub-endothelial vascular wall.

6.1.3. What now? - Translation of findings into treatment options

As shown, dysfunctions of the endothelium and the recruited cells type overlap with dysfunctional recruitment processes (**Figure 4**). Hence, treatment should aim to support all three entities. Especially enhanced inflammatory load - bringing about increased loads of reactive oxygen species and thereby tissue damage, but also an upregulation of proteases affording the degradation of the aforementioned mediators (Bradykinin, SDF-1) - needs to be lowered in these patients.

In a way, this recapitulates the current problem of stem cell therapy approaches in patients with cardiovascular disease: not only the functionality of stem/progenitor cells is hampered in patients with diabetes and/or cardiovacular disease *(Caballero et al, 2007; Hill et al, 2003; Tepper et al, 2002; Vasa et al, 2001)*, but in the setting of autologous transplantation, the same applies to the milieu conditions in the recipient *(Patel et al, 2013a)*. Thus, for transplantation-based therapies - as well as for employing endogenous recruitment processes - (pharmacologic) treatment approaches aiming to improve conditions within the target organ, as well as systemic inflammatory status need to complement *ex vivo* modifications of the transplanted cells in order to address all aspects of dysfunction - endothelial cell, transplanted cell and the recruitment/engraftment process itself (**Figure 4**).

Pharmacologic protection of kinin signalling: It has already been hypothesized that the beneficial actions of ACE inhibitors are in part due to their protection of bradykinin, thereby affording endothelial nitric oxide synthesis (*Ferrari & Fox, 2009*). Likewise, the vascular effects of ACE inhibitors might also in part be due to their support of recruitment of M2-like myeloid subsets. While ACE inhibitors are highly successfull, guideline-listed cardiovascular drugs, their side-effects (cough, edema) are also mediated by their protection of bradykinin. Moreover, our data indicate a downregulation rather at the level of the receptor (B2R) than at the level of the ligand. One mechanism of B2R downregulation in patients with CAD might be the accelerated senescence occurring in various cells with hematopoietic potential in those patients (*Giannotti et al, 2010; Nurmi et al, 2012; Samani et al, 2001; Satoh et al, 2008; Spyridopoulos et al, 2009*). The B2R molecule is usually recycled upon ligand binding (*Marceau et al, 2002*) A single B2R molecule can thus recycled and remain in the cell for several days, even upon activation (*Marceau et al, 2002*). If the molecule is damaged or dysfunctional, if modified ligands are linked to the receptor or if proteosomal vesicle formation is aberrant, the receptor will be subjected to proteosomal degradation (*Houle*

& Marceau, 2003). One might speculate whether increased levels of reactive oxygen species, which occurr in CAD and are considered to play a role in accelerated senescence (*Paneni et al, 2015*), might also be involved in an enhanced proteasomal degradation of the B2R. However, no studies have investigated this hypothesis yet. Another potential modulator of B2R trafficking and recycling are arrestins *(Khoury et al, 2014; Willets et al, 2015)*. The depletion of arrestin 2 or arrestin 3 in myometrial cells enhanced and prolonged bradykinin-induced B2R signalling and attenuated its desensitisation (*Willets et al, 2015*). However, arrestins regulate the turnover of several receptors of vasoactive peptides (*Khoury et al, 2014*) and induce differential effects in non-vascular cells (*Willets et al, 2015*), thus opening the possibility of unwanted systemic effects. It therefore remains unclear at the moment, how this mechanism might be exploited beyond the currently used ACE inhibitors.

Pharmacologic protection of SDF-1/CXCR4 signalling: DPP4-inhibitors, which can protect SDF-1, but also other peptides like GLP-1 from degradation, are currently used as anti-diabetic drugs (*Derosa et al, 2014*). Based on our results in animal studies (*Brenner et al, 2015; Brenner et al, 2014*) we had hypothesized that DPP-4 inhibitors might also be used as vascular protective drug, independently of its metabolic action, in cardiovascular disease. This has not been verified in human patients so far. Diabetic patients treated with the DPP-4 inhibitor saxagliptin did not suffer less cardiovascular events than the control group (*Scirica et al, 2013*). Neither did treatment with Alogliptin in patients with an acute myocardial infarction or unstable angina reduce the occurrence of another cardiovascular event (*White et al, 2013*). In light of our own findings and data by other working groups (*Virmani et al, 2005*) however, that the time point in the progression of the atherosclerotic disease could be important in determining the effect of DPP-4 inhibition: while M2-type macrophages in our model were associated to lower initiation of fatty deposits, later in plaque progression, similar types of non-classical macrophages might promote intra-plaque angiogenesis and thereby lead to plaque destabilization. In an acute vascular injury, or before the onset of atherosclerosis, the application of DPP-4 inhibition might therefore also be investigated in non-diabetic patients, while chronic treatment does not appear to yield a benefit in vascular therapy. A similar principle was used to employ bone marrow-derived stem cells for the reduction of myocardial infarct size: the CXCR4 antagonist AMD3100 was only given for a short time or even as a single application in order to facilitate their release from the bone marrow, but not jeopardize their recruitment to the infarcted myocardium (*Jujo et al, 2013*). Long-term treatment instead did not improve recruitment to the infarcted myocardium and resulted in equal infarct size (*Ruder et al, 2014*).

Exercise training: Physical exercise, performed regularly, is known to improve vascular function and lower inflammatory burden - beyond a number of metabolic effects - in patients with cardiovascular diseases (*Gleeson et al, 2011*). In a previous study we have observed that a 4 weeks exercise training - either below or above the ischemic threshold - enhances the expression of CXCR4 on CD34^{+} circulating progenitor cells and improves their capacity to integrate into endothelial cell networks, a function that is considered a measure of the pro-angiogenic capacity of these cells (*Sandri et al, 2005*). Those data suggest that exercise training might be one approach to improve the recruitment of supportive cell types to the injured endothelium. Exercise, however acts via a multitude of mechanisms, including metabolic and shear stress-induced signalling pathways leading to the improvement of nitric oxide syntesis and improved radical scavenging capacity, resulting in an increased bioavailability of nitric oxide *((Alessio & Goldfarb, 1988; Kanter et al, 1985), reviewed in: (Niebauer & Cooke, 1996))*. In the context of cardiovascular disease, however, exercise training is an interesting treatment option exactly because it works in a pleiotropic manner. We and others have before described an increase in vascular nitric oxide synthesis and a reduction of reactive oxygen species in patients with cardiovascular diseases after participating in an exercise training programm (*Adams et al, 2005; Hambrecht et al, 2003; Linke et al, 2005*). Thus, exercise training may indeed address all three components of dysfunctional vascular response to injury: the endothelium, the

circulating cell and the recruitment process itself. So far, evidence of exercise training reducing cardiovascular mortality on a long-term is lacking. This is usually attributed to several factors, most notably the control of adherence and (epi-)genetic factors. Moreover, the intensity and duration as well as the type of exercise (endurance or strength training) modulate the individual effects of exercise training. Thus, current and future studies need to provide better insight into the effects of modulating those parameters and into how motivation and thus adherence of the patient can be induced and maintained.

6.2. Methodological aspects

Cardiovascular research depends on the interaction between basic and clinical research, with translation in both directions. While in basic research, one usually aims to provide a well standardized experimental setup and only one parameter is modulated at a time, this strategy is not easily transferable to the study of human subjects, which differ by large number of variables, most of them unknown and thus not controllable. In addition, genetic and epigenetic aspects influence cellular behaviour. Those can either not be modified or require careful experimental setup (choice of control group/cases, intra-individual testing before versus after treatment). Moreover, the amount of biomaterial available from human subjects is often limited, restricting the number of experimental control groups as well as the choice of analysis methods. In my work, I have aimed to overcome some of those limitations and bridging the gap between highly standardized - but potentially artificial - basic research approaches and mainly associative, and thus merely descriptive clinical/epidepiological approaches. Together with my students and co-workers, we have developed assays to test adhesion and transmigration behaviour *ex vivo* and *in vivo (Ascione et al, 2015; Brenner et al, 2014; Krankel et al, 2010; Krankel et al, 2011)*.

The choice of the control group / control cases is a point of ongoing discussion in the cardiovascular field. Often, an "apparently healthy" group of subjects of similar age and gender distribution, but absence of the other "classical" cardiovascular risk factors, such as hypertension, dyslipidemia or family history is used. It is highly likely that also in those individuals, atherosclerosis is progressing, but to a slower/lesser degree. One might criticize that the identification of these control subjects in itself is a selection process, choosing individuals with a lower-than-average cardiovascular risk. In addition, these control subjects are usually not medicated, while patients with established cardiovascular or metabolic disease often already recieve pharmacological therapy according to the relevant guidelines. A selection of individualy with one risk factor, possibly even already successfully treated (e.g. hypertension) can therefore increase relevance of the findings. In contrast, the study of general biological principles or novel cell types might initially be assessed in young, healthy subjects before it is verified in advanced-aged individuals with/without established cardiovascular disease.

Basic biological principles are often tested in animal models. Those have the benefit of allowing control over a number of parameters, including diet and genetic background. However, differences between human and murine innate immunity *(Sun et al, 2016; Ziegler-Heitbrock, 2014)* as well as cholesterol metabolism *(Ishikawa et al, 2015; Takahashi et al, 2011)* limit translation. Advanced biotechnological methods, such as 3D printing and culture as well as the differentiation of more and more cell types from induced pluripotent stem cells allow for the usage of organoids for the *in vitro* study of complex cellular and molecular processes in a human-specific context. In addition, murine models only reflect human disease states with limitations. E.g. the study of plaque rupture in mice cannot be modelled satisfactorily, owing also to differences in scale, thus placing more relevance to diffusion distances and the thickness of the vascular wall as well as perivacular adventitial cells and vasa vasorum. Larger animal models (pig, sheep) may be more relevant to the human situation in this respect *(Laakkonen & Yla-Herttuala, 2015; Shim et al, 2016; Tsang et al, 2016; Watanabe et al, 2017)*, but require more complex maintenance conditions and more extensive funding.

6.3.   Study limitations

Several of the cited studies investigate a heterogenous cell population initially referred to as "endothelial progenitor cells" (EPCs), later as "early outgrowth cells" (EOCs). A comparable cell population has first been described by Kalka et al. *(Kalka et al, 2000)*. The paper extended earlier work performed in his group, which investigated the function of bone marrow-derived circulating cells *(Asahara et al, 1999)*. During the late 1990's and early 2000's, several groups have worked to support the hypothesis that blood vessel growth in the adult can be supported by these progenitor cells residing in the bone marrow, in a similar manner as vasculogenesis occurs in the pre-natal developing organism *(Crosby et al, 2000; Schatteman et al, 2000)*. Those studies have greatly contributed to scientific progress and understanding of the nature of the vascular system by including immature as well as hematopoietic cell types into the - at that time more static - conception of post-natal vascular homeostasis, development and regeneration. Yet, the studies were also flawed by methodological limitations which were only identified with hindsight, and which in themselves have helped to develop better mechanisms and concepts. The main limitation of those early studies was the lack of reliable antigenic markers for the hemangioblast, combined with a linear concept of cell differentiation and a small but relevant species heterogeneity between humans and rodents for cells of the hematopoietic and vascular system. Instead, cell lineages and stem cell development is now appreciated to be more flexible, with main cell types characterized by their ability to fulfill specific functions and the expression of only a small number of markers not shared by functionally different cell types. For example, expression of the T cell receptor, CD3, is limited to T cells, but certain adhesion molecules or growth factor receptors, such as the VEGF receptor 2, which were considered to be endothelial-specific, are now recognized to be expressed by other cell types as well. The difficulty of these definitions therefore lies in the potential of functional overlaps between different cell types *(Barnett et al, 2016)*, functional diversity between sub-types of a cell type, and variations in functional response as well as antigen expression due to external cues. All of those are displayed by myeloid cell types. Initially, detection of co-expression of the VEGF receptor 2 and the progenitor markers CD133 and CD34 was sufficient to accept an "endothelial progenitor" identity *(Peichev et al, 2000)*. We now know that monocytes/macrophages can very well upregulate VEGF receptors depending on the presence of the ligand, which is indeed added to widely used endothelial culture media. In addition to the expression of certain marker proteins, monocytes also exhibit functional characteristics which had been ascibed only to endothelial cells before, such as the network-like alignment in a three dimensional matrix *(Schmeisser et al, 2001)*.

A further limitation regards methods to follow cell fate in culture and in living organisms, as well as the resolution of biological detection methods, such as immunohistological sections, and their being hampered by autofluorescence in early studies. More sophisticated tools and models, such as conditional expression of fluorescent dyes or specific epitopes upon promotor activation or the parabiosis model allow for more precision. On the other hand, they are restricted to animal models. As mentioned above, the results may or may not be translatable to the human system. In essence, many of the limitations for the study of „endothelial progenitor cells" also apply for the various subtypes of myeloid cells. Functional and antigenic differences exist between species, hampering the direct translation of findings obtained in murine models onto the human system.

Moreover, myeloid cells show a high functional and phenotypic flexibility depending on milieu conditions. Hence, preanalytic sample preparation, especially speed of cell preparation after blood withdrawal, and temperature onditions, as well as buffer composition (e.g. presence of calcium chelators or protease inhibitors) are crucial when working with human primary samples.

6.4. Open questions and ongoing research

Based on the data presented before, several questions regarding the maintenance and improvement of endothelial integrity and function in patients with cardiovascular disease remain unanswered.

Extracellular vesicles with their complex composition and high stability in biological fluids, but especially microvesicles which bear surface markers of their cells of origin have been suggested as biomarkers for monitoring cardiovascular risk *(Amabile et al, 2014)*. Our own initial observations, as well as reports from the literature indicate a high inter-individual variation as well as high dependency of analysis protocols, thereby currently limiting the use of absolute SMV levels as predictors of CV risk *(Ayers et al, 2015)*. By carefully establishing and maintaining isolation and quantification protocols, it might be possible to monitor changes in SMV levels within individuals over time or in response to a challenge *(Ayers et al, 2015)*. Thereby, therapy success could be monitored and problems in adherence or tolerance towards a certain intervention could be identified, or therapy protocols could be personalized.

In addition to quantifying the heterogeneous spectrum of extracellular vesicles, it is also important to understand variations in their recruitment mechanisms. Our own preliminary data indicate a preferred recruitment of monocyte-derived microvesicles to endothelial areas subjected to non-laminar flow conditions as compared to endothelial areas growing under laminar flow, while T lymphocyte-derived microvesicles did not adhere in a flow-dependent way [*Kränkel & Kya et al. 2016, unpublished*]. This observation suggests that endothelial cells in areas of non-laminar flow receive "molecular input" by microvesicles in a different manner than endothelial cells in areas of laminar flow. This might well play a role in the localization of atherosclerotic plaques predominantly in areas of non-laminar flow. In a similar manner, this might affect endothelial erosion at the downstream "shoulder" of an atherosclerotic plaque. Moreover, activation of specific leukocyte types might bear more or less significance for endothelial function depending on the localization of the endothelial cell (e.g. in an area of disturbed flow, "input" from monocytes might prevail, while in an area of of laminar flow, molecular cues delivered by MVs derived from other cell types might carry more relevance to the individual EC´s function). Better characterization of the molecules involved might help to improve delivery strategies for therapeutics in the future.

Short, non-coding oligonucleotides, the so-called microRNAs (miRs), regulate the stability and translation of mRNAs and thereby affect the amounts of seceral target proteins *(Winter et al, 2009)*. We have identified the miR-483-3p as being present at higher levels in EOC/M2-macrophages of patients with diabetes mellitus type 2 in comparison to non-diabetic patients with comparable cardiovascular disease state, or in healthy controls (*Kränkel & Kuschnerus, 2012-2016, manuscript under review*). Subsequently, we have studied the role of the miR-483-3p in human *ex vivo* models and murine *in vivo* models of diabetic vascular response to injury. The results indicate a potential benefit of miR-483-3p inhibition in patients with diabetes mellitus type 2.

7. Summary

With the change of life style around the world – increased calorie uptake, less active lifestyle, increased uptake of substances and particles which increase oxidative and inflammatory stress for the endothelium –even greater efforts need to be put into prevention and treatment measures for cardiovascular diseases - the main non-communicable cause of death world-wide. This requires concerted efforts on a scientific as well as population-based pre-clinical and clinical scale.

The function of endothelial cells is crucial for both, physiologic as well as pathologic processes and underlies the initiation and progression of cardiovascular diseases. Endothelial cell function in turn is affected and modulated by a variety of cells with paracrine activity, both resident within the vascular wall as well as recruited to a site of vascular injury or to an ischaemic tissue from the circulation. Those „accessory" cells possess great functional and antigenic flexibility and may therefore be crucial in the pathogenesis of the disease, as well as a valuable tool in understanding disease mechanisms and in therapy.

It has been the focus of my work to better understand alterations in the function of cells with paracrine influence on endothelial cell function in patients with cardiovascular disease and in their recruitment to the vascular wall after a local injury. Together with my co-workers, I have developed and optimized ex vivo assays to enable and improve the phenotypic and functional characterization of circulating cell populations obtained from patients as well as healthy controls. Next, I have studied the recruitment of circulating cells to sites of tissue ischaemia as well as vascular injury on a molecular and cellular level. Here, we specifically investigated the role of kinin signalling and SDF-1/CXCR4 signalling as mechanisms to recruit specific subtypes of circulating cells, as well as the alteration of these recruitment processes in the context of acute vascular injury as well as atherosclerosis. In both cases, pharmacologic treatment is available to prevent the degradation of the messenger peptides/proteins by angiotensin-converting enzyme and dipeptidyl peptidase 4.

Our findings support the notion that modulating the function of myeloid cells and their selective recruitment can manipulate endothelial ability to mount capillarization and vascular repair processes, and that the altered paracrine profile of those „accessory" or „regulatory" cell types greatly contributes to the reduction of endothelial function in patients with cardiovascular diseases.

Our ongoing studies elucidate the molecular principles underlying microvesicle-mediated crosstalk between leukocytes and the endothelium, specifically the adherence of microvesicles derived from individual types of leukocytes to endothelial areas of laminar versus disrupted flow. Alterations in microvesicle release by distinct types of leukocytes during therapy might help to monitor therapy adherence and success. Together, these investigations might help optimize target specific delivery of therapeutics, thus enhancing therapy efficiency and limiting side effects.

8. Acknowledgement

9. References

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,

- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,

- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

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Datum   Unterschrift