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Author: Khairoun, Meriem

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Chapter 1

Introduction, aims and thesis outline

1. Endothelial dysfunction in renal disease

1.1 General introduction

Endothelial cells (ECs) line the lumina of all blood vessels and form the interface between the blood and tissue (1). A well-functioning endothelium is critical for the delivery of oxygen and nutrients (2). The endothelium forms an important physical barrier and regulates vasomotor tone and the control of tissue inflammation and of thrombosis (3). This process is tightly regulated by both autocrine and paracrine factors that influence the microvascular integrity in the kidney and other organs (4;5). As a consequence of chemical and mechanical injuries, the endothelium is constantly undergoing injury and repair. Upon EC injury there is a switch from a quiescent phenotype toward responsive activated endothelium. Most cardiovascular risk factors, including diabetes mellitus (DM) and chronic kidney disease (CKD) activate the endothelial layer that consequently results in upregulation of chemokines, cytokines, and adhesion molecules designed to prevent further damage (2;6). Repetitive exposure to injury/activation of the endothelium can ultimately exhaust these protective anti-inflammatory mechanisms within ECs (6). As a consequence, the microvascular endothelium loses integrity and its ability to maintain homeostasis, which results in disturbed capillary blood flow and detachment of ECs into the circulation (6;7). Eventually, if damaged endothelium is not repaired by replication of adjacent mature ECs or circulating endothelial progenitor cells (EPCs), ECs become dysfunctional and irreversibly damaged, resulting in a disease state (6).

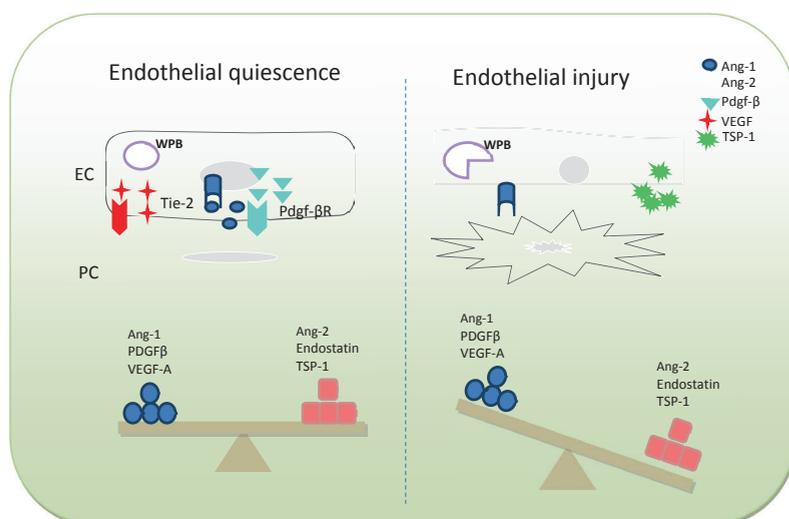


Figure 1: In healthy kidneys, there is a tightly controlled equilibrium between factors favoring (e.g. Angiopoietin-1 (Ang-1), Vascular Endothelial Growth Factors (VEGF), Platelet Derived Growth Factor (PDGF)) and inhibiting (e.g. Angiopoietin-2 (Ang-2), Thrombospondin-1 (TSP-1), Endostatin) EC proliferation and survival. When ECs become activated upon injury, a dysbalance in proangiogenic and antiangiogenic growth factors favors loss of renal microvascular ECs and loosening interaction with pericytes. EC: endothelial cells; PC: pericytes; WPB: Weibel Palade bodies.

1.2 Angiogenic factors play a central role in microvascular stability

The molecular mechanisms that lead to microvascular injury in organ failure are largely unknown. It has been suggested that several angioregulatory growth factors play a central role in the loss of vascular integrity (5;8). Under homeostatic conditions, there is a tightly controlled equilibrium between factors favoring (e.g. Angiopoietin-1 (Ang-1), Vascular endothelial growth factors (VEGF), Platelet derived growth factor (PDGF)) and inhibiting (e.g. Angiopoietin-2 (Ang-2), Hepatocyte Growth Factor (HGF), Endostatin) EC proliferation and survival in the kidney (5). A dysbalance in proangiogenic and antiangiogenic growth factors favors loss of renal microvascular ECs (Figure 1) (2;5). Recently, the angiopoietin/Tie2 system has been identified as playing a critical role in angiogenesis and inflammation of the renal microvasculature in progressive renal disease (5;9-12). Ang-1 and Ang-2 are growth factors that regulate endothelial function during angiogenesis and inflammation and are competitive ligands for the Tie-2 receptor. Ang-1 is produced by pericytes and has vasculoprotective effects by suppressing vessel leakage, inhibiting vascular inflammation, and preventing endothelial death. In addition, Ang-1 has a critical role in the crosstalk between ECs and perivascular stromal cells. In contrast, Ang-2 is released by ECs from Weibel Palade bodies (WPB) at sites of vascular remodeling and inflammation. Ang-2 has antagonistic effects and acts as a competitive inhibitor for Ang-1, with consequently vessel destabilization, inflammation and induction of the angiogenic response, with formation of abnormal tortuous capillary networks that lead to an abnormal blood flow and results in chronic hypoxia (8;12-15). Under pathological conditions, Ang-2 acts in concert with VEGF to induce inflammatory angiogenesis (16;17). By promoting pericyte dropout, Ang-2 will lead to loosening contacts between ECs and pericytes. In the presence of VEGF, Ang-2 can eventually lead to an active, sprouting state of ECs. Otherwise, when VEGF is absent, Ang-2 facilitates capillary regression (18).

1.3 Pericytes are the link between endothelial damage and renal fibrosis

Pericytes are contractile cells that wrap around ECs and have important roles in supporting the growth and maintenance of capillaries (19). In the microvasculature, communication between ECs and pericytes has been shown to stabilize capillaries and protect them from regression and rarefaction (20;21). Pericytes signal to the endothelium through secreted factors such as PDGF- β , TGF- β and Ang-1, as well as by direct endothelial/pericyte crosstalk which involves notch-3 signaling (22). Similarly, the endothelium signals to surrounding stromal cells using similar growth factors such as Ang-2 and VEGF as well as the notch ligand jagged-1. Bidirectional signaling between pericytes and ECs in disease states may result in pericyte detachment from capillary ECs, resulting in an angiogenic response and formation of unstable tortuous ECs, capillary rarefaction and exacerbation of tissue hypoxia and fibrosis (Figure 2) (8;12-15;23-25). The group of Duffield et al showed migration of pericytes from capillaries into the renal interstitium, already 9 hr after induction of unilateral ureter obstruction (UUO).

After loosening contact from the capillaries, pericytes became activated and proliferated into collagen producing myofibroblasts contributing to fibrosis (20;23;24). Using a transgenic mouse model of UUO, expressing green fluorescent protein in cells producing the collagen type I, the same group confirmed that pericytes are the main source for interstitial myofibroblasts during renal fibrosis (23). Transformation of pericytes into myofibroblasts also induced a switch in VEGF isomers from proangiogenic to antiangiogenic isoforms, which counted for ECs loss (25). The process of pericyte detachment has been suggested to be reversible. For example, blockade of PDGFR- β on pericytes attenuated recruitment of inflammatory cells and detachment of pericytes from ECs in mice subjected to renal I/R injury and unilateral ureter model. Conversely, inhibition of VEGF on ECs abolished pericyte detachment and attenuated both EC loss and development of fibrosis during kidney injury induced by UUO or renal I/R injury (20). These data suggest that targeting interaction between ECs and pericytes may provide a novel therapeutic targets to improve microvasculature and treat acute and chronic kidney injuries.

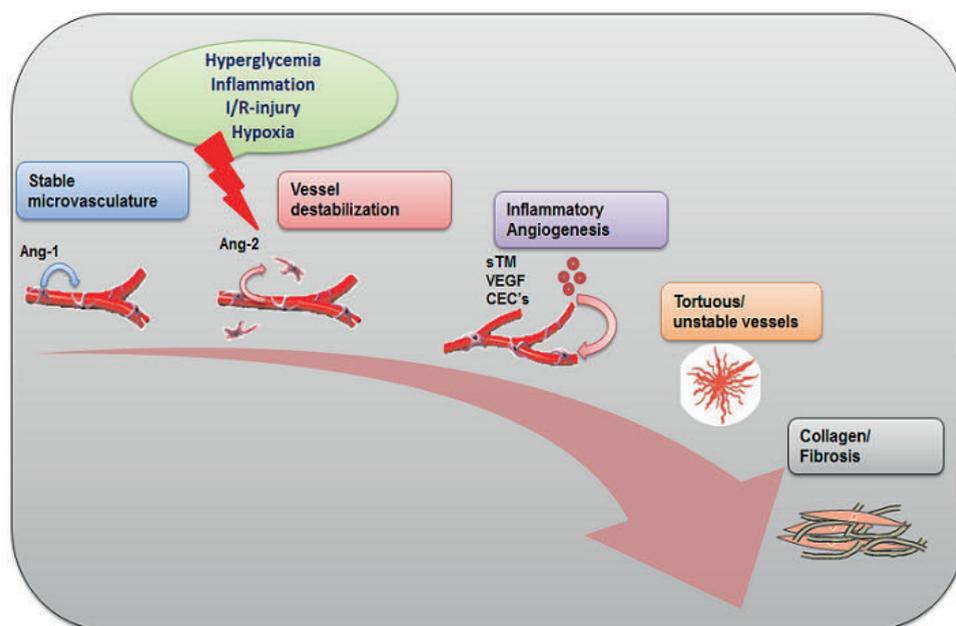


Figure 2: Angiopoietin-1 (Ang-1) produced by pericytes regulates vascular assembly and endothelial quiescence. Endothelial injury induced by different insults (e.g. diabetes mellitus, renal ischemia reperfusion (I/R) injury) leads to activation of endothelial cells (ECs) and release of Ang-2. Consequently, pericytes will detach from the capillary wall which results in vessel destabilization and induction of angiogenesis, partly in concert with other cytokines such as VEGF. When inflammation is sustained, formation of unstable tortuous capillaries occurs and finally microvascular rarefaction. This exacerbates tissue hypoxia and leads to transformation of pericytes into collagen producing myofibroblasts and ultimately scar formation with loss of renal function. sTM: soluble thrombomodulin; VEGF: Vascular Endothelial Growth Factor; CECs: Circulating Endothelial Cells.

2 Assessment of endothelial function

2.1 Circulating markers of endothelial function

The endothelium is relatively inaccessible to direct examination; therefore investigators have concentrated on various surrogate markers of endothelial function which include the measurement of specific plasma markers including the angiopoietins and sTM and measurement of circulating ECs and EPC's in peripheral blood (9;11;26-30). Elevated circulating levels of Ang-2 are reported to be associated with progression of renal disease and with systemic markers of micro-inflammation in chronic kidney disease (CKD) patients (9;26). Furthermore, elevated Ang-2 levels are shown to be strong predictors of long term mortality in CKD patients, independent of vascular stiffness and calcifications (31). Accordingly, renal angiopoietin dysbalance, in favor of Ang-2, has been found in different animal and human models of renal disease (both diabetic and non-diabetic) (13;32;33). In line, trombosmodulin levels are studied in detail in different vascular disease including CKD (29;34). Indeed, increased levels of sTM are demonstrated in CKD patients with and without diabetes and normalization after KTx is reported (29). In patients undergoing long-term hemodialysis treatment increased number of circulating ECs, as marker for systemic endothelial dysfunction, were found compared to healthy controls. EPCs in renal disease have been suggested to be useful for regenerating and maintaining the integrity of vascular structures. In patients with chronic renal disease, EPC numbers and function were decreased compared with healthy subjects, which may prevent physiological vascular repair and contribute to the increased risk for cardiovascular diseases observed in CKD patients (30;35;36).

2.2 Noninvasive visualization of human microcirculation

An additional method for the assessment of endothelial function in CKD includes the non-invasive monitoring of the microvasculature. Capillary nailfold videomicroscopy in advanced CKD patients showed an impaired functional and structural capillary density in the skin (37). These abnormalities may represent manifestations of ongoing systemic microvascular damage and underlie much of the organ dysfunction associated with cardiovascular disease in CKD patients (38). Another study has found a significant association between retinal microvascular abnormalities, using retinal fundus photographs, and renal function deterioration (39). Recently, sidestream dark field (SDF) imaging has been used to visualize the human microcirculation. Compared to conventional capillaroscopy, this method has been used to assess the microvasculature in vivo without injecting fluorescent dyes (40;41). The SDF imaging has opened a way to study the human microcirculation in more detail than previously. In this technique, the tissue is illuminated by light with a wavelength corresponding to the hemoglobin spectrum. Part of the light will be absorbed by the hemoglobin and the rest will be reflected from the background. Specific optical filtration allows the elimination of the light reflected at the surface of the tissue to produce high-contrast reflected light images of the

microcirculation. Hence, red blood cells will appear dark and white blood cells and platelets may be visible as refrinent bodies. Vessel walls are not visible and therefore, vessels will be visible only if they contain red blood cells (Figure 3). The SDF imaging device is particularly convenient for studying tissues protected by a thin epithelial layer such as mucosal surfaces (40). By using SDF imaging, capillary tortuosity and density can be measured to assess microvascular damage. In addition, this technique also enables measurement of the Endothelial Surface Layer (ESL) dimensions, calculated as the difference in Red Blood Cell (RBC) column width before (functionally perfused capillary diameter) and after (anatomical capillary diameter) leukocyte passage (42;43).

We previously used this validated technique to compare the labial mucosal tortuosity, as markers for microvascular damage, in DM patients and controls. We found increased capillary tortuosity in DM patients compared with healthy controls (41). However, there are no studies which have used SDF imaging for assessment of systemic microvascular alterations in CKD patients (including patients with diabetic nephropathy) before and after (pancreas) kidney transplantation.

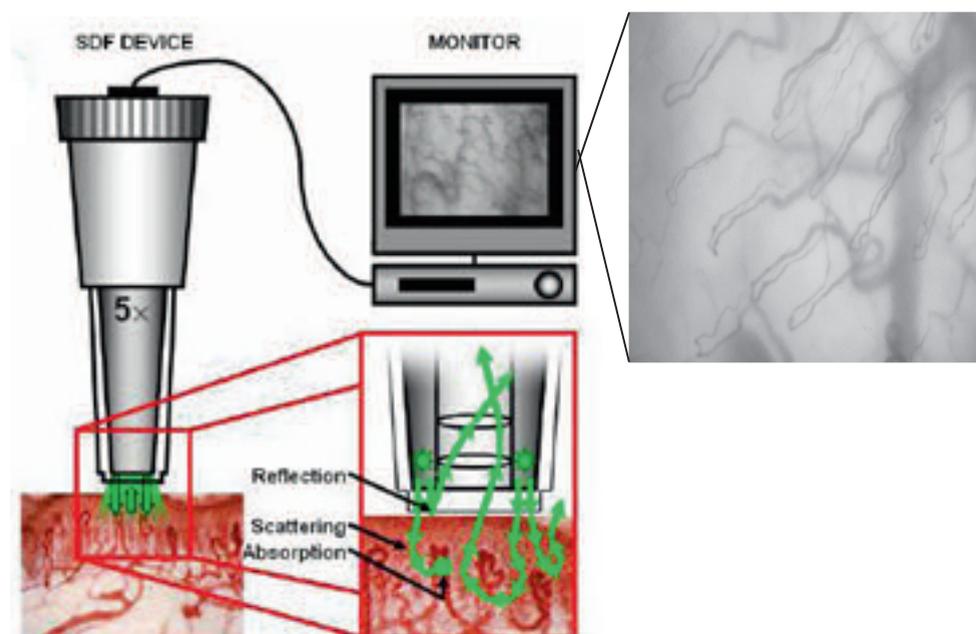


Figure 3: Visualization of the microcirculation using SDF imaging. The tissue is illuminated by light with a wavelength corresponding to the haemoglobin spectrum (548nm). Part of the light will be absorbed by the haemoglobin containing red blood cells and the rest will be reflected from the background. Specific optical filtration allows the elimination of the light reflected at the surface of the tissue to produce high-contrast reflected light images of the microcirculation. Vessels will be visible only if they contain red blood cells. Adapted from Goedhart et al 2007.

3. The microvasculature in disease

3.1 Endothelial injury in renal disease

Injury of microvascular ECs contributes to the impairment of renal perfusion and continued renal hypoxia/ischemia (44). Chronic hypoxia is an important trigger for profibrotic and inflammatory changes, from the early stages of chronic kidney disease (CKD), and finally scar formation and progression to end stage renal disease (5). A dysfunctional endothelium loses its ability to protect the microvasculature by reducing its anti-inflammatory and anti-coagulation effects (45). With decreasing renal function, the endothelium is exposed to pro-inflammatory cytokines which lead to upregulation of cell-surface adhesion molecules and impairs endothelium-dependent vascular relaxation, with further loss of ECs and development of fibrosis (5). In both experimental animal models and in humans, it has been shown that there is significant loss of peritubular capillaries and reduction in the capacity of EC proliferation as well as defective capillary repair in association with the development of renal interstitial fibrosis (2). Interestingly, there is intriguing evidence that stimulation of capillary repair may stabilize renal function and slow progression and that this benefit occurs independently of effects on blood pressure or proteinuria (4;5). Indeed, in patients with chronic renal failure, autopsy material revealed not only diffuse media calcifications and thickening of the arteries, but above all changes in the myocardial microcirculation including rarefaction of the intramyocardial capillaries (46).

3.2 Microvascular disease in diabetes mellitus

Diabetes mellitus is strongly associated with the development of macrovascular and microvascular disease, including retinopathy, neuropathy and nephropathy (DN), which is the leading cause of CKD (47;48). The development of these complications have been correlated with glucose mediated endothelial damage, oxidative stress due to accumulation of toxic intracellular products such as sorbitol or advanced glycation end products (AGEs) (49). As a response to hyperglycemia, microvascular ECs undergo several metabolic derangements, which cause activation of ECs, increased expression of different growth factors, altered blood flow and permeability of the endothelium and coagulation resulting in structural and functional alterations in microvascular integrity (50;51). Endothelial dysfunction and increased oxidative stress are reported in patients with prediabetes and in the early course of DM as well, indicating a prominent role of microvascular EC damage in the onset of early microangiopathy (52). Different angiogenic growth factors have been shown to be increased in DM including VEGF and Ang-2 (53). Several studies suggest that Ang-2 and VEGF act together inducing sprouting angiogenesis and ultimately leading to vascular destabilization and dysfunction (17). In diabetic retinopathy, loss of pericytes is reported to be one of the first morphological changes that occurs in the capillaries with concomitant endothelial alterations, such as ECs loss, irregular shaping of capillaries and local areas of hypoxia (19;54).

3.3 Transplantation and microvasculature

Kidney transplantation has improved survival and quality of life for patients with end-stage renal failure. Despite dramatic improvements in short-term survival, long-term survival of renal allografts has changed little during the past decade (55). Most late renal allograft loss has been attributed to progressive renal dysfunction, histological characterized by interstitial fibrosis and renal atrophy (IFTA) (56). The fibrosis in the transplanted kidney represents cumulative and incremental damage to nephrons from various immunological (antibody-mediated and T cell-mediated rejection, sensitization, HLA mismatch) and non-immunological (nephrotoxicity of calcineurin inhibitors, ischemia-reperfusion (I/R) injury, donor age, hypertension, infections) insults (57). Among these factors, acute rejection and I/R injury primarily target the endothelium and disrupt microvascular homeostasis. Activation of pro-inflammatory and pro-coagulant pathways lead to irreversible injury of the endothelium with ensuing renal fibrosis and loss of allograft function (2;58).

3.3.1 Ischemia-reperfusion injury

Renal I/R injury is an inevitable consequence of organ transplantation, and a major determinant of patient and graft survival (59). The pathophysiology of I/R injury is complex and incompletely understood (60). Although the role of tubular cell injury in post-transplantation graft dysfunction is widely acknowledged, damage to microvascular endothelial cells is emerging as increasingly important (61). Inflammation after reperfusion is considered to be the most crucial initiator of EC injury in kidneys subjected to I/R. This will lead to production and release of pro-inflammatory and pro-angiogenic factors by ECs (e.g. tumor necrosis factor, VEGF, Ang-2) and upregulation of different adhesion molecules, that facilitate leukocyte-endothelial cell interactions with consequently ECs dysfunction and peritubular capillary loss (62;63). If early capacity of EC repair and protection is limited, ongoing local ischemic injury will result in persistent cell death and chronic allograft injury (2). Indeed, it is demonstrated that early loss of peritubular capillaries predicts the development of tubulointerstitial fibrosis and rejection after transplantation and can be predictive of long-term graft survival (64). Therapeutic interventions aiming at enhancement of the microvasculature and promoting repair early after transplantation may be beneficial for graft health and prevent chronic injury and rejection.

3.3.2 Allograft rejection

In the pathogenesis of rejection, ECs respond to cytokines and growth factors produced in association with alloimmune responses (64;65). Activated microvascular ECs express adhesion molecules, cytokines, chemokines and growth factors that mediate the recruitment of recipient leukocytes (2;64;65). In addition, activated ECs express MHC class I and II molecules which are critical for the presentation of alloantigen to infiltrating lymphocytes (64). It is proposed that repetitive insults target the microvasculature and induce loss of the microvasculature, which

may result in impaired delivery of oxygen and nutrients to renal tubular epithelial cells, chronic ischemia and cell death (58). In vascularized solid organ allografts, such as the kidney, the degree of injury and microvascular EC loss has been reported to predict the development of IFTA and later chronic rejection and strategies which can protect the endothelium have potential to improve long-term graft survival (58;66;67).

4. Restoration of the renal microvasculature

The concept that injury to the endothelium may precede renal fibrosis strongly suggests that interventions to maintain vascular integrity are of major importance in the treatment of renal disease (5). On the beneficial effects of preservation of the microvasculature in renal disease several studies have been reported using different therapeutic strategies. Promising results about the administration of different growth factors have been shown in experimental animal models to enhance angiogenic sprouting and subsequently repair the injured kidney microvasculature. The use of recombinant COMP-Ang-1 (a soluble, stable and more potent form of Ang-1) and VEGF-A in kidney models of progressive renal failure, improved renal function, preserved renal microvasculature and decreased inflammation and fibrosis. Another important factor for angiogenic therapy to be successful is the stage of disease. Indeed, initial stages of DN are associated with upregulation of VEGF and increased number of vessels, implicating that at this stage antiangiogenic therapy may be beneficial. In contrast, advanced stage of DN is associated with loss of peritubular capillaries, and proangiogenic therapies have shown promising results at this stage (5;10;68;69). An alternative strategy is the use of EPCs. In different animal models of CKD, transplantation of EPCs resulted in preservation of peritubular capillaries, improvement in renal function and reduction glomerulosclerosis and development of fibrosis, by homing to areas of injury and inflammation in the kidney. Furthermore, there is also evidence that resident stem/progenitor cells in the kidney might contribute to the maintenance and repair of renal endothelium and interstitium (2;5;35;36;70). Finally, mesenchymal stromal cells (MSCs) have been reported to have potent immunomodulatory and reparative effects. In different experimental animal models of renal disease, infusion of MSCs decreased fibrosis and promoted angiogenesis. For example, in renal ischemia, the process of microvascular loss was hampered in postischemic kidneys after injection of MSCs (71;72).

5. Aims and outline of this thesis

The growing literature indicates that endothelial injury and repair are most important concepts for our understanding of renal disease and that manipulation of the response might have major consequences for therapeutics in the future. The use of SDF imaging to measure

microvascular damage and the measurement of endothelial dysfunction markers may be useful diagnostic tool for monitoring the microvasculature before and after transplantation. This could ultimately lead to a better understanding of the pathogenesis, earlier diagnosis and effective treatment of microvascular damage in CKD and renal (pancreas) transplant recipients. This thesis focuses on the mechanisms involved in the process of endothelial damage and repair in CKD, (early) DM, renal I/R injury and after transplantation

The aims of this thesis are:

1. Study in detail the effect of renal I/R injury on angiotensin expression and its association with pericytes and fibrosis development, using a rat I/R injury model (**chapter 2**).
2. Study damage to the microvasculature and the role of angiotensins in human renal I/R injury and compare living-donor (LD) and deceased-donor (DD) kidney transplantation (**chapter 3**).
3. Investigate the early effects of atherogenic diabetes on systemic microvasculature and its association with renal damage using a streptozotocin -induced model of diabetes and atherogenesis in pigs (**chapter 4**).
4. Assess effect of simultaneous pancreas kidney transplantation in DN patients on microvascular alterations using SDF imaging and endothelial dysfunction markers (**chapter 5**).
5. Investigate systemic microvascular alterations in CKD patients before and after living kidney transplantation, using SDF imaging and endothelial dysfunction markers (**chapter 6**).
6. Investigate whether acute rejection after kidney transplantation is associated with systemic microvascular damage (**chapter 7**).

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