# Painting proteins to study cardiovascular pathology

**ir. A. Dickhout, PhD**
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Universiteit Maastricht

*Promotores:*
Prof. T.M. Hackeng, PhD
R. Koenen, PhD

*Co-promotor:*
ir. I. Dijkgraaf, PhD

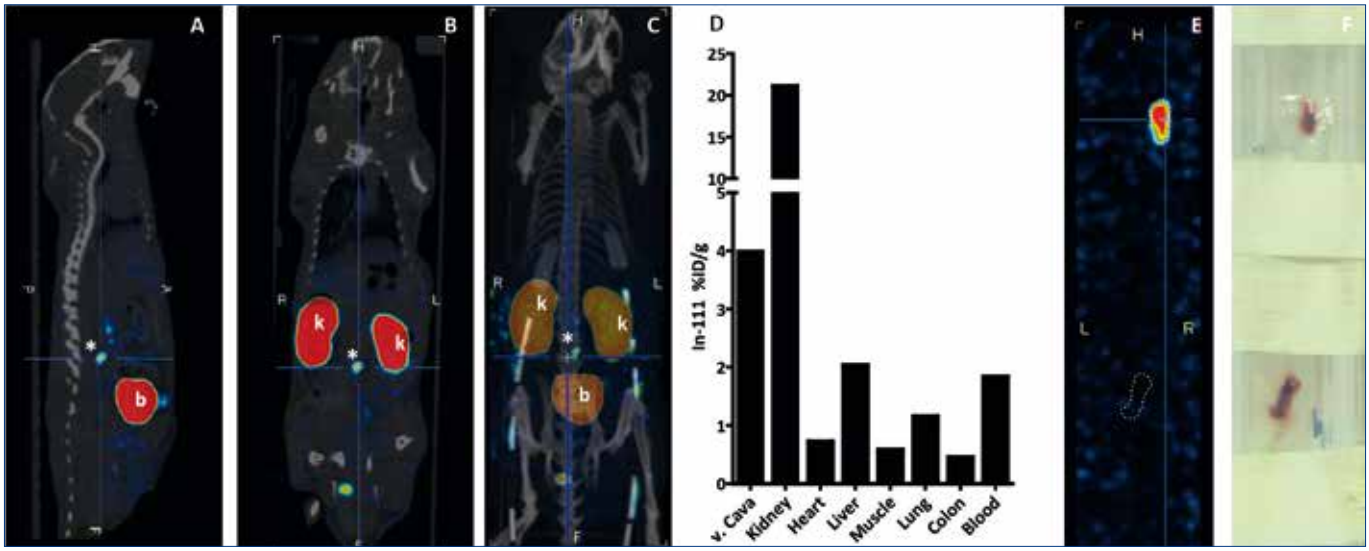
## Cardiovascular disease

Cardiovascular disease causes eminent morbidity and mortality across the globe. As the aetiology is complex and diagnosis is often in a late stage of disease, deciphering molecular mechanisms underlying cardiovascular pathology is vital to advance diagnosis and prevention. In the first part of this thesis, we use different molecular imaging strategies to elucidate molecular and cellular communication underlying cardiovascular pathology. These molecular mechanisms have the potential to pose as excellent imaging targets, which we exploit in the second part of this thesis to image thrombosis in a mouse model using SPECT/CT.

## Cellular communication

A class of proteins playing a major role in the communication between cells of the blood and the vasculature are chemokines. The chemokines CCL5 and CXCL4 can form heterodimers and with CXCL4 enhancing the CCL5-mediated monocyte attraction, drive atherosclerosis. Understanding the localization and behaviour of these two chemokines is thereby important in understanding atherosclerosis formation. We use different high-resolution microscopy techniques to analyse the localization of CXCL4 and CCL5 in resting platelets. Both are α-granule proteins, but previous studies suggest differential release upon different stimuli. In this thesis, we show that CCL5 and CXCL4 in resting platelets can be imaged with light microscopy, electron microscopy, and combined light and electron microscopy. The results suggest there is differential storage of CCL5 and CXCL4 in α-granules, possibly explaining differential release. We also investigate the fate of CCL5 and CXCL4 after platelet release and deposition on endothelial cells *in vitro*. Chemokines are incubated with primary endothelial cells and an endothelial cell line, and chemokine localization is studied. CXCL4 is localised partly on the membrane, and partly internalized in endothelial cells, in contrast to CCL5 which is all internalized quickly, and no CCL5 is left on the cellular membrane. Intracellular, chemokines localise partly to the nucleus. The internalization is rapid and active endocytosis dependent on dynamin, clathrin, and G protein-coupled receptors (GPCRs), but not on surface proteoglycans. Intracellular calcium signals are found to be increased by chemokine treatment, however, monocyte recruitment under laminar flowing conditions is unaltered. Studying blood cell interactions under laminar flow is extremely important, as different interactions take place under different shear stress. This thesis harbours a methodologic paper, in which we provide a detailed protocol that can be adjusted based on the specific interactions to be studied, for both human and mouse cells. As a representative example, human monocytic cells are flowed over healthy primary endothelial cells at venous shear. Monocyte adhesion is markedly increased after endothelial stimulation with TNFα. Human neutrophil to human platelet adhesion is increased after platelet stimulation with the PAR1 ligand TRAP-6. Using platelets of JAM-A$^{+/+}$ and JAM-A$^{-/-}$ mice, and the mouse monocytic cell line RAW264.7, JAM-A deficiency increases the adhesion of mouse monocytes. This increased adhesion is likely based on the GPIbα-$\alpha_M\beta_2$ axis, as blocking of both receptors decreases monocyte adhesion.

Platelets can be activated by different stimuli. In this thesis, we focus on platelet stimulation by galectin-1 and CXCL4. We show that both proteins stimulate platelets in a complementary matter, as galectin-1 stimulation results in robust activation of integrin $\alpha_{IIb}\beta_3$ without expression of P-selectin, whereas CXCL4 stimulation fails to induce an $\alpha_{IIb}\beta_3$ response but does lead to P-selectin expression. Pre-incubation of the




*Figuur 1.* Representative SPECT/CT overlays of sagittal (A) and coronal (B) views, and a 3D model (C) of a mouse 6 hr after IVC ligation, injected with $^{111}$In-A16. High uptake of the tracer is seen in kidneys (k), bladder (b) and thrombus (*). (D) accompanying biodistribution of this mouse showing high uptake in the v. cava and kidneys, expressed as percentage injected dose per gram tissue (%ID/g). (E) Ex vivo SPECT scan of the thrombus (top dotted line). The lower dotted line outlines the IVC of a sham-surgery mouse injected with $^{111}$In-A16. (F) Embedded thrombi.

galectin and chemokine leads to additive platelet activation rather than a synergistic response. Desialylation of platelets leads to a higher galectin-1 binding and response in contrast to expectation, and despite unaltered P-selectin and higher PS-exposure on desialylated platelets stimulated with CXCL4, aggregation is completely abolished.

## Imaging thrombosis

With a globally ageing population, the lifetime risk of thrombo-embolic and ischemic diseases is increasing. Survival rates after thrombo-embolic disease, such as pulmonary embolism (PE) but also ischemic stroke and myocardial infarction, are inversely correlated with time to treatment and therefore it is important to diagnose early after onset. Fibrinolytic therapy (lysis) is the first line of treatment for these ischemic diseases, but resistance to lysis increases with the age of the thrombus, while the hazard of severe side effects such as gastrointestinal bleeding or intracerebral haemorrhage remains. The first hours after thrombus formation is the timeframe in which thrombolytic treatment with tissue plasminogen activator (tPA) is indicated for PE, ischemic stroke, and ST-segment elevation myocardial infarction (STEMI), as well as other thrombosis-related off-label indications. Therefore diagnosing early thrombus formation will aid in selecting patients that will benefit from fibrinolytic therapy.

During early thrombogenesis, activated factor XIII (FXIIIa) cross-links $\alpha_2$-antiplasmin to fibrin to protect it from early lysis. This feature is exploited in the second part of the thesis to develop an $\alpha_2$-antiplasmin-based imaging agent (A16) to detect early clot formation likely susceptible to thrombolysis treatment. First, efficient *in vitro* fluorescent- and $^{111}$In-labelled imaging probe-to-fibrin crosslinking is obtained. Next, thrombus formation is induced in C57Bl/6 mice by endothelial damage ($FeCl_3$) or by ligation (stenosis) of the infrarenal vena cava (IVC). Two or six hours post-surgery respectively, mice are injected with $^{111}$In-DTPA-A16 and ExiTron™ Nano12000, and binding of the imaging tracer to thrombi *in vivo* is assessed by SPECT/CT. Subsequently, *ex vivo* IVCs are subjected to autoradiography and histochemical analysis for platelets and fibrin. *In vivo* IVC thrombosis models yields stable platelet-rich thrombi with $FeCl_3$, and fibrin and red cell-rich thrombi with stenosis. Clot formation in the vena cava corresponds with a SPECT hotspot using the A16 imaging probe as a molecular tracer, as shown in figure 1. The fibrin-targeting A16 probe shows specific binding to murine thrombi in *in vitro* assays and the *in vivo* DVT model. The use of specific and covalent fibrin-binding probes might enable clinical non-invasive imaging of early and active thrombosis.

*annemiekdickhout@hotmail.com* ♦