Molecular Imaging of Arterial and Venous Thrombosis

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June 3, 2020

Abstract

Thrombosis contributes to one in four deaths worldwide and is the cause of a large proportion of mortality and morbidity. A reliable and rapid diagnosis of thrombosis will allow for immediate therapy, thereby providing significant benefits to patients. Molecular imaging is a fast-growing and captivating area of research, in both preclinical and clinical applications. Major advances have been achieved by improvements in three central areas of molecular imaging: 1) Better markers for diseases, with increased sensitivity and selectivity; 2) Optimised contrast agents with improved signal to noise ratio; 3) Progress in scanner technologies with higher sensitivity and resolution. Clinically available imaging modalities used for molecular imaging include, magnetic resonance imaging (MRI), X-ray computed tomography (CT), ultrasound, as well as nuclear imaging, such as positron emission tomography (PET) and single photon emission computed tomography (SPECT). In the preclinical imaging field, optical (fluorescence and bioluminescent) molecular imaging has provided new mechanistic insights in the pathology of thrombembolic diseases. Overall, the advances in molecular imaging, driven by the collaboration of various scientific disciplines, have substantially contributed to an improved understanding of thrombotic disease, and raises the exciting prospect of earlier diagnosis and individualised therapy for cardiovascular diseases. As such, these advances hold significant promise to be translated to clinical practice and ultimately to reduce mortality and morbidity in patients with thromboembolic diseases.

Molecular imaging for thrombosis

Thrombosis is a complex event, usually a result of undesired and uncontrolled platelet adhesion and aggregation, as well as increased thrombin formation and fibrin generation, within a vessel (Ziegler et al., 2019; Montague et al., 2020). Therefore, biomarkers of platelet activation and fibrin generation have attracted major attention as molecular targets for molecular imaging of arterial and venous thrombosis. As microthrombosis plays an important role in ischemic-reperfusion (I/R) injury, these targets have also been used to image, for example cardiac I/R injury (Ziegler et al., 2019).

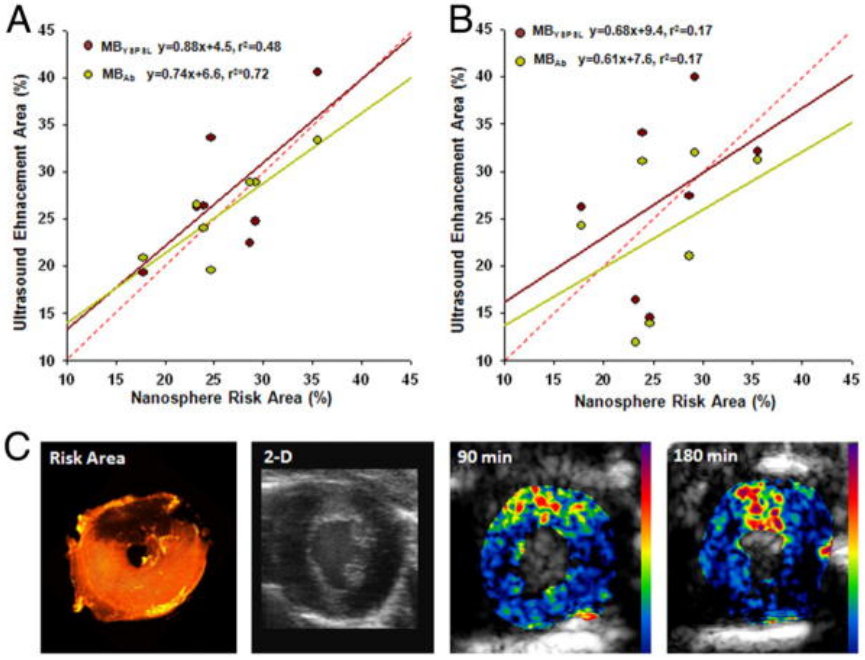
Platelet imaging

Platelets express several platelet-typical receptors on their surface. For molecular imaging, those receptors are of particular interest, which can be used to differentiate between resting and activated platelets. Thereby, selective targeting of activated platelets present in thrombi as compared to non-activated platelets in the circulation can be achieved. The two most attractive biomarkers for platelet activation used in molecular imaging are P-selectin and Glycoprotein (GP) IIb/IIIa.

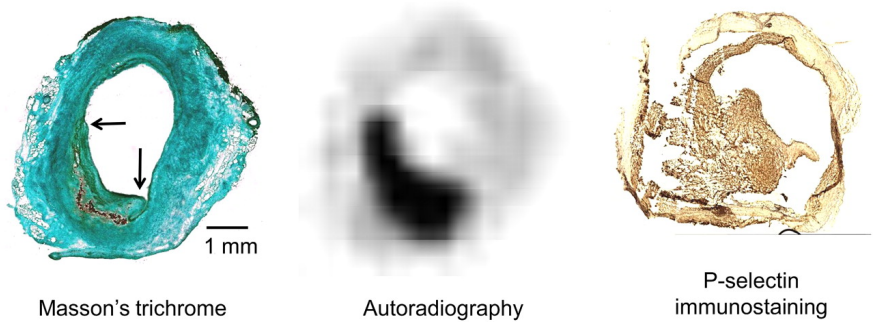
P-selectin

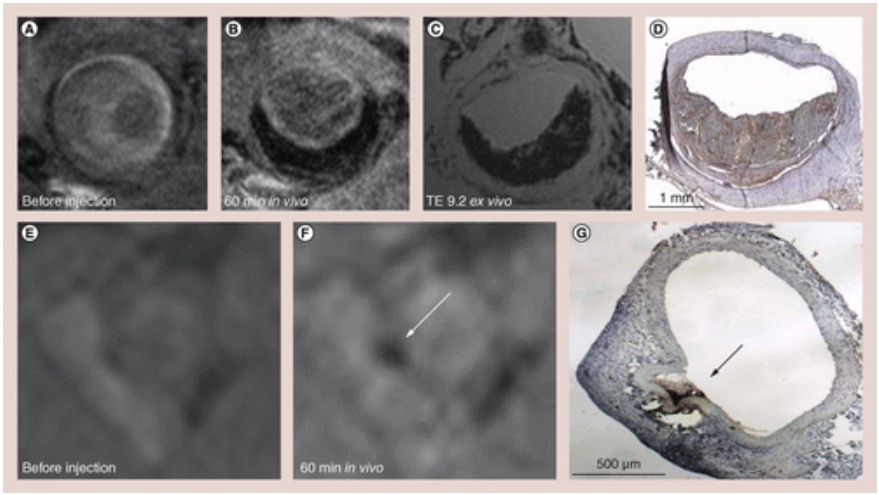
Upon platelet activation P-selectin, which is also known as CD62P, is rapidly mobilised from alpha granules to the platelet surface membrane, thereby allowing for the discrimination between non-activated and activated platelets. The expression of P-selectin also leads to leukocyte recruitment into the vessel via P-selectin glycoprotein ligand-1 (PSGL-1) (Furie and Furie, 2004). By conjugating microbubbles with either a P-selectin monoclonal antibody or a PSGL-1 recombinant targeting ligand, Davidson et al. visualised

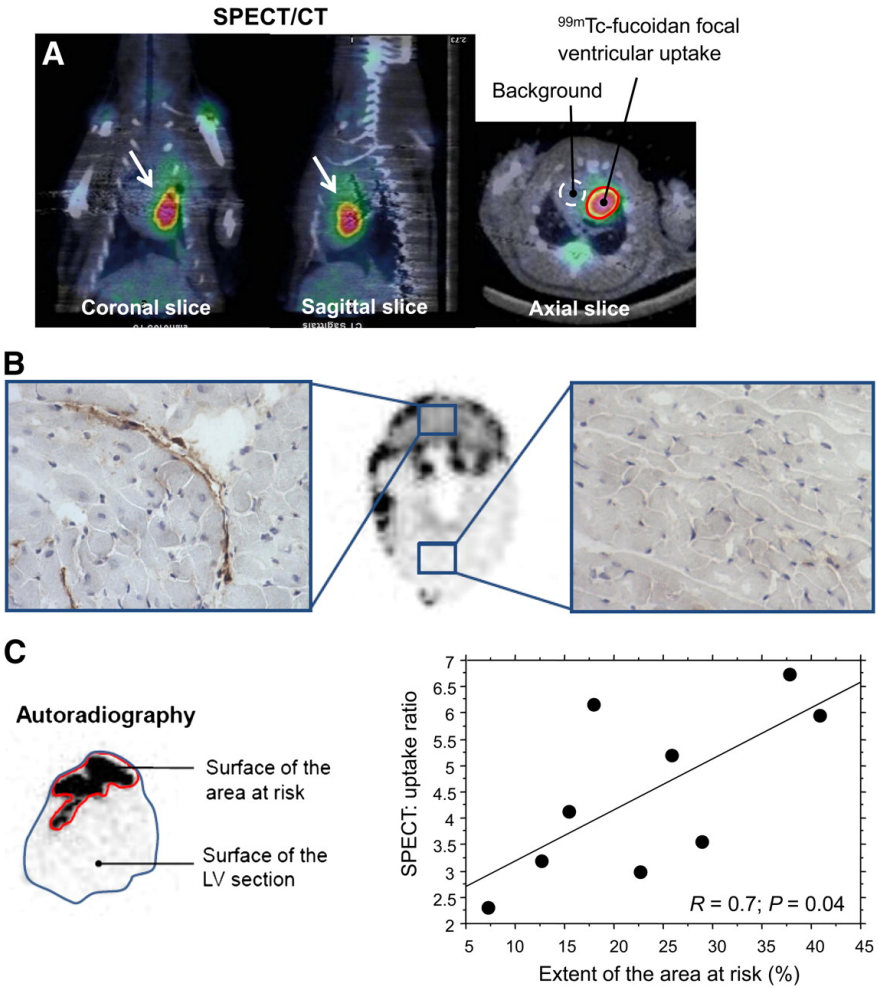
enhanced ultrasound imaging in the post-ischemic regions using a hind-limb ischemia murine model, as well as myocardial I/R injury in both a murine and a non-human primate model (Davidson et al., 2012, 2014). These microbubbles produced an increase in signal enhancement at the 90 min and 3 h time-points post-cardiac I/R injury (Figure 1) (Davidson et al., 2012). The area of contrast enhancement on ultrasound correlated with the area at risk of ischemia on histology.



Several research groups have used fucoidan, a polysaccharide that has a high affinity towards P-selectin, functionalised to contrast agents for visualisation of thrombi via SPECT, PET, MRI and ultrasound imaging (Rouzet et al., 2011; Sabourel et al., 2014; Suzuki et al., 2015; Li et al., 2019). Using rat models, Rouzet et al. demonstrated the binding of fucoidan radiolabeled with ^{99m}Tc to the intraluminal thrombus in an elastase rat model of abdominal aortic aneurysm (AAA). Histologic analysis using Masson trichrome and immunostaining using P-selectin showed co-registration with the autoradiography of^{99m}Tc-fucoidan uptake within the mural thrombi (Figure 2) (Rouzet et al., 2011). Using the same AAA rodent model, Suzuki et al. investigated the binding of ultrasmall superparamagnetic iron oxide (USPIO) particles coated in fucoidan to intraluminal thrombi via MRI. The successful targeting of USPIO-FUCO resulted in hyposignals at the location of the thrombi, whereas no change of signal was observed using the non-binding USPIOs control (Figure 3) (Suzuki et al., 2015). Via SPECT imaging, the group also observed an uptake of^{99m}Tc-fucoidan in the intramyocardial vasculature in a cardiac I/R injury rat model (Figure 4) (Rouzet et al., 2011; Sabourel et al., 2014). The uptake of radioactivity in the mid ventricular section of the heart (area at risk) also matched their results from immunohistological staining of P-selectin (Rouzet et al., 2011). However, P-selectin can be shed from the surface of activated platelets and is also expressed on other cells such as endothelial cells. Therefore, it is not specific for platelets, thereby limiting its suitability as a target epitope of activated platelets.

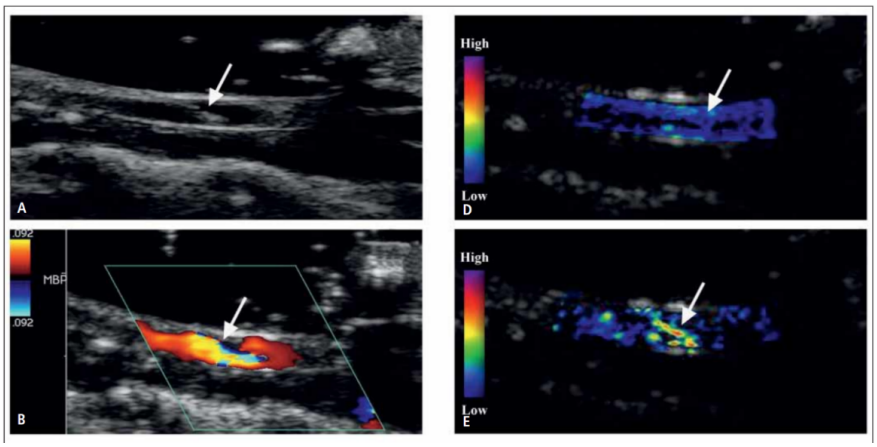






Glycoprotein (GP) IIb/IIIa

GPIIb/IIIa receptor, also known as integrin $\alpha_{IIb}\beta_3$ and CD41/CD61, is the most abundant receptor on the membrane surface of platelets with 60,000 to 80,000 receptors per platelet. The expression of GPIIb/IIIa is platelet-specific and upon platelet activation, the GPIIb/IIIa receptor undergoes a conformational change from a low affinity to a high affinity receptor for its natural ligand fibrinogen/fibrin. The arginine-glycine-aspartic (RGD) peptide mimics the binding epitopes of GPIIb/IIIa's natural ligand fibrinogen, and has therefore been used for the detection of vascular thrombosis via a range of imaging technologies (Schumann et al., 2002; Klink et al., 2010; Zhou et al., 2011; Hu et al., 2012; Unger et al., 2014; Guo et al., 2015; Kang et al., 2015; Rix et al., 2016). Hu et al, demonstrated that microbubbles conjugated with cyclic RGD peptides, facilitated the visualisation of thrombosis via molecular ultrasound imaging (Figure 5) (Hu et al., 2012). In this study, the authors demonstrated that brightness-mode ultrasound imaging could be used to visualise the echogenic thrombi in the abdominal artery of a rodent model. Colour Doppler imaging was also used to confirm the vascular stenosis created by the thrombi (Hu et al., 2012).

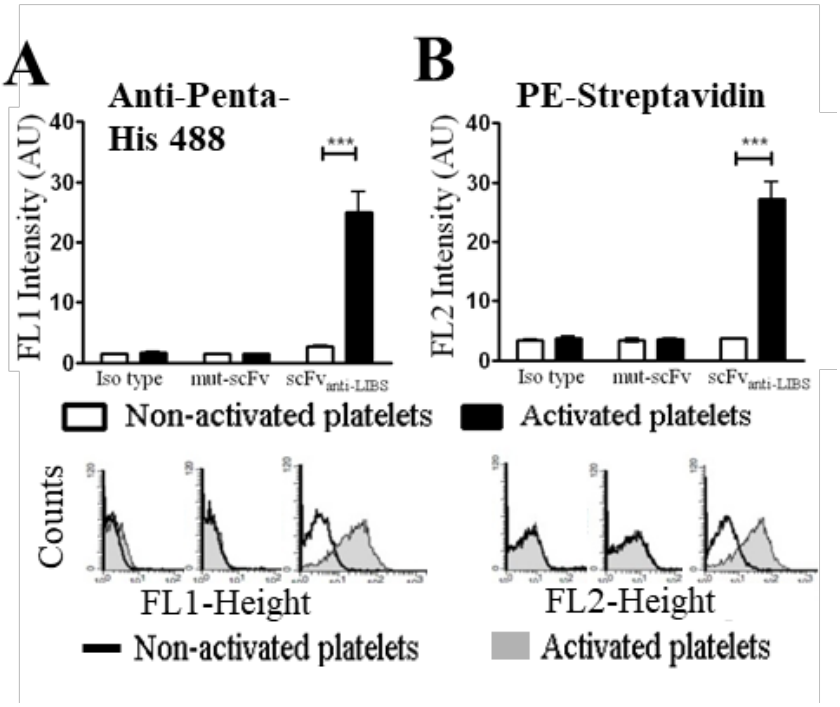


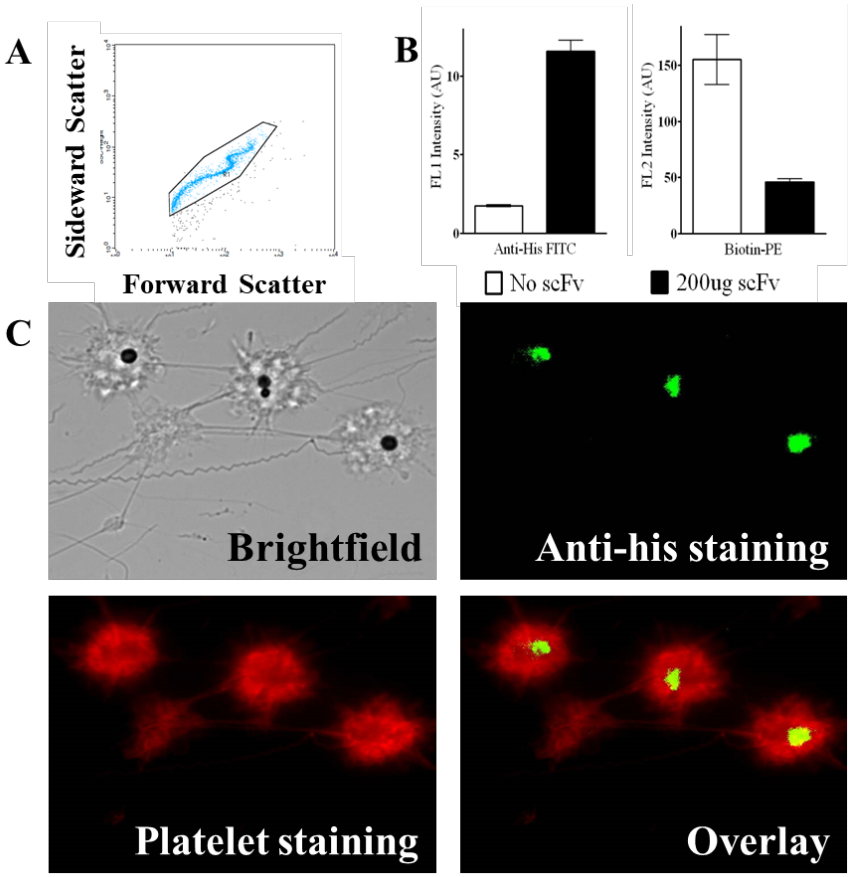
Using a cyclic RGD peptide, P975 conjugated to a Gd-DOTA chelate, Klink et al. demonstrated enhanced MRI signal of the carotid artery *in vivo* using a murine model of arachidonic acid induced thrombosis. T1-weighted MRI was used to image the black blood of the healthy left carotid and an occluded right carotid artery. After injection of P975, increased in Gd signal intensity was observed at 30 min and 120 min, where no signal enhancement was noted for the untargeted Gd or in the healthy carotid artery (Klink et al., 2010). Two other radiolabelled RGD-based constructs have proceeded to clinical trials for the diagnosis of deep vein thrombosis (DVT) via SPECT imaging (Muto et al., 1995; Lister-James et al., 1996; Taillefer et al., 1999, 2000; Klem et al., 2000). Unfortunately, both ^{99m}Tc-P280 and^{99m}Tc-DMP444 have been discontinued because of their low sensitivity and specificity for the diagnosis of PE, contributed by prolonged radioactivity readout in both the chest region and the blood pool, as well as their low uptake by the thrombus (Carretta et al., 1999; Taillefer et al., 2000; Oliveira and Caravan, 2017). However, RGD-based contrast agents have two major limitations. 1) As RGD peptides do not provide specificity for activated GPIIb/IIIa , RGD-based contrast agents will bind to all circulating platelets. Thrombus imaging can be only achieved by the comparison of the static thrombus signal to the moving blood signal. 2) Several other integrins act as receptors for RGD containing ligands (Wang and Peter, 2017). Indeed, this has been used for molecular imaging approaches since RGD peptides have been used to target the vitronectin receptor (integrin $\alpha_v\beta_3$, CD51/CD61) (Golestani et al., 2015; Sun Yoo et al., 2015) and are widely used in cancer imaging (Alonso et al., 2009; Melemenidis et al., 2015; Withofs et al., 2015).

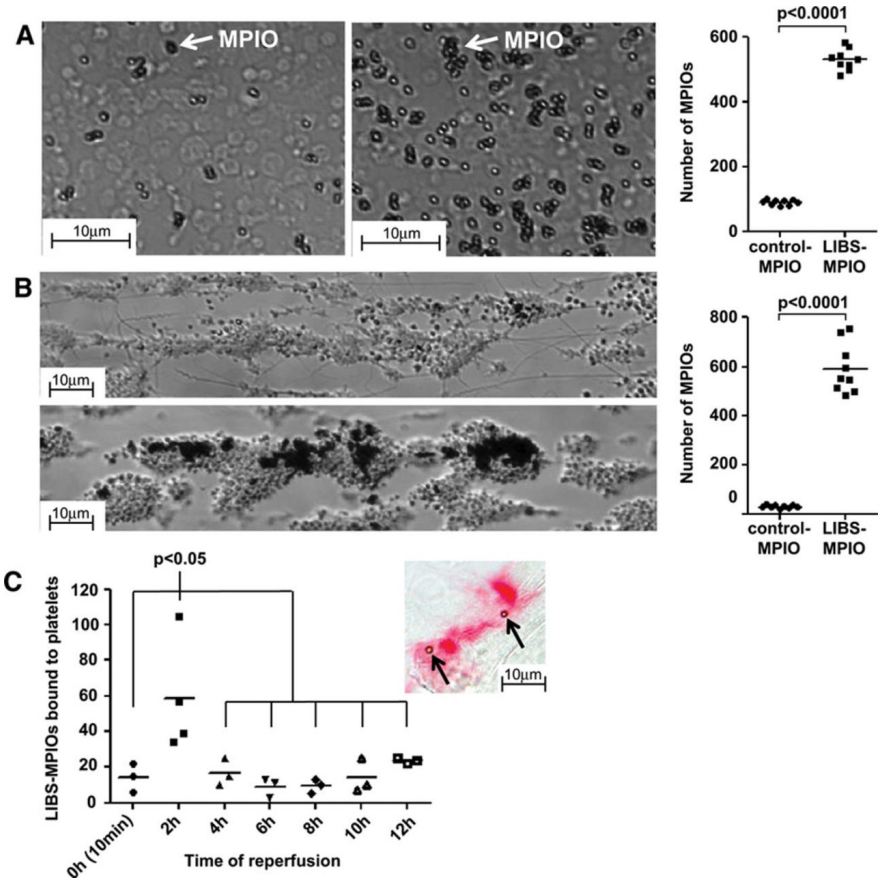
Using the antibody fragment abciximab (Reopro®), Alonso et al. demonstrated that preincubation of abciximab-microbubbles with human thrombi generated *ex vivo* resulted in enhanced ultrasound signals after i.v. injection. Visualisation of the human clot in the artery was achieved using ultrasound imaging, and as a negative control, upon ultrasonic bubble destruction, the clot lost its echogenicity/brightness (Alonso et al., 2007). However, Reopro has been developed as an anti-platelet drug and is known to bind to GPIIb/IIIa on all human platelets, independent of their activation state (Coller, 1999; Schwarz et al., 2002). The use of Reopro as an imaging agent faces the same limitations as RGD-based contrast agents with binding to all circulating platelets and therefore the excistence of a circulating blood background signal. Also abciximab blocks fibrinogen binding and therefore inhibits the function of circulating platelets, potentially resulting in an increased bleeding risk (Peter et al., 2000; Lele et al., 2001).

The development of a single-chain antibody (scFv) that specifically targets the activated form of the GPIIb/IIIa receptor ($scFv_{anti\text{-}GPIIb/IIIa}$) has provided the unique opportunity to do molecular imaging of activated platelets. This approach has been successfully used to image diseases with central involvement of activated platelets such as thrombosis, inflammation and malignant diseases (Wang and Peter, 2017; Yap et al., 2017). This $scFv_{anti\text{-}GPIIb/IIIa}$ does not bind to the resting platelets (Figure 6) in the circulation and does not cause bleeding complications (Schwarz et al., 2006; Wang et al., 2012). The $scFv_{anti\text{-}GPIIb/IIIa}$ has been successfully used with a wide range of contrast agents, such as ultrasound microbubbles (Wang et al.,

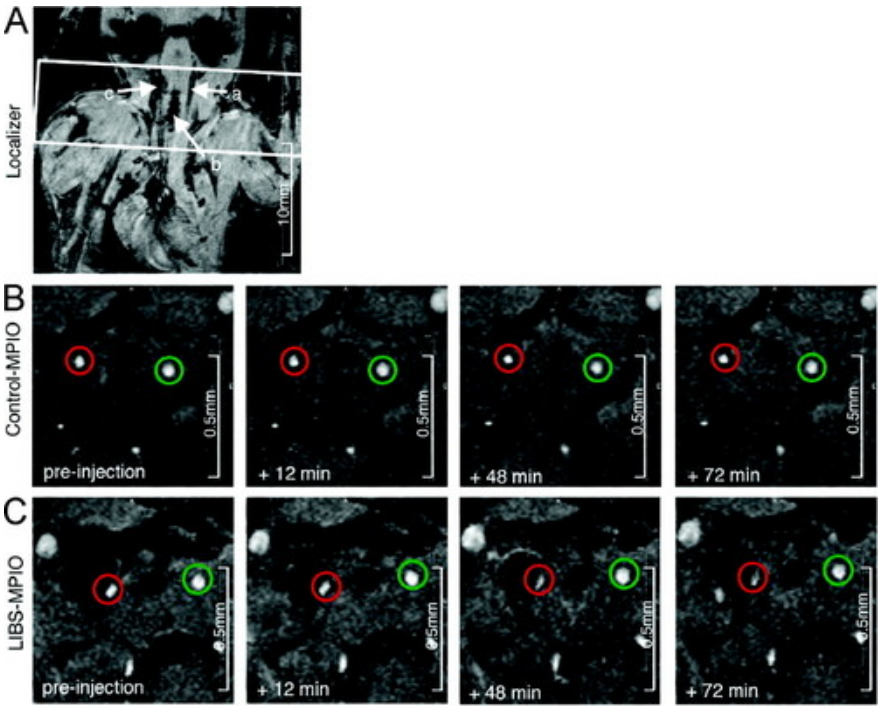
2012, 2014, 2016) and MPIOs (von Elverfeldt et al., 2014), and demonstrated highly favourable binding capabilities to microthrombi under flow conditions (Figure 7 and 8).

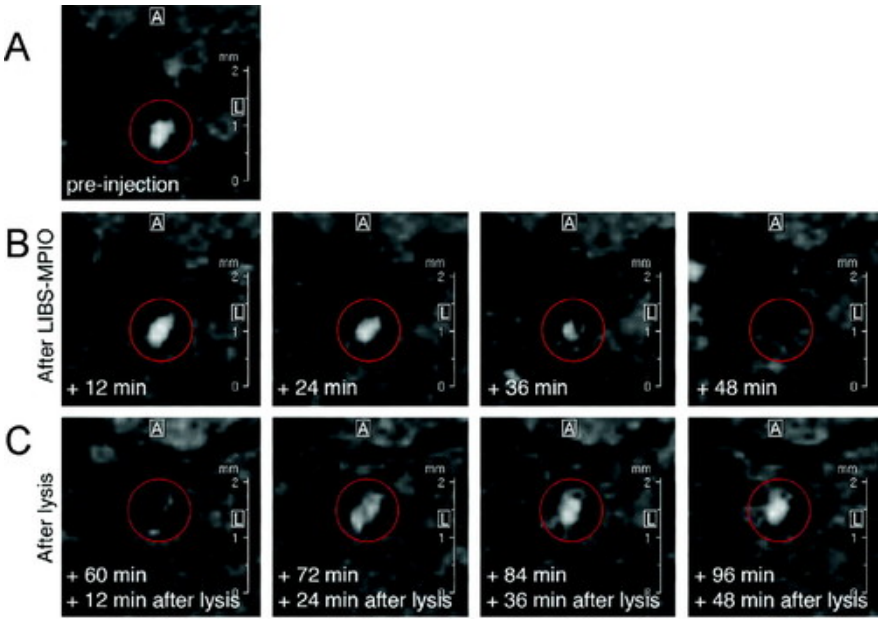


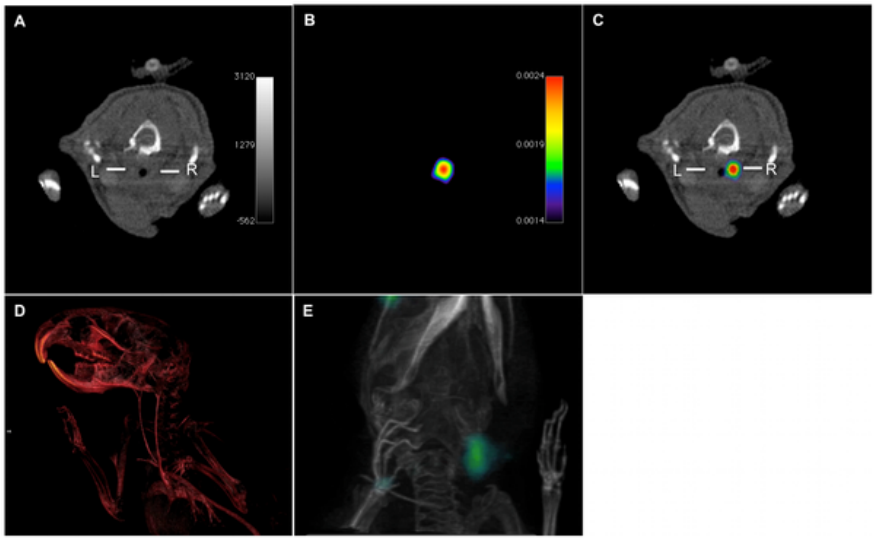


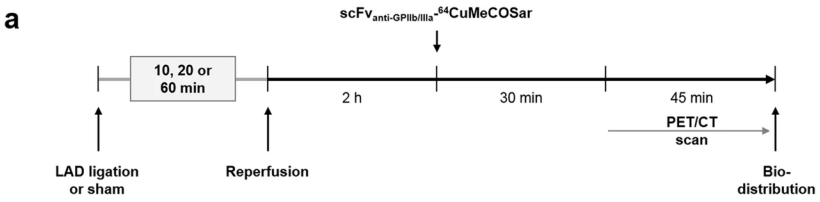


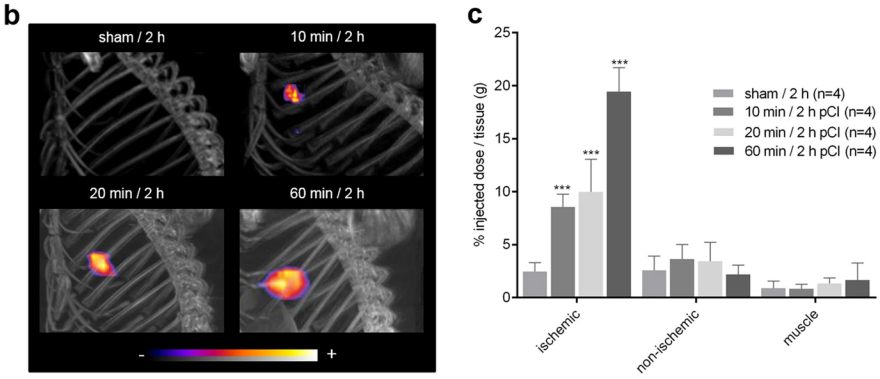
Using an acute mouse thrombosis model, where the carotid artery is exposed to ferric-chloride to induce thrombosis, thrombi were successfully visualised via MRI (von zur Muhlen et al., 2008; Ta et al., 2011, 2017), nuclear imaging (Heidt et al., 2011; Ardipradja et al., 2014; Hagemeyer et al., 2014), ultrasound (Wang et al., 2012, 2014, 2016), and optical imaging (Lim et al., 2017). Upon the injection of microparticles, iron oxide (MPIOs) coupled with $\text{scFv}_{\text{anti-GPIIb/IIIa}}$, these targeted MPIOs were shown to bind at the site of blood clots, resulting in a decrease of signal intensity in T2-weighted MRI (Figure 9) (von zur Muhlen et al., 2008). The authors further demonstrated that this diminution in signal could be reversed after the injection of urokinase as a fibrinolytic drug, indicating successful thrombolysis (Figure 10). Similarly, $\text{scFv}_{\text{anti-GPIIb/IIIa}}$ radiolabelled with Indium-111 (^{111}In) and ^{18}F has been used for the imaging of activate platelets in thrombi using SPECT (Figure 11 and Video 1) (Heidt et al., 2011) and PET (Ardipradja et al., 2014), respectively. In another murine study, Ziegler et al. radiolabelled $\text{scFv}_{\text{anti-GPIIb/IIIa}}$ with ^{64}Cu, and visualised the accumulation of activated platelets in ischemic and then reperfused myocardium (Ziegler et al., 2016). This PET imaging approach showed an uptake of radioactivity in the myocardium of mice, after just a- 10 min period of ischemia (Figure 12).(Ziegler et al., 2016). Noteably, after 10 min there was no increase of classical markers of cardiac ischemia, indicating that the imaging of platelets provides a means of detection of cardiac ischaemia with an unprecedented sensitivity. Using ultrasound, Wang *et al.* demonstrated direct and immediate visualisation of thrombi using microbubbles coupled with $\text{scFv}_{\text{anti-GPIIb/IIIa}}$ (Figure 13) (Wang et al., 2012). Realtime molecular ultrasound imaging can be used to image the success or failure of thrombolysis (Figure 14), and importantly, the dislodgment of a thrombus can be directly visualised (Video 2) (Wang et al., 2012). Lim et al. conjugated near-infrared dyes onto $\text{scFv}_{\text{anti-GPIIb/IIIa}}$, which allowed early and sensitive detection of thrombi using *in vivo* three-dimensional optical imaging (Lim et al., 2017).

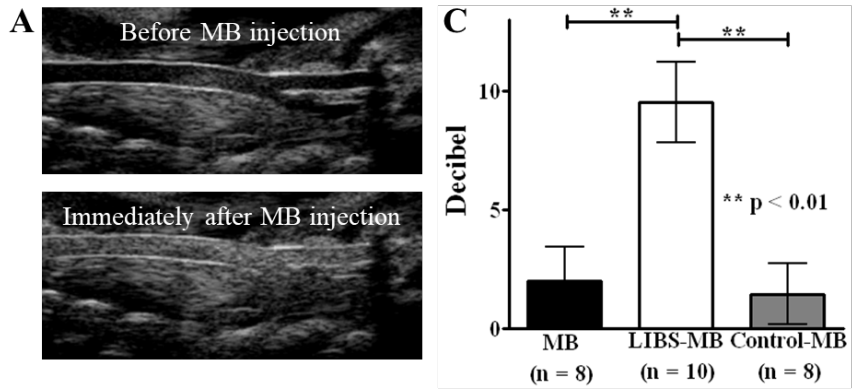


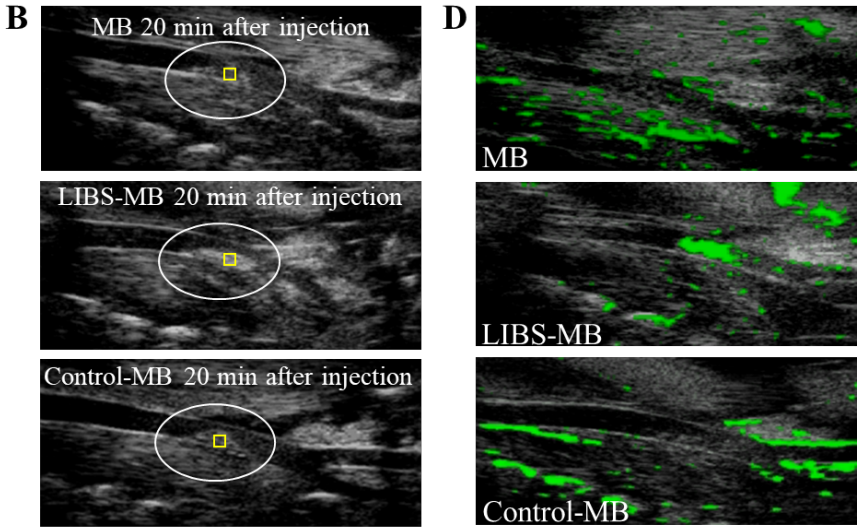


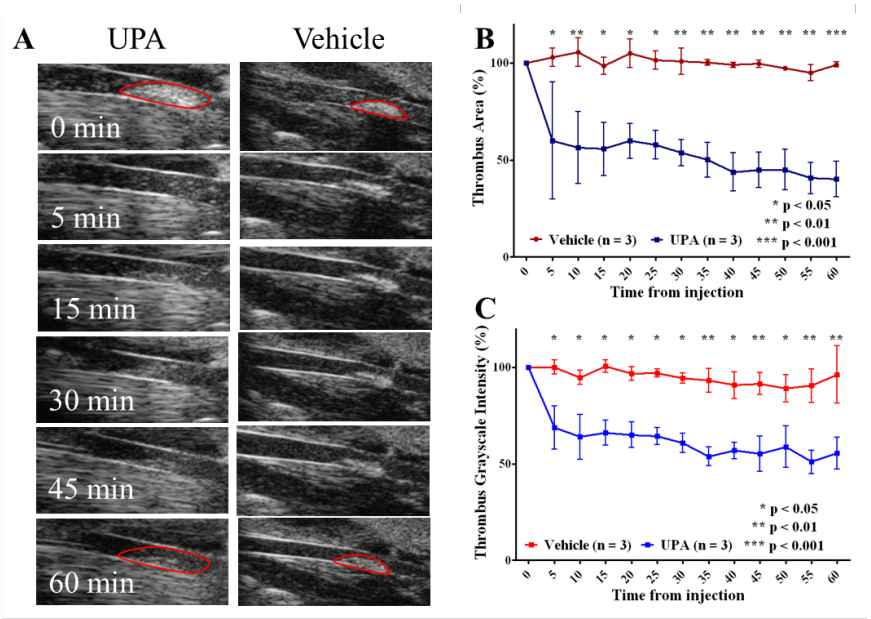




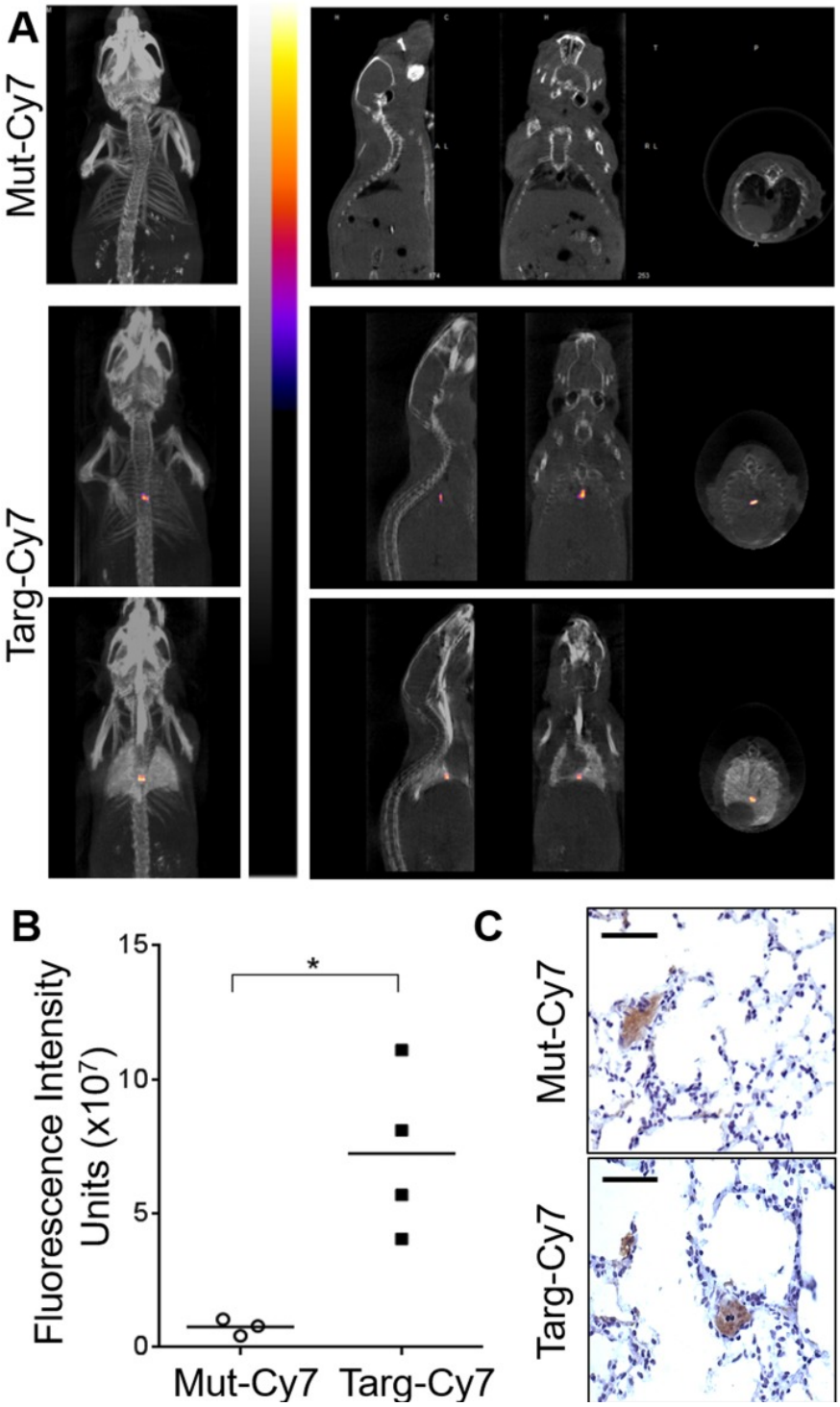


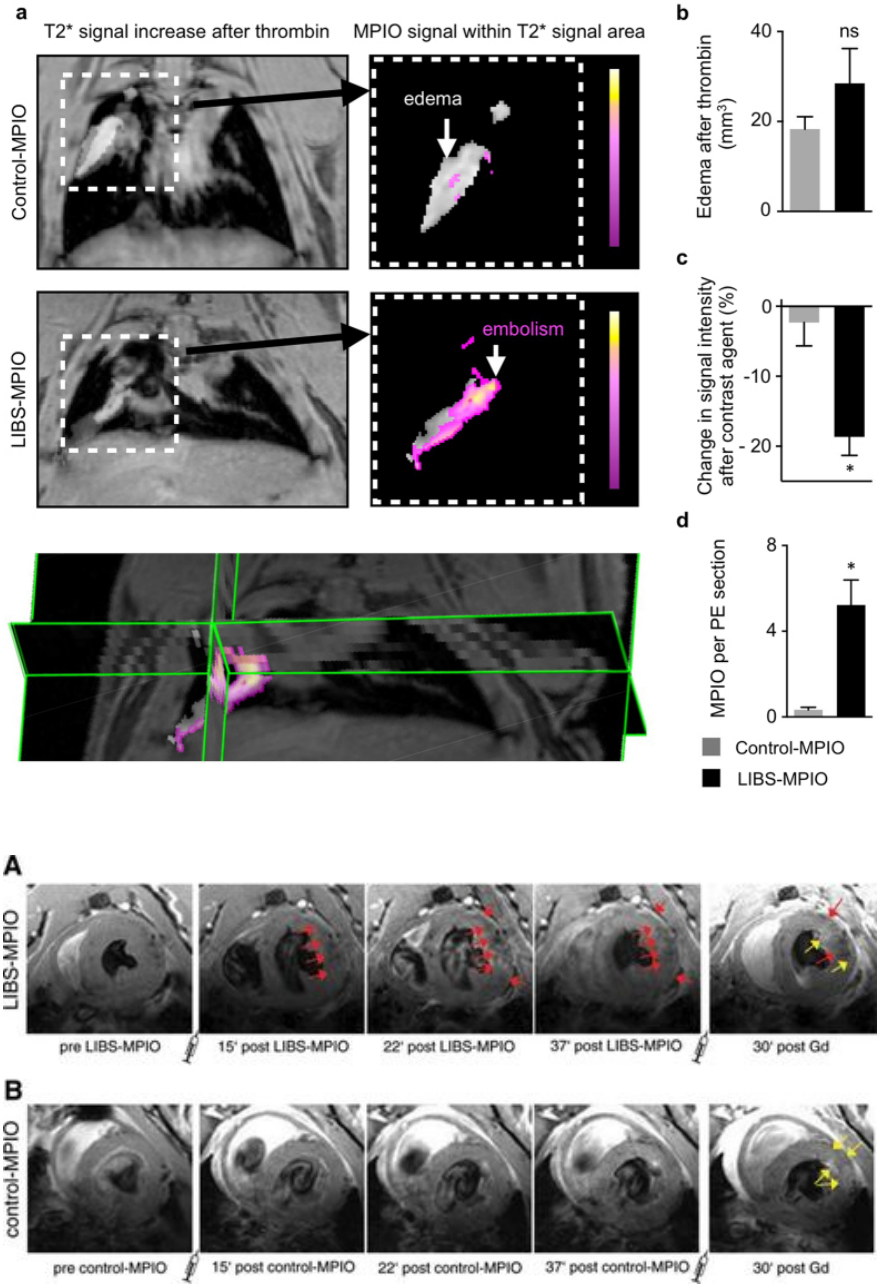






An increased fluorescence signal was also detected in the lung area, indicating successful imaging of PE in a murine model (Figure 15) (Lim et al., 2017). In another *in vivo* study of PE, these activated GPIIb/IIIa targeted MPIOs demonstrated focal accumulation within the edematous area in the lung via T2 MRI (Figure 16) (Heidt et al., 2016). Using the same particles, von Elverfeldt et al. successfully imaged platelet-driven inflammation in the myocardium of an I/R injury mouse model (von Elverfeldt et al., 2014). This activated-platelet targeted MPIOS compared favourably to a standard gadolinium imaging of myocardial necrosis (Figure 17) (von Elverfeldt et al., 2014).



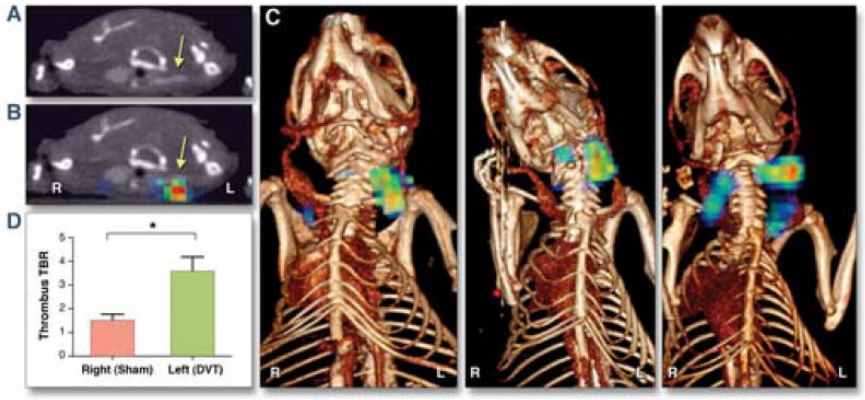


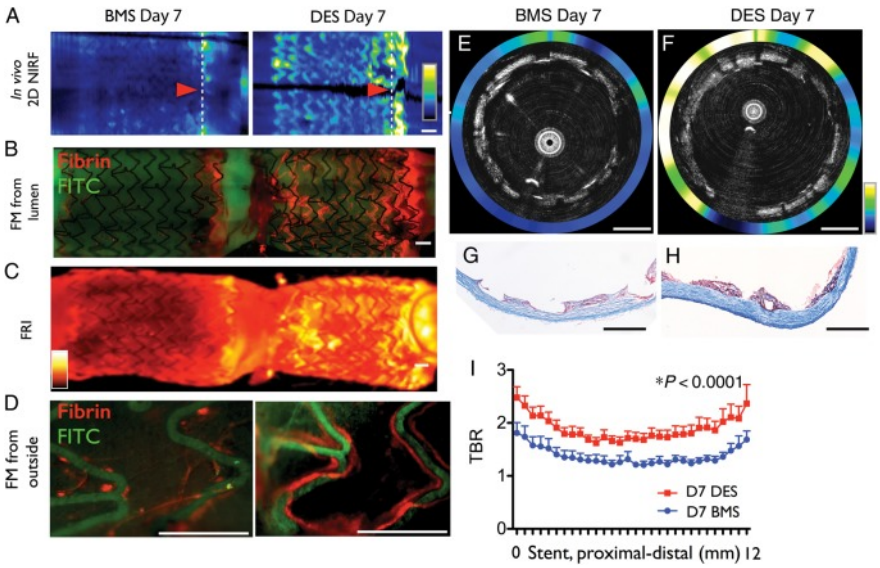
Fibrin imaging

Fibrin is another crucial target that is frequently used for molecular imaging of thrombosis. Botnar *et al.* developed a novel fibrin-binding gadolinium-labelled peptide (EP-1873) for MRI of thrombosis and tested this in an experimental rabbit model of plaque rupture (Botnar et al., 2004). This probe was further optimised (EP-2104R) and successfully validated for MRI-based diagnosis of coronary thrombosis and PE (Spuentrup et al., 2005a, 2005b; Overoye-Chan et al., 2008). In another study using a murine model of venous thrombosis in the inferior vena cava, Andia et al. demonstrated the maximal increased of signal at day 7 of thrombosis and gradual reduction from day 10 onwards (Andia et al., 2014). After radiolabelling ^{64}Cu with EP-2104R, the construct was injected into a rat model of crush injury-induced thrombosis. A clear increase in uptake of

radioactivity was observed in the location of the thrombi via PET imaging (Ay et al., 2014). Furthermore, the administration of fibrinolytic therapy resulted in a loss of radioactivity signal, indicating that the thrombi have been thrombolysed, indicating the suitability of this imaging approach for the monitoring of success or failure of thombolytic therapy (Ay et al., 2014). Bimodal imaging performed with ^{64}Cu radiolabeled EP-2104R provided direct visualisation of thrombi via PET and MRI (Uppal et al., 2011). This method also allowed the detection of multisite thrombi located in the carotid artery and the femoral vein in a rat model via a whole-body PET scan (Blasi et al., 2015). In another study, EP-2104R was radiolabelled with ^{68}Ga or^{111}In, whereas a non-binding control was radiolabeled with ^{64}Cu, for multimodal SPECT/PET/CT imaging (Oliveira et al., 2015). ^{125}I-fibrinogen was injected prior to ferric chloride induced thrombosis of the common carotid artery. Multimodal imaging showed a hot spot corresponding to the^{125}I-fibrinogen labelled thrombi with fibrin-targeted^{68}Ga or ^{111}In isotopes, but not the non-binding radioisotopes (Oliveira et al., 2015).

Using EP-2104R, Hara et al. synthesised a fibrin-binding peptide (FTP11) conjugated to a near-infrared dye and demonstrated successful *in vivo* optical imaging of thrombi in a jugular DVT murine model (Figure 18) (Hara et al., 2012). Using the same construct, the group further demonstrated successful imaging of fibrin deposition on stents that were implanted in rabbits using invasive optical coherence tomography (Figure 19) (Hara et al., 2015). EP-2104R has also been evaluated in Phase II trials, where molecular MRI was performed on patients who were previously diagnosed with thrombosis in the arteries, veins and/or the heart (Spuentrup et al., 2008; Vymazal et al., 2009). Nevertheless, none of these imaging products has reached approval for clinical use (Lanza et al., 2019).





Using a fibrin-binding peptide (FibPep) radiolabelled with 111In, Starmans et al. demonstrated the successful location of thrombi in the carotid artery of an acute murine thrombosis model using SPECT (Starmans et al., 2013). In the same animal model, the group also demonstrated significantly increased thrombus uptake when they injected iron oxide nanoparticle-micelles, which were conjugated with Fibpep, via magnetic particle imaging (Starmans et al., 2015).

Conclusion

Molecular imaging holds strong promise for early and sensitive diagnosis of thromboembolic diseases. The choice of epitopes and biomarkers for molecular targeting, as well as the selection of imaging modalities and their respective contrast agents, are especially important for clinical translation. A better understanding of thrombotic diseases and their progression will provide the clinicians with information to determine the pharmacological treatment most suited for an individual, taking us closer towards personalised medicine. Furthermore, the ability to determine the success or failure of antithrombotic or fibrinolytic treatment will also enable better decisions for invasive interventions to be performed. Therefore, molecular imaging of thrombi has an enormous potential to provide benefits for patients suffering from thromboembolic diseases.

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