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The Tandem Stenosis Mouse Model: Towards Understanding, Imaging and Preventing Atherosclerotic Plaque Instability and Rupture.

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KP is inventor and Chief Medical Officer of NIRTEK, a start-up company developing an intracoronary device for the detection of unstable plaques.

Abstract

The rupture of unstable/vulnerable atherosclerotic plaques is the major cause of cardiovascular mortality/morbidity. Despite significant limitations in our understanding and ability to identify unstable plaque pathology and prevent plaque rupture, most atherosclerosis research utilises preclinical animal models exhibiting stable atherosclerosis. Here, we introduce the Tandem Stenosis (TS) mouse model that reflects plaque instability/rupture as seen in patients. The TS model involves dual ligation of the right carotid artery, leading to locally predefined unstable atherosclerosis in hypercholesterolaemic mice. It encapsulates key characteristics of human unstable plaques, including plaque rupture, luminal thrombosis, intraplaque haemorrhage, large necrotic cores, thin/ruptured fibrous caps and extensive immune cell accumulation. Ultimately, the TS model represents an ideal preclinical tool for improving our understanding of human plaque instability/rupture, for the development of imaging technologies towards the identification of unstable plaques, and for the development and testing of plaque stabilising therapeutics for the prevention of atherosclerotic plaque rupture.

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An Introduction to Atherosclerosis

Atherosclerosis is a disease characterised by the development of lipid laden and chronically inflamed plaques in medium and large arteries (Chen et al., 2016; Libby et al., 2019). These plaques are cellularly complexes, with their development involving the interaction between vascular resident cells, such as endothelial cells, smooth muscle cells, and tissue macrophages, as well as recruited immune cells. Broadly, atherosclerosis predominantly advances along three pathways: Firstly, stable atherosclerotic plaques can become larger over time, narrowing the vessel lumen and restricting blood flow to organs supplied by affected arteries. In the case of the growing atherosclerotic plaque residing in the coronary arteries, this typically results in angina pectoris upon exercise. Secondly, the sudden rupture of unstable atherosclerotic plaques (often referred to as vulnerable or rupture-prone plaques) can lead to thrombotic events, which precede and directly cause stroke and the majority of myocardial infarction (MI), the dominant clinical outcomes that make cardiovascular disease the leading cause of death globally (Bentzon Jacob Fog et al., 2014; Chen et al., 2016). Thirdly, the denudation of plaque endothelium, known as plaque erosion, also leads to such cardiovascular events (Bentzon Jacob Fog et al., 2014; Libby et al., 2019).

Atherosclerosis Mouse Models and a Lack of Plaque Rupture

Animal models are critical tools for the exploration of biological mechanisms, the differentiation of cause from effect, the establishment of imaging technologies, and for the development and testing of therapeutics. The most commonly used animal models for

studying atherosclerosis are the Apolipoprotein E and Low-Density Lipoprotein Receptor deficient strains of mice ($ApoE^{-/-}$ and $LDLR^{-/-}$, respectively); a broad overview of atherosclerosis mouse models is excellently reviewed by Veseli *et al.* (Emini Veseli et al., 2017). These animal models represent the foundation upon which we have built our mechanistic understanding of atherosclerosis. However, there is a significant limitation with these models; plaque instability as found in patients is not reflected in these mouse models and, importantly, spontaneous plaque rupture does not occur in $ApoE^{-/-}$ or $LDLR^{-/-}$ mice. Whilst being an important and necessary stage in the aetiology of atherosclerosis, stable plaques are rarely a cause of hospitalisation or death. This is illustrated in **Figure 1**, graphically summarising the relevance of broad atherosclerotic disease stages to clinical practice and the use of animal models reflecting these disease states.

Ultimately, of several decades of preclinical animal research on atherosclerosis, nearly all studies have focused on the stable phenotype of atherosclerosis, despite plaque instability and rupture underpinning the majority of cardiovascular morbidity and mortality. Moreover, until recently, there was a paucity of reliable models for studying the mechanisms of plaque rupture. To address this experimental limitation, a study led by Dr Chen in our laboratory succeeded in developing a model of plaque instability and rupture in mice (Chen et al., 2013). This model, the Tandem Stenosis (TS) model, replicates the key features of unstable human atherosclerotic plaques and will be the focus of this review. We will discuss the current, albeit limited, understanding of plaque rupture; the long-standing need for preclinical models of

unstable atherosclerosis and plaque rupture; the utility of the TS approach for addressing this need; and the overarching translational importance of incorporating animal models of plaque instability and rupture in atherosclerosis research.

Atherosclerotic Plaque Rupture and Limitations in the Current Paradigm

Clinical imaging data and post-mortem histology supports the critical relevance of plaque rupture to the morbidity and mortality of atherosclerosis (Palasubramaniam et al., 2019). In an assessment of 800 cases of sudden coronary death, for example, 55-60% of deaths were attributed to atherosclerotic plaque rupture, with the remaining deaths linked to plaque erosion (30-35%) or calcified nodules (2-7%)(Finn et al., 2010). Moreover, a recent study utilising optical coherence tomography identified plaque rupture as being responsible for 68.6% of ST-segment Elevation Myocardial Infarction's (STEMI) in a study of 822 STEMI patients (Dai et al., 2018). Whilst the relevance of plaque rupture to outcomes is clear, the majority of our understanding of rupture-prone plaques is inferred from a small number of post-mortem histopathological examinations of human coronary arteries combined with studies of carotid endarterectomy samples (surgically resected atherosclerotic plaques from carotid arteries). These studies have contributed to a histopathological definition of rupture-prone plaques, defined as those with thin fibrous caps (defined by a cap thickness below 65 μm), leukocyte infiltration, scarcity of smooth muscle cells, local proteolytic activity, large lipid/necrotic cores and presence of intraplaque haemorrhage (Burke et al., 2001; Virmani et al., 2006; Narula, 2009; Davies and Thomas, 2010; Finn Aloke V. et al., 2010; Finn et al., 2010;

Libby, 2013; Bentzon Jacob Fog et al., 2014; Hansson et al., 2015). The obvious limitation in the data gained from these post-mortem studies is that the plaques examined are obtained from patients with advanced atherosclerosis that have caused symptoms or death, introducing an inherent selection bias into our understanding. Furthermore, they lack information on how a plaque may have progressed from a stable to an unstable phenotype capable of causing clinical symptoms. Consequently, atherosclerotic plaques remain defined by crude histological features, which do not necessarily reflect the complex cellular or molecular mechanisms underpinning the potentially heterogeneous pathology or, most importantly, relevant timelines of events.

Several clinical studies have attempted to translate the understanding gained from post-mortem examinations in order to identify rupture-prone plaques and predict major adverse cardiac events (MACE), including MI and stroke. Highlighting the clear limitations of histological definition of unstable plaques; there has been a distinct lack of success in this endeavour. The PROSPECT study (Providing Regional Observations to Study Predictors of Events in the Coronary Tree), a longitudinal imaging trial utilising intravascular ultrasound, highlighted that plaques with thin fibrous caps and large necrotic cores (thin-cap fibroatheromas; TCFA), were associated with an increased risk of clinical events (Stone et al., 2011). However, only 4.9% of plaques observed as TCFA led to MACEs. Though this confirms TCFA are more likely to rupture, it also highlights that the criteria used in this study are not sufficient for identifying plaques at risk of rupture and on its own without any other criteria

would not be suitable for risk stratification. Consequently, this study made clear that there must be other factors dictating plaque rupture beyond gross morphology.

In addition to placing doubt upon the use of morphology as a sole predictor of plaque rupture, an important outcome of PROSPECT was the uncoupling of the degree of stenosis from plaque instability, identifying that plaques identified as not requiring treatment based on a low degree of stenosis, judged by angiography, were as likely to be the source of plaque rupture and MI as those with a high degree of stenosis. This insight clarified that plaque size does not necessarily predict risk of rupture, with the underlying morphology, and likely biology, more predictive of outcome (Yun et al., 2012). A more recent examination of the SWEDEHEART data (Swedish Web System for Enhancement and Development of Evidence-Based Care in Heart Disease Evaluated According to Recommended Therapies) has identified that the risk of a second MI was twice as likely to result from the rupture of a non-culprit lesion, than a culprit lesion (Varenhorst et al., 2018). Again, this highlights the great challenges currently associated with identifying unstable, rupture-prone atherosclerotic plaques that will subsequently cause MACEs and reflects our poor understanding of the pathobiology underlying plaque instability and rupture.

The Need for a Suitable Animal Model of Plaque Instability and Rupture

Given plaque size, morphology and the degree of luminal stenoses caused by an atherosclerotic plaque poorly predict cardiovascular events, many hypotheses have been

presented as to the potential mechanisms governing plaque instability and rupture. The pathways presented broadly overlap with those governing atherogenesis, including alterations in vessel haemodynamics, lipid profiles, and inflammation, alongside additional features such as intraplaque haemorrhage (IPH) (Chen et al., 2016). In respect to haemodynamics; it is well known that atherosclerosis preferentially develops in areas of the arterial tree experiencing non-laminar blood flow and low/oscillating shear stress, such as at branching points or curvatures (Perdersen et al., 1999; Stone et al., 2003; Papafaklis et al., 2015; Gijsen et al., 2019). Importantly, alterations in haemodynamics have been shown to induce plaque rupture in animal models, including in the TS model (Chen et al., 2013). Mechanistically, changes in blood flow haemodynamics have a wealth of impacts on the arterial wall and circulating/recruited immune cells (Bryan et al., 2014; Baratchi et al., 2017; Baratchi et al., 2020). Endothelial cells, for example, experience increased proliferation and apoptosis in atheroprone regions of the vasculature, which is compounded by an increased inflammatory phenotype and permeability as a result of altered haemodynamics (Matharu et al., 2008; Zhou Jing et al., 2014). As a critical component of vessel integrity, inflammatory changes in endothelial cells or disruption of the endothelial cell layer are highly plausible mechanisms of plaque destabilisation.

Inflammation itself has been highlighted as a potential therapeutic target and diagnostic/molecular imaging marker of unstable, rupture-prone plaques, as well as a likely critical player in plaque destabilisation processes (Hansson et al., 2015; MacRitchie et al.,

2018; Noonan et al., 2018; Bäck et al., 2019; Maffia and Guzik, 2019). As early as 1994, van der Wal and colleagues identified that the sites of plaque ruptures were often associated with inflammation, reflected by the presence of macrophages and T cells (van der Wal et al., 1994). An elegant histological study by van Dijk *et al.* more recently demonstrated that plaque progression coincides with increased plaque T cell numbers, peaking in plaques with a TCFA or ruptured phenotype (van Dijk et al.). The mechanisms by which immune-inflammatory responses could mediate plaque destabilisation and rupture are many (Galkina and Ley, 2009; Hansson et al., 2015; Kyaw et al., 2017, 2020; Geovanini and Libby, 2018; Bäck et al., 2019); one example would be the production of matrix metalloproteinases (MMPs), predominantly by macrophages, which can degrade the fibrous cap of atherosclerotic plaques (Amento et al., 1991; Galis et al., 1994; Mach et al., 1997). A recent study conducting parallel proteomic analysis of human and mouse plaques, focusing on the comparison between stable and unstable atherosclerosis, has further strengthened links between proteolytic degradation, inflammation and the rupture of atherosclerotic plaques (Vaisar Tomáš et al., 2020). Consequently, an immune cell driven bias towards extracellular matrix degradation in atherosclerosis is considered a likely key step in driving plaque instability and rupture.

Using recent technological advances, in particularly single-cell RNA sequencing (scRNA-seq) and mass cytometry, the extensive diversity of immune cell populations in both human and mouse atherosclerosis have been described (Zerneck et al., 2020). Winkels *et al.* were among the first to publish scRNA-seq data on mouse atherosclerosis, producing an immune cell 'atlas' of atherosclerotic disease (Winkels et al., 2018). Their analysis highlighted at least 11 broad

immune cell subsets based on scRNA-seq data, and 23 subsets based on mass cytometry (Winkels et al., 2018). Perhaps most interestingly, the authors further highlighted that the frequency of immune cell populations in human carotid endarterectomy samples may be predictive of event-free survival. Focusing entirely on human carotid endarterectomy samples; Fernandez *et al.* observed similar immune cell diversity within human plaques and identified relationships between cerebrovascular events and both T cells and macrophages (Fernandez et al., 2019). It is important to consider that inflammatory processes reach well beyond the immune system, and significant evidence highlights a critical role for non-immune cells in the development and, likely, rupture of atherosclerotic plaques (Shankman et al., 2015; Kalluri et al., 2019; Liu and Gomez, 2019; van Kuijk et al., 2019; Wirka et al., 2019). Whilst collectively these studies have provided a wealth of data on the cellular diversity in atherosclerosis, which is extensive, they do not define beyond association how each cell type may contribute to the plaque instability and rupture responsible for the majority of MACEs.

Clinically, the role of inflammation is often incorporated into 'residual cardiovascular risk'; in other words, the risk that remains when traditional risk factors, such as high low-density lipoprotein cholesterol (LDL-C) are controlled. Residual cardiovascular risk can be broadly separated into residual inflammatory, metabolic, or thrombotic risk (reviewed in Dhindsa et al., 2020). It is important to note that, while we have described some key data pertaining to inflammation and immunity in atherosclerosis, until recently it was unclear as to the relevance of immune-inflammatory responses in humans. However, as a result of the significant preclinical mechanistic evidence in models of stable atherosclerosis and

observational data in humans suggesting a causative role for inflammation in atherosclerosis, several landmark randomised double-blind clinical trials have now explored the potential for targeting inflammation in CVD, with mixed results.

Enrolling 10,061 patients with previous MI and residual inflammatory risk defined by ≥2mg/L high-sensitivity C-reactive protein (hsCRP); the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) identified that treatment with 150mg of the anti-IL-1β antibody canakinumab significantly reduced cardiovascular events, but did not alter all-cause mortality, at least partially as a result of increased fatal infection and sepsis (Ridker et al., 2017). Importantly, a secondary analysis suggested that canakinumab may be highly effective in a subset of patients, observing a 25% reduction in cardiovascular events and 31% reduction in all-cause mortality in patients achieving hsCRP levels below 2mg/L under canakinumab therapy (Ridker et al., 2018). In contrast, the Cardiovascular Inflammation Reduction Trial (CIRT), exploring the use of low-dose methotrexate, an immunosuppressant often prescribed for the treatment of inflammatory diseases such as rheumatoid arthritis, demonstrated no benefit in patients with stable atherosclerosis (Ridker et al., 2019). It is pertinent to note that the investigators found no reduction in inflammatory cytokines in patients treated with methotrexate, suggesting no anti-inflammatory effect was achieved in this trial. Colchicine, an inexpensive therapeutic most commonly used for the treatment of gout, has also been the focus of several clinical trials aimed at addressing residual inflammatory risk in cardiovascular patients, demonstrating great promise. Most recently, the LoDoCo2 trial demonstrated a significant reduction of ischemia-driven revascularisation, MI and cardiovascular death in

patients with coronary artery disease treated with colchicine (Nidorf et al., 2020). The COLCOT study, exploring colchicine treatment in patients after MI, also saw a significant reduction in cardiovascular events (Tardif et al., 2019). Furthermore, the COPS study investigated colchicine treatment in patients presenting with acute coronary syndrome, observing a trending but not significant improvement of cardiovascular events (Tong et al., 2020). However, despite evidenced use of colchicine dating back to ancient Egypt, the exact mechanisms by which it exerts its anti-inflammatory effects generally and in the cardiovascular setting are a subject of ongoing investigations (Eigsti and Dustin, 1955; Leung et al., 2015).

The varied success in these trials highlights our incomplete understanding of atherosclerosis, particularly in relation to plaque rupture and other plaque destabilising processes that incite cardiovascular events. It is perhaps not surprising that there have been such significant challenges in translating data from animal models of stable atherosclerosis to effective treatments in patients. In fact, the targeting of IL-1β genetically and pharmacologically in mouse models of stable atherosclerosis have failed to replicate the beneficial effects seen in patients, presenting conflicting protective and pathogenic pathways for this cytokine (Alexander et al., 2012; Maffia and Guzik, 2019). One might even consider it surprising that a clinical trial targeting this pathway has been completed with pre-clinical data still being a subject of debate (Maffia and Guzik, 2019). Ultimately, in our opinion; the present lack of mechanistic data pertaining to plaque instability, erosion and rupture represents a fundamental gap in knowledge that must be addressed if we are to facilitate a more informed

approach to drug and diagnostic development to prevent myocardial infarction and stroke. Therefore, the ability to model plaque instability and rupture in animals is a critically required tool for cardiovascular research to allow for the discovery and dissection of the fundamental biological mechanisms underpinning cardiovascular events. Indeed, transcriptomic and proteomic comparisons between the regions displaying stable and unstable atherosclerosis indicate that both are different disease entities (Chen et al., 2013; Vaisar Tomáš et al., 2020). With this in mind, we will now discuss the Tandem Stenosis model, which our laboratory designed and validated to provide a reliable, flexible and widely applicable model of plaque instability and rupture.

The Tandem Stenosis Model: Protocol and Characteristics

The TS model involves the dual ligation of the right common carotid artery of hypercholesterolaemic mice, including either $LDLR^{-/-}$ (unpublished data) or $ApoE^{-/-}$ mice (Chen et al., 2013). Briefly, mice are anesthetised and the fur at the neck is removed. A small (~1cm) incision is made through the skin on top of the right common carotid artery. The tissue underneath this incision is then bluntly dissected under a dissection microscope to expose the carotid artery. The vagus nerve is gently separated from the artery, before two stenosis points are placed 1mm and 4mm below the carotid bifurcation. To achieve the desired stenosis of 150μm diameter, a 150μm diameter needle is placed on top of the artery and sutures are then tied tightly around the artery and needle, blocking blood flow and reducing the vessel lumen to 150μm. Following this, the needle is carefully removed to reinstate blood

flow; this process is repeated for both ligations. **Figure 2** provides an overview of the surgical approach and representative images of the plaques which develop in the context of the TS model.

TS of the right common carotid artery results in the rapid formation of atherosclerotic plaques in this region (Chen et al., 2013). By 4 weeks post-TS, complex atherosclerotic lesions characterised by large necrotic cores, immune infiltrates and focal thin fibrous cap at plaque shoulder regions can be found. By 7 weeks, these unstable plaques rupture in ~50% of the animals. In direct comparison to stable plaques, specifically those found in the aortic arch in the same mice, unstable plaques in the TS model display: plaque rupture and luminal thrombosis; IPH defined by CD41 and fibrin staining; reduced intimal collagen content; increased immune infiltrates; reduced fibrous cap thickness; and increased necrotic core burden. Moreover, increased expression of inflammatory genes, such as the chemokine CCL2 and the inflammatory cytokine IL-1β, were also identified in unstable vs stable plaques. Ultimately, the TS model replicates key characteristics of human unstable plaques, inclusive of lipid accumulation, extensive immune infiltration, thin/disrupted fibrous caps, IPH and the presences of platelets / fibrin, luminal thrombosis, neovascularisation, vascular remodelling, and involving local haemodynamic alterations (Chen et al., 2013).

The ultimate aim of the TS approach was to provide an animal model that could support both discovery research and the preclinical development and testing of diagnostic and therapeutic

approaches. This aim was achieved, with the TS model being used in studies exploring the underlying biology of unstable atherosclerosis as well as testing novel diagnostics and plaque stabilising therapeutics. Perhaps the most important aspect of any new animal model is the ability for laboratories to incorporate the model into their work. In this respect, the TS model has now been implemented by several research groups both following collaboration/training from our laboratory and independently from our involvement. To highlight the variety of research now involving the TS model, we will discuss the insights gained from the TS model from our group and others.

Biological and Translational Insights from the Tandem Stenosis Model

In establishing the TS model, we gained several insights into the potential underlying biology of unstable atherosclerosis and plaque rupture. For example, ADAM Metallopeptidase With Thrombospondin Type 1 Motif 4 (ADAMTS4), a metalloproteinase capable of degrading extracellular matrix components including versican and collagen, had been identified in human atherosclerotic plaques and upregulated in the plasma of patients following MI (Wågsäter et al., 2008; Zha et al., 2010). As such, it was suggested that ADAMTS4 may play a role in plaque destabilisation. However, at the time of our initial TS study, it was unclear if this molecule was specifically enriched in rupture-prone atherosclerotic plaques. Using a microarray approach and the TS model, we were able to identify that ADAMTS4 gene expression was indeed highly upregulated in unstable rupture-prone plaques. This

demonstrates one of the first contributions the TS model made to the cardiovascular field, providing direct relevance of ADAMTS4 specifically to unstable rupture-prone atherosclerosis. Kumar et al. later supported a causative role for ADAMTS4 in atherogenesis in ADAMTS4$^{-/-}$ApoE$^{-/-}$ mice (Kumar et al., 2016). This highlights how findings from the TS model can be integrated with cardiovascular research generally and contribute to our overall understanding of atherosclerosis.

Platelets are of particular interest in respect to unstable atherosclerosis due to their central role in the (micro)thrombotic events that precede MI and stroke. In addition to inflammatory changes prominent at unstable plaques (e.g. increased vascular cell adhesion molecule-1 (VCAM-1) expression), platelets express potential molecular epitopes that can be targeted for molecular imaging and diagnosis of unstable plaques (Wang and Peter, 2017). In relation to this, we recently reported that a key lipid component of platelet-derived microvesicles, Lysophosphatidylcholine (LPC), plays an important role in platelet activation via interaction with the LPC receptor G2AR (Diehl et al., 2019). However, the relevance of this finding to stable vs unstable atherosclerosis was unclear. The TS model supported our discovery that LPC concentrations were highest in unstable atherosclerotic plaques. Importantly, this finding could be translated to unstable human carotid plaques, which demonstrated highest LPC concentrations at plaque regions associated with plaque instability. Ultimately, this study highlighted both LPC and G2AR as potential therapeutic and diagnostic targets for unstable atherosclerosis.

The accumulation of erythrocytes due to IPH has been proposed as a driver of inflammation and plaque destabilisation (Kolodgie et al., 2003; Michel et al., 2011). One consequence of IPH is an enrichment of iron in atherosclerotic plaques (Stadler et al., 2004; Kopriva et al., 2015). In relationship to this, deficiency in hepcidin, a peptide hormone which regulates iron homeostasis, has been found to protect from atherosclerosis in $LDLR^{-/-}$ mice (Malhotra et al., 2019). Given the enrichment of iron in IPH, the authors hypothesised that their results suggest a potential role for hepcidin in IPH (Malhotra Rajeev et al., 2019). Whilst they did not test this hypothesis, Li et al. later advanced these findings and strengthened the proposal of hepcidin as a viable therapeutic or imaging target, confirming that IPH and hepcidin do colocalise in unstable plaques using the TS model (Li et al., 2019).

Further demonstrating the importance of IPH; in 2017 we described a novel molecular imaging approach dependent upon IPH for the detection of unstable plaques, a discovery which was supported significantly by the TS model (Htun et al., 2017). More specifically, we identified that haeme degradation products, which are enriched in regions of IPH, are auto-fluorescent in the near infra-red spectrum. As demonstrated in **Figure 3**, this finding was consistent in both the TS model and human carotid endarterectomies containing vulnerable plaques. This discovery has since led to our establishing a company, Nirtek, which is developing a near infra-red catheter device for the detection of unstable plaques in the coronary artery of humans.

Focusing on smooth muscle cells, which are central to the integrity of the fibrous cap in atherosclerosis, Karamariti *et al.* used the TS model to explore the role of the secreted glycoprotein dickkopf 3 (DKK3) in rupture-prone atherosclerosis (Karamariti et al., 2018). DKK3 had previously been implicated in the differentiation of pluripotent and embryonic stem cells into smooth muscles cells. Consequently, the authors hypothesised that the DKK3 pathway may play a central role in the maintenance of plaque stability. Applying the TS model in DKK3$^{-/-}$ApoE$^{-/-}$ mice highlighted that DKK3 deficiency did not alter lesion size, but significantly reduced the numbers of plaque smooth muscle cells and extracellular matrix content. In other words, DKK3 deficiency resulted in reduced plaque stability. Importantly, the authors then identified that this phenotype could be rescued by administering exogenous DKK3 to TS'd vessels using pluronic gel.

Using the TS model, Yang *et al.* have also demonstrated a role for miRNA-216a in plaque instability (Yang et al., 2019). The authors reported that the treatment of ApoE$^{-/-}$ mice with miR-216a increased the inflammatory environment within unstable plaques, characterised by increased CCL2, TNFα, IL-1β, MMP9 and Nos2 in parallel with decreased IL-10 expression. At the cellular level, the authors presented data supporting a role for miR-216a in the promotion of pro-inflammatory M1-type macrophage phenotypes, further implicating a role of miR-216a in unstable atherosclerosis.

In 2016, Kojima and colleagues published an elegant paper in *Nature* detailing that CD47 blocking antibodies are able to overcome impaired efferocytosis in atherosclerosis and ameliorate disease by restoring phagocytic activity in atherosclerotic plaques (Kojima et al., 2016). CD47 is a "don't eat me" signal found to be upregulated in atherosclerotic plaques, which the authors hypothesised contributed to an efferocytosis deficit and inability to clear dead and dying cells from plaques (Gardai et al., 2005; Kojima et al., 2016). Using multiple *in vivo* approaches, the authors demonstrate that blocking CD47 stimulated efferocytosis, improving dead cell clearance and significantly reduced plaque burden. Importantly, the authors then used the TS model to specifically demonstrate that this approach successfully improved plaque stability, significantly reducing IPH. By combining models of stable and unstable rupture-prone atherosclerosis, the authors convincingly demonstrated that this therapeutic approach may be broadly applicable to many stages of atherosclerosis disease.

In addition to specific immunomodulatory approaches, derivatives of traditional medicines have also been explored in the TS model. The active alkaloid leonurine, an extract from the traditional Chinese medicine Herba leonuri, has been demonstrated to have protective effects in models of cardiovascular disease (Ning et al., 2020). Using the TS model, Ning *et al.* demonstrated that leonurine treatment increased plaque stability, observing increased collagen content and fibrous cap thickness and decreased inflammation. Continuing along the line of treating unstable atherosclerosis, Richardson and colleagues utilised the TS model to test the applicability of a targeted polymer-based drug delivery system (Richardson et al.,

2016). Though no therapeutic outcomes were assessed in this research, the authors demonstrate that the targeting of polymer capsules a towards degraded collagen IV may be a promising modality for delivering drugs to rupture-prone atherosclerotic plaques.

In a collaborative effort, we explored the utility of using myeloperoxidase (MPO), an inflammatory enzyme highly expressed in human atherosclerotic plaques, as a target for diagnostic molecular imaging and treatment (Rashid et al., 2018). In this study, we demonstrated that unstable rupture-prone plaques expressed higher levels of MPO than their stable counterparts in the TS model. Using an enzyme sensitive magnetic resonance imaging probe, *bis*-5HT-DTPA-Gd, we further demonstrated MPO as a potential target for the diagnosis of unstable plaques, which had significantly increased MPO activity vs stable plaques. Moreover, we also confirmed a role for MPO in plaque instability, showing that MPO deficient ApoE$^{-/-}$ mice had increased fibrous cap thickness in rupture-prone TS plaques. This finding was mirrored by treatment with the MPO inhibitor AMZ198, confirming MPO as a viable target for the stabilisation of vulnerable atherosclerotic plaques. Cheng *et al.* also utilised the TS model in a later study identifying that, mechanistically, AMZ198 attenuates endothelial function in unstable atherosclerosis (Cheng et al., 2019).

Most recently, we have explored the role of the microbiome derived metabolite trimethyl-amine-N-oxide (TMAO) in the context of unstable atherosclerosis (Koay et al.). TMAO has received significant attention as a potential biomarker of cardiovascular disease, as well as

being implicated in platelet activation and the development of atherosclerosis (Wang et al., 2011; Koeth et al., 2013; Tang et al., 2013; Zhu et al., 2016). In respect to atherosclerosis, such literature supports that diets rich in carnitine, lecithin or choline, which are then metabolised to Trimethyl amine (TMA) by gut bacteria, leads to increased TMAO via the conversion of TMA to TMAO. This increased TMAO is then proposed to elicit pro-atherogenic and thrombotic effects. However, much controversy exists as several other groups have found no links, and even inverse relationships, between TMAO and atherosclerosis (Mueller et al., 2015; Yin et al., 2015; Meyer et al., 2016; Lindskog Jonsson et al., 2018; Aldana-Hernández et al., 2020). Given the uncertainty in this area, we aimed to clarify directly whether high choline diets and TMAO influenced atherosclerotic plaque stability using the TS model. In this study, we identified that, whilst high choline diets did not influence the development of stable plaques, they did significantly increase intraplaque haemorrhage in the TS model (Koay et al.). This finding was paralleled by increased circulating monocytes, neutrophils and upregulated platelet P-selectin expression. Ultimately, in the context of the ongoing debate around TMAO in atherosclerosis, our results support a specific role for high TMAO levels in plaque instability and thus identifies the TMAO pathway as a potential target for plaque stabilisation approaches.

In summary, the TS model has been applied by our lab and several others to understand the underlying biology of atherosclerotic plaque instability and rupture, as well as for the testing of novel therapeutic and diagnostic strategies. These studies highlight the great flexibility of

the model as an experimental tool, being applied to address a wide variety of experimental questions. Most importantly, this body of research support the TS as a highly useful model for studying critical aspects of human atherosclerosis in mice, most of which are poorly represented in pre-clinical atherosclerosis studies to-date due to their absence in the most commonly used $LDLR^{-/-}$ and $ApoE^{-/-}$ mouse models.

Other Models of Plaque Instability/Rupture

It is important to recognise that the TS model is not the only approach that has been developed or proposed to allow for the study of unstable plaques in mice. As with all animal models, each has its strengths and limitations. **Table 1** provides a comparison of all mouse models of plaque instability that we are aware of, highlighting the classical features of vulnerable plaques each model reflects and the presence or absence of plaque rupture. We will now discuss three approaches which, in our opinion, possess the greatest utility alongside the TS model.

Firstly, it has been demonstrated that the brachiocephalic arteries in mice may be a useful site for the study of plaque rupture in $ApoE^{-/-}$ mice. This small artery (~2mm in length) was initially described to develop advanced lesions characterised by intraplaque haemorrhage, necrosis and fibrous cap thinning in chow fed aged $ApoE^{-/-}$ mice (42-54 weeks); though no plaque rupture was observed (Rosenfeld Michael E. et al., 2000). In 2001, a study by Johnson *et al*. further assessed the brachiocephalic arteries of male and female mice that had died

following long-term (37-59 weeks) HFD feeding (Johnson et al., 2001). The authors demonstrated that the addition of HFD led to brachiocephalic plaques demonstrating some, though in our opinion debatable, evidence of luminal thrombosis. In a later study, Johnson *et al.* then expanded on this work, presenting evidence of plaque rupture in 62% of male ApoE$^{-/-}$ mice following >8 weeks of HFD (Johnson et al., 2005). The plaques in the brachiocephalic artery after 8 weeks of diet displayed buried fibrous caps as well as intraplaque fibrin staining. However, observing no intraplaque neovascularisation, the authors hypothesised that episodic rupture and healing leads to the sequential incorporation of acute ruptures including the associated thrombi into brachiocephalic atherosclerotic lesions. In our opinion, there is a degree of speculation in this argument and further evidence would be required to confirm this hypothesis. If this rupture phenotype was present in standard ApoE$^{-/-}$ mice, then we would suggest analysis of the brachiocephalic artery in these mice would be a highly attractive entry point into the study of unstable plaques. However, perhaps the biggest limitation with the data supporting the potential to do so is the apparent requirement for ApoE$^{-/-}$ mice to be on a mixed C57BL/6 129SvJ genetic background in order for ruptures to be observed. There is clear evidence that the background on which ApoE mice are bred can influence atherosclerosis development (Stein et al., 2006; Maeda et al., 2007). However, the majority of researchers use ApoE$^{-/-}$ on a C57BL/6 background. Consequently, we believe it would be more beneficial to invest in more reliable models of plaque rupture, such as the TS model and those soon to be discussed. Notably, in a more recent study using a similar experimental design, rupture or thrombosis was not analysed and does not seem to be a prominent

histological feature in the presented data (Di Gregoli et al., 2020). A further limitation to consider with this approach is that the brachiocephalic artery contains only ~150μm of plaque length with the described features on instability/rupture, providing limited material to assess. To provide contrast; the TS model provides ~900μm of plaque length with reproducible plaque rupture, luminal thrombosis and intraplaque haemorrhage. However, despite these limitations, if it is possible to easily acquire or generate ApoE$^{-/-}$ mice on a mixed C57BL/6 129SvJ background, then the relative simplicity of also isolating and analysing the brachiocephalic artery is certainly an advantage. Moreover, the deletion of genes such as Galectin-3 enhances features of plaque instability, including fibrous cap thinning, increased immune cell recruitment and increased necrotic core size in the brachiocephalic artery (Di Gregoli et al., 2020). As such, this approach may be useful as a foundation to study gene manipulations or treatments that inhibit atheroprotective processes in the ApoE$^{-/-}$ mice.

A highly promising model for the study of unstable atherosclerosis and plaque rupture is the ApoE$^{-/-}$ Fbn1$^{C1039G\ +/-}$ mouse (Van Herck Jozef L. et al., 2009; Van der Donckt et al., 2015b). The introduction of a heterozygous mutation in the Fibrillin-1 (Fbn1) gene, promoting elastin fragmentation, leads to profoundly increased atherosclerosis and plaque instability when mice are fed a HFD. Following 20 weeks of HFD, the plaques in ApoE$^{-/-}$ Fbn1$^{C1039G\ +/-}$ female mice are highly unstable, demonstrating consistent neovascularisation and intraplaque haemorrhage (90%) complemented by large necrotic cores, fibrous cap disruption and luminal thrombosis. Importantly, this model recreates several clinical consequences of

atherosclerosis, including MI, stroke and a high incidence of spontaneous death (70% by 35 weeks). Similarly to the TS model, this approach has now been used to interrogate biological mechanisms, as well as testing diagnostic and therapeutic approaches (De Wilde et al., 2015; Roth et al., 2015, 2016b, 2016a, 2019; Van der Donckt et al., 2015a; Luyckx et al., 2018; Van der Veken et al., 2018; Kurdi et al., 2019; De Dominicis et al., 2020; Perrotta et al., 2020; Perrotta Paola et al., 2020).

In respect to potential limitations of this model; ironically, the high incidence of sudden death is perhaps the greatest experimental limitation. Whilst deaths were mostly observed between 15 and 35 weeks of HFD, the inability to predict exactly when death will occur may be challenging for studies that require a degree of synchronisation with respect to end points. We would further note that this model possesses features that go beyond atherosclerosis. $Fbn1^{C1039G\ +/-}$ mice develop abdominal aortic aneurysms, even in the absence of $ApoE^{-/-}$ deficiency, alongside mitral valve thickening, skeletal and lung defects effects (Judge et al., 2004; Ng et al., 2004). This added complexity and potential for confounding factors may be inhibitory to some studies. In respect to obtaining these animals, it doesn't appear possible to directly purchase these mice, but $Fbn1^{C1039G\ +/-}$ are commercially available, making it possible to generate $ApoE^{-/-}$ $Fbn1^{C1039G\ +/-}$ lines. Although the study of additional genetic phenotypes with the required crossbreeding might be time and cost intensive. We would suggest that this is a model of significant cardiovascular burden, also with potential non-cardiovascular related comorbidities. However, the consistency of a well characterised

genetically modified animal that recreates many features of plaque instability, albeit to the extreme, will be highly appealing to cardiovascular researchers and should encourage wider use of this model.

One other model we would draw attention to involves using transverse aortic constriction (TAC) surgery in atheroprone mice (Marino et al., 2019). TAC surgery introduces an aortic stenosis between the brachiocephalic artery and left common carotid artery. The consequence of TAC induced pressure overload in the coronary arteries and also right carotid artery is the development of advanced coronary atherosclerotic plaques with thin fibrous caps, which rupture or erode leading to occlusive thrombi and MI. An interesting postulated aspect of this model is the potential ability to induce an MI using a treadmill-based stress test. The authors demonstrated an 83% mortality rate in this study, with 80% of these deaths occurring shortly after physical stress tests. Whilst in this study deaths were over a 16-week period, with one stress test per week; it is possible that heightened physical stress may allow MI to be induced in a more synchronous fashion, which would likely be advantageous experimentally. In our opinion, this model represents an elegant approach with clear applicability to studies aiming to assess the fundamental biology of MI caused by plaque rupture. Current approaches to study MI commonly involve the permanent or transient surgical ligation of the left anterior descending coronary artery (Lindsey et al., 2018). As a result, they do not fully reflect the pathology that drives MI in patients. In contrast, this TAC approach overcomes this, causing MI as a direct result of atherosclerotic disease without

extensive confounding effects such as those observed with the ApoE$^{-/-}$ Fbn1$^{C1039G\ +/-}$ model. However, TAC does induce systolic dysfunction and cardiac fibrosis, which may also influence plaque development and rupture (Richards et al., 2019). As with the TS model, the obvious limitation is the requirement of small animal surgery skills and appropriate equipment. Unlike the TS and ApoE$^{-/-}$ Fbn1$^{C1039G\ +/-}$ models, it appears that the use of this approach so far is restricted to a single published study and therefore it remains to be determined how this model translates to other experimental settings and laboratories.

Limitations of the Tandem Stenosis Model

The translation of findings from animals to humans is rarely linear. As is the case with all animal models, any and all insights from the TS model should be validated using human samples. The cardiovascular community is conscious of the challenges to-date with translating preclinical data to clinically viable diagnostic or therapeutic approaches. We would speculate that perhaps a significant reason for this may be the dominant focus on animal models of stable atherosclerotic disease, which does not fully reflect human atherosclerosis and, in particular, its complications. However, it is also true that atherosclerosis is a highly heterogeneous disease. Consequently, it is unlikely that any one animal model will replicate perfectly the spectrum of atherosclerotic disease present in patient populations. Ultimately, the use of any animal model must be intricately related to the specific scientific questions being asked. As such, whilst plaque rupture is the main cause of MI, it is not the only implicated precursor to major adverse cardiac events. Plaque erosion and calcification have

also been directly associated with cardiovascular morbidity and mortality, and the TS model would not be suitable for studying these aspects.

To our knowledge, there are no well-established mouse models of plaque calcification. For plaque erosion, however, Franck *et al.* recently presented an attractive mouse model (Franck et al., 2017). Briefly, this model involves the surgical exposure and electrical injury of the left common carotid artery. Four weeks later, a second surgery introduces a cone-shaped polyethylene cuff below the initial injury site. The combination of electrical injury and disturbed flow incites key features of plaque erosion, providing a unique tool for the study of this atherosclerosis phenotype. Like the development of the TS and other models of plaque instability and rupture; this model of erosion is an exciting advance in preclinical models of atherosclerotic cardiovascular disease.

In respect to practical considerations of the TS model; utilising the TS approach does require access to basic surgical facilities, skill in small animal surgery and experience in post-surgical care and monitoring of animals. Though true for all models of plaque rupture that do not lead to clinical symptoms; assessing plaque rupture in the TS model requires careful histological analyses and experienced personnel to either conduct these analyses or provide appropriate training. In respect to the surgery; we would stress that this is minimally invasive. To-date, we have successfully trained several collaborators and members of our own laboratory to conduct the surgery. We would also point to the fact that several laboratories have adopted

this surgery without our input as an indicator of the relative ease of applying the TS model. Some may argue that the use of surgical manipulation dictates that the plaque ruptures observed are not 'spontaneous'. However, the surgical intervention in the TS occurs weeks before rupture and the site of plaque rupture is distant to the ligation sites. This is in contrast to, for example, a ferric chloride thrombosis model, where ferric chloride is applied directly to an exposed carotid artery to immediately induce thrombotic occlusion of the vessel. Despite this, it remains important to recognise that the TS model does require surgical manipulation of the carotid artery and, depending on the experimental context, this may be inhibitory to some research questions.

The TS model also does not induce cardiovascular events such as MI or stroke. This is a disadvantage in that the TS model does not fully recapitulate the consequences of unstable atherosclerosis beyond plaque rupture and other histopathological indicators of instability. However, experimentally, this can also be considered an advantage as the use of models which experience spontaneous death can be challenging from multiple stand points. For example, it can be difficult to assess animals at similar disease stages given that sudden death is unlikely to be synchronised across a cohort. We would stress, however, that this again relates directly to the experimental question. If the objective is to study the influence of a therapeutic or diagnostic in the context of plaque rupture which leads to MI, then the TS model would not be a suitable approach.

We would further comment that not all mice experience plaque rupture, although the percentage gets higher with increased time period post TS surgery. Therefore, while all plaques generally reflect an unstable, vulnerable phenotype, there is variation in the degree and timeline of vulnerability and susceptibility to plaque rupture between mice. In our hands, we embrace this variation as it may reflect differences in the underlying biology which dictates plaque rupture and also the heterogeneity seen in human disease. In this respect, some of our research now utilises molecular imaging to first differentiate ruptured plaques from those that are vulnerable but remain intact prior to further assessment in order to try and identify the biological differences between these phenotypes. However, it must be appreciated that in some experimental settings this variability may be unwanted. For example, should the experiment wish only to compare ruptured plaques in the tandem stenosed carotid artery to a healthy artery or the stable plaques found in the aorta, then the portion of TS animals that do not suffer plaque rupture would not be suitable for this purpose. We would also state that, while we have utilised modulation of haemodynamics to induce plaque instability, we have not yet identified the exact mechanisms by which this modulation causes plaque rupture. Indeed, this is one of the many interesting questions that the TS model is allowing our laboratory, and others, to explore.

Advantages of the Tandem Stenosis Model

The unique advantage of the TS model is its ability to recreate, in a simple approach, key features of vulnerable plaques seen in humans, including: lipid accumulation, extensive

immune infiltration, thin/disrupted fibrous caps, IPH and the presences of platelets and fibrin, luminal thrombosis, neovascularisation, vascular remodelling, being driven by local haemodynamic alterations, and also responsiveness to atheroprotective therapeutics such as atorvastatin. Whilst there are other models of plaque rupture, many of these either do not reflect this unique combination of characteristics of plaque instability and/or rupture, or these characteristics have not been reported (see **Table 1**).

In addition to reflecting many human disease-relevant characteristics, the TS model has several more distinct advantages as a model of plaque instability and rupture. For example, a clear advantage is the fact that the TS model has been established in both ApoE$^{-/-}$ and LDLR$^{-/-}$ mice (the latter unpublished), which are the mouse strains of choice for most cardiovascular researchers utilising pre-clinical models of atherosclerosis. From a practical point of view, as most researchers will have access to these mice, the purchase or generation of new mouse strains is not required to apply the TS approach. Moreover, inducing rupture-prone plaques in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice provides a 'two-for-one' model, where one can directly compare this understudied plaque phenotype to the stable plaques found in the aorta and aortic sinus of the same mice, which is an ideal setting for comparative profiling. This, in effect, allows investigators to put unstable plaque data from the tandem stenosed carotid arteries in the context of the same stable plaques on which we have over 30 years of pre-clinical research data. In respect to this point; we have no evidence that the stable plaques within ApoE$^{-/-}$ or LDLR$^{-/-}$ mice change following TS surgery. The use of individual ApoE$^{-/-}$ and LDLR$^{-/-}$ mice to study both stable and unstable phenotypes also negates any need to increase animal numbers

to accommodate for the study also of plaque rupture and limits additional practical burden that would be associated with using separate 'unstable' cohorts. This, of course, further avoids the costs associated with generating and/or using separate cohorts that would be required with some other models, and also has statistical advantages in that it is possible to compare stable to unstable plaques within animals, minimising inter-mouse variability.

Another advantage is the flexibility of the TS model's timeline. Most often we utilise 6 to 8-week-old mice undergoing 6 weeks of HFD, which are then subject to the TS surgery and continued on HFD for 4-11 weeks depending on the specific experimental objectives. As a result, we generally compare advanced unstable plaques in the tandem stenosed carotid arteries to early/moderate staged lesions in the aorta consistent with 10 – 17 weeks of high fat feeding. However, should the desire be to study advanced stable lesions in comparison with unstable/ruptured carotid plaques, it is entirely possible to use the TS approach in older animals and to subject mice to longer periods of HFD feeding prior/post-surgery. We would simply caution that carrying out the surgery in older animals may be more technically challenging due to increased adiposity in the surgical area, as well as considerations relating to the impact of anaesthesia on older and more obese mice. It is pertinent to make clear that the TS model provides an accelerated atherosclerosis, with plaques forming much faster than in unmanipulated arteries. Logistically, this can be considered an advantage given that the model is able to recapitulate a wide array of human-like plaque features quickly. However, in humans, atherosclerosis develops over many years. Therefore, consistent with arguably all

mouse modes of atherosclerosis, the accelerated timeline can also be considered a biological limitation as it does not fully reflect the human condition and should be taken into account.

In our opinion, perhaps the greatest advantage of the TS model is its tested, and also predicted, compatibility with other methodologies for manipulating biology in animal models. Firstly, we expect the TS model to work in most mouse models of atherosclerosis driven by hypercholesterolaemia. We would note that in our hands the TS model was not effective in ApoE$^{-/-}$ 3-Leiden mice, but highlight our own successful use of the TS in ApoE$^{-/-}$, LDLR$^{-/-}$ and ApoE$^{-/-}$Mpo$^{-/-}$ (Rashid et al., 2018) mice as well as, for example, DKK3$^{-/-}$ApoE$^{-/-}$ mice by Karamariti *et al.* (Karamariti et al., 2018).

We also expect the TS to be useful in the context of bone marrow chimera methodologies, and we are currently exploring the utility of this an approach in our own research. Generating bone marrow chimeras is a common approach for studying the relevance of immune-inflammatory mechanisms to health and disease, including in atherosclerosis, and it involves the irradiation of mice that subsequently receive a bone marrow transplant. Bone marrow, and potentially also foetal liver/neonatal thymocytes, are usually transferred from mice with genetic modifications (most commonly deficiencies) in cells or molecules of interest. This allows for one to directly test how a cell or molecule of interest influences an experimental scenario. It is also possible to restrict genetic modifications to distinct haematopoietic lineages using mixed bone marrow chimera approaches. For example, by transferring 80% of

bone marrow from a mouse deficient in a specific cell type, e.g. T cells, and 20% of bone marrow from mice deficient in a T cell relevant molecule, e.g. CD28. In this context, T cells will be replenished only from CD28 deficient bone marrow, and will themselves be CD28 deficient, whilst all other immune cells will be unaffected, being replenish predominantly from the T cell deficient but CD28 competent bone marrow. In atherosclerosis research, $LDLR^{-/-}$ mice are most commonly used as bone marrow recipients. $ApoE^{-/-}$ mice are generally not used for this purpose as the atherosclerotic phenotype in these mice partly relies on $ApoE^{-/-}$ deficiency in the bone marrow compartment (Sakai et al., 2002). Ultimately, we anticipate that the combination of bone marrow chimera approaches and the TS model will provide one option to test the mechanisms by which haematopoietic restricted cells or molecules influence unstable rupture-prone atherosclerosis.

Going further, we fully expect the TS model to be compatible with emerging models of atherosclerosis. For example, the recent development of an approach that induces hypercholesterolaemia through a single injection of an adeno-associated virus (AAV) mediating transgenic expression of pro-protein convertase subtilisin/kexin type 9 (PCSK9), and consequential downregulation of the LDLR and atherosclerosis may be particularly interesting in this respect (Roche-Molina et al., 2015; Goettsch et al., 2016; Emini Veseli et al., 2017). This approach, in effect, mimics the phenotype of $LDLR^{-/-}$ mice without the requirement of germline genetic modification, and is anticipated to allow for the induction of hypercholesterolaemia and atherosclerosis on most genetic backgrounds. Thus, in

combination with the TS model, the AAV-PSCK9 approach could allow for the study of both stable and unstable atherosclerosis in the context of the many genetically modified mouse models generated to date (e.g. those for studying immunity/inflammation) without the need or expense of generating new mouse lines on an $ApoE^{-/-}$ or $LDLR^{-/-}$ background. A general limitation in mechanistic atherosclerosis studies to-date is the rare use of approaches facilitating the temporal, spatial or cell type-specific deletion of genes in animal models, such as the Cre-Lox system. Such approaches would be a significant improvement upon the use of global genetic modifications in atherosclerosis research, and also chimeric mice which, though useful and arguably more flexible, are labour intensive and require irradiation of the host mouse. Importantly, this irradiation generally occurs in adult mice, which can cause issues with some cell types (particularly tissue resident immune cells), which are often seeded early in life and are potentially not replenished effectively following bone marrow transplantation (Gray et al., 2011; Haas et al., 2012).

While we cannot speak for all models of unstable atherosclerosis, we would highlight that the TS model has also been compatible with a very wide array of experimental techniques. Studies using the TS model published to date have generally used histological or imaging assessments of the unstable plaques. Whilst this may be somewhat reflective of biases in the tools predominantly used in the cardiovascular field; we can confirm from our ongoing research that the TS model is fully compatible with a much wider spectrum of experimental approaches. For example, across multiple studies (unpublished) we have recently utilised

extensive flow cytometry, proteomic, lipidomic and single cell genomic assessments of unstable plaques in tandem stenosed carotid arteries with great success.

Conclusions and Outlook

In this review we have focused on the utility of the Tandem Stenosis model, a preclinical model of unstable atherosclerosis that reflects the pathology of atherosclerosis as seen in humans. We have highlighted its use in published studies exploring the biology of unstable atherosclerosis, provided comparisons to other established models of unstable atherosclerosis, and outlined the model's strengths and limitations. We will now take the opportunity to advocate for the use of models of unstable atherosclerosis in the context of cardiovascular research.

Whilst it is well recognised that models of plaque instability are required, and indeed now exist, the adoption of these models has been limited in comparison to the ongoing extensive use of preclinical models of stable atherosclerosis. Unfortunately, medical advances relating to atherosclerosis have been scarce and certainly disproportionate to those made in other fields (e.g. oncology), which have arguably experienced medical revolutions in recent times. Whilst we have continued to develop therapeutics targeting hyperlipidaemia in cardiovascular disease, such as PCSK9 inhibitors (alirocumab/evolocumab; although these drugs have been developed based on observations in humans, not based on preclinical atherosclerosis models), there has been a failure to address residual cardiovascular risk,

particularly in relation to inflammation. There has been promise in the context of targeting inflammatory cytokines (CANTOS trial; anti-IL1β) alongside those trials utilising colchicine, which may elicit anti-inflammatory effects. However, it appears that anti-IL1β will not be approved and applied for secondary prevention of cardiovascular events, and it remains to be established whether colchicine will be approved and broadly used for secondary prevention. While there remain significant general challenges in translating preclinical animal model data to human disease, we would argue that the development of new therapeutics and diagnostics for atherosclerotic disease would likely be significantly improved if our understanding of plaque instability and rupture were enhanced. To achieve this, we strongly encourage the research community to adopt models of plaque instability, rupture, and also erosion as critical preclinical tools in the cardiovascular research arsenal. With the support of recent technological advances, such as single cell genomics and proteomics, we are now able to obtain unprecedented levels of information in both preclinical and clinical studies. Combining these technological advances with models of plaque instability, such as the TS model, we have a distinct opportunity to significantly improve our mechanistic understanding of plaque instability, define diagnostic biomarkers and imaging approaches with high prognostic value, support the development and preclinical testing of plaque-stabilising therapeutics, prevent MI and strokes and ultimately reduce cardiovascular mortality and morbidity.

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Figure Legends

Figure 1: Stages of atherosclerosis, their relevance to clinical practice and the use animal models reflecting these disease stages

This figure depicts different stages of atherosclerosis, ranging from a healthy vessel to an unstable/ruptured plaque. The initiation of atherosclerosis follows exposure to atherogenic stimuli, such as hyperlipidaemia or alterations in shear stress, inducing the expression of adhesion molecules and chemoattractants by arterial endothelial cells, which mediate the recruitment of immune cells. Concomitantly, extracellular lipids begin to accumulate in the arterial wall. Following their recruitment, monocytes differentiate into macrophages and phagocytose the accumulating lipids, becoming lipid-laden foam cells. Foam cells, alongside other recruited immune cells and the vascular stroma, secrete inflammatory cytokines and other factors which further enhance immune cell recruitment and drive the proliferation and migration of smooth muscle cells, forming the fibrous cap. A plaque with a thick fibrous cap separating the vessel lumen from the contents of the plaque can be considered stable. Factors implicated in plaque destabilisation and rupture include lipid accumulation, increased

immune cell diversity and density, heightened inflammation, and the expression of fibrous cap degrading enzymes, such as matrix metalloproteinases. The death of macrophages, and also smooth muscle cells, leads to the formation of acellular necrotic areas, referred to as necrotic cores. Intraplaque Haemorrhage, either caused by leaky neo-vessels or micro-ruptures of thin fibrous caps, further promote inflammation and plaque destabilisation. Major adverse cardiovascular events (MACE), such as myocardial infarction and stroke, are predominantly incited following acute rupture events, whereby the fibrous cap breaks, leading to occlusive thrombi.

Figure 2: Overview of the Tandem Stenosis Protocol and Atherosclerotic Plaque Phenotypes

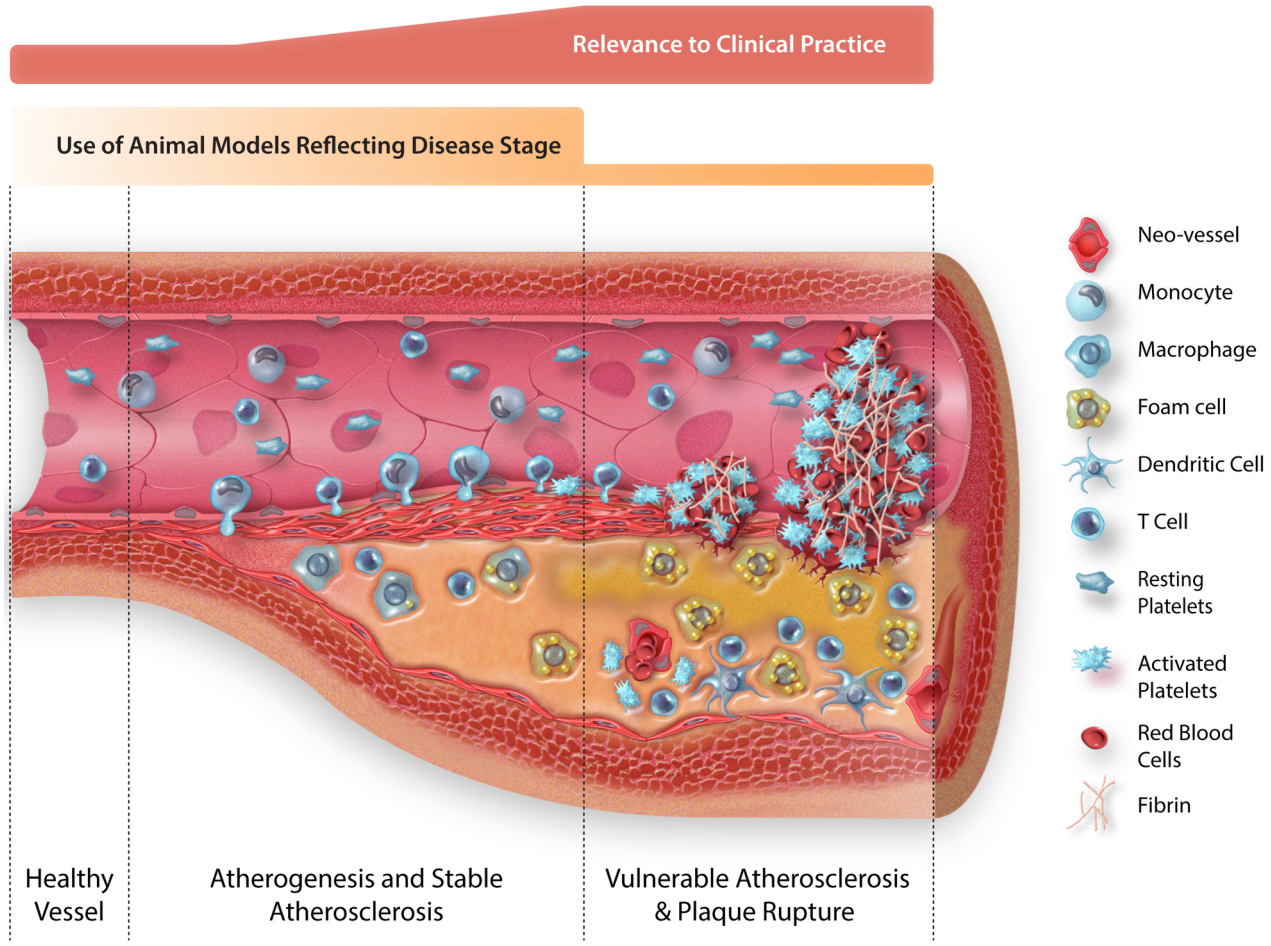
The TS model involves the dual ligation of the right common carotid artery of hypercholesterolaemic mice, such as $LDLR^{-/-}$ and $ApoE^{-/-}$ mice. Briefly, mice are anesthetised and the fur at the neck is removed. A small (~1cm) incision is made through the skin on top of the right common carotid artery to create a surgical window; this is shown as a red box in the mouse diagram. The tissue underneath this incision is then bluntly dissected under a dissection microscope to expose the carotid artery. The vagus nerve is gently separated from the artery before two stenosis points are placed 1mm and 4mm below the carotid bifurcation. These points are highlighted with an * on the diagram. To achieve the desired stenosis of 150μm diameter, a 150μm diameter needle is placed on top of the artery and sutures are then tied tightly around the artery and needle, blocking blood flow and reducing the vessel lumen to 150μm. Following this, the needle is carefully removed to reinstate blood flow; this process is repeated for both ligations. In the photograph of the exposed vasculature, the tied sutures can be seen in blue on the right carotid artery, alongside the unmanipulated left carotid artery and aortic arch. The insertion of these tandem stenosis points results in a reduction of vessel wall shear stress during both systole and diastole, which drives the development of rupture-prone atherosclerotic plaques. Computational modelling of the vessel wall shear stress for the aortic arch, left and right carotid arteries post TS surgery is shown, highlighting low shear stress in the right common carotid artery. [Pa] = Pascal. Following the TS surgery, accelerated plaques form in the right carotid artery, stable plaques form in the aortic arch (as well as the aortic

sinus and descending aorta) as is normal for atheroprone mice, whilst the left carotid artery remains healthy. Representative Haematoxylin and Eosin staining of Stable (**A**), Rupture-Prone (**B**) and Ruptured Plaques (**C**) are shown. Representative anti-Fibrin (**D**) and platelet (anti-CD41; **E**) immunohistochemical staining is also presented, highlighting intraplaque haemorrhage and luminal thrombosis, respectively. FC, fibrous cap; L, lumen; NC, necrotic core; and Th, thrombus. This Figure was adapted from Chen *et al.* (Chen et al., 2013)

Figure 3: Detection of Intraplaque Haemorrhage Induced Near Infra-Red Autofluorescence (NIRAF) as a Marker of Plaque Instability

Combining the TS model and the assessment of human carotid endarterectomy samples, our laboratory recently identified that the presence of intraplaque haemorrhage (IPH) results in strong autofluorescence signal in the near infra-red spectrum. To demonstrate this, representative white light images of exposed carotid arteries and aortic arch are presented alongside fluorescent images from near infra-red autofluorescence (NIRAF) in healthy arteries, and arteries originating from the TS model with and without IPH (**A**)**.** Firstly, comparison of the white light images highlights the presence of macroscopically visible IPH in the right carotid artery of a mouse with IPH (upper panel), which is not present in animals without IPH (lower panel). Focusing on the fluorescent images presented to the right of the white light images; arteries from the TS model that lack visible IPH, produce no NIRAF similar to healthy vessels. In contrast, arteries with IPH produce strong NIRAF signal allowing for their discrimination from healthy vessels and also, most importantly, stable plaques. Critically, these findings made in the TS model are reflected in the assessment of human carotid endarterectomy samples (**B**), where endarterectomy with gross IPH produces clear NIRAF signal in contrast to both normal tunica intima and plaque without haemorrhage. Inspired by these findings, we then demonstrated that it was possible to detect these NIRAF signals derived from plaques with IPH *in vivo* in mice using Fluorescence Emission Computed Tomography (FLECT) imaging, highlighting the translational potential of detecting NIRAF as a marker of plaque instability (**C**). w/o = without. Adapted from Htun *et al.,* Near-infrared autofluorescence induced by intraplaque haemorrhage

and haeme degradation as marker for high-risk atherosclerotic plaques. *Nat. Commun.* 2017;8:75. Under a Creative Commons Attribution 4.0.



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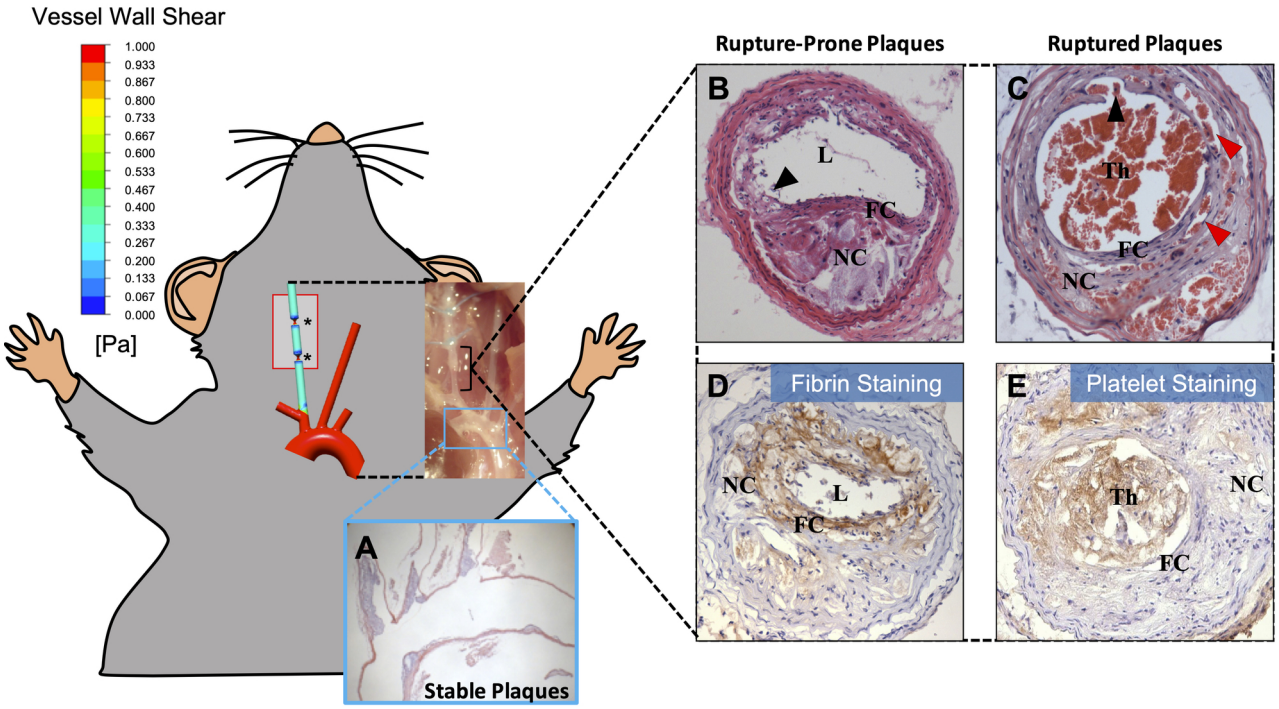

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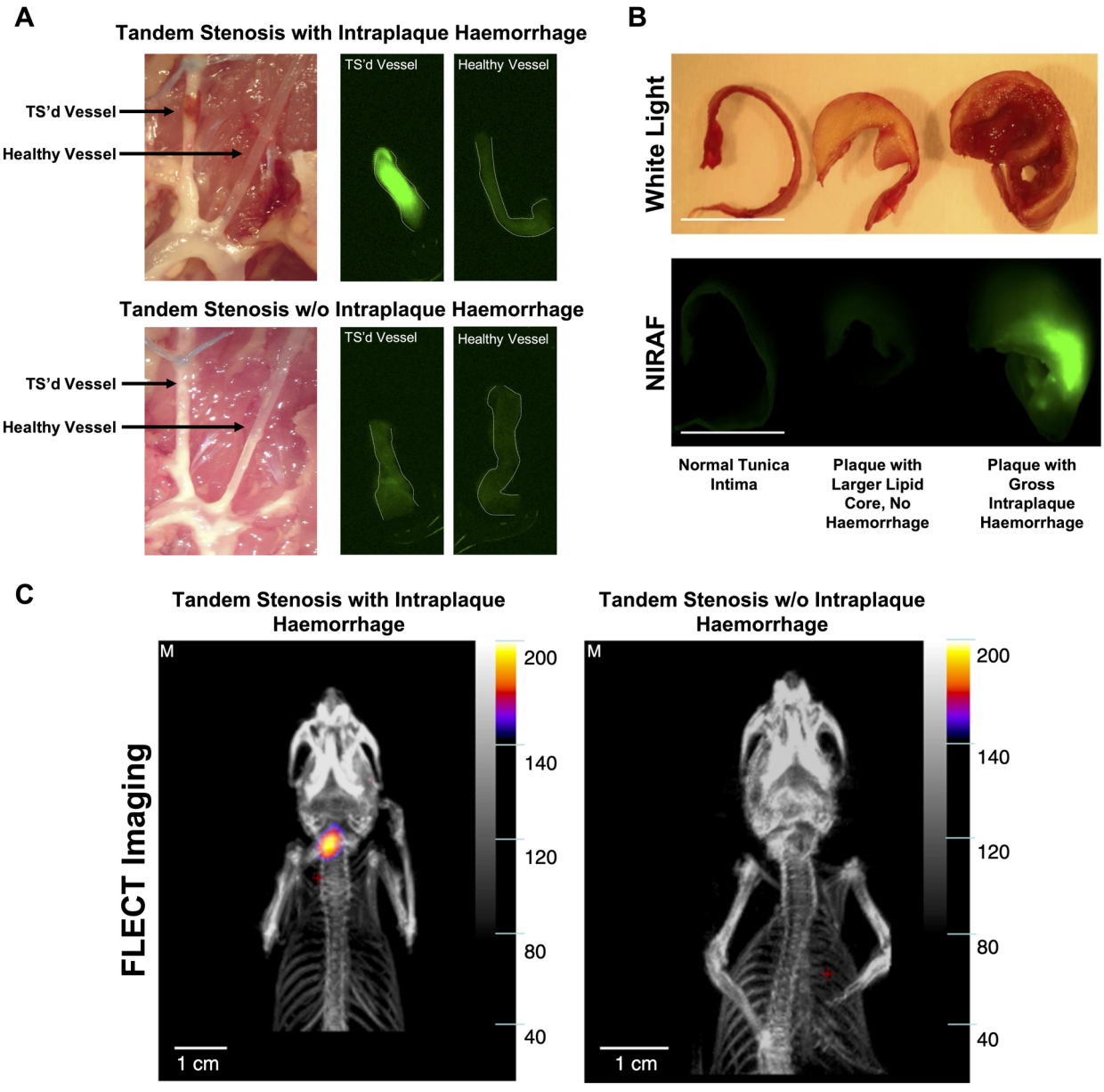

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