

REVIEW ARTICLE**Neuroprotection in stroke: the importance of collaboration and reproducibility****Ain A. Neuhaus,¹ Yvonne Couch,¹ Gina Hadley¹ and Alastair M. Buchan^{1,2,3}**

Acute ischaemic stroke accounts for 6.5 million deaths per year, and by 2030 will result in the annual loss of over 200 million disability-adjusted life years globally. There have been considerable recent advances in the gold standard of acute ischaemic stroke treatment, some aspects of which—aspirin to prevent recurrence, and treating patients in specialized stroke wards—are widely applicable. Recanalization of the occluded artery through thrombolysis and/or endovascular thrombectomy is restricted to only a small proportion of patients, due to contra-indications and the costs associated with establishing the infrastructure to deliver these treatments. The use of neuroprotective agents in stroke has been a notable failure of translation from medical research into clinical practice. Yet, with the advent of endovascular thrombectomy and the ability to investigate patients in much greater detail through advanced imaging modalities, neuroprotective agents can and should be re-examined as adjunct therapies to recanalization. In parallel, this requires appropriate planning on behalf of the preclinical stroke research community: there is a need to reinvestigate these therapies in a more collaborative manner, to enhance reproducibility through reduced attrition, improved reporting, and adopting an approach to target validation that more closely mimics clinical trials. This review will describe some of the novel strategies being used in stroke research, and focus on a few key examples of neuroprotective agents that are showing newfound promise in preclinical models of stroke therapy. Our primary aim is to give an overview of some of the challenges faced by preclinical stroke research, and suggest potential ways to improve translational success.

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Abbreviations: AIS = acute ischaemic stroke; EVT = endovascular thrombectomy; IVT = intravenous thrombolysis; MCA = middle cerebral artery; rt-PA = recombinant tissue-type plasminogen activator; STAIR = Stroke Academic Industry Roundtable

“Coming together is a beginning, staying together is progress, and working together is success”.

– Henry Ford

Introduction

Stroke is an umbrella term used for focal neurological deficits and CNS injuries of vascular origin. This simple

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definition encompasses a vast amount of heterogeneity regarding the nature of the vascular problem, location of the lesion, and severity of the insult. Stroke can be divided into two major categories, ischaemic and haemorrhagic. Acute ischaemic stroke (AIS) accounts for 87% of strokes (Mozaffarian *et al.*, 2016). It is the consequence of vascular occlusion, due to atherosclerotic large artery disease, small artery disease (lacunar strokes), or cardioembolic events (Adams *et al.*, 1993). In 2013, there were 6.5 million stroke-related deaths (11.75% of all deaths) and 10.3 million new strokes globally, with an overall cost of 113 million disability-adjusted life years (DALYs) (Feigin *et al.*, 2015). This burden is increasing, largely due to a rise in vascular risk factor prevalence in low- and middle-income countries, and will, according to current trends, reach 12 million deaths and 200 million DALYs lost by 2030 (Feigin *et al.*, 2014).

These figures clearly establish AIS as a leading contributor to morbidity and mortality worldwide, and developing more effective approaches for stroke prevention and therapy is a key objective in medical research (Hachinski *et al.*, 2010). To date, our successes in that aim have been limited. It is known that outcomes in AIS are improved by admission of patients into specialist stroke units (Trialists' Collaboration, 1997), the early administration of aspirin for secondary stroke prevention (Chen *et al.*, 2000) and hemicraniectomy in malignant middle cerebral artery (MCA) infarctions (Schwab *et al.*, 1998). Until 2015, there was only one widely available evidence-based therapy that directly targeted the occlusion—intravenous thrombolysis (IVT) through administration of recombinant tissue-type plasminogen activator (rt-PA), in order to disrupt the fibrin component of the clot and restore flow through the occluded vessel (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995; Wardlaw *et al.*, 2012, 2014; Balami *et al.*, 2013).

Despite strong evidence that IVT improves outcomes in AIS, it has notable limitations. First, IVT increases the risk of haemorrhagic transformation, where ischaemic damage to the vasculature itself results in disruption of vessels and extravasation of blood and associated circulating inflammatory cells (Alvarez-Sabin *et al.*, 2013; Whiteley *et al.*, 2016). Second, current licensing restricts the time window of rt-PA administration to within 4.5 h from stroke onset, although there is some evidence of its efficacy when administered up to 6 h from onset (Wardlaw *et al.*, 2014). Third, the effect of rt-PA is dependent on contact with the surface of the clot; therefore, larger thrombi are inefficiently lysed, with <1% likelihood of recanalization when clot length exceeds 8 mm (Riedel *et al.*, 2011).

Over the past 2 years, evidence in favour of acute endovascular intervention using stent-retriever devices has become compelling (Balami *et al.*, 2015). Indeed, recent NICE guidelines state that where available, IVT and endovascular therapy (EVT) are the gold standard of AIS treatment (NICE, 2016). With the advent of EVT and the flurry of research surrounding it, preclinical models are now perhaps more relevant than they have previously been

(Sutherland *et al.*, 2016). Intraluminal filament models may now be capable of more accurately recapitulating the clinical outcomes of patients who receive EVT, and when used with adjunct pharmacology, may be therapeutically relevant for neuroprotection. This concept warrants the clinical reinvestigation of compounds that failed after being successful in preclinical work, now in conjunction with EVT. In particular, the trials should feature more detailed imaging paradigms, which would allow us to reliably detect smaller changes in specific parameters, using a relatively small but highly homogenous cohort of patients. In the process of bridging the gap between both therapeutic and mechanistic preclinical work and clinical outcomes, we must strive to work from both ends, making preclinical trials more robust, but also investigating mechanistic aspects of therapy in human trials (Fig. 1).

Preclinical models of stroke

Modelling stroke in the preclinical setting is vital to the understanding of the basic mechanisms of action of novel therapeutics, as well as their potential effects on physiological parameters such as cerebral blood flow. Current preclinical research covers a spectrum of models, both *in vitro* and *in vivo*. While certain aspects of stroke are impossible to model in animals, such as the primary prevention strategies that have been so crucial in reducing stroke incidence, we can use these tools to develop a better understanding of stroke pathomechanisms and response to novel drugs. *In vitro* models of stroke provide valuable mechanistic insight into potential neuroprotective candidates. They can demonstrate the impact of oxygen and glucose deprivation on specific cell types (neuronal, glial, vascular, or immune) and provide valuable 'target validation' at the molecular level, in a very basic experimental paradigm. The major advantage of these reductionist systems is that it is possible to tightly regulate conditions. This greater control means that it is possible to more accurately deduce causality in relation to changes in specific variables. The corollary of this is that the more tightly controlled the experimental conditions, the further away they are from the complex, physiological interactions occurring in the brain *in vivo*.

The capacity for stroke therapy to have complex and distant physiological effects, in conjunction with an extremely heterogeneous patient population in most trials, highlights the need for relevant *in vivo* models. With all these caveats in mind, it is important to first consider whether the preclinical research being conducted is translatable in terms of clinical outcome and demonstrates all types of validity (face, construct, predictive), or whether it is mechanistic—that is to say, whether the authors are aiming to test the efficacy of a drug, or to determine the pathophysiological underpinnings of disease. To date, the approaches taken to address these two fundamentally disparate questions have broadly been the same. There has

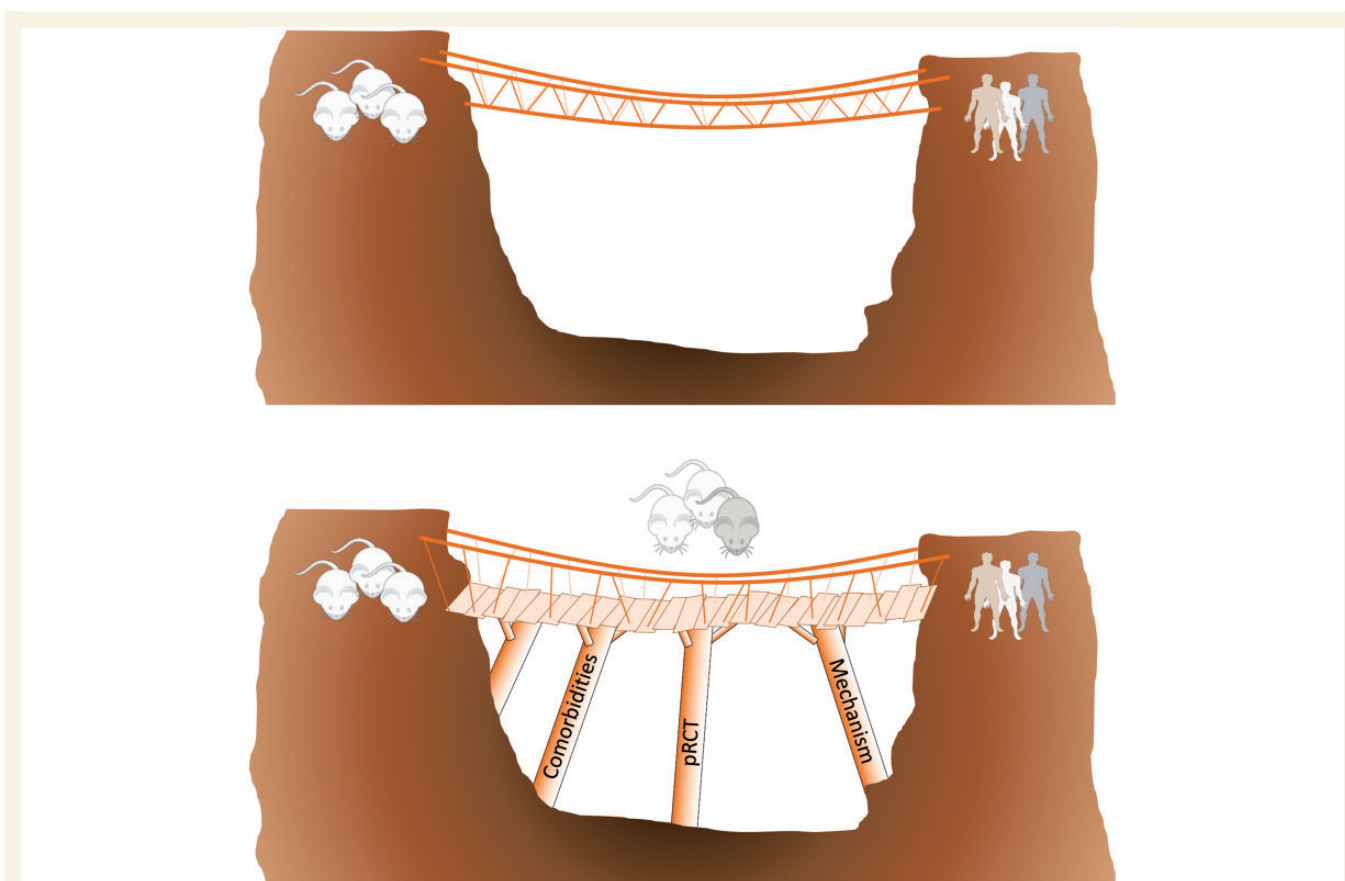


Figure 1 Bridging the gap. There is a significant gap between current preclinical approaches and recognized standards in clinical trials of stroke therapy. By bridging the gap from both ends, using both comorbidity studies and randomized controlled trials in the preclinical setting (pRCT), and by studying mechanism in the clinical setting, we may be able to bring about effective translational research.

been an abundance of literature suggesting that preclinical stroke research is failing to translate into the clinic, potentially because of the issues mentioned above (Sena *et al.*, 2007; Neuhaus *et al.*, 2014; Begley *et al.*, 2015), and therefore a new, more co-operative and clinically relevant approach for preclinical drug studies would be appropriate.

In vivo stroke studies are largely carried out in rodents, with 27% carried out in mice and 66% in rats (Emily Sena and Malcolm MacLeod, personal communication), although the widespread availability of transgenic mouse models has led to a recent increase in their use for stroke studies. As mentioned above, the choice of specific model will depend on the experimental question, whether it is translational preclinical work or fundamental mechanistic work. For example, models of global ischaemia cause selective neuronal death in particular regions of the hippocampus, and can be used to isolate pathways relating to neuronal vulnerability or survival (Papadakis *et al.*, 2013).

More commonly, stroke is modelled using focal ischaemia, where lesion reproducibility and location can be used to answer a variety of scientific questions, including the effects of therapies on lesion volume or behavioural outcomes. For example, the vasoactive peptide endothelin-1 can be injected intracranially into any location, allowing

the effect of infarct location on outcome to be specifically dissected with a consistent lesion (Macrae *et al.*, 1993). Transient and permanent middle cerebral artery occlusion (MCAO) models result in more variable lesions of known area and location, but are widely employed throughout the preclinical stroke field because of the prevalence of MCA territory strokes (Longa *et al.*, 1989; Macrae, 2011). Permanent occlusion provides a close approximation of most strokes, where recanalization is minimal; transient models are more akin to the effects of EVT, as discussed above, but only if the occlusion duration matches the onset-to-recanalization time seen in most patients. The two also differ in the pathophysiology of neuronal damage: with permanent occlusion there is a gradual loss of tissue, whereas transient models feature a delayed-type insult (Hossman, 2012). Embolic models using the autologous clot method more accurately mimic the human situation, but precise clot placement is challenging and mortality is high (Busch *et al.*, 1997). Modifications of the embolic model include photochemical (Watson *et al.*, 1985) and microsphere-induced (Mayzel-Oreg *et al.*, 2004) thrombus formation, as well as novel techniques involving local injection of thrombin (Orset *et al.*, 2007).

The variety of animal models and endpoints used, means that study quality and reporting has been remarkably inconsistent within the preclinical stroke field (Neuhaus *et al.*, 2014). There are a number of different approaches that can be taken to rectify this, some of which are already in use, such as the widespread implementation of STAIR (Stroke Academic Industry Roundtable) quality criteria (Fisher *et al.*, 2009). Others, most notably the adoption of phase III multicentre preclinical trials for validation of drug candidates before progressing to clinical trials, are still gaining traction (Dirnagl *et al.*, 2013; Llovera *et al.*, 2015). It is particularly important, when considering such studies, to include multiple species, and even different strains, as there is considerable heterogeneity between inbred rodents in factors that may confound outcome, such as differences in collateral circulation and behavioural responses (Bardutzky *et al.*, 2005; Zhang *et al.*, 2010; Kunze *et al.*, 2014). To further improve signal-to-noise in preclinical work aiming to study therapy, current rodent models need to be optimized and adherence to quality criteria must be improved in order to increase the likelihood of reproducible findings.

Optimizing rodent models

It is now widely agreed in the field of preclinical stroke research that the rodent population most commonly used does not mimic the typical AIS patient population (Dirnagl and Endres, 2014). Whilst risk factors such as age, diabetes and hypertension can be modelled in rodents (McColl *et al.*, 2010), the increased costs associated with comorbid animals (such as increased mortality and housing the animals for extended periods) often mean this crucial aspect of translational validity is neglected. The key factors to consider when modelling stroke are age, gender, comorbidities, and lifestyle (for a more detailed discussion of comorbidities in stroke models, see Howells *et al.*, 2010). Current work is principally conducted in young, healthy males, with relatively fewer studies using females, despite the fact that more than 60% of clinical stroke mortality is accounted for by females (Mozaffarian *et al.*, 2015). Preclinical stroke work, both *in vitro* and *in vivo*, is known to be affected by gender (Lang and McCullough, 2008), and this caveat should be considered in experimental design. Older animals potentially provide a more complex challenge, with money and time being limited in most institutes, and mortality in older animals being higher (Futrell *et al.*, 1991); as such, throughput in preclinical research is likely to be lower if laboratories are using aged co-morbid animals. Finally, lifestyle is the most challenging aspect of clinical stroke to model in rodents. As active social creatures, rodents might not represent the sedentary, solitary life experienced by many stroke patients. It is well known that patients suffering from depression, and those living alone prior to a stroke have a poorer prognosis (Aron *et al.*, 2015). In rodents, those who are socially isolated post-stroke fare worse than their group-housed counterparts (O'Keefe *et al.*, 2014). Whilst there is evidence that environmental

enrichment improves outcomes in rodents after stroke (Buchhold *et al.*, 2007), suggesting that standard conditions may in fact be 'sedentary' in these species, it is difficult to establish what constitutes adequate, 'natural' housing under fundamentally artificial conditions.

Much like the choice of model discussed above, outcome measures will depend on the experimental question. Most researchers will use infarct volume as an outcome measure, irrespective of how useful this is, but studies using MRI and detailed behavioural testing tend to be less common due to increased experimental complexity. Infarct location in patients will affect the specific neurological deficits produced, and thus using tests that cover a range of parameters, including sensorimotor, and learning and memory, are key when approaching preclinical rodent work.

Finally, clinical biomarker studies using specific metabolomic or proteomic panels provide an opportunity to back-translate clinical findings into the rodent population. This can be used to validate particular models of stroke (i.e. do we observe the same phenomena in our model as we do in patients), as well as provide a more direct point of comparison between patients and rodents when an experimental therapy is administered (i.e. does our therapy affect this biomarker, and the underlying processes that it reflects; Fig. 2). If we are to optimize rodent models of stroke, more rigorous thought needs to be applied to both choice of model and to the outcomes measures taken when conducting both mechanistic and therapeutic studies.

STAIR criteria

The updated STAIR preclinical recommendations (Fisher *et al.*, 2009) outline best practice in preclinical studies of novel stroke therapeutics. Now 17 years old, the original STAIR criteria outlined the importance of blinding, replication in more than one species, consideration of sex differences and clinical criteria such as route of administration and therapeutic window. Whilst these guidelines are now more widely accepted and acknowledged than they have ever been, there is still a significant gap between clinical and preclinical work that needs to be bridged. Maintaining consistency, reducing bias and increasing reproducibility are excellent ideas for preclinical science in general, but there is a paucity of funding available for such studies, compared to the rigorous quality control measures used in clinical research. Novel therapeutics discovered in one or two small collaborating laboratories are unlikely to progress to larger (phase II and III equivalent) preclinical randomized controlled trials, unless there is central funding to cover studies on this scale. The reality of preclinical randomized controlled trials is that they are likely to be thwarted at an early stage unless there is a fundamental change in policy priorities, to facilitate large-scale collaboration between preclinical laboratories.

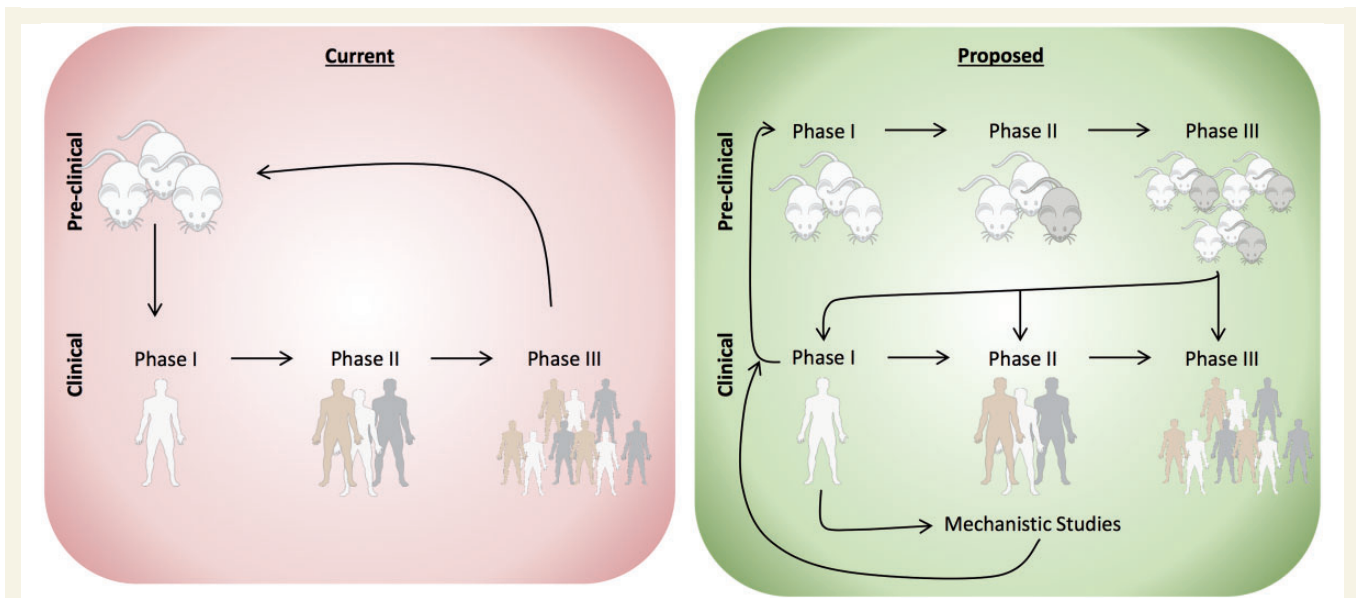


Figure 2 Changing perspectives. Current clinical trials have a very structured progression, coming as they do off the back of preclinical research. By altering the way preclinical trials are carried out, we may be able to feed information regarding mechanism and efficacy into all levels of the clinical trial pipeline. Finally, by studying mechanism in patients in clinical trials, we will be able to back-translate to ongoing preclinical work.

Bridging the gap: mechanism in patients

A corollary to replicating patient characteristics and clinical study design in our preclinical models, where highly homogenous groups of animals are compared, in terms of age, strain, stroke severity and other factors, is that clinical trial methodology should also more closely resemble some of the preclinical approaches. While the ultimate question of interest is whether patients derive a clinically meaningful benefit from the treatment, we should strive to validate the proposed mechanism of action in smaller, proof-of-concept trials. This approach can be contrasted with relying on the same metrics, such as the ubiquitous odds ratio of modified Rankin scale (mRS) 0–2, throughout the clinical trial stage. A notable example of this is EXTEND-IA, one of the five breakthrough EVT trials in 2015 (Campbell and Mitchell, 2015). Designed to recruit 100 patients, it was stopped prematurely due to the announcement of the MR CLEAN trial results. At that stage, only 70 patients had been enrolled. In a traditional neuroprotectant trial, this would have been an outrageously small sample size: differences in baseline lesion size, stroke severity and other variables would have made it impossible to identify an effect. However, EXTEND-IA had the most thorough characterization of its patients' baseline status seen in any major stroke trial to date. CT perfusion imaging was performed in all patients to determine blood flow deficit, and the inclusion criteria focused on the presence of salvageable tissue rather than clinical severity. As a consequence of these design choices, the authors were able to demonstrate

a highly significant improvement in outcome with EVT, despite the small sample size.

Previous generation thrombectomy trials, which failed to demonstrate an effect of the intervention (Broderick *et al.*, 2013; Ciccone *et al.*, 2013), used plain CT and/or clinical severity for patient selection. MR CLEAN—the first positive EVT trial that led to early cessation of recruitment in the other stent-retriever trials ongoing at the time—used a much less elaborate methodology (CT/digital subtraction angiography) for patient selection compared to EXTEND-IA, and the primary outcome was mRS 0–2 at 90 days. In many ways, MR CLEAN is a closer approximation of the tools available to most clinicians, as the advanced imaging modalities used in EXTEND-IA are not yet widely available. There are other magnetic resonance methods such as CEST (chemical exchange saturation transfer) imaging to measure pH that are currently purely experimental, but provide valuable insight into stroke pathophysiology and could serve as useful adjunct measurements and/or selection criteria in a trial context (Harston *et al.*, 2015). In addition, these techniques are often also available to preclinical researchers, and thus back-translation of mechanistic insight becomes much more feasible.

A broader point, therefore, is that clinical and preclinical trials should not necessarily aim to mimic conditions that are currently available, but rather to demonstrate what can be achieved under ideal conditions, which can subsequently be generalized to wider patient populations. Not only do the discoveries these trials provide justify application of the tested intervention, but they also guide us in terms of technical and infrastructural changes needed to achieve appropriate patient selection and ultimately improve the stroke treatment pathway.

Optimizing delivery of stroke therapies

In parallel to more thorough characterization of the patient population, there are improvements to be made in delivering therapies. Most preclinical trials use very early time points for treatment, e.g. upon reperfusion after 90 min of MCAO, as our group (Sutherland and Buchan, 2013) and many others have done. By comparison, clinical stroke trials almost always rely on longer intervals between stroke onset and treatment. For instance, the median time from stroke onset to IVT initiation was ~2 h in the 2015 EVT trials (with considerable heterogeneity between patients); however, the median interval from onset to groin puncture was ~3.5–4.5 h (Badhiwala *et al.*, 2015). Naturally, there are fundamental limits on what we can achieve in a real-world setting, and we should move towards later administration of therapeutics in animal models. However, that is not to suggest that further refinements in the stroke workflow are not available; for example, there are remarkable reports of reducing in-hospital IVT delays to 20–25 min (Schwamm *et al.*, 2009; Meretoja *et al.*, 2013). Of course, earlier recanalization will provide clinical benefit—as per the famous adage, ‘time is brain’—but it will also increase the likelihood of being able to intervene in ongoing pathophysiological cascades using adjunct therapies.

There is also considerable evidence that telemedicine (including both telestroke examination and teleradiology) methods can improve the uptake and speed of IVT without a neurologist present (as reviewed elsewhere; Schwamm *et al.*, 2009). This concept has been extended to pre-hospital evaluation and treatment, with a study demonstrating that CT-equipped ambulances carrying rt-PA significantly reduced the onset-to-IVT interval without increasing complication rates (Ebinger *et al.*, 2014). Pre-hospital treatment is even more feasible with less risky neuroprotective treatments. One notable example of this is magnesium sulphate in the FAST-MAG trial (Saver *et al.*, 2015), where almost three-quarters of patients received the drug within an hour from stroke onset. While the trial did not detect a benefit over placebo, other trials with similar designs are currently ongoing, including FRONTIER for NA-1 (discussed below).

Target discovery in the ‘omics era

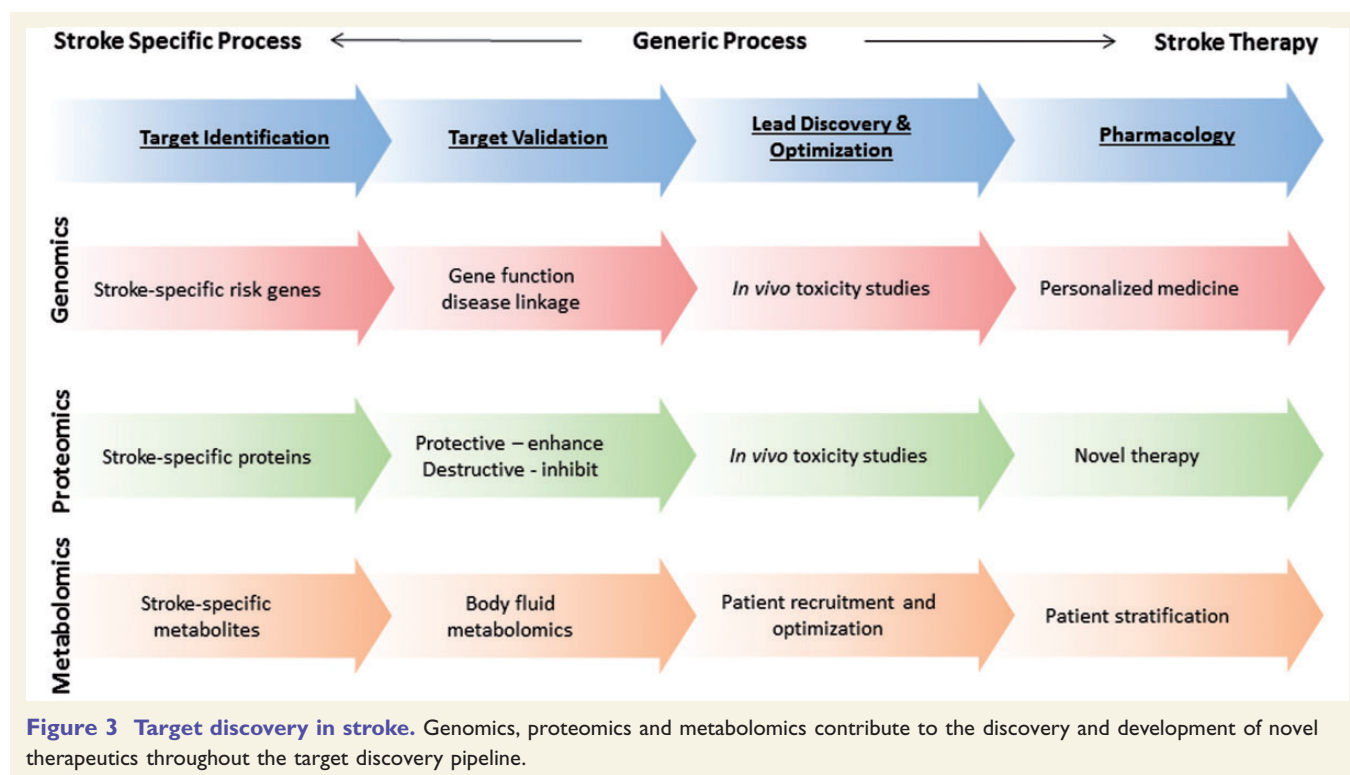
Targeting either clot mechanisms, repair mechanisms or damage mechanisms rapidly and effectively, in either rodent models or in the clinic, requires target discovery and validation. The emergence of ‘omics approaches as a way to pinpoint disease-specific targets has the potential to be the beginning of an exciting new field of target discovery

in stroke research (Fig. 3). These approaches include genomics and next generation sequencing, transcriptomics, proteomics and metabolomics. The utility of these studies is 2-fold: first, they provide us with insight into the basic mechanisms of stroke-related damage and repair processes in humans, but in addition, they provide novel biomarkers that can be adopted in clinical trials as rapid outcome measures for drug efficacy or toxicity.

Genome-wide association studies (reviewed in Black and Wang, 2015) have begun to identify a number of important genetic markers associated with the development of stroke, some of which are single nucleotide polymorphisms also associated with coronary heart disease. This provides us with the possibility of administering early therapeutic and lifestyle interventions in high-risk patients. Of greater diagnostic and prognostic interest are the rapid developments in proteomics and metabolomics. Small sets of metabolites, easily detected in bodily fluids, can be used to divide patients into specific subgroups based on particular pathophysiological changes (Dickens *et al.*, 2015). Indeed, in stroke patients, metabolomics has been used to enhance the sensitivity of imaging techniques (Laskowitz *et al.*, 2009), as well as to predict the likely occurrence of a stroke after an episode of transient ischaemia (Jove *et al.*, 2015). The importance of techniques such as these clinically, is the ease of use at the point of care. Rapid diagnostic tests provide clinicians with the capacity to more accurately stratify patients for treatment. Moreover, the availability of these techniques means that discoveries from clinical trials, such as the panel of markers used by Laskowitz and colleagues (2009), should now be fed back into animal studies to more thoroughly validate current preclinical models. These data would support mechanistic hypotheses generated in animals, and confirm the effects of therapeutics on clinically relevant biomarkers in a preclinical setting.

The metabolome in particular is highly sensitive to minute changes in physiology and in a diverse population, such as that presenting in stroke, variability and signal-to-noise may cause problems. Using the proteome, where changes are likely to be more stable, is a more common way to identify differences in heterogeneous patient groups. Whilst this approach does lack the rapid, on-the-ground diagnostic capabilities that metabolomics has, it may provide an important link between genome changes and protein changes. Studying proteases in patients before and after rt-PA using a proteomic approach has demonstrated significant changes in their activity (Ning *et al.*, 2010). The potential for haemorrhagic transformation after rt-PA is significant and may be the result of these changes. By taking this study back into animals, and determining whether any specific protease is responsible for blood–brain barrier breakdown, we may be able to provide selective inhibitors and expand the use of rt-PA in stroke.

The key to using the vast array of data now available to us, is effective integration from the point of biomarker discovery to mechanistic systems biology. By using a



cross-disciplinary approach to stroke research, it will be possible to bring together data analysis and modelling, with preclinical and clinical data to help better our understanding of the processes that underlie the damage caused by stroke.

Accentuate the negative

An important aspect of experimental design and planning in all preclinical and clinical research, is knowledge of the existing literature. Systematic reviews of the literature provide an excellent starting point; however, reports suggest that only ~10–15% of all published preclinical studies are of negative or null results (Fanelli, 2010). This provides a significant road block to progress in the preclinical stroke field. If the literature contains only a small number of very positive studies, and far fewer negative or null ones, there is no context on which to base validity. This is not a major issue blighting the clinical world, where negative trial data are seen as key to the refinement of therapeutic approaches. Whilst some journals now specifically contain a negative results section for preclinical work (Dirnagl and Lauritzen, 2010), the bias towards positive results, ‘hot’ topics and citation indices mean that researchers are often reluctant to publish their null data. Indeed, one recent review described this phenomenon by saying ‘our love of significance pollutes the literature with many a statistical fairy-tale’ (Horton, 2015). This problem extends beyond

publications, however, and requires coordinated efforts from journals, institutions (Begley *et al.*, 2015) and funding agencies (Collins and Tabak, 2014) to tackle effectively.

It has been well documented in the stroke field that publication bias skews outcomes towards the positive, overstating the efficacy of novel compounds (Sena *et al.*, 2010). Moreover, stroke research seems to be beset by a keener aversion to negative publication than other sciences (Fanelli, 2010), with some analyses estimating up to a third of all stroke research goes unpublished (Sena *et al.*, 2010). As with the inclusion of co-morbid animals to improve validity, the issues here are likely to be time and money. A small, underpowered yet extremely positive study is quick and easy to do, and relatively easy to publish with the correct ‘spin’. A negative study has to be properly powered in order for it to be taken seriously and often negative results are generated simply by researchers testing a hypothesis and finding it not to be true (Buchan *et al.*, 1991). Very few are then inclined to increase the power of this ‘failure’ merely to prove that it is definitively and significantly a failure. This issue will continue to be a problem until those who make decisions concerning policy, both at the journal editorial level, and at the funding level, reconsider the impact of publication bias (Macleod *et al.*, 2015).

In summary, the gap between preclinical and clinical work has severely hampered the development of novel neuroprotective strategies in stroke, and needs to be approached from both ends (Fig. 2).

Novel neuroprotective strategies

In spite of the many shortcomings that have affected the field, there is nevertheless an enormous clinical need that warrants continued preclinical research. The primary aim of stroke therapy is to restore blood flow to the brain in a manner that does not exacerbate the damage already caused by depriving the tissue of oxygen and nutrients. The secondary aim is to modulate any factors that may exacerbate this damage and if possible, repair the damage. The development of novel therapies, targeting both the primary and secondary causes of stroke damage, continues to be a major goal of preclinical research and translational medicine. Whilst there are a wealth of approaches under active investigation, far beyond the scope of any single review, some strategies are particularly promising due to the strength and quality of their existing preclinical and clinical data, as well as their translational promise.

Endovascular therapy

As discussed above, the first large-scale randomized controlled trial to unequivocally demonstrate a benefit with endovascular thrombectomy was the MR CLEAN trial, which found that thrombectomy within 6 h of AIS onset resulted in a significant increase in functional independence without increasing mortality (Rozeman *et al.*, 2016). Similar results have been found in several subsequent trials of stent-retrievers, or aspiration thrombectomy in some cases (reviewed in Balami *et al.*, 2015). The resounding benefit delivered by these trials demonstrates that much like rt-PA, the ‘art of patient selection’ (Balami *et al.*, 2013) is crucial to distinguish patients who stand to gain the most from intervention, and imaging is key in making the most appropriate therapeutic decision (Balami *et al.*, 2015). This has several implications. First, we must continue to develop infrastructure to enhance EVT availability on a 24-h basis. Second, we must perform further randomized controlled trials to investigate EVT in particular subgroups of patients, such as those with vertebrobasilar or distal MCAOs, or with extensive ischaemic changes (ASPECTS < 5), who are under-represented in the currently available data (Wardlaw and Dennis, 2015). Third, we must integrate neuroprotectant delivery with EVT for future trials, to provide the best possible chance of detecting an effect and developing clinically applicable adjunct therapies. This can also be investigated in the context of adverse effects: EVT carries a small but clinically relevant risk of distal embolization and ischaemia in a new territory (Ganesh *et al.*, 2016). Similarly to past trials investigating neuroprotection in iatrogenic stroke during cardiac endarterectomy (see below), this could therefore be a useful avenue for detecting neuroprotective effects.

Excitotoxicity

Even before the clot is removed, during tissue occlusion, death of neurons will be an ongoing process. One key mediator of this process is neuronal depolarization as a consequence of ATP depletion and failure to maintain membrane potentials. The resulting release of glutamate overwhelms the glial and neuronal mechanisms that remove glutamate from the synapse, and leads to excessive activation of ionotropic (AMPA, kainate, and NMDA) glutamatergic receptors. This causes uncontrolled calcium entry and disrupts a vast variety of physiological processes, ultimately resulting in DNA, lipid and protein damage (Sutherland *et al.*, 2012). In addition to this, depolarized cells will also release their neurotransmitter stores, leading to propagating waves of activity followed by electrical silencing (peri-infarct depolarization), which can exacerbate the severity of ischaemic damage (Dreier, 2011). Although excitotoxicity has been targeted in a variety of clinical trials—most notably using NMDA antagonists (Hoyte *et al.*, 2004), all of which failed to demonstrate benefit in patients—advances in our understanding of excitotoxicity have yielded more specific targets to pursue, rather than outright antagonism of all NMDA receptors.

One example is NA-1 (Tat-NR2B9c), a peptide that disrupts interactions between NMDA receptor subunits, post-synaptic density (PSD)-95, and neuronal nitric oxide synthase (nNOS). This uncoupling of nNOS from glutamate receptor activation decreases noxious nitric oxide (NO) production during excitotoxicity, rather than targeting either nNOS or its downstream effects directly. Cook *et al.* (2012) demonstrated that Tat-NR2B9c is protective in three non-human primate models of ischaemic stroke, an important step in the translational pathway. A phase II clinical trial of NA-1, administered to patients undergoing endovascular aneurysm repair (a procedure carrying a substantial risk of developing small ischaemic lesions), showed some evidence of protective properties (Hill *et al.*, 2012), and a larger trial using pre-hospital administration of NA-1 in stroke is currently recruiting (FRONTIER; registration NCT02315443).

Alternative approaches to excitotoxicity include directly targeting the downstream pathways associated with NR2B subunit activation. Coupling of NR2B with DAPK1, a death-associated kinase, during excitotoxicity results in apoptosis. Inhibition of this coupling either via direct interference, using compounds such as NR2B_{CT} (Tu *et al.*, 2010), or by interfering with downstream pathways such as p53 (Pei *et al.*, 2014), can reduce infarct size and improve neurological outcomes in animal models. Furthermore, targeting of the downstream molecular pathways associated with excitotoxicity is likely to result in a reduction in the side effects associated with direct targeting glutamatergic signalling: one key issue with NMDA antagonists was a high incidence of psychotic episodes and other neurological or psychiatric complications (Hoyte *et al.*, 2004).

Oxidative stress

A key step in the ischaemic cascade is the generation of free radicals, including reactive oxygen and nitrogen species (ROS/RNS) (Manzanero *et al.*, 2013). There is robust pre-clinical evidence to suggest that preventing generation of ROS/RNS or improving the antioxidant defences of the brain ameliorates damage in ischaemia-reperfusion; however, these results have not yet translated into clinical practice. Most notably, the nitron free radical scavenger NXY-059 failed in a high-profile clinical trial (Shuaib *et al.*, 2007), despite having fulfilled nearly all of the STAIR criteria for preclinical research quality. Similarly, uric acid failed to demonstrate an overall benefit, despite some evidence of efficacy in female patients (Chamorro *et al.*, 2014). Edaravone, another scavenger compound, is clinically used in parts of Asia and meta-analyses of trials suggest it is efficacious in reducing neurological impairment; however, it is not used in Europe or America, and there is a dearth of high-quality clinical evidence from large phase III trials in non-Asian populations (Feng *et al.*, 2011).

As stated above, a key mechanism through which NA-1 exerts a protective effect is NO production during ischaemia, resulting in nitrosative stress (Hill *et al.*, 2012). This is especially relevant to recanalization therapies because of the amount of ROS/RNS production associated with reperfusion (Manzanero *et al.*, 2013). Indeed, it has been suggested that the discrepancy between preclinical results and clinical trial findings may be accounted for by the speed of recanalization, which is extremely rapid in mechanical MCAO models, but much slower in thrombolysis, resulting in different pathophysiological effects (Hossmann, 2012). However, the advent of EVT, with its more rapid recanalization, has made findings from mechanical MCAO models much more clinically relevant (Chamorro *et al.*, 2016). As a result, much like with anti-excitotoxic compounds, it is worth reinvestigating compounds that had remarkable preclinical effects following transient mechanical occlusion, but yielded equivocal results in clinical trials, such as NXY-059 or uric acid (Chamorro *et al.*, 2016).

Neurorepair

An alternative approach to neuroprotection, and the prevention of secondary damage, is neurorepair. This has both the advantage and disadvantage of being used when damage has already occurred: we are not bound by short time windows, but key structures have already been lost and need to be repaired, either exogenously or through enhancement of endogenous processes. Cellular and pharmacological strategies have been used in animal models to stimulate neurogenesis, or regrowth and repair. There has been improved functional recovery and decreased infarct volume when treatments are administered 24 h or more following experimental stroke, considerably extending the window of current available therapies (Chopp *et al.*,

2009; Zhang and Chopp, 2009). There have now been phase I trials of allogeneic neural stem cells in patients, suggesting that these interventions are safe and potentially beneficial (Kalladka *et al.*, 2016). However, it is important to note that some aspects of safety are very difficult to assess in rodent models; for example, human stem cells are much less tumorigenic in mice than mouse stem cells, creating potential allograft versus xenograft concerns that cannot easily be addressed in the preclinical setting (Erdo *et al.*, 2003).

In addition to stem cell therapies, aimed at exogenously inducing repair through integration of cells into the CNS, or through donor cell-derived neurotrophic factors, one could 'remove the brake' by inactivating inhibitors of neuronal growth. Targets here have included myelin-associated glycoprotein, the Nogo family of proteins, and chondroitin sulphate proteoglycans in the glial scar; however, despite small-scale clinical trials, the clinical benefit of those therapies remains unproven (Cramer *et al.*, 2013). Again, more effective preclinical trials, in combination with mechanistic studies from the small-scale clinical trials may yield more information regarding the efficacy of this route of investigation.

Thinking outside the neuron

Whilst traditional preclinical stroke research has aimed target discovery and therapy design at preventing direct neuronal death or effecting neuronal repair, a considerable amount of the damage caused to CNS tissue post-stroke is via secondary injury, through microglial activation, capillary pericyte constriction, and numerous other mechanisms. Therapies directed towards non-neuronal cell populations aim to abrogate indirect neuronal degeneration, as well as enhance endogenous repair mechanisms. During ischaemic stroke there is significant CNS inflammation, blood-brain barrier disruption and infiltration of systemic immune cells, suggesting that targeting one or more of these processes may slow infarct development.

Targeting other cell types

The activation of the CNS immune system post-stroke has often been described as a double-edged sword (Patel *et al.*, 2013), with microglia actively removing dead cells and debris but also producing toxic substances that potentially exacerbate damage. This is largely due to the combination of factors that activate microglia during ischaemia. Neurons provide an 'off' signal, in the form of various ligand-receptor interactions (Biber *et al.*, 2007), which is switched off during ischaemic death, but microglia also react to damage-associated molecular patterns (DAMPs) (David, 2015), which switch them on during ischaemia. Here, there is a fundamental difference between the cellular processes that remove the brakes and those that apply the accelerator, and thus the therapeutic approaches to

specifically targeting microglial activation should take this into account. In practice, this means that microglia can produce pro-inflammatory cytokines (Gregersen *et al.*, 2000) and ROS/RNS, as well as anti-inflammatory cytokines (Parada *et al.*, 2013). Therapeutic targeting of the microglial response to ischaemia could therefore take any number of avenues; directly preventing ‘activation’ or reducing the pro-inflammatory response, or augmenting the anti-inflammatory response. Reduction of toll-like receptor signalling, one of the primary mechanisms by which DAMPs activate microglia, is known to reduce lesion volumes (Stevens *et al.*, 2008; Hua *et al.*, 2015). Pro-inflammatory cytokines are contentious, with TNF α being shown to be neuroprotective (Lambertsen *et al.*, 2009) and IL1 being shown to be deleterious (McColl *et al.*, 2007), and enhancing the anti-inflammatory activity of microglia is challenging, studies using IL10 (de Bilbao *et al.*, 2009) have shown some effect but results have tended to be expressed as lesion volumes and this may not necessarily be a useful outcome post-ischaemia.

Microglia represent only one aspect of reactive gliosis post-ischaemia. The glial scar, which forms after a stroke, is largely composed of reactive astrocytes (Stichel and Muller, 1998) and forms a dense barrier to neuronal outgrowth. Astrocytes are also capable of producing significant quantities of cytokines, regulating ion homeostasis and blood flow (Sofroniew and Vinters, 2010) and thus dysregulation of normal astrocyte physiology is likely to cause problems post-stroke. Much like microglia, this double-edged sword aspect of astrocyte physiology means that targeting astrocytes in the post-ischaemic period is challenging. For example, the astrocyte syncytium provides a network of communication channels throughout the brain. Following stroke, the gap junctions within this network remain open, and are liable to propagate injury signals (Lin *et al.*, 1998), but if these junctions are removed, the outcome is worsened (Nakase *et al.*, 2004). The diversity of astrocytic functions means that few studies have actively targeted this cell type post-stroke (Barreto *et al.*, 2011). In theory, targeting could be approached in a similar manner to that used to target microglia, i.e. enhancing the positive aspects of astrocyte physiology and reducing the detrimental ones.

An improvement in functional recovery has been shown using a non-selective inhibitor of cell proliferation (Wang *et al.*, 2008), in theory via the inhibition of reactive gliosis. However, the improvement in functional outcomes could also be via the reduction of proliferating systemic immune cells. Both neutrophilia (Buck *et al.*, 2008) and monocytosis (Kaito *et al.*, 2013) are known to occur after CNS injury and the ramifications of immune system activation are numerous. This is particularly relevant in stroke, where the risk of ischaemia-reperfusion injury after clot removal is high. Infiltration of systemic immune cells into the CNS can exacerbate ongoing damage (Jin *et al.*, 2010), but can also enhance the anti-inflammatory microglial population (Kim *et al.*, 2014). Increased systemic activity can protect

against pathogens, but stroke patients are often immunocompromised (Brambilla *et al.*, 2013). Thus, broad spectrum anti-inflammatory drugs are likely to be unhelpful at best. Much like with microglia and astrocytes, determining the mechanisms of activation, and the ways in which activation causes damage in patients, is essential in the development of novel therapeutics.

Targeting cerebral blood flow

Current stroke therapies are targeted at recanalization of the occluded vessel(s), and it is clear that radiologically defined recanalization improves outcomes in stroke (Rha and Saver, 2007). However, from a physiological viewpoint, the relevant change is reperfusion of the microvasculature, where nutrient exchange takes place. The two are not necessarily correlated: for example, collateral flow can result in tissue reperfusion without recanalization (Soares *et al.*, 2009). Conversely, there is an extensive body of literature outlining situations where microvascular flow is not restored following recanalization, termed the ‘no-reflow phenomenon’ (Ames *et al.*, 1968; del Zoppo and Mabuchi, 2003). Not only does this adversely impact the parenchyma supplied by the non-reperfused vessels, but an increase in capillary perfusion heterogeneity can reduce overall oxygen extraction from the microvascular network (Ostergaard *et al.*, 2013). *In extremis*, this can lead to a situation where increases in cerebral blood flow paradoxically reduce tissue oxygenation (Ostergaard *et al.*, 2013). While the no-reflow hypothesis is primarily based on preclinical studies, there is also clear evidence from clinical trials that angiographic and MRI measures of recanalization/reperfusion show only limited agreement (Khatri *et al.*, 2005), and that reperfusion has a stronger association with good outcome than recanalization does (Soares *et al.*, 2009, 2010; Eilaghi *et al.*, 2013). Even with the current gold standard methodology for EVT, only 71% of patients achieved reperfusion to 50% or more of the affected region (Goyal *et al.*, 2016).

The mechanisms underlying no-reflow, and related phenomena such as post-ischaemic hypoperfusion (Hossmann, 1997), are not well understood, with a number of theories being postulated. These include fibrin accumulation (Okada *et al.*, 1994), leucocyte adhesion (del Zoppo *et al.*, 1991), astrocyte swelling and oedema (Ito *et al.*, 2011) and microvascular pericyte constriction (Yemisci *et al.*, 2009; Hall *et al.*, 2014). Reducing no-reflow represents an exciting opportunity to further enhance the clinical benefits seen with EVT, but provides a mechanistic challenge. Indeed, while there have been numerous trials of drugs targeting some of the mechanisms that could account for no-reflow—including inhibitors of platelet activation (Adams *et al.*, 2008), antioxidants (Shuaib *et al.*, 2007) and Ca²⁺ antagonists (Zhang *et al.*, 2012)—these have mostly been conducted without concomitant recanalization. Therefore, any effect on the microvasculature may be masked by the larger effect of non-recanalization.

The increased perfusion and associated functional benefits associated with EVT over IVT have the potential to be enhanced by a number of strategies targeting both flow and neuronal death, which have proved unsuccessful in IVT trials. Advanced imaging studies deliver clear-cut answers on whether we can target no-reflow directly, or whether any putative benefit is derived through other mechanisms. Thus, smaller, more detailed trials are capable of providing proof-of-concept in man, based on endpoints that use physiological measurements or imaging, making a much stronger case to proceed into large phase III clinical trials studying functional outcomes. This would revolutionize the current approach, where progression from phase II to phase III is purely an exercise in increasing sample size rather than determining whether the proposed mechanism of action, based on preclinical evidence, holds true in patients.

Conclusion

It is clear that the field of stroke research is blighted by technical and experimental design issues, which can be corrected at the level of the researcher (inclusion of comorbidities, mixed cohorts, data sharing, etc.), but there are also considerable policy issues regarding the publication of negative data and the funding of preclinical stroke research. However, the revolutionary change brought about by EVT has nevertheless injected new hope into the field of stroke, and created a plethora of new research objectives for adjunct treatments, combining EVT, rt-PA and neuroprotective drugs. This has clear synergy with ongoing research in other fields, most notably cardiology, where similar approaches are being actively pursued (Gerczuk and Kloner, 2012). As a field, it is important that we strive to overcome the aforementioned barriers and take advantage of technological and clinical progress, in order to realise the full potential of bench to bedside research and make preclinical stroke research truly translatable.

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Conflict of interest

AMB is a senior medical science advisor and co-founder of Brainomix, a company that develops electronic ASPECTS (e-ASPECTS).

All other authors declare no conflict of interest.

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