LESSONS LEARNED REPORT

TITLE	**Crash 3 Mechanistic Study**
SHORT TITLE	Crash-3 MS
SPONSOR	University of Birmingham
SPONSOR REFERENCE NO	RG_15-194 (University of Birmingham)
HOST ORGANISATION	University Hospitals Birmingham, Birmingham B15 2TH
TRIAL REGISTRATION	IRAS 186446 - NIHR EME 14/205/01
CHIEF INVESTIGATOR	Prof Antonio Belli
CO-APPLICANTS	Prof Peter Hutchinson (Cambridge University), Mr Adel Helmy (Cambridge University), Prof Mark Wilson, Prof David Sharp, Prof David Menon, Prof Ian Roberts
DESIGN	Embedded observational study within a CTIMP study; Observational investigation into mechanistic aspects of any potential effect of tranexamic acid (TXA), within the context of an investigator, practitioner and participant-blinded placebo controlled trial (CRASH-3)
LOCAL R+D REFERENCE	RRK5587
PRODUCT / DOSE / DURATION	Intervention as per CRASH-3 (see protocol appendix 1)
RECRUITMENT TARGET	40 (revised target from initial 60)
START DATE	February 2016
PROGRAM MANAGER	Dr Komal Gupta
CLOSEDOWN DATE	On-going

Contribution summary

This report was drafted and compiled by Prof A Belli (Chief investigator of the CRASH-3 MS study), with contributions from Mr David Davies (Clinical lecturer in neurosurgery, University of Birmingham and investigator within the CRASH-3 MS team), and Dr Kamal Yakoub (Clinical research fellow University Hospitals Birmingham and investigator within the CRASH-3 MS team).

Nov 2018 – University of Birmingham

Disclaimer

Executive Summary

The CRASH 3 MS investigation was intended as an embedded observational study within the CRASH 3 randomised control study into the effects of TXA on patients who has sustained a traumatic brain injury with haemorrhage (blood) positive CT head imaging. TXA is an established pro-thrombotic agent used in many diseases involving prolonged and harmful blood loss and has demonstrated significant benefit within the context of poly-trauma (not head injury specific) in the CRASH 2 study[1]. Primarily, it was an opportunity to study the potential effects of TXA beyond the intuitive prevention of further bleeding. This would be achieved by the measurement of inflammatory mediator profiles and other biomarkers within both the circulating blood and directly from the brain tissue (extra cellular compartment) via the placement of invasive cerebral micro-dialysis catheters. Fundamentally it was an ideal and possibly unique opportunity for us to undertake a study within the framework of a randomised investigation into the use of TXA, as the outcome of such an investigation may remove the equipoise surrounding the use of such an agent and make it ethically difficult to reproduce such an investigation.

This would be a multi-centre collaboration involving the key specialist centres throughout the United Kingdom. The University Hospital Birmingham was (at that time) the highest recruiting UK centre to the CRASH 3 initiative (and the third highest worldwide) and therefore an ideal candidate for the leading centre into this embedded study. At the time of initial application, the recruitment rate for CRASH-3 at the University Hospital Birmingham (Queen Elizabeth) was approximately 9 patients per month. Three additional sites participating in the mechanistic study (Addenbrooke's, St. Mary's and University Hospital Southampton), were recruiting between 5 and 7 patients per months altogether. Suitable patients were identified on enrolment to the CRASH 3 investigation, and beyond the recruitment criteria for CRASH 3, there was the requirement for invasive monitoring (intra cranial pressure monitoring) within the critical care setting. Based on an audit, we estimated that 65% of CRASH 3 patients would be eligible for the mechanistic study.

At the time of designing the mechanistic study, patients could be recruited into CRASH 3 within 8 hours of injury. However, in November 2015, this was changed to 3 hours, as an analysis of the recruitment pattern showed that too few participants were receiving the IMP early. This profoundly affected the recruitment rate into CRASH-3 and consequently the mechanistic study at the participating centres. This made the original projection of 10-11 eligible patients per month unachievable. After the change in recruitment window, the average accrual rate of patients for CRASH-3 fell to approximately 1 patient per month (a reduction of approximately 80-90%). With limited resource and without specifically appointed clinical fellow (a condition of the original funding), the identification and recruitment of these now significantly rarer patients became

challenging. Unfortunately, despite significant efforts made by members of the team, the trial failed to recruit sufficient patients.

Delays in the commencement of the study due to specific requirements from the funding body were certainly disadvantageous (outlined in background), together with difficulty in establishing multi modal and enhanced monitoring modalities throughout our collaborative partners (again outlined below). However, even without this, the significantly reduced number of patients available for recruitment would have prevented adequate accrual.

The incorporation of a research fellow specifically into the grant framework may have allowed more rapid rollout of advanced monitoring modalities into the partnering centres. As it was, specific training involving study specific hardware was undertaken remotely or by nursing staff who had no experience in invasive neurological monitoring. However, it is unlikely that even immediate rollout of the trial specific technology, together with dedicated medically qualified and neurosurgically experienced members of staff is unlikely to have mitigated for the dramatic reduction in recruitment seen after November 2015.

Introduction

The Crash-3 MS study was intended as an embedded, observational cohort investigation into the theoretical (hypothesised) benefits of Tranexamic Acid (TXA) within the framework of the established CRASH-3 randomised placebo-controlled trial into the potential benefits of TXA in haemorrhagic traumatic brain jury vs placebo. The original application due to the formation of a multi-centre specialist collaboration representing the countries most experienced experts in traumatic brain injury tissue metabolomics (table. 1). The study was founded on the strong published track record within the co-applicant body particularly within the field of brain tissue microdialysis, along with the University Hospital Birmingham's (as then) positon as the leading UK recruitment centre for the CRASH-3 study.

Table 1. Clinical sites

Additional site (Institute)	Principle investigator
University Hospitals Birmingham, Queen Elizabeth Hospital	Prof Antonio Belli
University Hospital Cambridge, Addenbrooke's Hospital	Prof Peter Hutchinson
St. Mary's Hospital London	Prof Mark Wilson
University Hospital Southampton	Mr Diederik Bulters

The concept underlying the CRASH 3 investigation is that the administration of an established pro-coagulant (TXA, loading dose 1 g over 10 minutes then infusion of 1 g over 8 hours or matching placebo), within the context of a traumatic brain injury with haemorrhagic findings (blood) on CT imaging of the head could be beneficial to the clinical outcome of those patients. The CRASH 2 investigation[1] (a study that demonstrated a beneficial effect of administering TXA in cases of general extra-cranial trauma) had demonstrated a potential benefit in subgroup analysis indicating that this could potentially be the case. Intuitively it was primarily considered that the main mechanism of beneficial action ion these cases would be in the prevention or reduction of any further bleeding.

However, other potential mechanisms of effect were hypothesised including a modulatory effect on the neuro-inflammatory cascade (see appendix). The investigation of this proposed effect by the contemporaneous collection of both brain tissue micro-dialysate and blood forms the basis of the CRASH-3 Mechanistic Study.

Recruitment

Shortly prior to the commencement of the trial (before recruitment of patients began but after the initial trial design had been established) a change in the maximum recruitment time window for the CRASH 3 investigation was introduced. Initially the trial stipulated that the trial treatment drug should be given within an 8-hour window from the time of injury. However, from November 2015 this window was reduced to 3 hours. This significantly reduced recruitment rates due at the University Hospital Birmingham and at other centres throughout the UK due to the practicalities of identifying and recruiting target patients within that time. Average recruitment at the University Hospital Birmingham in the year leading up to November 2015 were approximately 9 per month. However, this fell to approximately 1 per month after this (as a mean, on some months no patients were recruited). One also has to consider that these are accrual numbers representative of the UK's lead recruitment centre. The number of patients recruited in other participating centres was significantly lower still. In addition to this, only a small proportion of these patients would require invasive neurological monitoring within the context of critical care. Therefore, within the participating centres only a small number of patients were eligible for CRASH-3 MS. Other sites were added to the study, namely the Royal London Hospital (RLH), Derriford Hospital, University Hospital of Coventry and Warwickshire and the John Radcliffe Infirmary. RLH contributed one patient but none could be recruited at the other centres due to the end of funding.

CRASH 3 was extended to the end of January 2019. On this basis, we requested a no cost extension to continue recruiting into the mechanistic study but this was denied. Funding for CRASH-3 MS therefore stopped at the end of January 2018. Recruitment is still on-going, using very limited available resources for consumables from internal reserves but, due to lack of funding, this has only been possible on a very small ad hoc basis and only at the Queen Elizabeth Hospital Birmingham. As of December 2018, within the collaboration, 14 patients were recruited, and all but one from the lead centre. Proportionally the number of patients recruited was actually higher than we would have expected if the proportional reduction in accrual was applied to our initial recruitment target. A reduction in accrual numbers from approximately 8-9 a month to approximately 1 a month as a scaled reduction applied to the original recruitment target would lead to a total recruitment population of 7-8 within the original time frame. However, there has been an extension to the CRASH-3 study beyond the original finish date as considered in the original proposal. The extension granted to the CRASH-3 collaborative from 31st December 2016 to Jan 2019 effectively doubled the recruitment window for the CRASH-3 MS; however, this would then translate to a figure of approximately 14-16 individuals. This still represents a considerable reduction from the original target of 40. It may serve however to illustrate how the recruitment of only 14 patients may be explained. It is difficult to say whether with an extension of funding and the mitigating strategies put in place, the recruitment target of 40 could have been achieved.

Commencement

Initial delays to the commencement of the trial were related to specific requirements of the trial funders. These chiefly concerned issues regarding the inclusion of a research fellow onto the grant, along with other funder/board specific requirements for staff and equipment. This was certainly not the central reason for the inadequate levels of recruitment seen in this investigation. However, it did

slow down the rolling out of the study to the clinical sites. This was a particular problem for this study due its embedded design, as the mechanistic study could not be extended beyond the end of CRASH 3 and time was of the essence.

Collaboration in specialist areas

The establishment of uniform advanced invasive monitoring techniques (uniform hardware required in order for comparable results) at centres other than the lead centre would have been dramatically aided by the addition of a medically qualified research fellow onto the staff of the study, as opposed to the research nurses and administrative assistants that were incorporated into the initial grant application. The funding body and board members specified that the funding of a research fellow was not permissible and plans to mitigate this were made by funding research nurses at clinical sites. However, due to the highly specialised and specific nature of brain tissue monitoring by micro-dialysis, together with the maintenance of the supporting equipment, no research nurse is qualified in the application or insertion of these devices. Therefore, distribution and training were delayed in the rollout of the study technology. Also, a research fellow would have been able to oversee the initial recruitment of the first patient at all clinical sites, taking pressure off clinical staff to maintain these monitoring devices and potentially significantly increased recruitment levels. These concerns were specifically discussed in the application process and a case for a medically qualified research fellow was made by the application collaborative. However, it was felt that at that time it would not have represented an effective use of funds by the review board.

Specific equipment issues

In order to minimise non-specific binding of cytokines and other large molecules with the tubing/conduit matrix of the micro-dialysis hardware, previous research by the original applicants/collaborative group advocate the use of a colloid (rather that crystalloid as standard) dialysis fluid solution. This highly technical point may seem extraneous; however, it did lead to a certain component of the delay in starting the investigation. Reducing the non-specific binding within the dialysis device/apparatus reduces the level of noise that one sees in the output data. Thereby reducing the number of patients required in the cohort. Original power calculations were made taking this into account. However, the only available pharmacy that had previously supplied this dialysis fluid had now ceased trading and no replacement product could be sources or replaced from anywhere. It was therefore decided that although a potentially more noise-prone recovery process, that the investigation would use the crystalloid dialysis fluid.

Late addition sites

In an effort to mitigate the reduction in accrual a number of additional recruitment centres were brought on board with the study. Unfortunately, these centres invariably did not have experience with the advanced monitoring modalities. Therefore, there were significant time and resource limitations encountered whilst attempting to introduce these techniques. Universally, a process of individual trust approval had to be undertaken for the specific equipment, together with a rollout of training to both medical and non-medical staff. This was undertaken ad-hoc by member of the CRASH-3 MS collaboration. Unfortunately, this did not lead to any substantial additional recruitment.

Positive contributions and outputs

The failure of the CRASH-3 MS study to recruit sufficient patients was a great disappointment to all involved. A number of members of staff had worked extremely hard to establish and maintain

effective recruitment. Along with providing a great deal of medical support, frequently out of hours and during holidays to ensure multi modal monitoring devices were inserted and maintained. Frequently clinical nursing staff would be unfamiliar with the equipment and round the clock support was required to maintain an effective stream of data together with patient safety. The failure of this study has catalysed an effort to strengthen the trial design process at the University of Birmingham. Together with being learning opportunity, to highlight the requirements of any future similar investigation to avoid failure the CRASH-3 MS study did produce a number of positive future output.

The involvement of and discussion with the Patient Participation Initiative (PPI) at the University Hospital Birmingham increased their knowledge and understanding of the principles and reasoning behind advanced neurological monitoring. The added insight gained by the PPI together with the recruitment of a number of patient relatives from related concurrent studies on to the initiative has strengthened the PPIs understanding of the subject. This in turn has allowed for more considered and informed guidance and council from the group.

The education of non-medical members of research and clinical staff in the application and principles of neurological monitoring was dramatically advanced by the introduction of the CRASH-3 MS study. Members of the research nursing staff in particular who had no specific knowledge or experience with intra-cranial pressure monitoring or micro-dialysis had the opportunity to learn about the techniques and set up and maintain a great deal of the supporting hardware and software. This has been of particular benefit in planning future similar investigations.

After the eventual expected end to recruitment of the CRASH-3 investigation in January 2019 an analysis of the existing patients' data will be undertaken. The results from this will be reviewed and disseminated.

Key Lessons learned

- **The key lesson that we have observed here is to carefully consider the timeframe and accrual rate in which any future similar (embedded) trials are undertaken. Also, a rapid proactive reaction to any change in recruitment criteria must be undertaken to mitigate and compensate for any effect it may have.**

- Trails which rely on complex monitoring modalities that can only be undertaken by medically qualified staff should incorporate a dedicated medically qualified and adequately experienced research fellow from conception.

- Prior to finalisation and protocol development, ensuring the availability of all potential hardware reagents and consumables should be undertaken at all sites. This will prevent any delay in rollout/initiation of the study after approval for commencement has been granted.

- Early training of non-medical staff in the maintenance and rollout of complex trial equipment is helpful and should be undertaken wherever possible

- When there is the foreseeable potential for a shortfall in recruitment, the addition of other recruitment centres should be undertaken as early as possible in the process together with accompanying training.

References

1) Crash-2 Collaborators, 2011. The importance of early treatment with tranexamic acid in bleeding trauma patients: an exploratory analysis of the CRASH-2 randomised controlled trial. The Lancet, 377(9771), pp.1096-1101.
2) Helmy A, De Simoni MG, Guilfoyle MR, Carpenter KL, Hutchinson PJ. Cytokines and innate inflammation in the pathogenesis of human traumatic brain injury. Prog Neurobiol. 2011 Nov;95(3):352-72

Appendix 1. Protocol

Project title

Mechanism of action of tranexamic acid in acute traumatic brain injury

Background

Brief description of main clinical study that this proposal relates to

The proposed project is an observational, mechanistic study of patients randomised into the CRASH-3 trial.

The CRASH-3 trial is a randomised, double blind, placebo-controlled trial. Trial participants receive the best available emergency care for traumatic brain injury (TBI) but in addition are randomised to tranexamic acid (TXA) or placebo. TBI patients are at high risk of intracranial bleeding and this can cause death or disability. Based on the available evidence from trials of TXA in TBI, there is a reasonable possibility that TXA will reduce intracranial bleeding, thus reducing death and disability. The objective of the CRASH-3 trial is to quantify the effectiveness and safety of a short course (8h) of TXA in adults with TBI. This multicentre, randomised controlled trial will quantify the effects of early administration (within 8h of injury) of TXA on death, disability and vascular occlusive events in TBI patients. TXA reduces surgical bleeding and reduces mortality in extra-cranial bleeding, but it also has anti-inflammatory and potentially neuroprotective properties. TBI is a major cause of death and disability worldwide but especially in Africa and Asia. Intracranial bleeding is common after TBI and is associated with increased mortality and disability. Our two pilot studies valuated the effect of TXA on intracranial bleeding after TBI and provide a strong basis to expect that TXA could improve patient outcome after TBI. The CRASH-3 trial will recruit 10,000 TBI patients from hospitals in low, middle and high-income countries. As of this month, more than 3,000 patients have been recruited in centres worldwide, of which 40 are based in the UK.

CRASH-3 is funded by NIHR and MRC, and it is important to point out that the trial Data Monitoring Committee (DMC) specifically requested a mechanistic study into the action of TXA.

Existing research

The injured brain elicits a vigorous inflammatory response characterised by activation of glial cells, microglia and astrocytes and leucocyte infiltration. There are increases in pro-inflammatory cytokines (IL-1, Tumour Necrosis Factor and IL-6) and chemokines that promote the accumulation of immune cells in the injured brain (1). Cytokines are signalling molecules produced by several immune system cells as well as by brain cells, including microglia, astrocytes and neurons. They are pivotal mediators in several central nervous system (CNS) pathologies. There are several characteristics that are common to most cytokines: they act in cascades, they are endowed with pleiotropic actions and they function synergistically. An additional emerging concept is that cytokines, besides being involved in virtually all CNS conditions, may also have physiological, neuromodulatory and restorative functions. It is now generally accepted that when their concentration exceeds certain levels, they contribute to tissue damage and neurodegeneration (2). Thus cytokines can be roughly divided into pro- and anti-inflammatory; however, for most of them these opposing roles seem to coexist and toxic or trophic actions can be observed depending on the exact context. Chemokines are a diverse group of heparin-binding proteins with molecular weights in the range of 8–12 kDa, which are produced by a range of inflammatory cells and act to recruit and attract leucocytes. Together with cytokines and chemokines, a range of other mediators have been implicated in the inflammatory response to TBI, including Matrix-Metalloproteinases (MMPs) (3). MMPs interact with cytokines and chemokines (CCKs) to

modulate the inflammatory response. In the CNS, MMPs have been shown to have roles in neurogenesis, myelinogenesis, axonal growth and guidance, synaptic plasticity, memory and learning, but also blood-brain barrier (BBB) disruption, demyelination, cytotoxicity and oxidative stress. The inflammatory response after TBI, therefore, involves a complex interplay of mediators and a fine balance of pro-inflammatory and anti-inflammatory processes. Not only is the 'double-edged sword' of the neuroinflammatory response a key mechanism in determining tissue fate immediately after injury, but it is also responsible for modulating the long-terms effects of TBI. TBI is in fact to be regarded as a dynamic process of changing disability over time, with chronic neuroinflammation being detectable years after the injury and potentially being linked to progressive neurodegeneration, epilepsy and neuropsychiatric sequelae (4). Modulating this balance in favour of reparative processes is regarded as a potential therapeutic target of neuroprotective drugs (5, 6).
Systematic reviews of the literature are enclosed as Appendix 2.

Risks and benefits

In addition to CRASH 3 trial procedures, patients enrolled in this mechanistic sub-study will undergo invasive neuromonitoring. This is part of routine clinical care in all four participating centres and so this sub-study involves no additional burden or risk to patients. As discussed in the study design section, the planned recruitment is realistic. As this study will analyse multiple CCKs and MMPs that have complex interactions, there is a risk that the effects of TXA will be hard to interpret. To mitigate this, we will utilise a Principal Component Analysis as a strong statistical method to analyse a relatively large number of variables in a relatively small sample of patients, as well as more traditional statistical methods to illustrate the mechanistic action of TXA.
The benefits of the new knowledge generated by the mechanistic sub-study would be two-fold: 1) if the anti-neuroinflammatory effects of TXA were confirmed, the indications for TXA or at least further clinical trials would be extended to non-haemorrhagic TBI. This represents the vast majority of TBI presentations but is currently virtually excluded from CRASH 3. It is important to point out that neuro-inflammation has been shown to be responsible for acute and long-term sequelae in all grades of severity of TBI, including mild and moderate non-haemorragic cases. 2) Neuro-inflammation is implicated in a large number of acute and chronic neuropathologies, from epilepsy to Alzheimer's disease. The mechanistic insights provided by this study could therefore potentially benefit research in the wider neurosciences arena.
An example of these benefits is indirectly provided by CRASH-2, the precursor of CRASH-3. The CRASH-2 trial showed that TXA reduces mortality in patients with extra-cranial bleeding. Giving TXA within 8 hours of injury reduces death from bleeding (RR=0.85, 95% CI 0.76–0.96; p=0.008), and all-cause mortality (RR=0.91, 95% CI 0.85–0.97; p=0.0035), without increasing vascular occlusive events. If given sooner, the reduction is even larger. Giving TXA to patients with extra-cranial bleeding is highly cost-effective in low and middle-income countries. Indeed, the demonstration that an antiplasmin (TXA) reduces mortality from extra-cranial haemorrhage has already stimulated a plethora of studies (reverse translation) aimed at understanding the role of plasmin both as a mediator of both fibrinolysis and inflammation. Studies in cardiovascular surgery provide an excellent example of this (7, 8).

Rationale for the proposed study

The CRASH-3 trial hypothesis is that TXA reduces death and disability by inhibiting fibrinolysis, thereby reducing intracranial bleeding. TXA crosses the BBB and its action is to block the lysine binding sites on plasminogen, thus inhibiting its binding to fibrin and its consequent break-down into fibrin degradation products. The cross-talk between coagulation and inflammation is well described (9) (http://dx.doi.org/10.5772/55998), as is the interaction between plasmin and MMPs (10, 11). CCKs and MMPs operate both up- and down-stream of the plasminogen-plasmin axis to modulate its proteolytic action. The net effect of this interaction can either promote tissue repair and angiogenesis

or lead to further tissue injury through deleterious effects that include BBB disruption, amplification of inflammatory infiltrates, demyelination, and possibly interruption of cell–cell and cell–matrix interactions that may trigger cell death.

In addition to its role in fibrinolysis, the plasminogen-plasmin system has diverse non-fibrinolytic effects in the CNS, both physiological and pathophysiological. It has effects on neuronal sprouting, neuronal plasticity, microglial activation, BBB permeability, excitotoxic injury and extracellular matrix-related neuronal death (12). Plasminogen is constitutively expressed in several areas of the brain (including the cortex and hippocampus) and spinal cord. Its expression is modulated by cytokines IL-6, TNF, TGF-β and IL-1, and glucocorticoids. (13). The effects of the plasminogen-plasmin axis on neuronal survival, excitotoxicity, oxidative stress and apoptosis are largely fibrin-independent and are instead directly mediated by the plasmin-MMP interaction on the extracellular matrix (10), cell signalling and microglial activation (14). Plasminogen receptors also include the annexin A2-S100A10 heterotetramer, α-enolase, histone H2B and the trans-membrane plasminogen receptor. By inhibiting plasminogen binding, TXA could have major anti-inflammatory effects in the CNS. We cannot assume that giving TXA will only affect intracranial bleeding: it could affect patient outcome though other important biological mechanisms mediated by neuroinflammation.

Research objectives

We will study the mechanisms of action of the plasminogen inhibitor tranexamic acid (TXA) in patients with acute traumatic brain injury (TBI) by collecting specimens and imaging data from participants enrolled in the NHIR funded CRASH-3 trial. Understanding the mechanism of action of TXA in TBI will enable clinicians to apply TXA in the care of NHS patients. However, because TXA impacts on diverse biological mechanisms (neuroinflammation, neuroprotection, permeability of the neurovascular unit and bleeding) that are highly relevant to other major CNS diseases, including Alzheimer's disease and stroke, insights gained could lead to new treatments with major patient and economic benefits.

This is a unique opportunity to investigate the effect of plasminogen inhibition in the CNS. The trial is recruiting in 40 UK hospitals, including all major trauma centres (over 80 patients per month). Randomisation is as essential in determining the neurobiological effects of TXA, as it is in determining its effect on patient outcomes. However, if shown to improve outcome, it could be impossible to conduct further randomised studies of mechanisms. Because patients with extra-cranial injuries are excluded, the effects of TXA on the injured brain can better be discerned. Importantly, after reviewing un-blinded data from 1200 patients, the data monitoring committee has recommended further studies to examine "why and how patients are affected by tranexamic acid."

As discussed above, we cannot assume that TXA, a potent anti-plasmin that crosses the BBB, will only affect intracranial bleeding. It could affect patient outcome through other important biological mechanisms. Neuroinflammation is an important secondary injury mechanism that contributes to on-going neurodegeneration and neurological impairments associated with TBI, triggering a vicious self-perpetuating cycle of damaging events that lead to prolonged microglial dysregulation. Human and animal studies indicate that microglia remain chronically activated for weeks, months and even years after the initial brain trauma, and may contribute to chronic neurodegeneration and related neurological deficits following injury (15, 16).

TXA could be neuroprotective both through plasminogen-mediated and plasminogen-independent mechanisms. The aim of this project is to elucidate if TXA modulates the neuro-inflammatory response after TBI exploiting the ideal set-up of an observational study embedded in a randomised controlled trial. Our hypothesis is that TXA activates neuroprotective, neuroinflammation-modulating mechanisms that involve both plasminogen-dependent and plasminogen-independent pathways.

Research design

Design: This is a mechanistic observational study embedded within a CTIMP (CRASH-3)
Study procedures: The eligibility criteria will be checked by the Research Nurses in each clinical centre. Consent for inclusion in the mechanistic study will be sought in addition to the CRASH-3 main study consent (see below). To be included, patients will need to have invasive neuromonitoring consisting of intracranial pressure ICP) and cerebral microdialysis. The latter is a minimally invasive procedure that monitors cerebral biochemistry via a catheter implanted via the same bolt as the ICP transducer and is in routine use in the four participating clinical centres. Invasive neuromonitoring is standard practice in most UK neurosurgical units for the management of patients with severe or complicated TBI, and is inserted as soon as possible after injury.
Microdialysis produces hourly samples that are first analysed by the intensive care nurse, using a bedside analyser, for markers of tissue injury and dismetabolism. Instead of being disposed of after the bedside analysis, the samples will be stored at -80°C and will then be transferred in batches to the Biochemistry Lab of the University of Cambridge. The sample collection will continue until day 5 for the purpose of the study or as long as possible if the bolt has to be removed before this time. Normal CRASH-3 procedures and data collection will continue as normal. As part of the substudy, minute-by-minute ICP, daily Therapy Intensity Level and vital parameters will be logged. Brain CT scans on admission and follow-up (routinely done in these units within 72h) will be classified according to the Marshall grade and will be transferred to Addenbrooke's on CD for automated intracranial blood volumetric analysis.

Inclusion/exclusion criteria: Adults with TBI, who are within eight hours of injury, with any intracranial bleeding on CT scan **or** who have a Glasgow Coma Score (GCS) of 12 or less, and do not require blood transfusion for extra-cranial bleeding, are eligible for inclusion into the main CRASH 3 study. Patients with major extra-cranial bleeding are excluded from the CRASH-3 trial because the CRASH-2 trial showed that TXA is indicated in these patients. The main eligibility criterion is the doctor's 'uncertainty' as to whether or not to use TXA in a particular patient.
Eligibility for the mechanistic substudy will be determined by the clinical indication for invasive neuromonitoring, including cerebral microdialysis, which means that not all patients currently recruited into CRASH-3 will be eligible. An audit of CRASH-3 trial patients recruited at the Queen Elizabeth Hospital shows that about 65% (102 out of 156) underwent invasive neuromonitoring, including cerebral microdialysis.

Type of analyses: The mechanistic study will focus on the acute inflammatory response after administration of TXA. In the acute phase, we will investigate whether TXA modulates neuroinflammation by measuring cerebral concentrations of pro-inflammatory and anti-inflammatory cytokines and chemokines, as well as markers of tissue injury (Lactate/Pyruvate ratio (L/P), Glycerol, Glutamate, S100B and Glial Fibrillary Acidic Protein (GFAP)). Cerebral microdialysis is ideal for this purpose, as it allows continuous sampling of cerebral biochemical compounds over several days and has specifically been recommended as a technique to assess the mechanistic effects of candidate neuroprotective drugs 'at the point of action' by consensus group workshops for the reorientation of TBI research organised by the European Commission and the National Institutes of Health/National Institute of Neurological Disorders and Stroke (NIH/NINDS) (17).

Microdialysis and plasma samples will be analysed using the Milliplex™ Multi-Analyte Profiling Human Cytokine/Chemokine 41-plex premixed kit and Human MMP Panel (Millipore Corp, Missouri, USA) on the Luminex 200 system (Luminex Corporation, Austin, TX, USA). Thirty of the 41-plex of the Milliplex cytokine/chemokine panel's calibration standards have been referenced to NIBSC standards and the rest to Milliplex's "gold standard" to which all standard lots are referenced to ensure lot-to-lot reproducibility.

In order to control for differences in intracranial blood load between intervention groups as a potential confounder, an automated blood volumetric analysis will be carried out on the initial and follow-up CT scans (routinely performed within 72h of admission in the participating centres) with ad hoc software at the Division of Neurosurgery, University of Cambridge. This is important, as the anti-fibrinolytic effect of TXA is at this stage the main putative mechanism of action of this drug and without controlling for intracranial bleeding, any anti-inflammatory effects could simply be attributed to a reduction in the intracranial blood load. This will allow us to discern the non-fibrinolytic effects of TXA in the CNS.

Study population

Patients with TBI recruited into CRASH-3 at four high recruiting UK centres, Queen Elizabeth Hospital Birmingham (QEH), Addenbrooke's Hospital Cambridge (AHC), Southampton General Hospital (SGH) and St. Mary's Hospital London (SMH), will be considered for inclusion in the mechanistic substudy. QEH is the third highest recruiting centre into CRASH-3 worldwide and enrols on average 8-9 patients a month. AHC, SGH and SMH joined the trial more recently and recruit on average 2, 1-2 and 4 patients a month respectively.

The four clinical centres have more than enough capacity to carry out cerebral microdialysis on all patients recruited to this study. QEH has the capacity to monitor up to 6 patients with microdialysis at any given time and over the last 12 months (July 14-June 15) performed microdialysis on 36 patients. AHC performs microdialysis on an average of 2.7 per month (2007-2015 audit). SMH has acquired microdialysis more recently and figures are not available; however, this site can monitor up to three patients at any given time.

The biochemical analyses will be performed in the Neurochemistry Laboratory in the Division of Neurosurgery, Dept. of Clinical Neurosciences, in the University of Cambridge.

Proposed outcome measures

CRASH-3 collects in-hospital deaths, disability and complications assessed at death, hospital discharge, or one month following randomisation, whichever occurs first. The mechanistic study will examine the acute inflammatory response in patients who receive TXA and placebo. We will investigate how TXA modulates neuroinflammation by measuring cerebral concentrations of pro-inflammatory and anti-inflammatory CCKs, MMPs and markers of neuronal and astroglial injury. Specifically, the latter group will include markers of metabolic dysfunction (L/P), cell membrane degradation (glycerol), excitotoxicity (glutamate), and biomarkers of tissue injury (GFAP and S100B).

The principal outcome measure will be levels of CCKs and MMPs on day 2 (reported with the Principal Component Analysis described below), as this time follows completion of the IMP infusion and corresponds to the peak time of many CCKs of interest.

Proposed sample size

Overall, CRASH-3 aims to recruit 10,000 patients, based on 90% power (two sided alpha of 1%) to detect a 15% relative reduction (20% to 17%) in all-cause mortality. The expected loss to follow-up (<1%) will not impact importantly on study power. The sub-study proposed here will select 40 patients for in-depth assessments of the neuroinflammatory response to TXA administration. To ensure balance between the two arms of the study, we will initially collect samples from 60 patients. After unblinding, the first consecutive 20 patients in each arm will undergo biochemical analysis. The remaining samples will be banked and will be available for future research.

The literature suggests that likely mechanistic targets of TXA are MMPs and CCKs such as TNF, VEGF, IL-1, IL-6, IL-8 and IL-10 (7, 8, 18). There may be many more, as other CCKs have not been so extensively studied. A sample size of 40 is likely to detect an effect of TXA on neuroinflammation and markers of tissue injury. There are no pre-existing data on TXA and brain CCKs, but a recent study by 3 of the co-applicants (AH, PJ and DM) was able to show a clear differentiation between 10 patients treated with recombinant IL-1ra and 10 controls using the same statistical (Principal Component Analysis) and analytical methodology (cerebral microdialysis and Luminex) proposed here. In this study, for, example, treated patients had 81% increase and 75% reduction in the levels of TNFα and macrophage-derived chemoattractant (MDC) respectively, compared to controls. When considering the sample size it is important to remember that brain CCKs are barely detectable in the normal brain but increase several thousand-fold in response to injury. Previous studies have shown differences of several orders of magnitude in CCKs microdialysate and CSF concentrations in TBI patients with different responses to injury or treatment (1, 19) (e.g. a 6-fold higher MD concentration of IL-6 in TBI survivors vs. non survivors (20)). Based on means and SDs deviations derived from data published by co-applicants (2, 21), a sample of 40 patients will be able to detect an effect size of approximately 50% in CCKs known to be mechanistically linked to the action of TXA with equal sample sizes, alpha=0.05 and power 0.80 (see table below). As we expect TXA to have an effect on MD CCKs at least comparable to recombinant IL-1ra or to differences seen between good and poor outcome groups after TBI (around 75%-80%), we are likely to detect meaningful effects in our sample size. In addition, the Principal Component Analysis methodology is likely to make the differentiation between treatment and control groups even clearer, as this is a strong statistical method for looking at a relatively large number of variables in a small sample of patients.

CCK	Mean concentration on day 2 (pg/ml)	SD	Detectable effect with power 0.80	Percentage smallest detectable effect with power 0.80	Detectable effect with power 0.90	Percentage smallest detectable effect with power 0.90
TNF	3.25	2.81	2.6	80%	3	92%
VEGF	37.03	27.25	24.75	67%	28.75	78%
IL-10	15.14	10.66	9.68	91%	11.2	105%
1L-6	1345	1027	958	71%	1079	80%
IL-1ra	59.48	47.74	43.35	91%	50.16	105%

Statistical analysis

Microdialysis generates hourly samples. Bedside analysis consumes less than 3µL and the leftover microdialysates are then stored at -80°C for subsequent multiplex analysis of cytokines and chemokines in the laboratory, on the Luminex analyzer. This multiplex methodology generates a large number of data points requiring the utilisation of specific statistical techniques. We will employ a set of methodological procedures already successfully utilised by our co-applicants (19) in a Phase II RCT of recombinant IL-1ra in a population of severe TBI patients. In that study all cytokines and chemokines of interest to be included in our proposed study were investigated. In order to have sufficient volume for Luminex analysis, as well as to reduce the number of biochemical analyses to be performed, the microdialysate samples are pooled in relation to time of injury. In the proposed study, we plan to pool microdialysates into 12-hourly time epochs starting from 0-12 hours up to 120 hours. Blood samples will be collected at 12h intervals to measure the systemic inflammatory response using the same Luminex analysis. Previously published data by our group showed that brain cytokines and chemokines steadily rise and fall over relatively long time periods in the first few days of injury and therefore shorter epochs would only increase the costs and labour without necessarily adding further mechanistic insights (21). TXA concentrations in the brain microdialysates will also be measured with High Performance Liquid Chromatography, providing an important pharmacokinetics insight into mechanism of action (TXA crosses the BBB; however intracerebral concentration of drugs, in general, has been shown to be highly variable between patients after TBI).

A Principal Component Analysis (PCA) will then be utilised to select candidate cytokines that are impacted by the administration of TXA. PCA is an unsupervised dimension reduction technique that generates latent variables designated principal components and provides an unbiased representation of the complex multivariate cytokine and chemokine data set (22). The first Principal Component (PC) is a linear combination of each of the original variables, which incorporates the greatest sources of variation within a dataset. The second and subsequent PCs are further latent variables that explain the greatest sources of variation that are left over beyond the first PC and lie orthogonal to it. As well as a separation on the scores plot between the control and intervention group along, this analysis will enable us to select the CCK with the score of the greatest magnitude to compare between groups using conventional statistical methods. To distinguish between groups or classes we will perform a regression extension of PCA (Partial Least Squares Discriminant Analysis, PLS-DA). This is a more advanced method that pre-specifies groups and looks for variation that can specifically separate between these two groups. We utilise SIMCA software (Umetrics, Sweden), which uses an iterative method for deriving principal components andcan carry out further advanced analyses including orthogonal-partial least squares discriminate analysis as well as an in built module for a classification algorithm with the capacity for prediction.

To show whether a difference exists between control and intervention groups, the concentration of the candidate CCK identified from the PCA stage will be analysed in each group for each study day using a mixed (repeated measures and between group) factorial ANOVA to explore the effects of both time and drug administration on mean compound concentration on each day of the study period. Mauchly's test of sphericity will be used for all contrasts. If the assumption of sphericity is violated, then the Greenhouse-Geisser correction will be applied.

We have also approached and secured the collaboration of Prof John Aston, Professor of Statistics, Statistical Laboratory, Dept. of Pure Mathematics and Mathematical Statistics (DPMMS), University of

Cambridge) as an advanced statistics expert. We will have access to his group's expertise in machine learning and advanced statistics. Prof Aston's research group has a number of collaborations with the Wolfson Brain Imaging Centre: a joint statistics postdoctoral research associate between the Statistical Laboratory and Wolfson Brain Imaging Centre has recently been appointed and will start in June, based at Addenbrooke's Hospital, to further enhance this collaboration. In addition, Prof Jean-Baptiste Cazier, Professor of Bioinformatics and Director of the Centre for Computational Biology of the University of Birmingham, will collaborate on this project.

Ethical arrangements

Informed consent will be impossible to obtain in this TBI patient population. Patients are unlikely to regain full capacity within the time space of the sample collection (5 days). The study protocol is time critical. Proxy consent by a doctor not involved in the study or the next of kin will be sought for inclusion in this study, which is a model that we have successfully adopted in all our other TBI studies. In the event that an enrolled patient regains capacity, they will be asked (verbally) if they are willing to remain in the study. Consent will be sought from patient or personal legal representative at the earliest appropriate time after hospital admission. If consent is declined, no further data collection will take place. Data that has already been collected up to this point will be analysed. Invasive neuromonitoring is carried out as part of clinical care of TBI in all four participating hospitals. Only patients who require invasive neuromonitoring as part of their routine care will be included in this study.

Research Governance

CRASH-3 is carried out under a Clinical Trial Authorisation in accordance with the Medicines for Human Use Clinical Trials regulations and LSHTM CTU Standard Operating Procedures. The proposed project is an observational study within the said CTIMP and will benefit from a data sharing agreement with CRASH 3.
University of Birmingham will act as trial sponsor and will manage the full REC application, public registration and all other Governance aspects. Site agreements will be required between the sponsor and the NHS Trusts and Boards for the recruiting hospitals.
1. **Sponsorship and Indemnity:** NHS Trusts participating in this trial will provide clinical negligence insurance cover for harm caused by their employees.

2. **Study oversight:** Study Steering Committee (SSC). The SSC will be convened for the study comprising an independent chair, independent clinical members and patient representatives. Representatives of the Sponsor and EME programme will be invited to all SSC meetings. The SSC will meet every 12 months. Day-to-day study management will be supported by the NIHR Surgical Reconstruction and Microbiology Research Centre (SRMRC - Trauma Research), which enlists a programme manager, 2 trial coordinators, 2 systematic reviewers, 2 medical statisticians and 7 research nurses operating a 24/7 service. The SRMRC has a monthly Trial Management Group meeting to review accruals, recruitment issues and adverse events of its portfolio studies.

3**. Confidentiality of personal data and long-term storage**: Participant's personal data collected during the study will be held by each Trust on a secure database server in line with the Data Protection Act 1998. Data processed outside the generating centre will be anonymised. Data will be archived for 10 years at the University of Birmingham. Before identifying grouped consecutive samples for the

biochemical analysis, the randomisation identifier of the participants will be matched with the randomisation data held by the LSHTM CTU via a data sharing agreement (already approved).

Project timetable and milestones

After award confirmation - prior to release of funding	0-2	Protocol finalised Study documents developed Contracts arranged	
	3-4	NRES REC and local R&D applications Investigator/Staff training (QEH, AHC, SGH, SMH) Clinical fellows recruitment Equipment checks and service if necessary Consumables procurement	
After release of funding	November 2015	Study opens and recruitment commences	**Research Nurses appointed and trained**
	3 - end of January 2016	Review of first milestone	**9 patients recruited across all sites**
	6 - end of April 2016	18 patients recruited	
	12 - November 2016	36 patients recruited Non-clinical Research Fellow in post (University of Cambridge)	
		Laboratory analyses commence	
	15 - End of December 2016	Patient Recruitment ends	**40 patients recruited**
	16 - End of January 2017	Randomisation and clinical data released by CRASH 3 team	
	21	Laboratory and data analysis completed	
	21-24	Reporting, publication and dissemination	

Deliverability

From a dry run carried out at QEH, we project that out of all patients recruited across the four study centres 7-8 a month meet the inclusion criteria for the sub-study, and that it would be realistic to

perform the mechanistic assessments in 4-5 patients a month and to meet the overall target of 60 patients in 13 months. CRASH-3 will close to recruitment on 31st December 2016, by which time recruitment into the sub-study will also stop. From the recommendations of the Data Monitoring Committee, who looked at the unblinded data on the first 1,200 patients, it is very unlikely that CRASH-3 will close prematurely. The necessary equipment to carry out sample collection and analysis is already in place.

Service users

Although TBI presents challenges for patient involvement, patients and the public had a key role in planning the CRASH 3 trial. Victim organisations (The European Federation of Road Traffic Victims, and RoadPeace, the UK national charity for road crash victims) represent patients on the Trial Steering Committee and we will work closely with victim organisations in disseminating results.
A summary of the background to the sub-study and our proposal was circulated to our trauma Patient & Public Involvement (PPI) group. Feedback was sought via a structured questionnaire. The study was unanimously considered "essential". A public engagement event and PPI focus group were held. There was universal support for the study. Potential approaches to the relatives of study participants were discussed and the plain English summary was prepared at this event. We will ask our Patient & Public Involvement group to review our consent documents to ensure that they are comprehensible to the lay reader. Two members of the group have agreed in principle to join the Trial Steering Committee. Public engagement meetings will be arranged at the end of the study, as we have experience of this being particularly successful at engaging the public in our research. Our study findings will be presented at academic conferences, via public "showcase" events at hospital and university and at public engagement events organised by our various partners. We intend to involve our PPI group in publicising both the study and its results.

Project identifiers

IRAS ID	186446
University of Birmingham Study ID	ERN_15-1213
University of Birmingham Sponsorship ID	RG_15-194
University Hospitals Birmingham NHS Foundation Trust R&D	RRK5587
NIHR EME (Funder's) Reference	14/205/01
REC ID	15/EM/0544

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