Evaluation of Metabolism of Adenosine Triphosphate as a Novel Biomarker of Tissue Ischaemia *in vivo.*

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List of abbreviations

AAA	Abdominal aortic aneurysm
ADP	Adenosine diphosphate
Ag	Silver
AgCl	Silver chloride
ALI	Acute limb ischaemia
AMP	Adenosine monophosphate
AOPCP	alpha-beta-methylene-ADP
ATP	Adenosine triphosphate
Au	Gold
BBB	Blood brain barrier
c-AMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium
CBF	Cerebral blood flow
CEA	Carotid endarterectomy
CFA	Common femoral artery
CI	Confidence interval
CLTI	Critical limb threatening ischaemia
$CMRO_2$	Cerebral oxygen uptake
CNS	Central nervous system
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CPP	Cerebral perfusion pressure
CRF	Case report form
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computer tomography
CTA	Computer tomography angiography
DBD	Donation after brainstem death
DCD	Donation after circulatory death
DIEP	Deep inferior epigastric perforator
DVT	Deep vein thrombosis
e	Electron
ECG	Electrocardiogram
ED	Emergency department

EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalography
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine
ENT	Equilibrative nucleoside transporter
EVAR	Endovascular aortic aneurysm repair
FESS	Functional endoscopic sinus surgery
FiO_2	Inspired oxygen concentration
GA	General anaesthetic
GCP	Good clinical practice
GCS	Glasgow coma scale
GP	General practitioner
GST	α-glutathione S-transferase
GTN	Glyceryl trinitrate
H^+	Hydrogen
H_20_2	Hydrogen peroxide
HDU	High dependency unit
HPLC	High performance liquid chromatography
IC	Intermittent claudication
ICP	Intra cranial pressure
IFABP	Intestinal fatty acid binding protein
IFU	Instruction for use
IP	Ischaemic preconditioning
LA	Local anaesthetic
LD	Living donor
MAP	Mean arterial pressure
Mg^{2+}	Magnesium
MI	Myocardial infarction
MRA	Magnetic resonance angiography
Na^+	Sodium
NHS	National health service
NIRS	Near infra-red spectroscopy
NSTEMI	Non ST-Elevation myocardial infarction
O_2	Oxygen
OSR	Open surgical repair
PAD	Peripheral arterial disease
PB	Prussian blue

PCI	Percutaneous coronary intervention
PIS	Participant information sheet
PRISM	Purines for rapid identification of stroke mimics
Pt	Platinum
REE	Resting energy expenditure
rGMR	Relative glucose metabolic rate
RP	Ruthenium Purple
RTC	Road traffic collision
SaO_2	Oxygen saturation
SFA	Superficial femoral artery
$SJVO_2$	Jugular venous oximetry
SSEP	Somatosensory evoked potentials
STEMI	ST-elevation myocardial infarction
TBI	Traumatic brain injury
TcD	Transcranial Doppler
TIA	Transient ischaemic attack
TKA	Total knee arthroplasty
UHCW	University Hospitals Coventry & Warwickshire NHS Trust
UHNM	University Hospital of North Midlands NHS Trust
UKA	Uni-compartmental knee arthroplasty

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Abstract

Introduction

Ischaemia is often a medical emergency, clinical signs and symptoms vary dependant on organ or organ system affected. Timely restoration of oxygen delivery to affected tissues is paramount to preventing morbidity and mortality. Current diagnostic aids are non-specific and often late markers of ischaemia where there has been irreversible tissue damage.

Metabolism of adenosine triphosphate (ATP) is altered during periods of ischaemia and intermediate nucleosides such as adenosine, inosine and hypoxanthine are released into the systemic circulation in demonstrable quantities. Development of an amperometric biosensor sensitive to these purine nucleosides, produced early in ischaemia prior to cell death, gives potential for purine nucleosides to be a clinically relevant biomarker.

Methods

A series of prospective observational cohort studies have been designed to provide pilot data on purine nucleoside changes, measured in real-time *in vivo* in populations of both surgical and non-surgical patients where a simple test for early identification of ischaemia would be clinically useful expedite diagnosis and prevent patient harm.

Results

There was no significant variation from baseline readings in purine nucleosides in systemically obtained samples intra-operatively in patients who are anaesthetised with general anaesthetic. There were significant increases in nucleosides in blood samples obtained locally within acutely ischaemic tissue. Elevated purine nucleosides were also identified in patients with critical limb ischaemia and following some types of brain injury.

Conclusions

Reliable determination of purine nucleosides *in vivo* remains challenging; multiple factors may interfere with quantification *in vivo*. Results here have identified purine nucleosides may have a role in acute and chronic limb ischaemia as well as traumatic brain injury. This requires further validation in populations of patients in clinical practice. Expansion to further areas of ischaemia may also be of clinical value.

Introduction

Ischaemia is defined as a restriction in blood flow (and therefore oxygen delivery) to tissues. The effect of prolonged ischaemia is ultimately cellular death, the effect of which will vary dependant on the anatomical location and metabolic requirement of tissue in relation to the extent of ischaemic insult. In general, the mainstay of treating ischaemic events is the restoration of blood flow. To offer patients the best prognosis this should be achieved as quickly as possible. Reliable biomarkers of ischaemia are of clinical importance to aid diagnosis and guide management in a timely manner. Currently there are very few biomarkers of ischaemia that are widely used in clinical medical practice.

The purinergic role *in vivo* is multifaceted and varies significantly throughout the human body. Their role includes regulatory, retaliatory and anti-inflammatory functions as well as neurotransmission and regulation of blood flow (1-4). There is also evidence of an acute rise in purine nucleoside concentrations in response to hypoxia and ischaemia in an experimental setting (5, 6).

This body of work sets out proof-of-concept data on the evaluation of adenosine triphosphate (ATP) metabolism in the form of the purine nucleosides adenosine inosine and hypoxanthine as biomarkers of ischaemia across a range of ischaemic conditions in clinical medical practice where a diagnostic aid would be of potential clinical utility.

1. Review of ATP metabolism in ischaemia and its potential use as a biomarker *in vivo*

1.1 Mechanisms of ATP metabolism in ischaemia

Adenosine triphosphate (ATP) is the essential currency in mammalian cellular metabolism (7). In resting conditions a continuous supply of ATP is generated within mitochondria via oxidative phosphorylation in the electron transport chain (8). During periods of hypoxia and/or ischaemia ATP can be generated by both glycolysis or production of lactate but this is inefficient and short lived, therefore ATP generation ceases within minutes (9). To continue energy production intracellularly, ATP is metabolised along enzymatically regulated cascades ending with the formation of uric acid (10). The mechanism and pattern of purine production and release varies between cell types within the same tissue or organ and between different tissue types throughout the body (11). In general, after the onset of ischaemia oxidative phosphorylation to produce ATP ceases. Existing ATP and adenosine diphosphate (ADP) are cleaved of their remaining phosphate(s) by hydrolysis to form adenosine monophosphate (AMP). Activation of 5'nucleosidase and AMP-deaminase in the cytosol leads to metabolism of adenosine monophosphate into adenosine and inosine.

Both adenosine and inosine can be transported across cell membranes by equilibrative nucleoside transporters (ENTs) (12) into the interstitial space where further metabolism, catalysed by purine nucleoside phosphorylase, to hypoxanthine can occur. These purines have low molecular weight and therefore are free to pass into the systemic blood circulation relatively unimpeded allowing their detection at sites distant to the area of ischaemia (13). Further metabolism to xanthine and uric acid are both oxygen dependant (via xanthine oxidase) and therefore inhibited whilst hypoxia and/or ischaemia are ongoing and as such not produced during ischaemia (14).

In addition to this intracellular metabolism cascade there is also an extracellular pathway for adenosine production known as the cyclic-AMP (c-AMP) – adenosine pathway (15). There is active transport of c-AMP into the extracellular space as well as transport of ATP across cell membranes, this can occur directly via exocytosis or gap junction hemi channels within the central nervous system (CNS) (16). c-AMP and adenosine can then be metabolised to liberate adenosine via membrane bound ecto-phosphodiesterase and ecto-nucleosidases (CD39 and

CD73) (17-19). A diagrammatic representation of the purine metabolism pathways is shown in Figure 1.

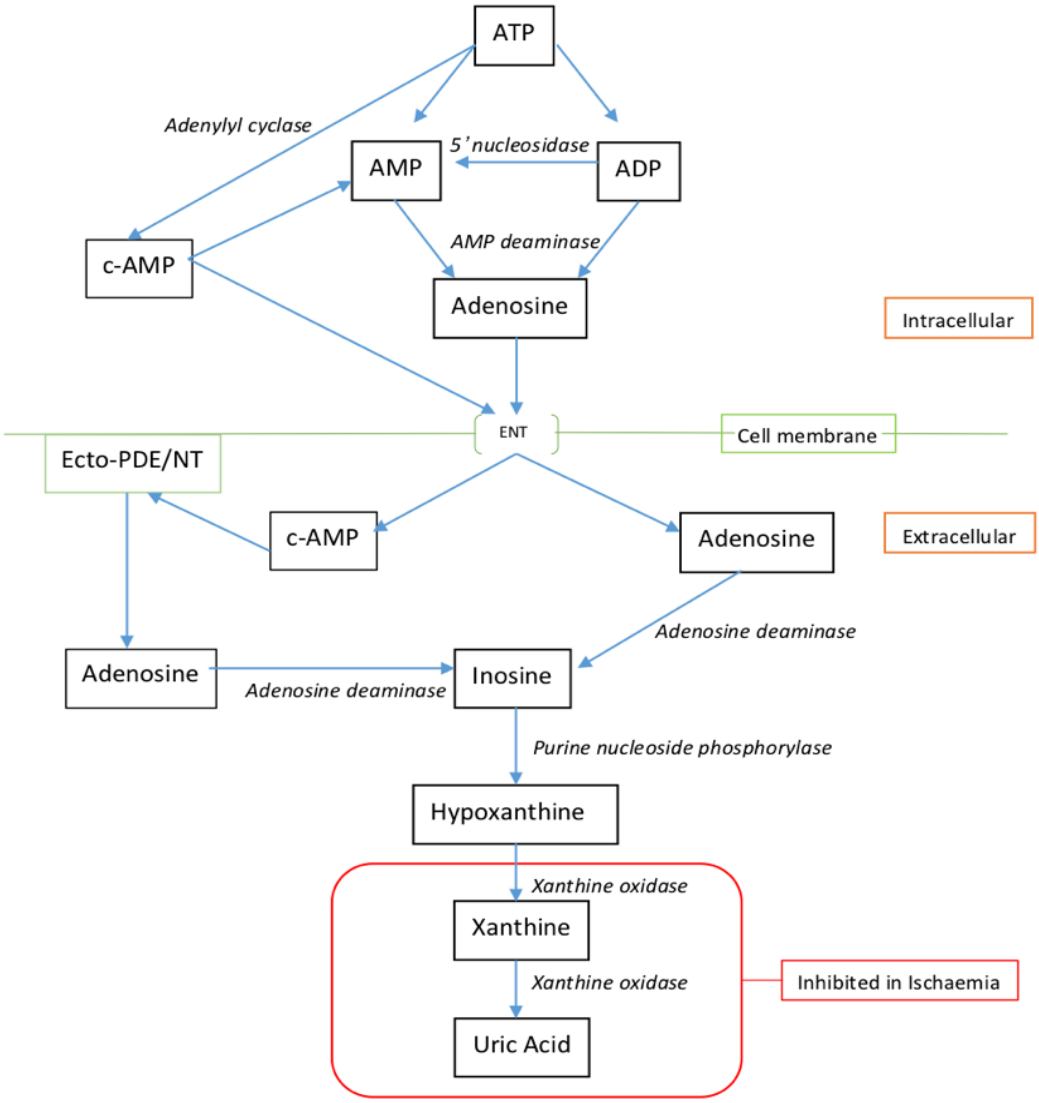

Figure 1 *Diagrammatic representation of intra and extracellular purine metabolism in ischaemia.*

1.2 Evolution of techniques for assessment of purine concentrations

1.2.1 Frozen tissue

Historically, the short half-life and multiple sites of re-utilisation of purines (20-22) lead assessment techniques to revolve around rapidly freezing tissue samples with liquid nitrogen prior to analysis. This technique instantly stops ongoing purine metabolism therefore accurate determination of nucleoside concentrations can be achieved. The need for excision of tissue and rapid freezing is destructive and has restricted its use to animal models as well as limiting its potential application in human research settings and clinical medical practice (23, 24).

1.2.2 Interstitial fluid (via microdialysis)

Tissue interstitial purine concentrations became of interest with the advent of microdialysis (25). Fine bore cannulae can be inserted into tissues between cells and flushed with a perfusate. Over time purine concentrations equilibrate between perfusate and interstitial fluid that then can then be collected and analysed. Microdialysis allows direct assessment of extracellular purine nucleosides and when compared to rapid freezing, cannula insertion is significantly less traumatic. Transient rises in purine concentrations attributed to local release following insertion have been identified though these are minor and short lived (26). It also requires the site of ischaemia to be readily accessible for cannula insertion and is therefore limited to peripheral sites of ischaemia. Sample collection can be time consuming as a period must be allowed for equilibration between interstitial fluid and dialysate. Though periods of less than one minute have been reported, typical collection periods used can be around ten minutes (27). Recovery percentage of the nucleosides is inversely proportional to dialysate flow rate. Percentages can be increased by slowing flow rate but collection time periods may then become further extended (28, 29).

1.2.3 Cerebrospinal fluid

Analysis of purine nucleosides is possible from cerebrospinal fluid (CSF) samples (30-32) in humans via lumbar puncture. Though this mechanism of sampling is limited by its invasive nature, risk of significant complications (such as paraplegia) and requires skilled clinicians to perform it in a controlled setting. This again limits its widespread use, especially in the context of ischaemia where results are time critical.

1.2.4 Blood

Arterial, venous and capillary blood sampling is part of routine clinical practice and therefore obtaining samples is minimally invasive and easily achievable. Analysis of blood samples was initially confounded by ongoing enzymatically mediated metabolism as well as purine release from, and uptake into, platelets and erythrocytes after sampling but before sample analysis. This necessitated development of blocker solutions to preserve samples prior to analysis. A blocker solution should contain an adenosine deaminase inhibitor, an adenosine reuptake inhibitor and a 5'ecto-nucleosidase inhibitor. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), dipyridamole and alpha-beta-methylene-ADP (AOPCP) or ethylenediaminetetraacetic acid (EDTA) have been used respectively, in various combinations, to achieve this (33-36). In order for the blocker solution to achieve maximum efficacy it has to be introduced to blood at the earliest possible opportunity after sampling. Dedicated cannulae have been designed for mixing of the blocker and sample to occur immediately after sampling (4, 37). This mixing can then be followed by deep freezing until analysis.

1.3 Analysis of biological samples

1.3.1 High performance liquid chromatography

The first widely used analysis technique for samples was high performance liquid chromatography (HPLC). HPLC is both sensitive and specific for determination of concentrations of constituents of complex biological samples, such as blood, interstitial fluid or CSF. In addition it requires small volumes to analyse and is faster than traditional chromatography (38). There are also multiple drawbacks to the use of HPLC in clinical medicine samples require preparation prior to analysis (deproteinisation) and analysis requires specialist equipment and operator skills to ensure reliable results (39). This means it is not scalable into hospital laboratories and remains a method of analysis in a research setting only.

1.3.2 Optical detection

Optical detection methods such as fluorescent and chemiluminescent analysis techniques have been widely used (40, 41). These both rely on enzymatic metabolism of purines by adding adenosine deaminase and/or xanthine oxidase to the sample to produce hydrogen peroxide (H_2O_2). This H_2O_2 can then react with reagents that will fluoresce when light is applied (e.g. difluorescin) or luminesce without an external light source (e.g. luminol). The

strength of the light emitted can then be measured and converted to an integer by comparison with a calibration measurement from a signal given by known concentration of purine nucleosides. Both of these techniques have been validated as highly sensitive and specific. Analysis time is significantly shorter than HPLC and detection equipment is cheaper (5, 42). Results can be affected by background interference and signals emitted from other biological compounds. Results can also be disturbed by alterations in reaction conditions such as temperature and pH (43-45). As with HPLC, samples often require deproteinisation prior to analysis. A chemiluminescent sensor has been validated *in vivo* in patients presenting to the emergency department with chest pain (5).

1.3.3 Electrochemical detection

Enzyme based electrochemical (amperometric) biosensors (6, 46) function in a similar way to their optical counterparts. Enzymatic formation of H_2O_2 induces a change in current around an electrode which can then be interpreted as an integer. The advantages of electrochemical sensors are short analysis duration (in the order of seconds) and allowing analysis of unprepared samples (47, 48). They remain susceptible disturbance in sensing due to interference from other biological compounds within the sample, electrode fouling and variations in temperature or pH (46, 49). Amperometric biosensors have been utilised to observe changes in purine concentration in patients undergoing carotid endarterectomy (6).

Other techniques such as radioimmunoassay, nuclear magnetic resonance spectroscopy and mass spectroscopy have been described for the quantification if purine nucleosides *in vitro* with reliable results (50-52). However, their use has limitations context of time-critical assessment of ischaemia. These include use of radioactive isotopes, expensive specialist equipment, the need for sample preparation and prolonged analysis duration.

1.4 Purines as biomarkers

There has been interest in purines as an early clinical biomarker in ischaemia and hypoxia as traditional biomarkers of tissue ischaemia (e.g. lactate and acid-base disturbance) are late and non-specific (53). Studies have focused on a wide range of ischaemic and hypoxic scenarios given the multifaceted role purines play *in vivo.*

Early animal models

1.4.1 Cerebral Ischaemia

The brains' unique role, relative physiological isolation by means of the blood brain barrier (BBB) and requirement for high energy substrate to function leaves it particularly vulnerable to injury caused by periods of hypoxia or ischaemia (54). Purine release in response to cerebral metabolic stress has been investigated since the 1960s (55, 56).

Rapid, significant rises in adenosine concentrations were recorded in brain tissue and interstitial fluid in rats, measured by cortical freeze blowing, up to 60 seconds after inducing hypotension and subsequent brain ischemia by aortic transection (mean peak adenosine 1.28 nmol/g control vs 5.50nmol/g at 60 seconds, $p<0.001$) (23). No significant changes in hypoxanthine were noted. The same group measured a ten-fold increase in cerebral interstitial concentrations of adenosine during ten minute periods of hypoxia and a four-fold increase ten minutes after ceasing respiration in anaesthetised rats (56).

In order to measure extracellular changes Laudignon et al and Park et al (32, 57) identified increases in adenosine in CSF samples obtained from direct puncture of the cisterna magna in piglets. These results were supported by Phillis et al (31) who found adenosine increased in the CSF of rats during hypoxia and that this increase was dependant on increasing hypoxia (Adenosine peak at 14% inspired oxygen concentration (FiO_2) 261±130nmol/L vs FiO_2 5% peak 331.9±133.65nmol/L). Progressive asphyxia of foetal lambs (58) resulted in a four- to six-fold increase in hypoxanthine from central venous samples. Results from this study showed no difference in peripheral arterial or venous concentrations. Plaschke et al (59) showed that in rats having undergone staged (over the course of five days) bilateral carotid and vertebral artery ligation exhibited sustained two-fold increase above control values in adenosine within cerebral tissue at two weeks post completion of four vessel occlusion.

1.4.2 Cardiac Ischaemia

Adenosine was first proposed separately in the early 1960s by Berne (60) and Gerlach and Deuticke (61) to play a role in regulating blood flow in cardiac tissue in order to balance supply and demand of oxygen (O_2) via its action as a potent vasodilator (62, 63). This theory became known as the adenosine hypothesis was generally accepted for a significant period of time. It has since been observed that administration of adenosine deaminase fails to reduce reactive hyperaemia to decreased O_2 saturations in myocardial tissue (64) leading to the

theory that although adenosine and other nucleosides increase during ischaemia they cannot not be the sole regulator to changes in myocardial blood flow. It is now accepted that endothelial release of nitric oxide is responsible for initial changes in coronary blood flow in response to hypoxia (65).

There has been extensive investigation into changes in cardiac purines. It was theorised that rises in purines would be pronounced in cardiac ischaemia as mitochondria account for up to 50% of myocyte cellular mass (13) in order to meet energy demand. Current gold standard cardiac enzyme tests (Troponin I and troponin T) used for diagnosis of myocardial ischaemia are indicative of irreversible cellular damage and tissue necrosis (38). As purine release occurs from viable cells under ischaemic stress it was considered purines may be useful as an earlier indicator of cellular ischaemic stress that the current tests.

Canine studies (66, 67) measured changes in adenosine, hypoxanthine and inosine in cardiac interstitial fluid via microdialysis catheters implanted into left ventricle myocardium. In the first study regional myocardial hypoxia was induced by perfusing the left anterior descending artery with deoxygenated venous blood. This allowed systemic SaO_2 to remain normal so changes in purine concentrations were solely in response to reduced myocardial oxygen supply. A significant rise in adenosine was only identified in dialysis catheters primed with an EHNA containing stopping solution. Dialysate hypoxanthine and inosine increased in a similar fashion to adenosine. In a second study, reactive hyperaemia was prevented with blood flow being restricted to normoxic levels in periods of hypoxia, preventing extra O_2 delivery with extra blood flow. This induced a rise in adenosine greater than that when a normal physiological response to hypoxia in coronary blood flow was permitted (14% vs 53% increase $p<0.05$). In this study levels of hypoxanthine and inosine were elevated on administration of exogenous adenosine deaminase that also caused a fall in adenosine. These findings were replicated in HPLC measurements of canine pericardial fluid by Kekesi et al (68) during periods of coronary artery spasm induced by intracoronary administration of endothelin-1 (ET-1).

Porcine experimental models have been used widely as their coronary circulation closely mimics humans. Unlike in canine hearts, occlusion of a single vessel induces ischaemia due to a lack of adequate collateral circulation. Studies of coronary venous and arterial purines after ischaemia induced by a snare or clamp around the left anterior descending artery all

produced similar results. De Jong (69) recorded significant increases in inosine and hypoxanthine in venous samples during ischaemia but arterial samples obtained at the same time points remained at base values. Backstrom et al. (70) divided pigs into cohorts undergoing varying lengths of cardiac ischaemia and analysed samples obtained from the great cardiac vein, external jugular vein and pulmonary artery. Results indicated the only significant rise occurred in samples from the great cardiac vein. Rises in hypoxanthine were related to duration of ischaemia, +150% at 15 minutes ($p<0.01$ vs baseline) and +415% at 45 minutes of ischaemia ($p<0.001$ vs baseline). A plateau was demonstrated after 45 minutes of ischaemia followed by a further rise within the first 15 minutes of reperfusion peaking at +590% against baseline ($p<0.001$). This plateau phase may indicate complete depletion of mitochondrial ATP stores followed by a degree of reperfusion injury on restoration of flow. This late rise has been demonstrated in other experimental settings (see later). Wikstrom (71) identified an initial rise in adenosine after 15 minutes of ischaemia in interstitial myocardial dialysate this rise was not sustained and declined to pre-ischaemic levels after 50 minutes of ischaemia. Hypoxanthine and inosine were elevated during the whole of the ischaemic period. Ramani et al. (72) set out to identify differences in purine metabolism with increasing age in rats. They found older rats showed significantly faster ATP depletion and a significantly lower adenosine rise than in younger rats. A changing profile of nucleoside release with increasing age would be an important factor when considering interpretation of purine concentrations as an investigation in a clinical setting.

1.4.3 Global hypoxia

There has been significant interest in purines as a biomarker against a background of global hypoxia alongside conventional markers. It was hypothesised that purines may be a more specific biomarker as both lactate and base deficit may be elevated in pathological conditions other than hypoxia (53).

Initial canine studies into the relationship between purine changes and hypoxia revealed very little change in arterial concentrations but did show significant central venous increases, subsequently high xanthine oxidase activity was found in canine lungs promoting degradation of hypoxanthine to uric acid (73) thus accounting for low arterial concentrations. Unlike canines rats, pigs and humans lack lung xanthine oxidase (74). Rodent studies have shown a mean arterial adenosine increase from 79.2±12.7μmol/l to 190.2±32.2μmol/l on induction of

hypoxia (FiO_2 8%)(75). Saugstad and Assen (74) also recorded significant rises in arterial hypoxanthine in pigs with severe hypoxia (FiO_2 7%). Conde (20) investigated adenosine release from rat carotid bodies, the primary mammalian chemoceptor involved in response to hypoxia, during progressively decreasing oxygen partial pressures (10%, 5% and 2% FiO_2). Adenosine release was positively associated with increasing duration of hypoxia, a 31% increase at ten minutes' vs 44% increase at 30 minutes ($P<0.0001$) In addition, it was also demonstrated that increasing severity of hypoxia induced greater adenosine release from the Carotid artery endothelium.

1.4.4 Skeletal Muscle and Peripheral Tissues

Skeletal muscle and other peripheral tissues are relatively more resistant to ischaemic injury when compared to myocardium or cerebral tissue (62, 76). It has also been hypothesized that tissue injury occurs both during ischaemia and on reperfusion by oxygen free radical production in energy depleted cells (77, 78). Much research has been performed into adenosine and the purine nucleosides as regulatory and retaliatory substances in peripheral ischaemia, comparatively less work has been undertaken as purines as marker of ischaemia and/or tissue damage. Ihnken et al (77) induced acute hind limb ischaemia in pigs followed by restoration of flow to simulate femoral artery embolectomy and found uncontrolled reperfusion lead to a greater drop in ATP with subsequent increased purine nucleosides when compared to graded reperfusion over a number of minutes. Mo and Ballard (79) measured arterial, venous and interstitial adenosine changes in the gracilis muscle of anaesthetised dogs in hypoxia and during exercise (by direct electrical muscle stimulation) and found venous concentration of adenosine significantly increased 350±52ηmol/l to 518±107ηmol/L during hypoxia whereas interstitial adenosine significantly increased during exercise. There was no change in arterial purines during hypoxia or exercise.

Progression to Purinergic role in human ischaemia

1.4.5 Cerebral Ischaemia

There have been several studies into ischaemic production of purine nucleosides *in vivo* in humans. Initially these used carotid endarterectomy (CEA) as a model of controlled reversible, reproducible cerebral ischaemia. Weigand et al (80) measured changes in ipsilateral jugular venous adenosine and hypoxanthine in patients undergoing CEA with a general anaesthetic (GA). They demonstrated significant rises in adenosine and hypoxanthine

when compared to time-matched peripheral arterial samples in patients exhibiting loss of somatosensory evoked potentials (SSEP) during interruption of carotid blood flow (229-338nM). Loss of SSEPs suggests inadequate collateral blood supply, rendering the cerebral hemisphere ipsilateral to the CEA ischaemic. No significant changes were noted in patients where SSEPs were maintained. Tian et al (6) found a 2.8-fold increase from baseline in median peripheral arterial purine concentrations in patients undergoing CEA with loco-regional anaesthesia (LA) regardless of shunt use (2.4μM-6.7μM). A subgroup of patients within this study lost consciousness soon after carotid clamping and required shunt insertion. In this group baseline purines were significantly higher than those not requiring shunt insertion and shunt use was associated with a fall to below baseline levels of purines.

Elevation in purine nucleosides in patients presenting to secondary care with radiologically confirmed stroke, when compared with healthy controls, has been demonstrated using the same biosensor as used in the Tian et. al. study. Mean baseline purines were 7.1±4.2μM in controls and 11.6±8.9μM in stroke patients (81). This elevation was universal to mild moderate or severe stroke, graded by the national institute of health stroke scale. The same group also noted a correlation between baseline purines and early neurological deterioration in stroke patients (82). Prior to this, Laghi-Pasini et al (83) conducted a study measuring peripheral venous adenosine daily in patients presenting with clinically confirmed transient ischaemic attack (TIA) or stroke. They found adenosine was significantly raised on presentation to hospital and continued to rise, peaking on day two and day three of admission before declining to a stable level by days five and 15 post event respectively.

Purine release from the brain during ischaemia is not in doubt. Whilst ischaemia induced during CEA is reversible and milder than in stroke, peak adenosine was higher in the CEA study of Tian than that of stroke patients in the Pasini study (6.7μM vs 290nM). The discrepancy in results between these studies is likely to be related to advancement in detection methods i.e. increasing sensitivity in the detection of adenosine by electrochemical enzyme assay compared to HPLC. Peak adenosine in the Pasini and Weigand studies are comparable (290nM vs 229nM) and used similar analysis techniques. The CEA studies identify purine release is rapid following onset of ischaemia with rapid return towards baseline values with restoration of flow. Prolonged elevation identified in stroke patients may ether be representative of greater degree of injury or ongoing ischaemia as there was no

intervention to restore cerebral perfusion in any of the patients studied. The pattern of purine release in the patients requiring shunt insertion in the study of Tian et al lends support to other work in rodents (59) that identified raised baseline purine concentrations in subjects with chronically ischaemic tissues compared to those with normal tissue perfusion.

Carotid endarterectomy carries a 2% procedural risk of stroke (84). Large scale meta analyses have been unable to demonstrate superiority of routine vs selective shunting to protect against hypoperfusion injury of the brain during CEA (85) and current methods of intraoperative cerebral monitoring have not been proven effective (86). Stroke remains a clinical diagnosis via the face, arm speech test. Larger scale studies in stroke patients are needed as a rapid reliable peripheral blood marker may serve to expedite diagnosis and therefore treatment in stroke patients where early intervention can be critical to limiting ischaemic injury and improving neurological outcomes (87). The amperometric biosensor used by Tian et. al. and Dale et. al. study allows analysis in real time from whole unprocessed blood. Results above would suggest that purines are a sensitive marker of ischaemia that can be detected by peripheral sampling of whole blood and, with further investigation, may be able to offer a simple clinical test to assess cerebral perfusion in a range of settings.

1.4.6 Cardiac ischaemia

Current gold standard cardiac enzyme tests (Troponin I and troponin T) are indicative of irreversible cellular damage and tissue necrosis (38). As purine release occurs from viable cells under ischaemic stress it was considered purines may be useful as an earlier indicator that the current tests. Early human studies identified significant elevation in coronary venous inosine and hypoxanthine during rapid atrial pacing and exercise in patients with coronary artery disease after the onset of anginal chest pain. These purine changes were not replicated in patients without angina symptoms or in those with cardiac failure (88-90). These findings suggested purine release is specific to ischaemic myocardium rather than a general response to increased metabolic demand. Bardenheuer et al. (91) went on to identify purine rise is proportional to duration of coronary artery occlusion (30, 60 or 90 seconds) in patients with coronary artery disease undergoing percutaneous transluminal angioplasty. Farthing et al. (13) recently developed a chemilluminescence method of testing to identify rapid purine changes in venous plasma in patients presenting to the emergency department with non-traumatic chest pain. They found significantly elevated inosine and hypoxanthine in peripheral venous sampling in patients with confirmed myocardial infarction (MI).

This study is unique in validating the findings of the previous experimental models in a clinical setting with direct application to myocardial infarction. Furthermore, their novel method of testing for purines was able to appreciate significant increases via peripheral venous sampling with a short analysis time. Combined, these two factors offer promise for testing of purines as a useful biomarker. A summary of the findings of the discussed studies is shown in Table A.

Study	Year	Sampling	Patient group(s)	Mean baseline purine	Mean peak purine
Fox et al.	1974	Coronary venous effluent	Patients under investigation of coronary artery disease. Rapid pacing	Unable to detect	400nmol/l Unable to detect in non-angina sufferers
Remme et al.	1976	Coronary venous effluent	Patients under investigation of coronary artery disease. Rapid pacing	Hypoxanthine 0.8nmol/l	Angina sufferers: 2400nmol/l * No change in non-angina sufferers
Kugler et al.	1979	Coronary venous effluent	Angina sufferers only: Rapid pacing & exercise	Inosine: 575nmol/l Hypoxanthine: 1180nmol/l	Pacing: Inosine: 1510nmol/l* Hypoxanthine: 1640 nmol/l Exercise Inosine: 1120 nmol/l * Hypoxanthine:1995 nmol/l *
Bardenheuer et al.	1994	Coronary venous effluent	Patients with known coronary artery disease. Balloon occlusion of left anterior descending coronary artery.	Adenosine: 176nmol/l	Coronary vessel occlusion 30sec: 326nmol/l* 60sec: 531nmol/l* 90sec: 793nmol/l*
Farthing et al.	2011	Peripheral venous blood	Patients presenting to emergency department with chest pain.	Not given	Not given

*Table A: Summary of studies reporting purine release during cardiac ischaemia. For ease of interpretation given values have been standardised to nmol/l. *=statistical significance*

1.4.7 Systemic hypoxia

Following studies identifying large rises in arterial adenosine during induced systemic hypoxia in rodent and porcine studies (20, 74, 75) human studies have been less conclusive. Tuchschmid et al. (92) failed to demonstrate any significant increase in venous hypoxanthine during hypoxia of simulated 6000m altitude (FiO_2 10%) or during hypoxia combined with exercise. Saito et al. (34) measured peripheral adenosine concentration changes during normoxia and moderate hypoxia (SaO_2 80%). Significant rises in adenosine were observed at 20 minutes of hypoxia although this was only noted in participants commenced on an intravenous dipyridamole infusion, acting as an adenosine reuptake inhibitor, prior to induction of hypoxia. Maclean et al. (26) measured interstitial hypoxanthine from catheters inserted between fibres of the vastus lateralis muscle in healthy males at normoxia and periods of hypoxia (FiO_2 10.5%). Adenosine levels doubled from baseline within 15 minutes and remained elevated as hypoxia continued.

There are methodological differences to account for the discrepancy between the human and animal studies. The degree of hypoxia induced in the animal studies was more severe and resulted on the death of the study subjects (FiO_2 below 10% compared to SaO_2 maintained at 80% by Saito et al). Increases following pre-treatment with dipyridamole indicates purine release does occur in humans but there may be systemic reuptake of nucleosides confounding analysis of study samples. From this it could be concluded that demonstrable purine release may only be achieved at a level of systemic hypoxia that it is not possible to replicate safely in humans under experimental conditions. If this is the case in clinical practice, pre-existing clinical measures, such as peripheral saturation probes or arterial blood gas analysis, are currently available to quickly and safely identify patients who are hypoxic.

1.4.8 Exercise

Patterson et. al. (93) identified significant increases in venous hypoxanthine during exercise from peripheral venous samples in healthy adults undergoing forearm exercise testing with a grip dynamometer. Two further studies reported similar results in “semi ischaemic” forearm testing whereby a tourniquet on the upper arm was inflated to mean arterial pressure (MAP) before exercise was started (29, 94). Hellsten et al. (95) demonstrated increases in interstitial fluid adenosine are proportional to intensity of exercise

1.4.9 Peripheral arterial disease

Sørlie et al. (96) demonstrated a rise in popliteal venous hypoxanthine on exercise in both patients with peripheral arterial disease (PAD) and healthy controls. However, the PAD patients had significantly higher basal hypoxanthine than the controls and exhibited a similar rise as the controls with less strenuous exercise. Similarly, Rexroth et al. (97, 98) studied changes in arterial and venous hypoxanthine in patients with peripheral arterial disease (PAD) compared to healthy subjects during exercise. Venous hypoxanthine levels increased significantly during the recovery period post exercise in the PAD group compared to no change in the control group, again after undertaking less strenuous exercise.

1.4.10 Surgery

Karg et al. (99) investigated purine metabolism in ischaemic tissue in patients undergoing total knee arthroplasty (TKA) with the use of a lower limb tourniquet inflated to twice systolic blood pressure, which serves to make the limb ischaemic. They sampled both venous effluents from the operated leg and from an antecubital upper limb vein. Results demonstrated a peak hypoxanthine in the femoral vein samples 5 minutes' post release of the tourniquet. Elander et al (76) analysed biopsies of microvascular free transfer flaps prior to dissection, during ischaemia and after flap reperfusion. Mean hypoxanthine concentration increased seven-fold from baseline at 1 hour of ischaemia.

The multitude of studies clearly identify purine release from peripheral tissues under physiological stress from hypoxia, exercise, ischaemia or surgery. Purine release has been demonstrated to be sustained during ischaemia and proportional to degree of cellular metabolic stress. The study of Karg et al is of interest as it demonstrated a peak purine concentration after tourniquet release representing restoration of outflow from the limb on reperfusion. Peak nucleoside concentrations were also significantly higher compared to the other studies discussed. The mean ischaemic time was 85 minutes; this was a significantly longer period of ischaemia than the studies using an upper arm tourniquet. Purine release from surgically "damaged" tissue may also be a confounding factor in these results, though no samples were obtained from tissue distal to the tourniquet whist it was inflated to assess evolution of purine release in relation to stage of procedure. In the Rexroth study there was no intervention to induce ischaemia therefore the delay in purine nucleoside peak may be representative of reduced inflow to the limb and therefore delayed efflux. As in the CEA

studies, there was again the finding of increased resting purine nucleosides in those with confirmed chronic tissue ischaemia. Study results are summarised in Table B.

Study	Year	Subject	Hypoxia/ischaemia	Baseline purine	Peak purine
Patterson et al.	1982	Peripheral venous sample during forearm exercise	Exercise only	1.58μmol/l	25.2μmol/l* exercising arm 7.8μmol/l non-exercising arm
Sorlie et al.	1982	Lower limb venous hypoxanthine in PAD and healthy controls	Exercise only	Not given	Higher resting hypoxanthine in PAD* 2.4-fold increase* during exercise
Bolhuis et al.	1988	Peripheral venous sample during forearm exercise (with tourniquet)	Arm tourniquet inflated to MAP	5.85μmol/l	12.1μmol/l*
Rexroth et al.	1989	Lower limb venous hypoxanthine in patients with PAD	Normoxic exercise 20 minutes	4.02μmol/l	5.04μmol/l*
Maclean et al.	1992	Interstitial fluid from vastus lateralis	Hypoxia: 30 minutes at FiO_2 10.5%	0.44 μmol/l	1.03μmol/l*
Elander et al.	1994	Tissue biopsy of free transfer flaps	pre, intra and post flap transfer	Not given	100% increase in inosine after 1hr of ischaemia*, return to baseline within 1hr of reperfusion
Karg et al.	1997	Lower limb and upper limb venous hypoxanthine in surgery with a tourniquet)	Lower limb surgery with tourniquet at 2x MAP	Not given	100-fold rise at 5 minutes post tourniquet release*
Helsten et al.	1998	Interstitial fluid from vastus lateralis	Normoxic exercise 10watts power	0.22μmol/l	1.14μmol/l
Tanaka et al.	2003	Peripheral venous sample during forearm exercise (with tourniquet)	Arm tourniquet inflated to MAP	Not detected	26.1μmol/l*

*Table B: Summary of results of studies reporting purine nucleoside changes during peripheral ischaemia or hypoxia. For ease of interpretation all results have been standardised to μmol/l. *=statistical significance.*

1.4.11 Other tissues and clinical applications

There has been work carried out into purine release in other tissues, though comparatively less than the work described above.

Saugstad et al have published extensively on the clinical application of purine nucleoside measurements in the perinatal period (14, 73, 74, 100, 101). They identified significantly elevated placental and CSF hypoxanthine in perinatal hypoxia and cerebral haemorrhage however, the results were confounded by significant overlap with normal infants (102). As maternal hypoxanthine is known to increase during labour, with potential for transfer to the foetus, the significance of these results has been disputed (103).

It has been hypothesised that purines may be used as a marker of renal transplant graft function and survival (104). Studies have shown an increase in purine concentrations during renal transplantation, in samples obtained from the graft renal vein (105, 106). This increase was linear in relation to time of ischaemia and returned to baseline on restoration of perfusion in the transplanted kidney. Whilst purines are sensitive for renal ischaemia in this context, purine rise during transplantation failed to correlate with future decline in graft function. No studies to date have examined purine changes in grafts known to be failing.

Work was started into the potential for purines as an early biomarker of intestinal ischaemia as serum lactate levels may only be raised late in the disease process. Akgur (107) examined changes in peritoneal purine concentrations in guinea pigs with induced intestinal ischaemia. No significant elevation of hypoxanthine was noted despite increases in lactate. This was attributed to xanthine oxidase activity within the peritoneal cavity. No analysis of blood samples to examine serum changes in purines associated with intestinal ischaemia was undertaken.

Purine release in response to ischaemia and hypoxia has been studied in many contexts. Results have demonstrated high sensitivity for ischaemia in a variety of tissues placed under varying degrees of ischaemic or hypoxic stress. Early attempts at purine assessment were hampered by limited understanding of the complex role of purine nucleosides *in vivo*, technological limitations in obtaining samples, prolonged analysis techniques and complex analysis equipment. Advances such as chemiluminescent and amperometric biosensors can now offer relatively simplified analysis techniques and rapid ascertainment of results from

unprocessed biological samples. These studies indicate there is potential for assessment of purine nucleosides to be a clinically useful and cost-effective point-of-care biomarker of ischaemia with potential to improve and expedite diagnoses ultimately leading to improved patient outcomes following a variety of ischaemic events. Currently their use in clinical practice remains in its infancy. The studies described are small single centre studies that are heterogeneous in sampling sites and techniques with differing target tissues and experimental aims. In addition, these studies were mainly performed under strict experimental conditions. Given this, their results may not be directly transferable into clinical practice. Significant future work is required before purine nucleosides can be used routinely in clinical practice.

1.5 Potential application of purine nucleosides within clinical practice

Ischaemia is often a medical emergency. Adequate oxygen delivery to tissues is essential to maintain normal cellular activity and therefore organ function. Tissues deprived of blood (and therefore oxygen) have a finite period within which ischaemia can be tolerated, after which cells will undergo apoptosis and necrosis (108). Though this "critical" ischaemic time varies between tissues, prolonged ischaemia may in turn lead to a cascade of sequelae including temporary or permanent organ dysfunction the clinical significance of which will vary significantly dependant on tissue or organ involved. Restoration of blood flow to the affected area as quickly as is safe and practical is paramount to minimising the deleterious effects of ischaemia in many situations (109).

Delay in intervention to re-establish tissue perfusion may be secondary to failure to recognise ischaemia, diagnostic uncertainty or diagnostic delay (110, 111). Many modalities of investigation already exist for the detection and diagnosis of ischaemia. Direct clinical assessment remains the cornerstone of diagnosis in any clinical scenario though the sensitivity of clinical signs can be variable and reliability of clinical examination may be variable dependant on the experience of the clinician assessing the patient (112, 113). Non-specific indicators such as serum lactate can be used as markers of ischaemia but results can be confounded by sepsis, shock or lactate metabolism (114). Organ specific blood tests are often late markers of end organ dysfunction (troponin assays in suspected myocardial infarction are indicative of myocyte death (115) and thus significant myocardial damage occurs prior to elevations of cardiac enzymes in serum). Radiological assessment via computer tomography (CT) or computer tomography angiography (CTA) of a vascular disease or occlusion leading to ischaemia is widely and routinely available in secondary care

but can result in delay and carries risk in the form of iodine-based contrast and radiation (116). In some situations, arterial duplex is of clinical value in ischaemia but results are operator dependant and specialised vascular ultrasound is not universally available.

Scenarios also arise where the usual clinical signs of ischaemia are attenuated or absent completely, i.e. a sedated or anaesthetised patient or in patients unable to communicate due to illness or trauma. In addition to this, in surgical specialities, vascular control or temporary interruption of blood flow to tissues is necessary in order to operate safely or ischaemia is a recognised as a significant risk of a surgical procedure, for example spinal cord ischaemia following thoracic aortic aneurysm repair (117). Whilst precautions are taken to minimise time of interruption of flow and to ensure any collateral flow is preserved where possible, unrecognised or late recognition of ischaemia may result in poorer outcomes for patients.

There remains a place for a simple, reliable, rapid test that could be used as either an aid in diagnosis of ischaemia, intra- or post-operative monitoring where there is potential for ischaemic complications. In conjunction with the University of Warwick, Sarissa Biomedical (Warwick, UK) have developed SMARTchip, an amperometric biosensor with the potential to detect purine nucleosides from whole, unprocessed blood with results obtained in a time frame in the order of minutes. This in turn opens the possibility of purine nucleosides being a clinically useful biomarker. Details of sensor mechanism of action, development and validation are discussed later.

Encouraging results were obtained using an earlier developmental version of SMARTchip (SMARTcap) (6). This study identified significant purine nucleoside changes in peripheral arterial blood samples in line with cerebral ischaemia secondary to interruption and subsequent restoration of carotid blood flow in patients undergoing carotid endarterectomy with local anaesthetic. To build on these results a series of prospective observational cohort studies using SMARTchip were designed to assess purine nucleoside use in a wider range of clinical scenarios where ischaemia and the risks associated with ischaemia are a necessary intraoperative step or ischaemic complications as a result of disease process, intervention or injury may occur.

2. Rationale for choice of current study

2.1 Phase one - Systemic Sampling

2.1.1 General Anaesthetic Carotid Endarterectomy

Neurological complications of Carotid endarterectomy (CEA) caused by embolism of plaque material from the operative site or cerebral hypoperfusion during carotid cross clamping combined with inadequate collateral perfusion can cause significant neurological morbidity (118). Procedural risk of stroke has been a source of much debate, currently it is quoted at around 2% (84, 119, 120). Many preoperative and intraoperative cerebral protection strategies have been postulated in an attempt to identify those at risk of hypoperfusion cerebral injury. Pre-operatively, radiological assessment of collateral flow can be undertaken by computer tomography (CT) or magnetic resonance (MR) angiography. An incomplete circle of Willis is associated with increased incidence of neurological events (121, 122) however sensitivity of radiological imaging drops significantly as vessels decrease in size (123). Intra operatively, local anaesthetic affords clinicians the luxury of direct neurological assessment of the awake patient (124), decline in neurological functioning can be easily identified at an early stage by assessing response to commands or questions, therefore whilst a purine nucleoside concentration change in local anaesthetic (LA) CEA is of interest, its usefulness in clinical assessment of cerebral adenosine triphosphate (ATP) metabolism is limited.

Local anaesthetic procedures are not desirable in every patient. The requirement for the patient to lie still with the neck extended and rotated away from the surgeon for a prolonged period of time can be challenging and this can be compounded by challenging soft tissue, neurological or arterial anatomy. In these situations, general anaesthetic (GA) is preferable. Many mechanisms of monitoring cerebral perfusion during GA exist currently including electroencephalography (EEG), somatosensory and motor evoked potentials (SSEP), transcranial Doppler (TcD), carotid stump pressure monitoring, and cerebral oximetry. None of these modalities have been universally accepted or validated in clinical practice as all have drawbacks in application and interpretation of results intraoperatively (86).

The gold standard method to ensure adequate ipsilateral cerebral blood flow intraoperatively is insertion of a temporary shunt. Shunting involves the use of a plastic tube inserted into distal and proximal ends of the carotid arteriotomy during the endarterectomy and patch (if used) phase of CEA procedures. The shunt is inserted after opening the carotid artery and removed prior to completion of closure of the vessel. Shunts allows ipsilateral carotid blood flow to be maintained throughout the procedure with only very short duration of interruption of ipsilateral blood flow during shunt insertion and removal. Some would advocate routine shunting in all patients undergoing GA CEA to negate risk of hypoperfusion-ischaemia injury. However, the intraoperative use of shunts is not without risk of air or plaque embolism and vessel wall endothelial trauma which may cause neurological morbidity in the perioperative period. Published large scale pooled and meta-analyses have failed to clearly advocate superiority, in terms of reduction in neurological events, of either selective (based on pre or intra operative assessment of ipsilateral cerebral perfusion), routine or never shunting strategies (85, 125, 126). This may indicate a proportion of shunts inserted are unnecessary, exposing patients to risk (126). SMARTchip potentially may offer a rapid, simple intraoperative test to identify evolving cerebral ischaemia during GA CEA and thus those patients at risk of neurologic injury due to hypoperfusion and thus require shunt insertion.

2.1.2 Abdominal Aortic Aneurysm: open and endovascular repair

Abdominal aortic aneurysm (AAA) repair is defined as major plus surgery and is undertaken in high risk a population of patients. As such, mortality rates are approximately 4% following elective open surgical repair (OSR) and 1% following endovascular aneurysm repair (EVAR) (127). Mortality rates following repair after rupture are significantly higher. In addition to this, there is potential for significant complications and thus morbidity in the perioperative period.

During OSR cross clamping of the infra-renal aorta is mandatory in order for graft implantation. This clamping renders the pelvis and lower limbs ischaemic as well as interruption of inflow to the descending and sigmoid colon via the inferior mesenteric artery and inferior and middle rectal arteries for a period of time until completion of graft implantation. Whilst the inferior mesenteric artery is universally sacrificed during aneurysm repair and collateral flow via the marginal artery is relied upon, ischaemic complications in the form of renal, gut or lower limb ischaemia represent a significant proportion of surgical

complications necessitating unplanned interventions or re-interventions (128, 129) either during the index procedure or in the perioperative period. This may be due to loss of a native vessel with inadequate collateral supply, thrombosis-in-situ of the graft or native vessels or embolism to distal vessels on reperfusion. Whilst there is no formal vascular "clamping" in a traditional surgical sense during EVAR, ischaemic complications are well recognised. Endograft position may be suboptimal and inadvertently completely occlude or shutter renal or visceral vessels (130-132). There is also potential for displacement or kinking of endografts resulting in thrombo-embolic complications there is potential for this as early or late phenomena (133). There is also repeated short duration arterial occlusion during endograft deployment and moulding. Ischaemia of lower limbs intra-operatively due to insertion of large calibre (16f or greater) introducers into the common femoral vessels has also been reported (134).

Significant biochemical stress response to AAA surgery has been demonstrated in the form of perioperative increases in inflammatory cytokines, glucocorticoids, cortisol and oxygen free radicals which may represent a degree of ischaemia-reperfusion injury (135, 136) and increases in purine nucleosides have been identified in serum pre-operatively in patients awaiting AAA repair when compared to age and sex matched controls without AAA (137, 138). Perioperative changes in purine nucleosides as a marker of intraoperative ischaemic cellular stress, including differences between OSR and EVAR, or as potential markers of ischaemic complications of aneurysm surgery have not been studied.

2.1.3 Peripheral arterial disease and critical limb ischaemia

Peripheral arterial disease (PAD) is thought to affect more than 200 million people worldwide (139). A significant proportion (between 20 and 50%) of patients with PAD are asymptomatic or symptoms are masked by concomitant disease (cardio-respiratory or musculoskeletal) affecting their ability to walk (140, 141). Classical symptoms of intermittent claudication (IC) such as reproducible, consistent exercise induced lower limb discomfort and fatigue are only present in a small number of patients, diagnosis can be confounded by non-arterial lower limb pain such as neuropathy, arthritis of the spine, hip or knee, vasculitis or venous insufficiency (142). Development of critical limb threatening ischaemia (CLTI) with either rest pain, night pain or development of ischaemic ulcers or gangrene has been reported at 7.5% of patients within one year of referral to vascular services with intermittent claudication (IC) and has an annual incidence 0.35% (143, 144).

Untreated, PAD can result in significant morbidity due to lifestyle limiting claudication, limb loss or ultimately death (145). Patients with PAD require aggressive lifestyle adjustment and medical management of co-morbidities including cardiovascular risk factors to prevent disease progression (146). Despite this literature cites up to 25% of patients with IC will progress to need intervention with a primary amputation rate of between 40 and 70% at five years after diagnosis (147-149). Decision making around when it is most appropriate to intervene can be challenging for clinicians. Both surgical and radiological intervention are not without risk, with potential to worsen ischaemia and/or accelerate the necessity for amputation (150, 151). As such, intervention is commonly reserved for those with CLTI (152).

Historic work has identified ongoing abnormal, elevated purine release in venous samples from chronically ischaemic tissue (59, 98) and specifically in PAD patients during exercise, compared against non-PAD controls. No work has been undertaken to assess the correlation of PAD severity and/or disease progression and purine nucleosides or effect of revascularisation on purine release from chronically ischaemic tissue. These factors must also be correlated with clinical outcomes.

2.1.4 Microvascular free tissue transfer flap

Free tissue transfer flap procedures, only possible with the advent of microvascular anastomotic techniques, have greatly expanded reconstructive options available to surgeons and patients. Common clinical applications include following major traumatic injury and radical oncological resection to provide soft tissue coverage of deep structures, restore anatomical form and in some cases restore function (153). Transplantation of tissue based around its own vascular pedicle by definition requires a primary period of ischaemia between excision and implantation. Prolonged primary ischaemia has been postulated to play a role in ischaemia-reperfusion injury of the flap and poorer long-term outcomes i.e. flap survival (154-156). A secondary ischaemic insult to the flap can occur postoperatively, this can be caused by arterial or venous occlusion and again may threaten viability of the flap in part or as a whole (157).

Overall rates of flap success are excellent, but identification of a potentially ischaemic flap relies on clinical assessment. This can be difficult and confounded by clinician experience,

temperature, anaemia, use of coloured skin preparation solutions and availability of equipment such as Doppler ultrasound. Identification of an ischaemic flap is especially difficult if a significant proportion of the flap is positioned subcutaneously under native skin (158, 159). A peripheral serum biomarker of evolving flap ischaemia may be of clinical use where there is clinical uncertainty regarding perfusion of a flap intra or post-operatively. Significant elevation in purine nucleosides have previously been identified in interstitial fluid samples and in tissue biopsies during primary flap ischaemia and initial reperfusion (1hr post revascularisation) (76, 160).

2.1.5 Renal transplantation

In 2018, 3272 renal-only transplants were performed in the UK. Despite this only 61% of patients receive a transplant within three years of being added to the waiting list (161). Whilst five year transplant survival rates for living and deceased donors are 93 and 86% respectively the shortage of donated organs compared to potential recipients highlights the need to ensure every possible measure is taken to maintain and improve function and survival rates in the long term.

As with free tissue transfer flaps a period of renal ischaemia during transplantation is unavoidable. This occurs in the kidney between completion of nephrectomy in the donor and completion of arterial anastomoses in the recipient. This time can vary significantly dependent on relative locations of donor and recipient (i.e. living related/unrelated donors where nephrectomy and transplant can take place almost in parallel vs deceased donors where significant time may be needed for transport), type of donor (the need for cross-matching and tissue typing in deceased donors), theatre availability and technical and anatomic challenges intraoperatively (162). Investigation into the effect of ischaemia on transplant outcomes has been extensively studied (163-166). This has led to development of protocols to minimize ischaemic time that include therapeutic hypothermia (packing the kidney in ice) and dedicated preservative solutions (167) all aimed at decreasing ischaemia stress to the kidney resulting in improved function and longevity.

Studies in both animal models and human transplanted kidneys have demonstrated rises in purine nucleosides in renal vein samples during transplantation (104-106). This has then been extrapolated to potentially be a marker of renal ATP stores failing to meet metabolic demand

despite measures taken to reduce energy expenditure during transplantation. This purine rise has yet to be correlated to function or survival.

The aim of these studies is to replicate the published LA CEA study and investigate changes in systemic (arterial) purine nucleosides in response to iatrogenic ischaemia in populations of patients undergoing surgery to provide proof-of-concept data that purine nucleosides are of clinical value in situations where an early biomarker of ischaemia would potentially be of clinical interest.

2.1.6 Mixed "non-ischaemic" controls

Given all of the studies described above are to be undertaken in populations of patients undergoing procedures where periods of tissue ischaemia are a necessity. Participants undergoing a variety of general anaesthetic procedures without an ischaemic component were recruited to act as "non-ischaemic" controls in order to investigate changes in peripheral serum purine concentrations related to physiological stress associated with surgery without superimposed ischaemia.

2.2 Phase two: Local Sampling

In addition to, and based on the results of, the first phase of SMARTchip studies, a second phase of studies was designed to investigate variation in purine nucleoside concentrations locally within a given ischaemic area. As SMARTchip has the capability to determine purine nucleoside concentrations from extremely small sample volumes there is potential for the use of capillary blood, obtained via a finger prick (or equivalent) to provide adequate blood volumes for analysis. The advantage of this is that it extends the applicability of SMARTchip beyond that where a facility for venous blood sampling exists. Capillary blood sampling is easily achieved in community and pre-hospital settings. It is regularly used in the monitoring of blood glucose levels in diabetic patients and requires minimal training to perform. Clearly direct sampling for cerebral, aortic or renal ischaemia is not practical or possible, therefore, these studies focused on limb ischaemia as samples could be obtained easily directly via toe prick samples with time-matched finger prick samples as non-ischaemic comparators.

2.2.1 Acute Limb Ischaemia

Acute limb ischaemia (ALI) has been defined internationally as "any sudden decrease in limb perfusion causing a potential threat to limb viability" can be caused by embolus, situ thrombosis or trauma and is a surgical emergency (168). It remains a common presentation to secondary care with an incidence of 10-16 per 100 000 population per annum. Mortality rates of 9-22% have been reported with three month amputation free survival of only 59% (169). Timely recognition and prompt intervention are paramount to avoiding sequelae of ischaemia such as reperfusion injury secondary to free radical formation, compartment syndrome, limb loss and death (170).

Traditionally ALI was diagnosed by the presence of "the six p's" – pain, pallor, perishing cold, pulselessness, paraesthesia and paralysis. In clinical practice this sextet of signs and symptoms can be present or absent in varying degrees and primary assessment may often be carried out by non-vascular specialists. This may result in subtle changes in a limb not being appreciated (111, 171). Palpation of peripheral pulses and Doppler ultrasound evaluation is often performed and interpreted poorly with significant inter-observer variability, potentially providing false reassurance (113). This can lead to diagnostic uncertainty and delay. In the current hub-spoke vascular network design, a patient with suspected ALI may present to services away from vascular specialists. Acute ischaemia leading to limb loss is a significant cause of litigation against the NHS with delay in diagnosis stated as a significant contributory factor in actions reported by the NHS litigation authority (111).

Previous work (99) has identified elevation in purine nucleosides in venous washout following a period of acute limb ischaemia. A simple test of peripheral blood marker to identify patients suspected of having ALI would be of use to non-vascular specialists such as general practitioners (GPs), allied healthcare professionals such as paramedics and emergency department clinicians in order to expedite appropriate triage and referral to specialist secondary services which may in turn lead to improved patient outcomes in terms of morbidity and mortality associated with ALI. This study investigates the use of purine nucleosides as a biomarker of ALI in an experimental model.

2.2.2 *PAD & Critical Limb Ischaemia*

In a similar fashion to the phase one systemic sampling of patients with CLTI, this study aims to characterise perioperative and medium-term changes in serum purine concentrations in patients undergoing lower limb revascularisation.

The key difference between this and the previous study is blood sampling will be directly from the affected limb via toe prick samples. As previously stated, elevation in purine nucleosides has been documented in samples obtained from the popliteal vein in PAD patients at rest and whilst undertaking exercise (98). The pattern of purine release following definitive revascularisation has not been studied although logic would lead to the hypothesis that once adequate flow to meet oxygen demand has been restored purine metabolism should cease and mechanisms for normal cellular energy production would be restored.

The aim of this study is to evaluate local purine changes within critically ischaemic limbs and the effect of surgical revascularisation. In comparison to this study and the critical limb ischaemia arm of the dynamic control group in this arm the range of surgical procedures considered eligible has been narrowed significantly to include only femero-popliteal, femero-distal and popliteal distal bypass grafts. The effect of this is homogenisation of the study cohort compared to the systemic CLTI study and offers the ability to study purine nucleoside changes in a single limb in isolation.

2.3 Non-surgical populations

2.3.1 Traumatic brain injury

Approximately 1.4 million people attend hospital following head injury in the UK every year (172). Outcomes following traumatic brain injury (TBI) remain poor (173). Primary injury triggers a number of secondary inflammatory cascades that are yet to be fully understood. These include pro-inflammatory cytokine release, formation of oxygen free radicals and excitotoxin release (174-176). This can lead to blood brain barrier (BBB) dysfunction, cellular swelling, vasogenic and cytotoxic cerebral oedema and neuronal apoptosis and necrosis. These pathological processes create phenomena of either localized or generalized cerebral hypoxia and ischaemia. Untreated, both hypoxia and ischaemia have been shown to be associated with poorer neurological outcomes following TBI (177).

Whilst this cohort of patients differs significantly in terms of diagnosis and potential intervention strategies from the other study cohorts. The mechanism of cerebral injury is similar to both carotid endarterectomy and previous studies in patients suffering stroke with cerebral ischaemia a predominant factor in neurological injury, thus it was felt that there is potential to deploy purine nucleosides in the evaluation of patients who have suffered TBI.

Given that presentation to the emergency department following a fall is common (178), significant head injury following a fall is relatively uncommon (179). The consequences of unidentified head injury can be catastrophic. Increasing population age (180) combined with increasing use of antiplatelet and anticoagulation agents (181) provides a "perfect storm" scenario whereby patients at increased risk of falls are also at increased risk of intra cranial bleeding. In an emergency department (ED) setting the most effective mechanism for diagnosis of TBI is CT imaging of the head. Anecdotal evidence suggests a significant proportion of CT imaging requested following suspected head trauma do not reveal any intracranial injury (unpublished observations). It would seem logical these scans have been requested based on mechanism of injury and bleeding risk factors rather than clinical signs and symptoms of TBI.

Numerous biomarkers have been studied in an attempt to diagnose and predict severity and/or outcomes following TBI. S100 calcium binding protein b and glial fibrillary acidic protein have been shown to correlate well with acute traumatic radiological lesions and clinical outcomes (182-184). Expansion of their use has been limited in clinical practice by the need for specialist processing techniques meaning analysis times are relatively prolonged and prohibitively expensive. Purine nucleosides have also previously been studied in the context of TBI. Significant transient increases in adenosine, inosine and hypoxanthine have been identified in experimental models and in patients with TBI (185, 186).

Potential remains for a simple, reliable test to identify those at risk of TBI following head trauma. Pre-hospital point-of-care testing to best triage patients appropriately or ED point of care testing to guide further investigation and management are both potential applications of SMARTchip. This study was designed to assess peripheral serum adenosine changes using SMARTchip in patients with radiologically confirmed TBI.

3. Methods 1 – Design and validation of the biosensor

3.1 Amperometric Biosensors

There is much interest in the field of biosensors and their application in clinical medicine (187). Their role focuses on the rapid recognition of a given substance (e.g. physiological metabolite or drug) that can allow snapshot measurements or continuous monitoring *in vivo*. An ideal biosensor can respond continuously, and reproducibly over wide concentrations of a given analyte. The analyte should also be able to be detected in a variety of biological fluids such as blood, plasma, sweat, cerebrospinal fluid or saliva (188, 189). Sensor activity must be robust with the ability to be maintain sensitivity over time, pH and changes in temperature (190). Increasing automation in production has allowed miniaturisation of components and production of sensors sufficiently robust to be used outside of the controlled environment of research laboratories. This combined with their inherent ability to provide rapid results increases their usefulness in clinical medicine.

3.1.1 Mechanism of action

An electrochemical biosensor allows recognition of a biological compound and conversion, via an electrode transducer, into an electrical signal that can then be interpreted as a decimalised number. Two common types of electrochemical biosensors are amperometric and potentiostatic (188).

Amperometric biosensors use oxidation of an electroactive substrate, namely hydrogen peroxide (H_2O_2). Oxidative enzymes specific to the analyte in question are encapsulated in a matrix, forming a thin layer, surrounding a working electrode. The reaction catalysed by the enzyme produces H_2O_2. This H_2O_2 is then oxidised by initiating and maintaining a potential between the working electrode and a reference electrode. The current generated will increase with increasing concentrations of the substrate as more H_2O_2 is produced (Figure 2: oxidation of a substrate to liberate H_2O_2). This current can be converted into a numerical value with relation to a reference electrode via a transducer and appropriate software. Selection of the working electrode is dependent on its reactivity to H_2O_2 commonly used working electrodes are platinum (Pt), gold (Au), carbon and metalized carbon (190). Reference electrodes are commonly silver (Ag) or silver chloride (AgCl)(191).

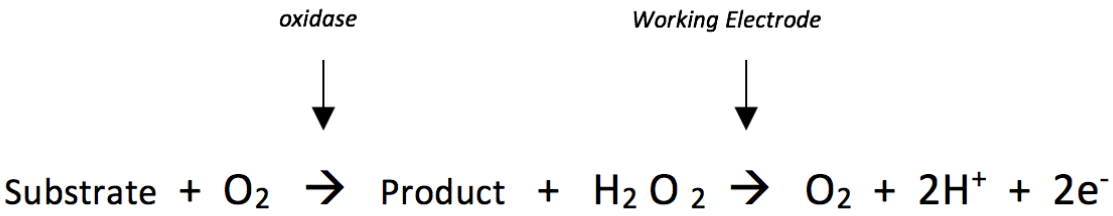

Figure 2: Oxidation of a substrate to liberate H_2O_2

In order for oxidation of H_2O_2 to be detected oxidative enzymes must be held locally to the surface of the working electrode. This occurs due to trapping of the enzymes in a biologically inert matrix applied to the surface of the electrode. Various mechanisms have been attempted to achieve this such as adsorption, solvent casting, covalent binding and electropolymerisation (192). Sol-gel matrices have achieved much attention in recent times, this is a process whereby small (nanometer sized) inorganic particles are suspended in a liquid then deposited onto the desired surface (the electrode). They have increased in popularity due to their tuneable porosity (the ability to manipulate the matrix structure to limit particle passage through its structure), thermal and chemical stability and biocompatibility (192, 193). Commonly used sol-gels include silica and titanium (192, 194).

Another mechanism for achieving reliable and reproducible sol-gels is via electrodeposition of pyrrole derivatives (195, 196). An electropolymerisable monomer solution containing a known quantity of a desired enzyme is oxidised via a current generated at the electrode surface. This leads to precipitation and polymerisation of the solution to create layers of matrix, entrapping enzymes on the electrode surface (195). Repeated current cycling leads thicker layers of matrix deposited. This is advantageous as additional enzyme is available to catalyse substrate. Although it has been shown that large increases in matrix depth may cause the matrix to delaminate from the electrode and can cause a decrease in signal due to increasing distance of the reaction with H_2O_2 from the electrode surface (190).

3.1.2 Interfering actions and reactions

In order to create a sensor of sufficient sensitivity a relatively high anodic potential can be needed (>0.6V) (46), This in turn can lead to interference. *In vivo* electroactive substances of a similar molecular weight to the substrate in question such as ascorbic acid, uric acid and paracetamol. These have potential to cause interference as they may be oxidised at the working potential of the biosensor, thus amplifying the signal from the sensor that will lead to overestimation of the result obtained. Biofouling and electrode passivation are further potential sources of inaccurate results. The concept of biofouling refers to large molecules such as cellular debris, proteins or whole cells as well as naturally occurring reactions within biological fluid (such as coagulation in blood) that impede or prevent analyte diffusion to the biosensor surface. Passivation occurs when small molecules adhere to the sensor surface, decreasing its active surface area. Both will lead a decrease in in sensor reaction to a given concentration of analyte (197).

Artificial electron mediators are now used to lower the working potential required for reactivity to occur thus negating the effect of interfering substances (46, 198). An additional benefit of electron mediators is the biosensor is no longer oxygen dependant (197, 198) and therefore sensitivity is maintained where oxygen tension is decreased. An ideal electron mediator allows direct electron transfer whilst not altering sensor response time and remain chemically stable in both oxidised and reduced forms (188). Commonly used electron mediators belong to the family of ferrocyanides (197) such as Prussian Blue (PB) or its analogues; palladium hexacyanoferrate, chromium hexacyanoferrate and cupric hexacyanoferrate as they have strong electrocatalytic action towards H_2O_2, and exhibit a poor response to coexisting oxidizable substances (199).

3.2 Development of the Purine Specific Biosensors

An amperometric biosensor (SMARTchip, Sarissa biomedical, Coventry, UK) was developed and refined over a number of years to be sensitive specifically to purine nucleosides formed following the metabolism of adenosine triphosphate (ATP). These are namely adenosine, inosine and hypoxanthine. SMARTchip has the ability to analyse samples of unprocessed whole blood in real time with the aim of developing a test that can be used at point-of-care in a clinical environment.

3.2.1 Design and Manufacture

The current biosensor utilises Ruthenium Purple (RP), another analogue of PB, as an electron mediator (46) Unlike PB, RP's electrochemical properties are unaffected by cations commonly found *in vivo* such as sodium (Na^+), hydrogen (H^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) (6). The biosensor is then able to be reliably used as a sensor in Na^+ based physiological fluids *in vivo.*

Firstly, a gold (Au) electrode (to become the working electrode) has to be coated with RP as an electron mediator. The electrode is pre-treated in 1M sulfuric acid and polarised between 0 and 1.5V at a sweep rate of 50mV/s to form a gold oxide (Au_2O_3) coating. The electrode is then cycled at 0.1V to 1.0V at a rate of 50mV/s in a mixture of iron chloride ($FeCl_3$), potassium hexacyanoruthenate ($K_4Ru(CN)_6$) and potassium chloride (KCl). Finally, RP is electrodeposited onto the pre-treated gold electrode from an acidic (pH 2) solution of ruthenium chloride ($RuCl_3$) in cycles at the same voltages and sweep rate. At approximately

30 cycles, two well defined redox peaks are noted with further cycles decreasing the peak current indicating complete coverage of the Au with RP (46).

The second step in sensor formation is deposition of the sol-gel, enzyme containing matrix onto the pre-treated electrode. To form the solution; acidic solutions of hydrolysed silanes (inorganic compounds of silicon and hydrogen such as tetramethoxysilane and (3-aminopropyl) trimethoxysilane) are formed then added to a buffer solution to partially neutralise the pH (47). The enzymes; adenosine deaminase, nucleoside phosphorylase and xanthine oxidase (1U of each) are added individually to 10µ/l of the neutralised solution (198). The working and null electrodes are introduced to each of these enzyme containing solutions in turn and polarised to between -900 and -1200 mV for between ten and 40 seconds (46). This leads to precipitation and polymerisation of the solution to form a uniform, thin layer of gel matrix entrapping enzymes around the working electrode. Null sensors can also be produced by replicating the process in solutions not containing enzyme. Sensors are then washed in pure water to remove any unbound enzyme (46).

Current SMARTchip design incorporates four sensors per chip. As a blood sample is introduced onto the sensor it is polarised for 180 seconds. The values for the final ten seconds for each electrode are averaged to give an integer for each active electrode. Raw results give a total of four readings (one for each sensor), these can then be averaged to provide a single final value.

3.3 Validation of sensor design

Details of sensor design, development and validation of its performance have been published in detail previously (6, 46, 47, 198).

3.3.1 Sensitivity and selectivity

Performance of the biosensor was analysed in a flow system for 1 hour with 40µM of ATP in a phosphate buffer (pH 7.4, NaCl 0.1M). Response of the sensor was linear from 0.3-80µM ATP, after which response plateaued resulting in sensitivity to ATP of 126nAµM^{-1}cm^{-2}. Response to hypoxanthine was analysed by adding increasing concentrations of hypoxanthine to phosphate buffer. Response was linear from 0.2-40µM hypoxanthine with a sensitivity of 334nAµM^{-1}cm^{-2}.

3.3.2 Sensitivity in the presence of confounders

Sensor performance was tested in the presence of potentially interfering substances found *in vivo* (Substances with a similar molecular weight to ATP that may competitively inhibit sensor performance). To test sensitivity to ATP, buffer solutions were prepared with concentrations of 40μM ascorbic acid, dopamine and paracetamol. No interfering signals were detected on testing with addition of 40μM ATP at -50mV. Sensitivity to 40μM hypoxanthine was tested in samples prepared with addition of 100μM 5HT, 100μM ascorbic acid, 1mM paracetamol and 1mM uric acid, again no interfering signals were detected. These results infer the use of RP as an electron mediator allow sensitivity and selectivity of the biosensor to be active at sufficiently low potentials as not to be affected by physiological substances or drugs *in vivo.*

Response of the sensor in changing oxygen saturations was achieved by testing performance in saline solutions firstly saturated with 95%O_2 and 5% CO_2 and secondly with 95% N_2 and 5% CO_2 (47). The reduction in current in total absence of O_2 was 3.2±1.3% of the total response meaning sufficient responses were maintained in order for SMARTchip to function in low oxygen (O_2) scenarios such as ischaemia. Such low oxygen partial pressures will not be encountered *in vivo*.

3.3.3 Effect of pH on sensor characteristics

Significant variation in pH may be encountered *in vivo* during ischaemia as acid-base homeostatic mechanisms become overwhelmed. The biosensor could be affected by pH disturbance either via the electron mediator or by denaturation of the entrapped enzymes. Sensitivity of response of the biosensors to 10μM hypoxanthine and 10μM ATP respectively was tested at increasing pH of the buffer solution from 6.8 to 8.0 Response was stable between 7.0 and 8.0. pH outside of this range is extremely unlikely to be encountered in samples of physiological fluids (46).

3.3.4 Longevity of sensor

Another source of potential confounding results would be sensor degradation due to instability of the sol-gel or decreasing enzymatic activity over time. This was found not to be the case, 95% sensitivity was maintained after three months of dry storage at 4^{o}C (198).

3.3.5 Sensor performance in blood

The developed biosensor was validated for use in physiological fluids by adding known quantities of purines to samples of fresh, unprocessed whole blood and spun-down plasma. Recovery percentages were 54% and 88% for the whole blood and plasma respectively (6). It is well known that purines nucleosides are inherently unstable in whole blood with a half-life of seconds (21). The discrepancy between the values is due to uptake of purines by erythrocytes to be re-phosphorylated to ATP and/or degradation to urate/uric acid by enzymes on the cell membrane of red blood cells (200).

3.4 Sampling procedure and result validation

3.4.1 Sampling technique

SMARTchip is designed as a single use testing mechanism. Each measurement of purine nucleosides requires an individual SMARTchip to be calibrated prior to adding the blood sample to be analysed. First a buffer solution containing 10μM adenosine is added to the chip ensuring the whole of the sensor area is covered. A polarising cycle is run in order to calibrate each individual SMARTchip against a known concentration of purine. A null washing buffer is then added and polarised to clear the sensor of the adenosine buffer. Finally, the blood sample for analysis can be deposited onto the sensor surface. The volume of blood required to cover all four sensors is approximately 10-15μl.

Dependant on sampling method, transfer of blood from site of sampling to chip can be achieved in various ways. Arterial line blood samples, blood can be directly dropped onto the chip from a syringe. If using capillary blood samples, blood can be transferred via a micropipette and dropped onto the chip or dropped directly from the site of sampling if possible.

After the analysis polarising cycle is run specialist software can interpret the differences in current generated between the adenosine buffer and the blood sample and present this as a decimalised number. Once the analysis cycle is complete the sensor can be discarded.

3.4.2 *Pitfalls of sampling*

After production SMARTchips are dried and stored at 4°C. On addition of the first adenosine buffer adequate time must be allowed for rehydration of the biosensor, this need only be in the order of seconds. This can be achieved by adding the first buffer solution to the chip prior to entering chip and patient details into the analysis software, time taken to complete this is more than adequate for sensor rehydration.

Second, the biosensors on the chip are unprotected and physical contact to the sensor can damage the sensor surface meaning results may not be generated or results produced are unreliable. Care must be taken to avoid any contact with the sensor surface, buffer solutions and samples for analysis can be dropped from a small height above the sensor, avoiding formation of bubbles within the fluid on the sensor surface. Bubbling appears to only occur with blood samples and is likely due to its higher viscosity than the buffer solution. Bubbles within the analysis sample can have a confounding effect on results as there is the possibility of the sensor surface not being covered, i.e. contained within a bubble, despite seemingly complete sensor coverage.

3.4.3 Validation of individual results

Currently, each individual result requires manually validating before results can be interpreted. This is a three-step process. First, the analysis software produces a faradaic charging curve of the current against time for both of the active sensors and both of the null sensors during analysis and the final current readings should be between -20 and -30nA. Curves should be smooth, with little deviation. Samples exhibiting unexpected curves or positive currents were rejected. The second stage involves reviewing the raw data. Each sensor calibration factor must be between -1 and -10 μM/nA. This indicates the SMARTchip is calibrated properly and is able to give a valid result. Finally, the sample difference, i.e. the difference in current between the null and active sensor should always be a negative integer, this can be of any value. Only if these three conditions are met can the final value obtained be considered valid. As previously stated, each SMARTchip contains four sensors. Results from each sensor have to be validated individually. The final purine concentration in μM is obtained from the mean of all valid results obtained from the SMARTchip. Given each SMARTchip has four sensors there is a degree of latency built in and a reading can still be obtained if one or more of the sensors fails validation. If no valid result is obtained from any

of the four sensors the result must be discarded. This may mean some data points will be missing as results appearing valid may be lost on retrospective data validation.

4. Methods 2 - Current study design and protocols

4.1 Ethics

Ethical approvals for the described studies were obtained from the West Midlands Research Ethics committee REC ref: 15/WM/0341 and 16/WM/0164. IRAS IDs: 189877 and 198854.

4.2 Funding Source

This work was funded by a National Institute for Health Research (NIHR) Invention for innovation (i4i) grant. Reference II-LA-0313-20002.

4.3 Study design

Phase one of this series of studies was a prospective, multi-arm, observational, cohort study to investigate potential clinical applicability of determination of systemic purine nucleosides and validate SMARTchip during ischaemia in both surgical cohorts of patients. These were namely:

1- Elective carotid endarterectomy (CEA) with a general anaesthetic (GA).
2- Elective non-cerebral surgery involving iatrogenic ischaemia including; abdominal aortic aneurysm (AAA) repair (both open and endovascular), renal transplantation, autologous free tissue transfer flap (deep inferior epigastric artery perforator (DIEP) flap), and surgery to treat critical limb ischaemia (dynamic controls).
3- Elective non-vascular surgery (non-vascular controls).

The second phase of study ran concurrently with phase one. This study was also an observational cohort study with two arms to assess purine concentration changes locally within the ischaemic tissue (i.e. within the lower limb) and the effect of revascularisation in patients undergoing:

1- Elective total or uni-compartmental knee arthroplasty with the use of a surgical tourniquet, Acute limb ischaemia (ALI).
2- Lower limb bypass surgery for critical limb threatening ischaemia (CLTI).

A similar third study assessed purine changes in non-surgical patients with radiologically confirmed traumatic brain injury (TBI).

These studies have commonality in terms of assessment and quantification of changes in purine nucleosides. Each sub arm was subtly different in terms of populations of patients, site and duration of ischaemia and as such each arm varied in terms of the details of its design.

4.4 Populations & data collection

The numerous study groups represented heterogeneous populations of patients undergoing elective surgical procedures, with the exception of the patients recruited to the traumatic brain injury study sub arm where presentation to hospital was emergency in nature.

Men and women aged 18 years and over, scheduled for elective carotid endarterectomy at University Hospital Coventry and Warwickshire (UHCW, Coventry) and University Hospital of the North Midlands (UHNM, Stoke-on-Trent), were recruited to take part in the CEA study. Non-vascular controls were recruited from men and women, aged 18 years and over, scheduled for surgery under a general anaesthetic without planned iatrogenic ischaemia at the same hospitals. These non-vascular controls were matched to CEA cases with respect to age and sex.

Participants recruited to the dynamic control arms were recruited into the relevant systemic sampling arm from UHCW only. Patients recruited to the local sampling CLTI and ALI arms were recruited from UHCW only. Surgical procedures in the ALI arm were carried out at both UHCW and the hospital of St Cross (Rugby).

Participants presenting with radiologically confirmed TBI following head injury were recruited from UHCW and St. Mary's Hospital (Paddington, London).

Participant recruitment and data collection in the GA CEA and non-vascular control sub studies was distributed between the researcher and a wider team of research nurses given multiple centres had been enrolled as recruiting sites. In the dynamic control, ALI, CLTI and TBI sub studies recruitment and data collection was performed solely by the researcher.

4.5 Recruitment & consent

Potentially eligible participants (men and women, aged 18 years and over) were identified and approached initially by a member of their direct healthcare team. Patients who express an interest in taking part were contacted by a member of the research team, who visited the

participants. For CEA participants, dynamic controls and local sampling CLTI participants' initial approach was made upon their presentation at their pre-assessment visit, typically between ten days and 48 hours prior to their scheduled surgery. In some cases, potential participants were approached to take part in the study as hospital inpatients if their presentation to secondary care was as an emergency and their surgery deemed as urgent or emergency in nature and is expedited. In this case the initial approach was made the day prior to the participants' surgery and consent taken on the day of surgery. Potential non-vascular control participants were approached at their pre-operative clinic, typically seven days prior to enrolment in the study. For logistical reasons local sampling ALI participants were initially contacted by telephone after their pre-operative assessment.

An appropriately trained member of the research team explained the study in detail and provided potential participants with a comprehensive participant information sheet (PIS) specific to the arm of the study into which they were suitable to be enrolled. This was via post (after initial telephone contact) for ALI participants followed by a second telephone call once suitable time had been allowed to receive and study the PIS. Following this, typically the day of or day prior to their surgery, patients were approached by the researcher to re-check their eligibility for the study and to obtain informed consent for participation. Those meeting eligibility criteria and agreeing to participate were formally enrolled into the study and assigned a study ID number.

Informed, written consent was sought from all participants prior to enrolment in the study. Consent was taken by a researcher who has been trained in the principles of good clinical practice (GCP) and the study consent procedures. Upon the participants first contact (in person or via telephone) with the research team, the researcher explained the study in detail and provide them with a comprehensive PIS specific to the study arm for which they are potentially eligible. Participants were encouraged to read the PIS, ask questions of the researcher and discuss their involvement with their friends, family and (where practical) their GP before deciding whether to take part. Potential participants were given a minimum of 24-hours to consider their involvement before they were approached to give consent wherever possible.

Potential participants were approached for informed consent when they present on the day of their surgery providing they meet the eligibility criteria. All participants were made aware that they are free withdraw their consent to participate and/or their data at any time without

giving a reason and that their decision to participate would not influence their current or future care in any respect. Participants were provided with a copy of the consent form; a second copy was stored with the participants' medical notes and a third copy was stored securely at each site.

The recruitment and consent processes for enrolment into the TBI sub-arm differed significantly from the other study arms. Given the emergency nature of their admission to hospital and time-sensitive nature of sample timing post injury. Attaining the first study sample took place immediately following consent, confirmation of eligibility and enrolment. Potentially eligible participants were identified by a member of their direct healthcare team upon the patient's admission to hospital or post confirmatory radiological imaging. If admission was designated "major trauma" the research team was be pre-notified via in house systems and attended the emergency department in readiness for patient arrival with the major trauma team. Initial approach was directly to the participants, provided the participants retained the capacity to provide informed consent in line with the Mental Capacity Act (201). Assessment of capacity was undertaken by the researcher in real time in conjunction with the direct healthcare team. If the participant lacked capacity to consent, consent was sought from persons attending with the patient acting as a consultee. If patients attended hospital alone and lack capacity to consent they were not approached for recruitment. With the participants' or consultees' agreement, a research team member visited the patient as soon as possible after admission to assess their eligibility to participate in the study. Patients that meet the eligibility criteria were enrolled into the study.

4.6 Inclusion and exclusion criteria

Given the heterogeneity of the populations recruited to each sub study there were number of universal inclusion and exclusion criteria and some sub-study specific inclusion/exclusion criteria for each subgroup within the study.

Systemic Sampling – inclusion criteria

CEA:

- Aged 18 years and over.
- Scheduled for carotid endarterectomy.

Dynamic controls

- Aged 18 years and over.
- Scheduled for:
    - Aortic aneurysm repair, either open or endovascular.
    - Critical limb ischaemia surgery. Eligible procedures include femoral endarterectomy, axillo-(bi)femoral bypass graft, Aorto-(bi)iliac or (bi)femoral bypass graft, femoral-femoral crossover graft, femoral-popliteal bypass and femoral-distal bypass graft.
    - Renal transplantation.
    - Autologous microvascular free tissue transfer flap surgery.

Non-vascular Controls

- Aged 18 years and over.
- Scheduled for surgery under a general anaesthetic.

Traumatic brain injury

- Aged 18 years and over.
- Admitted to hospital with radiologically confirmed traumatic brain injury.
- Time since injury <4.5 hours.

Local sampling- inclusion criteria

Acute limb ischaemia (ALI)

- Aged 18 years and over.
- Scheduled for elective Lower limb surgery with the use of a surgical pneumatic tourniquet: total knee arthroplasty or uni-compartmental knee arthroplasty.

Chronic limb threatening ischaemia (CLTI)

- Aged 18 years and over.
- Scheduled for lower limb arterial bypass surgery; including femoral-popliteal, femoral-distal or popliteal-distal bypass graft.

Exclusion criteria:

General

- Unable/unwilling to provide informed consent.
- History of myocardial infarction
- History of gout.
- Current chemotherapy.
- History of cerebral ischaemia (except for CEA patients).
- History of cancer (except for free flap patients).

Subgroup specific

- Time of injury >4.5hours (TBI only).
- History of peripheral vascular disease (ALI only).

4.7 Sample size justification

Sample size in the GA CEA and Non-vascular control studies was based on the observed differences identified in the previous LA CEA. Purine release in GA CEA, analysed using SMARTchip has not been studied previously.

The dynamic control, local sampling and TBI studies were designed to extrapolate the findings of the LA CEA study into varying clinical situations of ischaemia and serve to provide pilot data for future larger scale studies. It was decided to recruit ten patients into

each of the five dynamic control arms of the study, the two local sampling (ALI and CLTI) and the TBI studies.

4.8 Individual study procedures

4.8.1 General anaesthetic carotid endarterectomy

Surgical procedures

CEA was performed in standard format. The use of intra operative shunting was not dictated by the study design and remained at the surgeons' discretion. Method of assessment of the need for shunting varied between recruiting sites. One site routinely employed the use of transcranial Doppler ultrasound (TCD). Indication for shunting was >50% loss of middle cerebral artery velocity. At the second site, decisions around shunting were on a surgeon-by-surgeon basis. Decision making tools used included incomplete circle of Willis on pre-operative computer tomography or magnetic resonance angiography, poor back bleeding, low carotid stump pressures or haemodynamic instability on clamping. This variation in practice serves to highlight the current lack of objective evidence of methods of assessing adequacy of cerebral perfusion when considering carotid shunt insertion intraoperatively.

Blood pressure was regulated between a systolic pressure of 110 and 180mmHg using glyceryl trinitrate (GTN) or metaraminol in order to maintain to maintain a mean arterial pressure (MAP), to give an adequate cerebral perfusion pressure. Choice of anaesthetic agent was at the discretion of the anaesthetist. All participants were managed in a high dependency unit (HDU) setting post-operatively.

Sampling

Participant flow through the GA CEA study is shown in Figure 3. Blood samples for analysis were obtained at baseline prior to anaesthetic, post anaesthetic but prior to commencement of surgery, during carotid artery exposure, at carotid cross clamp application and in ten minute intervals whilst the carotid clamp is applied, on clamp release (cerebral reperfusion) and every subsequent ten minutes until completion of the procedure, 1-hour post clamp release and 24-hours post clamp release. If intraoperative carotid shunting was pre-planned or deemed necessary intraoperatively additional blood samples were taken every ten minutes whilst the shunt remained in situ.

Pre-operative samples were capillary blood obtained via a finger prick; intraoperative samples for analysis were obtained via an arterial cannula, inserted into the radial artery as part of routine care for peri- and intra-operative blood pressure monitoring. Arterial cannulae are routinely removed at around 24-hours post operatively if the patient's condition allows therefore the one-hour post clamp samples were obtained from this. If the arterial cannula was still in situ at the time of the 24-hour sample, then to avoid unnecessary finger pricks the sample was obtained from this. If the cannula had been removed prior to sampling a finger prick sample was obtained.

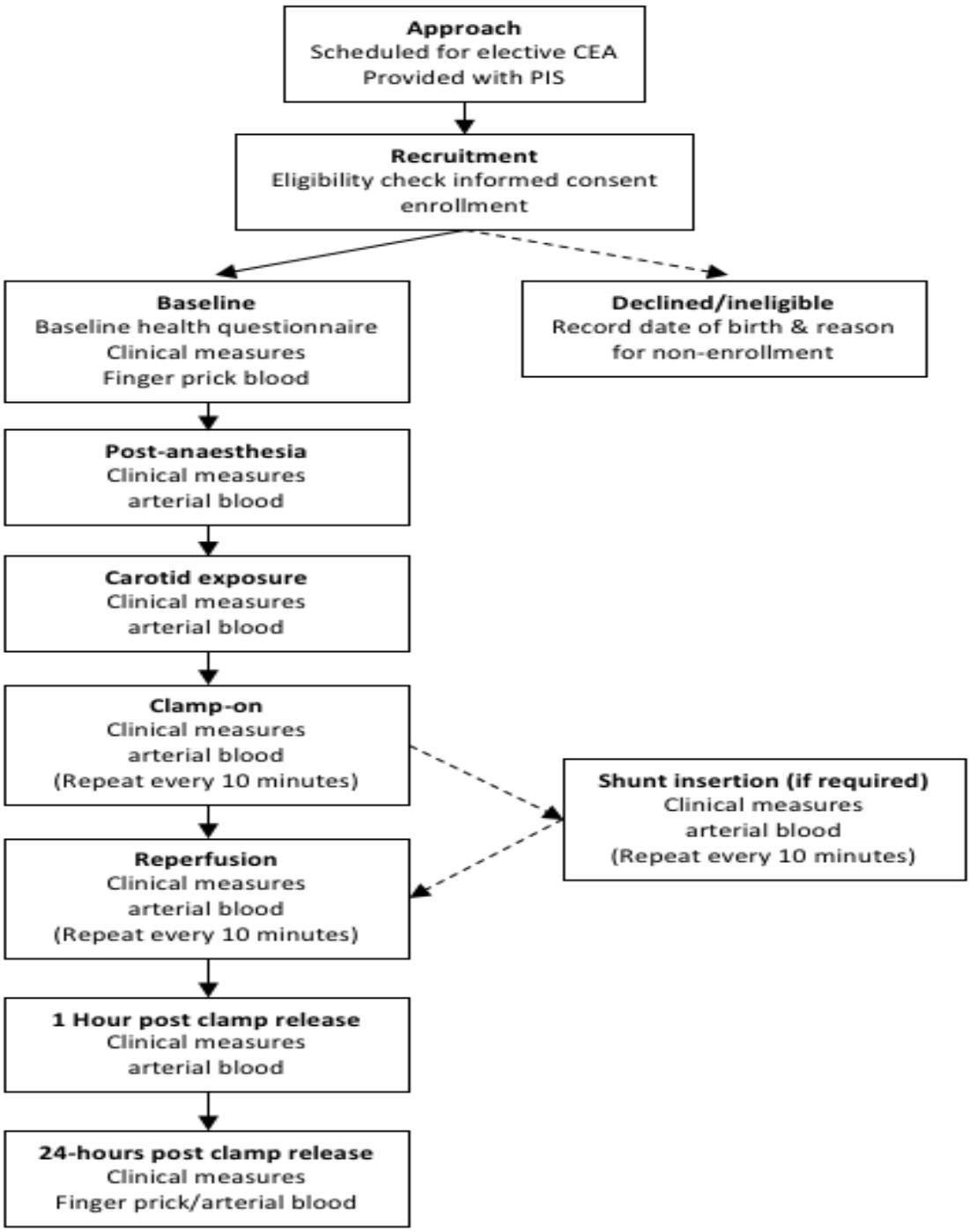

Figure 3: General anaesthetic carotid endarterectomy participant flow diagram.

4.8.2 Systemic sampling: dynamic controls

Each of the five dynamic control subgroups followed a similar sampling schedule to the GA CEA arm, with subtle variation to accommodate differences in patients, surgical procedures and duration of ischaemia. Participant flow through the "dynamic control" study is shown in Figures 4 & 5.

Abdominal aortic aneurysm repair

Surgical procedures

Elective infra renal aortic aneurysm repair including tube and bifurcated grafts were carried out using standard techniques. Supra-renal and further proximal top clamp positions were excluded as were ruptured, inflammatory and mycotic aneurysms. Endovascular repair of infra renal aneurysms was performed following standard procedure with a surgeon and interventional radiologist present. Fenestrated, branched, aorto-uni-iliac and iliac branch devices were not eligible to be recruited.

Sampling

For all aneurysm repairs blood samples for analysis were obtained at baseline prior to anaesthetic and post anaesthetic but prior to commencement of surgery. For open aneurysm repair intraoperative blood samples were obtained during aortic exposure, post aortic cross clamp (most proximal clamp) application, post aortic clamp release (after completion of distal vascular anastomoses and thus restoration of lower limb blood flow), one-hour post clamp release and 24-hours post clamp release. During EVAR intraoperative samples were obtained during femoral vessel exposure, during deployment of the main body of the graft after final graft moulding, 1-hour post completion angiogram and 24-hours post completion angiogram.

Pre-operative samples were capillary blood obtained via a finger prick; intraoperative samples for analysis were obtained via an arterial cannula, inserted into the radial artery as part of routine care for peri- and intra-operative blood pressure monitoring. Arterial cannulae are routinely removed at around 24-hours post operatively if the patient's condition allows therefore the 1-hour post completion samples were obtained from this. If the arterial cannula was still in situ at the time of the 24-hour sample, then to avoid unnecessary finger pricks the sample was obtained via this. If the cannula had been removed prior to sampling a finger prick sample was obtained.

Surgery for critical limb ischaemia

For all patients undergoing surgery for critical limb ischaemia (namely arterial bypass grafts or endarterectomy detailed in the inclusion criteria,) procedures were carried out in line with surgeon preference. The study did not impose any conditions on the procedure therefore there was variation in specific procedure, patient anatomy, choice of bypass conduit (vein vs prosthetic) and individual surgeon technique.

Sampling

Blood samples for analysis were obtained at baseline prior to anaesthetic and post anaesthetic but prior to commencement of surgery. Intraoperative blood samples were obtained during inflow artery exposure, post arterial clamp (most proximal clamp) application, post clamp release (after completion of distal arterial anastomosis and restoration of blood flow via the graft), one-hour post clamp release and 24-hours post clamp release.

Pre-operative samples were capillary blood obtained via a finger prick, intraoperative samples for analysis were obtained via an arterial cannula, inserted into the radial artery as part of routine care for peri- and intra-operative blood pressure monitoring. Arterial cannulae are routinely removed at around 24-hours post operatively if the patient's condition allows therefore the one-hour post clamp samples were obtained from this. If the arterial cannula was still in situ at the time of the 24-hour sample, then to avoid unnecessary finger pricks the sample was obtained via this. If the cannula had been removed prior to sampling a finger prick sample was obtained.

Microvascular free tissue transfer flap

All participants recruited to this arm underwent DIEP procedures with or without mastectomy as part of early or delayed breast reconstruction due to breast cancer. Blood samples for analysis were obtained at baseline prior to anaesthetic and post anaesthetic but prior to commencement of surgery. Intraoperative blood samples were obtained during isolation of the target vascular bundle around which the flap was to be formed, post arterial clamp application, post clamp release (after completion of distal arterial anastomoses and restoration of perfusion into the flap), one-hour post clamp release and 24-hours post clamp release.

Pre-operative samples were capillary blood obtained via a finger prick, intraoperative samples for analysis were obtained via an arterial cannula, inserted into the radial artery as part of routine care for peri- and intra-operative blood pressure monitoring. Arterial cannulae are routinely removed at conclusion of the procedure. Procedure duration after revascularisation of the flap (i.e. flap trimming, shaping and closure) dictated the mechanism of sampling for the one-hour post clamp samples. Sample timings were not adjusted to make use of the arterial cannula. If the arterial cannula was still in situ the sample was obtained from this if it had been removed, then a finger prick sample was taken. All 24-hour samples were obtained via finger prick.

Renal transplantation

Procedures

All transplant procedures were living donor (LD) transplants as these are performed on a largely elective basis. Recruitment of donation after circulatory death (DCD) or donation after brainstem death (DBD) donor transplants was not attempted due to their emergency nature; posing problems such as erratic procedure timing and truncated opportunity for and adequate consent process given clinical preoperative investigation that need to be undertaken by a transplant recipient pre-operatively especially when a preferable alternative (in LD transplants) exists.

Sampling

Blood samples for analysis were obtained at baseline prior to anaesthetic, post anaesthetic but prior to commencement of surgery, during iliac vessel exposure, at iliac artery cross clamp application, on iliac artery clamp release, one-hour post clamp release and 24-hours post clamp release.

Unlike the other dynamic control sub-arms, arterial line placement was not universally used intraoperatively. Where it was placed as part of with routine care, intra operative blood samples were obtained via the cannula. If an arterial cannula was not inserted capillary samples were obtained via finger pricks throughout.

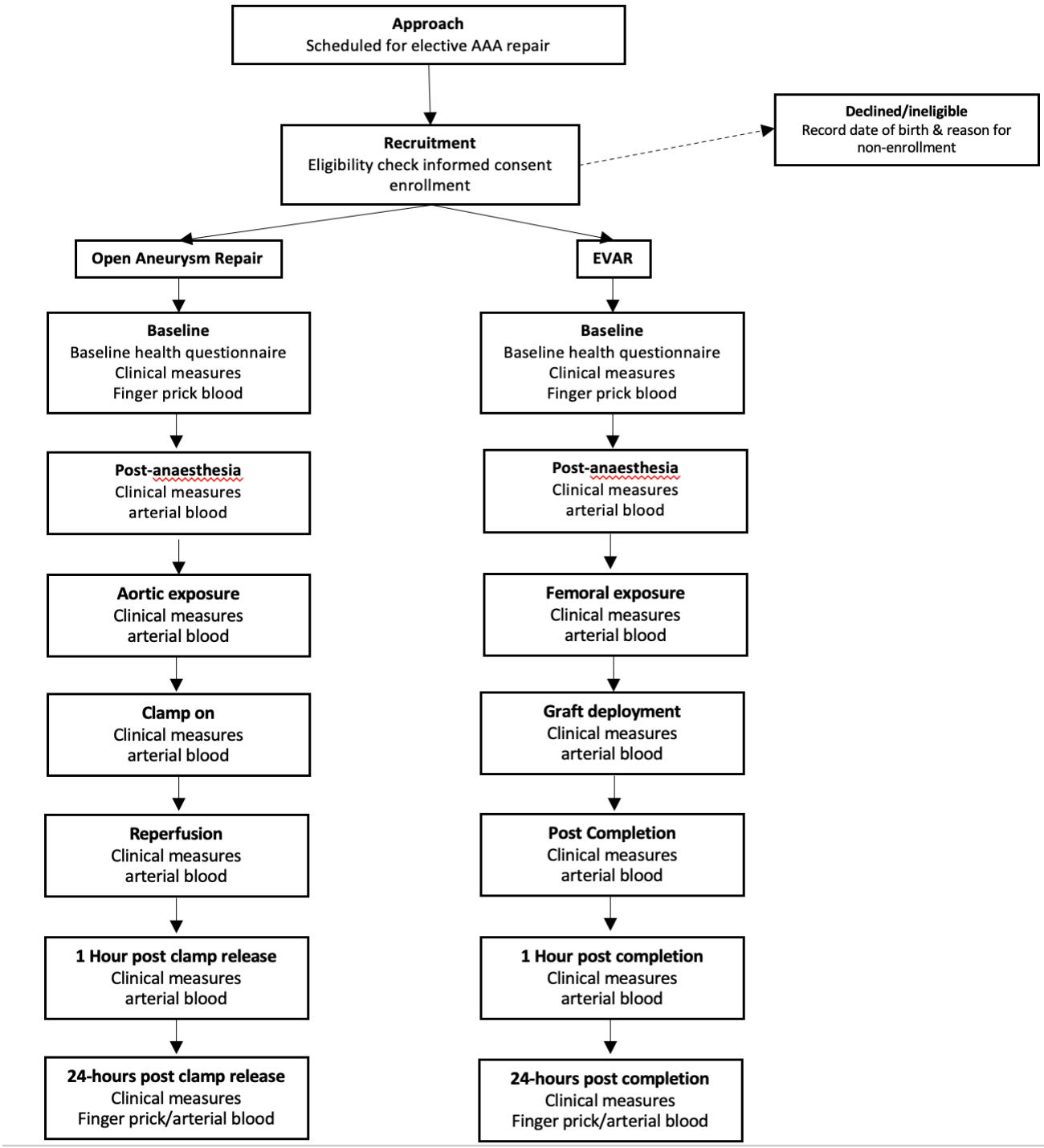

Figure 4 Participant flow diagram for dynamic control recruits undergoing aortic aneurysm repair (open and endovascular)

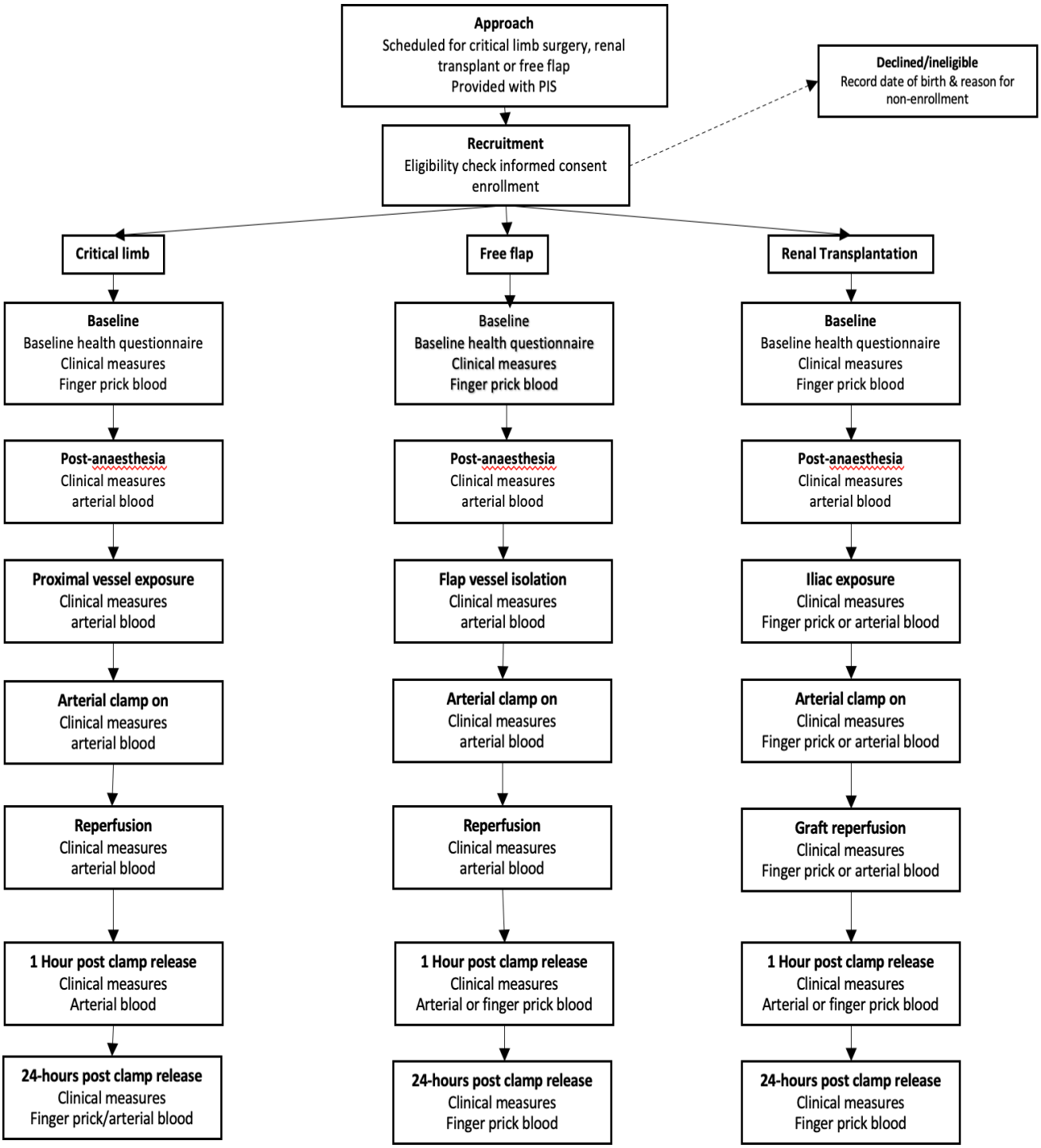

Figure 5 Participant flow diagram for dynamic control recruits undergoing surgery for critical limb ischaemia, renal transplant or microvascular free tissue transfer flap.

4.8.3 Non-vascular controls

These participants were recruited from mixed pre-assessment clinics and are thus heterogeneous in their nature. As there were no pre-defined intraoperative "ischaemic" points the sampling protocol was simplified to include: pre-operative baseline, post anaesthetic but prior to commencement of surgery, an approximate midpoint of the procedure as designated by the operating surgeon and at procedure completion. It was not anticipated that any of the procedures would require arterial cannula insertion therefore all samples were capillary blood obtained via finger pricks. Participant flow through the non-vascular control study is shown in Figure 6.

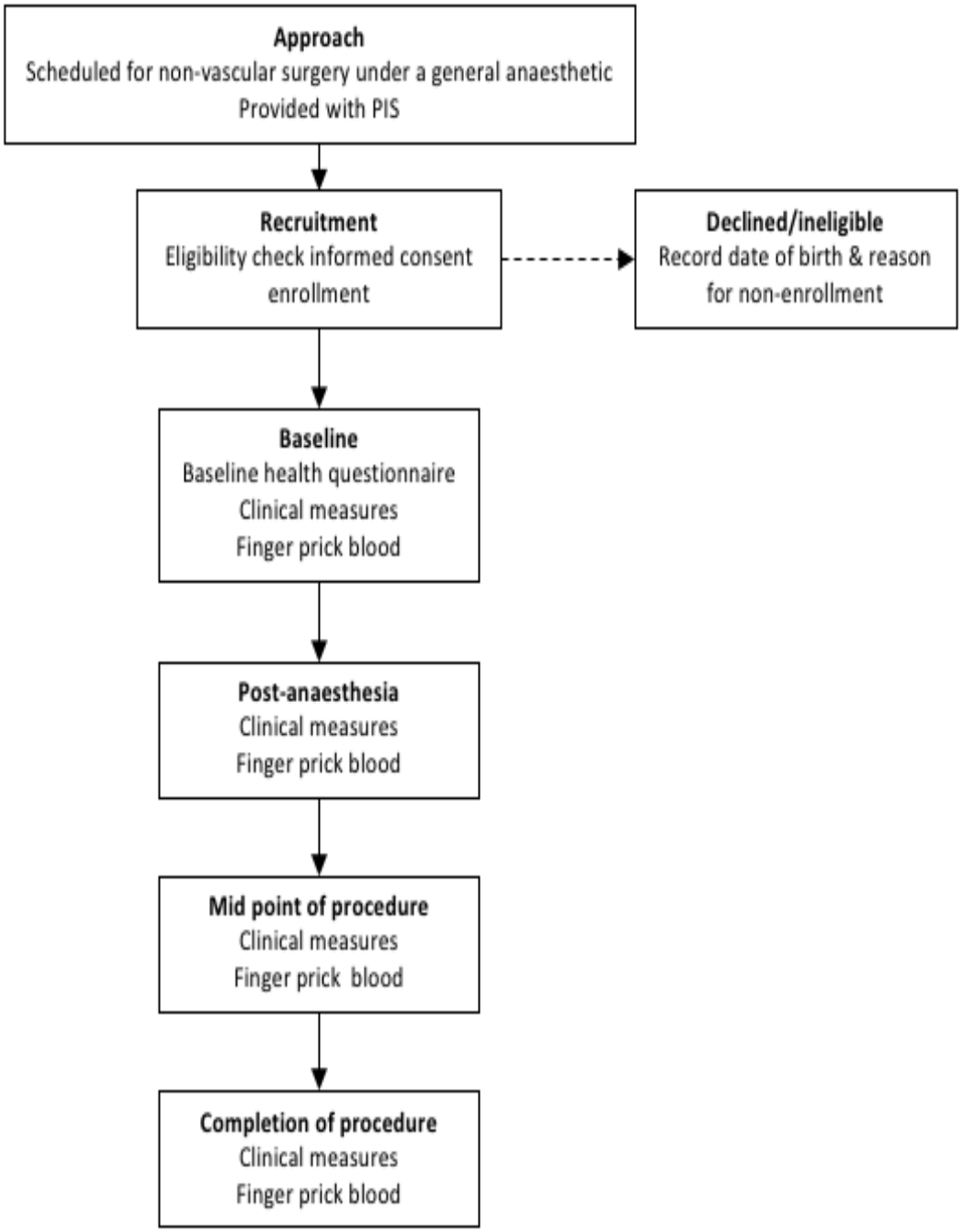

Figure 6 Non-vascular control participant flow diagram

4.8.4 Local sampling – acute limb ischaemia

Surgical procedures

Procedures included those performed under both general and spinal anaesthetic. All procedures were performed following standard arthroplasty technique by a single orthopaedic surgeon comprising of either total (TKA) or uni-compartmental (UKA) knee arthroplasty. Both cemented and uncemented prostheses were included. A pneumatic surgical tourniquet was inflated to 300 mmHg around the proximal thigh immediately after skin incision to completely occlude arterial inflow to the limb and render it ischaemic. This remained inflated throughout the procedure. Blood was not actively exsanguinated from the limb prior to tourniquet inflation.

Sampling

Samples were obtained directly from the operated limb pre, intra and post operatively with time-matched controls from capillary finger prick samples from the same subject. Due to availability of a single potentiostat, in order to allow accurate comparisons, the sequence of sampling limb sample first followed by finger sample was maintained throughout the study. In the operated limb pre-operative samples were obtained via capillary toe prick blood samples. The intra operative samples were obtained at set time points; immediately prior to tourniquet inflation, 20 minutes after tourniquet inflation, 40 minutes after tourniquet inflation and immediately post tourniquet deflation. Due to the need to maintain intraoperative sterility and the ability of the surgeon to manipulate the leg during prosthesis implantation the patients' foot was wrapped in sterile drapes and subsequently was not available as an intraoperative sampling site. The intra operative samples from the operated leg were obtained from blood within the surgical incision at appropriate sample time-points and analysed immediately. A final pair of capillary samples was obtained at 1-hour post tourniquet deflation.

4.8.5 Local sampling - critical limb ischaemia

Surgical procedures

Whilst this arm is very similar in nature to the dynamic control (critical limb threatening ischaemia) arm the breath of procedures included was narrowed to focus on a single limb. With regards to sample timings, at each time point samples were obtained directly from the operated limb with time-matched controls from capillary finger prick samples from the same

subject. Participant flow through the local sampling acute limb ischaemia (ALI) and chronic limb threatening ischaemia (CLTI) studies are shown in Figure 7.

Sampling

Pre-operative samples were obtained via capillary toe prick and finger prick blood samples. Intraoperative blood samples were obtained during most cranial (inflow) artery exposure, post arterial clamp (most proximal clamp) application, post clamp release (after completion of distal arterial anastomoses and restoration of blood flow via the graft). As in the ALI arm, due to the need to maintain intraoperative sterility and the ability of the surgeon to manipulate the leg during vein harvest (if necessary) and arterial anastomoses the patients' foot was wrapped in sterile drapes or placed within a sterile bag. This means the foot was not available as an intraoperative sampling site. The intra operative samples from the operated leg were obtained from blood within the most distal surgical incision at appropriate sample time-points and analysed immediately.

Post operatively samples were obtained one-hour post clamp release and 24-hours post clamp release, these samples, like the preoperative samples, were toe and finger prick samples. In this arm, one further sampling time point at the patients first surgical follow up appointment (approximately six weeks post operatively) was undertaken. Finger prick and toe prick samples were again obtained to evaluate the effect of reperfusion on chronically ischaemic tissue on purine beyond the perioperative period.

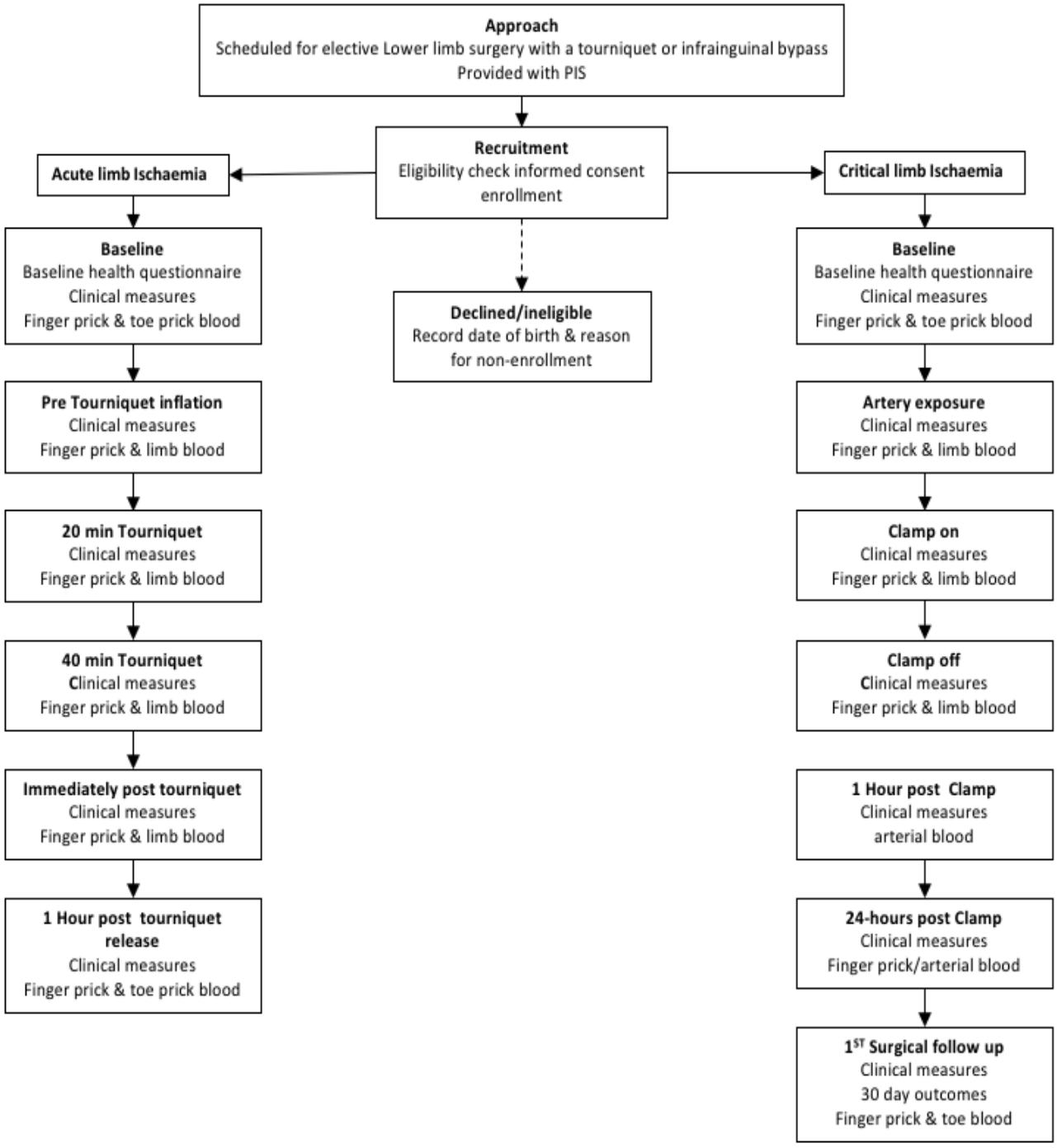

Figure 7 Local sampling (ALI and CLTI) participant flow diagram.

4.8.6 Traumatic Brain Injury

The first study sample was obtained as soon as possible after study enrolment. A second comparative sample was obtained at 24-hours (where possible) along with recording of diagnosis and any intervention to prevent or sequelae of the injury in the intervening time period between samples. Both samples obtained are capillary blood via finger prick.

4.9 Statistical analysis plan

4.9.1 Demographics

Continuous demographic and procedural data was assessed for normality and presented as mean ± standard deviation or range as appropriate. Comparisons of means of continuous demographic data between groups was undertaken using independent t-tests. Chi-squared tests were used to analyse differences in proportions between groups of binary categorical demographic or procedural data where appropriate.

4.9.2 Primary outcome

The primary outcome measure in the experiments in surgical populations described here is intra-cohort changes in purine concentrations from baseline in response to initiation, continuation and resolution of ischaemia. These are presented as median±95% confidence intervals. A linear mixed model was fitted to analyse changes in purine nucleosides. A mixed model was used in order to take into account multiple related samples and assess correlation between repeated samples within individual patients at different sampling sites, acquired over time. Linear mixed models allow for both fixed and random effects on purine concentrations as well as making use of all available data points collected. Time variables have been analysed in this model as categorical rather than continuous to allow for differing profiles of changes in purine nucleosides

In the non-surgical population (traumatic brain injury) the primary outcome is purine concentration on presentation to hospital. Purine concentrations are reported as median±95% confidence intervals. This purine concentration was compared to subsequent purine readings at 24-hours post admission using the Wilcoxon signed ranked test. Comparisons of purine concentrations between types of injury or other categorical measures were performed using the Mann-Whitney U test.

4.9.3 Secondary outcomes

Where applicable, differences in purine nucleoside profiles between similar cohorts of patients was evaluated (i.e. open AAA repair and EVAR and baseline purine concentrations in multiple study groups). This was performed using Mann-Whitney U test.

4.9.4 *Missing data*

Validation of results could only be performed post-hoc and was not available to the researcher at the time of sampling. The outcome of this was any results that are not valid are only detected after sampling was complete. Having four sensors per SMARTchip provides some inbuilt protection against no result being obtained. It was anticipated a proportion of results obtained will be completely invalid (no result from all four sensors) either due to fault with SMARTchip or error in sampling. As all data analysis of changes in purine nucleoside will be from pooled results of study subjects at any given sampling point there was tolerance for a small number of missing samples. Sampling points with <70% of total values available were excluded from subsequent analysis.

Even if result validation could be achieved in real time in the experiments performed here many of the samples are time critical, i.e. they were taken at set intra or post-operative time points. Given the unstable nature of purine nucleosides in whole blood, time delay in repeat sampling or repeat analysis of remaining blood from initial sample would invalidate that result.

4.9.5 Data Handling

Screening and recruitment logs of all patients approached to take part in this study and enrolled in the study are held at each site. Patients that agree to participate in the study were assigned a unique identifier, which were used to identify all documents associated with that participant for the duration of the study. Eligibility, baseline health data and clinical data was recorded contemporaneously, by hand, in a case report form (CRF) by the researcher. Participant consent was recorded, in triplicate, on an informed consent form and stored securely at each research site.

Access to study data was restricted to members of the study team and patient identifiable data was restricted to those members of staff that require it for the performance of their role. Direct access to data was granted to authorised representatives of the Sponsor, host institution and the regulatory authorities for the purposes of trial-related monitoring, audits and inspections.

Following the resolution of queries and confirmation of study close-out by the Chief Investigator, all essential documentation was transferred to a third-party archiving service, which provides suitable fire and water-resistant facilities. Patient identifiable data will be held for up to three years to facilitate any future food and drug administration or certification mark application. Anonymized data will be held for a period of 15 years.

4.10 End of study

The end of the study was defined at the date of the last patient's last visit.

5. Results and analysis

The combined studies recruited 199 participants. 190 participants underwent 191 surgical procedures. Nine participants in the traumatic brain injury study underwent no surgical procedure. In total 200 "ischaemic events" were assessed using SMARTchip. Results below detail each sub study individually.

5.1 General anaesthetic carotid endarterectomy

5.1.1 Demographics

Forty participants were recruited to the general anaesthetic (GA) carotid endarterectomy (CEA) study. On validation of the SMARTchip data, three patients were subsequently excluded from the analysis due to biochemical interference at the point of sampling leading to erroneous readings. The results for the remaining 37 patients are presented here.

Results for 28 males and nine females with a mean age of 72 (range 41-87) were analysed. Indications for surgery were as follows; stroke (n=20), TIA (n=15) and isolated ocular symptoms (n=2). Cardiovascular comorbidity as well as previous cerebrovascular disease was prevalent within the cohort. Full demographics of patient co-morbidity are shown in Table C with the demographic information from the published local anaesthetic (LA) CEA study for comparison. No intra operative shunt was used in 23 cases and in 14 cases were shunted intraoperatively.

5.1.2 Operative timings

Intra-operative timings are shown in Table D. There was no significant difference in procedural length between the GA shunted and GA unshunted cohorts (147±50mins vs 143±32mins, $p>0.05$) the LA procedures were significantly shorter than this (95±41 mins $p=0.09$ vs shunted and p=0.Ihunted). Relative ischaemic time, defined as the period of time where there was interruption in ipsilateral cerebral blood flow was, as expected, significantly longer in the GA unshunted group than the GA shunted group (51±2 mins vs 12±8mins $p<0.001$) and also significantly longer than the LA group (34±15 mins $p=0.014$).

DEMOGRAPHIC	N		P
	GA	LA	
TOTAL PARTICIPANTS	**37**	**17**	
SEX (M:F)	28:9	12:5	ns
AGE (mean)	72	72	ns
INDICATION FOR CEA			
STROKE	20	4	0.02
TIA	15	7	ns
AMAROSIS	1	3	0.06
OCCULAR ISCHAEMIA	1	0	ns
ASYMPTOMATIC	0	2	0.04
COMORBIDITY			
HYPERTENSION	27	12	ns
STROKE	25	6	0.01
HYPERLIPIDAEMIA	18	10	ns
DIABETES	8	0	0.03
IHD	5	4	0.41
PVD	2	3	ns
SMOKING STATUS			
Current	19	4	0.04
Ex	7	3	ns
Never	11	7	ns
ANTIPLATELET			
DUAL	19	6	ns
SINGLE	18	8	ns
NONE	0	3	0.01
STATIN			
YES	33	9	0.001

Table C Demographics from the recruited GA cohort. Data from the previously published LA cohort is included for comparison(6).

	PROCEDURE GROUP. MEDIAN (CI)		
	GA SHUNTED	**GA UNSHUNTED**	**LA**
OPERATIVE TIME (MINUTES)	147 (97-197)	143 (121-165)	95 (54-136)
RELATIVE ISCHAEMIC TIME (MINUTES)	12 (4-20)	51 (28-74)	34 (19-49)

Table D *Intra operative timings by CEA subgroup. There was no significant difference in total operative time between the GA CEA groups. LA CEA was significantly shorter (p=0.09 & 0.04 vs unshunted and shunted respectively). As expected, the shunted group experienced significantly shorter relative ischaemic time (p=0.001 & 0.014 vs unshunted and LA respectively).*

5.1.3 Intra operative blood pressure control

Intraoperative mean arterial pressure (MAP) profiles for all groups are shown in Figure 8. In both the LA and GA unshunted groups intraoperative MAP varied significantly from baseline readings. MAP in the LA Cohort was significantly increased after application of carotid clamps ($p=0.03$) whereas MAP in the unshunted GA cohort was significantly decreased after anaesthesia ($p=0.006$) though this difference was not sustained through the remainder of the procedure. MAP in the GA shunted group was significantly lower than the LA group ($p=0.018$). There was no statistically significant difference between the GA cohorts.

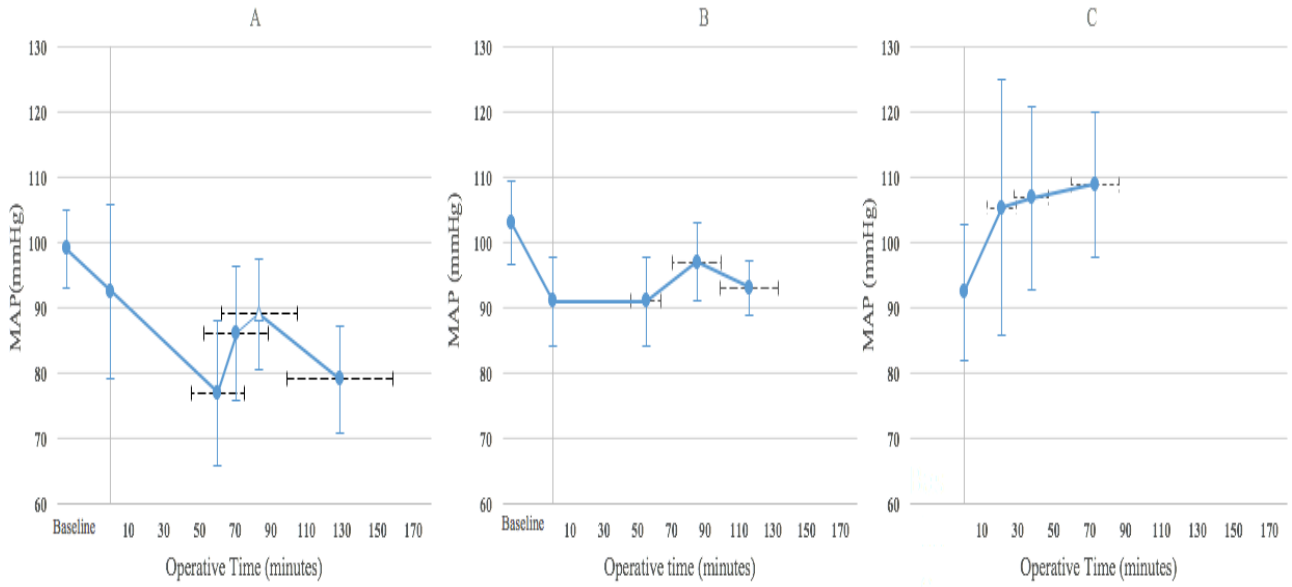

Figure 8 *Mean arterial pressure profiles for A- GA shunted, B-GA unshunted, C-LA subgroups. Data is presented as median with 95% confidence limits. Sampling points represent pre operative baseline, post anaesthetic prior to procedure starting, artery exposure, clamp on and reperfusion. As each intra-operative step may be reached at different chronological time points (minutes) for each individual participant the horizontal error bars during the intra opertaive sampling period represent median±95% confidence limits of the chronological time point at which each operative stage was achieved. Δ In Figure A represents an aditional data point obtained on shunt insertion. No pre operative MAP was obtained for the LA subgroup as there was no GA. MAP significantly decreased compared to baseline in the GA unshunted cohort (P=0.03). MAP was significantly increased in the LA cohort on application of the carotid clamp compared to baseline (P=0.018). MAP in the GA shunted group was significantly lower than the LA group(P=0.06).*

5.1.4 Intraoperative changes in peripheral arterial purine concentrations

There was no significant difference in baseline nucleoside concentrations between the GA cohorts; 3.02±1.11μM and 3.16±1.85μM in the unshunted and shunted cohorts respectively ($p>0.05$). These baseline readings were also comparable to the baseline values in the previously published LA cohort (2.4±1.35μM; $p=0.23$).

There was no significant variation within either of the GA groups from baseline after 30 minutes of carotid clamping when compared to baseline values; 2.07±0.89μM ($p=0.713$ compared to baseline) and 2.4 ±3.09μM ($p=0.474$ compared to baseline) in the unshunted and shunted cohorts respectively. In addition to this there was no significant difference in purine concentrations between the shunted and unshunted groups at any of the sampling points. The profiles of purine nucleosides against time from the shunted and unshunted GA cohorts are presented in Figures 9 & 10.

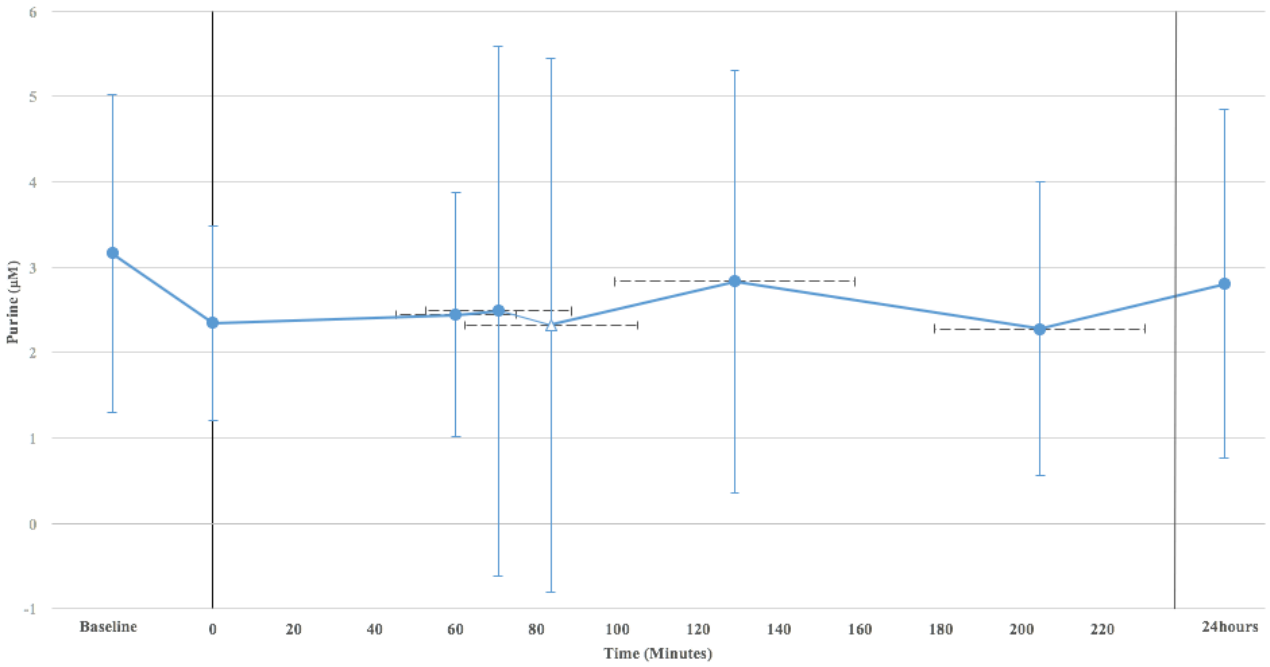

Figure 9 *Purine nucleoside changes in the GA shunted cohort (median & 95% CI). As each intra-operative step may be reached at different chronological time points (minutes) for each individual participant the horizontal error bars during the intra opertaive sampling period represent median±95% confidence limits of the chronological time point at which each operative stage was achieved. Compared to the unshunted cohort (Figure 10) Δ represents an additional data point on shunt insertion. Observed changes in purines were not significant ($p>0.05$).*

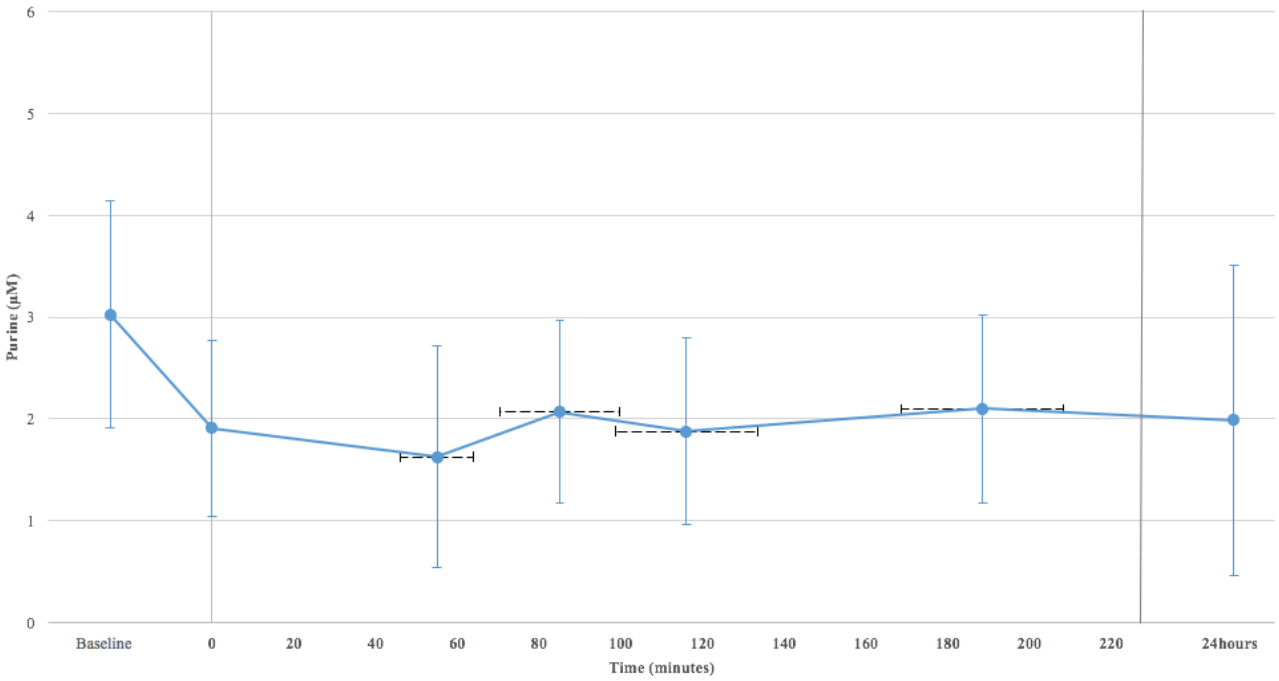

Figure 10 *GA Unshunted cohort purine nucleosides (Median & 95% CI) presented at median time points whereby operative stages for sampling were reached. As each intra-operative step may be reached at different chronological time points (minutes) for each individual participant the horizontal error bars during the intra opertaive sampling period represent median±95% confidence limits of the chronological time point at which each operative stage was achieved. Observed changes in purine concentrations were not significant ($p>0.05$).*

The LA CEA study(6) demonstrated a peak 2.8-fold increase during carotid clamping; 2.4μM at baseline rising to 6.7μM during clamping. This did represent a significant increase compared to both GA cohorts at comparable time points, i.e. 30 minutes of carotid clamping, (shunted 2.4 ±3.09μM vs 6.7±3.4μM and unshunted 2.07±0.89μM vs 6.7± 3.4μM p=0.004).

No significant difference was noted in the post-operative (both 1-hr and 24-hr) purine nucleosides between either of the GA groups and the LA group. In addition to this no statistically significant changes in purine nucleosides was demonstrated in differing anaesthetic agents used or with increasing degree of ipsilateral or presence of contralateral carotid stenosis.

There were no reported peri-operative neurological events in either the GA or LA cohorts. Four patients reported transient cutaneous sensation alteration in the distribution of the great auricular nerve (two GA and two LA).

5.1.5 Analysis

The results of this study are directly comparable to the previous LA work (6) as the studies were identical except for choice of anaesthesia, however, the anticipated rise during carotid cross clamping was not identified.

Differences in cerebral metabolism between LA and GA CEA have been identified previously. McLeary et al (202) analysed results of continuous jugular venous oximetry ($SJvO_2$) and near infrared spectroscopy (NIRS) in patients undergoing GA or LA CEA. They demonstrated a smaller fall in $SJvO_2$ in the GA cohort than the LA cohort (9% vs 13%), meaning cerebral oxygen consumption was decreased to a greater extent in the GA cohort. The NIRS measurement of oxidised cytochrome oxidase (caa_3) as a proxy marker of cellular ischaemia showed a greater fall in the LA cohort suggesting a greater degree of tissue hypoxia in patients who were conscious during CEA. This is consistent with the contrasting findings of the LA and GA cohorts in our studies; namely cerebral metabolic rate is higher in patients undergoing LA CEA than GA CEA. This in turn may lead to greater cellular stress during LA CEA on carotid cross clamping, appreciated as detectable rise in purine nucleosides that is not replicated in the GA cohort.

The lack of any appreciable rise in purine concentration during ischaemia in the GA CEA cohort is at odds with previously reported measures by Weigand (80), this study did demonstrate a significant rise in adenosine that was measured in samples from the ipsilateral jugular bulb during the clamped phase of the CEA using GA. Sampling from the jugular bulb represents direct venous drainage of the brain (203) whereas the radial artery sampling in our study represents blood that has circulated through the heart twice and lungs before being distributed (and diluted) to the periphery. In addition, other historic studies noted differences in changes in purine concentrations between arterial and venous samples and between differing location of venous samples in the same participant (34).

The direct effect of local anaesthetic agents on purine production within tissues has not been investigated. Hypothetically the results obtained by Tian et al (6) could be attributed (wholly or in part) to changes in purine metabolism induced by local anaesthetic agents within (or immediately adjacent to the operative field. This would seem unlikely given the rises in purine nucleosides obtained directly from the main venous outflow of the brain under GA that was demonstrated in the Weigand study (80) however they did not report if adjuvant LA was used. In addition to this significant changes purine nucleosides in peripheral arterial samples would be unlikely to occur from the small volume of tissue (Skin, subcutaneous adipose and connective tissue of the neck) on which local anaesthetic agents are used.

As previously stated, CEA carries an approximate 2% risk of stroke (84). Intra-operative carotid shunting is used to provide ongoing ipsilateral cerebral perfusion during the clamped phase of CEA. The use of shunts in carotid surgery remains contentious as shunting itself carries risk of stroke via embolization, vessel endothelial trauma or un-noticed intraoperative occlusion. A Cochrane review published in 2014 (85) of six studies involving 1270 patients and a retrospective pooled analysis of 28475 CEA procedures performed in the United States (125) concluded there was insufficient robust evidence to support the use of either routine or selective shunting. As such, there is significant variation in practice globally and selective, routine and no shunt use all have staunch supporters. The absence of a significant purine rises in the GA cohort, compared to the previous LA cohort may indicate that the decrease in cerebral metabolic rate afforded by general anaesthetic agents gives an element of cerebral protection against clamp induced cerebral hypoperfusion-ischaemia. Therefore, in a proportion of cases the perceived protection offered by shunting may be unnecessary and introduce risk than confer benefit.

In this study shunting remained at surgeons' discretion. Individual surgical practice will have produced variation in necessity and timing of shunt insertion. In addition to this, methods for determination of the need for shunting varied between surgeons and between recruiting sites. This potentially serves to confound changes in purine nucleosides as it would be expected shunt insertion would attenuate ischaemic purine nucleoside production as cerebral flow is maintained. This is demonstrated in results obtained in patients requiring shunt insertion in the previously published LA study. Given rises in purine nucleosides in the LA cohort were appreciated prior to neurological symptoms it was not deemed appropriate to require surgeons to deviate from their preferred mechanisms of cerebro-protection. A lack of appreciable purine rise in conjunction with no perioperative neurological events could be interpreted as appropriate prophylactic shunt insertion based on existing selection mechanisms despite a lack of objective validation. There is also potential the results are a consequence of the relative rarity of neurological events combined with a small study population.

Adequate cerebral perfusion pressure (CPP) to maintain normal cerebral functioning is defined as being between 50 and 70 mmHg (204). CPP is derived from the difference between MAP and intra cranial pressure (ICP). Maintenance of CPP intraoperatively during CEA is critical to minimise risk of ischaemic cerebral insult. In this study the assumption was made that all recruited patients had normal ICP as none had a history of raised ICP, prior neurosurgical intervention or were displaying symptoms of raised ICP at the time of CEA. Intraoperative MAP was maintained at a level to prevent ischaemic injury secondary to malperfusion, this would also be expected to prevent purine production as a result of hypotension rather than reduction in blood flow caused by carotid clamping. The relatively lower MAP readings in the GA shunted cohort may be attributable to less rigorous BP control given cerebral perfusion via the shunt. The relative increase in MAP in the LA study may also be explained by discomfort or anxiety experienced by the patient during an LA procedure.

As the results obtained in the LA study were not replicated in the GA cohort, currently it has not been demonstrated that purine nucleosides can be used as an intra-operative biomarker of evolving or ongoing cerebral ischaemia in patients under GA. This study does offer further information into differences in cerebral ATP metabolism between LA and GA. It can be hypothesised that the lack of a rise in purine nucleosides in GA CEA may be caused by a

decrease in cerebral metabolic rate and loss of metabolic rate-blood flow coupling caused by general anaesthetic agents.

5.2 **Dynamic controls**

Whilst for clarity of reading these five subgroups have been defined collectively as dynamic controls, they actually represent separate surgical cohorts. Their commonality is all procedures included involve a period of intraoperative iatrogenic ischaemia. The primary analysis is of intra-group changes in purine concentrations at set operative time-points. Comparisons between these subgroups is of little value as they represent very different cohorts of patients undergoing different surgical procedures, with the exception of the open (OSR) and endovascular (EVAR) aortic aneurysm repair subgroups.

5.2.1 Demographics & Surgical procedures

Open aneurysm repair

This sub-group consisted of nine males and one female, mean age at surgery was 66±14 years. Mean aneurysm diameter was 5.9±1.3cm. Cardiovascular co-morbidity was prevalent in the cohort as was smoking, five participants were current smokers with a further two identifying as ex-smokers.

All procedures were performed in an elective setting in a conventional transperitoneal approach via a midline laparotomy. Procedures included implantation of five tube grafts and four bifurcated grafts. One patient underwent aortic endartectomy rather than aneurysm repair; they have been included here as the technical steps of induction and duration of lower extremity ischaemia by aortic cross clamping is comparable to aneurysm repair. There was no significant difference in total procedure time or ischaemic time between tube and bifurcated grafts.

EVAR

This subgroup consisted of six males and four females, mean age at surgery was 75±11 years. All procedures were elective infra-renal endovascular aneurysm repairs with open access to the femoral vessels. Aneurysm size was 6.0±0.3cm All procedures were performed by a combination of a vascular surgeon and an interventional radiologist and were implanted within the manufacturer's instructions for use (IFU).

Critical limb threatening ischaemia

This *Critical limb threatening ischaemia (CLTI)* group was exclusively male. Mean age at surgery was 64±9 years. All patients were staged clinically by the treating team using either the Fontaine or Rutherford peripheral arterial disease (PAD) classification scoring systems. Both scoring systems use an increasing numerical value to indicate increasing severity of PAD. A detailed breakdown of both scoring systems is shown in Table E. Whilst the indication for intervention was broadly the same, (intervention for critical limb ischaemia secondary to peripheral arterial disease) nature of arterial disease is shown in Table F, this is the most heterogeneous of the sub-groups in terms of pattern of disease and procedure undertaken. Procedures included five femoral endarterectomies, two femoral-femoral cross over grafts, two femoral to below knee (one popliteal and one posterior tibial) bypass grafts and one axilo-unifemoral graft.

Clinical Features	Rutherford score	Fontaine stage	Notes
Asymptomatic	0	I	No pain
Mild claudication	1	IIa	Can complete standard treadmill exercise test
Moderate claudication	2	IIb	Decreasing distance to onset of pain on testing
Severe Claudication	3	IIb	Cannot complete standard exercise test
Rest pain	4	III	Continuous ischaemic pain without tissue loss
Minor tissue loss	5	IV	Digital ulcers distal to metatarsal-phalangeal joint only
Major tissue loss	6	IV	ulcers proximal to metatarsals or gangrene, viability of limb is uncertain

Table E Overview of clinical grading classification systems used in Peripheral arterial disease.

MOST SIGNIFICANT ARTERIAL DISEASE	NUMBER
CFA STENOSIS	5
CFA OCCLUSION	1
SFA OCCLUSION	2
POPLITEAL OCCLUSION	1
AORTIC OCCLUSION	1
RUTHERFORD SCORE	
3	2
4	3
5	5
PROCEDURE	
COMMON FEMORAL ENDARTERECTOMY	5
FEMORAL-FEMORAL CROSSOVER	2
FEMORAL-POPLITEAL BYPASS	2
AORTO-BIFEMORAL BYPASS	1

Table F Pattern of disease and procedures undertaken within the dynamic control CLTI study.

Microvascular free tissue transfer

All Procedures were deep inferior epigastric artery perforator (DIEP) flap procedures for breast reconstruction, therefore this cohort was exclusively female with a mean age at surgery of 52±5 years. One participant underwent bilateral procedures and therefore each flap was treated individually. The group consisted of eight mastectomy combined with immediate reconstruction and two delayed reconstruction following previous mastectomy. Excluding a history of breast cancer, the cohort was free of major co-morbidity.

Transplant

As previously stated, all transplant procedures were elective live donor renal-only procedures. This subgroup consisted of six males and four females, mean age at surgery 40±11 years. Indication for transplant varied and included; IgA nephropathy (n=2), chronic pyelonephritis (n=2), reflux nephropathy, uromodulin disease, medullary sponge kidney, polycystic kidney disease, chronic pyelonephritis and glomerulosclerosis (all n=1). This group were significantly younger than any other of the dynamic control cohorts ($p<0.001$) and the demographic data reflects this; significant co-morbidity, other than indication for transplant, was not prevalent within the subgroup.

Demographic information for all dynamic control participants is shown in Table G.

DEMOGRAPHICS	SUBGROUP					WHOLE COHORT N (%)
	OSR	EVAR	CLTI	DIEP	TRANSPLANT	
TOTAL PARTICIPANTS	10	10	10	9	10	49
TOTAL PROCEDURES	10	10	10	10	10	50
SEX (M:F)	9:1	6:4	10:0	0:9	6:4	31:18 (63:37)
AGE (MEAN)	66	75	64	52	40	59
COMORBIDITY						
HYPERTENSION	5	8	5	0	4	22 (45)
STROKE	0	1	2	0	0	3 (6)
HYPERLIPIDAEMIA	5	6	4	0	0	15 (31)
DIABETES	0	3	2	0	0	5 (10)
IHD	0	5	2	0	0	7 (14)
PVD	2	4	10	0	0	16 (33)
OTHER	3	0	1	2	9	15 (31)
SMOKING						
CURRENT	5	5	6	2	1	19 (39)
EX	2	1	2	0	3	8 (16)
NEVER	3	4	2	7	6	22 (45)
ANTIPLATELET						
YES	7	7	7	0	1	22 (45)

Table G Dynamic control subgroup demographics.

5.2.2 Ischaemic times

Time of ischaemia, between clamp application and restoration of flow, was available for 47 of the 50 patients. As anticipated, duration of ischaemia varied significantly in line with procedure. OSR patients underwent a significantly longer period of intraoperative ischaemia than any other subgroup (98±14 minutes). Transplant recipients underwent the shortest duration of ischaemia (18±5 minutes). This transplant ischaemic time is the time between iliac clamp application and completion of anastomoses in the recipient, total duration of graft ischaemia (nephrectomy to completion of anastomoses) was not recorded. Ischaemic times for each subgroup are shown in Table H.

5.2.3 Intra-operative purine changes

Baseline purine and post clamp release purine readings in each of the cohorts are shown for reference in Table I. Each of the subgroups were found to have baseline purines within normal resting range (i.e. <3μM). There was no significant difference in baseline purines between any of the cohorts. In addition to this, the linear mixed model analysis revealed there was no significant variation in purine concentrations from baseline at any subsequent sampling point, in any of the cohorts. Perioperative purine profiles for each of the subgroups are presented in Figure 11(i-v). This was again true if the analysing the "dynamic control" cohort as a whole (n=50).

As previously mentioned, the only appropriate inter-group comparisons to make are between open and endovascular aortic aneurysm repair groups. As with other analyses in this "dynamic control" study there was no significant difference between these two cohorts at any point in the pre, intra or post-operative period.

SUB-GROUP	NUMBER	ISCHAEMIC TIME (minutes)
OPEN AAA	10	98±14.1
EVAR	9	58±17.8
CLTI	9	47±23.9
DIEP	9	35±8.04
TRANSPLANT	10	18±5.11

Table H *Ischaemic times of each dynamic control subgroup. Open aneurysm repair patients underwent a significantly longer period of ischaemia than the other cohorts.*

SUBGROUP	NUMBER	BASELINE PURINE μM (median±95% CI)	POST CLAMP PURINE μM (median±95% CI)	P-VALUE
WHOLE COHORT	50	2.32 ±0.51	1.67±0.46	0.586
OPEN AAA	10	2.54±2.09	1.74±1.25	0.905
EVAR	10	2.49±0.65	1.31±1.28	0.43
CLTI	10	1.44±7.32	1.14±1.98	0.416
DIEP	10	2.64±0.8	1.50±0.66	0.167
TRANSPLANT	10	1.53±0.98	1.73±1.24	0.329

Table I *Summary of peri-operative purine nucleoside changes within with in each dynamic control group. Baseline and post clamp (reading at which largest increase was anticipated) readings are displayed for reference. P value given is obtained from linear model of all sampling points. No statistically significant change identified within each cohort between sampling points.*

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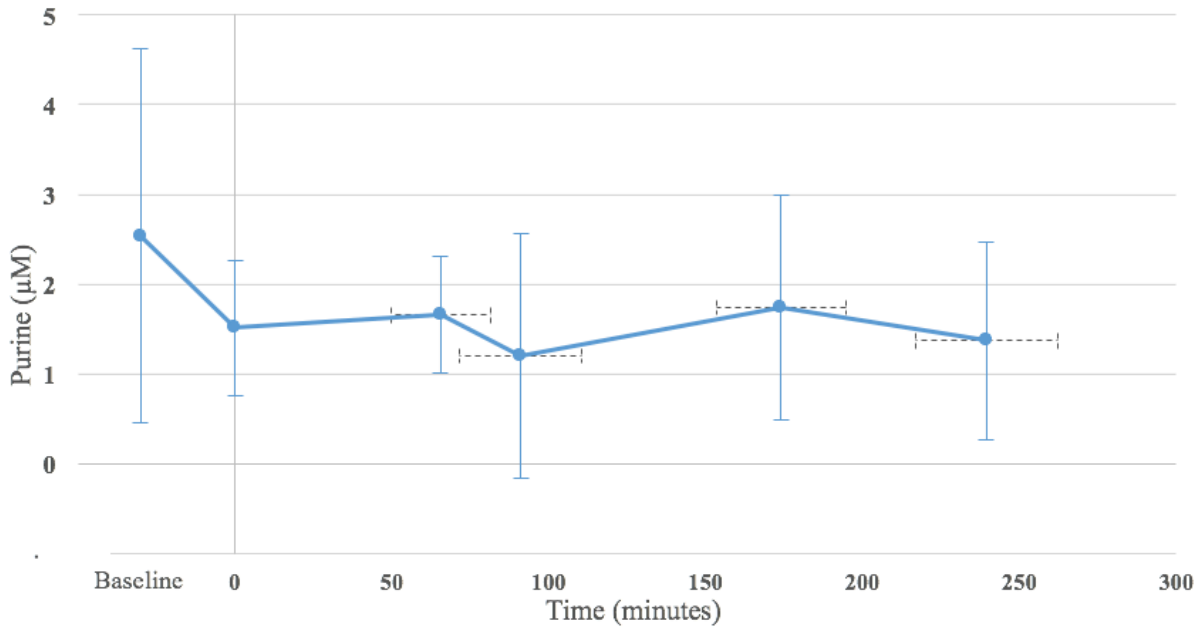
5
4
3
2
1
0
Purine (µM)
Baseline
0
50
100
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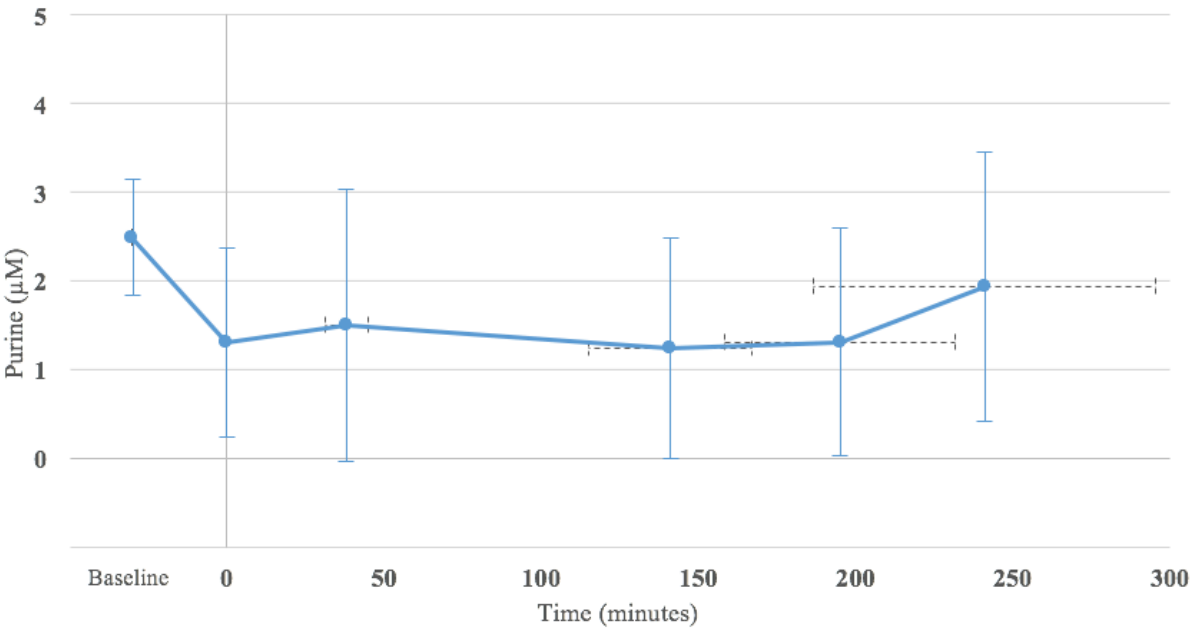
10ii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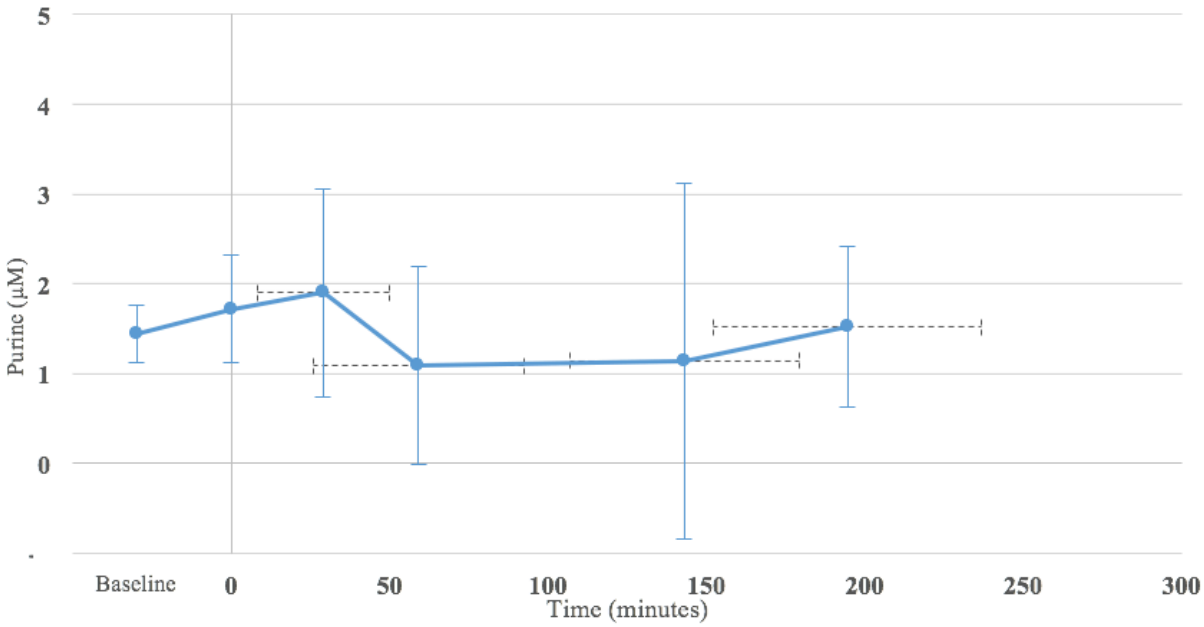
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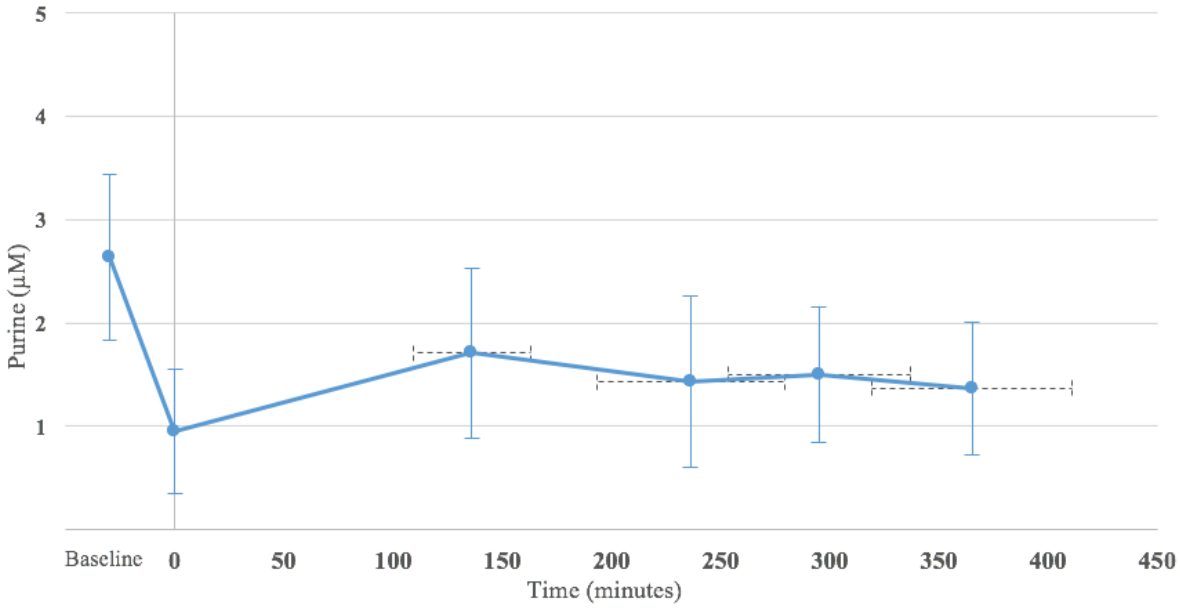
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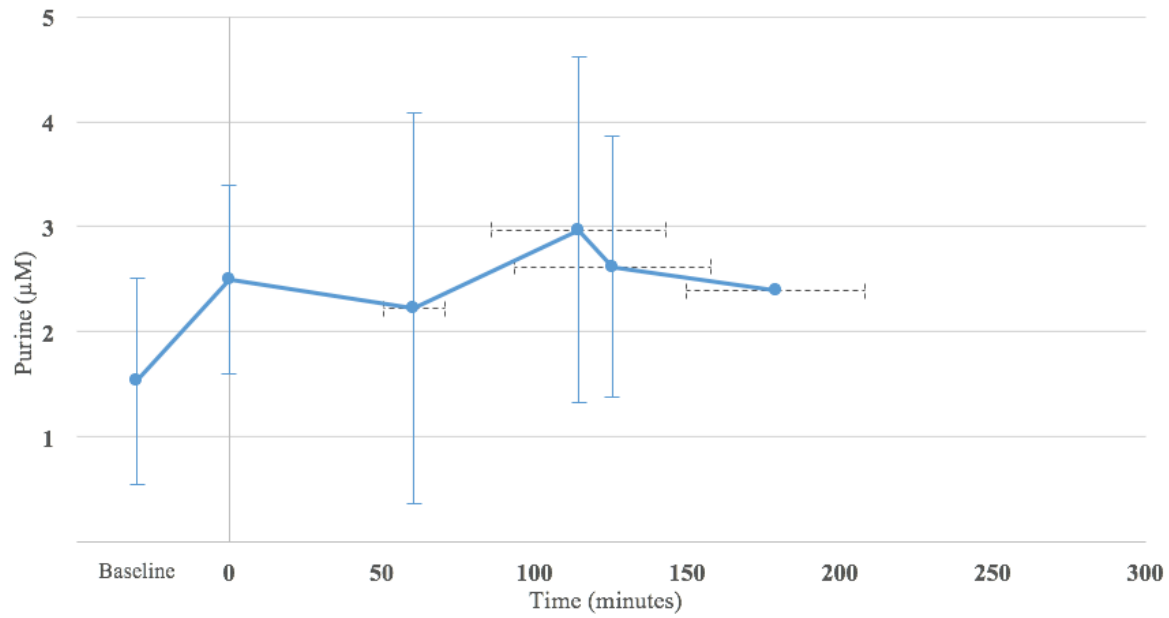

Figure 11 *Scatter plots of intra subgroup perioperative purine changes. i-open aneurysm repair, ii-EVAR, iii-CLTI, iv-DIEP, v-Transplant. As each intra-operative step may be reached at different chronological time points (minutes) for each individual participant the horizontal error bars during the intra opertaive sampling period represent median±95% confidence limits of the chronological time point at which each operative stage was achieved. Sampling time points were: Baseline, post anaesthesia, clamp application, clamp release and 1hr post clamp release. Baseline readings were taken prior to first case of the theatre list and therefore are not time-continuous with the intraoperative readings as such the post anaesthesia sample is marked as time point zero. Purine results are presented as medians with 95% CI. No significant intra-group variation was noted.*

5.2.4 Analysis

The hypotheses that there would be significant perioperative purine nucleoside change in these surgical cohorts was based on extrapolation of the findings in the LA CEA study. As with the GA CEA study, results from these dynamic control studies may be confounded by GA. However, there may be other factors other than choice of anaesthesia that may influence the results obtained.

Patients undergoing open aneurysm repair underwent the longest ischaemic time and had the largest mass of ischaemic tissue of any of the study populations. Despite this no significant changes in purine concentrations were noted. During EVAR there is no formal vascular "clamping" where flow is completely occluded for a prolonged duration. Instead, there is repeated, intermittent short duration (seconds only) aortic occlusion in addition to longer duration partial occlusions secondary to large bore (16f or greater) intra-arterial delivery sheaths which in theory restrict blood flow to the lower extremities for the duration of the procedure. It is, maybe, not unexpected that no significant purine rises were noted intraoperatively within participants undergoing EVAR. Ischaemic complications, though rare, are potentially devastating for patients and therefore are a significant issue facing clinicians undertaking endovascular aneurysm repair. This study was not expected to identify or have sufficient power to investigate ischaemic complications but form pilot data for future studies into purine nucleosides as markers of ischaemic complications post EVAR. Clinical need for a simple, reliable diagnostic adjunct to aid diagnoses remains. Whether SMARTchip can be used in these situations remains unclear.

In comparison to other "dynamic" cohorts a microvascular free tissue transfer flap has a significantly smaller mass of tissue that is rendered ischaemic and the composition of that tissue is primarily adipose, which has a lower metabolic rate than the majority of other tissues studied in this series of studies. It was anticipated a purine rise would be noted on reperfusion whereby purine nucleosides produced during ischaemia may be detected in systemic circulation on reperfusion. Given the lack of rise in the GA CEA study it is unsurprising that no appreciable purine rise was noted in a small mass of relatively low metabolically active tissue.

During renal transplantation the aim of the study was to attempt to identify and quantify the effect of ischaemia in the kidney to be transplanted. It was noted that vascular control of the ipsilateral iliac vessels during arterial anastomosis provided a period of time within which to study a period of limb ischaemia. Therefore, it was anticipated there may be a "double peak" in purine nucleoside concentrations the first representing limb ischaemia during iliac cross clamping and a second, or superimposed, peak after complete restoration of blood flow through the transplanted kidney representing washout of nucleosides accumulated within the kidney during the period between nephrectomy and implantation. Whilst there was a small increase after clamp application 2.97±1.65μM vs 1.53±0.98μM at baseline, this result was not statistically significant but may represent acute-onset ischaemia in a previously normally perfused limb without any peripheral arterial disease.

In both the microvascular free tissue transfer flap and the transplant studies the anticipated peak (or continuation of a purine concentration raised above baseline) after graft reperfusion was not identified. In both studies venous anastomoses are completed prior to arterial there may be gradual "leak" of purine nucleosides into the systemic venous system prior to restoration of arterial inflow rather than an anticipated "surge" of accumulated purines once arterial inflow is restored. In addition, mechanisms to decrease metabolic rate within the kidney such as cooling in ice with may be effective in reducing purine production, there is also repetitive flushing with cold preservative solutions immediately prior to transplantation to prevent in-situ arterial or venous thrombosis may mean purine nucleosides was washed away from the graft and are lost.

5.3 Non-vascular controls

5.3.1 Demographics & surgical procedures

This heterogeneous subgroup consisted of 80 patients undergoing a variety of surgical procedures with general anaesthetic this consisted of 46 males and 34 females. On validation of the case report form data 13 patients' results were invalid. Purine nucleoside data was not sufficient for eight participants. Demographic and procedural data was missing for a further five participants, this was deemed sufficient to affect interpretation of any purine nucleoside value changes. These participants were excluded from subsequent analyses. The analysed cohort consisted of 38 males and 29 females. Mean age at time of surgery was 55±16 years.

Mean operative time was 138±81 minutes. Full cohort demographics are shown in Table J and procedures undertaken are shown in Table K.

DEMOGRAPHIC	**N (%)**
TOTAL PARTICIPANTS	67
SEX (M: F)	38:29 (57:44)
AGE (mean)	55
COMORBIDITY	
HYPERTENSON	16 (24)
STROKE	0 (0)
HYPERLIPIDAEMIA	9 (13)
DIABETES	7(10)
IHD	3 (4)
PVD	0 (0)
OTHER	20 (30)
SMOKING	
CURRENT	12 (18)
EX	14(21)
NEVER	41(61)

Table J Group demographics for the non-vascular control cohort.

PROCEDURE	NUMBER
Open inguinal hernia repair	18
Laparoscopic cholecystectomy	7
Dental extraction	5
Excision of skin lesion	6
Circumcision	3
Ocular surgery (vitrectomy/trabeculectomy)	3
Tonsillectomy	3
Laparoscopic gynaecological surgery (Salpingo-oophrectomy/cystectomy)	3
ENT surgery	3
Hysterectomy	2
Transvaginal tape	2
Open varicose vein stripping	2
Revision of amputation	2
Arthrodesis	1
Cerclage	1
Haemorrhoidectomy	1
Laparoscopic hiatus hernia repair	1
Cerebrosinal fluid leak repair	1
Laparoscopic inguinal hernia repair	1
Mandibular plate removal	1
Wide local excision (Breast)	1

Table K Breakdown of procedures included in the non-vascular control cohort.

5.3.2 Perioperative purine nucleoside changes

Baseline purine nucleosides were 2.79±0.66μM. This was not significantly different to either the GA CEA or dynamic control groups.

There was no statistically significant variation in purine nucleosides intraoperatively (p=0.43, 0.72 and 0.37 at each sequential intraoperative sampling point when compared to baseline). Perioperative purine changes for the whole cohort are shown in Figure 12. There was also no significant variation by procedure or by laparoscopic/open procedure type.

Given the heterogeneous group of surgical procedures recruited from subgroup analysis cannot be undertaken for many due to insufficient numbers. It was possible to perform subgroup analyses for those undergoing open inguinal hernia repair and laparoscopic cholecystectomy (n=18 and 7 respectively).

Eighteen male patients underwent open inguinal hernia repair. Mean age at surgery was 63±13 years. Mean procedure duration 116±80minutes. Seven patients underwent laparoscopic cholecystectomy. Mean age 60±6 years, procedure duration 130±81 minutes. No significant intra-group variation in purine concentration was identified in either of these two cohorts. Individual purine profiles are shown in Figures 13 & 14. As in the renal transplantation cohort the laparoscopic cholecystectomy group did exhibit a small rise in purines over the duration of the procedure though this was not statistically significant.

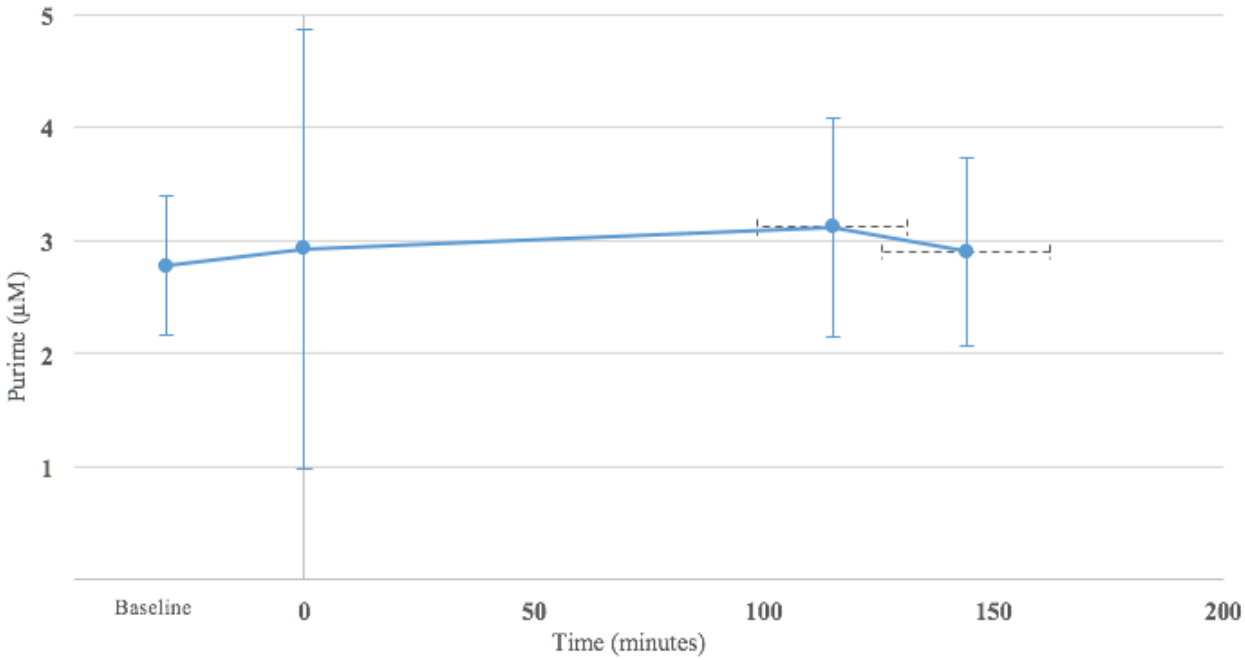

Figure 12 *Non-vascular control whole group intra operative purine changes. Purine nucleosides are median values with 95%CI at time points baseline, post anaesthesia, mid procedure and post operatively. As each intra-operative step may be reached at different chronological time points (minutes) for each individual participant the horizontal error bars during the intra opertaive sampling period represent median ± 95% confidence limits of the chronological time point at which each operative stage was achieved. Baseline readings were taken prior to first case of the theatre list and therefore are not time-continuous with the intraoperative readings as such the post anaesthesia sample is marked as time point zero.*

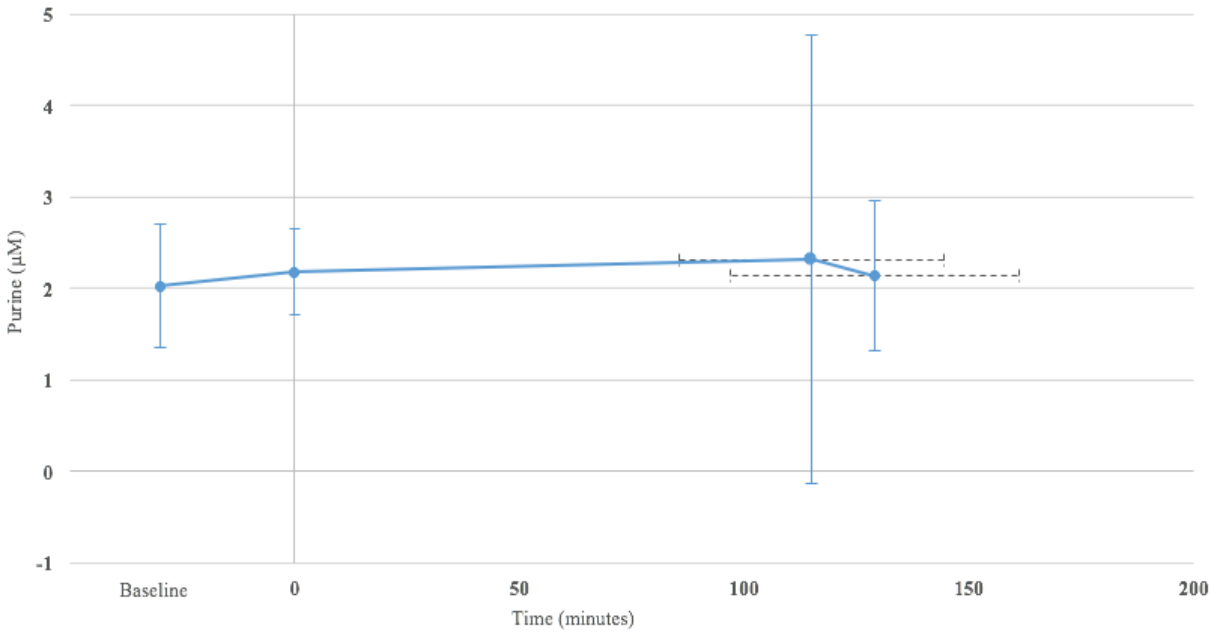

Figure 13 *Inguinal hernia intra-operative purine changes. Purine values are median values with 95%CI at time points baseline, post anaesthesia, mid procedure and post operatively. As each intra-operative step may be reached at different chronological time points (minutes) for each individual participant the horizontal error bars during the intra opertaive sampling period represent median±95% confidence limits of the chronological time point at which each operative stage was achieved. Baseline readings were taken prior to first case of the theatre list and therefore are not time-continuous with the intraoperative readings as such the post anaesthesia sample is marked as time point zero.*

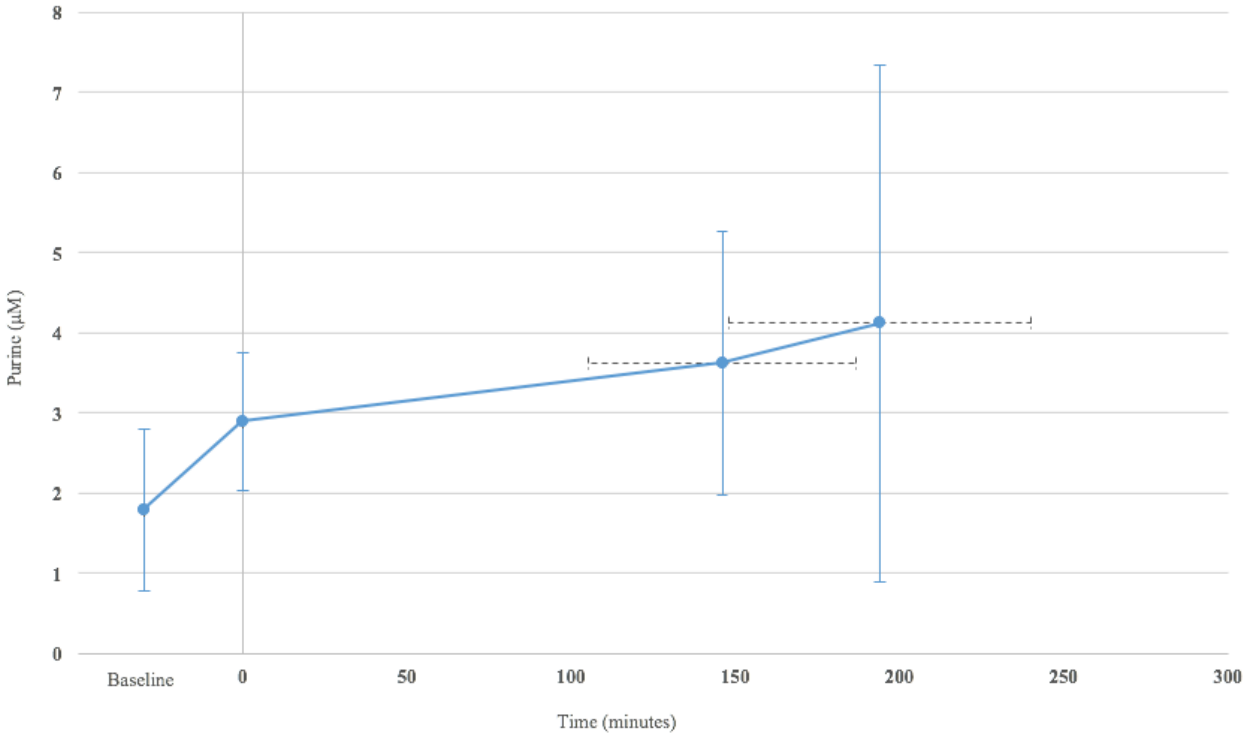

Figure 14 *Laparoscopic cholecystectomy intra-operative purine changes. Purine values are median values with 95%CI at time points baseline, post anaesthesia, mid procedure and post operatively. As each intra-operative step may be reached at different chronological time points (minutes) for each individual participant the horizontal error bars during the intra opertaive sampling period represent median±95% confidence limits of the chronological time point at which each operative stage was achieved. Baseline readings were taken prior to first case of the theatre list and therefore are not time-continuous with the intraoperative readings as such the post anaesthesia sample is marked as time point zero.*

5.3.3 Analysis

Results in this cohort of patients undergoing "non-vascular, non-ischaemic" surgery with general anaesthetic were as anticipated; showing no significant variation from baseline throughout the surgical procedure in line with the hypothesis that procedures without ischaemia intraoperatively will not produce a rise in purine concentrations. Given the results obtained in the GA CEA and dynamic control cohorts the lack of a rise may be related to GA rather than surgical procedure and the significance of the results obtained must be questioned.

It would be possible to investigate potential changes in some of these procedures performed under LA to exclude GA as a confounder in nucleoside production. This may be of little clinical significance as it would be limited to minor, superficial procedures where risk of significant ischaemic insult is minimal in a conscious patient. In addition to this all procedures in this cohort were day case or short stay procedures. Whether peripheral purine concentration changes would be different in surgery deemed major or major plus has not been identified in this cohort although given no significant rises were noted in major plus procedures with intra operative ischaemia (dynamic controls) it would seem unlikely that "non-ischaemic" procedure would cause a significantly different intraoperative purine profile.

Following the results obtained from systemic samples in the studies above. It was hypothesised that obtaining samples of blood from within an ischaemic area may be able identify smaller rises in purine nucleoside concentrations locally prior to systemic dilution that are not detectable in sampling at sites distant from the ischaemic area. This limited study to areas of ischaemia that are readily accessible.

5.4 Acute Limb Ischaemia

5.4.1 Demographics

Ten participants underwent lower limb orthopaedic surgery with a pneumatic tourniquet. This cohort included nine females and one male. Indication for surgery was exclusively symptomatic, radiologically proven, osteoarthritis of the knee joint. Mean age at surgery was 58±7 years. The cohort was relatively free from major co-morbidity. Importantly, all patients were free from peripheral arterial disease. Full demographic details are shown in Table L.

DEMOGRAPHIC	NUMBER
TOTAL PARTICIPANTS	10
SEX (M:F)	9:1
AGE (MEAN±SD)	58±7
COMORBIDITY	
HYPERTENSION	3
STROKE	0
HYPERLIPIDAEMIA	0
DIABETES	2
IHD	0
PVD	0
OTHER	4
SMOKING STATUS	
CURRENT	2
EX	3
NEVER	5
PROCEDURE	
LEFT:RIGHT	6:4
UNI-COMPARTMENTAL	5
TOTAL	4
NO IMPLANT	1

Table L Demographic and surgical details of patients undergoing lower limb surgery with a tourniquet.

5.4.2 Surgical Procedures

Five uni-compartmental (UKA) and four total knee arthroplasties (TKA) were included. In one patient, scheduled for a total knee arthroplasty, turbid effusion was identified on opening the joint capsule. The arthroplasty was abandoned and synovectomy undertaken.

Mean limb ischaemic time, defined as the time between tourniquet inflation and deflation, was 73±18 minutes. Patients undergoing TKA had a significantly longer ischaemic time (94±13 minutes vs 63±10 minutes. p=0.009). Ischaemic time in the patient in whom no implant was used was equivalent to those who did undergo UKA. Mean time between the "ischaemia 40" sample and "reperfusion" sample was 28±20 minutes.

5.4.3 Perioperative purine changes

There were no significant differences in baseline purine nucleosides, these were 2.15±1.03μM and 3.01±2.82μM in the finger prick and operated limb samples respectively (p=0.71). These baseline values were not significantly different to the dynamic control or non-vascular control study groups.

After 20 minutes of tourniquet inflation operated limb purine nucleosides exhibited a significant increase from baseline to 15.91±6.69μM (p<0.0001). At 40 minutes of tourniquet inflation (ischaemia) the operated limb purines increased further to 25.57±9.98μM (p<0.0001). This represents a 5.2 and 8.5-fold increase respectively. On tourniquet deflation purine values fell to 9.82±9.57μM and within one hour of tourniquet release purine concentrations within the operated limb had returned to baseline values 2.26±2.25μM (p=0.89 vs baseline). Intraoperative comparisons between finger and limb comparisons cannot be made as the tourniquet prevents venous outflow from the limb. It should be noted there were no statistically significant changes in finger prick purine values at any sampling point. Finger pick values at 40 minutes of limb ischaemia were 3.59±2.22μM (p=0.55 vs baseline). The profile of perioperative purine nucleoside change is shown in Figure 15, Of note there were no significant differences in changes in purine nucleoside values between UKR and TKR or between spinal and general anaesthetic procedures.

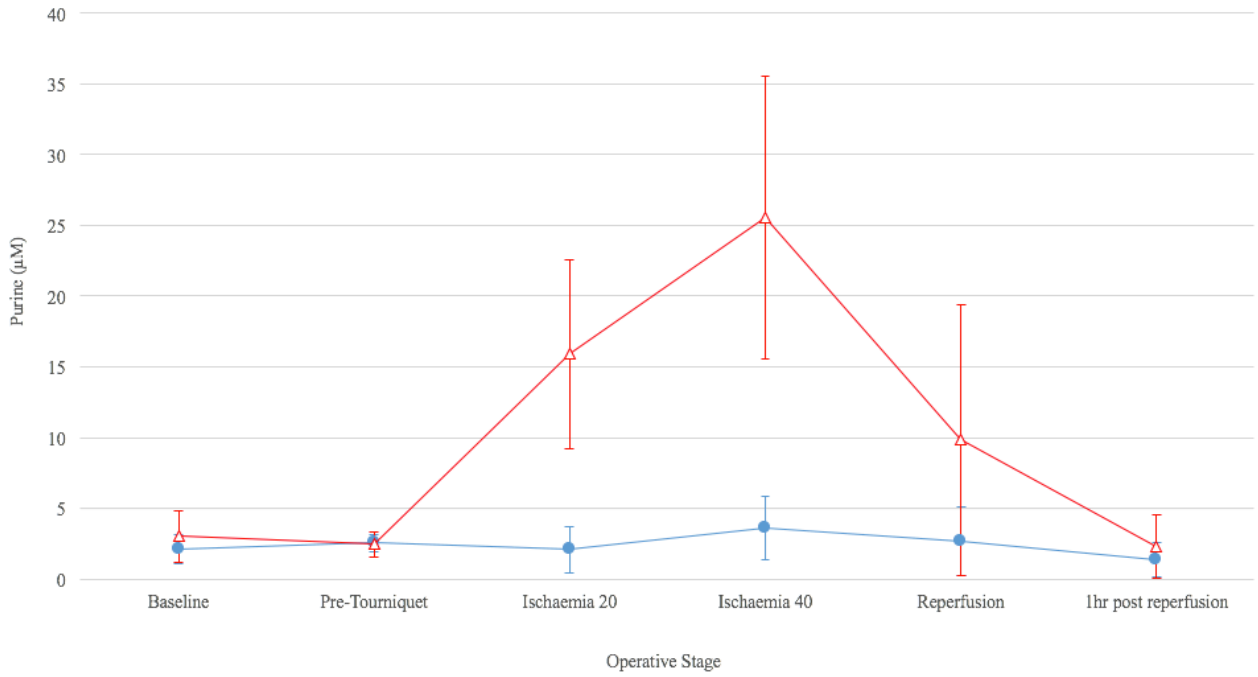

Figure 15 *Median purine nucleosides in both the operated limb and time, matched systemic samples •=systemic samples, Δ=operated limb samples. Significant increases in purine nucleosides were observed in the operated limb at 20 minutes and 40 minutes of ischaemia (both $p<0.0001$).*

5.4.4 Analysis

The results obtained in this study clearly demonstrate a significant rise in purine nucleosides within the ischaemic limb compared to limb baseline and time-matched systemic sampling. Purine nucleosides in the limb rise rapidly (in the order of minutes) and then fall to baseline levels on restoration of arterial inflow. These results are in line with the results of Karg et. al. (99) who demonstrated a purine rise during knee arthroplasty. In that study peak purine nucleosides were identified five minutes after tourniquet deflation, in ipsilateral femoral vein samples. This represents venous washout from the limb in line with restoration of arterial inflow. A smaller (non statistically significant) rise was identified in samples obtained from an antecubital vein at the same time point. This supports the hypothesis of rapid degradation/reuptake of nucleosides in the systemic circulation that influence determination of nucleosides from a sampling point distant to the site of ischaemia. The key difference between that study and this is the mechanism and location of sampling to observe evolution of changes of purines within the limb in real time.

This study has demonstrated rise in purine nucleosides is rapid, a 5.2-fold increase at 20 minutes and an 8.5-fold increase at 40 minutes of ischaemia with purine nucleosides returning to baseline values within one hour of restoration of normal blood flow. Indicating purine assessment may be sensitive to limb ischaemia in its earliest phases. Patients with ALI are highly likely to have an ischaemic time far in excess 40 minutes by the time they are assessed by a healthcare professional. Studies have reported median time from onset of symptoms to first contact with a healthcare professional of 24-hours (205) purine nucleosides at this time are likely to be grossly elevated. Purine released during prolonged acute ischaemia has not been studied, and at late stages of ischaemia clinical signs are likely to be readily apparent.

Lower limb surgery with a tourniquet is a good fit initial experimental model of ALI as it provides a homogenous cohort of healthy patients with normal arterial supply to the lower limb undergoing a procedure with a consistent degree of ischaemia, where both time of onset and time of resolution are documented. It must be noted there are some differences between this experimental model and ALI occurring clinically. First, a pneumatic surgical tourniquet completely occludes arterial flow into the limb. In patients presenting with ALI there may still be inflow to the limb via collaterals from branches of the internal iliac system or via the profunda femoris if the level of arterial occlusion is distal to the common femoral bifurcation.

This remaining inflow, in a limb exhibiting signs of acute ischaemia, is by definition not sufficient to maintain normal oxygen delivery therefore metabolism of adenosine triphosphate and purine nucleoside production is likely to occur. The effect of limited ongoing inflow is not currently known but may have an attenuating effect on purine production.

Second, the tourniquet restricts venous outflow for the limb meaning there is stasis of blood within the limb. The effect of this on the measured purine concentration is uncertain. Free purine nucleosides are taken up by platelets and erythrocytes to reform high energy phosphate compounds. There is also further metabolism downstream to urate and uric acid, (206) though this only occurs in the presence of xanthine oxidase and is oxygen dependent (14). The true total purine nucleosides released may be higher than is able to be ascertained. In addition, the tourniquet will obstruct venous outflow whilst it is inflated which will prevent purines reaching distant sampling sites meaning intraoperative comparisons between limb and finger samples are not applicable. No rise in finger prick purine values was identified on release of the tourniquet in this study, confirming purine release is related to the ischaemic limb.

Third, Patients undergoing total knee arthroplasty for osteoarthritis are not a representative cohort of patients who will present with acute limb ischaemia. Whilst both conditions become more prevalent with age they do not have significant pre-existing PAD are less likely to suffer from ischaemic heart disease and diabetes than patients with PAD (207-209). In addition, a significant proportion of patients present with ALI on a background of peripheral vascular disease and chronic limb ischaemia. This is in contrast to the study population, all of whom do not have clinically significant peripheral vascular disease. The theoretical effect of ischaemic preconditioning will be discussed later, although this has not been previously studied. In animal models nucleoside release was sustained in chronically ischaemic tissues subjected to acute-on-chronic exacerbation ischaemia (59) and the observed effect on purine nucleoside production of acute-on chronic ischaemia in PAD patients is discussed in the next section.

A final factor to be considered is the impact of surgery itself on purine release. Knee replacement requires significant soft tissue dissection, bone resection and metal prothesis implantation. The prosthesis is then fixed in place with polymethyl methacrylate (commonly

known as bone cement). This cement hardens over a period of minutes after implantation in an exothermic chemical reaction. The significant changes in purine nucleoside demonstrated have been presumed to originate primarily due to limb ischaemia created by the use of a tourniquet. However, as previously discussed many factors may influence purine nucleoside release from tissues other than ischaemia. Surgical tissue trauma and/or use bone cement may exert an influence on local purine nucleoside release. Strategies were employed in the study protocol in an attempt to mitigate theses potential confounders by seeking fresh venous bleeding for sampling. In addition no significant rises were noted in systemic samples in the dynamic control studies where potentially there was a greater degree of surgical tissue trauma (e.g. laparotomy). Clearly the promising results obtained here require clinical validation in true populations of patients with ALI to eliminate potentially confounding surgical factors as a source of purine nucleoside rise.

5.5 Chronic Limb ischaemia

5.5.1 Demographics

Eight male and two female participants were recruited with a mean age of 74±9years. All participants recruited were scheduled to undergo lower limb bypass grafting for critical limb ischaemia. As anticipated cardiovascular co-morbidity was prevalent within the cohort as was current or previous smoking history five of ten participants were currently taking both an antiplatelet agent and a statin.

Participants disease was again clinically graded using the Rutherford and Fontaine classifications. Nine participants had critical limb ischaemia with tissue loss or gangrene with a Fontaine score of four. Of the participants with tissue loss; five patients had a Rutherford score of five and four had a score of six. One participant underwent bypass for severe claudication (Fontaine two, Rutherford three). five participants had an SFA occlusion, three had popliteal occlusions and two had crural vessel disease only. Two participants had three vessel run off, three had two crural vessels patent and five had single vessel patent. Demographics are shown in Table M.

5.5.2 Surgical Procedures

All surgical procedures were performed at a single vascular hub by one of six vascular surgeons. Procedures were limited to infra inguinal arterial bypass grafts using autologous vein. Four patients underwent bypass of the femoral-popliteal segment, four patients underwent femoral-pedal bypass, and two patients underwent popliteal-distal bypass. Nine grafts used reversed vein and one bypass was left in-situ.

There was a large range in duration of ischaemic time, defined as the period of time between application of the most cranial cross clamp and completion of the distal anastomosis, 34-136 minutes. Mean ischaemic time was 66±40 minutes. Patients undergoing popliteal-distal bypasses sustained the longest period of limb ischaemia 92±19 minutes but this was not significantly longer than either femoral-popliteal or femoral-distal procedures ($p=0.35$).

DEMOGRAPHIC	NUMBER
SEX (MALE:FEMALE)	9:1
AGE (MEAN±SD)	74 (9)
COMORBIDITY	
HYPERTENSION	10
ISCHAEMIC HEART DISEASE	4
DIABETES	3
COPD	4
NATURE OF VASCULAR DISEASE	
SURGICAL INDICATION	
MAJOR TISSUE LOSS	4
MINOR TISSUE LOSS	5
CLAUDICATION	1
FONTAINE SCORE	
2b	1
4	9
RUTHERFORD SCORE	
3	1
5	5
6	4
MOST SIGNIFICANT ARTERIAL DISEASE	
SFA	5
POPLITEAL	3
CRURAL	2
NUMBER OF PATENT CRURAL VESSELS	
3	2
2	3
1	5

Table M Demographic and disease burden details for participants recruited to the CLTI study arm.

5.5.3 Perioperative purine nucleoside changes

Baseline purine nucleoside values were 5.55±4.42 μM and 5.68±6.21 μM in the systemic and operated limb samples respectively. In comparison to the ALI cohort (2.15±1.03 μM and 3.01±2.82 μM in the systemic and operated limb samples respectively) and the non-vascular control study group systemic sample baseline purines were significantly raised (p=0.007).

There was marked inter-subject variation in pattern of purine nucleoside changes in both the systemic and operated limb samples. As such the overall changes in purine nucleoside concentrations were not statistically significant. Whole group purine nucleoside changes are shown in Figure 16.

Four of the ten participants exhibited a similar purinergic response to that observed within the ALI cohort i.e. a rise in purine nucleoside concentrations within the operated limb on application of vascular clamps. These changes are shown in Figure 17. Three patients exhibited a late rise in purine nucleosides at the 24-hour post-operative sampling time point, (6.42±6.14 μM vs 2.63±0.82 μM). This trend is shown in Figure 18. The remaining three patients displayed no trend in perioperative nucleoside concentrations. These differing responses were not linked to procedure, ischaemic time or number of run off vessels.

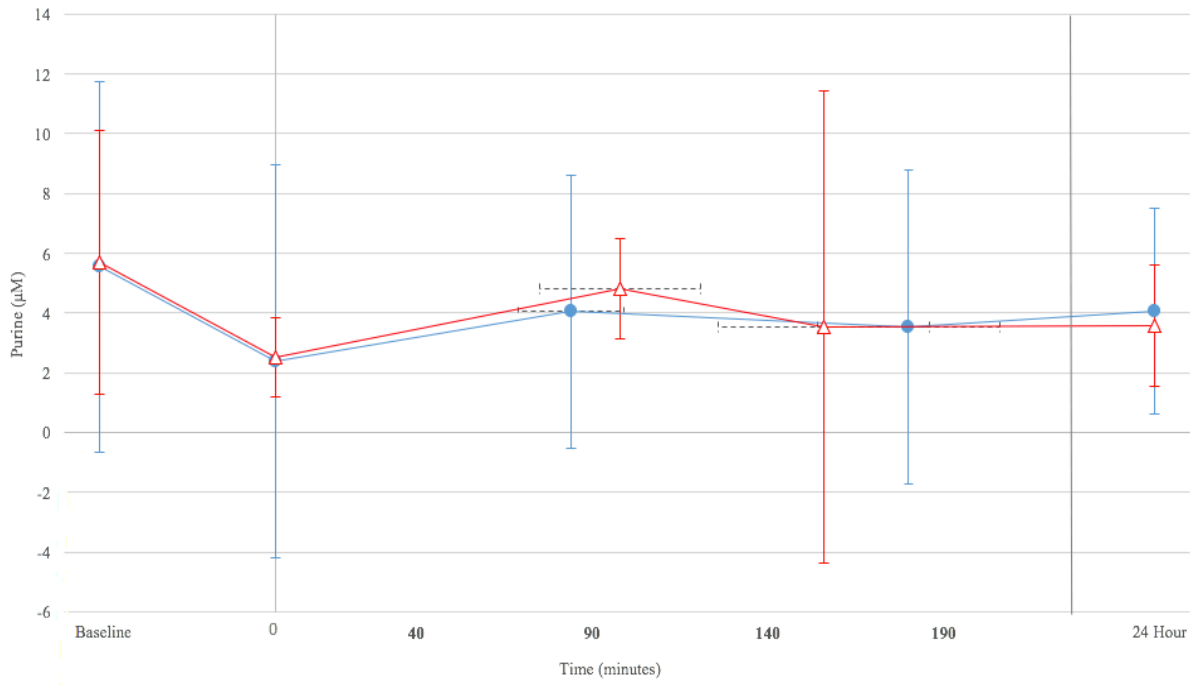

Figure 16 *Median purine nucleosides (with 95% CI) at pre, intra and post-operative sampling points (baseline, vessel exposure, vascular clamp application, clamp release and 24 hours post operatively) given at median time at which this sampling point occurred (with 95%CI) sampling points. ●=systemic samples, Δ=operated limb samples. Purine nucleoside concentrations were not significant.*

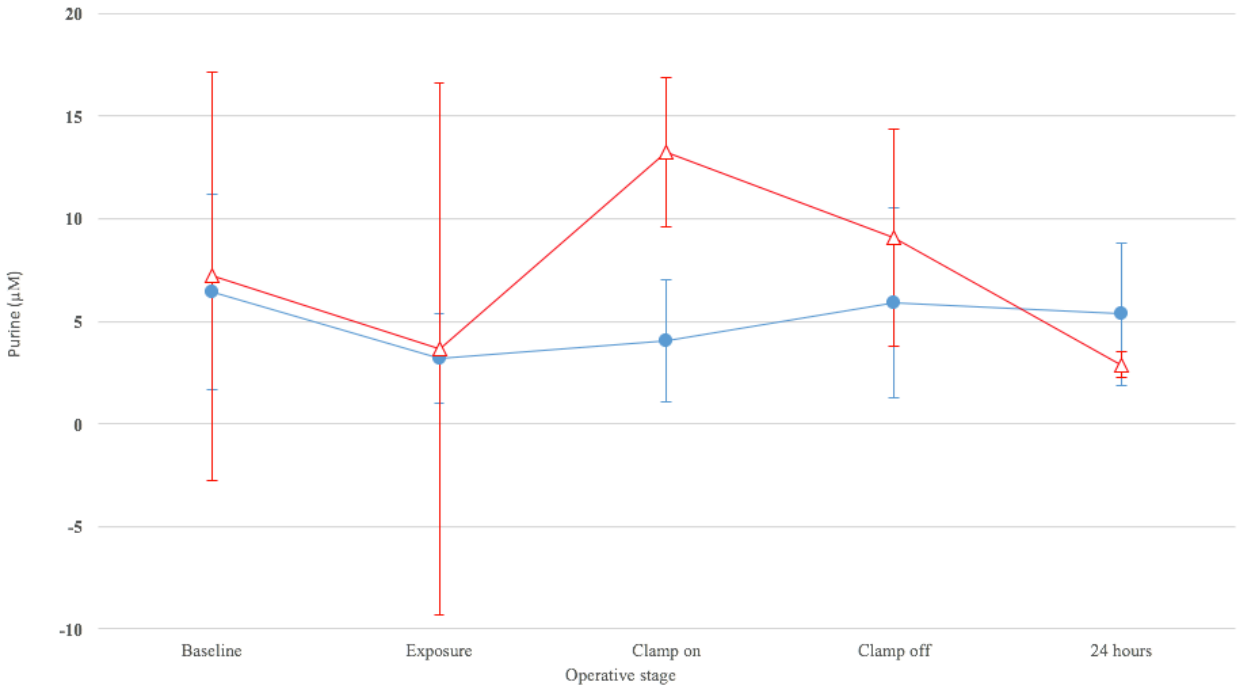

Figure 17 *Median purine values with 95% CI in Participants (n=7) displaying an intraoperative purine nucleoside concentration rise (acute-type responders).*
●= systemic samples, Δ= operated limb samples.

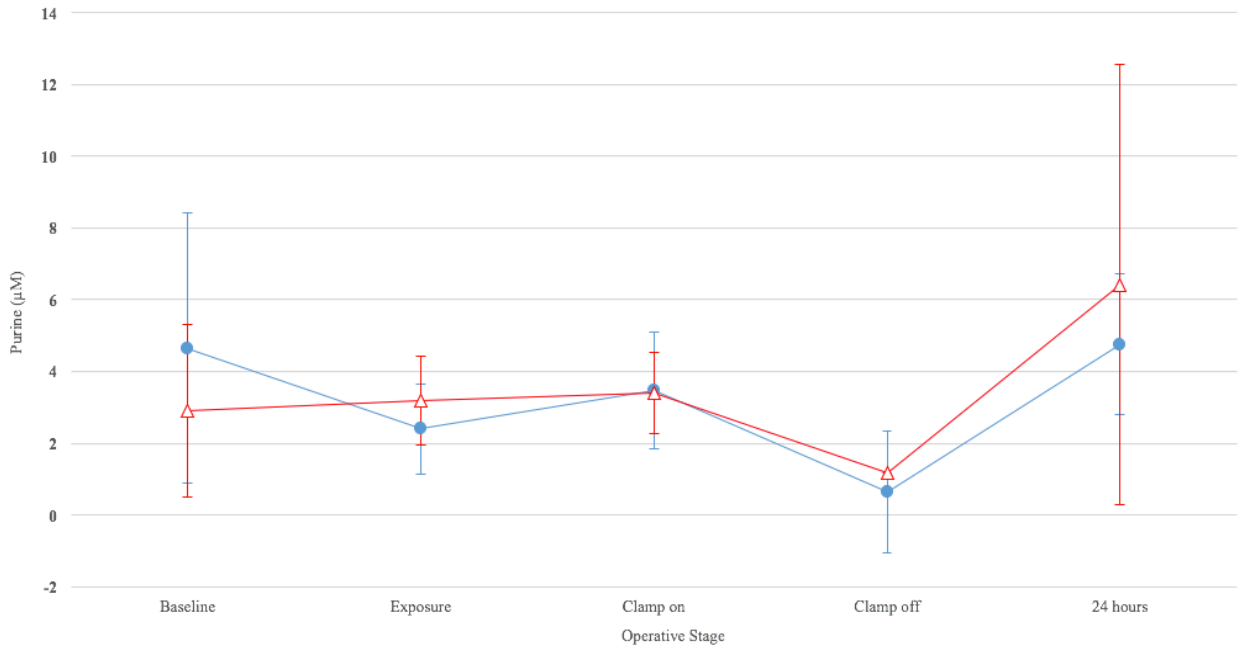

Figure 18 *Median purine values with 95% CI in Participants (n=3) displaying a rise at 24 hours only (late responders). •= systemic samples, Δ= operated limb samples.*

Due to technical limitations systemic and operated limb purine concentrations at first surgical follow up were only undertaken in one patient. Concentrations were 1.18μM and 1.5μM respectively.

5.5.4 Clinical Outcomes

At 30 days post operatively all bypass grafts were patent. At 90 days four grafts were occluded. One participant underwent further endovascular revascularisation and two participants underwent major amputation. One participant was managed conservatively. Retrospective analysis of participants who suffered graft occlusion revealed there was no correlation between pre-operative purine nucleoside concentrations and early graft occlusion although three of the four graft occlusions occurred in participants who exhibited a "late-type" purine response at 24-hours post operatively. In comparison, all grafts in those who displayed an "acute-type" response remained patent to 90 days.

5.5.5 Analysis

Perioperative changes in purine nucleosides in both systemic and operated limb samples in this cohort of participants were clearly the most heterogeneous of all the groups of participants studied.

In animal models it has been demonstrated chronically ischaemic tissue exhibits higher baseline values of purine nucleosides than normally perfused tissue (59). In humans, specifically in patients with PAD, baseline purine nucleosides were also raised compared to healthy controls (96, 98) this was identified in the local sampling CLTI study here, with both baseline limb and systemic samples significantly raised compared to other study populations. In the historical studies purine nucleosides increased above the elevated baseline on induction of acute-on-chronic ischaemia or increasing metabolic demand through exercise. In the PAD studies this rise was achieved through exercise and was noted at lower exercise intensity than in healthy controls.

That single model of raised baseline purines with a preserved response to exacerbation of ischaemia to chronic and acute-on chronic ischaemia would seem overly simplistic in the context of the nucleoside release demonstrated here. Some participants exhibited an intra operative trend towards a purine rise in the operated limb, akin to both the historic PAD

studies and the current local sampling ALI study, i.e. on application of arterial clamps. Other participants exhibited little intra operative response but appeared to show a trend of a rise in operated limb purine concentrations at 24-hours post operatively.

It must be noted there is a difference between the baseline purine nucleosides in the CLTI arm of the dynamic control study and the baseline systemic samples of CLTI local sampling study these were 1.44±7.32 μM and 5.55±4.42 μM respectively. This variation in response to acute-on-chronic ischaemia between the systemic and local sampling studies as well as between participants within each study is likely to be multifactorial. Differences in inclusion criteria to each study may have resulted in differences in severity of PAD between the groups. Although indication for surgical intervention was most commonly "chronic limb ischaemia with tissue loss" this is a broad category that will encompass significant heterogeneity in pattern and anatomic location (single lesion vs multilevel disease), severity and duration of disease. The dynamic control CLTI study included a wide variety of surgical procedures whereas the local CLTI study recruited exclusively unilateral infra-inguinal bypass grafts.

The dynamic control group included no patients with a Rutherford score of six and did include two patients with a Rutherford score of three whereas the local sampling group included a significant proportion of patients with a preoperative Rutherford score of six indicating clinical stage of PAD was more severe in patients in the local sampling study. This may account for the noted differences in baseline systemic purine nucleosides. Interestingly, in the dynamic control CLTI study one patient underwent a femoral-popliteal bypass graft for an SFA occlusion with a pre-operative Rutherford score of five. Baseline systemic purine values was 6.4μM. this patients result is consistent with both the preoperative disease pattern and the purine nucleoside profile of those patients recruited to the local sampling ALI study.

In addition to variation in disease, the potential effects of GA that have already been discussed are also applicable to these studies. The increased ischaemic stress on the operated limb by vascular clamping may be negated by the decreased metabolic rate caused by GA. It is also possible chronic ischaemia secondary to PAD may offer a degree of protection against acute-on-chronic ischaemia via an ischaemic preconditioning phenomenon. The observed 24-hour rise may be indicative of oxygen free radical generation and subsequent localised

cellular injury and hypoxia due to the inflammatory response as part of ischaemia-reperfusion phenomenon. It is already known that clinically significant ischaemia-reperfusion injury does not occur universally following infra-inguinal bypass (210). Its clinical significance is of uncertain importance compared to potentially life threatening reperfusion syndrome following revascularisation of an acutely ischaemic limb.

Of further interest in this CLTI cohort was the effect of reperfusion on purine nucleoside concentrations in the operated limb beyond the perioperative period. Full data collection at first surgical follow up (approximately six weeks) was not possible due to available supply of SMARTchip. In the patient in which six-week purine concentration was recorded purine nucleosides were lower than pre-operative baseline values. Hypothetically this may be due to decreased ischaemic stress on the operated limb post revascularisation. Given the incomplete data in this part of the study this hypothesis requires significant further investigation to demonstrate causality related to surgical intervention. In addition, the significance of an apparent trend towards early graft occlusion in patients displaying a purine nucleoside rise at 24-hours in this small sample is unknown. Whilst a predictive marker of impending graft failure would be extremely useful, graft failure is often multifactorial (211) and the concept a single biomarker may be overly simplistic. The trend observed in this small cohort requires significant further study to investigate purines as a predictive biomarker.

5.6 Traumatic Brain Injury

5.6.1 Demographics

Ten participants with radiologically confirmed TBI were enrolled into the study. This group included five males and five females. Mean age at presentation was 68 years (range 31-96). Four participants were taking either antiplatelet or anticoagulant medication. Demographic information is displayed in Table N.

5.6.2 Mechanisms of injury, types of injury, clinical measures and outcomes

The majority of participants recruited sustained injuries as a result of falls. These varied significantly in mechanism from fall from standing height to fall from significant height including stairs and scaffolding. One participant sustained injuries as a result of a road traffic collision.

In addition to analysis of purine nucleoside concentrations in the cohort as a whole, participant injuries were divided into two natural groups; those with “contained” intracranial bleeding (this group included extra and subdural haemorrhage) and “free” intracranial bleeding (subarachnoid haemorrhage, intraparenchymal bleeding and cerebral contusion). Four participants sustained contained injuries and six patients sustained free bleeding injuries. Four participants were identified as taking oral antiplatelet or anticoagulant medication; three of these four sustained free bleeding injuries whereas this only occurred in three of the six patients’ not taking antiplatelet or anticoagulant medication.

There was significant variation in clinical signs indicating severity of injury, four patients showed no evidence of cognitive disturbance with a Glasgow coma scale (GCS) of 15 and a further four patients exhibited minor cognitive impairment (GCS 14/15). Two patients exhibited severe cognitive impairment (GCS 5/15 and 3/15 respectively) on admission. Three patients (two free and one contained) exhibited midline shift on their initial brain imaging. One patient underwent craniotomy and evacuation of haematoma, all other patients were managed conservatively.

DEMOGRAPHIC	NUMBER
SEX (M:F)	5:5
AGE (mean±SD)	68±23
COMORBIDITY	
ISCHAEMIC HEART DISEASE	3
DIABETES	1
COPD	2
PREVIOUS STROKE	0
HISTORY OF BLEEDING	0
ANTIPLATELET/ANTICOAGULANT	4
MECHANISM OF INJURY	
FALL FROM HEIGHT	2
FALL FROM STANDING	7
RTC	1
NATURE OF INJURY	
SUBDURAL	4
SUBARACHNOID	4
INTRAPARENCHYMAL	2
MIXED	1
GCS ON ADMISSION	
13-15	8
8-12	0
<8	2

Table N TBI Cohort demographics and injury details.

5.6.3 Admission purine nucleosides

Whole group admission purines were 3.07±3.13μM. Free haemorrhage injuries presented with higher a purine concentration than contained haemorrhage injuries (6.08±4.67 μM vs 1.65±0.88 μM) respectively, although this difference did not meet statistical significance (P=0.11) (Figure 19). Patients taking antiplatelet or anticoagulant medication had an admission purine value of 6.05±5.70 μM whereas those not taking antiplatelet or anticoagulant medication median value on admission was 1.65±2.45 μM (P=0.11) (Figure 20). Admission purine nucleosides were weakly correlated with GCS (r^2=0.12) (Figure 21).

5.6.4 24hr purine nucleosides

Second readings at 24-hours were available for five of the participants recruited. Of the patients for which readings were not available; one patient was deceased prior to sampling, technical failure prevented readings in two patients and two patients were discharged prior to sampling. Whole group admission vs 24-hour purine nucleosides are shown in Figure 22. In those suffering contained bleeding injuries purine nucleosides at 24-hours was no different to baseline (1.72±0.99μM, p=0.66). Patients having sustained free bleeding injuries 24-hour purine values had fallen to 1.35±2.28μM, again this decrease was not statistically significant (p=0.11) Figure 23.

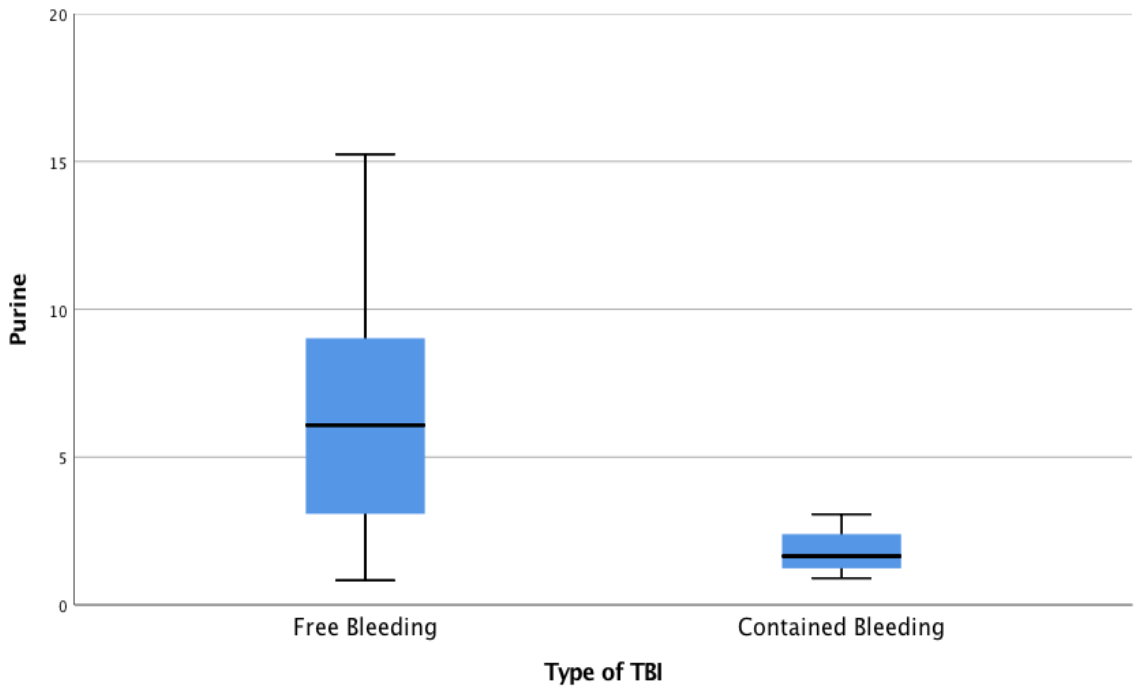

Figure 19 Admission purine nucleosides (μM) by type of injury.

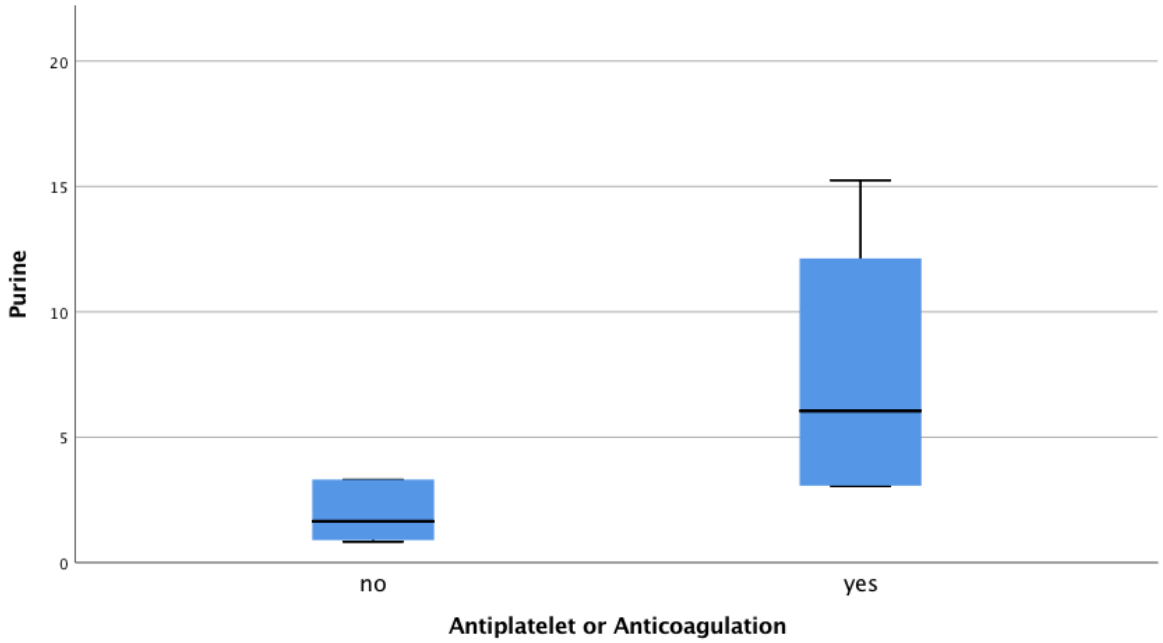

Figure 20 *Admission purine nucleosides (μM) in those taking or not taking antiplatelet or anticoagulant agents.*

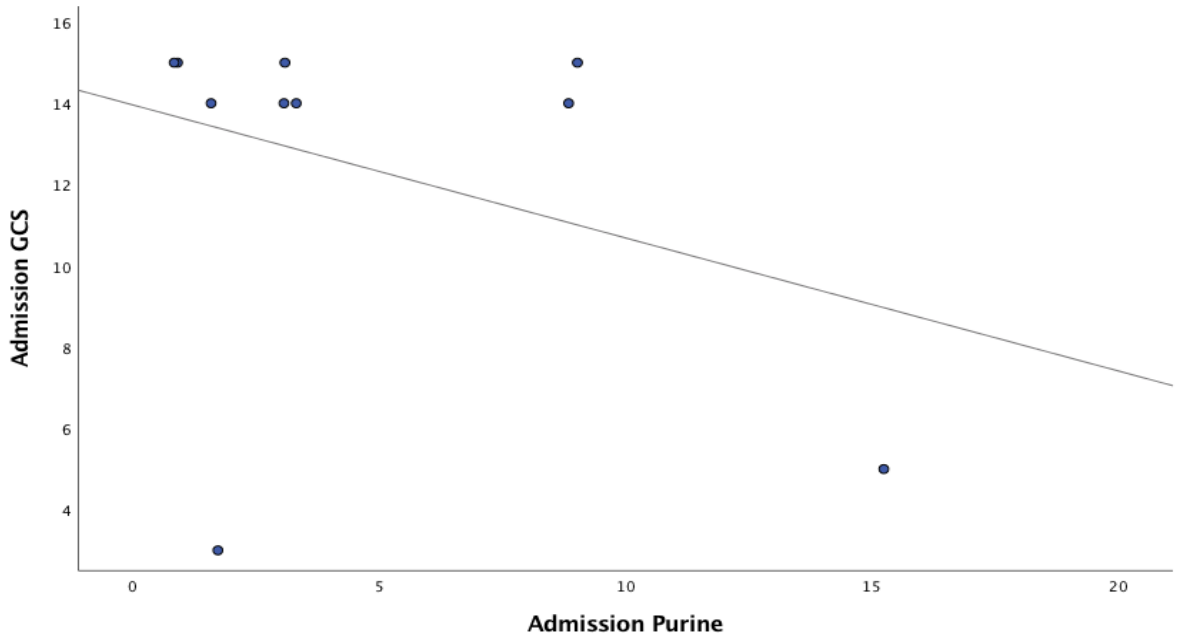

Figure 21 Admission purine nucleosides showed a weak correlation with GCS (r^2=0.12).

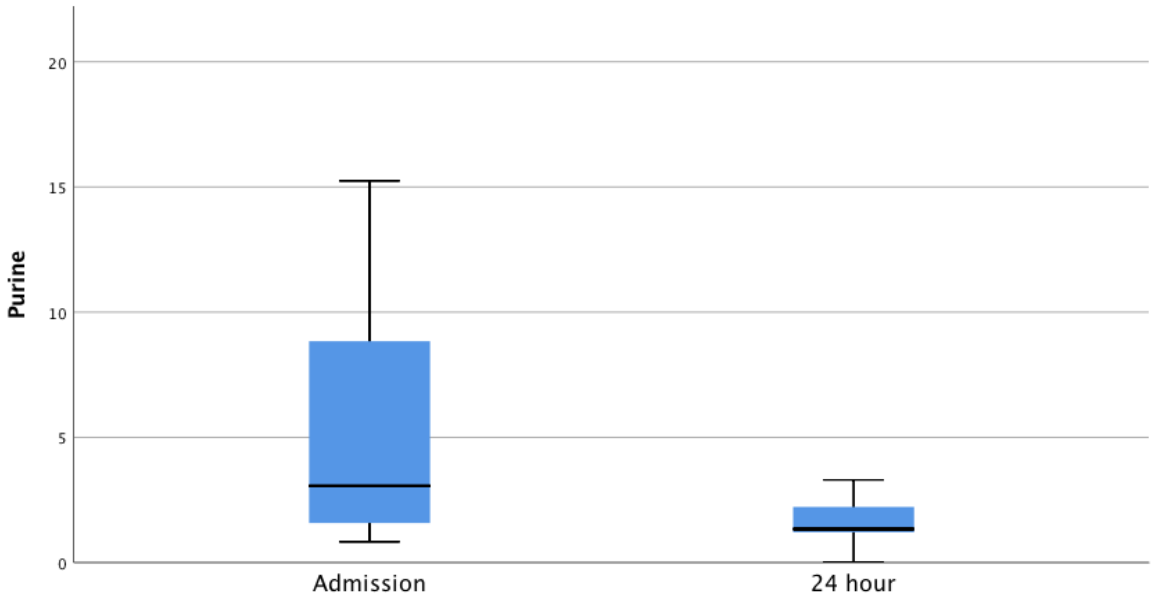

Figure 22 *Whole cohort purine nucleosides (μM) on admission and 24-hours post admission.*

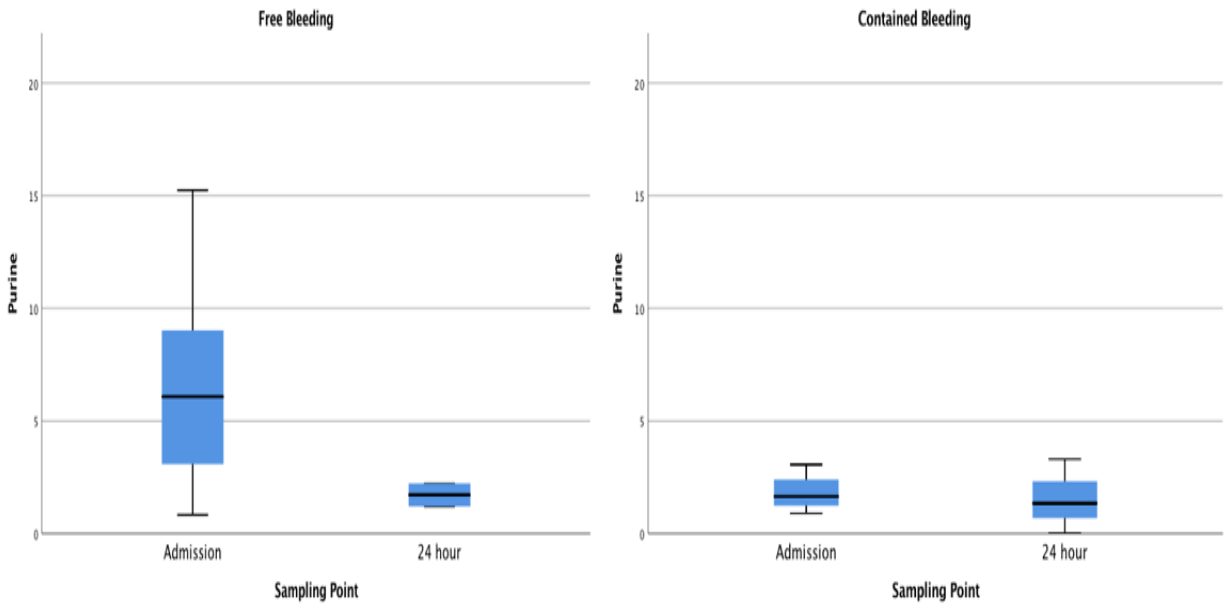

Figure 23 *Comparison of admission and 24-hour purine values (μM) in the free and contained bleeding injury subgroups.*

5.6.5 Clinical Outcomes

As previously mentioned, one patient was deceased less than 24 hours after admission, one patient was repatriated to their native country after a prolonged hospital stay (48days). All other patients have made a full neurological recovery to their pre-injury neurological status. Mean length of hospital stay was five days.

5.6.6 Analysis

This study enrolled patients with any radiologically diagnosed traumatic intracranial injury and thus encompasses a wide range of injury type and severity. This included both extra and sub-dural haemorrhage as well as subarachnoid haemorrhage, intraparenchymal bleeding and cerebral contusion. Patients presented with a wide variety of clinical signs and symptoms from no symptoms or mild confusion (GCS of 14-15/15) through to comatose (GCS of 3/15). In retrospect, recruiting such a variety of intracranial injuries to a single study cohort may have been an overly simplistic model of TBI. This combined with a small study size has led to difficulty in drawing firm conclusions from the data obtained.

Changes in purine nucleosides following TBI were not demonstrated to be significant, however the data does suggest that peripheral serum purine nucleosides may increase following some types of TBI. As such this pilot data may serve to pose further research questions rather than satisfy the hypothesis posed by this study.

First, TBI that was categorised as contained bleeding (i.e. sub-dural and extra-dural haemorrhage) would appear not to influence nucleoside production to the same extent as "free" (subarachnoid and intraparenchymal) intracranial bleeding. Second, purine release may be related to severity of injury sustained (defined by GCS). Anatomically these findings are logical. Sub-dural and extra-dural venous bleeding, though intracranial, are not truly brain injuries. Whilst large volume dural bleeds can cause neurological symptoms or death; this is through volume and subsequent pressure effects in the fixed volume space of the cranial vault. In these injuries blood does not come into direct contact with cerebral tissue as it is contained by layers of dura. Increasing severity of injury can be defined as either a more severe injury in one area of the brain or increasing volume of brain affected as a proportion of the whole. Logically, both of these mechanisms may lead to an increased local inflammatory response, increasing cellular ischaemia and driving purine nucleoside production. Often minor injuries are isolated to a single category of bleeding in a discrete area of the brain

whereas, in contrast, major brain injuries often involve multiple categories of injury i.e. combined dural and parenchymal bleeding potentially involving multiple lobes of the brain.
In the current study purine nucleosides were weakly correlated with admission GCS. Larger scale study is required to fully investigate this potential correlation with regard to severity and anatomic location of injury.

Observed increases in admission purines in patients taking antiplatelet or anticoagulant agents are potentially a consequence of a higher proportion of "free" bleeding injuries sustained in this group compared to those not taking antiplatelet or anticoagulant agents. Anticoagulation is known to confer a risk of increased bleeding post traumatic brain injury but the proportion of patients suffering intracranial bleeding following head injury remains small (212). Rises in purine nucleosides in patients taking antiplatelet or anticoagulant medication may be a simple screening test to identify those patients needing brain imaging.

Clearly no firm conclusions regarding the influence of anatomic location, extent of injury, GCS or effect of medications on purine production can be drawn from this study. Significant further study with greater stratification of participants by injury type and/or extent is required to investigate whether trends identified here are genuine.

Concomitant injury is also a factor in interpretation of purine results. Although in this cohort mechanism of injury was predominantly fall from standing, TBI is often a high energy injury and therefore additional extracranial injury can occur. As purine nucleosides are not specific to the brain purine release can occur from concomitant extra-cranial trauma. Injuries sustained in the study cohort were limited to localised minor soft tissue injuries that are unlikely to influence systemic purine nucleoside production. In any future study the confounding influence of significant polytrauma should be considered and investigated.

6. Discussion

The results from the general anaesthetic (GA) carotid endarterectomy (CEA), dynamic control and non-vascular control cohorts were significantly different to the reported results from the previously published local anaesthetic (LA) CEA cohort (6), the results of which formed the theoretical basis of this work. Periods of significant intraoperative ischaemia are common to all these procedures. The only methodological differences between the cohorts were the anatomical location of ischaemia, duration of ischaemia and the choice of anaesthesia chosen under which to perform the procedure.

6.1 Factors influencing nucleoside determination

6.1.1 General anaesthesia

It would appear general anaesthesia may exert an effect on purine nucleoside production and release confounding any interpretation of results from intra-operative purine determination. The lack of significant systemic (arterial) rise in purine nucleosides in any of the general anaesthetic subgroups (especially the GA CEA study) may indicate GA decreases cellular metabolic rate to a point where ischaemic purine nucleoside production does not occur, or their production is limited to a point where they can no longer be detected at significant concentrations in peripheral circulation. The influence of GA on purine production and systemic determination of concentrations of purine nucleosides released was unexpected though in retrospect, entirely predictable.

The effect of GA on cerebral energy use has been extensively studied. The mechanism of action of many anaesthetic agents is not fully understood but it is well established that cerebral neuronal activity is depressed by general anaesthesia (213). It is extremely difficult to directly measure cerebral metabolism *in vivo* and a number of proxy measures are widely used. The vast majority cerebral metabolism is via aerobic glycolysis (214, 215) therefore changes in cerebral uptake of oxygen ($CMRO_2$) and relative glucose metabolic rate (rGMR) are commonly used as markers for changing cerebral metabolic rate (216, 217). Multiple studies have used positron emission tomography techniques to study $CMRO_2$ and rGMR in a variety of different anaesthetic agents. Sevoflurane was found to decrease global cerebral rGMR by 56% ($p<0.01$) (218) and propofol induced a decrease in rGMR of 54% ($P<0.01$) (217). Propofol also induced a total reduction in cerebral blood flow (CBF) of 47%. Xenon was shown to decrease rGMR but increase CBF (213). Volatile induction of anaesthesia has

also been shown to have less impact on cerebral oxygenation when compared to total intravenous anaesthesia (219).

In addition to changes in metabolic rate, GA also affects cerebral blood flow. Under normal physiological conditions cerebral blood flow and cerebral glucose metabolism are tightly linked (220, 221). The studies above demonstrated that with many commonly used anaesthetic agents this coupling is lost (216). rGMR has been found to be decreased disproportionately (i.e. to a greater extent) when compared to CBF (217). The term luxury perfusion was first coined by Lassen (222, 223) in the 1960s to describe a phenomenon of oxygen delivery to cerebral tissue in excess of metabolic requirement. This can occur due to relative hyperaemia such as in response to a period of hypoxia or a profound decrease in cellular metabolic rate with preserved or moderately decreased CBF such as in the case of cellular necrosis following stroke or brain injury. Changes in metabolic rate and blood flow induced by GA may mimic this phenomenon by reducing cerebral metabolic rate on induction of anaesthesia. It can be hypothesised that the reduction in flow caused by carotid clamping is compensated for (in terms of ischaemic stress) by the concurrent decrease in cerebral metabolic rate. GA has also been shown to offer favourable neurological outcomes in experimental models of cerebral ischaemia and traumatic brain injury when compared to an remaining concious, This finding has been attributed to a lower metabolic demand on the brain in the short term after injury (224).

There is comparatively less evidence of the effects of anaesthesia on metabolic rates of non-cerebral tissue. Both flurane-based and high-dose fentanyl anaesthetic choices have been shown to decrease cardiac oxygen consumption, evidenced by increased coronary sinus oxygen partial pressures; 8.3±0.5 $ml.dl^{-1}$ at baseline to 9.1±0.5 $ml.dl^{-1}$ after induction of anaesthesia, and falls in myocardial oxygen volume, 9.9±4.0 ml/min^{-1} at baseline to 6.9±4.1 ml/min^{-1} after induction of anaesthesia, in patients undergoing coronary artery bypass grafting (225, 226). These changes were not replicated with low-dose fentayl techniques (227) indicating the effect of fentanyl on metabolic rate may be dose-dependent. Glucose uptake and production have also been shown to fall during periods of anaesthesia (228). Spinal anaesthesia, whilst having no effect on cerebral metabolic rate does have a similar metabolic effect of peripheral tissues, has been shown to decrease resting metabolic rate of skeletal muscle measured via indirect calorimetry. Oxygen uptake was reduced by 25% from

baseline and was correlated with decreased resting-muscle activity recorded by mechanomyography in the anterior thigh (229).

The anaesthetic agents mentioned above, or derivatives of them, are all commonly used in current anaesthetic practice and were used to anaesthetise participants in all of the studies described here. In all of the study subgroups anaesthetic choice was not standardised in the study protocol but was at the discretion of the anaesthetic team. As such there was variation within and between cohorts. The aim of the studies was not to investigate the effect of choice of anaesthetic agent on changes in intraoperative metabolic rate. The potential significance (and therefore confounding influence) of anaesthetic choice became apparent during the study period. In retrospect, it stands to reason that the decreases in metabolic rate induced by anaesthesia may be sufficient to prevent or significantly attenuate ischaemic purine nucleoside production as previously discussed in relation to cerebral metabolic rate. Future work around purine nucleosides should seek to standardise anaesthetic regimen to avoid this as a potential confounder.

A number of factors other than anaesthetic that may influence all *in vivo* applications of purine nucleoside and SMARTchip testing have been identified. These should be considered during sampling and in interpretation of any results achieved.

6.1.2 Mass of ischaemic tissue

The mass of ischaemic tissue clearly has an important bearing on total adenosine triphosphate (ATP) metabolism and subsequent purine nucleoside release. In the experiments here; the procedure and the vascular inflow dictates the site of ischaemia. This in turn will result in varying volumes of ischaemic tissue from which purine nucleosides may be released. Studies performed by the united states of America military have reported the mean mass of an adult male leg at 10.5kg (230) an open abdominal aortic aneurysm repair would render both legs and the pelvis ischaemic. The total mass of ischaemic tissue is likely to be in excess of 20kg. Compare this to the mass of an average male human brain of 1.33kg (231) During CEA one cerebral hemisphere will undergo a reduction in blood but will not be rendered completely ischaemic so as not to induce stroke. Continued flow is either via supply via the circle of Willis from the contralateral hemisphere, ipsilateral and/or contralateral vertebral arteries or an ipsilateral intraoperative shunt.

A deep inferior epigastric artery perforator (DIEP) flap has a mean mass of 644g (232). Therefore, at average mass, an abdominal aortic aneurysm repair may have upwards of 17.5 times the amount of ischaemic tissue of a DIEP flap. In analysis of samples obtained from the systemic circulation (i.e. capillary or arterial line samples) it is possible that total purine release may influence determined concentrations in the samples. Whilst the aim of the dynamic control studies was not inter-group comparisons the differences in tissue mass, as well as tissue composition and metabolic rate (discussed next), serve to illustrate that a larger volume of ischaemic tissue may produce a greater rise in purine nucleosides than a smaller volume of tissue as a greater number of cells would be physiologically stressed and forced to metabolise ATP rather than re-phosphorylate it for reuse.

6.1.3 Metabolic rate of ischaemic tissue

In addition to mass, metabolic rate of the tissue in question must be considered. Energy requirements of specific tissues vary significantly throughout the body. Tissues with high metabolic rates are vulnerable to irreversible ischaemic damage in shorter time periods than tissues with lower metabolic rates; for example in the brain is most susceptible to injury given its high metabolic rate and limited pool of energy substrate (8, 233). Irreversible cerebral cellular damage is detectable in less than 20 minutes of ischaemia. Myocardium and kidney show irreversible damage at 20 and 30 minutes respectively (108).

In vivo metabolic rate of individual organs and tissues has been estimated. First reported by Elia in 1992 as organ specific *Ki* values, in the format of resting energy expenditure (REE) in $KcalKg^{-1}.d^{-1}$ (Kilocalories per kilogram per day) (234). This initial estimate has been validated in young adults by examining oxygen consumption and carbon dioxide production at rest with radiological assessment of organ mass (235). Heart, kidney, liver and brain are, as expected, large users of energy: 440 $KcalKg^{-1}.d^{-1}$ for heart and kidneys, 240 $KcalKg^{-1}.d^{-1}$ for brain and 200 $KcalKg^{-1}.d^{-1}$ for liver. Skeletal muscle and adipose tissue require significantly less energy, 12 and 4.5 $KcalKg^{-1}.d^{-1}$ respectively. These differences in tissue metabolic profiles are highly likely to influence the metabolism of ATP and subsequent purine nucleoside release. Tissues will comparatively lower energy expenditure may exhibit delayed or lower total release of purine nucleosides when compared to tissues with a higher metabolic demand in response to ischaemia.

Obesity has also been shown to influence REE. Although adipose tissue has a relatively low metabolic rate, its mass and distribution can vary significantly between individuals or within a single individual over time. The cumulative effect on energy requirement may be significant. Predicted REE was found to be significantly higher than measured REE in obese females. The hypothesis to explain this was fatty infiltration of organs and skeletal muscle served to decrease lean mass of the organ thus actually decreasing functional organ mass (236). There can also be significant differences in adipose distribution between males and females (237). This may exert some influence on purine production and release.

6.1.4 Subject age

Age has been shown to independently influence metabolic rate. REE in adults over 50 was found to decrease with increasing age by 1-2% per decade (235). This may be due to decreasing mass of tissue; Skeletal muscle mass has been found to decline at a rate of 3-8% per decade after 30 years of age (238) and cerebral tissue mass has been shown to decrease at 5% per decade after 40 years of age (239). Though this loss of mass is multifactorial a reduction in absolute cell numbers has been noted. This reduction in REE may influence purine response to ischaemia.

6.1.5 Body temperature

Increasing temperature is known to increase metabolic rate in tissues. Each degree increase results in a 10-13% increase in oxygen consumption (240). Similar changes in energy use occur with decreasing temperature. Whilst core body temperature is tightly regulated between 36.5 and 37.3°C peripheral and skin temperatures can be 2-4°C lower, influenced to a greater degree by a both physiological factors such as vasoconstriction and environmental factors such as ambient temperature, wind-chill and adequacy of clothing. General anaesthetic can influence temperature regulation via blunting autonomic physiological responses to hypo- or hyperthermia such as shivering or sweating, vasodilatory effects of the anaesthetic agents, reducing metabolic rate (and thus heat production) and skin exposure to allow adequate access to the surgical site (241). External temperature control in the form of heat mats or blankets are commonly used to regulate intraoperative temperature but significant perioperative temperature variation is common (242).

In body extremities changes in temperature are used as a clinical signs of ischaemia with decreased flow leading to cooler peripheries (171). Topical cooling of limbs during ischaemia to minimize reperfusion injury has been described (243), although this is not a strategy widely employed in clinical practice. Hypothermia is intentionally used in transplantation. Once the organ to be transplanted has been explanted from the donor it can be stored for short periods in ice and cold isotonic preservation fluid (known as the cold ischaemic time). A reduction of tissue temperature to 15°C has been shown to reduce tissue oxygen consumption to 12% of normothermic tissue (244). Decreasing temperature, and therefore metabolic demand, of tissues has been shown to reduce ATP depletion, oxygen free radical production and progressive intracellular acidosis all of which are known to contribute to cellular apoptosis and necrosis and therefore detrimental to graft function and survival (245, 246). Hypothermia does not completely halt ischaemic insult to tissues and prolonged cold ischaemia times are still associated with ischaemia-reperfusion injury and delayed graft function (247).

There are clear links between temperature and metabolic rate as well as metabolic rate and purine production. Therefore, a relationship will exist between ischaemic purine production and temperature that this series of studies was not designed to investigate. Decreased temperature *in vivo* during ischaemia will theoretically decrease purine production as metabolic demand is reduced with the reverse true for hyperthermia. Significant variation in temperature may require adjustment for when interpreting purine nucleoside concentrations clinically. If a patient has suffered prolonged exposure to temperature extremes immediately prior to testing this may confound any result obtained.

6.1.6 Heterogeneity of tissue within the ischaemic area

An ideal experimental model of purine release would be to study a single tissue type and its response to ischaemia. This is true for the carotid endarterectomy models (model of brain ATP metabolism) but is not for the other experiments. Ischaemia *in vivo* will affect an anatomical region, be that organ or limb, based around occlusion of the vascular supply. To use a lower limb as an example it is large volume and heterogeneous in its composition including; bone, muscle, adipose and skin. Each tissue type will contribute differently to purine nucleoside release with the most metabolically active tissue such as muscle

contributing a greater proportion of total purine nucleoside release than less metabolically active tissue such as adipose.

Whilst an important factor to consider in a research setting the aim of SMARTchip is to be used as a clinical diagnostic tool, where tissue-type heterogeneity will be almost universally apparent and therefore is of little consequence clinically.

6.1.7 Ischaemic preconditioning

Ischaemic preconditioning (IP) is a phenomenon whereby short cycles of total, but non-lethal, organ ischaemia followed by reperfusion offer a protective effect against cellular injury in subsequent prolonged ischaemia. This ischaemia can be local to the "target" organ or remote as periods of ischaemia of a given tissue has been demonstrated to confer protection against ischaemic injury in another tissue or organ (248). This phenomenon has been demonstrated in heart, brain, kidney (249) as well as improving muscle performance in trained cyclist and swimmers (250, 251). There are many hypotheses as to the mechanism by which IP offers protection from release of anti-inflammatory cytokines to activation of neuronal signalling pathways, though the exact protective mechanism remains unclear (252). Whilst interventional studies have focused on cycles of total ischaemia induced in either an upper or lower limb to offer protection against stroke or myocardial infarction the is also literature supporting a protective role for peripheral vascular disease against cerebral ischaemic damage (253). The authors hypothesised that chronic limb hypoperfusion caused by PAD may confer the same benefit as acute short duration ischaemia. Whilst it is known that PAD increases risk of myocardial infarction or stroke it would appear the extent of injury is decreased in patients with peripheral arterial disease compared to those without. IP has also been shown to extend time-to-pain in claudicants although this did not extend claudication distance (254, 255)

In the populations studied here the ischaemic insult observed in the study may not be the first ischaemic event experienced. All of the CEA patients had prior neurological manifestation of cerebral ischaemia and the chronic limb ischaemia arm were undergoing intervention for clinically significant and severely symptomatic critical limb ischaemia. The potential influence of IP on purine release from ischaemic tissues has not been previously studied though prior ischaemia may confer a degree cytoprotection decreasing purine release in those exposed compared to "ischaemia naïve" patients.

6.1.8 Collateral flow

In clinical practice, ischaemia is often not entirely absolute. Very few areas of the human body rely on a single arterial supply in order to reduce vulnerability to ischaemic events. Collateral vessels may provide ongoing inflow to an area of ischaemia. This is true within the brain, with potential for perfusion from the contralateral cerebral hemisphere via the circle of Willis or via leptomeningeal collaterals (256, 257) during periods of ischaemia. In the lower limb, development of collateral circulation is well documented and improvements in collateral circulation forms the basis of non-surgical management of patients with peripheral arterial disease either by enlargement of pre-existing collateral or new vessel formation (258). Cadaveric and radiological studies have identified collaterals in the lower limb originating from the profunda femoris or branches of the internal iliac system such as the superior gluteal or obturator arteries (259-261). It has been demonstrated peripherally that collateral supply will not provide full replacement of flow compared to a primary arterial supply (262) and development of significant collateralisation requires time. Clinically if there are signs or symptoms of ischaemia then the presence of collateral flow is inadequate to meet tissue demand for oxygen. In this circumstance ATP will be metabolised and purine nucleosides released. The impact of this collateral flow on total purine production is uncertain and has yet to be ascertained, its relevance to future use of SMARTchip in clinical medicine may be negligible.

6.1.9 Non-specificity of purine release.

Purine release is universal to all cells under ischaemic stress. Whilst this offers potential for purine nucleoside evaluation to be applied to a wide variety of clinical conditions elevated serum purine concentrations will not provide a diagnosis of tissue-specific ischaemia in isolation. This has led to comparisons with the use of D-dimer. Whilst D-dimer has been shown to have a high negative predictive value for deep venous thrombosis (DVT) it has a low specificity, having many confounding factors that may influence results obtained. This non specificity has led to D-dimer being much maligned in clinical practice, especially in comorbid surgical populations of patients. In reality D-dimer is useful, but can only be treated as a screening investigation, to rule out, rather than diagnose DVT (263, 264). It may be necessary to apply a similar logic to purine nucleoside use as a biomarker; its use and interpretation should be judicious and in line with strong clinical suspicion of a specific "ischaemic diagnosis".

6.1.10 Surgical stress response

The initiation of a catabolic endocrine state as part of the metabolic stress response to tissue trauma and surgery (265) could also influence increased purine nucleoside release. No significant rise was identified in the non-ischaemic surgery control cohort possibly meaning significant purine response to surgical trauma may not occur. Anaesthesia (both general, local and regional blockade) attenuate this stress response (265) and therefore may also exert an effect on purine nucleoside production.

6.2 Procedural and technical factors

In addition to *in vivo* patient and tissue factors influencing purine nucleoside determination there are a number of experimental procedural factors that may influence sampling and result interpretation in these studies.

6.2.1 Site of sampling

A factor that has become apparent in the experiments detailed here is the potential effect of sampling site in relation to the area of ischaemia. This is notable in the differences in results between the systemic and local sampling study designs whereby significant increases were only able to be identified from samples within ischaemic tissue. As previously documented purine nucleosides are inherently unstable within the circulation, half-lives of adenosine, inosine and hypoxanthine have been shown to be very short. Enzymatically driven metabolism to uric acid and as well as re-uptake and re-synthesis of ATP occurs rapidly (266). Whilst SMARTchip allows ease of sampling from capillary blood, most commonly obtained via finger prick sampling, results of the experiments detailed have raised the possibility that sampling from a site that is distant to the site of ischaemia may not produce a detectable rise in purine nucleosides.

Time taken for blood to circulate will influence the degree of metabolism and uptake. Under normal conditions circulation time would be in the order of seconds though there are multiple factors that can influence circulation time including cardiac output, peripheral vascular resistance, temperature and position of the sampling site relative to the heart (267, 268) all of which are influenced by a general anaesthetic. It may be that the extra time in circulation (i.e. time taken for circulation between jugular bulb and radial artery in the CEA study) is sufficiently long to enable significant metabolism/re-uptake to occur in a similar fashion to

the technical issues experienced in early experiments of purine concentration determination from serum prior to the development of "stopper" solutions to yielding negative results.

Given this, attempting to sample from (or as near as possible to) the ischaemic tissue would negate this time delay and thus minimise opportunity for purine metabolism between release from ischaemic cells and sampling. This is very dependent on site of ischaemia. The experiments here have examined ischaemia across a wide variety of tissue types. Whist it is not feasible to sample directly from vital organs, limb ischaemia does lend itself to this local sampling strategy.

6.2.2 Sample timing

Using SMARTchip, each sample analysis cycle takes a total of 345 seconds (5.75 minutes) to complete. This comprises of 165 seconds calibrating the SMARTchip and 180 seconds analysing. When comparing time matched samples, the minimum interval between sample timings that can be achieved is 510 seconds (8.5 minutes) comprising of one full analysis cycle and an additional calibrating cycle before the next analysis can begin. A further potentiostat, in order to run samples truly in parallel, was not available. The second time matched sample was not obtained until the analysis equipment had undergone calibration and was ready to analyse to prevent metabolism/re-uptake after sampling. In clinical practice as a diagnostic aid where a single sample is required at a given time this sample timing is not an issue. In the studies here, it must be noted that the paired samples are offset by approximately eight minutes. To standardise this difference paired samples were always obtained in a set order within each experiment.

6.2.3 Local tissue trauma

Purine release has been identified from traumatically injured tissues. Inflammatory response in reaction to tissue trauma results in local cellular ischaemia and subsequent purine release. This is a potential confounder within the local sampling studies. Initially sampling was attempted from within the sterile surgical field but distant to the operative site via small stab incisions or attempted direct puncture of visible skin veins. This was unsuccessful as bleeding from the skin was not sufficient to obtain samples. Where samples were obtained from within surgical incisions fresh venous bleeding from deep tissues was sought. Any pooled blood was not used for analysis in order to minimise the effect of tissue trauma on sample analysis.

Anecdotal evidence obtained during these studies would suggest where an initial finger prick does not yield sufficient blood for analysis excessive “milking” of blood from a sampling site may also produce a degree of tissue trauma to affect purine nucleoside concentrations and as such should be avoided.

Total purine production as a consequence of ischaemia is ultimately dependant on all of the factors discussed above. Each of this will influence nucleosides to different extents. Tissue volume and metabolic rate would seem to be the most important as they offer the greatest degree of differences between tissues investigated. Tissues with a higher metabolic rate will exhibit a rise in purines earlier in ischaemia than those with a lower metabolic rate, this is of clinical significance as a high metabolic rate is a feature of organs than fulfil essential functions for homeostasis. For example brain, heart, kidney and liver have the highest identified metabolic rates and all carry out functions essential to life (235). A diagnostic aid for the early detection of ischaemia is valuable in “high energy” organs in order to identify, investigate and intervene to relieve ischaemia (if clinically necessary) early as ischaemia of these organs will result in damage leading to loss of function at shorter durations of ischaemia than tissues with a lower metabolic rate.

6.3 Sensor development

6.3.1 *SMARTchip development*

Despite technological advances to enable SMARTchip development its production and use has remained challenging. Currently the sensor requires refrigerated storage at 4^{o}C. Ensuring safe, temperature controlled and monitored storage for a product that may be infrequently used may be challenging. An ideal sensor would need to retain functionality over a pronged period of time at ambient temperature.

The current biosensor is designed so that the biological sample is applied directly to the uncovered sensor, this mean the electrode ends are exposed and unprotected. Care must be taken during production, storage, handling prior to sampling, application of calibration solutions and application of the sample to be analysed. Any contact with the sensor prior to adding sample my cause damage to the electrode or polymer coating, leading to inaccurate or unreliable results. Use of SMARTchip clinical practice would require a more robust sensor and/or protective covering. An ideal protective cap is robust to direct contact, does not

contact the sensor and does not obstruct application of biological samples for analysis. Various iterations of sliding mechanisms and fixed covers that rely on hydrophilic coatings and capillary hydrostatic forces to transport sample from application site to sensor have been trialled, their refinement is an ongoing process. It has been noted that constituent of fluid is an important factor in how the cap behaves. Blood samples are more viscous than the buffer solutions and are more challenging to cover the whole sensing area than the buffer solutions. Further development is required prior to larger scale testing to ensure the biosensor is robust and reliable for results to be interpreted with the degree of confidence needed in clinical practice.

Relay of current along the chip body from sensor head to the potentiostat requires complete electrical isolation, this is achieved by a printed layer of an electrically inert polymer. Microscopic breaches in the covering leading to exposed electrode surface have been an ongoing issue during SMARTchip development. These breaches, leading to contact between electrodes and sample away from the correct position on the sensor lead to invalid results. As with sensor contact, in a research setting this can be adjusted for as results can be validated from the raw data and repeated if necessary though this is unlikely to occur in clinical practice especially in emergency situations. Work is ongoing to refine biosensor production and quality control will need to be validated prior to larger scale validation of SMARTchip.

Currently the biosensor equipment consists of a potentiostat connected to a laptop PC with the appropriate analysis software. Next generation biosensor development is already nearing completion. This improvement condenses the bulky, multi-piece equipment into a single battery powered, hand-held unit.

This progression and miniaturization of the technology is highly significant and represents a further step-change to open the door to potential of a validated biomarker that can be ascertained from capillary blood in a relatively short time frame on a portable device in a primary care, pre-hospital or outpatient setting Where its use (and results) may be of greater importance to aid diagnosis and triage patients appropriately to specialist services than within an acute hospital setting where other diagnostic methodologies are readily available.

6.3.2 Development of purines as a biomarker

As discussed previously purine nucleoside release occurs from a wide variety of cells and tissue types when physiologically stressed. This, whilst offering the advantage of applying the principle of purine nucleoside assessment to a wide variety of clinical scenarios, may present challenges in differentiating purine release by ischaemic tissues from potential purine release from concurrent physiological processes i.e. concurrent non ischaemic tissue injury or surgical stress response. Whether this potential for interference is a confounding factor in the presence of significant ischaemia is unknown and not conclusively demonstrated in this body of work. Quantification of and correction for any non-ischaemic or otherwise confounding purine release would necessary to validate purine nucleoside use as a biomarker.

This series of studies has grouped adenosine, inosine and hypoxanthine together as collective "purine nucleosides". SMARTchip currently does not differentiate between these nucleosides but gives an overall combined purine nucleoside result. This differentiation may offer an avenue of refinement in purine assessment *in vivo.* Some historical studies (23,77,88,97) have noted differences in changes between the different purine nucleosides during the same ischaemic stress event. Future development in purines as a biomarker may involve investigation into quantification of individual nucleosides rather than purine nucleosides as a group. If there is a differential increase in one nucleoside from a specific tissue it may be possible to negate (in part or whole) any confounding purine release, increasing test specificity thus making purine nucleoside determination more reliable as a biomarker of ischaemia in clinical practice.

As with interpretation of the result of any clinical investigation, the need for correlation of purine nucleoside assessment with a patients' physical symptoms and clinical signs will be required.

6.4 Potential clinical applications

All of the experiments detailed here are small scale studies providing proof-of-concept pilot data only. Results in acute limb ischaemia (ALI), chronic limb threatening ischaemia (CLTI) and traumatic brain injury (TBI) are encouraging. Further work is required in each case to validate the results obtained here in clinical populations of patients. Given the confounding effect of GA the use of SMARTchip as an intra operative biomarker of evolving ischaemia may be limited, however, its use in populations of patients with significant ischaemia away

from an intra-operative setting may still be of clinical value. The new sensing technology also may re-open avenues of research previously discontinued due to technical challenges.

6.4.1 Carotid Endarterectomy

Whist it appears GA confounded appreciable purine rise in this experiment; no neurological events were noted within the study cohort. Further work is needed specifically focusing on those patients in whom significant cerebral ischaemia is detected. It may be that in our relatively small cohort collateral cerebral blood supply was adequate or the practice of prophylactic shunt insertion prevented significant ischaemia from occurring. The concept of a study only investigating patients with significant intraoperative cerebral ischaemia has a significant problem as there is no validated gold standard comparator test and it would be unethical to impose a never shunt policy on a study population. In theory, as there is no demonstrated superiority of routine vs selective vs never shunting (125) a study with a routine shunt policy and intraoperative purine sampling, comparing patients with and without post-operative evidence of neurological insult would be possible. However, given the low incidence of perioperative stroke, numbers of patients required to reach statistical significance is likely to be prohibitively large.

6.4.2 Acute limb ischaemia

The use of a tourniquet has allowed investigation of ALI in this experimental model. It offers reproducible acute, absolute interruption of blood inflow to a limb that is appropriate for this proof-of-concept study. The differences in this form of ALI and ALI presenting clinically have already been discussed.

The next step towards the use of purine nucleosides as a biomarker of acute limb ischaemia is to validate the results obtained here in patients presenting to medical practitioners with acute limb ischaemia. A larger scale follow on study recruiting patients presenting to secondary care with suspicion of acute limb ischaemia is needed. This study may also be able to identify if purine nucleoside determination can differentiate ALI from other causes of acute onset limb pain including progression of chronic ischaemia, spinal cord or nerve root compression, neuropathy and deep vein thrombosis (140, 269). Purine release in these ALI mimics has not previously been studied. It must be demonstrated that purine nucleoside determination results in improved patient outcomes, in terms of limb salvage, following ALI above current diagnostic methods used.

Ultimately SMARTchip may be most useful in clinical practice outside of a hospital setting, in primary care or during pre-hospital emergency care in order to appropriately triage patients towards hospitals that are able to offer vascular surgical services. Use of purines may also be applied in an emergency department setting where there is diagnostic uncertainty; though this is less important given there is likely to be timely access to appropriate imaging modalities and vascular specialist clinical assessment.

In addition, further investigation is required into purine rises in systemic samples in patients with limb ischaemia. Without a tourniquet restricting venous outflow it may be possible to obtain significantly raised and reliable results from finger prick samples in the absence of GA.

6.4.3 Chronic ischaemia

Nucleoside determination intra operatively in patients undergoing revascularisation may be of little clinical value. The differences demonstrated between baseline purine values in patients with Peripheral arterial disease (PAD) and patients without PAD are of clinical interest. As discussed with reference to the ALI study raised purine nucleosides in finger (or toe) capillary blood samples in patients with suspected PAD/CLTI could be a useful screening tool for non-vascular specialists to initiate referral to specialist services. In addition, change in nucleoside concentrations over time may be of interest to vascular specialists as hypothetically nucleosides would increase as PAD progresses and ischaemic stress on the limb increases. Purine nucleosides potentially could aid identification of patients who may need surgical or endovascular revascularisation prior to significant tissue loss developing. Purine nucleosides as a marker of PAD progression has not been investigated in this series of studies or any historic work.

Future work into purine nucleosides in CLTI/PAD could focus on patients in primary care or those referred to vascular services with symptoms consistent with PAD and correlate purine nucleosides with clinical and radiological findings in order guide appropriate referral to vascular services. Serial purine determination over time may define pattern of purine nucleoside change over time in the presence of PAD to determine usefulness as a marker of disease progression and potentially effect of medical management strategies of ischaemic stress within the limb. Comparisons of pre and post-operative nucleoside concentrations may also be useful to gauge success of intervention although ultimately clinical assessment in

terms of symptom resolution and wound healing is most likely of greater importance to both patient and clinician.

6.4.4 Traumatic brain injury

The small study presented here has identified purine nucleoside release may occur following TBI and can be detected in the peripheral blood circulation, although results were not conclusive. For SMARTchip to be clinically useful continued recruitment of patients with TBI is required to validate the preliminary findings of this small study.

Patients with an altered GCS, significant cognitive impairment or focal neurological deficit suggestive of TBI will need radiological imaging to assess type, extent and location of TBI in order to adequately assess need and to plan for surgical or radiological intervention. In these cases, assessment of purine nucleosides may be superfluous. Purine assessment may be of greater value where there are no clinical signs of TBI or baseline cognitive state of the patient is unclear but there is clinical suspicion of TBI based on individual patient risk factors or mechanism of injury. For example, purine nucleoside concentrations may be of use in a patient, at increased risk of bleeding due to taking either an antiplatelet or anticoagulant medication, who has sustained a fall and potential brain injury but has a normal or mildly reduced GCS (i.e. 14) to guide the need for detailed radiological imaging and/or admission for observation.

If findings of this study are validated SMARTchip may have potential to be used pre-hospital to guide first responders as to whether attendance at hospital is necessary, if so, it may also influence choice of hospital towards one with appropriate neurological or neurosurgical facilities.

Correlation is needed between peripheral nucleoside concentration (on admission and during recovery) and radiological appearances, GCS, other biomarkers currently under investigation in the assessment of TBI such as S100 calcium binding protein b and glial fibrillary acidic protein as well as both short term and long term outcomes both neurophysical and neuropsychiatric in order to validate its use. Clearly a great deal of further work is required before firm conclusions can be drawn about the full role of purine nucleosides in TBI *in vivo.*

Extrapolation of results into other clinical scenarios

6.4.5 Intestinal ischaemia

Intestinal ischaemia both vascular and non-vascular in origin remains a diagnostic challenge for clinicians. It may affect 1 in 1000 hospital attendances (270) with significant mortality rates of up to 80%. Early clinical features can be non-specific which introduces potential for diagnostic delay, compromising patient outcomes (271). Both traditional and novel biochemical markers such as lactate, c-reactive protein, intestinal fatty acid binding protein and α-glutathione S-transferase have been extensively investigated as a potential biomarker but all have ultimately shown poor performance in aiding a diagnosis of bowel ischaemia in clinical practice (270, 272). CT imaging is also useful in aiding diagnosis but may be contraindicated in renal failure or contrast allergy. Laparotomy remains the gold standard in obtaining a diagnosis but carries significant morbidity in itself. The need for a simple marker of early ischaemia remains.

Whist no significant change in peritoneal purine nucleosides during intestinal ischaemia has been noted (107) changes in serum purine nucleosides have not been investigated. Purines may offer potential as an early diagnostic aid in patients with a suggestive history and/or other supportive investigations. A potential confounder to this is in cases of ischaemia that is non-vascular in origin e.g. bowel strangulation. This obstruction prevents venous outflow as well as arterial inflow therefore a rise in capillary or venous blood samples may not be appreciated due to restriction in venous outflow. Venous outflow is not restricted in cases of arterial embolism, in situ thrombosis or ischaemia due to hypoperfusion. These aetiologies are less common (273) but it may be possible to detect a rise in purine nucleosides more readily than non-vascular causes.

6.4.6 Acute compartment syndrome

Acute compartment syndrome following surgery, ischaemia or trauma is rare (274). Its diagnosis can be further complicated by non-specific symptoms, concomitant analgesia or patient sedation. Consequences of a missed or delayed diagnosis can be catastrophic for the patient with potential for permanent neurological dysfunction, limb loss or death (275). Diagnosis in clinical practice relies on clinical examination (tense compartment to palpation, pain on passive stretch) or compartment pressure monitoring, which is invasive and often not routinely available. Given the pathophysiology of acute compartment syndrome is vascular

compromise secondary to increased compartmental pressure (276), logically purine nucleoside concentrations would be increased in its early phases. As with many other potential applications discussed here the role of purine nucleosides in compartment syndrome has not been studied. The noted rise in purine nucleoside concentrations at 24-hours in the CLTI cohort, attributed to reperfusion combined with the acute rise during ischaemia in the ALI cohort would support purine nucleosides as potential marker of acute compartment syndrome. Restriction of outflow may confound systemic sampling though sampling directly from the limb in question may be possible. Any potential study to investigate this, may be hampered by the rarity of the condition and the judicious use of fasciotomies prophylactically to prevent the development of compartment syndrome in "at risk" patients.

6.4.7 Myocardial infarction

Purine nucleoside release as an early biomarker in response to myocardial ischaemia or infarction (MI) has been previously studied with encouraging results in clinical settings sampling peripheral venous blood (5, 13, 38). Effectiveness of SMARTchip has yet to be studied in the context of chest pain or myocardial ischaemia.

Early intervention in patients with ST-elevation myocardial infarction (MI) in the form of percutaneous coronary intervention (PCI) within 120 minutes of first contact with a health professional is essential to effectively treating myocardial infarction (277). Delays between symptom onset and/or first medical contact and percutaneous coronary intervention (PCI) have been shown to increase all-cause mortality and risk of heart failure (278). Whilst in patients with non ST elevation MI current guidelines advocate a medical rather than interventional approach to initial management timely assessment, via catheter angiography, of future risk and management planning is essential (279).

Theoretically, the potential advantage of SMARTchip over other commonly used biomarkers such as troponin assay or other purine detection mechanisms as reported by Farthing et. al. (5) is the use of battery powered analysis units and capillary blood sampling to provide rapid results in a community or pre-hospital setting. In conjunction with clinical assessment and existing out of hospital diagnostic aids such as electrocardiogram may allow healthcare professionals to expedite triage and appropriate transfer to secondary care cardiology services with PCI capabilities.

A study to obtain pilot data (purine nucleoside concentrations from finger prick samples) in patients presenting to cardiology services with confirmed myocardial infarction is needed to validate SMARTchip in this setting which can then be expanded to include patients presenting with chest pain to determine sensitivity and specificities against chest pain of non-cardiac origin.

6.4.8 Stroke

Building on the results of SMARTchip in cerebral ischaemia (6, 81) the purines for rapid identification of stroke mimics (PRISM) study (280) is currently investigating the potential of SMARTchip in a pre-hospital setting. Patients with suspected stroke will be recruited, with finger prick testing undertaken by paramedics in the community with the aim of identifying stroke vs conditions mimicking stroke. The results of this will be eagerly awaited.

7. Conclusions

The purinergic role *in vivo* is complicated. Despite a metabolic pathway universal to all cells during ischaemia multiple factors influence purine nucleoside formation, release, metabolism and reuptake. Obtaining results that are accurate, reproducible and reliable in both research and clinical settings has been exceptionally challenging. As such, their use as a biomarker, whilst recognised for over 50 years has not been realised. Advances in understanding of purine metabolism combined with technological advancement would appear to have offered potential for purine nucleosides to now be a useful biomarker in clinical practice. Challenges remain in sampling analysis and result interpretation in the context of varying duration, degree and anatomic location of ischaemia.

This body of work initially focused on purine nucleosides as an intraoperative biomarker of ischaemia from systemic arterial samples. Significant rises in nucleoside concentrations were not demonstrated. It would appear that a combination general anaesthetic agents and site of sampling prevent useful measurement of in changes nucleoside concentrations from peripheral sites intra-operatively. Local capillary sampling from within an area of ischaemia intraoperatively may avoid these confounding influences encountered in sampling from sites distant to ischaemia.

Whilst use of purine nucleosides as an intra-operative (with general anaesthetic) biomarker would appear to be limited to peripheral sites, ultimately meaning they may actually be of little value clinically, potential for their use in surgical populations away from the operating theatre as well as in non-surgical populations remains. A model of acute limb ischaemia has been shown to cause a significant rise in purine nucleosides. There is also evidence of purine release from chronically ischaemic limbs and in some types of brain injury although the nature of this release would appear to be complex and has not been fully explained in this work. Significant further work is required to fully understand the profile of purine nucleoside release in these contexts as well as to validate purine nucleosides as a biomarker of ischaemia in clinical practice and true patient populations.

As the pathways of purine metabolism in ischaemia are universal to all cells and cells have to be metabolically active (and therefore still viable) in order for purine nucleosides to be produced it would appear future potential of nucleosides as an early (and therefore clinically

useful) biomarker of ischaemia is widespread across a range of ischaemic scenarios in both pre-hospital and secondary care settings. Expansion of this initial proof-of-concept work is required to both validate the results obtained here and investigate further area of clinical utility before the use of purine nucleosides as a biomarker of ischaemia in clinical practice can be realised.

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