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Elevated blood purine levels as a biomarker of seizures and epilepsy

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| Complete List of Authors: | Beamer, Edward; Royal College of Surgeons in Ireland, Department of Physiology and Medical Physics  
Lacey, Austin; Royal College of Surgeons Ireland, Physiology and Medical Physics  
Alves, Mariana; Royal College of Surgeons Ireland, Physiology and Medical Physics  
Conte, Giorgia; Royal College of Surgeons in Ireland, Department of Physiology and Medical Physics  
Tian, Faming; Sarissa Biomedical Ltd, Neuroscience; University of Warwick, School of Life Sciences  
De Diego-Garcia, Laura; Royal College of Surgeons in Ireland, Department of Physiology and Medical Physics  
Khalil, Mohamed; Beaumont Hospital, Neurological Services  
Rosenow, Felix; Hospital of the Goethe University Frankfurt Center of Neurology and Neurosurgery, Department of Neurology and Neurosurgery  
Delanty, Norman; RCSI Research Institute Royal College of Surgeons in Ireland, and Division of Neurology, Beaumont Hospital, Department of Molecular and Cellular Therapeutics  
Dale, Nicholas; Sarissa Biomedical Ltd, Neuroscience; University of Warwick, School of Life Sciences  
El-Naggar, Hany; Beaumont Hospital, Neurological Services  
Henshall, David; Royal College of Surgeons Ireland, Physiology and Medical Physics; Royal College of Surgeons in Ireland  
Engel, Tobias; Royal College of Surgeons Ireland, Physiology and Medical Physics |
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Title: Elevated blood purine levels as a biomarker of seizures and epilepsy

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Authors: Edward Beamer¹, Austin Lacey²,³, Mariana Alves¹, Giorgia Conte¹, Faming Tian⁴,⁵, Laura de Diego-Garcia¹, Mohamed Khalil⁶, Felix Rosenow⁷,⁸, Norman Delanty²,³,⁶, Nicholas Dale⁴,⁵, Hany ElNaggar¹,⁶, David C. Henshall¹,³ and Tobias Engel*¹,³

Affiliations:
¹Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, University of Medicine and Health Sciences, Dublin, D02 YN77, Ireland
²School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, University of Medicine and Health Sciences, Dublin, D02 YN77, Ireland
³FutureNeuro, Science Foundation Ireland Research Centre for Chronic and Rare Neurological Diseases, Royal College of Surgeons in Ireland, University of Medicine and Health Sciences, Dublin, D02 YN77, Ireland
⁴Sarissa Biomedical Ltd., Vanguard Centre Sir William Lyons Road, Coventry CV4 7EZ, UK
⁵School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK
⁶Neurological Services, Beaumont Hospital, Dublin 9, Ireland
⁷Epilepsy Center Hessen, Department of Neurology, Baldingerstr, 35043, Marburg, Germany
⁸Epilepsy Center Frankfurt Rhine-Main, Center of Neurology and Neurosurgery, University Hospital Frankfurt, and LOEWE Center for Personalized Translational Epilepsy Research (CePTER) Goethe-University Frankfurt, Frankfurt on the Main, Germany
*Correspondence:* Tobias Engel, Ph.D., Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, University of Medicine and Health Sciences, 123 St. Stephen’s Green, Dublin D02 YN77, Ireland
Tel: +35314025199, Fax: +35314022447, E-mail: tengel@rcsi.ie

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Abstract

**Objective:** There is a major unmet need for a molecular biomarker of seizures or epilepsy that lends itself to a fast, affordable detection in an easy-to-use point-of-care device. Purines such as ATP and adenosine are potent neuromodulators released during excessive neuronal activity that are also present in biofluids. Their biomarker potential for seizures and epilepsy in peripheral blood has, however, not yet been investigated. The aim of the present study was to determine if blood purine nucleoside measurements can serve as a biomarker for the recent occurrence of seizures and to support the diagnosis of epilepsy.

**Methods:** Blood purine concentrations were measured via a point-of-care diagnostic technology based on the summated electrochemical detection of adenosine and adenosine breakdown products (inosine, hypoxanthine and xanthine) (SMARTChip). Measurements of blood purine concentrations were carried out using samples from mice subjected to intraamygdala kainic acid-induced status epilepticus and in video-electroencephalogram-monitored adult patients with epilepsy.

**Results:** In mice, blood purine concentrations were rapidly increased ~2-3 fold post-status epilepticus [2.32 ± 0.40 µM (Control) vs 8.93 ± 1.03 µM (post-status epilepticus)] and levels correlated with seizure burden and post-seizure neurodegeneration in the hippocampus. Blood purine concentrations were also elevated in patients with video-EEG-diagnosed epilepsy [2.39 ± 0.34 µM (Control, n = 13) vs 4.35 ± 0.38 µM (Epilepsy, n = 26)].

**Significance:** Our data provides the proof-of-concept that the measurement of blood purine concentrations may offer a rapid, low-volume bedside test to support the diagnosis of seizures and epilepsy.
**Key points:**

- Blood purine concentrations increase following status epilepticus in mice.
- Blood purine concentrations correlate with seizure severity and seizure-induced neurodegeneration in mice.
- Blood purine concentrations are elevated in patients with video-EEG confirmed epilepsy when compared to healthy controls.
- Blood purine nucleoside-measuring devices may represent a novel method to support the diagnosis of seizures and epilepsy.
Introduction

The diagnosis of epilepsy and acute seizures represents a major clinical challenge; particularly in settings were a rapid diagnosis is critical such as in the emergency department. To date, patient monitoring via conventional video-electroencephalogram (vEEG) recording at hospitals remains the gold standard but this is time-consuming, costly, low throughput, and requires a high level of specialist expertise. Misdiagnosis rates are high and clinical signs can easily be confused with disorders which present in a similar way, such as psychogenic non-epileptic attacks. Accordingly, there is significant interest in the discovery and validation of novel biomarkers of seizures and epilepsy.

An ideal biomarker for epilepsy should be as minimally invasive as possible, be measured via a reproducible, easy-to-use and economically feasible analysis platform, have a rapid readout to enable prompt treatment and, be translatable from experimental models to patients. Moreover, biomarkers should provide a high sensitivity and specificity and be associated with pathological changes occurring during seizures and epilepsy. Current biomarkers under investigation for seizures and epilepsy include genetic markers, imaging and electrophysiological measures and changes in gene expression and metabolite concentrations in tissues. Among these, circulating biomarkers detectable in biofluids have attracted particular attention. This includes markers of inflammation such as cytokines (e.g., Interleukins) and different members of the complement cascade, markers of neuronal injury [e.g., Neuron-specific enolase (NSE)] and astroglial response (e.g., Glial fibrillary acidic protein (GFAP), protein S100β) and, more recently, circulating non-coding RNAs such as microRNAs and transfer RNA fragments. Several limitations of current biomarkers remain, however. This includes the need for difficult-to-access biofluids [e.g., cerebrospinal fluid (CSF)], the need for large volumes of blood or further blood processing, possibly contributing to inter-hospital
variability, sensitivity to haemolysis, molecules being unstable potentially leading to variable results or the lack of a rapid and cost-efficient analysis platform.

Altered purinergic signalling has emerged as a mechanism during the development of epilepsy and a therapeutic target for the treatment of seizures and epilepsy. While it has long been known that adenosine accumulates extracellularly in the brain in response to high levels of neuronal activity, thereby functioning as an endogenous anticonvulsant, mounting evidence now also demonstrates a casual role for ATP-driven receptors during the generation of seizures and epilepsy. Although usually present at low extracellular concentrations, ATP and other nucleotides and nucleosides (e.g., adenosine) can be actively released from neurons and glial cells via exocytotic and non-exocytotic mechanisms or passively from damaged or dying cells during different pathological conditions such as increased neuronal activity (e.g., during a seizure). Extracellularly, ATP is rapidly broken down into different breakdown products, thereby contributing to increased extracellular levels of adenosine and adenosine metabolites.

Notably, adenosine and adenosine metabolites have been known for some time to rise in the blood following neurological insults, such as a stroke and other ischemic brain injuries. Adenosine is found to be elevated in the CSF following traumatic brain injury (TBI) in children and in interstitial fluid in adults, along with concomitant increases in xanthine, hypoxanthine and cyclic AMP (cAMP). Adenosine levels have also been reported to be increased in the CSF of rats following pentylenetetrazole (PTZ)-induced seizures, and data has shown increased hydrolysis of ATP, adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in the blood of rats after single and chronic injections of PTZ.

Until recently, the short half-life, low concentration and technical difficulty of purine detection and quantification in biofluids were the main reasons preventing the translation of blood purine measurements as a practical biomarker assay, particularly where a fast diagnosis
is crucial. This has now changed with the development of enzymatic amperometric detection
technology that allows adenosine and its metabolites, inosine, hypoxanthine and xanthine to be
rapidly detected in blood. Refinements have resulted in the SMARTChip detection system
which has the size of a glucose test strip and which enables the fast detection of summated
combination of these purines in small volumes of blood.

Here, we used the SMARTChip system to measure purine concentrations in the blood
in experimental and human epilepsy. We report that blood purine concentrations are elevated
following seizures and during epilepsy, providing the proof-of-principal that blood-based
purine detection kits may represent a useful easy, rapid and cost-efficient bedside test to support
the diagnosis of seizures and epilepsy.

**Methods**

**Blood purine measurement**

The SMARTChip (Sarissa Biomedical, Coventry, UK) consists of a Ruthenium purple
sol-gel layer anchored to a gold-plated electrode containing a layer of enzymes, which catalyse
an iterative sequence of reactions, leading to the formation of electroactive H$_2$O$_2$ proportional
to the summed concentration of adenosine, inosine, hypoxanthine and xanthine. Each
SMARTChip features two purine and two null sensors (see Fig. 1A). Measurements are taken
from whole, unprocessed blood extracted from mice or patients (~20 µl) and calibrated to a 10
µM adenosine buffer solution. HPLC for validation of SMARTChips was carried out using
an Agilent 1260 HPLC with an Agilent Poroshell 120 EC-C18 column. The mobile phase had
a flow rate of 1ml/min and comprised 96% ultrapure H$_2$O with 0.1% formic acid, and 4%
acetonitrile. Over 3.4 min the acetonitrile was increased to 50% during a total runtime of 3.8 min. Adenosine was detected via absorbance at 263 nm and eluted between 2.6-2.8 min.

**Blood sampling after evoked seizures in mice**

All animal experiments were performed in accordance with the principles of the European Communities Council Directive (2010/63/EU). Procedures were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (RCSI) (REC 1322) and Health Products Regulatory Authority (AE19127/P038; AE19127/P001). Studies were undertaken using 8-12-week-old C57BL/6 OlaHsd male mice bred and housed in a controlled biomedical facility at RCSI on a 12 h light/dark cycle at 22±1 °C and humidity of 40-60% with food and water provided *ad libitum*. Mice were anesthetized using isoflurane (5% induction, 1-2% maintenance) and maintained normothermic by means of a feedback-controlled heat blanket (Harvard Apparatus Ltd, Kent, UK). Next, mice were implanted with a guide cannula for intraamygdala injections and skull-based electrodes for EEG recording. For this, mice were placed in a stereotaxic frame and a midline scalp incision was performed to expose the skull. A craniotomy was performed and a guide cannula fixed with dental cement on the surface of the dura overlying the amygdala (Bregma: AP = -0.94 mm, L = -2.85 mm). Three partial craniotomies were then performed and three screw electrodes were placed (Bilaney Consultants Ltd, Sevenoaks, UK), two overlying each dorsal hippocampus and one above the frontal cortex as reference, also fixed in place with dental cement. Mice were removed from the frame and allowed to fully recover from anaesthesia in a warmed incubator. SE was then triggered via microinjection of 0.3 µg kainic acid (KA) (Sigma-Aldrich, Dublin, Ireland), diluted in 0.2 µl of phosphate-buffered saline (PBS) into the right basolateral amygdala, 3.75 mm below the dura in immobilized (hand-restrained) awake mice. Non-SE control animals received intraamygdala microinjection of 0.2 µl of PBS. In the
KA-injected mice, seizures typically began within 10-15 min and comprised long bursts of high amplitude, high frequency epileptiform activity. After 40 min post-intraamygdala injection, all mice (KA and PBS-injected), except mice used in specific experiments presented in Figure 3, received an intraperitoneal (i.p.) injection of lorazepam (6 mg/kg) (Wyetch, Taplow, UK) to reduce SE-induced morbidity and mortality. Blood purine measurements were made immediately after SE. Blood was collected from 0-24 h post-lorazepam administration via puncture of the saphenous vein once per mouse if not indicated otherwise.28

Seizure severity during SE was quantified by analysis of EEG recorded from the skull-mounted electrodes using an Xltek recording system (Optima Medical Ltd, Guildford, UK). Data were analyzed offline using Labchart7 (AD instruments Ltd, Oxford, UK). Total power of EEG, frequency bands from 0-100 Hz and the amplitude domain filtered from 0-50 mV, was calculated in 5 min bins and summed for the 40 min duration of SE between injection of KA and lorazepam.27 Seizure burden was calculated by summing the time within the 40 min period of SE in which electrographic seizures occurred (frequency >5 Hz, with polyspike discharges of ≥5 s in duration).29 Seizure duration was counted manually by a reviewer blinded to purine readings. Power spectral density heat maps were generated within LabChart7.

Effects of physical activity on blood purine levels in mice

To determine the impact of increased muscle activity on blood purine concentrations, additional mice (C57/Bl6 OlaHsd) were individually forced to swim in a transparent Plexiglas cylinder (30 cm high, 20 cm diameter) containing a 15 cm depth of water at 25±2°C for 10 min. Water was replaced fresh after each mouse was tested. Blood purine concentrations were measured before (baseline) and immediately following the test from the same mouse.

Histopathology
Seizure-induced neuronal loss was assessed 24 h post-SE using Fluoro-Jade B (FjB) (Chemicon Europe Ltd, Chandlers Ford, UK). Briefly, 12 μm coronal sections at the medial level of the hippocampus (Bregma AP = -1.94 mm) were cut on a cryostat. Tissue was fixed in formalin, rehydrated in ethanol, transferred to a 0.006% potassium permanganate solution and incubated with 0.001% FjB (Chemicon Europe Ltd). Sections were mounted in DPX mounting solution. Then, using an epifluorescence microscope by a reviewer blinded to purine concentration readings, FjB-positive cells within the CA3 subfield of the hippocampus of two adjacent sections were counted and averaged for each animal.

**Human studies**

Ethics approval was obtained from the medical research ethics committees at Beaumont Hospital (Dublin, Ireland) (DUB, 13/75), and written informed consent was obtained from all participants according to the Declaration of Helsinki principles. Patients (n = 26; age = 35.77±2.6; male = 42%) were recruited during in-patient video-EEG monitoring at the Department of Neurology, Beaumont Hospital, Dublin, Ireland. All patients admitted to the epilepsy monitoring unit (EMU) had a detailed clinical assessment on admission including seizure types and frequency. Detailed patient demographics can be found in Supplementary Table S1. Controls (n = 13; age = 34.92 ± 2.1; male = 38%) were healthy Hospital and University staff without any known underlying neurological condition. Blood was sampled via finger prick and immediately analysed for purine content in a room adjacent to the EEG monitoring room, giving real-time analysis at the point of care. Control samples were measured either in same room as epilepsy patients or at RCSI. Baseline measurement was taken following a seizure-free period of at least 24 h.

**Statistical analysis**
Statistical analysis of data was performed using Prism 5 (GraphPad) and STATVIEW software (SAS Institute). Data are mean ± standard error of the mean (SEM). One-way ANOVA parametric statistics with post-hoc Fisher's protected least significant difference test was used to determine statistical differences between three or more groups. Unpaired Student's t-test was used for two-group comparison. Correlations between variables were assessed using Pearson's correlation coefficient. Receiver Operator Characteristic (ROC) analysis was performed to investigate the diagnostic ability of purine measurements for SE in mice and epilepsy in patients. Significance was accepted at p < 0.05.

Results

**Increased purine concentrations in the blood following SE in mice**

Purines were measured as a single accumulative measurement consisting of adenosine, inosine, hypoxanthine and xanthine using an enzymatic amperometric detection system. Here, a drop of whole unprocessed blood (~20 µl) is placed directly onto a sensor (SMARTChip), consisting of two null biosensors and two purine-detecting biosensors, which is subsequently inserted into a reader (Fig. 1A). This allows for the rapid measurement (~3 min) of purine concentrations using a minimal volume of blood.\(^{18}\) Accuracy of SMARTChips was verified by measuring different concentrations of adenosine added to PBS or human serum (Fig. 1B,C). Recovery of added adenosine in serum (~80%) was similar to previously described recovery levels in plasma\(^{25}\). Moreover, SMARTChips measured accurately known concentrations of adenosine downstream purines inosine and hypoxanthine at similar sensitivity to adenosine (Fig. 1D). Very little interference with purine measurement was observed for ascorbate,
acetaminophen and urate (Fig. 1E), which collectively provide more than 97% of the interfering signal for electrochemical measurements.30

Differences in blood purine concentrations were first measured after focally-evoked SE in mice (Fig. 1F).27 In this model, seizures begin within a few minutes of intraamygdala KA, become increasingly severe and continuous and are terminated after 40 min by lorazepam to reduce morbidity and mortality. Blood purine concentrations measured 40 min post-intraamygdala PBS injection in vehicle control mice were 2.32 ± 0.40 μM. This is similar to data reported previously for normal human blood purine levels using the same detection technology.31 Induction of SE by intraamygdala KA resulted in a rapid 2-3-fold increase in blood purine concentrations when compared to vehicle-injected control mice (Fig. 1G). Purine levels were already increased 0-10 min after the termination of SE by lorazepam (8.93 ± 1.03 μM) and remained significantly elevated for up to 4 h (9.28 ± 1.71 μM) when compared to time-matched vehicle-injected control mice, returning to baseline levels by 8 h post-lorazepam treatment (4.47 ± 1.53 μM) (Fig. 1G). Treatment with lorazepam slightly lowered blood purine concentrations in vehicle-injected control mice [3.43 ± 0.67 μM (pre-lorazepam) vs 1.60 ± 0.27 μM (1 h post-lorazepam), n = 9 (pre-lorazepam) and 5 (post-lorazepam), p = 0.074], demonstrating that lorazepam treatment did not contribute to increased purine concentrations post-SE and in line with lower blood purine levels found in patients under general anesthesia.31

ROC analysis determined that blood purine concentration measurements taken within the first 10 min post-lorazepam at a cut-off rate of 2.96 μM had the highest sensitivity (97%) and specificity (80%) for differentiating seizing from control mice (Fig. 1H).

To test whether changes in muscle activity in mice lead to increases in blood purine concentrations, naïve C57/Bi6 mice were subjected to a forced swim test. Blood purine levels were measured shortly before and immediately following the test. No significant differences in
purine concentrations were observed between measurements [2.56 ± 0.24 µM (before test) vs 2.80 ± 0.49 µM (post-test); n = 4 per group; \( p = 0.67 \)].

**Blood purine levels correlate with seizure severity and brain damage after SE in mice**

We next explored the relationship between elevated blood purine concentrations, seizure burden and histopathological outcomes. First, by combining measurements from the first 4 h following termination of SE, we found that blood purine levels correlate strongly with the severity of electrographic seizures during the time from intraamygdala KA until treatment with lorazepam, as measured by EEG total power (Fig. 2A) or seizure burden (high frequency, high amplitude (HFHA) polyspiking) (Fig. 2B). Blood purine levels also showed a strong association with seizure-induced neurodegeneration in the model. Intraamygdala KA-induced SE leads to a characteristic cell death pattern involving the ipsilateral brain hemisphere including the cortex and the hippocampus, in particular the CA3 subfield.27 The elevation in blood purine levels due to SE positively correlated with the extent of neuronal death in the CA3 subfield of the ipsilateral hippocampus 24 h post-SE, as evidenced by more counts of FjB-positive cells, a marker of neurodegeneration, in mice with higher purine concentrations (Fig. 2C).

To further test whether purine concentrations change according to seizure severity and treatment, an additional set of mice were subjected to SE and split into two groups. One group received an i.p. injection of the anticonvulsant lorazepam 40 min post-KA injection (lorazepam group); the second group was treated with i.p. vehicle-only (vehicle group) (Fig. 3A). As expected, SE mice treated with vehicle after 40 min continued to seize during the subsequent 2 h recording period, whereas SE was gradually suppressed in mice treated with lorazepam (Fig. 3B). Blood purine concentrations were measured at the time of vehicle/lorazepam administration and 2 h later. Confirming our previous results, blood purine levels were
approximately ~2-3 fold increased after SE when measured at the time of vehicle/lorazepam treatment when compared to control mice (Fig. 3C). Blood purine levels returned towards baseline control levels in seizing mice when treated with lorazepam (3.39 ± 0.41 µM). In contrast, purine concentrations in the blood increased even further in SE mice given only vehicle 40 min post-KA (14.27 ± 2.76 µM) (Fig. 3C). Further demonstrating blood purine concentrations to be a read-out of seizure severity, blood purine levels correlated strongly with EEG activity during the 2 h recording period post-lorazepam and vehicle-treatment (Fig. 3D).

Together, these results indicate that SE causes an increase in blood purines which reflects seizure burden and neuropathological outcome.

**Increased blood purine concentrations during epilepsy in humans**

We next measured blood purine levels in healthy controls and patients undergoing video-EEG analysis in the EMU at Beaumont Hospital, Dublin, Ireland. This included 26 drug-refractory epilepsy patients with the majority having temporal lobe epilepsy (TLE) (Supplemental Table S1). Continuous video-EEG monitoring with the 10-20 standard international electrode placement system was carried out on all patients and recordings were manually reviewed by a neurologist with special training in epilepsy.

Baseline measurements, collected via finger prick upon arrival at the EMU with patients being seizure-free for at least 24 h (Baseline) (Fig. 4A), detected approximately 2-fold higher levels of blood purines compared to measurements in healthy controls [2.39 ± 0.34 µM (Control) vs 4.35 ± 0.38 µM (Epilepsy)] (Fig. 4B, Table 1). ROC analysis demonstrated that measurements of blood purine levels could differentiate between controls and patients with epilepsy with a relative high level of sensitivity (69%) and specificity (85%) (Fig. 4C). No obvious differences in blood purine levels were detected between different patient groups according to treatment or diagnosis (Table 1, Supplementary Figure S1A). Moreover, no


14
significant correlation was found between blood purine concentrations and disease onset ($r^2 = 0.1871, p = 0.0829$), duration of epilepsy ($r^2 = 0.073, p = 0.224$), seizure frequency ($r^2 = 0.126; p = 0.104$), generalized vs focal epilepsy ($p = 0.36$) or brain lesion ($p = 0.12$) (Supplementary Figure S1B-E). Analysis of potential co-variables found no significant differences in blood purine concentrations in controls between gender [2.45 ± 0.60 µM (males) vs 2.34 ± 0.43 µM (females)], the time of day [2.53 ± 0.76 µM (morning, am) vs 2.30 ± 0.33 µM (afternoon, pm)] or according to age ($r^2 = 0.1138, p = 0.26$) (Supplementary Figure S2).

**Discussion**

Here we report elevations in blood purine concentrations following experimental seizures and during epilepsy in humans. Blood purine levels correlated with seizure severity and brain damage in mice and could distinguish patients with epilepsy from controls. Thus, enzymatic detection of purines could offer a promising seizure biomarker and method upon which to build diagnostic tools for epilepsy.

Different neurotransmitter systems [e.g., glutamate, γ-aminobutyric acid (GABA)] are altered in brain tissue following seizures\(^{32, 33}\), and, although not shown to undergo concentration changes in the blood of epilepsy patients, this has been observed in the blood in other neurological conditions such as schizophrenia (glutamate)\(^{34}\) or post-traumatic stress disorder (GABA)\(^{35}\). While purines are also well-known to be released in the brain during seizures,\(^{36}\) whether this translates into changes in blood purine levels has, to our knowledge, not been proven to date. While there are long-standing links between adenosine and seizures, the short half-life of purines in blood has proved technically difficult and labour intensive to assay.\(^{37}\) Here we used a new technology, SMARTChip, which uses an electrochemical method for a rapid detection of purines in whole unprocessed blood via a user-friendly diagnostic
device, thereby overcoming several limitations of current biomarkers under investigation for epilepsy.6

Using the SMARTChip, we report that acute seizures and epilepsy are associated with increased blood purine levels. In mice, purine levels increased within minutes of an evoked seizure. They returned to baseline in mice given an anticonvulsant and levels correlated with seizure burden and neurodegeneration suggesting that purine measurements in the blood may be a useful tool in the stratification of patients according to seizure severity and seizure-induced brain damage. Importantly, blood purine concentrations in our study (low μM concentration range) were similar to previous studies using either the same detection technique31 or other commonly used techniques (e.g., HPLC).38, 39 Elevated blood purines were also found in patients with epilepsy. This is unlikely to be related to clinical events in patients as blood purines were measured at least 24 h after the last seizure in humans. Future studies using both experimental models and patients should, however, be designed to exactly establish whether epileptic seizures alter blood purine concentrations, to what extent and for how long.

An important feature of this biomarker is an ability to determine absolute concentration per volume blood and its translatability from animal models to patients, with baseline levels and epilepsy-related increases being very similar between species. This provides not only further proof of blood purine levels as valid biomarker for seizures and epilepsy but also suggests that blood purine levels may be a useful tool in drug development, monitoring treatment effects in experimental models of epilepsy.4

The source of increased purine levels detected in the blood remains uncertain. ATP and adenosine are released into the extracellular space in brain under pathological conditions including increased neuronal activity.40 In line with purines coming from the brain, adenosine increases in the CSF following PTZ-induced seizures in rats.21 We can, however, not discard epilepsy-induced changes in the blood contributing to increased purine levels. Peripheral
inflammation is a characteristic of patients with epilepsy\textsuperscript{41} and previous data has shown increased nucleotide hydrolysis in the blood after PTZ-kindling in rats.\textsuperscript{23} Muscle cells are another possible source of purine release. However, blood purine levels remained the same following the forced swim test in mice and data has shown only minimal increases in blood purines after high-intensity training.\textsuperscript{42} The forced swim test has, however, a major stress component which may contribute to circulating purine concentrations and previous work has shown adenosine release in the brain post-physical exercise.\textsuperscript{43} Thus, whether physical activity contributes to altered purine concentrations during convulsive seizures warrants further investigations. The SMARTChip analytes include adenosine and its metabolites, inosine, xanthine and hypoxanthine all detectable in blood.\textsuperscript{44, 45} Our technology does not, however, enable us to determine which of these contributes to the observed increase in purine concentrations. It is tempting to speculate, however, the signal is predominantly adenosine downstream purines such as inosine and hypoxanthine which have a longer half-live (minutes) when compared to adenosine (seconds).\textsuperscript{46, 47} Nevertheless, our data provides the proof-of-concept that changes in purine concentrations can be used for seizure and epilepsy diagnosis. Future studies, using different techniques (\textit{e.g.}, HPLC) or SMARTChips using different enzymatic cascades may be used to exactly establish which purines are increased following seizures.

It is important to keep in mind, that a major limitation of our study is the fact that increased blood purine levels are not unique to seizures and epilepsy and have been previously reported post-TBI, post-ischemia and hypoxia.\textsuperscript{15-19, 48} Nevertheless, while specificity is always desirable, it is likely that a biomarker would not be used as a stand-alone test and would be evaluated within a clinical context in combination with other measures. Finally, while very promising, our results should be validated in a multi-centre prospective study to allow for a better understanding of the relationship between blood purine levels, seizure types (\textit{e.g.}, motor
seizures vs non-motor seizures; epileptic seizures vs psychogenic seizures), underlying disease phenotype and the time-course following seizures in experimental models and humans. While purine biosensors have been shown to be highly selective against interferences such as serotonin, ascorbic acid, urate and acetamineophen\textsuperscript{24}, cross-reactivity of sensors should be further validated against other substances possibly interfering with measurements. Effects of purines on other organs (e.g., heart\textsuperscript{49}) and common co-morbidities such as sudden unexpected death (SUDEP)\textsuperscript{50} are well documented. Thus, future studies should establish the possible correlation of increased purine levels in the blood with common co-morbidities associated with epilepsy.

In summary, this study offers a proof-of-concept that the enzymatic detection of blood purines has a high level of promise as a diagnostic tool capable of supporting early clinical decisions regarding monitoring and treatment of seizures and epilepsy. Because purines can be measured in whole unprocessed blood immediately following sampling and of the minimal need for equipment, this technology could even be implemented outside the normal hospital environment (e.g., GP surgeries, ambulances, emergency wards) where an underlying epileptic condition is suspected.

**Conflict of interest**

Dr. Faming Tian is an employee of Sarissa Biomedical Ltd and is an inventor on patents that describe methods to measure purines in biofluids. Prof. Nicholas Dale is a Director and Founder of Sarissa Biomedical Ltd, holds equity in that company and is an inventor on patents concerning the detection of purines and their use in the diagnosis of neurological diseases that are owned or licenced by Sarissa Biomedical Ltd. The remaining authors declare no conflict of interest.
Ethical Publication Statement

We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Acknowledgments

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References


Figure Legends

Figure 1. Increased purine concentrations in the blood following status epilepticus in mice. (A) Enzymatic cascade used to detect blood purine concentrations. Enzymes are entrapped within a layer on a Ruthenium Purple-coated gold electrode. Enzymes used are: Adenosine deaminase (1); Nucleoside phosphorylase (2), and Xanthine oxidase (3) and (4). Each SMARTChip consists of two purine biosensors and two null biosensors. Null biosensors lack sensitivity to purines, thereby acting as a control for any non-specific interferences. Each result is the average of two simultaneous measurements, one from each purine biosensor, which is then compared to each null sensor giving a total of 4 readings ([Purine sensor 1] - [Null sensor 1], [Purine sensor 1] - [Null sensor 2], [Purine sensor 2] - [Null sensor 1], [Purine sensor 2] - [Null sensor 2]). (B) Linear concentration test showing the ability of purine sensors (SMARTChips) to measure a range of known adenosine concentrations validated via HPLC in phosphate buffered saline (PBS). Each adenosine concentration was tested with 10 different SMARTChips (each SMARTChip was only used once). \( r^2 = 0.997, \ y = 0.996x \). (C) Measurement of serum samples spiked with known concentrations of adenosine. The serum contained a concentration of approximately 5 \( \mu M \) purines (y-axis intercept), and approximately 80% of the added purines were recovered, comparable to the recovery reported from plasma as reported in\(^\text{25} \). \( n = 5 \) SMARTChip measurements for 0 and 10 \( \mu M \) added adenosine, and \( n = 4 \) SMARTChips for 20 \( \mu M \) added adenosine. Each SMARTChip was used once. (D) Measurement of 10 \( \mu M \) adenosine (Ado), inosine (Ino) and hypoxanthine (Hx) in PBS via SMARTChip (each SMARTChip was calibrated with 10 \( \mu M \) of adenosine). Of note, the SMARTChip is slightly more sensitive to hypoxanthine than adenosine or inosine. \( n = 8 \) SMARTChips per purine and each SMARTChip was used only once. (E) Effect of interferences on the measurement of 10 \( \mu M \) adenosine with
100 µM ascorbate (AA), 100 µM acetaminophen (AAP), 1000 µM urate (UA), all in combination (All) and without added substances (positive control, +) in PBS. n = 9 SMARTChips for each combination and each SMARTChip was used only once. (F)

Experimental design to measure blood purine concentrations in unprocessed, whole blood collected from mice post-SE. SE was induced via an intraamygdala injection of kainic acid (KA) and EEG recorded via cortical electrodes. The anticonvulsive Lorazepam (Lz) was administered 40 min following intraamygdala KA or PBS injection via i.p. to curtail seizures and reduce morbidity. Blood samples (~20 µl) were collected from the saphenous vein via cheek pouch either at the time of lorazepam administration or at different time-points post-SE (0-10 min, 30 min, 1 h, 4 h, 8 h and 24 h). Only one measurement was taken per mouse. (G)

Graph showing increased purine concentrations in the blood post-SE (n = 0-10 min post-SE: 20 (Control) and 33 (post-SE), 30 min post-SE: 3 (Control) and 4 (post-SE), 1 h post-SE: 3 (Control) and 8 (post-SE), 4 h post-SE: 3 (Control) and 6 (post-SE), 8 h post-SE: 5 post-SE, 24 h post-SE: 3 post-SE). One-way ANOVA with Fisher’s post hoc test: F = 5.391; df (between columns) = 9; 0-10 min: p < 0.0001; 30 min: p = 0.0057; 1 h: p = 0.0084; 4 h: p = 0.0135. (H)

ROC analysis showing that blood purine concentration changes have a 96.97% sensitivity and 80.00% specificity to diagnose SE with a cut off of 2.96 µM (n = 20 (Control) and 33 (0-10 min post-SE). Data are mean ± SEM. **p < 0.01, ***p < 0.001.

**Figure 2. Blood purine concentration changes diagnose seizure severity and predict brain damage in mice.** (A) Graph and representative EEG recordings presented as heat maps of frequency and amplitude data showing a strong association of blood purine concentrations with seizure total power during SE (F = 34.48, DFd = 36, r² = 0.489, p = < 0.0001, n = 38). (B) Graph and representative EEG traces of high frequency and high amplitude (HFHA) spiking showing a strong association of blood purine concentrations with seizure burden during SE (F
= 35.76, DFd = 36, \( r^2 = 0.498 \), \( p = < 0.0001 \), \( n = 38 \)). (C) Graph and representative photomicrographs depicting FjB-positive cells in the CA3 subfield of the ipsilateral hippocampus 24 h post-SE, showing a strong association of blood purine concentrations with SE-induced neurodegeneration (\( F = 12.47, \) DFd = 32, \( r^2 = 0.281 \), \( p = 0.0013 \), \( n = 34 \)). Scale bar = 10 µm. Pearson's correlation coefficient test for A, B and C.

**Figure 3. Blood purine concentration changes decrease following treatment with lorazepam in mice.** (A) Schematic showing experimental design. Mice subjected to intraamygdala KA-induced SE were divided into two groups. One group received an i.p. injection of the anticonvulsant lorazepam (Lz) 40 min post-KA injection, while the other group received i.p. vehicle at the same time-point. (B) Representative EEG recordings presented as heat maps of frequency and amplitude data and graph showing more sever seizures in mice treated with vehicle when compared to mice treated with lorazepam during a 2 h recording period starting at the time of vehicle/lorazepam treatment 40 min post-intraamygdala KA injection [\( n = 6 \) (vehicle-treated mice) and 12 (lorazepam-treated mice)]. Two-way ANOVA with Bonferroni correction: \( F = 2.26; \) df = 36; 45 min: \( p < 0.0001 \); 55 min: \( p < 0.05 \); 60 min: \( p < 0.05 \); 65 min: \( p < 0.001 \). (C) Blood purine concentrations were measured at the time-point of vehicle/lorazepam treatment 40 min post-KA injection and 2 h following i.p. vehicle/lorazepam treatment. While purine concentrations decrease in mice treated with lorazepam, purine levels increase in mice treated with vehicle [\( n = 11 \) (vehicle-treated mice) and 6 (lorazepam-treated mice)]. One-way ANOVA with Fisher's post hoc test: \( F = 11.84, \) df = 3; Veh 0h vs Veh 2h: \( p = 0.0013 \); Lz 2h vs Veh 2h: \( p < 0.0001 \). (D) Graph showing strong correlation between blood purine concentration and seizure severity measured as EEG total power during a 2 h recording period following vehicle/lorazepam administration 40 min post-intraamygdala KA (\( n = 17 \) [11
(vehicle-treated mice) and 6 (lorazepam-treated mice)]. Pearson's correlation coefficient (F = 126.9, df = 16, r² = 0.89, p > 0.001). *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4. Increased blood purine concentrations in patients with epilepsy (A) To measure blood purine concentrations in patients, blood was collected via finger prick and immediately analyzed via SMARTChip. (B) Increased blood purine concentrations in patients with epilepsy when compared to control [n = 13 (Control) and 26 (Epilepsy, baseline)]. Baseline measurements from patients with epilepsy were taken following a seizure-free period of at least 24 h. Unpaired Student's t test (t = 3.364, df = 37, p = 0.0018). (C) ROC analysis showing that blood purine concentration changes have a 69% sensitivity and 85% specificity to diagnose epilepsy with a cut off of 3.5 µM (n = 13 (Control) and 26 (Epilepsy). **p < 0.01.
Figure 1

112x118mm (300 x 300 DPI)
Figure 2

159x154mm (300 x 300 DPI)
**Figure 3**

181x97mm (300 x 300 DPI)
Figure 4

172x67mm (300 x 300 DPI)
Table 1: Clinical details of patients with corresponding blood purine concentrations at baseline.

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Abbreviations: BRIV, Brivaracetam; CBZ, Carbamazepin; CLOB, Clobazam; ESLI, Eslicarbazepine; LAC, Lacosamide; LEV, Levetiracetam; LTG, Lamotrigine; MID, Midazolam; NEAD, Non-epileptic attack disorder; PERM, Perampanel; PHENB, Phenobarbital; TPN, Topiramate; VAP, Valproate; ZNS, Zonisamide