Elevated blood purine levels as a biomarker of seizures and epilepsy.

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FULL-LENGTH ORIGINAL RESEARCH

Elevated blood purine levels as a biomarker of seizures and epilepsy

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Abstract

Objective: There is a major unmet need for a molecular biomarker of seizures or epilepsy that lends itself to fast, affordable detection in an easy-to-use point-of-care device. Purines such as adenosine triphosphate 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 whether 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 intra-amygdala kainic acid-induced status epilepticus and in video-electroencephalogram (EEG)-monitored adult patients with epilepsy.

Results: In mice, blood purine concentrations were rapidly increased approximately two- to threefold after status epilepticus (2.32 ± .40 μmol·L^{-1} [control] vs. 8.93 ± 1.03 μmol·L^{-1} [after status epilepticus]), and levels correlated with seizure burden and postseizure neurodegeneration in the hippocampus. Blood purine

concentrations were also elevated in patients with video-EEG-diagnosed epilepsy ($2.39 \pm .34$ μmol·L^{-1} [control, $n = 13$] vs. $4.35 \pm .38$ μmol·L^{-1} [epilepsy, $n = 26$]).
Significance: Our data provide 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.

KEYWORDS
diagnosis, epilepsy, point-of-care device, purines, seizures

1 | INTRODUCTION

The diagnosis of epilepsy and acute seizures represents a major clinical challenge, particularly in settings where a rapid diagnosis is critical, such as in the emergency department. To date, patient monitoring via conventional video-electroencephalographic (EEG) recording at hospitals remains the gold standard, but this is time-consuming, costly, and low-throughput, and requires a high level of specialist expertise.[1] Misdiagnosis rates are high, and clinical signs can easily be confused with disorders that present in a similar way, such as psychogenic nonepileptic attacks.[2] Accordingly, there is significant interest in the discovery and validation of novel biomarkers of seizures and epilepsy.[1]

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.[1] Moreover, biomarkers should provide 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) and astroglial response (e.g., glial fibrillary acidic protein, protein S100β), and more recently, circulating non-coding RNAs such as microRNAs and transfer RNA fragments.[3–7] 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 interhospital variability; sensitivity to hemolysis; molecules being unstable, potentially leading to variable results[8]; and the lack of a rapid and cost-efficient analysis platform.

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

Altered purinergic signaling has emerged as a mechanism during the development of epilepsy and a therapeutic target for the treatment of seizures and epilepsy.[9,10] Although 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,[11,12] mounting evidence now also demonstrates a causal role for adenosine triphosphate (ATP)-driven receptors during the generation of seizures and epilepsy.[9] 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 nonexocytotic mechanisms or passively from damaged or dying cells during different pathological conditions such as increased neuronal activity (e.g., during a seizure).[13] Extracellularly, ATP is rapidly broken down into different breakdown products, thereby contributing to increased extracellular levels of adenosine and adenosine metabolites.[14]

Notably, adenosine and adenosine metabolites have been known for some time to rise in the blood following neurological insults, such as stroke and other ischemic brain injuries.[15–18] Adenosine is found to be elevated in the

CSF following traumatic brain injury (TBI) in children[19] and in interstitial fluid in adults, along with concomitant increases in xanthine, hypoxanthine, and cyclic adenosine monophosphate (AMP).[20] Adenosine levels have also been reported to be increased in the CSF of rats following pentylenetetrazole (PTZ)-induced seizures,[21] and data have shown increased hydrolysis of ATP, adenosine diphosphate, and AMP in the blood of rats after single and chronic injections of PTZ.[22,23]

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.[24] Refinements have resulted in the SMARTChip (Sarissa Biomedical) 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.[18,25,26]

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 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.

2 | MATERIALS AND METHODS

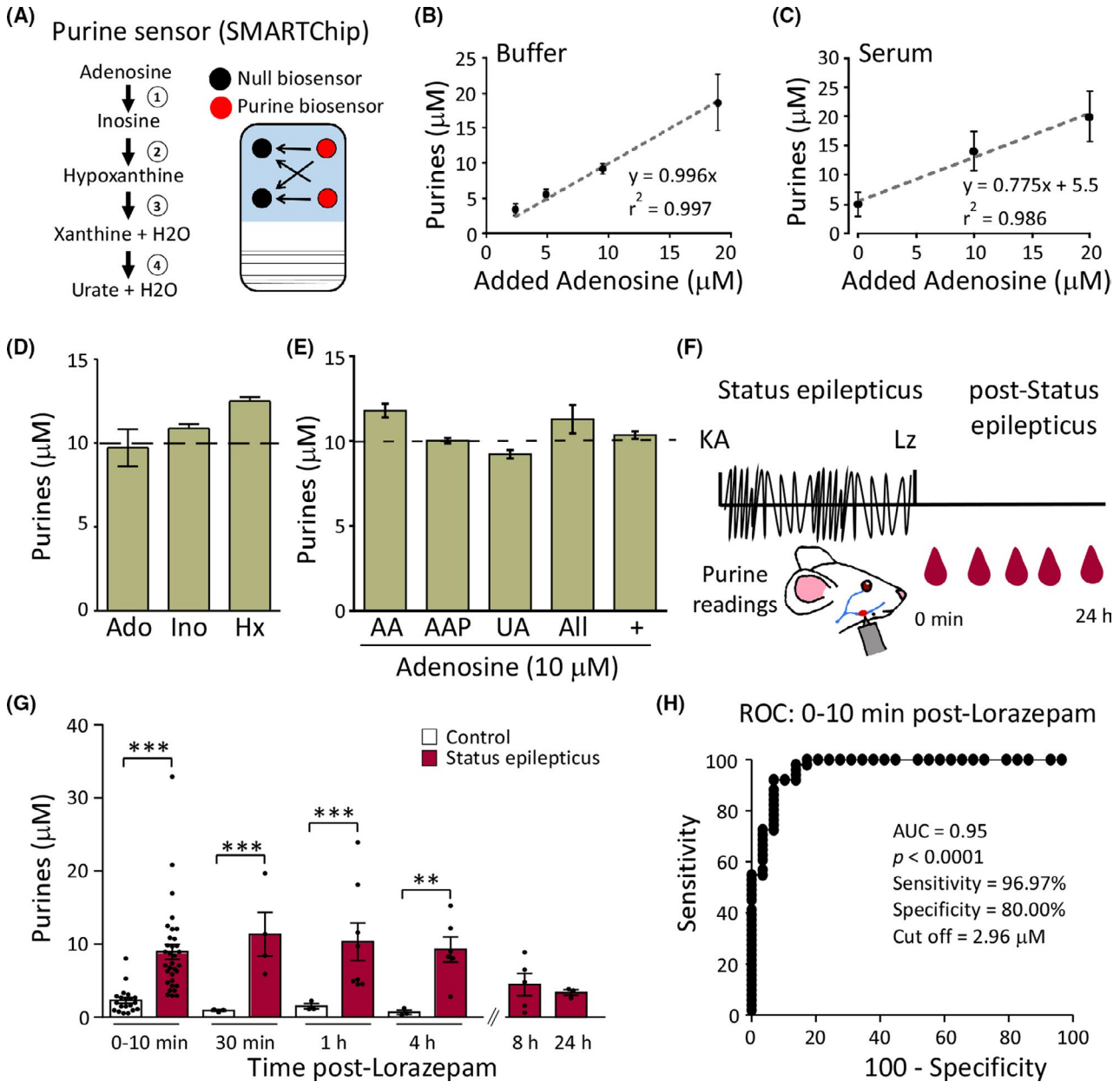
2.1 | Blood purine measurement

The SMARTChip consists of a ruthenium purple sol-gel layer anchored to a gold-plated electrode containing a layer of enzymes, which catalyze an iterative sequence of reactions, leading to the formation of electroactive H_2O_2 proportional to the summed concentration of adenosine, inosine, hypoxanthine, and xanthine.[25] Each SMARTChip features two purine and two null sensors (see Figure 1A). Measurements are taken from whole, unprocessed blood extracted from mice or patients (~20 μl) and calibrated to a 10-μmol·L^{-1} adenosine buffer solution.[18] High-performance liquid chromatography (HPLC) for validation of SMARTChips was carried out using an Agilent 1260 HPLC system with an Agilent Poroshell 120 EC-C18 column. The mobile phase had a flow rate of 1 ml/min and comprised 96% ultrapure H_2O with .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 and 2.8 min.

2.2 | 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 ± 1ºC and humidity of 40%–60%, with food and water provided ad libitum.[27] Mice were anesthetized using isoflurane (5% induction, 1%–2% maintenance) and maintained normothermic by means of a feedback-controlled heat blanket (Harvard Apparatus). Next, mice were implanted with a guide cannula for intra-amygdala 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 was fixed with dental cement on the surface of the dura overlying the amygdala (bregma: anteroposterior = −.94 mm, lateral = −2.85 mm). Three partial craniotomies were then performed, and three screw electrodes were placed (Bilaney Consultants), 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 anesthesia in a warmed incubator. Status epilepticus (SE) was then triggered via microinjection of .3 μg kainic acid (KA; Sigma-Aldrich), diluted in .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 intra-amygdala microinjection of .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. Forty minutes after intra-amygdala injection, all mice (KA- and PBS-injected), except mice used in specific experiments presented in Figure 3, received an intraperitoneal (ip) injection of lorazepam (6 mg/kg; Wyetch) to reduce SE-induced morbidity and mortality. Blood purine measurements were made immediately after SE. Blood was collected from 0 to 24 h after 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). Data were analyzed offline using Labchart7 (AD Instruments). Total power of EEG, with frequency bands from 0 to 100 Hz and the amplitude



domain filtered from 0 to 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.

2.3 | 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 in 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.

2.4 | Histopathology

Seizure-induced neuronal loss was assessed 24 h post-SE using Fluoro-Jade B (FjB; Chemicon Europe).[27] Briefly, 12-μm coronal sections at the medial level of the hippocampus (bregma: anteroposterior = −1.94 mm) were cut on a cryostat. Tissue was fixed in formalin, rehydrated in ethanol, transferred to a .006% potassium permanganate solution, and incubated with .001% FjB (Chemicon Europe). Sections

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 nonspecific 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 four 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 high-performance liquid chromatography in phosphate-buffered saline (PBS). Each adenosine concentration was tested with 10 different SMARTChips (each SMARTChip was used only once); $r^2 = .997$, $y = .996x$. (C) Measurement of serum samples spiked with known concentrations of adenosine. The serum contained a concentration of approximately 5 $\mu mol \cdot L^{-1}$ purines (y-axis intercept), and approximately 80% of the added purines were recovered, comparable to the recovery reported from plasma as reported in Tian et al.[25]; $n = 5$ SMARTChip measurements for 0 and 10 $\mu mol \cdot L^{-1}$ added adenosine, and $n = 4$ SMARTChips for 20 $\mu mol \cdot L^{-1}$ added adenosine. Each SMARTChip was used once. (D) Measurement of 10 $\mu mol \cdot L^{-1}$ adenosine (Ado), inosine (Ino), and hypoxanthine (Hx) in PBS via SMARTChip (each SMARTChip was calibrated with 10 $\mu mol \cdot L^{-1}$ 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 $\mu mol \cdot L^{-1}$ adenosine with 100 $\mu mol \cdot L^{-1}$ ascorbate (AA), 100 $\mu mol \cdot L^{-1}$ acetaminophen (AAP), 1000 $\mu mol \cdot L^{-1}$ 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 after status epilepticus (SE). SE was induced via an intra-amygdala injection of kainic acid (KA) and electroencephalogram was recorded via cortical electrodes. The anticonvulsive lorazepam (Lz) was administered 40 min following intra-amygdala KA or PBS intraperitoneal injection via to curtail seizures and reduce morbidity. Blood samples (~20 µl) were collected from the saphenous vein via cheek pouch either at the time of Lz administration or at different time points post-SE (0–10 and 30 min, 1, 4, 8, and 24 h). Only one measurement was taken per mouse. (G) Graph showing increased purine concentrations in the blood post-SE (0–10 min post-SE: $n = 20$ [control] and $n = 33$ [post-SE]; 30 min post-SE: $n = 3$ [control] and $n = 4$ [post-SE]; 1 h post-SE: $n = 3$ [control] and $n = 8$ [post-SE]; 4 h post-SE: $n = 3$ [control] and $n = 6$ [post-SE]; 8 h post-SE: $n = 5$ [post-SE]; 24 h post-SE: $n = 3$ [post-SE]). One-way analysis of variance with Fisher post hoc test: $F = 5.391$; *df* (between columns) = 9; 0–10 min: $p < .0001$; 30 min: $p = .0057$; 1 h: $p = .0084$; 4 h: $p = .0135$. (H) Receiver operator characteristic (ROC) analysis showing that blood purine concentration changes have 96.97% sensitivity and 80.00% specificity to diagnose SE with a cutoff of 2.96 $\mu mol \cdot L^{-1}$ ($n = 20$ [control] and $n = 33$ [0–10 min post-SE]). Data are mean ± standard error of the mean; $^{**}p < .01$, $^{***}p < .001$. AUC, area under the curve

were mounted in DPX mounting solution. Then, with an epifluorescence microscope employed 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.[27]

2.5 | 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 years, male = 42%) were recruited during in-patient video-EEG monitoring at the Department of Neurology, Beaumont Hospital. 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 Table S1. Controls ($n = 13$, age = 34.92 ± 2.1 years, male = 38%) were healthy hospital and university staff without any known underlying neurological condition. Blood was sampled via finger prick and immediately analyzed 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 the same room as epilepsy patients or at RCSI. Baseline measurement was taken following a seizure-free period of at least 24 h.

2.6 | 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. One-way analysis of variance parametric statistics with post hoc Fisher protected least significant difference test was used to determine statistical differences between three or more groups. Unpaired Student *t*-test was used for two-group comparison. Correlations between variables were assessed using Pearson 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 $p < .05$.

3 | RESULTS

3.1 | 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 (Figure 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 (Figure 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 (Figure 1D). Very little interference with purine measurement was observed for ascorbate, acetaminophen, and urate (Figure 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 (Figure 1F).[27] In this model, seizures begin within a few minutes of intra-amygdala 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 after intra-amygdala PBS injection in vehicle control mice were 2.32 ± .40 µmol·L^{-1}. This is similar to data reported previously for normal human blood purine levels using the same detection technology.[31] Induction of SE by intra-amygdala KA resulted in a rapid two- to threefold increase in blood purine concentrations when compared to vehicle-injected control mice (Figure 1G). Purine levels were already increased 0–10 min after the termination of SE by lorazepam (8.93 ± 1.03 µmol·L^{-1}) and remained significantly elevated for up to 4 h (9.28 ± 1.71 µmol·L^{-1}) when compared to time-matched vehicle-injected control mice, returning to baseline levels by 8 h after lorazepam treatment (4.47 ± 1.53 µmol·L^{-1}; Figure 1G). Treatment with lorazepam slightly lowered blood purine concentrations in vehicle-injected control mice (3.43 ± .67 µmol·L^{-1} [prelorazepam] vs. 1.60 ± .27 µmol·L^{-1} [1 h postlorazepam], $n = 9$ [prelorazepam] and $n = 5$ [postlorazepam], $p = .074$), 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 postlorazepam at a cutoff rate of 2.96 µmol·L^{-1} had the highest sensitivity (97%) and specificity (80%) for differentiating seizing from control mice (Figure 1H).

To test whether changes in muscle activity in mice lead to increases in blood purine concentrations, naïve C57/Bl6 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 ± .24 µmol·L^{-1} [before test] vs. 2.80 ± .49 µmol·L^{-1} [posttest], $n = 4$ per group, $p = .67$).

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

We next explored the relationship between elevated blood purine concentration, seizure burden, and histopathological outcome. 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 intra-amygdala KA until treatment with lorazepam, as measured by EEG total power (Figure 2A) or seizure burden (high-frequency, high-amplitude polyspiking; Figure 2B). Blood purine levels also showed a strong association with seizure-induced neurodegeneration in the model. Intra-amygdala 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 higher counts of FjB-positive cells, a marker of neurodegeneration, in mice with higher purine concentrations (Figure 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 ip injection of the anticonvulsant lorazepam 40 min post-KA injection (lorazepam group); the second group was treated with ip vehicle only (vehicle group; Figure 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 (Figure 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 two- to threefold increased after SE when measured at the time of vehicle/lorazepam treatment when compared to control mice (Figure 3C). Blood purine levels returned toward baseline control levels in seizing mice when treated with lorazepam (3.39 ± .41 µmol·L^{-1}). In contrast, purine concentrations in the blood increased even further in SE mice given only vehicle 40 min post-KA (14.27 ± 2.76 µmol·L^{-1}; Figure 3C). Further demonstrating blood purine concentrations to be a readout of seizure severity, blood purine levels correlated strongly with EEG activity during the 2-h recording period after lorazepam and vehicle treatment (Figure 3D).

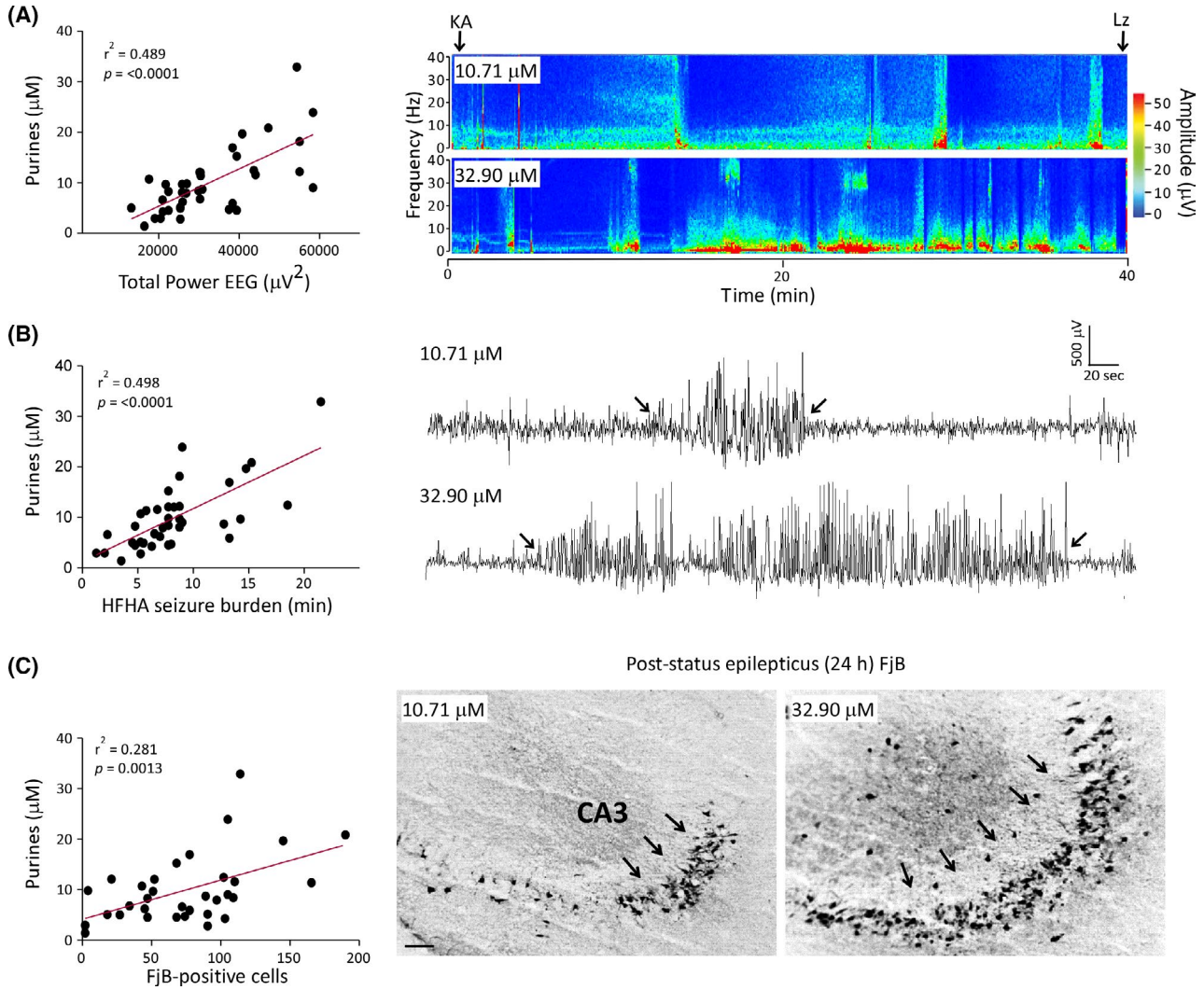


FIGURE 2 Blood purine concentration changes diagnose seizure severity and predict brain damage in mice. (A) Graph and representative electroencephalographic (EEG) recordings presented as heat maps of frequency and amplitude data showing a strong association of blood purine concentrations with seizure total power during status epilepticus (SE; $F = 34.48$, Degree of freedom [DFd] = 36, $r^2 = .489$, $p < .0001$, $n = 38$). (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, $r^2 = .498$, $p < .0001$, $n = 38$). (C) Graph and representative photomicrographs depicting Fluoro-Jade B (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, $r^2 = .281$, $p = .0013$, $n = 34$). Scale bar = 10 μm. Pearson correlation coefficient test was used for A–C. KA, kainic acid; Lz, lorazepam

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

3.3 | 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. This included 26 drug-refractory epilepsy patients, with the majority having temporal lobe epilepsy (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; Figure 4A), detected approximately twofold higher levels of blood purines compared to measurements in healthy controls (2.39 ± .34 μmol·L^{-1} [control] vs. 4.35 ± .38 μmol·L^{-1} [epilepsy]; Figure 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%; Figure 4C). No obvious differences in blood

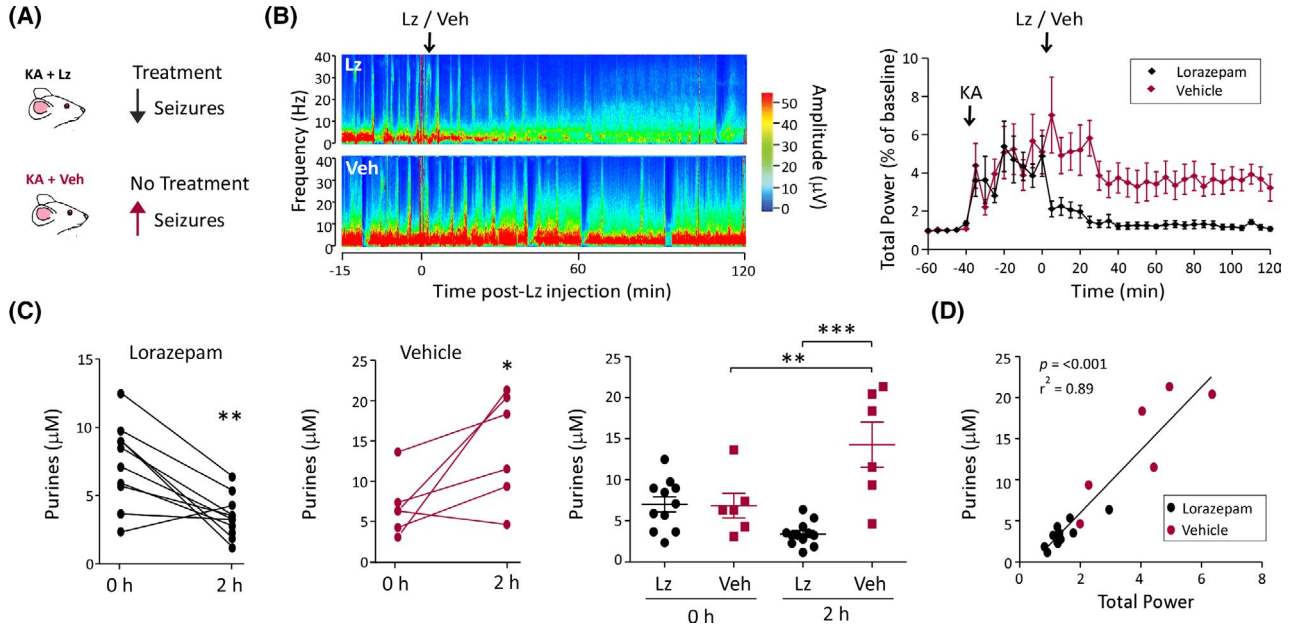


FIGURE 3 Blood purine concentration changes decrease following treatment with lorazepam (Lz) in mice. (A) Schematic showing experimental design. Mice subjected to intra-amygdala kainic acid (KA)-induced status epilepticus were divided into two groups. One group received an intraperitoneal (ip) injection of the anticonvulsant Lz 40 min after KA injection, whereas the other group received ip vehicle (Veh) at the same time point. (B) Representative electroencephalographic (EEG) recordings presented as heat maps of frequency and amplitude data and graph showing more severe seizures in mice treated with Veh when compared to mice treated with Lz during a 2-h recording period starting at the time of Veh/Lz treatment 40 min after intra-amygdala KA injection ($n = 6$ [Veh-treated mice] and $n = 12$ [Lz-treated mice]). Two-way analysis of variance (ANOVA) with Bonferroni correction: $F = 2.26$; $df = 36$; 45 min: $p < .0001$; 55 min: $p < .05$; 60 min: $p < .05$; 65 min: $p < .001$. (C) Blood purine concentrations were measured at the time point of Veh/Lz treatment 40 min after KA injection and 2 h following ip Veh/Lz treatment. Whereas purine concentrations decrease in mice treated with Lz, purine levels increase in mice treated with Veh ($n = 11$ [Veh-treated mice] and $n = 6$ [Lz-treated mice]). One-way ANOVA with Fisher post hoc test: $F = 11.84$, $df = 3$; Veh 0 h versus Veh 2 h: $p = .0013$; Lz 2 h versus Veh 2 h: $p < .0001$. (D) Graph showing strong correlation between blood purine concentration and seizure severity measured as EEG total power during a 2-h recording period following Veh/Lz administration 40 min after intra-amygdala KA ($n = 17$; 11 Veh-treated mice and six Lz-treated mice). Pearson correlation coefficient: $F = 126.9$, $df = 16$, $r^2 = .89$, $p > .001$. *$p < .05$, **$p < .01$, ***$p < .001$

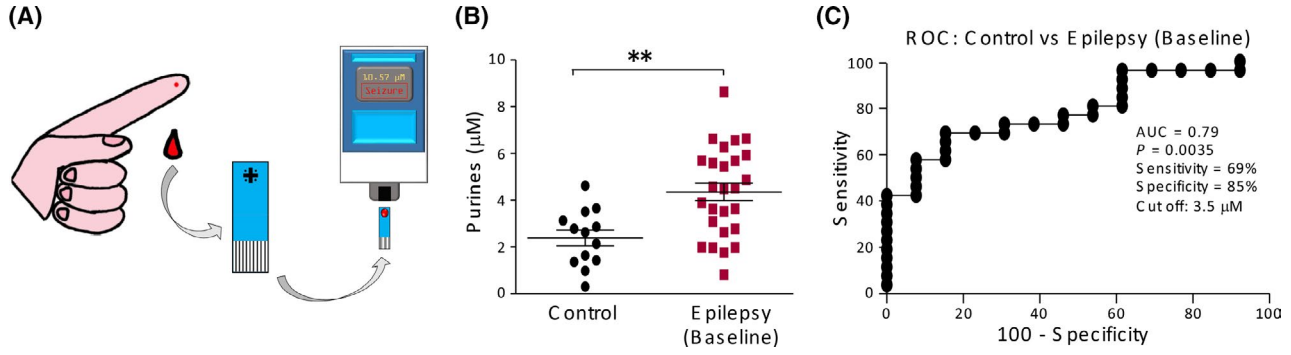


FIGURE 4 Increased blood purine concentrations in patients with epilepsy. (A) To measure blood purine concentrations in patients, blood was collected via finger prick and immediately analyzed via SMARTChip. (B) Increased blood purine concentrations in patients with epilepsy when compared to control ($n = 13$ [control] and $n = 26$ [epilepsy, baseline]). Baseline measurements from patients with epilepsy were taken following a seizure-free period of at least 24 h. Unpaired Student t-test: $t = 3.364$, $df = 37$, $p = .0018$. (C) Receiver operator characteristic (ROC) analysis showing that blood purine concentration changes have a 69% sensitivity and 85% specificity to diagnose epilepsy with a cutoff of 3.5 μmol·L^{-1} ($n = 13$ [control] and $n = 26$ [epilepsy]). **$p < .01$. AUC, area under the curve

purine levels were detected between different patient groups according to treatment or diagnosis (Table 1, Figure S1A). Moreover, no significant correlation was found between blood purine concentrations and disease onset ($r^2 = .1871$, $p = .0829$), duration of epilepsy ($r^2 = .073$, $p = .224$), seizure frequency ($r^2 = .126$, $p = .104$), generalized versus focal

TABLE 1 Clinical details of patients with corresponding blood purine concentrations at baseline

ID	Sex	Age, years	Onset	Purines, μmol·L^{-1}	Diagnosis	AEDs
1	F	54	17	3.52	TLE	PHENB
2	F	54	52	5.93	TLE	LEV, LTG, LAC
3	M	21	19	6.27	TLE	ESLI
4	F	23	21	5.45	TLE	LTG, LEV
5	F	58	16	3.62	TLE	LTG, LEV, CLOB
6	F	55	31	4.48	TLE	LEV, ESLI
7	F	27	16	1.76	TLE	LTG, LEV
8	M	54	20	3.89	TLE	ESLI, LTG
9	M	44	9	2.63	TLE	BRIV, CBZ, PERM
10	M	20	13	4.52	TLE	LTG, OXC, PHY
11	M	27	16	8.64	TLE	LTG, MID
12	F	45	3	2.77	TLE (heterotopia)	LTG
13	F	53	Unknown	3.09	TLE and NEAD	LAC
14	F	37	Unknown	.81	TLE and NEAD	LEV
15	F	22	21	5.59	Generalized epilepsy with focal features	LEV, CLOB, ZNS, PERM
16	F	28	1	4.56	Generalized epilepsy and NEAD	LEV, TPN
17	M	35	15	6.56	Parietal lobe epilepsy	ZNS, LEV
18	M	25	16	1.99	Likely frontal lobe epilepsy	LTG, LEV
19	F	22	18	3.63	Frontal lobe epilepsy	BRIV, ESLI, LAC
20	M	38	34	4.87	Frontotemporal epilepsy	ZNS, MID
21	M	45	34	5.69	Focal epilepsy	ZNS
22	M	23	13	1.97	Focal epilepsy secondary to cavernoma	LEV, LAC
23	F	42	32	5.7	Focal epilepsy (cortical dysplasia) and NEAD	BRIV, LTG, CLOB
24	M	20	Unknown	6.63	Idiopathic generalized epilepsy	BRIV, VAP
25	F	36	17	6.64	Idiopathic generalized epilepsy	BRIV, LAC
26	F	22	16	1.98	Idiopathic generalized epilepsy	LAC, LEV, TPN

Abbreviations: AED, antiepileptic drug; BRIV, brivaracetam; CBZ, carbamazepine; CLOB, clobazam; ESLI, eslicarbazepine; F, female; LAC, lacosamide; LEV, levetiracetam; LTG, lamotrigine; M, male; MID, midazolam; NEAD, nonepileptic attack disorder; OXC, oxcarbazepine; PERM, perampanel; PHENB, phenobarbital; PHY, phenytoin; TLE, temporal lobe epilepsy; TPN, topiramate; VAP, valproate; ZNS, zonisamide.

epilepsy ($p = .36$), or brain lesion ($p = .12$; Figure S1B–E). Analysis of potential covariables found no significant differences in blood purine concentrations in controls by gender (2.45 ± .60 μmol·L^{-1} [males] vs. 2.34 ± .43 μmol·L^{-1} [females]), time of day (2.53 ± .76 μmol·L^{-1} [morning] vs. 2.30 ± .33 μmol·L^{-1} [afternoon]), or age ($r^2 = .1138$, $p = .26$; Figure S2).

4 | 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] and posttraumatic stress disorder (GABA).[35] Although 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. Although there are long-standing links between adenosine and seizures, the short half-life of purines in blood has proved technically difficult and labor intensive to assay.[37] Here, we used a new technology, SMARTChip, which uses an electrochemical method for

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 micromolar concentration range) were similar to previous studies using either the same detection technique[31] 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 not only provides further proof of blood purine levels as a valid biomarker for seizures and epilepsy but also suggests that blood purine levels may be a useful tool in drug development for 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 cannot, however, discard epilepsy-induced changes in the blood contributing to increased purine levels. Peripheral inflammation is a characteristic of patients with epilepsy,[41] and previous data have shown increased nucleotide hydrolysis in the blood after PTZ kindling in rats.[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 have shown only minimal increases in blood purines after high-intensity training.[42] The forced swim test has, however, a major stress component that may contribute to circulating purine concentrations, and previous work has shown adenosine release in the brain after physical exercise.[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 of which are detectable in blood.[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, that the signal is predominantly adenosine downstream purines such as inosine and hypoxanthine, which have a longer half-life (minutes) when compared to adenosine (seconds).[46,47] Nevertheless, our data provide the proof of concept that changes in purine concentrations can be used for seizure and epilepsy diagnosis. Future studies, using different techniques (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 the major limitation of our study that increased blood purine levels are not unique to seizures and epilepsy and have been previously reported post-TBI, postischemia, and posthypoxia.[15–19,48] Nevertheless, although specificity is always desirable, it is likely that a biomarker would not be used as a standalone test and would be evaluated within a clinical context in combination with other measures. Finally, although very promising, our results should be validated in a multicenter prospective study to allow for a better understanding of the relationship between blood purine levels, seizure types (e.g., motor seizures vs. nonmotor seizures, epileptic seizures vs psychogenic seizures), underlying disease phenotype, and the time course following seizures in experimental models and humans. Although purine biosensors have been shown to be highly selective against interferences such as serotonin, ascorbic acid, urate, and acetaminophen,[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[49]) and common comorbidities such as sudden unexpected death in epilepsy[50] are well documented. Thus, future studies should establish the possible correlation of increased purine levels in the blood with common comorbidities associated with epilepsy.

In summary, this study offers 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 because of the minimal need for equipment, this technology could even be implemented outside the normal hospital environment (e.g., general practice surgeries, ambulances, emergency wards) where an underlying epileptic condition is suspected.

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CONFLICT OF INTEREST

F.T. is an employee of Sarissa Biomedical and is an inventor on patents that describe methods to measure purines in biofluids. N.Da. is a Director and Founder of Sarissa Biomedical, 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 licensed by Sarissa Biomedical. None of the other authors has any conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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