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Additional Declarations: **Yes** there is potential Competing Interest. RS BG and YD have filed patents on cfDNA methylation technology.

Brain-derived cell-free DNA as a marker for neuroapoptosis following prolonged general anesthesia in infants and children: results of the BRAIN study*.

***The BRAIN study = Biomarker Response to Anesthesia-Induced Neuroapoptosis.**

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ABSTRACT (150 words exactly)

Preclinical studies in rodents and non-human primates demonstrate anesthesia-induced neuroapoptosis in the developing brain. This prompted an FDA black-box warning for anesthetic agents in children <3yrs, despite no robust molecular or clinical data in humans. Here we report results of the BRAIN study, which used validated brain-specific cell-free DNA (cfDNA) methylation biomarkers to detect neuroapoptosis during prolonged general anesthesia. Following IRB approval and parental consent, serial plasma samples were collected from infants (≤ 12 m) and young children (1–3yrs) undergoing anesthesia ≥ 3 hrs; older children and adults served as controls. We quantified cfDNA derived from neurons, astrocytes and oligodendrocytes, with hepatocyte-derived cfDNA as a reference. General anesthesia was associated with a mild temporal increase in astrocyte-derived cfDNA ($p=0.001$) with a modest age interaction ($p=0.003$). No significant changes were observed for neurons or oligodendrocytes, or non-brain biomarkers. Signals were markedly lower than in established neuroinjury states, suggesting no widespread or clinically meaningful neuroapoptosis.

Prolonged exposure to any of numerous anesthetic drugs triggers accelerated neuroapoptosis in fetal and infant rodents, piglets, and non-human primates¹⁻⁸. The degree and distribution of neuroapoptosis appear to correlate both with the age of the animal at the time of exposure and with the cumulative duration of exposure⁹⁻¹¹. Animal studies further suggest that this neuroapoptosis may be associated with adverse neurologic and behavioral development^{9,12-16}.

The impact of anesthetic drugs on the developing human brain is not known. In experimental models, neuroapoptosis is demonstrated by sectioning and staining the brains of animal subjects; this is obviously not possible with human subjects, and human studies have thus been limited to neurodevelopmental, rather than molecular or cellular, testing.

Retrospective studies in children are subject to considerable bias and have yielded conflicting results¹⁷⁻¹⁹. The only randomized prospective study conducted to date concluded that a single, brief (<1 hour) exposure to general anesthesia in infancy does not introduce measurable neurodevelopmental changes²⁰. Despite the paucity of human data, the Federal Drug Administration warned that “repeated or lengthy use of general anesthetic and sedation drugs during surgeries or procedures in children younger than 3 years or in pregnant women during their third trimester may affect the development of children’s brains.”²¹

Dying cells release DNA fragments into the bloodstream, and the presence of circulating cell-free DNA (cfDNA) in plasma can serve as an indicator of ongoing cell death. Tissue-specific DNA methylation patterns allow identification of the source tissue of cfDNA²²⁻²⁵. Since the half-life of cfDNA is about 15 minutes^{26,27}, the presence of cfDNA fragments from a specific tissue provides indirect evidence of recent organ-specific cell death. We and others have previously

demonstrated tissue-specific cfDNA elevations in multiple pathologies, including elevations in brain-derived cfDNA associated with ischemia²⁸, inflammation²², cancer²⁵, and acute psychosis³⁰. We designed the BRAIN study (Biomarker Response to Anesthesia-Induced Neuroapoptosis) to use brain-derived cfDNA biomarkers to objectively assess molecular evidence of neuroapoptosis in humans following prolonged general anesthesia in infants and young children.

RESULTS

Population

Sixty patients were recruited for data and blood collection between June 2019 and November 2023 (patient recruitment was halted during the COVID19 pandemic). Technical difficulties precluded extraction of eDNA from baseline and/or end-of-surgery samples in three patients, leaving data from 57 patients for analysis.

Demographics

Demographic data are summarized in Table 1. Among the 57 patients included, 41 (72%) were male and 16 (28%) were female. Ages ranged from 2 months to 45 years (median 19 months, IQR 14-59.5). Eighteen (31.6%) were infants (≤ 12 months), 21 (36.8%) were toddlers (13-36 months), and 18 (31.6%) were older children (3-12 years) or adults (>12). One toddler was a former premature infant (born at 34 weeks gestation). Twenty-one patients (37%) had been previously exposed to one or more (range 1 to 7) general anesthetics. Forty-six (81%) of the analyzed anesthetics were for urological procedures (most commonly pyeloplasty or hypospadias repair), of which 33 also received caudal anesthesia. The remaining 11 patients underwent either plastic or dental surgery. Mean $\pm$ standard deviation of anesthesia duration

was 196 ± 53 minutes (median 195, IQR 160-235, range 90-300). Cumulative exposure ranged from 1.3-9.4 MAC-hours (median 4, IQR 2.9-5). Six (11%) received total intravenous anesthesia (TIVA); in these cases, 1 hour of propofol was counted as 1 MAC-hour. All patients except for one received opioids (fentanyl and/or morphine). Anesthetic drugs received included any combination of nitrous oxide, sevoflurane, isoflurane, propofol, midazolam, fentanyl, morphine, and epidural (caudal) bupivacaine. Ten patients (18%) developed hyperthermia $\geq 38^{\circ}\text{C}$ (up to 39°C) at some point during their intraoperative course; 28 patients (49%) received steroids (dexamethasone, used for nausea prophylaxis). There were no recorded incidents of sustained hypoxemia or hemodynamic instability.

For cfDNA analysis, patients were categorized into three age groups: ≤ 12 months, 13–36 months, and >36 months.

Cell free DNA (cfDNA)

We obtained 324 blood samples from 57 patients. We extracted 324 analyzable cfDNA results for each of astrocytes and hepatocytes, 318 analyzable cfDNA results for neurons, and 292 for oligodendrocytes. As described above, cfDNA for each of astrocytes, oligodendrocytes and hepatocytes was averaged from 3 loci, and cfDNA for neurons was averaged from 4 loci. Hence, in total, we obtained 1,258 analyzable average cfDNA data points from 4,092 cell-specific PCR-amplified sequences.

The entire raw data spreadsheet is available as Supplementary File 1; the full SPSS output file as Supplementary File 2, and the summary easy-to-read table of all data assessments as Supplementary File 3.

Data for each patient were analyzed using a baseline sample plus at least one intraoperative and an end-of-surgery sample. There were 57 baseline data points, 51 at 1 hour, 51 at 2 hours, 33 at 3 hours, 10 at 4 hours, 3 at 5 hours, and 55 at the end of surgery. Where an end-of-surgery sample was not available, the last intraoperative sample served as substitute. Additionally, we obtained PACU samples in 31 patients.

Background signals of brain-derived cfDNA were detected in all patient groups at baseline (before any impact of anesthesia). Baseline cfDNA signals for each cell type by age are depicted in Supplementary Figure 1. Baseline levels of brain-derived cfDNA appear visually to be most prominent in the youngest cohort and decline with age, although this trend did not approach statistical significance: neurons, $p = 0.53$; astrocytes, $p = 0.22$; and oligodendrocytes, $p = 0.48$.

The data comparing baseline cfDNA, *average* intraoperative cfDNA, and end-of-surgery cfDNA are presented in Figure 1 for the markers of the 3 brain cell types (neurons, astrocytes and oligodendrocytes) and the non-brain cfDNA references (hepatocytes and total cfDNA).

The data comparing baseline cfDNA, *maximum* intraoperative cfDNA and end-of-surgery cfDNA are presented in Supplementary Figure 2.

(A) Brain-derived cfDNA

(i) Neurons: Log-transformed data using the average of intraoperative data points did not show an effect of time ($p = 0.13$). There was also no evidence of a time $\times$ age interaction ($p = 0.21$). Age grouping exerted no significant effect on neuron cfDNA levels ($p = 0.045$). Scheffé post hoc comparisons revealed no significant differences between any groups. The effect of

time was still not significant when RM-ANOVA used the maximum rather than the average intraoperative data.

(ii) Astrocytes: Log-transformed data using the average of intraoperative data points showed a significant effect of time ($p = 0.001$). There was a significant time $\times$ age interaction ($p = 0.003$). Age grouping exerted a significant effect on astrocyte cfDNA levels ($p = 0.005$). Scheffé post hoc comparisons revealed significant differences between the youngest and oldest children's groups only; ≤ 1 year vs > 3 years: mean difference $+1.09$ ($p = 0.006$); none of the other between age-group comparisons were significant. When we controlled for gender, fever, steroids, caudal anesthesia and prior general anesthesia, the effect of time was no longer significant ($p = 0.069$). The effect of time was still not significant when RM-ANOVA used the maximum rather than the average intraoperative data.

(iii) Oligodendrocytes: Log-transformed data using the average of intraoperative data points did not show an effect of time ($p = 0.13$). There was also no evidence of a time $\times$ age interaction ($p = 0.39$). Age grouping exerted no significant effect on oligodendrocyte cfDNA levels ($p = 0.072$). Scheffé post hoc comparisons revealed no significant differences between any groups. The effect of time was still not significant when RM-ANOVA used the maximum rather than the average intraoperative data.

(B) Non-brain-derived cfDNA:

(i) Hepatocytes

Log-transformed data using the average of intraoperative data points did not show an effect of time ($p = 0.96$). There was also no evidence of a time $\times$ age interaction ($p = 0.83$). Age grouping exerted no significant effect on liver-derived cfDNA levels ($p = 0.14$). Scheffé post hoc

comparisons revealed no significant differences between any groups. The effect of time was still not significant when RM-ANOVA used the maximum rather than the average intraoperative data.

(ii) Total cfDNA concentration

Log-transformed data using the average of intraoperative data points did not show an effect of time ($p = 0.089$). There was also no evidence of a time $\times$ age interaction ($p = 0.22$). Age grouping exerted no significant effect on total cfDNA levels ($p = 0.11$). Scheffé post hoc comparisons revealed no significant differences between any groups. The effect of time was still not significant when RM-ANOVA used the maximum rather than the average intraoperative data.

(C) PACU

Among the subset of 31 patients for whom PACU (1-hour postop) data were available, no significant difference was noted for any of the cell types between end-of-surgery and PACU signals (Supplementary Figure 3).

(D) Frame of reference:

In order to provide a quantitative frame of reference for the brain-derived cfDNA signals observed in this study, we display graphically the signals observed in the BRAIN study alongside those observed in other clinically-relevant scenarios including brain metastasis²⁵, and pediatric cardiac surgery with cardiopulmonary bypass⁴⁵ (see Figure 2). For the demographics of these two control populations see Supplementary File 5.

DISCUSSION

At clinically relevant doses and durations of general anesthesia, we found a very modest rise in select subtypes of brain-derived cfDNA following surgery under general anesthesia. In all age groups, astrocyte biomarkers rose with time, whereas neurons, oligodendrocytes and non-brain reference biomarkers (liver and total cfDNA) did not. Except for astrocytes, where the rise in astrocyte cfDNA was highest in infants, the degree of change over time was independent of age.

This study constitutes the first use of molecular biomarkers (cfDNA) to detect neuroapoptosis following exposure to general anesthesia in humans. Previous clinical studies attempting to assess neurotoxicity following general anesthesia have been limited to neurodevelopmental testing¹⁷⁻²⁰, typically long after the exposure. Tissue-specific cfDNA facilitates “liquid biopsy”³⁴ of human brains to evaluate cellular, and not only behavioral, responses to anesthetic exposure.

In brain-derived cfDNA, we observed either no change (neurons and oligodendrocytes) or very modest change (astrocytes). It is unclear how to interpret the modest changes detected in the latter cell type. There is no standardized threshold to distinguish clinically significant from clinically insignificant levels of brain-derived cfDNA, or to suggest what level may be indicative of accelerated neuroapoptosis. Direct comparison between the subtle perioperative changes in cfDNA observed in the BRAIN study versus the robust rise observed in the clinical settings of brain metastasis, and cardiopulmonary bypass, suggest that the perioperative changes in cfDNA biomarkers are negligible, when present at all.

The medical community, and the wider community of patients and families, has been concerned with anesthesia exposure primarily in infancy and early childhood, when physiologic cell turnover is highest³⁵. The molecular and behavioral studies that demonstrated anesthesia-induced neuroapoptosis in mice, rats, and monkeys emphasized the unique susceptibility of fetal and neonatal brains³⁶⁻³⁹. Consequently, the FDA warning was limited to children under 3 years of age. In the BRAIN study, even among cell types in which we detected an increase in cfDNA, the increase was age-sensitive only in astrocytes.

Our overall findings are reassuring. The minimal rise in perioperative brain-derived cfDNA and the general lack of an interaction with age are both consistent with neurobehavioral clinical data in humans, which found no evidence that anesthesia exposure in early life adversely affects subsequent neurodevelopment^{17,18,20}.

Important limiting factors highlighted in prior clinical studies were incorporated into the design of the BRAIN study. In contrast to animal models of anesthesia-induced neuroapoptosis, clinical patients never receive anesthesia in the absence of an accompanying diagnostic or therapeutic procedure. It is difficult to distinguish between the effects of anesthesia, the effects of surgery, and the effects of the underlying condition that necessitated surgery. Surgery induces a complex inflammatory response⁴⁰, and that inflammatory response may itself alter a variety of molecular and other biochemical processes in the brain. We limited the BRAIN study to patients undergoing anesthesia for lengthy surgical procedures associated with minimal tissue damage and minimal perioperative cardiorespiratory lability (typically urological procedures). The ideal procedure might have been a non-invasive diagnostic imaging study with no surgical stimulus; however, these procedures are typically brief. Since the animal studies that sparked concern

about anesthesia-induced neurotoxicity relied on longer exposure times, and since the FDA advisory explicitly references an exposure time of ≥ 3 hours²¹, we deliberately selected surgical procedures predicted to last at least three hours. Indeed, the mean and median anesthesia times in our study were well in excess of three hours. By contrast, median anesthesia time in the GAS study was 54 minutes²⁰.

A potential limitation of the BRAIN study relates to the ability of a clinical biomarker, sampled a finite number of times, to reliably capture the time course of anesthesia-induced neuroapoptosis. A key element to this limitation is the delayed appearance of plasma cfDNA after apoptosis. By contrast with infarction or direct tissue injury, where cfDNA is detectable in plasma within minutes⁴¹⁻⁴³, apoptosis involves a series of programmed, methodical, stepwise molecular processes that culminate in cell death, with cfDNA typically not appearing before 30 minutes and peaking only at 2-6 hours^{38,44}. Two other factors that undermine confidence in the ability of intermittent biomarker sampling to capture a neuroapoptosis signal are the short half-life of cfDNA in plasma (15-60 minutes^{26,42}) and uncertainty regarding the critical anesthesia exposure time required to trigger neuroapoptosis. To address the possibility that we missed peak cfDNA signals that occurred after the perioperative sampling frame was completed, we sampled patient blood, where possible, one hour after emergence from anesthesia. There were no differences in cfDNA signals between end-of-anesthesia samples and the one-hour PACU samples in any cell type. Nonetheless, we cannot exclude the possibility that, had we sampled blood later, or more frequently, we may have detected a significant rise in neuron-derived or oligodendrocyte-derived cfDNA (as detected in animal studies^{1,3,8}), or that the peak astrocyte-derived cfDNA would have been higher.

General anesthesia was associated with small but measurable elevations in brain-derived cfDNA from astrocytes only, notably not neurons or oligodendrocytes. With the exception of astrocytes, there were no age-related changes. This biomarker-based approach reinforces the reassuring findings of large neurobehavioral studies and suggests that prolonged general anesthesia in infants and children does not precipitate widespread or clinically meaningful neuroapoptosis.

ON-LINE METHODS

Study design

We conducted a prospective, observational cohort study of children undergoing general anesthesia to assess changes in brain-derived cfDNA during the immediate perioperative period. All subjects had blood samples drawn at the beginning of anesthesia and at repeated intervals during and after surgery. We designed the study using the STROBE checklist (Supplementary File 4).

Ethics

The institutional review board (Helsinki Committee) of Hadassah Hebrew University Medical Center approved the BRAIN study (IRB reference HMO-0198-14). Informed consent was obtained from all patients ≥ 18 yrs and from parents or legal guardians for all patients < 18 . In addition, when possible, informed consent was obtained from children 14-18 yrs and verbal assent was obtained from younger children.

Population

We included healthy (ASA I-II) children younger than three years of age scheduled for non-

neurosurgical, non-cardiac, non-orthopedic procedures meeting the following criteria: (1) anticipated duration of general anesthesia would reach or exceed 3 hours, and (2) associated tissue damage, systemic inflammatory response, and perioperative respiratory and hemodynamic fluctuations were all predicted to be minimal.

We excluded children who had undergone another surgical procedure, who had been hospitalized in an intensive care unit, who had been pharmacologically sedated within seven days prior to surgery, or who had underlying neurological diagnoses.

We divided study patients into infants (≤ 12 months of age) and toddlers (13-36 months). The control population included older children (>36 months) and adults scheduled for procedures meeting the same criteria.

Anesthesia management

Treating anesthesiologists induced and maintained anesthesia for the duration of surgery according to standard clinical practice; this included choice of anesthetic drugs, depth of anesthesia, mode of ventilation, and fluid administration.

Demographic variables

We recorded the following demographic and clinical information: age (months), sex, gestational age at birth, prior exposures to general anesthesia, surgical procedure, anesthesia duration, anesthetic drug used, cumulative anesthetic dose (measured in MAC, minimum alveolar concentration), use of regional (caudal) anesthesia, administration of opioids and corticosteroids, presence of intraoperative hyperthermia $\geq 38^{\circ}\text{C}$, and whether there were any significant intraoperative respiratory or hemodynamic events.

Blood sampling

The investigating anesthesiologist drew blood samples for measurement of baseline cfDNA fragments either just prior to or, more frequently, immediately following the induction of anesthesia. For most children, an intravenous cannula was inserted after mask induction. Occasionally, patients arrived in the operating room with an indwelling venous catheter *in situ* or required venipuncture for clinical reasons prior to anesthetic induction; in such cases, blood was aspirated immediately prior to anesthetic induction. Following anesthesia, blood samples were drawn at hourly intervals and at the end of the procedure. When blood could be readily drawn from an indwelling intravenous catheter without fresh venipuncture, we obtained an additional sample in the post-anesthesia care unit (PACU) at one hour after surgery.

Extraction and analysis of cfDNA

Venous blood (1-2 mL) was collected in cfDNA BCT tubes (Streck), processed via a double-centrifuge protocol (1,500g for 10 minutes followed by 3,000g for an additional 10 minutes), and stored at -80°C until assay. cfDNA was extracted using the QIAasympohony Circulating DNA Kit and quantified via Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). cfDNA was treated with bisulfite (EZ DNA Methylation-Gold, Zymo Research) and PCR-amplified with primers specific for bisulfite-treated DNA but independent of methylation status at the monitored CpG sites, as described³¹. Treatment with bisulfite led to degradation of 60–90% of the DNA (average, 75% degradation), consistent with previous reports³². Primers were bar-coded using TruSeq Index Adapters (Illumina), permitting the mixing of samples from different patients when sequencing PCR products with NextSeq sequencers (Illumina). Sequenced reads were

separated by barcode, aligned to the target sequence, and analyzed using custom scripts written and implemented in R Statistical Software (v4.1.2; R Core Team 2021). Reads were quality-filtered and classified as methylated (CG) or unmethylated (TG). Tissue-specific genome equivalents per milliliter (GE/mL) were calculated by multiplying the tissue-specific unmethylated fraction by the total pre-conversion cfDNA concentration.

We assessed ten previously validated methylation markers specific to neurons, oligodendrocytes or astrocytes; the development and validation of these markers were previously described^{25,30}. In addition, we assessed three hepatocyte-specific DNA methylation biomarkers and total plasma cfDNA, as previously described³³. In order to provide a quantitative frame of reference for cfDNA signals, we graphically compared the cfDNA signals obtained in our study to those observed in other clinically-relevant conditions; brain metastasis²⁵ and pediatric cardiac surgery⁴⁵.

Statistics

The primary endpoint was the change over time in copies of brain-derived cfDNA for each of the following tissue types: (1) neuron, (2) astrocyte, and (3) oligodendrocyte. As our primary end point was comprised of 3 brain-derived biomarkers, we used the Bonferroni correction; $p = \alpha / n = 0.05 / 3 = 0.017$ to determine statistical significance.

To assess age-related trends in baseline cfDNA levels (prior to the induction of anesthesia), comparisons between the three age groups (0–12 months, 13–36 months, >36 months) were performed using a one-way ANOVA with Tukey's multiple comparisons test.

Changes over time were assessed using repeated measures ANOVA (RM-ANOVA) to assess measures at three time points: baseline (start of anesthesia), intraoperative (calculated from the average of the hourly intraoperative samples), and end-of-surgery. We used simple contrast to baseline. Data are presented separately for each cell-type. Time was the “within-subject” variable and age category (0-12 months, 13-36 months, >36 months) was the primary “between-subject” categorical variable. If the interaction of time and patient age was positive, we used Scheffe’s post hoc test to assess the difference between the different patient age groups. Secondary between-subjects variables included sex, prior exposure to general anesthesia, regional (caudal) anesthesia, administration of corticosteroids, and intraoperative fever (>38°C). As cfDNA concentrations were right-skewed, the Greenhouse-Geisser correction was applied, and all inferential statistics were performed on log-transformed values.

As a secondary analysis, we repeated RM-ANOVA as above, but where the intraoperative time point represented not the *average* intraoperative values, but rather the *maximum* intraoperative values. This secondary analysis was inevitably biased in favor of elevated intraoperative cfDNA, but (a) it would ensure that the highest intraoperative cfDNA samples would be considered, and (b) lack of significant signal rise using maximum intraoperative cfDNA would be even more convincing than lack of significant signal rise using average intraoperative cfDNA.

For those patients in whom both PACU samples and end of surgery samples were available (n=31), paired t-tests were performed to compare end-of-surgery and PACU levels for each tissue type.

Summary graphs for each cell type illustrate the dynamic changes across the three age groups, corresponding to the statistical analysis of the effects of time, age, and the time x age interaction; graphs present untransformed data to more clearly illustrate the direction of change.

Sample-size power calculations were not performed prior to embarking on this study because of a lack of data necessary to perform such calculations, such as the upper level of normal of brain-derived cfDNA methylation products in this age-group, or the threshold levels indicative of neuroapoptosis. Instead, we compared the findings in this study to results from other relevant studies of neural injury using the same assay and similar population sizes.

REFERENCES:

1. Brambrink AM, Back SA, Riddle A, et al. Isoflurane-induced apoptosis of oligodendrocytes in the neonatal primate brain. *Ann Neurol* 2012;72:525-35.
2. Brambrink AM, Evers AS, Avidan MS, et al. Ketamine-induced neuroapoptosis in the fetal and neonatal rhesus macaque brain. *Anesthesiology* 2012;116:372-84.
3. Creeley CE, Dikranian KT, Dissen GA, Back SA, Olney JW, Brambrink AM. Isoflurane-induced apoptosis of neurons and oligodendrocytes in the fetal rhesus macaque brain. *Anesthesiology* 2014;120:626-38.
4. Noguchi KK, Johnson SA, Dissen GA, et al. Isoflurane exposure for three hours triggers apoptotic cell death in neonatal macaque brain. *Br J Anaesth* 2017;119:524-31.
5. Broad KD, Hassell J, Fleiss B, et al. Isoflurane Exposure Induces Cell Death, Microglial Activation and Modifies the Expression of Genes Supporting Neurodevelopment and Cognitive Function in the Male Newborn Piglet Brain. *PLoS One* 2016;11:e0166784.
6. Istaphanous GK, Howard J, Nan X, et al. Comparison of the neuroapoptotic properties of equipotent anesthetic concentrations of desflurane, isoflurane, or sevoflurane in neonatal mice. *Anesthesiology* 2011;114:578-87.
7. Istaphanous GK, Ward CG, Nan X, et al. Characterization and quantification of isoflurane-induced developmental apoptotic cell death in mouse cerebral cortex. *Anesth Analg* 2013;116:845-54.
8. Ikonomidou C, Bosch F, Miksa M, et al. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 1999;283:70-4.
9. Zhu C, Gao J, Karlsson N, et al. Isoflurane anesthesia induced persistent, progressive memory impairment, caused a loss of neural stem cells, and reduced neurogenesis in young, but not adult, rodents. *J Cereb Blood Flow Metab* 2010;30:1017-30.
10. Walters JL, Zhang X, Talpos JC, et al. Sevoflurane exposure has minimal effect on cognitive function and does not alter microglial activation in adult monkeys. *Neurotoxicology* 2019;71:159-67.
11. Jevtovic-Todorovic V, Absalom AR, Blomgren K, et al. Anaesthetic neurotoxicity and neuroplasticity: an expert group report and statement based on the BJA Salzburg Seminar. *Br J Anaesth* 2013;111:143-51.
12. Rappaport BA, Suresh S, Hertz S, Evers AS, Orser BA. Anesthetic neurotoxicity--clinical implications of animal models. *N Engl J Med* 2015;372:796-7.
13. Jevtovic-Todorovic V. Exposure of Developing Brain to General Anesthesia: What Is the Animal Evidence? *Anesthesiology* 2018;128:832-9.
14. Jevtovic-Todorovic V, Brambrink A. General Anesthesia and Young Brain: What is New? *J Neurosurg Anesthesiol* 2018;30:217-22.
15. Jevtovic-Todorovic V, Hartman RE, Izumi Y, et al. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003;23:876-82.
16. Paule MG, Li M, Allen RR, et al. Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. *Neurotoxicol Teratol* 2011;33:220-30.
17. Sun LS, Li G, Miller TL, et al. Association Between a Single General Anesthesia Exposure Before Age 36 Months and Neurocognitive Outcomes in Later Childhood. *JAMA* 2016;315:2312-20.
18. Warner DO, Zaccariello MJ, Katusic SK, et al. Neuropsychological and Behavioral Outcomes after Exposure of Young Children to Procedures Requiring General Anesthesia: The Mayo Anesthesia Safety in Kids (MASK) Study. *Anesthesiology* 2018;129:89-105.
19. Wilder RT, Flick RP, Sprung J, et al. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology* 2009;110:796-804.

20. McCann ME, de Graaff JC, Dorris L, et al. Neurodevelopmental outcome at 5 years of age after general anaesthesia or awake-regional anaesthesia in infancy (GAS): an international, multicentre, randomised, controlled equivalence trial. *Lancet* 2019;393:664-77.
21. FDA Drug Safety Communication: FDA review results in new warnings about using general anesthetics and sedation drugs in young children and pregnant women. 2016. at <http://www.fda.gov/Drugs/DrugSafety/ucm532356.htm>.)
22. Lehmann-Werman R, Neiman D, Zemmour H, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc Natl Acad Sci U S A* 2016;113:E1826-34.
23. Sun K, Jiang P, Chan KC, et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci U S A* 2015;112:E5503-12.
24. Gai W, Ji L, Lam WKJ, et al. Liver- and Colon-Specific DNA Methylation Markers in Plasma for Investigation of Colorectal Cancers with or without Liver Metastases. *Clin Chem* 2018;64:1239-49.
25. Lubotzky A, Zemmour H, Neiman D, et al. Liquid biopsy reveals collateral tissue damage in cancer. *JCI Insight* 2022;7.
26. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 1999;64:218-24.
27. Sherwood K, Weimer ET. Characteristics, properties, and potential applications of circulating cell-free dna in clinical diagnostics: a focus on transplantation. *J Immunol Methods* 2018;463:27-38.
28. Pollak U, Zemmour H, Shaked E, et al. Novel cfDNA Methylation Biomarkers Reveal Delayed Cardiac Cell Death after Open-heart Surgery. *J Cardiovasc Transl Res* 2023;16:199-208.
29. Ben-Ami R, Loyfer N, Cohen E, et al. Epigenetic liquid biopsies reveal endothelial turnover and erythropoiesis in asymptomatic COVID-19. *Life Sci Alliance* 2025;8.
30. Lubotzky A, Pelov I, Teplitz R, et al. Elevated brain-derived cell-free DNA among patients with first psychotic episode - a proof-of-concept study. *Elife* 2022;11.
31. Neiman D, Gillis D, Piyanzin S, et al. Multiplexing DNA methylation markers to detect circulating cell-free DNA derived from human pancreatic beta cells. *JCI Insight* 2020;5.
32. Grunau C, Clark SJ, Rosenthal A. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res* 2001;29:E65-5.
33. Lehmann-Werman R, Magenheimer J, Moss J, et al. Monitoring liver damage using hepatocyte-specific methylation markers in cell-free circulating DNA. *JCI Insight* 2018;3.
34. Pages M, Rotem D, Gydush G, et al. Liquid biopsy detection of genomic alterations in pediatric brain tumors from cell-free DNA in peripheral blood, CSF, and urine. *Neuro Oncol* 2022;24:1352-63.
35. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007;35:495-516.
36. Deng M, Hofacer RD, Jiang C, et al. Brain regional vulnerability to anaesthesia-induced neuroapoptosis shifts with age at exposure and extends into adulthood for some regions. *Br J Anaesth* 2014;113:443-51.
37. Hofacer RD, Deng M, Ward CG, et al. Cell age-specific vulnerability of neurons to anesthetic toxicity. *Ann Neurol* 2013;73:695-704.
38. Yon JH, Daniel-Johnson J, Carter LB, Jevtovic-Todorovic V. Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways. *Neuroscience* 2005;135:815-27.
39. Wang C, Liu S, Liu F, Bhutta A, Patterson TA, Slikker W, Jr. Application of Nonhuman Primate Models in the Studies of Pediatric Anesthesia Neurotoxicity. *Anesth Analg* 2022;134:1203-14.
40. Watt DG, Horgan PG, McMillan DC. Routine clinical markers of the magnitude of the systemic inflammatory response after elective operation: a systematic review. *Surgery* 2015;157:362-80.
41. Yamamoto R, Asano H, Tamaki R, et al. Dynamics and Half-Life of Cell-Free DNA After Exercise: Insights from a Fragment Size-Specific Measurement Approach. *Diagnostics (Basel)* 2025;15.

42. Kustanovich A, Schwartz R, Peretz T, Grinshpun A. Life and death of circulating cell-free DNA. *Cancer Biol Ther* 2019;20:1057-67.
43. Fatouros IG, Jamurtas AZ, Nikolaidis MG, et al. Time of sampling is crucial for measurement of cell-free plasma DNA following acute aseptic inflammation induced by exercise. *Clin Biochem* 2010;43:1368-70.
44. Rostami A, Lambie M, Yu CW, Stambolic V, Waldron JN, Bratman SV. Senescence, Necrosis, and Apoptosis Govern Circulating Cell-free DNA Release Kinetics. *Cell Rep* 2020;31:107830.
45. Pollak U, Zemmour H, Shaked E, et al. Novel cfDNA Methylation Biomarkers Reveal Delayed Cardiac Cell Death after Open-heart Surgery. *J Cardiovasc Transl Res.* 2023;16:199-208.

Table 1. Demographic data

Age group	N	Sex	Surgical procedure	Anesthetic duration (minutes; mean $\pm$ SD)	Cumulative MAC hours (mean $\pm$ SD)	Caudal anesthesia	Steroids
≤ 12 months	18	14 M 4 F	16 urology 2 other	192 $\pm$ 58	4.0 $\pm$ 1.8	13 (72%)	7 (39%)
1-3 yrs	21	19 M 2 F	19 urology 2 other	209 $\pm$ 51	5.0 $\pm$ 2.0	18 (86%)	4 (19%)
>3 years	18	8 M 10 F	11 urology 7 other	186 $\pm$ 47	3.6 $\pm$ 1.0	2 (11%)	11 (61%)
Total	57	41 M 16 F	46 urology 11 other	196 $\pm$ 53	4.2 $\pm$ 1.7	33 (58%)	28 (49%)

Figure 1

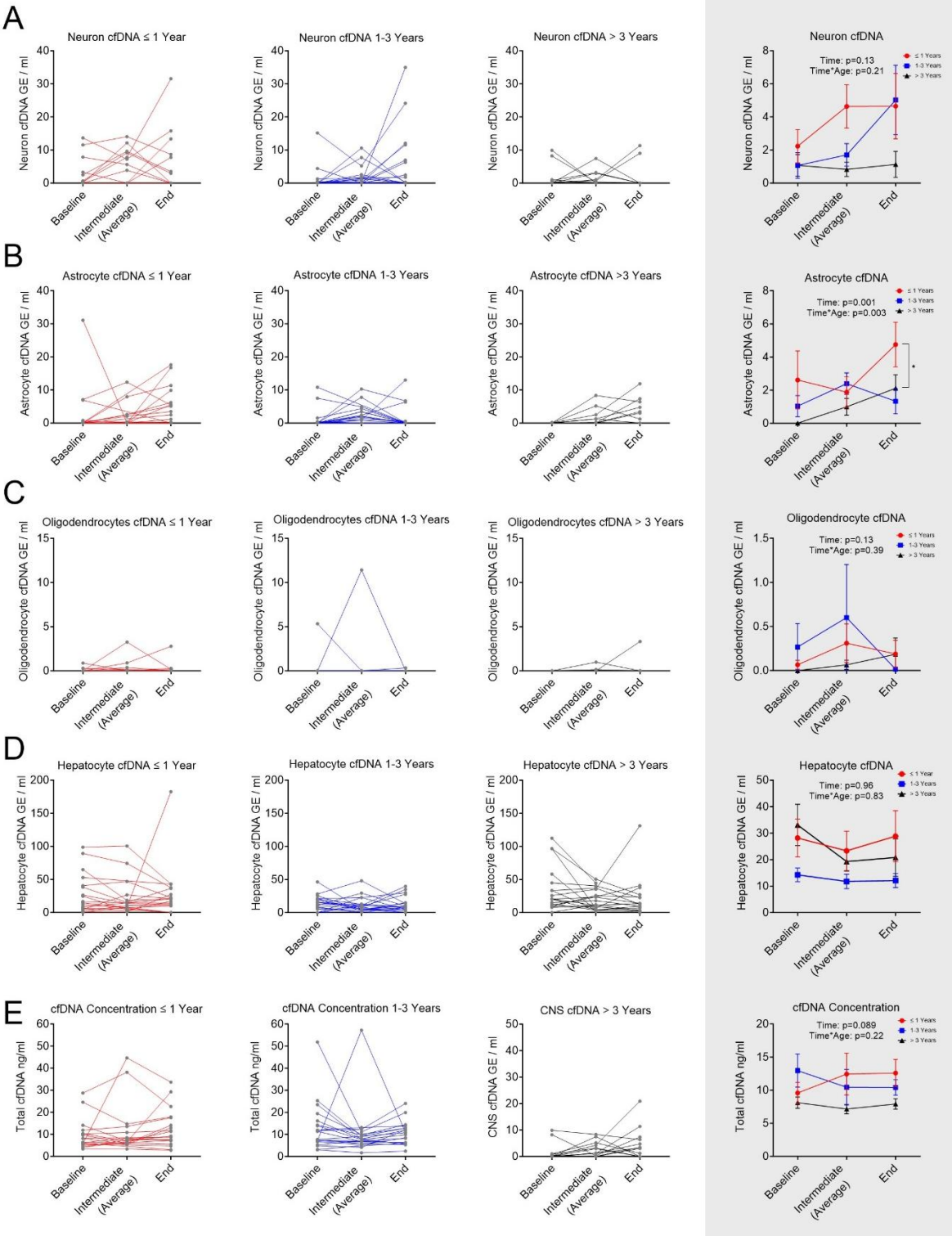


Figure 1. Perioperative dynamics of tissue-specific and total cell-free DNA. (A-D)

Concentration of cfDNA derived from neurons, astrocytes, oligodendrocytes, and hepatocytes (Genome Equivalents [GE]/ml). **(E)** Total cfDNA concentration (ng/ml). **Timepoints:** Baseline (pre-induction), intermediate (intraoperative average), and end of surgery. **Left Panels (unshaded):** Individual patient trajectories stratified by age: infants ≤ 1 year (red), toddlers 1–3 years (blue), and children >3 years (black). **Right Panels (shaded):** Summary data presented as mean $\pm$ SEM shows the pooled data for each age group. **Statistics:** Repeated measures ANOVA on log-transformed data. **Results:** Astrocyte markers **(B)** exhibited significant Time ($p=0.001$) and Time $\times$ Age interaction ($p=0.003$) effects. Post-hoc Scheffé analysis: Infants ≤ 1 year vs >3 years ($p=0.006$). No significant main effects or interactions were observed for any other cell types **(A, C, D, E)**.

Figure 2

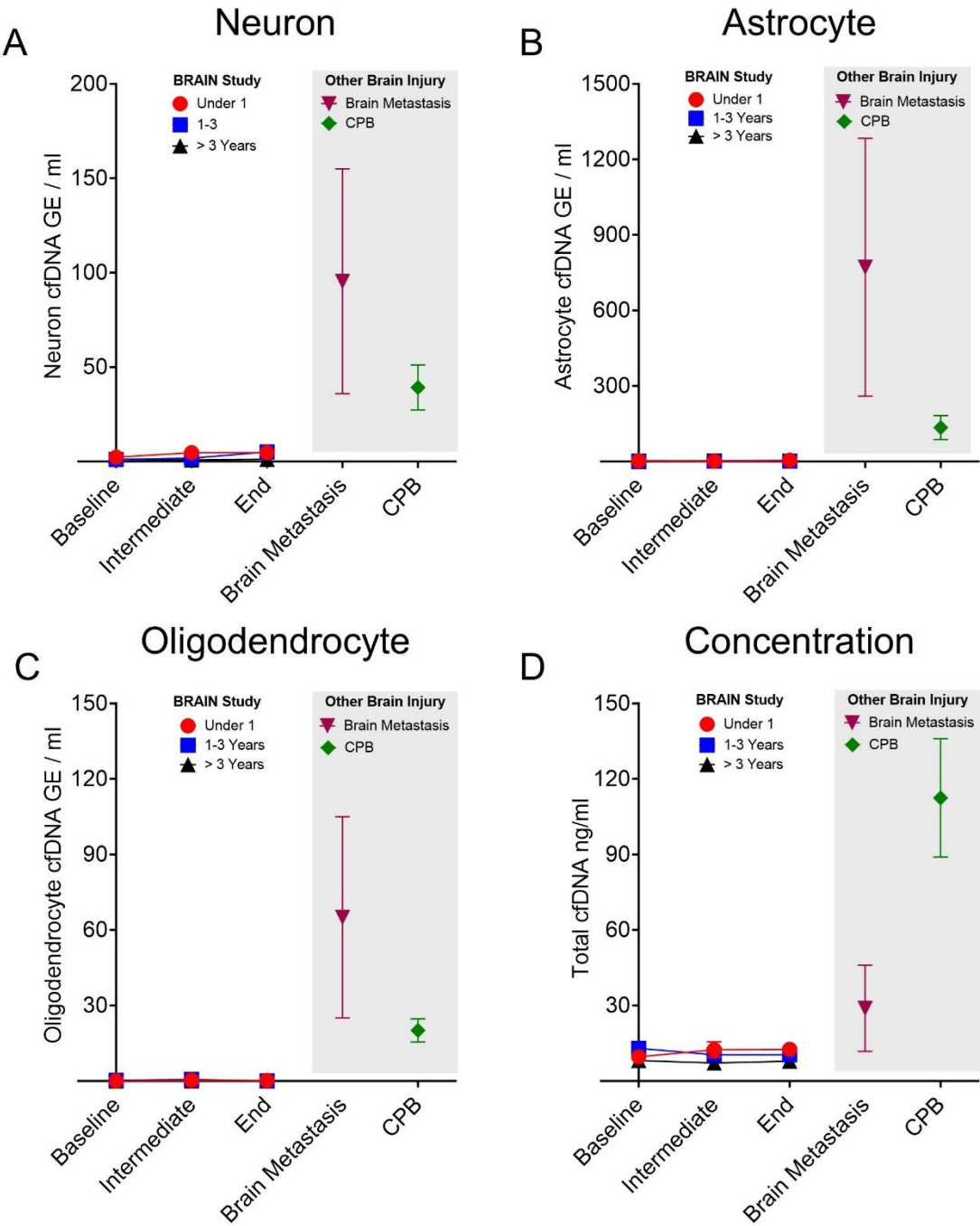


Figure 2. Contextualizing perioperative cfDNA dynamics against established brain injury

models. Perioperative cohort signals (Baseline, Intermediate and End) are benchmarked against independent pathological states associated with neural injury and neuroapoptosis: brain metastasis and pediatric cardiopulmonary bypass (CPB). Quantification of (A) neuron, (B) astrocyte, (C) oligodendrocyte and (D) total cfDNA concentration. Data are presented as Mean $\pm$ SEM. **Results:** Y axis scales are optimized to accommodate the robust release observed in metastasis²⁵ and CPB⁴⁵. Notably, the statistically significant elevations observed during routine anesthesia in Figure 1 are biologically negligible relative to established neuro-injury states, appearing as a near-flat trajectory when placed on the same quantitative scale. These findings suggest that exposure to clinically relevant concentrations of volatile anesthetic do not induce the neuroapoptotic release characteristic of organic brain injury.

Supplementary Files

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