ABSTRACT

BACKGROUND: Extracellular vesicles (EV) released from neurons into the blood can reflect the state of nervous tissue. Measurement of neuron derived EV (NDE) may serve as an indicator of brain injury.

METHODS: A sandwich immunoassay was established to measure plasma NDE using anti-neuron CD171 and anti-EV CD9 ([CD171 + CD9+]). Plasma samples were obtained from commercial sources, cross-country (n = 9), football (n = 22), soccer (n = 19), and rugby (n = 18) athletes over time. Plasma was also collected from patients undergoing total aortic arch replacement (TAR) with selective cerebral perfusion during cardiopulmonary bypass before and after surgery (n = 36).

RESULTS: The specificity, linearity, and reproducibility of NDE assay (measurement of [CD171 + CD9+]) were confirmed. By scanning electron microscopy and nanoparticle tracking, spherical vesicles ranging in size from 150 to 300 nm were confirmed. Plasma levels of NDE were widely spread over 2 to 3 logs in different individuals with a significant age-dependent decrease. However, NDE were very stable in each individual within a ±50% change over time (cross-country, football, soccer), whereas rugby players were more variable over 4 years. In patients undergoing TAR, NDE increased rapidly in days post-surgery and were significantly (P = .0004) higher in those developing postoperative delirium (POD) (n = 13) than non-delirium patients (n = 23).

CONCLUSIONS: The blood test to determine plasma levels of NDE was established by a sandwich immunoassay using 2 antibodies against neuron (CD171) and exosomes (CD9). NDE levels varied widely in different individuals and decreased with age, indicating that NDE levels should be considered as a normalizer of NDE biomarker studies. However, NDE levels were stable over time in each individual, and increased rapidly after TAR with greater increases associated with patients developing POD. This assay may serve as a surrogate for evaluating and monitoring brain injuries.

KEYWORDS: Extracellular vesicles, brain injuries, total aortic arch replacement, sports concussion, CD171, CD9, ELISA
After the detection of neuron-derived EV (NDE) in human plasma, the neuronal cell surface marker CD171 (L1-cell adhesion molecule) has been widely used to isolate and characterize plasma NDE in various neurological disorders. NDE carry critical intracellular biomolecules that originate directly from neurons and may reflect changes and biological events that occur in the brain.

In previous studies, various NDE biomarkers were quantified by standard immunoassays after NDE were isolated and lysed. Results are dependent on the purity and yield of the NDE isolation procedure and lysis efficiency, and the assumption that conditions are identical among all tested subjects. Currently, limited data is available for the plasma level of NDE, even though this is a critical parameter to normalize NDE-associated biomarkers measured by any means. Therefore, we have developed a blood test to quantify plasma levels of NDE by a sandwich enzyme-linked immunosorbent assay (ELISA) using anti-CD171 antibody as an NDE capture agent and antibody against the EV common marker CD9 as an EV detection probe. This study tests the specificity, linearity, and reproducibility of the optimized assay to detect CD171^+ CD9^+ double positive signals ([CD171^+ CD9^+]). This study also examines the association of neuron biomarkers and confirmation of the vesicle by scanning electron microscopy (SEM) and nanoparticle tracking analysis (NTA). Then, the utility of this assay to monitor post-brain injuries is tested.

**Materials and Methods**

**Antibodies**

Mouse monoclonal antibody against human CD171 (IgG2a, Thermo Fisher Scientific, Waltham, MA), CD9 (Bio-Rad, Hercules, CA), CD81 (BD Biosciences, Franklin Lakes, NJ), control mouse IgG2a (BioLegend, San Diego, CA), normal mouse IgG (Equitech-Bio), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, BD), aldolase (ALDO, Santa Cruz Biotechnology, Dallas, TX), surfactant protein B (SFTPB, US Biologicals, Salem, MA), presenilin 1 (PSEN, Santa Cruz), β-secretase (BACE, Santa Cruz), S100B (R&D Systems, Minneapolis, MN), acetylcholinesterase (AchE, Santa Cruz), neurogranin (NRGN, BioLegend), cathepsin D (CTSD, Santa Cruz), synaptic vesicle glycoprotein 2A (SV2A, Abcam, Cambridge, United Kingdom), synaptotagmin (SYNPO, Santa Cruz), and synaptosome associated protein 25 (SNAP25, Santa Cruz), and enolase 2 (ENO2, R&D) were purchased from designated suppliers.

**Antibody immobilization**

Antibodies used for immobilization (anti-CD171 and anti-CD81) and control mouse IgG2a and purified normal mouse IgG were diluted in ELISA coating buffer (BioLegend) in a final concentration of 2.5 μg/mL, and 50 μL was applied to ELISA wells (Corning #3923, Sigma Aldrich, St Louis MO). After 1 hour incubation, each well was washed once with phosphate buffered saline (PBS, Thermo Fisher), and incubated with undiluted blocker casein (Thermo Fisher) for another 1 hour. After each well was washed twice with PBS, ELISA wells were stored in a refrigerator.

**ELISA**

The protocol was similar to our previous publication with substantial improvement. In brief, 40 μL samples were applied to ELISA wells and incubated for 1 hour at room temperature. After the first washing step, each well was reacted with various biotinylated probes (all 50 ng/mL) supplemented with 0.8% bovine serum albumin (BSA, Thermo Fisher), 40 μg/mL mouse IgG (Equitech-Bio) for another 1 hour. In order to maintain captured EV intact, tween-20 was not used at any time during the whole procedure. Biotinylation was carried out by EZ link Sulfo-NHS-LC-Biotin (Thermo Fisher) followed by the spin column procedure to remove free biotin. After the second washing step, each well was reacted with a 1/4000 dilution of poly-horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fisher) supplemented with 10% BSA (Equitech-Bio) and 30% blocker casein (Thermo Fisher), and incubation was continued for 20 minutes. After the third washing step, each well was incubated for 5 minutes with 0.0006% H2O2 (CVS pharmacy, Irvine, CA) diluted in PBSwater (1:1) solution to remove non-specifically bound HRP conjugates. After aspiration of H2O2, each well was mixed with one-third dilution of chemiluminescent substrate (Super Signal Thermo Fisher) for 4 minutes, then relative light units (RLU) were determined by a luminometer (Active GLO, ANSH Labs, Webster, TX). Using our standard plasma, arbitrarily assigned to 100 units/mL (U/mL), ELISA readings of RLU were converted to U/mL by a 4 parameter logistic formula.

**SEM**

After EV were captured and washed, each ELISA well bottom was removed by a puncher, and suspended in 2% glutaraldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4 (Sigma Aldrich), they were treated with the SEM preparation previously published. Each well bottom was immersed in 1% tannic acid (Nacalai Tesque, Kyoto, Japan) in 0.1 M PB for 1 hour, rinsed in the buffer for 1 hour and soaked in 1% osmium tetroxide (OsO4) in 0.1 M PB for 1 hour. The specimens were then dehydrated through a graded ethanol series, transferred to t-butyl alcohol and dried in a freeze dryer (ID-2; Eiko Co., Tokyo, Japan). Subsequently, dried specimens were mounted onto aluminum bases and coated with platinum-palladium in an ion-sputter coater (E1030; Hitachi, Tokyo, Japan). Finally, the specimens were observed in a field emission scanning electron microscope at an accelerating voltage of 5 kV (S 4100; Hitachi, Tokyo, Japan).

**NTA**

We first tested that [CD171^+ CD9^+] was not irreversible but dissociated with pH 2.5 to 2.0. We also found appropriate
paramagnetic beads (PM-50, Spherotech, Lake Forest, IL) with the same surface characteristic as ELISA wells, so that the procedure developed for ELISA was immediately applied to the beads. After the assay condition was established and validated, plasma samples were applied to the beads. Captured EV were then eluted off the beads with a pH 2.5 solution and immediately neutralized by adding appropriate amounts of 1M Tris, pH 8.0. NTA analysis was carried out at Particle Technology Labs (Downers Grove, IL) using NanoSight NS300.

**Plasma samples**

Control adult EDTA-plasma samples were purchased from 3 different commercial sources (Innovative Research, Novi, MI, BioIVT, Westbury, NY, and Equitech Enterprise, Kerrville, TX). Plasma samples from high school cross-country (n=9, all male), high school football (n=22, all male), and adult soccer players (n=19, 12 male and 7 female, age between 18 and 26 (average ± standard deviation = 20 ± 2 years old) were obtained from Indiana University, with Indiana University Institutional Review Board approval (protocol #1904461516), and all participants and their legal guardians gave written informed consent. These athletes were free of concussion for at least the past 12 months and did not have any elevated baseline symptoms. Plasma samples from college rugby players were collected at Keio University (Japan) (n=18, all male) with the Ethics Approval by The Ethics Review Committee of Sports Medicine Research Center, Keio University, Yokohama, Japan. The approval number was 2022-04. Pre-registration screening was performed by questionnaire concerning past medical history including injury and illness. Rugby players in this study had no pre-existing conditions. Plasma samples from patients who underwent total aortic arch replacement (TAR) surgery were collected at 5 time points (pre-operation, post-operation, postoperative day 1, 2, and 5) at National Cerebral and Cardiovascular Center (Japan). The study was approved by Research Ethics Committee of National Cerebral and Cardiovascular Center, Suita, Osaka, Japan with approved number of R19084-4. All plasma samples were frozen and stored in a –80°C freezer until analysis.

**Statistical analysis**

For the comparison of 2 groups, matched and unmatched pair Student t-test was employed using both linear and log values. For the comparison of 2 groups with multiple datasets (TAR cases), a mixed-effects regression model was used with random intercept for each patient to adjust its variation.

**Results**

**ELISA validation**

Capture antibody specificity: Mouse monoclonal antibody (IgG2a) against human CD171, control mouse IgG, and control mouse IgG2a were immobilized on the ELISA wells. Three different control human plasmas and buffer alone were applied to the designated wells and the ELISA was carried out using anti-human CD9 probes. As shown in Figure 1A, all 3 plasmas showed higher CD9 signals on anti-CD171-immobilized wells than the control mouse IgG and isotype control mouse IgG wells. Although plasma #3 showed a limited signal in the IgG2a control wells, the levels on anti-CD171 were much higher than those of IgG2a. Plasma #3 is a good example of the non-specific binding of IgG and donor-to-donor variation.

Probe specificity: Three control plasmas and buffer alone were applied to mouse IgG or anti-CD171-immobilized ELISA wells, then reacted with biotinylated probes of mouse IgG, mouse IgG2b, or anti-human CD9 (mouse monoclonal IgG2b). As shown in Figure 1B, all 3 plasma samples showed high anti-CD9 signals on anti-CD171-immobilized wells, but minimal signals from control wells, control probes, and buffer controls, demonstrating CD9 specificity.

**EV specificity I: EV reduction.** Control plasma samples were incubated with anti-CD81- or control mouse IgG-immobilized paramagnetic beads, respectively, for 1 hour to remove EV. Beads were removed by magnetic separation and supernatant plasma samples were applied to 3 different ELISA wells, where anti-CD171, anti-CD81-, or control mouse IgG were previously immobilized. After extensive washing, each well was reacted with biontynlated anti-CD9. As shown in Figure 1C, CD9 signals on anti-CD81 immobilized ELISA wells were decreased in anti-CD81-treated plasma (w/, dark gray columns) by approximately 50% compared to the control IgG-treated plasma (w/o, light gray columns), indicating that approximately half of EV were removed by this procedure. [CD171 + CD9+] was also reduced by a similar degree by the treatment of EV reduction, indicating that [CD171 + CD9+] was derived from EV.

**EV specificity II: Spiked EV.** EV were prepared from control plasma using the ExoQuick (System Biosciences, Palo Alto, CA) procedure and suspended in PBS. Three different diluted EV and PBS were spiked into plasma or plasma diluents, and [CD171 + CD9+] was determined. As shown in Figure 1D (Δ), prepared EV showed [CD171 + CD9+] in a dose-dependent manner. The addition of EV into plasma samples also increased [CD171 + CD9+] in a dose-dependent manner (●) with a similar slope to that of EV alone (Δ). These data indicate that [CD171 + CD9+] is EV-dependent.

**Plasma dilution studies:** As shown in Figure 1E, [CD171CD9] in 3+ different control plasmas showed linear increases in a plasma volume-dependent manner on anti-CD171-immobilized wells (●), whereas signals stayed very low when samples were applied to control mouse IgG-immobilized ELISA wells (○). These data support that the results of ELISA data in each sample can be converted to U/mL using the dilution curve of a standard plasma.
Assay reproducibility: The assay was always performed in duplicate, and as shown in Figure 1F, intra-assay duplicate variation (n = 134 controls, athletes, and TAR patients) was extremely small and inter-assay (n = 12 runs) variation (Figure 1G) remained between 90% and 158% variance from the first determination.

Confirmation of EV

SEM: Since SEM requires a flat surface, we removed each ELISA well bottom carefully without damaging the surface. We tested 2 different EV, NDE using anti-CD171 (Figure 2A and B) and pan-EV using anti-CD81 (Figure 2C and D) as capture agents, respectively. These exosomes were prepared from control plasma samples. SEM images showed the visualization of putative EV with clear surface characteristics. All were spherical with a size of 100 to 200 nm. CD171 EV were slightly smaller than CD81 EV. Although Figure 2A to D showed a single typical EV in each panel, many similar EV were observed.

NTA: Control plasma samples were applied to anti-CD171- and control mouse IgG-immobilized paramagnetic beads, then captured EV were eluted as described in the Methods. As shown in Figure 2E, the eluted solution from anti-CD171-immobilized paramagnetic beads showed 150 to 300 nm particles, equivalent to the size observed by SEM, whereas the solution from control IgG-immobilized beads failed to show such particles. Anti-CD171 also showed small peaks around 380, 500, 580, and 720 nm, indicating the presence of large molecules/aggregates in the sample.

Assessment of neuronal origin

Control plasma samples were applied to both anti-CD81- and anti-CD171-immobilized ELISA wells, and probed with antibodies against various neuron-associated and non-specific markers. As shown in Figure 3, the CD171/CD81 ratio of non-specific biomarkers, such as CD9 (exosome common marker), GAPDH (general housekeeping protein),
ALDO (muscle- and liver-associated), SFTPB (surfactant proteins in lung), BACE and PSEN (β−, γ-secretase, expressed in brain as well as other tissues), were all less than 2. In contrast, the CD171/CD81 ratio of neuron-associated biomarkers, such as S100B, AchE, NRGN, CTSD, SV2A, SYNPO, and SNAP25 were all above 5 with the exception of ENO2.

**Range of control values**

Total 210 subjects including controls, athletes, and TAR patients were used to measure [CD171+CD9+]. All gender and ethnicity were included. Surprisingly, [CD171+CD9+] was widely spread among individuals and such spread was almost 2 to 3 logs wide (Figure 4). However, the variation was
not random and showed a normal-like distribution in log scale in each age group. Therefore, statistical analysis was done using log values. [CD171^+CD9^+] in teen's was not different from that of 20's, and 30's were not different from 40's. However, [CD171^+CD9^+] in 30's-40's group was significantly ($P=3e^{-4}$) lower than that of teen's-20's group (Figure 4), and >70's groups was significantly ($P=4e^{-9}$) lower than 30's-40's group (Figure 4). Moreover, [CD171^+CD9^+] in >80's was significantly ($P=.02$) lower than that of 70's (Figure 4).

**Monitoring of the levels of [CD171^+CD9^+]**

Controls: [CD171^+CD9^+] was stable within the same individual over time in high school cross-country athletes (non-contact sports, n = 9) from July to November, and values stayed within ±50% range from the values obtained in July, with the exception of one individual who showed a large fluctuation (Figure 5A). This was similar to the results as reported in our previous publication,7 even though substantial improvements had been made to the assay.

Contact sports athletes: [CD171^+CD9^+] in high school American football athletes (n = 22) was also stable from July to November, although %Change from pre-season values showed >150% increase in 2 players toward the end of the season (Figure 5B). None of these players suffered a concussion during this season. Adult soccer players (n = 19) were also very stable for 3 days after intensive heading practice, although a small increase was seen 2 hours after the practice (Figure 5C). In contrast, college rugby players (n = 18) who received much heavier head impacts than high school football players with much less protective equipment, showed an upward trend of [CD171^+CD9^+] toward the end of 4 years of rugby play, and some players showed %Change over 200% (Figure 5D). Alog [CD171^+CD9^+] in the third to forth year (2016-2017) was significantly ($P=.002$) higher than that of the first to second year (2014-2015). None of the rugby players had any clinical brain-related issues.

**TAR**: We collected plasma samples from patients who underwent TAR (n = 36, Figure 5E and F), because various neuronal problems have been reported after TAR surgery.9,10 In fact, postoperative delirium (POD) assessed using Intensive Care Delirium Screening Checklist (ICD-SC) $\geq 4^{11}$ was found in 13 patients (13/36, 36%) within 1 to 5 days after TAR. In most patients, [CD171^+CD9^+] showed a huge jump immediately after TAR, and slowly decreased (Figure 5E and F, e-f). Interestingly, [CD171^+CD9^+] in log scale was significantly ($P=.0004$) higher in those developing POD (n = 13) than non-delirium patients (n = 23) using a mixed-effects regression model with random intercept for each patient to adjust its variation (Figure 5E and F). Age is one of risk factors of POD, and in fact, POD (+) patients were significantly ($P=.0037$) older than POD (-) patients (Table 1). Interestingly, parameters associated with surgeries, such as duration of anesthesia, operation, and cardiac pump, and volume of total fluid, transfusion, urine, and bleeding were not different between POD (+) and (-) patients (Table 1).

**Discussion**

Antibodies against CD171 have been used widely to isolate and analyze NDE.4-6 However, in spite of the provocative results with CD171^+NDE, there have been some controversies around CD171-based NDE as highlighted in a critical paper.12 In the current study, the specificity of CD171 and CD9 has been carefully validated (Figure 1A and B) to demonstrate that [CD171^+CD9^+] is EV dependent (Figure 1C and D), plasma volume dependent (Figure 1E), and reproducible (Figure 1F-G). Captured EV were clearly visualized on ELISA wells by SEM (Figure 2A–D), and dissociated EV were confirmed by NTA (Figure 2E).

When EV are isolated by ultracentrifugation, they may be deformed through a high gravitational pressure. When EV are eluted from antibody-bound solid supports, EV are influenced by elution conditions such as low pH. Detergents such as tween-20 are often used in ELISA to block non-specific binding, however, the assay developed here did not include any detergents, so that captured EV were intact, and not lysed or permeabilized. Therefore, photographs shown in Figure 2A to D would represent the natural appearance of EV under physiological conditions.

One of the critical questions to address is whether [CD171^+CD9^+] truly represents NDE. This is not a simple question, since various biomarkers may be bound on the surface of EV. Moreover, EV are known to fuse or aggregate to other EV,13 and such aggregate-like size was also seen in our NTA.
This means that a single pure NDE population may aggregate with non-NDE. Due to the difficulty in isolating pure EV core components without membrane contamination, we measured EV surface binding of various biomarkers in both anti-CD81 and anti-CD171-immobilized ELISA wells. Although all of these biomarkers were cytosolic or secretory proteins, ELISA signals were clearly detected, indicating that EV surface was sticky. Interestingly, CD171/CD81 ratio of many non-specific biomarkers were all $<2$, whereas many brain-specific biomarkers were $>2$ (Figure 3). When NDE is in the endosomes in neuronal cells, various neuronal proteins may bind to the surface of NDE. After NDE are released to the extracellular space, additional neuronal proteins present in the microenvironment may bind to the surface of NDE, then in the blood stream plasma proteins may bind to NDE sequentially. Therefore, a CD171/CD81 ratio may be one of the parameters to differentiate between pan-EV and NDE. Although this does not provide information on the purity of NDE, Figure 3 showed that the performance of CD171 was different from that of CD81 and preferentially more neuron-like.

One of surprising results was the widespread of $[\text{CD171}^{+}\text{CD9}^{+}]$ among human subjects (Figure 4). This is not a simple technical error since this is beyond the variability of the assay itself as shown in Figure 1. Moreover, the values were quite stable in each individual over considerable periods (Figure 5A–C). Since we do not know the actual number of neurons and synapses in each individual, such a big variation of $[\text{CD171}^{+}\text{CD9}^{+}]$ may reflect a variation of brain activities among subjects. It was also apparent that $[\text{CD171}^{+}\text{CD9}^{+}]$ gradually decreased with increasing age (Fig. 4). The brain shrinks with age, and cerebral blood flow velocity which peaks at age 6, declines with age. Various
proteins in plasma NDE lysates also change significantly with aging. Because [CD171 + CD9+] is substantially simpler than the analysis of brain size, cerebral blood flow, or NDE lysate preparation, [CD171 + CD9+] may be applicable to future studies of aging.

Unlike sports concussion, traumatic brain injuries, and stroke where blood samples are collected only after the event, blood samples can be collected before and after a scheduled surgery. Moreover, TAR is known to induce various neuronal problems. Therefore, TAR is an ideal research model and was used in this study. As shown in Figure 5E and F, e-f, [CD171 + CD9+] showed a huge 100 to 1000-fold jump immediately after TAR, and then slowly decreased. This means that [CD171 + CD9+] is clearly and widely changeable. Such a sudden increase of [CD171 + CD9+] may be due to the release of NDE through the leakage of the blood brain barrier (BBB). BBB is a continuous endothelial membrane and plays as a boundary structure between central nervous tissue and circulating blood and maintains the highly regulated homeostasis of the central nervous system. Previous research reported that the BBB was disrupted after cardiovascular surgery with cardiopulmonary bypass (CPB) as visualized by dynamic contrast enhancement MRI and severity of the disruption revealed a correlation with postoperative cognitive decline (POCD). In addition, BBB disruption has also been associated with increases in neuronal damage and the development of POD. In this study, [CD171 + CD9+] was significantly higher in patients with POD than patients without POD. Therefore, the increase in post-TAR [CD171 + CD9+] may directly be the result from BBB disruption.

As shown in Table 1, none of parameters associated with surgeries, such as duration of anesthesia, operation, and cardiac pump, and volume of total fluid, transfusion, urine, and bleeding were not different between POD (+) and (-) patients. Although MRI is capable of assessing BBB leakage, it is not practically applicable after TAR surgeries. Thus, [CD171 + CD9+] represents an interesting clinical tool for the monitoring of POD.

After seeing the stability of [CD171 + CD9+] in control subjects (Figure 5a) and substantial changes in TAR patients (Figure 5e-f), we considered that detecting even slight changes in contact sports athletes (Figure 5b-d) may be important. Over time, repetitive head impact and episodes of sub concussive conditions are known to cause various forms of dementia and physical/mental problems in later stages of life. Although many athletes, such as American football, soccer, rugby, ice hockey, boxing, water polo, various martial arts, and combat soldiers have the potential to deliver a damaging head impact, there are limited resources currently available for the objective and quantitative assessment of these conditions. Various blood biomarkers are studied for traumatic brain injuries, but these are not sensitive for the assessment of asymptomatic sub concussive conditions and long term monitoring. In the current study, high school athletes in the absence of concussion showed that [CD171 + CD9+] remained relatively constant over months but were more variable over years in high impact college sports such as rugby. Since this test requires a very small volume of blood, fingertip blood may be acceptable and could be applicable for field studies.

There are several limitations to this study. According to Figures 1 to 3, we indicated that [CD171 + CD9+] was NDE, but more research is needed to fully validate the nature of NDE. This study was an international research collaboration, and is not immediately applicable to diagnostics. The samples were collected by various institutions for their own studies that varied significantly in length and sample collection times (from hours in soccer heading practice to years with rugby).

### Table 1. TAR Patient characteristics.

|                  | TAR-POD (-) | TAR-POD (+) | P VALUE |
|------------------|-------------|-------------|---------|
| N                | 23          | 13          |         |
| Age (year)       | 67.4 (5.9)  | 78.7 (7.0)  | .0037   |
| Height (cm)      | 164.5 (3.5) | 157.9 (4.5) | .0389   |
| Weight (kg)      | 62.8 (6.0)  | 63.3 (8.2)  | .9030   |
| Sex (Male/Female)| 17/6        | 5/8         | .0732   |
| Anesthesia duration (min) | 489.1 (35.6) | 500.2 (49.0) | .7514   |
| Operation duration (min) | 426.5 (39.7) | 448.5 (37.4) | .3001   |
| Pump duration (min) | 229.3 (22.7) | 233.5 (17.5) | .5101   |
| Total fluid (mL) | 17777.6 (4964.3) | 20138.0 (5669.6) | .3789   |
| Total transfusion (mL) | 7431.7 (1637.5) | 9879.5 (2382.8) | .0646   |
| Urine (mL)       | 1438.3 (429.9) | 1712.3 (405.6) | .2362   |
| Bleeding (mL)    | 2030.6 (516.4) | 2829.1 (1525.3) | .8139   |

Mean (95% Confidence interval) POD: Postoperative Delirium.

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Biomarker Insights
which help to identify trends but cannot be considered directly comparable. Further work will be needed to fully define the potential application of this test in clinical testing as well as to enhance our understanding of the relationship of changes in [CD171 + CD9+] with trauma, neurologic disorders, and treatment.

**Conclusion**

The blood test to determine plasma levels of NDE was established by a sandwich immunoassay using 2 antibodies against neuron (CD171) and exosomes (CD9). NDE levels varied widely in different individuals and decreased with age, indicating that NDE levels should be considered as a normalizer of NDE biomarker studies. However, NDE levels were stable over time in each individual, and increased rapidly after TAR with greater increases associated with patients developing POD. This assay may serve as a surrogate for evaluating and monitoring brain injuries.

**Declarations**

**Ethics Approval and Consent to Participate**

Plasma samples from high school cross-country, high school football, and adult soccer players were obtained from Indiana University, with Indiana University Institutional Review Board approval (protocol #1904461516).

Plasma samples from college rugby players were collected at Keio University (Japan) with the Ethics Approval by The Ethics Review Committee of Sports Medicine Research Center, Keio University, Yokohama, Japan. The approval number was 2022-04.

Plasma samples from patients who underwent total aortic arch replacement (TAR) surgery were collected at National Cerebral and Cardiovascular Center (Japan). The study was approved by Research Ethics Committee of National Cerebral and Cardiovascular Center, Suita, Osaka, Japan with approved number R19084-4.

**Consent for Publication**

The written informed consent was obtained from each participant.

**Author Contributions**

NH: sample collection, data analysis, manuscript writing and editing, TT: experiment design, data analysis, manuscript writing and editing, JH: sample analysis, data analysis, DK: sample analysis, data analysis, reviewing manuscript, KK: Sample collection, data analysis, reviewing manuscript, HI: Sample collection, data analysis, reviewing manuscript, YO: Sample collection, data analysis, reviewing manuscript, AH: Sample collection, data analysis, reviewing manuscript, MM: experiment design, data analysis, manuscript writing and editing, and KY: experiment design, sample collection, data analysis, reviewing manuscript.

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**Availability of Data and Materials**

Not available.

**REFERENCES**

1. Shahim P, Politis A, van der Merwe A, et al. Neurofilament light as a biomarker in traumatic brain injury. Neurology. 2020;95:e610-e622.
2. Shahjouei S, Sadeghi-Naini M, Yang Z, et al. The diagnostic values of UCH-L1 in traumatic brain injury: A meta-analysis. Brain Inj. 2018;32:1-17.
3. Papa L, Zonfrillo MR, Welch RD, et al. Evaluating glial and neuronal blood biomarkers GFAP and UCH-L1 as gradients of brain injury in concussion, subconcussive and non-concussive trauma: a prospective cohort study. BMJ Paediatr. Open. 2019;3:e000473.
4. Fiandaca MS, Kapogiannis D, Mapstone M, et al. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: a case-control study. Alzheimer's Dement. 2015;11:600-7.e1.
5. Goetzl EJ, Peltrz CB, Mustapic M, Kapogiannis D, Yaffe K. Neuron-derived plasma exosome proteins after remote traumatic brain injury. J Neurol. 2020;37:382-388.
6. Jiang C, Hopftner F, Katrakoudi A, et al. Serum neuronal exosomes predict and differentiate Parkinson's disease from atypical parkinsonism. J Neurol Neurosurg Psychiatry. 2020;91:720-729.
7. Kawata K, Mitsuhashi M, Aldert R. A preliminary report on brain-derived extracellular vesicle as novel blood biomarkers for sport-related concussions. Front Neurol. 2018;9:239.
8. Koga D, Ushiki T, Watanabe T. Novel scanning electron microscopy methods for analyzing the 3D structure of the Golgi apparatus. Ani Sci Int. 2017;92:37-49.
9. Glumac S, Kardum G, Karanovic N. Postoperative cognitive decline after cardiac surgery: A Narrative Review of current knowledge in 2019. Med Sci Monit. 2019;25:3626-3270.
10. Hendy A, Hall R. Cardiac Surgery and the blood-Brain Barrier. Anesthesiol Clin. 2019;37:87-900.
11. Laertz A, Weiss B, Boertcher S, Burmeister J, Wenecke KD, Spies C. Routine delirium monitoring is independently associated with a reduction of hospital mortality in critically ill surgical patients: A prospective, observational cohort study. J Crit Care. 2016;35:168-173.
12. Norman M, Ter-Ovanesyan D, Trieu W, et al. LiCAM is not associated with extracellular vesicles in human cerebrospinal fluid or plasma. Nat Methods. 2021;18:631-634.
13. Beit-Yannai E, Tabak S, Stanner WD. Physical exosome:exosome interactions. J Cell Mol Med. 2018;22:2001-2006.
14. Peters R. Ageing and the brain. Postgrad Med J. 2006;82:84-98.
15. Schöning M, Hartig B. Age dependence of total cerebral blood flow volume from childhood to adulthood. J Cereb Blood Flow Metab. 1996;16:827-833.
16. Ahnber EL, Jicha GA, Shaw LM, Trojanowski JQ, Goetzl EJ. Plasma neuronal exosomal levels of Alzheimer’s disease biomarkers in normal aging. Ann Clin Transl Neurol. 2016;3:399-403.
17. Sweeney MD, Sagare AP, Zlokovic BV. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. Nat Rev Neurol. 2018;14:133-150.
18. Abrahamow D, Levran O, Naparstek S, et al. Blood-brain barrier disruption after cardiopulmonary bypass: Diagnosis and correlation to cognition. Anesthesiol Clin. 2017;104:161-169.
19. Lopez MG, Hughes CG, DeMatteo A, et al. Intraoperative oxidative damage and delirium after cardiac surgery. Anesthesiology. 2020;132:551-561.
20. Montenegro PH, Ahoco ML, Martin BM, et al. Cumulative head impact exposure predicts later-life depression, apathy, executive dysfunction, and cognitive impairment in former high school and college football players. J Neurol. 2017;34:328-340.
21. Bogoslovsky T, Gill J, Jeromin A, Davis C, Diaz-Arrastia R. Fluid biomarkers of traumatic brain injury and intended context of use. Diagnostics. 2016;6:37.
22. Dadas A, Washington J, Diaz-Arrastia R, Janiero D. Biomarkers in traumatic brain injury (TBI): a review. Neuropsychiatr Dis Treat. 2018;14:2989-3000.
23. Hiskens MI, Schneiders AG, Angoa-Pérez M, Vella RK, Fenning AS. Blood biomarkers for assessment of mild traumatic brain injury and chronic traumatic encephalopathy. Biomarkers. 2020;25:213-227.