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Pediatric reference intervals for serum neurofilament light and glial fibrillary acidic protein using the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) cohort

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Abstract

Objectives: Blood biomarkers have the potential to transform diagnosis and prognosis for multiple neurological indications. Establishing normative data is a critical benchmark in the analytical validation process. Normative data are important in children as little is known about how brain development may impact potential biomarkers. The objective of this study is to generate pediatric reference intervals (RIs) for serum neurofilament light (NfL), an axonal marker, and glial fibrillary acidic protein (GFAP), an astrocytic marker.

Methods: Serum from healthy children and adolescents aged 1 to <19 years were obtained from the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) cohort. Serum NfL (n=300) and GFAP (n=316) were quantified using Simoa technology, and discrete RI (2.5th and 97.5th percentiles) and continuous RI (5th and 95th percentiles) were generated.

Results: While there was no association with sex, there was a statistically significant (p<0.0001) negative association between age and serum NfL (Rho −0.400) and GFAP (Rho −0.749). Two statistically significant age partitions were generated for NfL: age 1 to <10 years (lower, upper limit; 3.13, 20.6 pg/mL) and 10 to <19 years (1.82, 7.44 pg/mL). For GFAP, three statistically significant age partitions were generated: age 1 to <3.5 years (80.4, 601 pg/mL); 3.5 to <11 years (50.7, 224 pg/mL); and 11 to <19 years (26.2, 119 pg/mL).

Conclusions: Taken together with the literature on adults, NfL and GFAP display U-shaped curves with high levels in infants, decreasing levels during childhood, a plateau during adolescence and early adulthood and increasing levels in seniors. These normative data are expected to inform future pediatric studies on the importance of age on neurological blood biomarkers.

Keywords: neurofilament light (NfL); glial fibrillary acidic protein (GFAP); blood biomarker; pediatric; reference intervals; neurology

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Introduction

Blood biomarkers are low-cost, objective, relatively non-invasive tools that provide insights to complex biological processes underlying both health and disease. Due to technological advances, it is now possible to accurately quantify protein biomarkers in blood that are specific to or highly enriched in central nervous system (CNS) tissue [1, 2]. This has enabled rapid growth in a wide array of research studies for multiple neurological indications aiming to identify and validate blood biomarkers for potential diagnostic, therapeutic, and prognostic contexts of use [1–3].

A critical step towards advancing neurological blood biomarkers from research-use-only toward clinical practice is the generation of valid normative reference intervals

(RIs), which are fundamental to test interpretation as age and biological sex are common modifiers [4]. Given the magnitude and rate of change in brain organization and maturation during development from birth through to adolescence [5, 6], we hypothesize that the concentration of neurological blood biomarkers will reflect these processes. As such, pediatric-specific RIs are particularly important for neurological biomarkers to help assess their utility and refine potential contexts of use ranging from diagnostic indications related to neuroimaging and neuromonitoring, to prognostication of neurodevelopmental outcome, disease progression, and therapeutic efficacy spanning critical care to outpatient settings. The objective of this study was to generate discrete and continuous RIs for serum neurofilament light (NfL) [1], an axonal marker, and glial fibrillary acidic protein (GFAP) [2], an astrocytic marker, in healthy children aged 1 to <19 years old to inform future research studies on the marked effect of age on biomarker levels in children.

Materials and methods

Participant and specimen selection

Serum specimens from healthy children and adolescents 1 to <19 years of age were obtained from the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER: www.caliperproject.ca) cohort [4]. This study is a secondary analysis of NfL and GFAP using banked serum specimens obtained for the purposes of generating discrete RI for serum total tau as previously reported [7]. Participants were recruited through community initiatives, such as schools, community centres, and daycares. Due to challenges associated with recruitment and blood collection from children under 6 years of age, additional children aged 1 to <6 years who were metabolically stable were recruited through outpatient clinics at The Hospital for Sick Children, excluding all orders from infectious disease, hematology and oncology, bone marrow transplant, rheumatology, and allergy and immunology clinics, with written informed consent. The frequency distribution of recruitment source for participants age 1 to <6 years is detailed in Supplemental Table 1. Exclusion criteria included a history of chronic illness, acute illness within the past week, pregnancy, and use of prescription or non-prescription medication within the past two weeks. All specimens were collected using a serum separator tube (SST – 3.5 mL, BD Vacutainer), centrifuged at 4,000 rpm for 10 min, and divided into 500 µL aliquots within 4 h of collection, after which specimens were stored at −80 °C until analysis.

Quantification of serum GFAP and NfL

Serum NfL analysis was performed between April 19 to July 4, 2017, and serum GFAP analysis was performed between February 20 to 27, 2019. Specimens were analyzed on the Quanterix HD-1 platform (Quanterix Corp., Billerica, MA) using the NfL Advantage Kit (cat. #103186, lot 500740) and GFAP Discovery Kit (cat. #102336, lot 501618) following the manufacturer's directions. All assays included an 8-point calibrator curve, two kit controls and 3 naïve serum controls used to confirm assay performance, and up to 84 serum specimens, all assayed in duplicate with the average of those values used for analysis. Specimens were analyzed using an on-board 4-fold dilution strategy, as recommended, in a randomized order with each assay run containing between 36 and 84 specimens that covered the age range with both sexes, for a total of 4–5 assay runs per analyte. Quality control information for all assay runs was recorded and is summarized in Supplemental Table 2. Values from all specimens fell within the assay limits of quantification.

Cross-lot and cross-assay analysis

Recognizing that these are research-use-only (RUO) grade assays, to expand the generalizability of these results, we show a subset of cross-lot and cross-assay analyses using a selection of CALIPER specimens that covered the dynamic analyte concentration and age range to the fullest extent possible given limited specimen volumes. Using different lots of the same singleplex assay; 23 specimens were crossed from NfL Advantage lot 500740 to lot 502036 and 149 specimens were crossed from GFAP Discovery lot 501618 to lot 501418. Additionally, 79 specimens (n=72 for GFAP) were crossed to the Neurology-4-Plex-A Advantage assay from Quanterix (cat. # 102153, lot 500760), which measures NfL, GFAP, total tau, and ubiquitin C-terminal hydrolase L1. All analyses were carried out on the same HD-1 analyzer.

Statistical analysis and generation of reference intervals

Analysis was conducted using Microsoft Excel Software, R Statistical Programming (V 4.1.2), and Graph Pad V9.4. Discrete RIs were generated in accordance with Clinical and Laboratory Standards Institute (CLSI) EP28-A3c guideline as described previously [7, 8]. Scatterplots of NfL or GFAP by age and sex were generated and extreme outliers identified based on visual inspection were removed. Age and sex-specific partitions were identified and statistically confirmed using the Harris and Boyd Method. Following Box-Cox transformation, and the normality of each age partition was tested using the Shapiro-Wilk test and Quantile-Quantile plots. Outliers were removed using the Tukey or adjusted Tukey test twice for normally distributed and skewed data, respectively. Lower (2.5th) and upper (97.5th) reference limits and the corresponding 90 % confidence intervals (CI) were calculated using either the robust method of Horn and Pesce (40<n<120) or the nonparametric rank method (n≥120). Continuous RIs were created using the quantreg growth R package to create smoothed regression curves at the median and 5th and 95th percentiles without exclusion of any data points [9–11]. The smoothing factor for each regression function was determined by cross validation, and visually adjusted based on biological expectations. 95 % CI were also created for both upper and lower limit regression curves. Point intervals for each year of age from 1 to <19 years old were then created using the predict function. The percentiles corresponding to the lower (2.5th) and upper (97.5th) limits for discrete RIs were selected based on the CLSI EP28-A3c guidelines. However, to create continuous RIs, the 5th and 95th percentiles were used. This was because the 2.5th and 97.5th percentiles were highly susceptible to skewing by outliers and low sample numbers at the oldest and youngest ages, regardless of what smoothing factor was used to create the curves. Presenting the 5th and 95th percentiles still capture an accurate representation of the

population, while reducing the susceptibility to skewing of the curve. Associations between serum tau (previously reported), NfL, and GFAP were tested using a Spearman correlation. Cross-lot and cross-assay agreement were determined using Spearman correlation and Bland-Altman analysis and plots, reported as the percent bias and 95 % limits of agreement.

Results

A total of 300 specimens (149 males, 151 females) from CALIPER participants with a median age [interquartile range (IQR)] of 11.3 years [7.0, 15.1] were selected for analysis of serum NfL. One specimen from a 1.98-year-old male with a NfL value of 1,390 pg/mL was removed as a visual extreme outlier; insufficient volume remained for repeat testing. In the remaining 299 specimens, median serum NfL was 4.43 pg/mL, with a range of 0.85–62.9 pg/mL (Figure 1A). While there were no sex effects, there was a negative association between serum NfL and age (Spearman Rho −0.40, p<0.0001). Generation of continuous RIs (Figure 1B) and point intervals on a per annum basis (Table 1) demonstrated a maximum decrease of 10 % per annum up to age 5, 5 % for ages 5–11 years of age, and 2 % change per annum thereafter. Generation of discrete RIs yielded two statistically significant age partitions: age 1 to <10 years, with NfL ranging from a lower limit of 3.13 pg/mL to upper limit of 20.6 pg/mL; and 10 to <19 years, ranging from 1.82 to 7.44 pg/mL (Figure 1C; Supplemental Table 3). Sex was not a modifier of NfL levels.

GFAP was quantified in a total of 316 specimens (158 male, 158 female) from CALIPER participants with a median age [IQR] of 10.3 years [5.54, 15.5]. Median GFAP was 84.9 pg/mL, with a range of 25.0–670 pg/mL, and there was a negative association between GFAP and age (Spearman Rho −0.749, p<0.0001) (Figure 2A). In children ages 4 to <6 years, where a combination of specimens recruited from both community initiatives and outpatient clinics were used, there was no statistically significant difference in serum GFAP between groups based on sample source (Supplemental Figure 1), consistent with our previous report on serum total tau [7]. Generation of continuous RI (Figure 2B) and point estimates per annum (Table 2) demonstrated that GFAP continuously decreases with age. Over the first five years of life, median serum GFAP decreases at a rate of 15–25 % per annum, slowing to a decrease to 7–8 % per annum between ages 5 and 13 years, 4–5% for ages 14–16 years, and finally 1 % per annum for ages 17 to <19 years. Generation of discrete RIs yielded three statistically significant age partitions: age 1 to <3.5 years (lower limit, upper limit; 80.4 pg/mL, 601 pg/mL); 3.5 to <11 years (50.7 pg/mL, 224 pg/mL); and 11 to <19 years (26.2 pg/mL, 119 pg/mL) (Figure 2C, Supplemental Table 3). Sex was not a modifier of GFAP levels.

Between serum analytes, across all ages, the following statistically significant (p<0.0001) associations were noted: NfL and GFAP (Rho 0.503), NfL and t-tau (Rho 0.308), and GFAP and t-tau (Rho 0.221).

Lastly, we determined cross lot, for both NfL and GFAP singleplex assays, and cross-assay, single-plex vs. the Neurology-4-Plex-A assay, performance to determine generalizability of the data (Supplemental Figure 2). For the

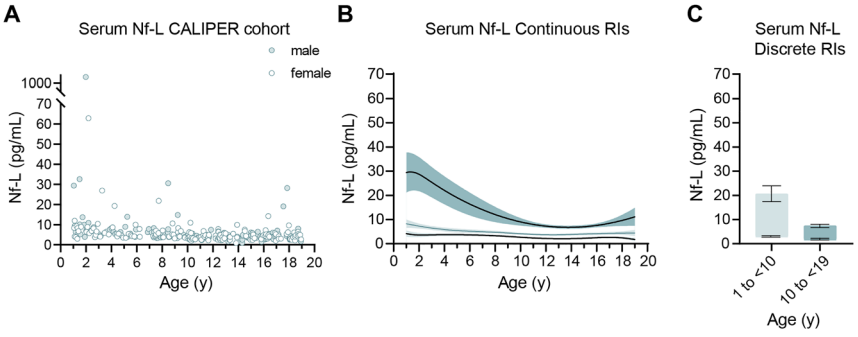

Figure 1: Pediatric reference intervals for serum NfL. (A) Serum NfL levels were measured in 300 male (n=149) and female (n=151) healthy children aged 1 to <19 years (median age 11.3 years) from the CALIPER cohort. One specimen from a 1.98 year old male with a serum NfL concentration of 1,390 pg/mL was identified as an extreme outlier and subsequently removed from all further analysis, leaving 299 specimens for RI calculations. (B) Continuous RIs (n=299) were generated using quantile regression. Upper and lower reference limits (95th and 5th percentile) and median are shown with 95 % CI. (C) Discrete RIs: two significant age partitions were generated and outliers (n=9; serum NfL values of 0.85, 0.98, 9.65, 9.92, 11.0, 14.4, 19.1, 28.2 pg/mL) removed; age 1 to <10 years (n=119), and 10 to <19 years (n=171). No partitions were required for sex. Upper and lower reference limits (97.5th and 2.5th percentile) and 90 % CI for each RI were defined. CALIPER=Canadian Laboratory Initiative on Pediatric Reference Intervals; CI=confidence interval; NfL=neurofilament light; RI(s)=reference interval(s).

Table 1: Point estimates for the lower limit (5th percentile), median, and upper limit (95th percentile) with corresponding 95 % confidence interval (CI) for serum NfL per year from age 1 to <19 determined using quantile regression.

Age, years	Lower limit, pg/mL	Lower limit 95 % CI		Median, pg/mL	Median 95 % CI		Upper limit, pg/mL	Upper limit 95 % CI	
1 to <2	3.78	4.84	2.72	7.78	9.11	6.45	29.6	37.3	22.0
2 to <3	3.59	4.30	2.88	6.87	7.75	5.99	27.3	33.9	20.6
3 to <4	3.68	4.23	3.13	6.15	6.95	5.35	23.8	29.8	17.7
4 to <5	3.72	4.21	3.23	5.62	6.42	4.82	20.6	26.2	15.0
5 to <6	3.68	4.11	3.25	5.28	6.04	4.52	17.7	22.8	12.7
6 to <7	3.58	3.97	3.19	5.06	5.75	4.37	15.2	19.6	10.8
7 to <8	3.40	3.75	3.05	4.84	5.45	4.23	13.0	16.6	9.41
8 to <9	3.15	3.46	2.84	4.58	5.13	4.03	11.2	13.9	8.44
9 to <10	2.84	3.13	2.55	4.27	4.76	3.78	9.65	11.6	7.71
10 to <11	2.50	2.77	2.23	3.96	4.39	3.53	8.45	9.70	7.20
11 to <12	2.24	2.51	1.97	3.75	4.16	3.34	7.57	8.39	6.75
12 to <13	2.10	2.39	1.81	3.67	4.10	3.24	7.01	7.66	6.36
13 to <14	2.07	2.38	1.76	3.73	4.20	3.26	6.77	7.44	6.10
14 to <15	2.17	2.50	1.84	3.89	4.40	3.38	6.86	7.55	6.17
15 to <16	2.38	2.73	2.03	4.07	4.62	3.52	7.26	7.99	6.53
16 to <17	2.59	2.94	2.24	4.22	4.77	3.67	7.98	9.00	6.96
17 to <18	2.56	2.93	2.19	4.32	4.93	3.71	9.03	10.8	7.23
18 to <19	2.10	2.67	1.53	4.39	5.35	3.43	10.4	13.4	7.38

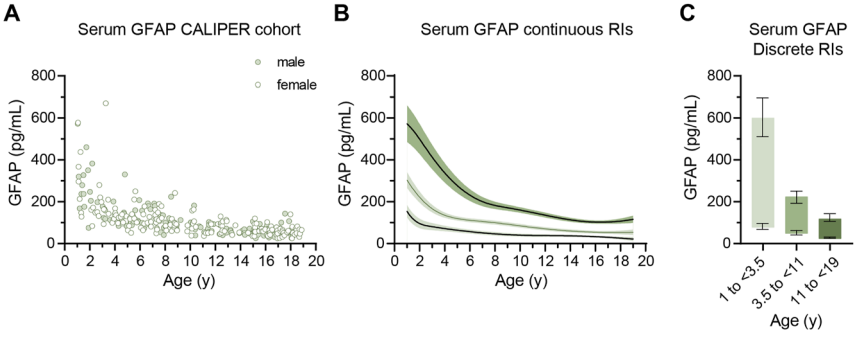

Figure 2: Pediatric reference intervals for serum GFAP. (A) Serum GFAP levels were measured in 316 male (n=158) and female (n=158) healthy children aged 1 to <19 years (median age 10.3 years) from the CALIPER cohort. (B) Continuous RIs were generated using quantile regression. Upper and lower reference limits (95th and 5th percentile) and median are shown with 95 % CI. (C) Discrete RIs: three significant age partitions were generated and outliers (n=3) removed; age 1 to <3.5 years (n=41), 3.5 to <11 years (n=121), and 11 to <19 years (n=150). No partitions were required for sex. Upper and lower reference limits (97.5th and 2.5th percentile) and 90 % CI for each RI were defined. CALIPER=Canadian Laboratory Initiative on Pediatric Reference Intervals; CI=confidence interval; GFAP=glial fibrillary acidic protein; RI(s)=reference interval(s).

NfL Advantage Assay, there was a strong positive association (Rho 0.919, p<0.0001) between lots 500740 and 502056, with a mean bias of −5.2 %; similar results were yielded for the GFAP Discovery Assay, with a Spearman Rho of 0.925 (p<0.0001) and mean bias of −17.4 % for lot 501618 to 501418. When compared to the Neurology-4-Plex-A assay, for NfL the Spearman Rho was 0.921 (p<0.0001) with a mean bias of 13.4 %; for GFAP the Spearman Rho was 0.967 (p<0.0001) with a mean bias of −7.0 %.

Discussion

The brain undergoes prolific growth and development through childhood, doubling in size during the first year after birth and reaching 80 % of adult volume by age 3, with up to twice as many synapses than are present at adulthood [5]. Astrocytes play major roles in bi-directional communication with neurons to regulate the formation of new synaptic pathways, modulate the plasticity of existing circuits,

Table 2: Point estimates for the lower limit, median, and upper limit with corresponding 95 % confidence interval (CI) for serum GFAP per year from age 1–18 determined using quantile regression.

Age, years	Lower limit, pg/mL	Lower limit 95 % CI		Median, pg/mL	Median 95 % CI		Upper limit, pg/mL	Upper limit 95 % CI	
1 to <2	118	145	91.9	262	294	229	542	622	461
2 to <3	86.2	106	66.4	192	214	170	458	523	392
3 to <4	76.1	91.2	61.0	149	164	134	374	427	322
4 to <5	67.5	79.5	55.5	126	137	115	306	347	265
5 to <6	60.0	70.2	49.8	115	124	105	252	284	220
6 to <7	53.6	62.8	44.4	108	118	98.8	214	239	189
7 to <8	48.4	57.2	39.6	102	112	93.0	190	209	170
8 to <9	44.2	52.4	36.0	95.8	105	86.6	176	192	160
9 to <10	41.1	48.7	33.5	88.3	97.3	79.3	165	179	151
10 to <11	39.1	46.2	32.0	80.3	88.7	71.9	154	167	141
11 to <12	38.2	44.5	31.9	73.0	81.2	64.8	141	153	129
12 to <13	37.9	43.6	32.2	66.8	74.6	59.0	129	140	117
13 to <14	37.3	42.4	32.2	61.8	69.4	54.2	117	128	107
14 to <15	35.9	40.4	31.4	57.8	65.1	50.5	109	118	99.5
15 to <16	33.9	38.0	29.8	55.0	61.7	48.3	104	112	95.5
16 to <17	31.1	35.4	26.8	53.2	59.7	46.7	102	110	94.3
17 to <18	27.5	33.2	21.8	52.7	60.5	44.9	105	116	94.5
18 to <19	23.2	31.4	15.0	53.2	64.6	41.8	112	127	96.7

and direct removal of unnecessary or non-functional pathways [6]. Thus, it is not surprising to observe an age-dependent response for NfL, and to an even greater extent GFAP, such that biomarker levels were highest and most variable in young children, with a plateau observed by early adolescence in normally developing children. Blood biomarker levels reflect the combined processes of production, transport from the CNS into the systemic circulation, and normal clearance by the liver and kidneys, all of which may be influenced by age and physiological development. While both serum NfL and GFAP were negatively associated with age in children, the change of GFAP on a per annum basis was almost twice that of NfL on a percentage basis. While differences may be expected between GFAP, an astrocytic, and NfL, an axonal marker, it is also interesting to compare age-related effects between NfL and total tau, both axonal markers. As previously published using CALIPER specimens, serum total tau also negatively associates with age, with the greatest rate of change observed before age 4 [7]. There is only a weak positive association between serum NfL and total tau across ages, potentially due to analyte or assay differences, as tau is also expressed in the liver, heart, and kidneys, and some studies report a poor association of serum vs. cerebrospinal fluid (CSF) total tau [12] compared to the excellent association of serum and CSF NfL [1].

Our data are consistent with those published by Reinert et al. [13], who quantified NfL in 301 serum specimens from German children age 3-months to 18 years who attended a hospital due to a non-neurological disease, and those recently published by Abdelhak et al. [14], who generated an age-adjusted reference database for serum NfL in a cohort of 2,667 healthy children and adolescents, age 0–22 years, from Europe and North America. The median and IQR for serum NfL was 5.1 pg/mL (3.7, 6.7) in Reinert's study, 4.8 pg/mL (3.7, 6.2) in Abdelhak's study, and 4.4 pg/mL (3.5, 6.1) for the CALIPER data presented here; all studies reported an age-related decrease of NfL most pronounced in young children up to age 6–10, and a relative plateau throughout adolescence, while none of the studies found an effect of sex on NfL. Using a non-parametric smoothing line, Reinert et al. reported a 90th percentile of 7.7 pg/mL and 99th percentile of 12.6 pg/mL for serum NfL in children aged 6.5–18 years, while Abdelhak et al. used a generalized additive model for location, scale, and shape, reporting a 95th percentile between 7.2 and 7.5 pg/mL for ages 10–18 years, all which aligns with our predicted 95th percentile of 6.77–9.03 pg/mL between ages 10–18. We do acknowledge that the 95th percentiles for children under 10 years of age are lower in the study by Abdelhak compared to CALIPER; variations in the statistical modelling approaches used to generate the reference percentiles, study populations and notably sample size, in addition to analytical variation, may explain the differences observed. Two studies have also reported on plasma NfL levels in healthy children: Simren et al. reported a 95th percentile of 7 pg/mL for plasma NfL in a cohort of 27

Swedish children age 5–16 years [15] and in a cohort of 119 healthy Chinese children, Jin et al. reported 95th percentiles ranging from a high of 19.96 pg/mL in children age 1–3 years, to a low of 13.8 pg/mL in children age 13–18 years [16]. Compared to NfL, until very recently, there was a relative paucity of data pertaining to serum GFAP in healthy children. Tybirk et al. recently published continuous RI for serum GFAP in 391 Danish children age 0.4–17.9 years [17], demonstrating a strong negative association between age and GFAP, with an estimated median decrease of 66 % between ages 4 months and 5 years and 65 % between 5 and 18 years. By comparison, median serum GFAP in CALIPER decreased by 56 % between age 1 and 5 years and 54 % between age 5 and 18 years, demonstrating excellent agreement with the Danish cohort. While the concentration of GFAP reported is higher in the Danish cohort, Tybirk et al. stated that they observed a ~50 % increase between different reagent lots used compared to their previous publication in adults [18], highlighting the challenge of working with research-use-only reagents that are not cross-lot validated nor harmonizable in the absence of quality control materials. Furthermore, while the majority of RI studies to date have focused on either the pediatric or adult population separately, Cooper et al. generated discrete and continuous RI for plasma NfL and GFAP from ages 3–79 years using 900 specimens from the Canadian Health Measures Survey (CHMS) [19]. Despite differences in matrix (i.e., serum in CALIPER vs. plasma in CHMS) and assay format (i.e., singleplex vs. multiplex), data CALIPER and CHMS data show marked agreement with respect to association with age and rate of change and threshold concentration.

Clinically, serum GFAP and NfL are approved for use in specific indications in adult neurology. Serum GFAP, measured in combination with serum ubiquitin C-terminal hydrolase L1, is US FDA approved to assist clinicians to decide whether adults with mild traumatic brain injury (TBI) or concussion should undergo head computed tomography (CT). While CT imaging can be a useful diagnostic tool, there are valid concerns about potential CT overuse in children, as radiation exposure and the potential need for sedation in young children both carry known health risks [20]. While not as well characterized as the adult population, serum GFAP was found to be higher in mild TBI compared to orthopedic [21, 22] and healthy controls [23, 24], elevated in response to TBI severity [23, 24], and discriminated between CT positive and CT negative TBI patients [22], prompting the need for further studies. Our data enable future studies to refine at what ages serum GFAP may have potential utility to rule out the need for CT in children with mild TBI. Serum NfL has been clinically validated and is currently being used to monitor disease progression and therapeutic response in adult patients with relapsing remitting multiple sclerosis (RRMS) in parts of Canada and Europe, with the US FDA granting breakthrough device designation for the Quanterix Simoa NfL test in 2022 [25–27]. Literature suggests that serum NfL is also elevated in pediatric MS patients compared to controls, reflects disease activity and severity, and may be able to monitor therapeutic responses [13].

Our data also inform future studies to assess the potential utility of serum NfL and GFAP for neuromonitoring contexts of use and neurodevelopmental outcomes. Invasive neuromonitoring using implanted probes is challenging in critically ill children and feasible only at select sites, and blood biomarkers that report on neurological status may provide invaluable diagnostic and prognostic insights into pediatric neonatal encephalopathy, brain function after cardiac arrest, and neurological responses after pediatric surgery. For example, serum GFAP and/or NfL are reportedly higher in critically ill neonates with hypoxic ischemic encephalopathy [28, 29] and children following cardiac arrest [30] compared to age-matched controls, with biomarker levels associated with the degree of neurological injury observed on magnetic resonance imaging [28], adverse neurodevelopmental or neurological outcome [29, 31], and death [30]. Serum GFAP has also been reported to prognosticate impaired neurodevelopmental outcome 1–5 years following surgical repair of congenital heart disease in neonates and infants [32, 33]. Together, this literature supports that both serum NfL and GFAP are sensitive markers of neurological injury in critically ill children and may also yield prognostic insights on neurodevelopment.

Strengths of this study include leveraging of CALIPER specimens, which have been used extensively to implement pediatric RIs for more than 200 laboratory tests in children's hospitals worldwide, and the generation of both discrete and continuous RIs for pediatric serum NfL and GFAP. Continuous RIs have the benefit of enabling smooth transitions between ages, whereas discrete RIs can have marked changes between age partitions. However, a limitation is that continuous RIs may be influenced by outlying data points as outliers are not removed prior to analysis. One limitation of this study is the use of the Quanterix HD-1 analytical platform, which is research-use-only and now discontinued and replaced by the HD-X model. A second limitation is that this study (and many other RI studies to date for these biomarkers) lack matrix-appropriate reference materials and methods to allow for cross laboratory and cross platform validation that will be essential to allow these results to be transferable and applicable in both research and eventually clinical contexts. A third limitation is the use of serum specimens collected from children

attending outpatient hospital clinics, as we do not have a detailed medical history. While this is part of the parent CALIPER study protocol, and data from these children have been used for the generation of RI for multiple clinically used analytes, there is the potential for underlying injury that may alter these neurological markers, even with the exclusion criteria in place.

In conclusion, we generated both discrete and continuous RI for two promising neurological blood biomarkers, NfL and GFAP, using the Canadian CALIPER cohort. Our data demonstrate that while sex does not modify biomarker levels, there is a significant impact of age, particularly for GFAP with more modest effects observed for NfL. Thus, although additional studies will be required to transfer NfL and GFAP onto clinical analytical platforms using clinical grade assays and quality control calibration materials, our study provides valuable insights into the need to account for age in future studies of these biomarkers in pediatric populations.

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Research ethics: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration and has been approved by the institutional review boards of The Hospital for Sick Children (#1000010867) and Mount Sinai Hospital, Toronto, Canada and The University of British Columbia, Vancouver, Canada (H16-02548).

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

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