

Age specific reference intervals for plasma biomarkers of neurodegeneration and neurotrauma in a Canadian population

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ABSTRACT

Introduction: In this study, we aimed to create reference intervals (RI) using a large Canadian population-based cohort, for plasma protein biomarkers with potential utility to screen, diagnosis, prognosticate and manage a variety of neurological diseases and disorders. RIs were generated for: the ratio of amyloid beta 42 over 40 (A β 42/40), phosphorylated tau-181 (p-tau-181), neurofilament light (NFL), and glial fibrillary acidic protein (GFAP).

Methods: 900 plasma specimens from male and female participants aged 3–79 years old were obtained from the Statistics Canada Biobank, which holds specimens from the Canadian Health Measures Survey. Analysis of A β 42/40, p-tau-181, NFL and GFAP was performed on the Quanterix Simoa HD-X analyzer using the Neurology 4-plex E and p-tau-181 assays. Discrete RIs were produced according to Clinical Laboratory Standards Institute guidelines (EP28-A3c). Continuous RIs were created using quantile regression.

Results: For discrete RIs, significant age partitions were determined for each biomarker. No significant sex partitions were found. The following ranges and age partitions were determined: A β 42/40: 3–<55y = 0.053–0.098, 55–<80y = 0.040–0.090; p-tau-181: 3–<12y = 1.4–5.6 pg/ml, 12–<60y = 0.8–3.1 pg/ml, 60–<80y = 0.9–4.0 pg/ml; NFL: 3–<40y = 2.6–11.3 pg/ml, 40–<60y = 4.6–17.7 pg/ml, 60–<80y = 8.1–47.1 pg/ml; GFAP: 3–<10y = 47.0–226 pg/ml, 10–<60y = 21.2–91.9 pg/ml, 60–<80y = 40.7–228 pg/ml. Continuous RIs produced smooth centile curves across the age range, from which point estimates for each year of age were calculated.

Conclusions: Discrete and continuous RIs for neurological plasma biomarkers will help refine normative cut-offs across the lifespan and improve the precision of interpreting biomarker levels. Continuous RIs are recommended for use in age groups, such as pediatrics and older adults, that experience rapid concentration changes by age.

1. Introduction

Blood-based biomarkers, predominantly derived from the central nervous system (CNS), are promising tools to screen, diagnose, prognosticate and manage a variety of neurological disorders. These biomarkers are considered to be a low cost, minimally invasive proxy measurement of CNS pathologies. Many prominently investigated biomarkers focus on Alzheimer's disease (AD), the most common form of

dementia. AD is neuropathologically defined by amyloid plaques of aggregated β -amyloid (A β) peptides and neurofibrillary tangles of hyperphosphorylated tau. In plasma, the ratio of A β 42/40 can be measured as an indicator of amyloid plaque pathology [1,2]. Phosphorylated tau at residue 181 (p-tau-181), a neuronal microtubule-associated protein phosphorylated at amino acid position 181, has recently emerged as a promising plasma biomarker that closely tracks with amyloid burden in AD patients [3,4]. Neurofilament light (NFL) is

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an axonal intermediate filament protein that, when measured in plasma, is elevated in AD [5], other neurological conditions such as multiple sclerosis (MS) [6], frontotemporal dementia [7], and neurotrauma [8]. Glial fibrillary acidic protein (GFAP) is an astrocytic intermediate filament protein that is presumed to reflect astrocyte activation when measured in plasma. Similar to NFL, plasma GFAP levels are increased in several neurodegenerative conditions as well as after in neurotrauma and acute cerebrovascular insults [9,10].

These four plasma biomarkers are at various stages of analytical and clinical validation depending on the specific context of use being examined. An important step in the clinical validation process is to establish cut-offs to discriminate normal from abnormal values. This can be done by creating reference intervals (RI) to define normal concentrations for a healthy or normative population based on a person's age or sex. As many of the above biomarkers have been found to associate with age [11,12], it is important to develop age-dependent RIs across the lifespan to facilitate interpretation of their plasma levels. In this study, we developed discrete RIs using methodology recommended by the Clinical Laboratory Standards Institute guidelines (CLSI) (EP28-A3c) [13] and continuous RIs using smoothed quantile regression, to provide age specific cut-offs for plasma A β 42/40, p-tau-181, NFL, and GFAP reflective of the Canadian population.

2. Methods

2.1. Ethical approval

This study was reviewed and approved by The University of British Columbia Clinical Research Ethics Board, Vancouver, Canada (H19-01445). The Canadian Health Measures Survey (CHMS), which provided plasma specimens for this study, was reviewed and approved by the Health Canada and the Public Health Agency of Canada Research Ethics Board, Ottawa, Canada.

2.2. Participant and specimen selection

Specimens were acquired from the Statistics Canada Biobank which holds specimens from CHMS [14]. CHMS is a population-based national survey that collects information on the health of Canadians [15]. Specimens were from CHMS cycle 5 where plasma specimens were collected in 2016 and 2017 from Canadians aged 3 to 79 years old. The CHMS study excluded individuals who live in the three Canadian territories (Northwest Territories, Yukon and Nunavut), live on reserves and other Indigenous settlements in Canadian provinces, are full-time members of the Canadian Forces, are institutionalized, or are residents of remote regions with under 10,000 people in a 75 km radius. These exclusions represent approximately 4% of the targeted Canadian population [16]. No further exclusion criteria for this study were applied beyond CHMS eligibility criteria. Blood was collected in EDTA vacutainers and centrifuged at 8° Celsius for 15 min at 1,800 g-force. Plasma was separated and stored directly into a -80° Celsius freezer within a maximum of 4 h from collection. N = 5,232 specimens were obtained from the Statistics Canada Biobank, and N = 900 participant specimens across the full age range were randomly selected for analysis by a Statistics Canada methodologist to ensure CHMS participant information was protected and anonymized. A subset of N = 384 specimens was initially selected to determine how many additional specimens would need to be analyzed to establish RIs, which were selected by Statistics Canada methodologists based on an even distribution across CHMS age groupings (3–5, 6–11, 12–19, 20–39, 40–59, 50–79 years). For each age group, 4 participants (2 males, 2 females) were selected from each of the 16 collection sites across Canada, for a total of 64 subjects per age group. A Statistics Canada representative then selected an additional 516 specimens across age bins with an even distribution of sex and geographical region of collection to ensure a large enough sample size to comply with CLSI EP28-A3c guidance [13]. Although our results are

based on unweighted population data and are therefore not fully representative of the Canadian population, our data exceed CLSI recommendations for RI generation.

2.3. Biomarker analysis

Biomarker concentrations were quantified using the single-molecule array enzyme linked immunoassay (Simoa) HD-X platform from Quanterix (Billerica, MA) following the manufacturer's protocol. Plasma GFAP, NFL, A β 40, and A β 42 were measured using the Neurology-4-plex E advantage assay (catalogue #103670, lot 503105), and plasma p-tau-181 was measured using the ptau-181 V2 advantage assay (catalogue #103714, lot 502923). Each assay included an 8-point calibrator curve, two internal kit controls, and three plasma controls. All specimens were assayed in duplicate and the mean value was reported as the result. Analysis was completed using a single lot of each assay to reduce analytical variability that can be introduced by harmonizing lots. For all analytes, quality control information was documented across all runs. Standard curve concentrations had an average absolute error of 2.7–5.8% and an average recovery of 96.5–100%. Internal kit control concentrations had an average absolute error of 7.6–24.7% and an average recovery of 97.3–124%. The inter-plate CVs, calculated using plasma control concentrations analyzed in duplicate across every plate, were between 8.4 and 14.4%. The average intra-plate CVs, calculated using participant specimen, were between 2.0 and 7.0%. Analysis was conducted approximately 5 years after sample collection. It has been shown that A β 40 and A β 42 are stable over this time period [17]. While NFL, GFAP and p-tau-181 have not been investigated for their stability in plasma over 5 years, they have been shown to be robust, stable analytes in plasma that are resilient to varied storage conditions and freeze thaw cycles [18].

2.4. Cross lot analysis

Although all specimens were analyzed using the same HD-X instrument and assay lot, a cross lot analysis was conducted to interpret the generalizability of the results. N = 80 CHMS specimens were randomly selected across the dynamic range of the original N = 900 to be re-analyzed on two separate lots of reagents. N = 40 of the cross lot specimens were re-analyzed on a different lot (N4PE: 503212, p-tau-181: 503199) using the same HD-X instrument as the original study. Another N = 40 specimens were re-analyzed on a different lot (N4PE: 503811, p-tau-181: 503545) and a different HD-X instrument from the original study. In addition to re-analyzing CHMS specimens across lots and instruments, we also tracked 3 plasma controls that were used in the original study to monitor inter-plate variability. These controls were generated from healthy plasma, with a low concentration control being unaltered plasma, and medium and high concentration controls created by spiking the plasma with the top calibrators provided with the assay kits. These plasma controls were carried over onto 10 additional plates, 5 plates using a different lot (N4PE: 503212, p-tau-181: 503199) and the same HD-X instrument as the original study, and another 5 plates using a different lot (N4PE: 503811, p-tau-181: 503545) and a different HD-X instrument from the original study. We then determined if each control value was within the acceptable ranges, which were determined by taking the 95% CI of the results obtained from each of the 26 plates analyzed in the original study.

2.5. Statistical analysis

Discrete RIs were produced according to CLSI EP28-A3c guidelines [13]. Scatterplots for each biomarker and age were used to visually estimate break points for partitioned RIs. Breakpoints were established using the Harris and Boyd method [19]. Within each age partition, a Shapiro-Wilk test for normality was performed; all partitions contained non-normally distributed data. A Mann-Whitney *U* test and Harris and

Boyd method [19] was used to test for a statistical difference between sexes within each partition. For all biomarkers, significant partitions were required for age but not sex. Within partitions, RIs were created using the *referenceIntervals* package [20] for the R Statistical Programming Language. Outliers were removed from the dataset using Tukey’s test for outliers. Upper and lower limits of the RIs were determined using the non-parametric method [13], where the upper limit is denoted by the 97.5th percentile of the population and the lower limit by the 2.5th percentile of the population. 90% confidence intervals (CIs) of upper and lower limits were also generated using the non-parametric method.

Continuous RIs were created for each analyte with the *quantreg-Growth* package [21,22] for the R Statistical Programming Language. No outliers were removed for this analysis. Smoothed regression curves were generated at the 95th and 5th percentiles to represent the upper and lower limits of the RIs, respectively. A regression curve was also generated at the 50th percentile to indicate the population median. The smoothing factor lambda for each regression function was determined by cross validation, and visually adjusted to determine fit based on biological expectations. 95% CIs were also created for both upper and lower limit regression curves. Point intervals for each age from 3 to 79 were then created using the predict function.

Bland-Altman analysis and plots were used to determine agreement between lots for the cross lot analysis. Agreement was determined as the percentage bias and the 95% limits of agreement. All statistical analyses were performed using RStudio version 4.1.2 and GraphPad Prism version 9.5.1.

3. Results

3.1. Reference intervals

A total of N = 900 specimens from CHMS participants aged 3 to 79 years were analyzed to create discrete and continuous RIs. The sex distribution of participants was equal, with N = 450 (50%) males and females. N = 369 participants were under 20 years of age, N = 342 participants were between 20 and 60 years of age, and 189 participants were over 60 years of age. The distribution of participants by age and sex is shown in Fig. 1.

For discrete RIs, significant partitions were determined to create age bins for each biomarker. The age bins for each analyte were: Aβ42/40: 3 to <55 years, and 55 to <80 years; p-tau-181: 3 to <12 years, 12 to <60 years, and 60 to <80 years; NfL: 3 to <40 years, 40 to <60 years, and 60 to <80 years; GFAP: 3 to <10 years, 10 to <60 years, and 60 to <80 years. No significant sex differences were determined, allowing the same

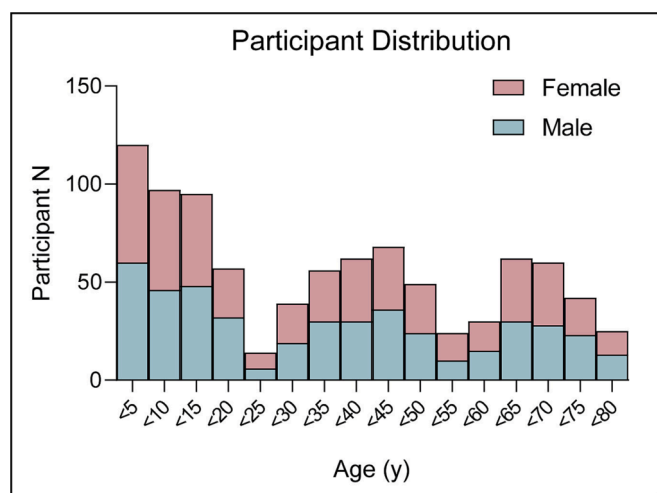


Fig. 1. Distribution of participants by age and sex. N = 900 participants from CHMS, cycle 5 collected in 2016/2017.

RIs to be used for both sexes. The values for the upper (97.5th) and lower (2.5th) limits for the RIs with associated 90% CIs for each age bin are displayed in Table 1 and Fig. 2.

Continuous RIs were created to determine the upper and lower limits for each year of age. As no sex differences were determined, data were combined to create continuous RIs to be used for both sexes. Graphs displaying continuous RIs are displayed in Fig. 3. The upper and lower limits are displayed as smoothed curves across the 95th and 5th percentiles of the population. The 50th percentile is also displayed to show the population median. Exact values for the upper and lower limits of the RI at every year of age with associated 95% CIs can be found in Supplemental Tables 1–4.

3.2. Assay lot agreement

To determine the generalizability of our results, we conducted cross lot investigations by reanalyzing a subset of N = 80 CHMS specimens to compare results across another lot of reagents and a separate HD-X instrument. The first cross lot analysis involved re-analyzing N = 40 specimens on the same HD-X instrument as the original study with different assay lots. For the Neurology 4-Plex E assay there was an average bias of 8.4%, and for the p-tau-181 assay there was a bias of 22.1%. The second cross lot analysis involved re-analyzing a separate set

Table 1

Discrete reference intervals for plasma Aβ42/40, p-tau-181, NfL, and GFAP. Significant age partitions were determined by Harris and Boyd method, no significant sex partitions were detected. Tables display N per each age bin after outliers were removed using Tukey test, values for upper (97.5th) and lower (2.5th) limits with corresponding 90% confidence.

Aβ42/40					
Age Bin (y)	N	Lower Limit	90% CI	Upper Limit	90% CI
3 to <55	661	0.053	0.050, 0.057	0.098	0.096, 0.099
55 to <80	221	0.04	0.028, 0.045	0.09	0.088, 0.095

p-tau-181					
Age Bin (y)	N	Lower Limit (pg/ml)	90% CI (pg/ml)	Upper Limit (pg/ml)	90% CI (pg/ml)
3 to <12	239	1.4	1.4, 1.5	5.6	5.0, 6.6
12 to <60	444	0.8	0.7, 0.8	3.1	2.9, 3.6
60 to <80	202	0.9	0.9, 1.0	4	3.6, 5.7

NfL					
Age Bin (y)	N	Lower Limit (pg/ml)	90% CI (pg/ml)	Upper Limit (pg/ml)	90% CI (pg/ml)
3 to <40	522	2.6	2.5, 2.7	11.3	10.7, 12.8
40 to <60	165	4.6	4.3, 4.9	17.1	15.5, 18.0
60 to <80	202	8.1	7.5, 9.1	47.1	37.5, 56.6

GFAP					
Age Bin (y)	N	Lower Limit (pg/ml)	90% CI (pg/ml)	Upper Limit (pg/ml)	90% CI (pg/ml)
3 to <10	200	47	36.5, 55.2	226.3	190.0, 231.9
10 to <60	491	21.2	19.7, 22.7	91.9	86.2, 96.5
60 to <80	199	40.7	38.0, 44.0	227.5	184.4, 255.8

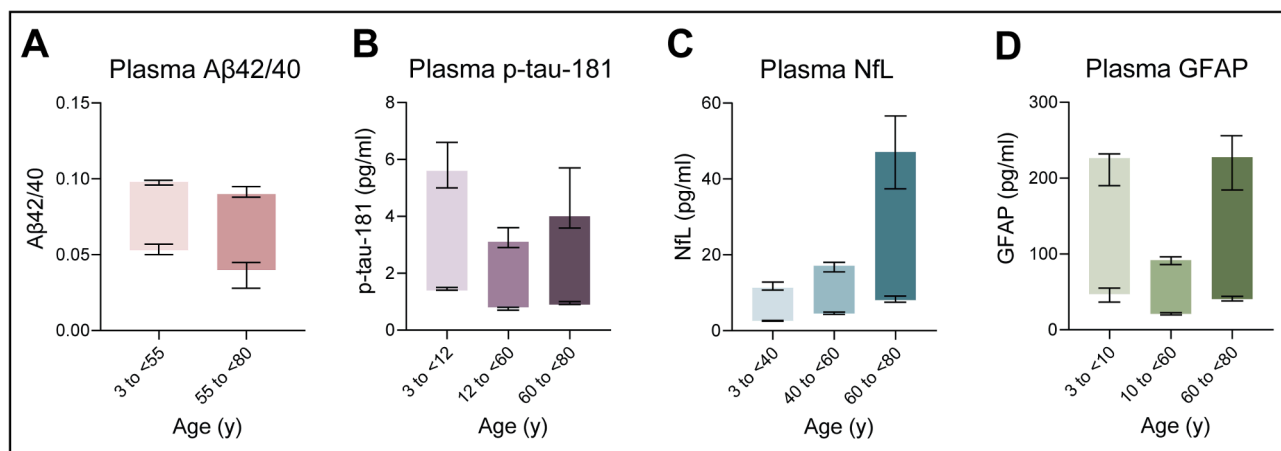


Fig. 2. Discrete reference intervals for plasma Aβ 42/40 (A), p-tau-181 (B), NfL (C), and GFAP (D). Significant age partitions were determined by Harris and Boyd method, no significant sex partitions were detected. The shaded regions display the reference interval with upper (97.5th percentile) and lower (2.5th percentile) limits of reference range, error bars indicate the 90% CI of the limits.

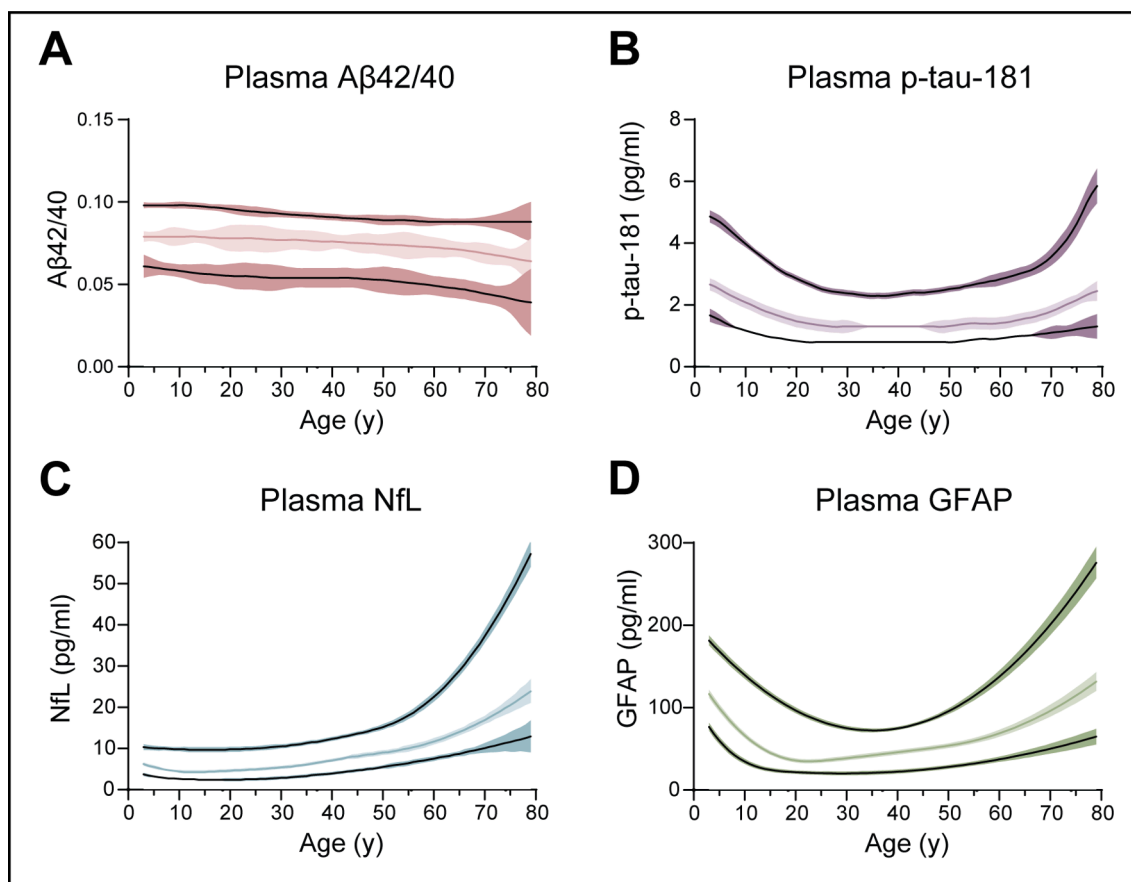


Fig. 3. Continuous reference intervals for plasma Aβ 42/40 (A), p-tau-181 (B), NfL (C), and GFAP (D). N = 900 for all figures. Black lines are 95th (upper limit) and 5th (lower limit) percentiles. Lighter coloured line is the 50th percentile. Shaded area around lines are the 95% CIs.

of N = 40 specimens on a different HD-X instrument than the original study with different assay lots. For the Neurology 4-Plex E assay there was an average bias of 9.7%, and for the p-tau-181 assay there was a bias of 13.7%. The bias for each individual analyte can be found in [Table 2](#) and [Supplemental Fig. 1](#).

Plasma controls were also tracked onto different lots to determine their agreement with the ranges generated on the original lot ([Supplemental Fig. 2](#)). Acceptable ranges were determined by calculating the 95% CI of plasma control results across 26 plates analyzed on the

original lot. When analyzed on the same HD-X instrument as the original study using a different assay lot, plasma controls were within range for 80% of Aβ40 data points and 93% of GFAP data points, with 100% of the data points for Aβ42, AB42/40, p-tau-181 and NfL falling in acceptable ranges. When analyzed on a different HD-X instrument and using a different assay lot than the original study, plasma controls were within acceptable ranges for 67% of Aβ42 data points and 80% of p-tau-181 data points, and 100% of the data points for Aβ40, Aβ42/40, NfL, and GFAP fell within acceptable ranges. Thus, an average of 92% of plasma

Table 2

Cross lot comparison for Quanterix Neurology-4-plex E (N4PE) advantage and ptau-181 V2 advantage assays. N = 80 samples were randomly selected across the dynamic range of the sample set to be re-analyzed on two separate lots of reagents. One lot was analyzed on the original HD-X that this study was conducted on, and the other lot was analyzed on a different HD-X instrument. Bland-Altman analysis was conducted to determine agreement between lots and reported here as the percentage bias.

Compared to original study analysis:	Different Lot	Different Lot
	Same HD-X Instrument	Different HD-X Instrument
Cross-Lot N	40	40
Analyte	Mean Bias, %, (SD)	Mean Bias, %, (SD)
A β 40	9.8 (12.9)	4.4 (14.7)
A β 42	3.7 (11.9)	7.4 (5.4)
A β 42/40	-6.2 (13.4)	-2.9 (14.5)
NfL	11.0 (32.4)	-21.8 (20.0)
GFAP	9.4 (24.3)	-5.3 (18.4)
p-tau-181	-22.1 (17.6)	-13.8 (11.8)

control data points per analyte fell within acceptable ranges.

4. Discussion

4.1. Interpretation of reference intervals

We created RIs for plasma A β 42/40, p-tau-181, NfL and GFAP that included cut-offs for age bins and for each year of age. Sex was not identified as a modifying variable for any biomarker. At least one significant age partition was required for each biomarker. For A β 42/40, only one partition point was determined at age 55. A β 42/40 continuous RI showed little change over the lifespan, with an average yearly change of <1%. To our knowledge, no other study has created RIs for plasma A β 42/40, though RIs for the individual markers have been investigated [23,24]. p-tau-181 had two partition points at the ages of 12 and 60 years. Examination of continuous RI reveals that median p-tau-181 concentrations decreased by an average of 3% per year for participants under 20 years of age, experienced no significant change during middle age, and increased by an average of 3% per year in participants over 60 years old. The increase in p-tau-181 aligns with previous findings showing a positive association of p-tau-181 levels with age in controls without AD from an American cohort [25]. However, to our knowledge, no study has produced age specific reference intervals for p-tau-181. To our knowledge, this is the first study characterizing normative levels of p-tau-181 in pediatric participants.

For NfL, two partition points were determined at the ages of 40 and 60 years. The continuous RI indicated median NfL concentration increased by an average of 3% per year from age 13 to 60 years, increasing to a 4% per year increase after the age of 60 years. NfL RIs have been the most extensively investigated compared to the other biomarkers this study [6,26–28]. Our results align with previous findings that show median levels of NfL increase by approximately 3% per year of age in American adults [26], and that this increase becomes steeper after the age of 50 years [6]. Similar to a study by Benkert et al., which created continuous cut offs for serum NfL in a European and American cohort [6], we also observed a general increase in the 50th and 95th percentiles and increasing separation between the 50th and 95th percentiles as age increases. Our discrete RI data has similar age cut points to a study by Simren et al. that utilized data from multiple European cohorts to create cut-offs for plasma NfL [28]; our upper limits are quite similar. Our discrete RI upper limits are within an average of 4 pg/ml from each other under the age of 60, however, they do differ by up to 27 pg/ml in age bins over 60 years old.

For GFAP, two partition points were determined at the ages of 10 and

60 years. For participants under the age of 20 years, continuous RI indicated that median GFAP concentrations decreased by an average of 7% per year. Above 20 years old, median GFAP concentrations increased by an average of 2% per year, with a 3% yearly increase over 60 years old. Tybirk et al. previously published two studies on discrete and continuous RIs for serum GFAP in Danish pediatric and adult populations [29,30]. Compared to their pediatric cohort, although our raw GFAP concentrations are different in terms of absolute levels, the overall trajectory is similar with an approximately 7% decrease per year of age. For adults, absolute GFAP levels differs between our results and those of Tybirk, yet a similar trajectory and percent change with age for both the median and upper RI are observed.

The results of our study for both NfL and GFAP are also consistent with the findings of another study conducted by our group in which Stukas et al. created pediatric RIs for serum NfL and GFAP using a separate Canadian cohort [31]. Although the studies were conducted using a different matrix, cohort, and assay formulation, our results largely overlap in terms of cut-offs and changes in median concentrations. For GFAP, both studies observed a decrease in GFAP of 7–8% per year between ages 3 to 18 years old. The lower limits of the RI differed by an average of 4 pg/ml and the upper RI differed by an average of 33 pg/ml, with results being closest in the older ages. For NfL, both studies observed an initial yearly decrease of median NfL by 4–6% from 3 to 12 years old, followed by a yearly increase of 1–3% from 13 to 18 years old. The lower limits of the RIs differed by less than 1 pg/ml at every age and the upper limits differed by an average of 1.5 pg/ml.

Some patterns were observed in biomarker trajectories across continuous RIs. P-tau-181 and GFAP had U-shaped curves with high concentrations in pediatric specimens, lowest levels in early adulthood and increasing concentrations in later adulthood. Although higher concentrations in pediatric age ranges were not observed for NfL or the A β 42/40 ratio, this pattern was observed for A β 42 and A β 40 as individual markers (Supplemental Fig. 3), and for NfL in the separate pediatric study conducted by our group [31], where serum NfL levels were highest in 1-year olds, with an average decrease in concentration of 10% per year up to 3 years old. A pattern of high biomarker levels in young children likely reflects neurodevelopment, with brain growth, synaptic pruning and maturing CNS clearance pathways [32] all potentially contributing to high biomarker levels in blood. Although A β 42, A β 40, and p-tau-181 are often reported as AD-specific markers, their high concentrations in pediatric specimens indicate that other factors can influence their plasma concentrations. Except for A β 42/40, all biomarkers increased with age in adulthood. This association with age has been previously reported, however, most previous studies determined only a single cutoff for these biomarkers for all ages [2,33–35]. Because of the dynamic changes noted in biomarker concentrations, especially under 20 and over 60 years old, it is recommended to use continuous rather than discrete RIs for improved interpretation of biomarker concentrations. We provide yearly cut points for each biomarker in Supplemental Tables 1–4 that were derived from the continuous RI regression curve.

4.2. Current and future utilities of biomarkers

Age-specific RIs can help refine reporting and interpreting cut-off values to improve interpretation of laboratory tests in a variety of contexts. Currently, the United States Food and Drug Administration has approved all four biomarkers as breakthrough devices for distinct contexts of use. Though the approvals are on different devices, both plasma p-tau-181 and cerebrospinal fluid A β 42/40 have been approved to aid in the diagnostic evaluation of AD [3,36–38]. Plasma NfL has been clinically validated to monitor disease progression and therapeutic response in patients with multiple sclerosis [6,39]. Plasma GFAP is approved for use in adults with mild traumatic brain injury or concussion to determine the need for head computed tomography (CT) [9]. In terms of future utility of these biomarkers, A β 42/40, p-tau-181, NfL and GFAP

are all of interest for diagnostic and prognostic utility in neurodegenerative diseases [40,41], and NfL and GFAP are also of interest for neurotrauma and other acute neurological insults [8,9,42]. In 2022, the Alzheimer's Association published recommendations for appropriate use of blood biomarkers in AD, which outlined how these biomarkers are currently being used for screening in clinical trials and may be used in memory clinics to assist in diagnosis when confirmed by secondary testing [43]. One of the research priorities identified by the group was to establish cut points for different contexts of use [43], a gap which this study helps fill.

4.3. Generalizability of reference intervals

The RIs generated here can only be used for interpretation of data analyzed using the Simoa HD-X platform. Importantly, compared to other studies mentioned in section 4.1 of this manuscript, our results align well with those of others despite analyses being performed on different cohorts from different countries using different assay lots and instruments. When investigating the applicability of our own results to other HD-X users, we conducted cross lot experiments that showed a bias between 3% and 22% for all analytes investigated in this study. We also found that the use of a different HD-X did not increase the overall bias across the assays used. Over both lots A β markers and GFAP tended to have a lower bias <10%, while NfL and p-tau-181 had larger bias. NfL had a bias of 11% and 21% and p-tau-181 had a bias of 22% and 14% on each lot. The results of this study suggest that there may be a mild bias of 10–20% between results produced on different assay lots for certain analytes. However, use of a different instrument will likely not increase this bias. Although a bias over 20% may be cause for concern, there are ways to mitigate this bias such as harmonizing the data through transformation if lots have a large bias with small limits of agreement. When tracking plasma controls over different lots, we observed that an average of 92% of data points fell in the acceptable ranges defined on the original lot. The only analyte that was found to have less than 80% of data points in the acceptable range was A β 42. Though this also may be some cause for concern, the corresponding A β 42/40 ratio had 100% of data points deemed acceptable, raising confidence in the translation of A β 42/40 over lots. As a mild bias between assay lots is not uncommon in laboratory medicine, the RIs shown in this study be used as a guideline for research-use-only interpretation of biomarker levels, and values that fall within the 95% CI of the RI should still be interpreted with caution.

Future studies will be conducted to incorporate a larger number of samples on multiple lots, and to develop widely available reference materials to facilitate calibration of results by other investigators to increase the utility of using RI to improve biomarker interpretation across the lifespan. Prior to the creation of reference materials for calibration, we recommend transference studies are conducted by other laboratories to improve confidence in use of these RIs. When utilizing the same analyzer and assay type, CLSI EP28-A3c guidelines recommend validation of a reference population to accomplish transference [13]. This may be conducted on a small group of N = 20, that represent the laboratories normative population, where it is expected that 90% of specimens fall within the reference limits. More robust validation can also be conducted with a larger reference population.

4.4. Limitations

One limitation of this study is its reliance on a population-based sample, making these RI representative of normative biomarker concentrations rather than concentrations in healthy persons rigorously screened to exclude neurological disease. Participation in the CHMS is voluntary. CHMS participants are not excluded from the CHMS study based on any neurological indications or cognitive impairment. Nevertheless, participants with cognitive impairment severe enough to limit their ability to provide informed consent would have been ineligible, and participants from whom blood could not safely be drawn or who did

not provide consent for sample storage would not have their samples stored in the Statistics Canada Biobank. Thus, it is possible that underlying neurological disorders in study participants may underlie some of the increased data spread between the median and 95th percentile especially in those over 60 years old for p-tau-181, NfL and GFAP. However, using a normative population also has its advantages, particularly for biomarker studies on acute neurological insults that can happen at any age and can occur with co-morbid neurodegeneration in the elderly. As the biomarkers investigated here have multiple potential contexts of use, having cut-offs that represent normative population levels can improve diagnostic precision in a broader population. A second limitation is that data is not adjusted for survey weights of CHMS, and thus our results are not fully representative of the Canadian population. Previous studies have shown that A β 42/40, p-tau-181, NfL and GFAP levels in blood are modified by kidney function and body mass index, and diabetes and cardiovascular disease may be additional modifiers [12,25]. Future studies will identify variables that modify the concentration of these biomarkers and how to account for them when interpreting RIs. A third limitation is that the Quanterix instruments are not clinically approved, and the RI data presented here cannot be used to aid biomarker interpretation for assays performed on other instruments. Finally, reference materials will be required to calibrate future results to the RIs developed here, a limitation that also exists for all published RI studies on these biomarkers.

5. Conclusion

Discrete and continuous RIs for neurological plasma biomarkers will help refine normative cut-offs that can be used for interpretation in research settings to improve the precision of interpreting biomarker levels. These RIs also help us to understand the dynamic changes these biomarkers display across the lifespan.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clinbiochem.2023.110680>.

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