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ALTERNATE FORMAT RESEARCH ARTICLE

Characterization of cerebrospinal fluid biomarkers associated with neurodegenerative diseases in healthy cynomolgus and rhesus macaque monkeys

Emma L. Robertson[1] | **Susan E. Boehnke**[1,2] | **Natalia de M. Lyra e Silva**[1,2] | **Brittney Armitage-Brown**[1,3] | **Andrew Winterborn**[3] | **Douglas J. Cook**[1,4] | **Fernanda G. De Felice**[1,2,5,6,7] | **Douglas P. Munoz**[1,2]

[1]Centre for Neuroscience Studies, Queen's University, Kingston, Ontario, Canada

[2]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario, Canada

[3]Animal Care Services, Queen's University, Kingston, Ontario, Canada

[4]Department of Surgery, Kingston Health Sciences Centre, Kingston, Ontario, Canada

[5]Institute of Medical Biochemistry Leopoldo de Meis, Federal University of Rio de Janeiro, Cidade Universitaria – Rio de Janeiro, Rio de Janeiro, Brazil

[6]D'OR Institute for Research and Education, Rio de Janeiro, Brazil

[7]Department of Psychiatry, Providence Care Hospital, Kingston, Ontario, Canada

Correspondence
Susan Boehnke, Centre for Neuroscience Studies, Queen's University, 2nd Floor Botterell Hall, Kingston, ON K7L 3N6, Canada.
E-mail: susan.boehnke@queensu.ca

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Abstract

Monkeys are becoming important translational models of neurodegenerative disease. To facilitate model development, we measured cerebrospinal fluid (CSF) concentrations of key biomarkers in healthy male and female cynomolgus and rhesus macaques. Amyloid beta (Aβ40, Aβ42), tau (total tau [t-tau], phosphorylated tau [pThr181]), and neurofilament light (NfL) concentrations were measured in CSF of 82 laboratory-housed, experimentally naïve cynomolgus (n = 33) and rhesus (n = 49) macaques. Aβ40 and Aβ42 were significantly higher in rhesus, and female rhesus were higher than males. NfL and t-tau were higher in males, and NfL was higher in rhesus macaques. p-tau was not affected by species or sex. We also examined whether sample location (lumbar or cisterna puncture) affected concentrations. Sample acquisition site only affected NfL, which was higher in CSF from lumbar puncture compared to cisterna magna puncture. Establishing normative biomarker values for laboratory-housed macaque monkeys provides an important resource by which to compare to monkey models of neurodegenerative diseases.

KEYWORDS
Alzheimer's disease, amyloid beta, biomarkers, cerebrospinal fluid, cynomolgus macaque, neurofilament light, non-human primate, rhesus macaque, tau

1 | NARRATIVE

1.1 | Introduction

Cerebrospinal fluid (CSF) biomarkers are increasingly used to diagnose and track progression and evaluate treatment of various human neurological disorders. For example, core CSF biomarkers involved with Alzheimer's disease (AD) include amyloid beta 1-40 (Aβ40), amyloid beta 1-42 (Aβ42), total tau (t-tau), and phosphorylated tau (p-tau).[1,2] Additionally, neurofilament light (NfL), a protein providing structural support to the neural cytoskeleton, has been identified as a marker of general axonal degradation and is also being investigated in a variety of neurodegenerative disorders such as AD,[3–7] Parkinson's disease,[5,8,9] multiple sclerosis,[10–13] and Huntington's disease.[3,14]

Old-world monkeys, such as cynomolgus (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*), are important models for human neurological disorders due to their similarities in brain architecture, anatomy, physiology, and behavior.[15,16] As many neurodegenerative disorders involve impairment of higher cognitive functions, monkeys have the potential to advance our knowledge of these disorders and validate treatments. Several non-human primate (NHP) models of AD and aging are being developed,[17–26] but only a subset have characterized biomarker levels in CSF and how they change in the disease model.[17–19,21–23,25]

Macaques reach sexual maturity between 3 and 4 years in females[27,28] and 3 to 6 years in males[28,29] and maturity is typically earlier in captivity than in the wild.[30,31] They enter old age by 20 to 25 years with a lifespan of 30 to 40 years in captivity, but lifespan is far shorter in the wild.[32] Natural aging in NHPs is associated with amyloid plaque deposition and p-tau accumulation in the brain parenchyma in some animals older than 20 years,[25,33–38] with some studies showing age-associated changes in CSF biomarkers.[25,33] Despite this progress in development of NHP models, little is known about the normative range of CSF concentrations of Aβ, tau, and NfL in healthy young NHPs in laboratory housing conditions. While natural aging in NHPs is a robust model exhibiting features similar to human AD (see Frye et al.[38] for a review), aging animals for > 20 years when AD-related pathology becomes evident is inefficient for mass preclinical drug testing—housing and maintenance of NHPs is expensive and not all will go on to develop the hallmarks of AD. Therefore, induced models being developed[26] (e.g., Beckman et al.[23] and Forny-Germano et al.[24]) may provide another option and could use younger animals. Given the limited number of animals often used in many NHP studies, it is difficult to interpret pathological CSF changes in models of neurodegenerative disease without having good reference ranges with which to compare. It is also unknown how factors such as species and sex affect biomarker levels in NHPs, independent of neurological disease.

Methodological issues may also affect biomarker concentrations in NHPs. One example is sample acquisition site. Lumbar punctures (LPs) can be performed in NHPs to collect CSF, providing translation to human clinical work, which typically uses LPs. However, it is also common for CSF to be obtained from a cisterna magna puncture in NHPs. Therefore, it is important to compare CSF samples acquired from these two sites. For example, we have previously demonstrated that LPs, but not cisterna magna punctures, significantly elevate NfL for several weeks in NHPs possibly due to damage of the cauda equina (see Boehnke et al.[39]).

To address these knowledge gaps, we characterized concentrations of Aβ40, Aβ42, t-tau, p-tau (pThr181), and NfL in the CSF of a laboratory-housed colony (n = 82) of experimentally naïve cynomolgus and rhesus macaques of both sexes in an age range that would likely be used for inducible models of disease (e.g., adolescent/young adult). For all animals, CSF was obtained by LP, and in a subset of animals we also obtained CSF via cisterna magna puncture (see Detailed Methods section). To measure concentrations of Aβ40, Aβ42, p-tau (pThr181), and t-tau, a MILLIPLEX Human Amyloid Beta Tau Magnetic Bead Panel was

RESEARCH IN CONTEXT

1. **Systematic review**: We reviewed reports characterizing CSF biomarkers of neurodegenerative diseases in non-human primates, an increasingly important model of disease - revealing that studies with laboratory housed macaque monkeys were of small sample size, with a paucity of data about how biomarkers varied as a function of species, sex, age, and site of acquisition.
2. **Interpretation**: To address this gap, we collected CSF from 82 experimentally naïve laboratory housed male and female macaques of two species and measured Aβ40, Aβ42, tTau, pTau, and NfL. In addition to providing normative statistics for concentrations of these biomarkers, we described various species and sex differences.
3. **Future directions**: Establishing normative values of biomarkers is an important step to the efficient development of cynomolgus and rhesus macaques as models of neurodegenerative disorders, including Alzheimer's disease and tauopathies. Reference values reduce the need for large control groups by which to compare with disease model animals.

HIGHLIGHTS

- Aβ40 and Aβ42 concentrations were higher in rhesus vs. cynomolgus macaques
- Aβ40 and Aβ42 concentrations were higher in female vs. male rhesus macaques
- tTau and NfL concentrations were significantly higher in males
- pTau was not affected by species or sex
- NfL concentrations from the cisterna area were lower when compared to lumbar CSF

used, while NfL concentration was measured using the Uman sandwich enzyme-linked immunosorbent assay (ELISA). This choice of measurement platform provides a way to optimize CSF usage because the volume of CSF that can be acquired from NHPs is limited (1 to 2 ml). It is also a potential limitation as few studies in NHPs have used multiplex assays.

1.2 | Species and sex differences in biomarker concentrations

Concentrations of Aβ40, Aβ42, and NfL were significantly higher in rhesus macaques compared to cynomolgus macaques (Figures 1A, 1B, 1E). t-tau and NfL concentrations were significantly higher in males compared to females (Figures 1C, 1E). There were interactions between species and sex for both Aβ40 and Aβ42, with the species difference being greater for females than for males (Figure 1A-B). For p-tau, there was no effect of species or sex (Figure 1D). Summary statistics for all groups are available in Table 1.

As would be expected, strong correlations were observed between Aβ40 and Aβ42 and between t-tau and p-tau, but we observed no other correlations between biomarkers (Figure 2, see full correlation matrix in Table 2). Finally, CSF samples from cisterna magna puncture were lower in NfL compared to CSF obtained from LP (Figure 3E-F). No other biomarker was affected by sampling location (Figure 3A-D).

Overall, these data help establish normative values for these biomarkers in two commonly used species of macaques and provide reference points for efficient use and development of primate models of neurodegenerative diseases. Normative ranges are crucial for interpretation of clinical biomarkers—without them, interpretation of values from a given individual is difficult. This is particularly important in NHP research, for which the number of subjects available for studies is often limited, so having a reference database may help minimize the number of animals required in control groups for such studies. This is also an issue in human clinical research in which obtaining proper control groups can be difficult. There remain challenges, however, such as interlab differences in collection and storage methods and assays used. As NHP model development expands, we recommend standardization in collection protocols and analysis methods to allow for better comparison across studies. Such challenges were met for biomarker measurement in humans by development of rigorous standards,[40] a strategy that NHP research may benefit from moving forward. We used a multiplex assay for Aβ and tau biomarkers, as it has the advantage of measuring all analytes with only a small amount of CSF. This solves a challenge in NHP research, as only 1 to 2 ml (sometimes less) can be collected during a given LP.

1.3 | Translation to human studies

In humans, concentrations of Aβ40 have been found in the CSF of cognitively normal adults with mean values ranging from 4003[41] to 8958 pg/ml.[42] Our mean values reported in cynomolgus (1603 pg/ml) and rhesus (2700 pg/ml) macaques were somewhat lower, which could be due to assay, age, or species-specific differences (see Table 1 for a summary of biomarker values). For Aβ42 concentrations, a multicenter sample of cognitively normal human subjects reported values of Aβ42 ranging from 200 to 1250 pg/ml across the age span of 40 to 80+ years.[43] The mean values we report for both cynomolgus (290 pg/ml) and rhesus (504 pg/ml) macaques were in this range.

For t-tau, humans aged 40 to 80+ ranged from 50 to 1250 pg/ml.[43] The mean t-tau concentrations for cynomolgus (224 pg/ml) and rhesus (281 pg/ml) macaques in our sample fell in this human range. Our observation of higher t-tau in male macaques is not consistent with reports in humans, which have observed no consistent sex difference.[44] For p-tau, concentrations in humans ranged from 10 to 150 pg/ml.[42] Again, our cynomolgus (37 pg/ml) and rhesus (37 pg/ml) macaque mean values were in the human range, indicating CSF tau values are comparable across species. We also saw no sex difference in p-tau, consistent with human studies.[44]

Finally, median values for NfL in CSF acquired from healthy young individuals aged <30 years were 187 pg/ml,[45] which would be most comparable to NfL values obtained for our young adult NHPs (cynomolgus: 275.52 pg/ml; rhesus: 476.02 pg/ml). Furthermore, our observation of higher NfL concentrations in the CSF of male macaques is consistent with that observed in healthy controls in several human studies.[3,44,46]

Due to the current colony demographics available to us, CSF samples were collected in NHPs 12 years and younger (Figure 4A). These were adolescents and young adults, with most having reached sexual maturity. While we saw some decrease in Aβ40 and Aβ42 with age within the colony, we had limited animals of older age, and the ranges of ages between male and female cynomolgus and rhesus macaques were inconsistent (Figure 4). We were not expecting age effects across this narrow range, and used age as a covariate when examining sex and species effects. With this caveat, we found that Aβ42 biomarkers decreased with age, which is consistent with human data,[47,48]. Our measured concentrations of Aβ42 (and p-tau) were similar to those reported in studies using African green monkeys[25] and they also found a negative correlation of Aβ42 with age in an older age group (8 to 23 years). Given Aβ plaque deposition is not typically observed until macaques are older than 20 years, it is unknown why Aβ biomarkers decreased with age in our younger cohort. Therefore, these results should be interpreted with caution given our lack of older animals. While increased t-tau levels have been found in aged humans,[49,50] we did not observe any significant effect of age across our colony. This probably reflects our relatively young colony, and so age effects on tau biomarkers would require further investigation. In addition, increased levels of CSF NfL in healthy individuals have been found in humans.[45] We observed no significant correlation of NfL with age, which may also suggest we would require more aged NHPs to see this effect.

In conclusion, we characterized Aβ40, Aβ42, t-tau, p-tau, and NfL biomarkers in CSF collected from a large colony of cynomolgus and rhesus macaques as a function of species, sex, and age, providing the largest reference values for laboratory-housed animals of these species to date. Trends observed, such as a decrease in Aβ42 with age

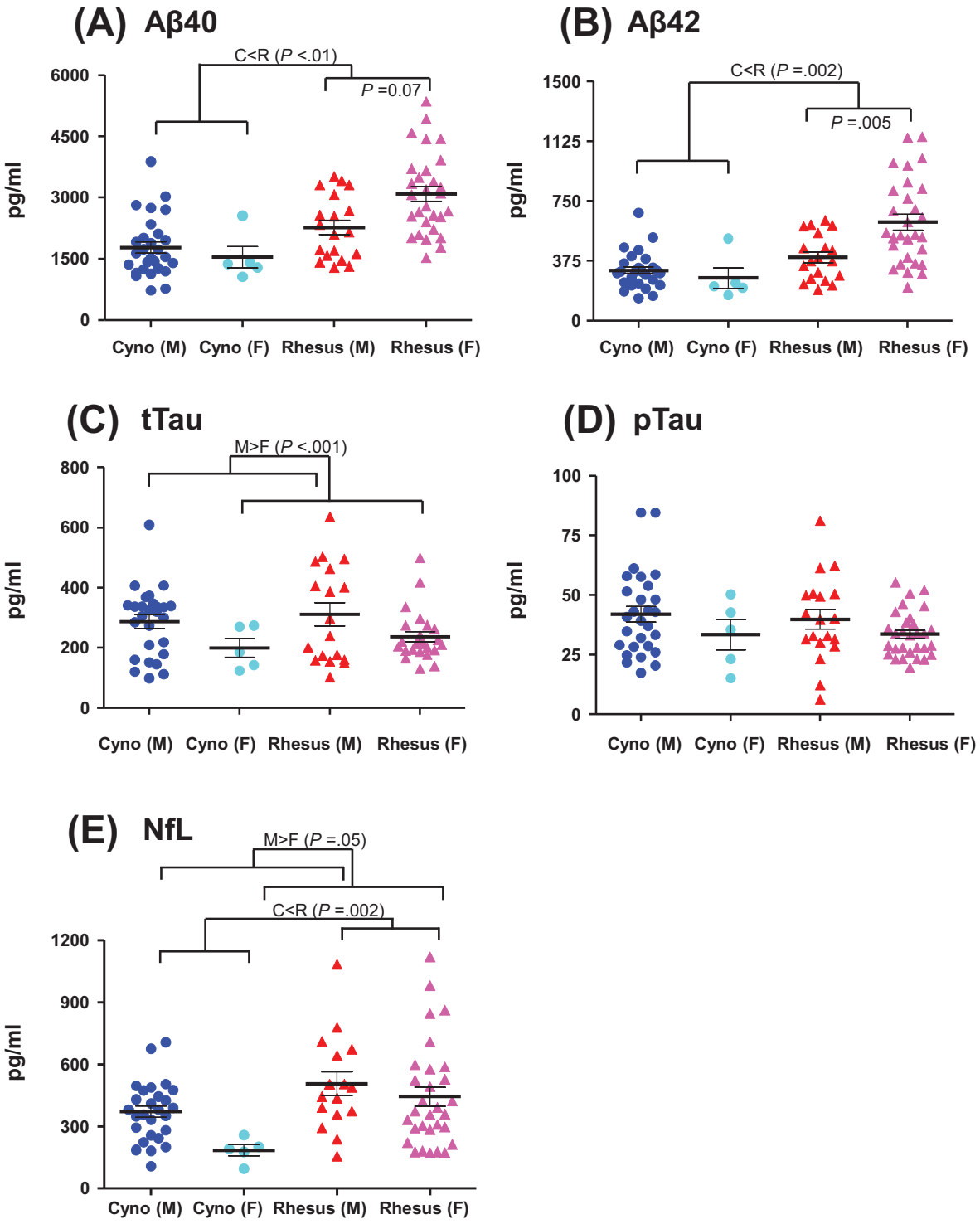

FIGURE 1 Species and sex comparison for cerebrospinal fluid (CSF) amyloid beta (Aβ), tau, and neurofilament light (NfL) biomarkers. CSF Aβ, tau, and NfL biomarkers measured in CSF samples obtained by a lumbar puncture (LP) in male and female cynomolgus (Cyno) (C) and rhesus macaques (R). Two-way analyses of covariance indicate that (A) Aβ40 concentrations were higher in rhesus macaques, and the female rhesus were higher than male rhesus. B, Aβ42 concentrations were higher in rhesus macaques compared to cynomolgus macaques. They also tended to be higher in female rhesus compared to male rhesus, but that trend was not significant ($P = .07$). C, Total tau (tTau) concentrations were higher for males. D, Phosphorylated tau (pTau) concentrations did not differ between groups. E, NfL concentrations were higher in rhesus macaques, and males were higher than females

TABLE 1 Means, age-adjusted means, standard deviations, and standard errors for Aβ40, Aβ42, t-tau, p-tau, p-tau/t-tau ratio, and NfL concentration in cynomolgus and rhesus macaques, with sample human data from the literature provided for comparison

		Cynomolgus		Rhesus		Human
		Male	Female	Male	Female	From published control data
Aβ40 pg/mL	*N*	28	5	19	29	4003[40]–8959 pg/ml[41]
	M	1779.27	1539.33	2271.48	3083.87	
	(*SD*)	(721.64)	(585.17)	(774.97)	(1006.50)	
	M_{adj}	1813.91	1391.52	2426.73	2974.20	
	(*SE*)	(159.18)	(384.73)	(215.35)	(169.80)	
Aβ42 pg/mL	*N*	28	5	19	29	200–1250 pg/ml[42]
	M	317.29	266.68	397.60	608.89	
	(*SD*)	(125.17)	(141.25)	(143.89)	(274.60)	
	M_{adj}	318.27	262.50	401.99	605.78	
	(*SE*)	(37.82)	(91.41)	(51.17)	(40.37)	
t-tau pg/mL	*N*	27	5	18	25	50–1250 pg/ml[42]
	M	286.71	199.37	310.71	237.03	
	(*SD*)	(114.28)	(70.09)	(162.23)	(81.99)	
	M_{adj}	293.85	156.23	355.69	205.56	
	(*SE*)	(21.24)	(50.90)	(29.42)	(24.04)	
p-tau pg/mL	*N*	28	5	19	30	10–150 pg/ml[42]
	M	40.93	33.43	39.87	33.67	
	(*SD*)	(17.36)	(14.21)	(17.82)	(9.47)	
	M_{adj}	41.29	31.94	41.439	32.60	
	(*SE*)	(2.84)	(6.87)	(3.85)	(2.98)	
p-tau/t-tau ratio	*N*	27	5	18	25	0.1–0.4[52,53]
	M	0.18	0.16	0.15	0.15	
	(*SD*)	(0.14)	(0.03)	(0.09)	(0.04)	
	M_{adj}	0.18	0.18	0.13	0.16	
	(*SE*)	(0.02)	(0.04)	(0.03)	(0.02)	
NfL pg/mL	*N*	27	5	16	30	187 pg/mL[45]
	M	372.22	184.67	505.42	443.19	humans <30 years
	(*SD*)	(142.15)	(58.39)	(231.01)	(251.74)	
	M_{adj}	375.17	175.87	515.08	436.96	
	(*SE*)	(40.83)	(96.21)	(58.30)	(41.62)	

Abbreviations: Aβ, amyloid beta; CSF, cerebrospinal fluid; NfL, neurofilament light chain; p-tau, phosphorylated tau; SD, standard deviation; SE, standard error; t-tau, total tau.

TABLE 2 Spearman's rho correlation matrix of all biomarkers measured[a]

	Aβ40	Aβ42	t-tau	p-tau	NfL
Aβ40	1	-	-	-	-
Aβ42	**0.96 (*P* < .001)**	1	-	-	-
t-tau	−0.03	−0.01	1	-	-
p-tau	0.08	0.11	**0.40 (*P* < .001)**	1	-
NfL	0.11	0.12	0.09	−0.01	1

[a]Corrected for multiple comparisons the threshold *P*-value was .005.
Notes: Significant correlations were only seen between the pairs of Aβ and tau biomarkers (in bold). All other correlations had associated *P*-values > .05.
Abbreviations: Aβ, amyloid beta; NfL, neurofilament light chain; p-tau, phosphorylated tau; t-tau, total tau.

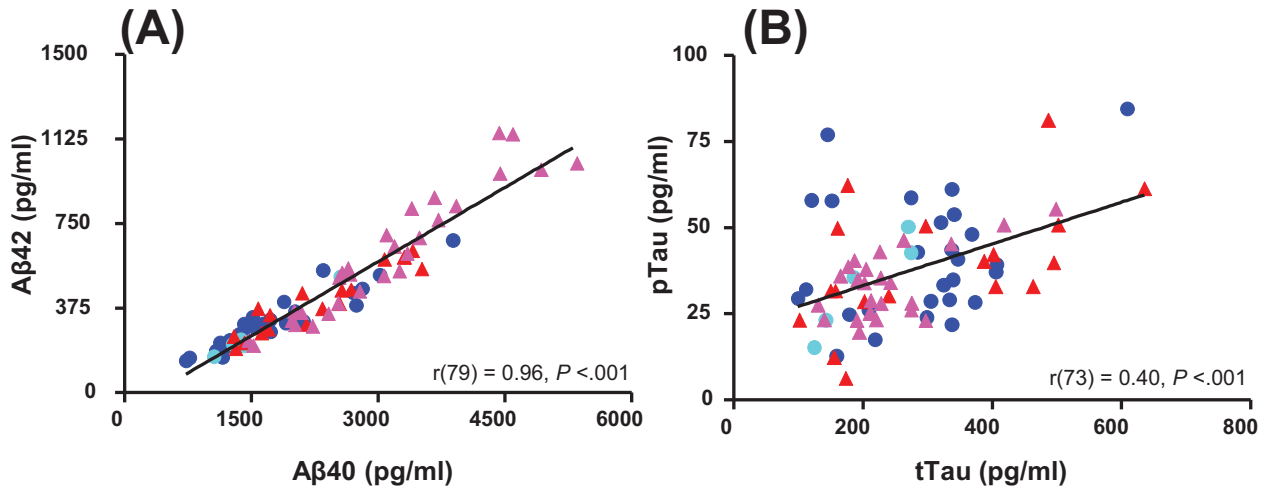

FIGURE 2 Amyloid beta (Aβ) and tau biomarkers are correlated. Each point represents values from a single animal. A, Aβ40 and Aβ42 have a significant positive correlation. Spearman's rho r (79) = 0.96, $P < .001$. B, Total tau (tTau) and phosphorylated tau (pTau) have a significant positive correlation. Spearman's rho r (73) = 0.40, $P < .001$. No other biomarker combinations were correlated (all P's > .09)

and higher NfL concentrations in males, were concordant with observations in humans and support the validity of macaque monkeys as a model for human neurodegenerative diseases. Overall, these reference values will provide useful benchmarks by which to compare CSF from primate models of neurological disorders generated using these species.

2 | CONSOLIDATED RESULTS AND STUDY DESIGN

2.1 | Design

We obtained CSF from 82 cynomolgus and rhesus macaques with the goal of obtaining reference ranges for core biomarkers of neurodegenerative disease for NHPs. LPs were performed, and CSF was allowed to drip into low retention polypropylene tubes and then was aliquoted into smaller tubes for biobanking storage (see Detailed Methods section). In a subset of these animals for which we had obtained CSF via LP, we obtained CSF on another occasion via cisterna magna puncture to determine whether biomarker concentrations were affected by the sampling location. We analyzed Aβ and tau biomarkers in CSF samples using a multiplex assay (see Detailed Methods). After quantification of biomarker concentrations (see Detailed Methods), we wanted to determine whether any of the biomarkers varied across our relatively narrow range of ages (2 to 12 years) represented within the colony (Figure 4A-F). When corrected for multiple comparisons and collapsed across species and sex, there was a negative correlation in Aβ40 and Aβ42 with age (Figure 4B, C; r = 0.28, $P = .01$; r = 0.33, $P < .01$). No significant correlation of t-tau, p-tau, or NfL was found with age (Figure 4D-F; all $P > .05$). A two-way analysis of covariance (ANCOVA) was conducted on each of the biomarkers to assess the role of species and sex. The age range was small, thus we removed age effects by adding age as a covariate in the analysis. The means, age-adjusted means, standard deviations, and standard errors of each biomarker are presented in Table 1. The raw values for each animal are plotted separated by species and sex for each biomarker in Figure 1A-E.

2.2 | Results

2.2.1 | Aβ40 and AB42

For both Aβ40 and Aβ42 (Figure 1A, B), there was a main effect of species (Aβ40: F [1, 76] = 20.53, $P < .001$, $\eta^2 = 0.21$; Aβ42: F [1, 76] = 13.748, $P < .001$, $\eta^2 = 0.15$) with rhesus macaques (Aβ40: 2700.46 pg/ml; Aβ42: 503.89 pg/ml) having higher concentrations than cynomolgus macaques (Aβ40: 1602.72 pg/ml; Aβ42: 290.43 pg/ml). There was no main effect of sex in either biomarker ($P = .82$; $P = .26$) but there was a statistically significant interaction between species and sex (Aβ40: F [1, 76] = 4.14, $P = .045$, $\eta^2 = 0.052$; Aβ42: F [1, 76] = 5.252, $P = .025$, $\eta^2 = 0.065$). Analysis of simple effects indicated that the effect of species was greater for females (Aβ40: rhesus 2974.2 pg/ml vs. cynomolgus 1391.52 pg/ml; $P < .001$, Aβ42: rhesus 605.78 pg/ml vs. cynomolgus 262.50 pg/ml; $P < .001$) than males (Aβ40 rhesus 2426.73 pg/ml vs. cynomolgus 1813.91 pg/mlL; $P = .02$, Aβ42: rhesus 401.99 pg/ml vs. cynomolgus 318.37 pg/ml; $P = .179$). Analyzed by sex, female rhesus had significantly higher levels of Aβ42 ($P = 0.005$) and marginally higher levels of Aβ40 ($P = .07$) compared to male rhesus. There was no sex difference for cynomolgus macaques.

2.2.2 | t-tau and p-tau

For t-tau (Figure 1C), there was a main effect of sex (F [1, 70] = 14.88, $P < .001$, $\eta^2 = 0.175$), with males (324.77 pg/ml) having higher concentrations than females (180.89 pg/ml). There was no significant main effect of species (F [1, 70] = 2.911, $P = .092$, $\eta^2 = 0.040$) or an interaction

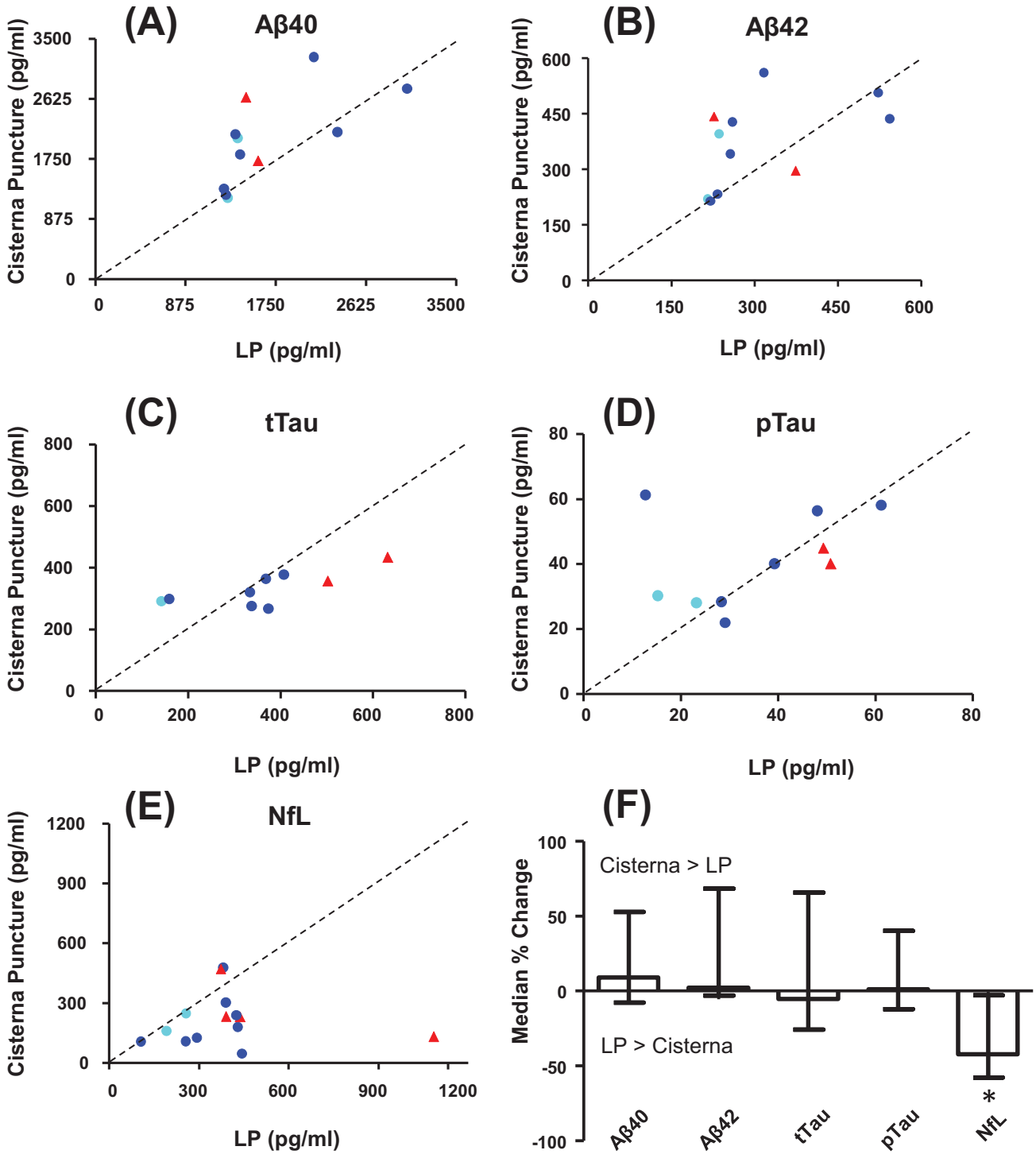

FIGURE 3 Comparison of biomarkers taken from non-human primates (NHPs) that had both lumbar punctures (LPs) and cisterna magna punctures. A subset of NHPs from the colony had both LPs and cisterna punctures conducted at least 5 months apart. Scatterplots of values measured from cerebrospinal fluid (CSF) obtained from each location are plotted for each biomarker: (A) amyloid beta (Aβ40) (B) Aβ42 (C) total tau (tTau), (D) phosphorylated tau (pTau), and (E) neurofilament light chain (NfL). Each dot represents the values from LPs and cisterna punctures from a single animal. The dotted line is the line of unity. Colors are as defined in Figure 1, note there are no data from female rhesus. F, The median percent change ([lumbar puncture – cisterna puncture]/cisterna puncture × 100) is plotted for each biomarker with the interquartile range. NfL in CSF taken from the cisterna magna area was significantly lower than of CSF taken from the lumbar area (Mann Whitney $U = 45.5$, $z = -2.779$, $P = .004$). All other P's > .05

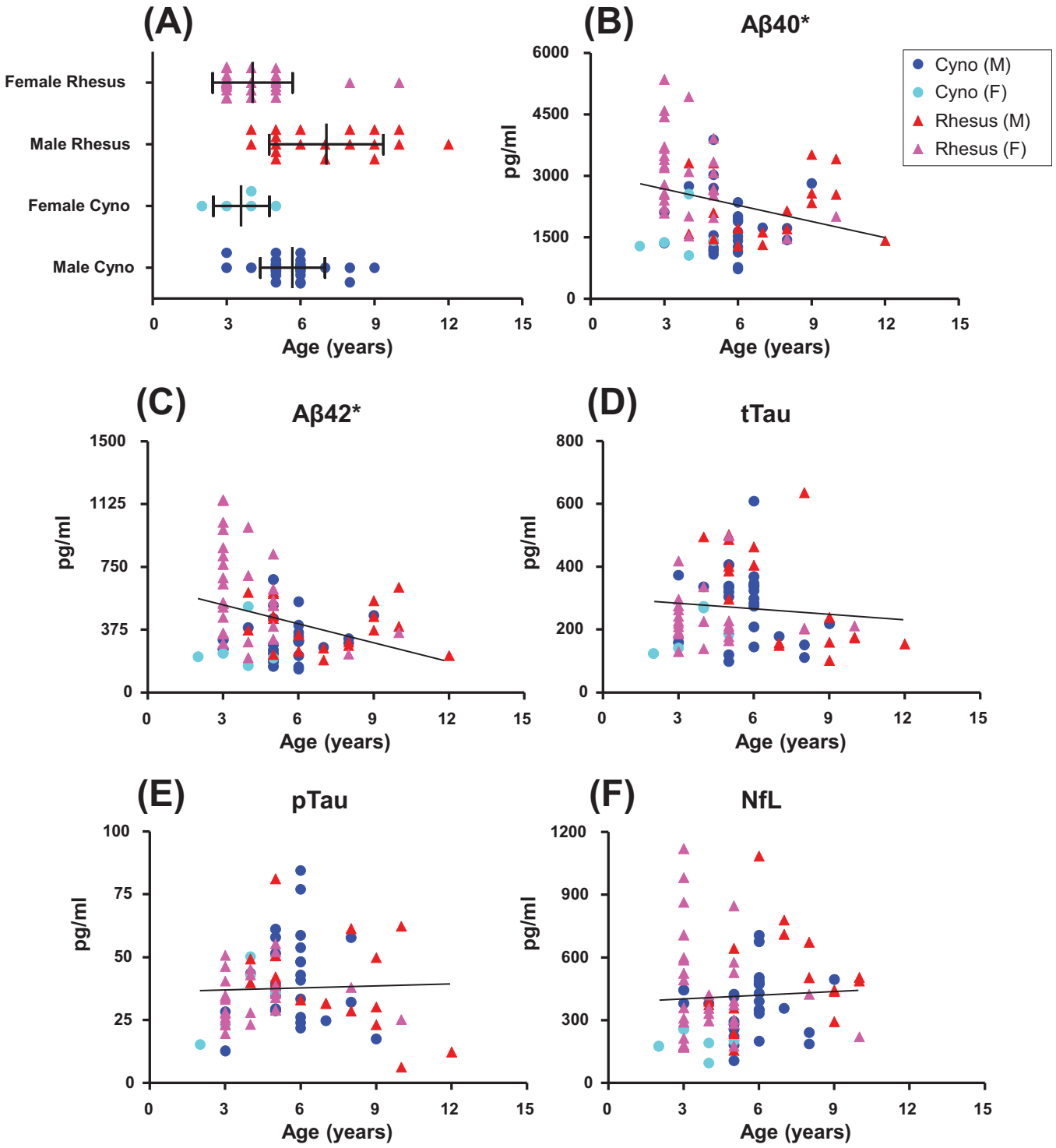

FIGURE 4 Colony demographics and correlations of biomarkers with age. A, Cerebrospinal fluid (CSF) from 82 animals was analyzed: 28 male and five female cynomolgus (Cyno) macaques (*Macaca fascicularis*, ages: 2–9 years, body weight: 3.4–10.7 kg) and 19 male and 30 female rhesus macaques (*Macaca mulatta*, ages: 3–12 years, body weight: 5.4–18 kg). All animals were experimentally naïve. B, Amyloid beta (Aβ40) was negatively correlated with age ($r = 0.28$, $P = .01$). C, Aβ42 was negatively correlated with age ($r = 0.33$, $P < .01$). No correlation with age (all P's> .05) was observed for (D) total tau (tTau), (E) phosphorylated tau (pTau), or (F) neurofilament light chain (NfL)

(F [1, 70] = 0.038, $P = .845$, $\eta^2 = 0.001$). For p-tau (Figure 1D) there was no main effect of species or sex and there was no interaction (all P values > .05). We further analyzed the ratio of p-tau/t-tau and observed no main effect of species or sex, and no interaction (all P's > .05, see Table 1).

2.2.3 | NfL

For NfL, there was a main effect of species (F [1, 73] = 10.64, $P = .002$, $\eta^2 = 0.127$) with rhesus (476.02 pg/ml) having higher concentrations than cynomolgus (275.52 pg/ml). There was also a main effect of sex (F [1, 73] = 3.85, $P = .05$, $\eta^2 = 0.050$) with males (445.12 pg/ml) having higher values than females (306.42 pg/ml). There was no interaction between species and sex (F [1, 73] = 1.006, $P = .319$, $\eta^2 = 0.014$).

2.2.4 | The effect of housing status on biomarkers

We did not systematically study the effect of housing status in this study, and one limitation is that more females were group housed than males. Further, housing status prior to arriving in our colony was typically unknown. With those caveats noted, when collapsed across the colony, group housing was associated with higher Aβ40 ($P = .001$) and Aβ42 ($P < .001$) concentrations, which appears to be driven by the fact that all (but one) females were group housed, and females had higher levels of Aβ biomarkers than males. There was a more even split in housing status for males, and there was no effect of housing status on Aβ biomarkers for males, which suggests that our sex difference for Aβ may not be simply due to the group housing of females. Group-housed males had somewhat elevated levels of t-tau (*t*-test, t [43] = 2.034, $P = .048$) and NfL (*t*-test, t [41] = 2.322, $P = .025$). Given that we did not systematically examine the effect of housing status in a controlled study, we are hesitant to draw any conclusions from these results.

2.2.5 | Effect of CSF acquisition site on biomarkers

In a subset of the NHPs (n = 16) for whom CSF had been obtained by LP, CSF was collected through the cisterna magna (see Detailed Methods). In a series of scatterplots, biomarker values from CSF obtained by LP are plotted against those obtained by cisterna puncture (Figure 3A-E). In Figure 3F, the median percent change ([lumbar puncture – cisterna puncture]/cisterna puncture × 100) is plotted because the difference scores were not normally distributed for Aβ42, t-tau, and p-tau (Kolmogorov–Smirnov test = Aβ40: D [11] = 0.21, $P > .05$; Aβ42: D [11] = 0.26, $P = .03$; t-tau: D [8] = 0.36, $P < .005$; p-tau: D [10] = 0.38, $P > .005$; NfL: D [14] = 0.14, $P > .05$). Using non-parametric statistics, only NfL was significantly lower in cisterna samples (median percent change: −42%, interquartile range [IQR] = 51%; Mann Whitney $U = 45.5$, z = −2.779, $P = .004$). This result is consistent with our previous report,[39] but with an increased sample size. For Aβ40, the percent change between LP and cisterna puncture was not significant with non-parametric statistics (Mann Whitney $U = 46$, z = −0.919, $P > .05$). However, given Aβ40 and NfL percent change values were normally distributed, we analyzed them with a single-sample *t*-test. There was a small effect whereby the percent change in Aβ40 was significantly greater than zero (cisterna puncture >LP; single sample *t*-test, t [10] = 2.410, $P = .037$) and the percent change in NfL was significantly lower than zero (cisterna puncture <LP; single sample *t*-test, t [13] = 3.439, $P < .005$). While we are confident in the result for NfL, the Aβ40 effect should be replicated in a larger sample size.

2.2.6 | Aβ and tau biomarker correlations

Aβ40 and Aβ42 were positively correlated with each other (r [79] = 0.956, $P < .001$; Figure 2A). This tight correlation reveals that although there was considerable between-animal variability in levels of Aβ40 and Aβ42, within-animal variability was much smaller. t-tau and p-tau were also positively correlated (r [73] = 0.404, $P < .001$; Figure 4B). No other biomarker correlations reached significance (see Table 2 for correlation matrix).

3 | DETAILED METHODS

3.1 | Subjects

All monkeys were housed at the Centre for Neuroscience Studies at Queen's University (Kingston, Ontario, Canada) under the care of a lab animal technician and the institute veterinarian. All procedures were approved by the Queen's University Animal Care Committee and were in full compliance with the Canadian Council on Animal Care (Animal Care Protocol Munoz, 2011-039-Or).

LPs were performed on a total of 82 animals (Figure 4A): 19 male and 30 female rhesus macaques (*Macaca mulatta*, ages: 3–12 years, body weight: 5.4–18 kg) and 28 male and 5 female cynomolgus macaques (*Macaca fascicularis*, ages: 2–9 years, body weight: 3.4–10.7 kg). Animals were housed in a laboratory setting in small groups (n = 49) or individually (n = 33), and kept on a 12:12-hour light:dark cycle starting at 7 a.m. They were fed a standard diet of high-protein or high-fiber monkey chow and supplemented with fresh fruit and vegetables. On CSF collection day, animals were fasted with access to water ad libitum. Daily enrichment was provided through foraging, puzzle toys, swings, ropes, perches, mirrors, etc. All animals were experimentally naïve when the CSF samples were obtained except for three animals for which we were unable to acquire CSF on the first attempt. On a separate day, a second attempt was required to obtain a sample.

Cisterna magna punctures were performed in 16 of the 82 animals for which CSF was acquired by LP (seven male and three female cynomolgus macaques [ages: 2–6 years, body weight: 3.4–10.7 kg]) and six male rhesus macaques (ages: 4–9 years, body weight: 5.4–18 kg). All 16 samples were analyzed for NfL and 11 samples were analyzed for

Aβ40, Aβ42, t-tau, and p-tau. The cisterna puncture was performed on a separate occasion at least 5 months before (n = 4) or after a LP (n = 12).

3.2 | CSF collection and storage

CSF was collected as previously described.[39] A trained veterinarian or veterinary technician performed LPs and cisterna magna punctures between 9 a.m. and 1 p.m. Animals were sedated with ketamine (5–15 mg/kg, intramuscular) and masked briefly, only if required, with isoflurane (1% to 3%) and oxygen (2%) to minimize movement. If additional procedures were planned (e.g., magnetic resonance imaging, surgery), anesthesia was induced with ketamine (10 mg/kg, intramuscular) and diazepam (5 mg/kg, intramuscular). Glycopyrrolo adverse neurological effects were observate (0.013 mg/kg, intramuscular) was given, and the animal was intubated. Anesthesia was maintained using isoflurane (1% to 3%) and oxygen (2%). In these relatively rare cases (n = 3), CSF sampling was the first procedure conducted to minimize any isoflurane anesthetic effects.

For LP sampling, animals were placed in lateral recumbency and the superior iliac crest was palpated. The lumbar area was shaved and cleaned using chlorhexidine, alcohol, and betadine. Depending on animal size, a 20 or 22 g Quincke spinal needle (BD) was inserted into the intrathecal space between L4/5, or in some cases, L3/4 or L5/6. CSF was allowed to drip by gravity into a sterile 1.5 mL polypropylene Eppendorf tube (Axygen Maxymum Recovery) and then immediately placed on ice.

For cisterna magna sampling, animals were placed in lateral or sternal recumbency and the area between the occipital protuberance to the third cervical vertebrae was shaved and cleaned. A 23 g needle connected to a 1 ml (BD Luer-Lok) polypropylene syringe was inserted into the midline of the neck, with the tip of the needle pointing toward the nose.[51] No adverse neurological effects were observed after LP or cisterna magna punctures.

If the CSF sample was visibly contaminated with blood, it was centrifuged at 1800 g for 10 minutes at 4°C to remove blood cells. Samples were stored as 120 ul aliquots within 30 minutes of collection in 0.6 ml sterile polypropylene tubes (Axygen Maxymum Recovery) at −80°C. Prior to biochemical analysis, samples did not undergo any freeze–thaw cycles.

3.3 | CSF biomarker analysis

CSF samples were thawed in a biological safety cabinet just before analysis. To measure Aβ40, Aβ42, p-tau (pThr181), and t-tau, a MILLIPLEX Human Amyloid Beta Tau Magnetic Bead Panel (HNABTMAG-68K, EMD Millipore) was completed on a Biorad Luminex platform according to the manufacturer's instructions. NfL was measured using a commercial sandwich ELISA (NF-light ELISA kit, UmanDiagnostics) performed according to the manufacturer's instructions. Reference samples were included on all multiplex and NfL ELISA plates for comparison across plates. Within-plate and interplate coefficients of variation were <15% for the multiplex; and <10% for NfL.

3.4 | Exclusion criteria

Exclusion criteria included: (1) values below detection limits; (2) for NfL analysis, animals that received a recent LP, as this procedure was shown to elevate NfL (but not other Aβ/tau biomarkers) for several weeks;[39] (3) values that were more than four standard deviations above the mean and flagged by Grubb's test as outliers (P <.01). For CSF collected via LP, one female rhesus was removed from Aβ40/Aβ42. One male cynomolgus, one male rhesus, and five female rhesus macaques were removed from t-tau. One male cynomolgus and three male rhesus macaques were removed from NfL. For CSF collected via cisterna magna, one male and female cynomolgus macaque were removed from t-tau, one male cynomolgus macaque was removed from p-tau, and another was removed from NfL.

3.5 | Data/statistical analysis

Statistical analyses were performed with SPSS version 26. Pearson correlations were used to test for relationships between age and biomarker levels. To examine the effects of species and sex on Aβ40, Aβ42, t-tau, p-tau, and NfL while controlling for age, a two-way ANCOVA was conducted. A Spearman's rho correlation was used to assess the association between each biomarker. To compare location differences of LP versus cisterna magna puncture, the Mann-Whitney U test for independent samples was used because the data were not normally distributed. P-values <.05 were considered significant.

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CONFLICTS OF INTEREST

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ORCID

Emma L. Robertson https://orcid.org/0000-0003-4285-2706
Susan E. Boehnke https://orcid.org/0000-0002-0726-6659

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