Cerebrospinal Fluid Total Protein Reference Intervals Derived from 20 Years of Patient Data

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BACKGROUND: Reference intervals are vital for interpretation of laboratory results. Many existing reference intervals for cerebrospinal fluid total protein (CSF-TP) are derived from old literature because of the invasive nature of sampling. The objective of this study was to determine reference intervals for CSF-TP using available patient data.

METHODS: Twenty years of hospital database information was mined for previously reported CSF-TP results. Associated demographic, laboratory, and clinical diagnosis (International Classification of Diseases 9/10 codes) details were extracted. CSF-TP results included 3 different analytical platforms: the Siemens Vista 1500, Beckman Lx20, and Roche Hitachi 917. From an initial data set of 19591 samples, the following exclusion criteria were applied: incomplete data, white blood cells (WBCs) >5 × 10^6/L, red blood cells (RBCs) >50 × 10^6/L, and glucose <2.5 mmol/L. Patient charts were reviewed in detail to exclude 60 different conditions for which increases in CSF-TP would be expected. A total of 6068 samples were included; 63% of the samples were from females. Continuous reference intervals were determined using quantile regression. Age- and sex-partitioned intervals were established using the quantile regression equation and splitting age-groups into 5-year bins.

RESULTS: CSF-TP showed a marked age dependence, and males had significantly higher CSF-TP than females across all ages. CSF-TP results from the 3 different instruments and manufacturers showed small (approximately 0.04 g/L), but statistically significant, differences. CSF-TP showed weak, but again statistically significant, correlation with WBC and RBC but was independent of serum total protein and creatinine.

CONCLUSIONS: The age dependence of CSF-TP supports that age-partitioned reference intervals will be more accurate than a single cutoff, particularly in patients with advancing age.

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Cerebrospinal fluid total protein (CSF-TP) is widely used in the differential diagnosis of neurological disorders, including autoimmune, neoplastic, inflammatory, and infectious diseases (1). CSF-TP is largely derived from protein entry via pinocytotic transfer by endothelial cells (2). CSF-TP is increased in pathological conditions that increase white blood cell (WBC) or red blood cell (RBC) counts, involve bacteria, or obstruct CSF flow.

To make effective clinical decisions, it is necessary to have appropriate reference intervals. Reference intervals are typically derived from healthy population studies, recruiting willing volunteers that are intended to represent the population being tested. The invasive nature of CSF sampling precludes typical normal value studies. As a result, it is common that CSF reference intervals are sourced from textbooks (3) or other external references. Textbook sources are often referenced from older studies, for which both technology and the populations themselves may differ. Further, older studies at times do not partition reference intervals by age or sex or may have small sample sizes, and as such they may not serve as an optimal representation of the population.

As a resolution of the concerns of invasive sampling of healthy populations, there are several studies that have attempted to derive reference values from patient samples (4, 5). The reference patient population is defined by removal of samples with high CSF WBC counts, CSF RBC counts, and other laboratory data, such as low glucose. Ideally, patient chart review is used to exclude patients with disorders that are known to increase CSF-TP, such as Guillain-Barré, brain tumors, and meningitis. One of the most recent studies showed a distinct age-dependent increase in CSF-TP (4), which indicates that many of the widely used existing nonpartitioned intervals

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4 Nonstandard abbreviations: CSF, cerebrospinal fluid; TP, total protein; WBC, white blood cell; RBC, red blood cell.
may overestimate the frequency of abnormal CSF-TP in older patients.

In alignment with early published CSF-TP reference intervals and existing clinical guidelines (7), our laboratory has used the same interval of ≤0.45 g/L for >20 years across 3 different instruments sourced from a reference textbook (6). The striking difference of age-partitioned intervals reported in recent studies prompted both our laboratory and neurologists to consider using age-partitioned intervals instead of the existing interval. However, the published studies used different methodology (biuret or protein precipitation) than that used in our laboratory (see Materials and Methods). In addition, the Ottawa Hospital has a unique resource in the form of a data warehouse with >20 years of patient data, including clinical history, diagnostic coding, and laboratory results. Given the availability of a large volume of high-quality data, the objective of this study was to derive reference intervals from hospital patient data.

Materials and Methods

STUDY POPULATION

This was a retrospective analysis of CSF-TP samples from patients with encounters at the Ottawa Hospital between January 1, 1996 and December 1, 2016. All data were extracted from the Ottawa Hospital Data Warehouse, and the study was approved by the Research Ethics Board (protocol 20160863-01H). In addition to CSF-TP results, associated demographic (age and sex), clinical diagnosis (International Classification of Diseases 9/10 codes), and laboratory data were extracted. Laboratory data included CSF glucose, CSF WBC, and CSF RBC, serum creatinine, and serum total protein. To be included in the analysis, laboratory results needed to be performed on the same sample or be collected within 24 h of the CSF-TP sample.

CHART REVIEW

Because of the potentially unreliable nature of International Classification of Diseases coding for diagnosis (7), individual clinical charts were reviewed for conditions known or suspected to cause increased CSF-TP (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue12); charts were reviewed only for patients with CSF-TP >0.45 g/L, the existing cutoff used by the laboratory. Laboratory data, demographics, and clinical history were used as exclusion criteria as described in Fig. 1. When multiple exclusion criteria were met, the sample was excluded and counted based on the first instance encounter in the sequence outlined in Fig. 1.

CSF-TP ANALYSIS

CSF-TP was analyzed on 3 different instruments over the course of the 20 years included in the study as follows: Roche Hitachi 917, January 1, 1996 to September 30, 2001; Beckman Lx20, September 30, 2001 to April 1, 2009; and Siemens Vista 1500, April 1, 2009 to December 1, 2016. The Roche method is based on a benzethonium-chloride turbidimetric analysis, whereas the Beckman and Siemens methods use a pyrogallol red-molybdate complex, which is measured at 600 nm. In all cases, analyses were performed according to manufacturer’s directions.

Other laboratory values were measured on different instruments across the 2 decades included in the study. Serum creatinine, total protein, and CSF glucose were measured on the platforms described above for the same time frames; creatinine was measured by the Jaffe method from September 30, 2001 through April 13, 2013 and by the enzymatic method thereafter. CSF WBC and RBC counts were determined using the Beckman Coulter between 1996 and 2009 and the Sysmex XE5000 from 2009 to 2016. All laboratory analyses were determined according to manufacturer’s instructions throughout the study in a routine clinical laboratory in an academic medical center (The Ottawa Hospital).

REFERENCE INTERVAL DETERMINATION

Based on significant correlation ($r^2 = 0.1, P < 0.00001$) and visual identification of the relationship between age and CSF-TP, we established age-partitioned reference intervals for CSF-TP. Reference intervals were determined using quantile regression in the statistical programming language R (8) as previously described (9); quantile regression is a nonparametric method without assumptions about the distribution or shape of the data. Both lower 2.5th and upper 97.5th percentiles were calculated. Because continuous regression-based reference intervals cannot be readily implemented into most laboratory information systems, we also determined 5-year age-binned intervals. Age-binned intervals were calculated by averaging the highest and lowest values derived from the quantile regression equations in each bin (as opposed to making discrete age bins and calculating percentiles, which does not account for the continuous effect of age between bins). We established 95% CIs for each partition using bootstrap resampling (10). Separate reference intervals were determined for combined age and sex partitions using the methods described above.

STATISTICAL ANALYSIS

To determine the strength of association and statistical significance between individual laboratory values and CSF-TP, we used univariate linear regression. Where data were heteroscedastic and not normally distributed, we used Box–Cox transformation and applied the linear
Fig. 1. Flowchart of patient inclusion and exclusion criteria.

Expected increase in CSF-TP was based on chart review by neurologists. A detailed list of exclusions is included in Table 1 of the online Data Supplement. Hx, history; IQR, interquartile range.
regression after satisfying the assumptions of the method (independent, normally distributed, homoscedastic). To determine whether different reference interval partitions (sex, age bins) were statistically significant, we used ANOVA. Where statistically significant differences were observed by ANOVA (comparison of all groups), Tukey honest significant differences tests were applied to determine which age-groups were different. All analyses were done using the statistical programming language R (11).

Results

STUDY POPULATION SUMMARY
After excluding 13523 samples according to the criteria outlined in Fig. 1, there were 6068 samples, each from a unique patient. The most common clinical exclusions were neuroinflammation (e.g., multiple sclerosis; n = 254), polynuropathy (e.g., demyelination; n = 215), and seizure (n = 204) (see Table 1 in the online Data Supplement for the complete list). In cases of repeat samples on a patient, the first instance was selected and follow-up samples were excluded (n = 456); there was a maximum of 11 samples from a given patient over a 5-year period. After exclusions, there were 3804 females with a median age of 43 years (SD, 16.9; range, 18–97 years) and 2264 males with a median age of 44 years (SD, 17.4; range, 18–94 years).

REFERENCE INTERVALS
CSF-TP showed a strong age dependence prompting partitioning of reference intervals by age (Fig. 2). We selected 5-year age bins and calculated nonparametric upper and lower reference intervals as shown in Table 1; 95% CIs are also shown. Most, but not all, of the upper 97.5th percentile age bins were significantly different from each other (P < 0.001). The equations for a continuous lower 2.5th and 97.5 upper reference intervals for males and females combined are:

\[
2.5\text{th percentile} = 0.111 - 1.69 \times 10^{-3} \times \text{age} + 2.31 \times 10^{-4} \times \text{age}^2 + 4.53 \times 10^{-6} \times \text{age}^3 - 2.58 \times 10^{-8} \times \text{age}^4
\]

\[
97.5\text{th percentile} = 0.124 + 0.0284 \times \text{age} - 7.08 \times 10^{-4} \times \text{age}^2 + 8.23 \times 10^{-6} \times \text{age}^3 - 3.35 \times 10^{-8} \times \text{age}^4
\]

SEX DIFFERENCES
A significant sex difference was observed for CSF-TP (Fig. 3). The mean female CSF-TP was 0.32 g/L (SD, 0.10; n = 3804), whereas the mean male CSF-TP was 0.38 g/L (SD, 0.11; n = 2264). This difference was statistically significant (P < 0.00001) with a moderate effect size (Cohen d approximately 0.5) (12). The sex differences were:
Table 1. Age-partitioned reference intervals.a

| Age, years | All | | | | | | Males | | | | | | Females | | |
|------------|-----|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|            | 2.5th | 97.5th | n | 2.5th | 97.5th | n | 2.5th | 97.5th | n |
| 18–25      | 0.14 (0.13–0.16) | 0.47 (0.46–0.49) | 724 | 0.17 (0.16–0.18) | 0.50 (0.47–0.52) | 273 | 0.13 (0.12–0.15) | 0.46 (0.44–0.47) | 451 |
| >25–30     | 0.16 (0.15–0.17) | 0.52 (0.51–0.53) | 527 | 0.18 (0.17–0.2) | 0.54 (0.53–0.55) | 211 | 0.15 (0.14–0.16) | 0.47 (0.45–0.50) | 316 |
| >30–35     | 0.17 (0.16–0.18) | 0.54 (0.53–0.55) | 622 | 0.19 (0.18–0.21) | 0.57 (0.55–0.58) | 214 | 0.17 (0.15–0.18) | 0.49 (0.47–0.52) | 408 |
| >35–40     | 0.19 (0.18–0.19) | 0.56 (0.55–0.57) | 602 | 0.20 (0.19–0.21) | 0.59 (0.57–0.6) | 223 | 0.18 (0.17–0.19) | 0.51 (0.49–0.54) | 379 |
| >40–45     | 0.19 (0.18–0.20) | 0.57 (0.57–0.58) | 717 | 0.20 (0.19–0.22) | 0.60 (0.59–0.61) | 259 | 0.19 (0.18–0.20) | 0.54 (0.51–0.56) | 458 |
| >45–50     | 0.20 (0.19–0.20) | 0.59 (0.58–0.6) | 636 | 0.21 (0.19–0.23) | 0.61 (0.60–0.63) | 227 | 0.19 (0.18–0.20) | 0.56 (0.53–0.58) | 409 |
| >50–55     | 0.20 (0.19–0.20) | 0.60 (0.59–0.61) | 584 | 0.21 (0.19–0.23) | 0.62 (0.61–0.64) | 188 | 0.19 (0.18–0.20) | 0.58 (0.55–0.60) | 396 |
| >55–60     | 0.20 (0.18–0.20) | 0.62 (0.60–0.62) | 425 | 0.21 (0.19–0.23) | 0.63 (0.62–0.65) | 157 | 0.19 (0.18–0.20) | 0.60 (0.57–0.62) | 268 |
| >60–65     | 0.19 (0.18–0.20) | 0.63 (0.61–0.65) | 302 | 0.22 (0.19–0.24) | 0.64 (0.62–0.66) | 135 | 0.18 (0.17–0.20) | 0.61 (0.59–0.64) | 167 |
| >65–70     | 0.19 (0.17–0.20) | 0.65 (0.63–0.67) | 270 | 0.22 (0.19–0.24) | 0.66 (0.63–0.68) | 112 | 0.17 (0.15–0.20) | 0.63 (0.60–0.66) | 158 |
| >70–75     | 0.19 (0.16–0.21) | 0.67 (0.65–0.69) | 234 | 0.23 (0.19–0.24) | 0.68 (0.65–0.70) | 101 | 0.17 (0.14–0.20) | 0.64 (0.62–0.68) | 133 |
| >75–80     | 0.19 (0.16–0.21) | 0.69 (0.66–0.72) | 189 | 0.23 (0.18–0.25) | 0.70 (0.66–0.73) | 87 | 0.17 (0.14–0.20) | 0.67 (0.64–0.70) | 102 |
| >80–85     | 0.20 (0.18–0.23) | 0.71 (0.68–0.76) | 149 | 0.25 (0.18–0.28) | 0.72 (0.67–0.80) | 51 | 0.19 (0.15–0.22) | 0.68 (0.65–0.76) | 98 |
| >85        | 0.21 (0.17–0.26) | 0.73 (0.68–0.82) | 87 | 0.26 (0.18–0.31) | 0.76 (0.67–0.93) | 26 | 0.22 (0.17–0.27) | 0.72 (0.65–0.89) | 61 |

*a Values represent nonparametric reference intervals (95% CIs in parentheses) partitioned by age and combined age/sex; n, represents the number of samples per partition.
difference was consistent across all ages, with an average absolute effect of approximately 0.06 g/L. Sex- and age-partitioned reference intervals are shown in Table 1.

INSTRUMENTATION COMPARISON
Included in the 20 years of the study were results from 3 different instruments: the Roche 917, the Beckman LX20, and the Siemens Vista (Fig. 4). There were statistically significant \((P < 0.05)\) differences between all instruments. These differences were relatively modest (mean absolute difference of 0.04 g/L). An analysis of reference intervals partitioned by age and instrument was largely similar (data not shown). The 95\% CIs for age- and instrument-partitioned intervals overlapped for ages <65 years; for >65 years, there was relative data scarcity for the Roche 917 method, resulting from a smaller sample size (<60 samples/bin).

ADDITIONAL LABORATORY CORRELATIONS WITH CSF-TP
Linear regression was used to analyze the association between CSF-TP and other available laboratory data (Fig. 5). In particular, we were interested in the effects of CSF WBC and CSF RBC counts, which are widely used as exclusion criteria for CSF-TP studies. CSF WBC \((r^2 = 0.018)\), CSF RBC \((r^2 = 0.047)\), and serum/plasma total protein \((r^2 = 0.0027)\) were all statistically significantly correlated with CSF-TP. However, the \(r^2\) value for these correlations was low and the effect sizes are extremely small (Cohen \(d' < 0.1\)). CSF-TP correlation with creatinine was examined to determine whether renal function affected CSF-TP; no significant correlation was found between serum/plasma creatinine and CSF-TP \((r^2 < 0.0001)\).

Discussion

Herein we describe age- and sex-partitioned CSF-TP reference intervals derived from patient samples using a large sample size. The reference intervals established in this study are roughly similar to those recently published by Hegen et al. \((4)\). The main differences are in older age-groups, which may reflect different upper limits (97.5\% percentile here vs 95\% percentile in the other study) and bin sizes. Although we used smaller bins than the previous study (5 years vs 10 years), the reference intervals from both studies support a strong age-dependent increase in CSF-TP. This finding is also consistent with several older studies \((13–15)\) but conflicts with 1 report that examined age-related reference intervals in patients >50 years old. In that study \((5)\), there were not significant differences between 10-year age bins despite using similar CSF-TP measuring methods (pyrogallol red/molybdate) and a fairly large sample size (>1100). It is not clear why an age-dependent effect was not observed.

In general, there appears to be a lack of consistency in methodology used to establish upper limits and what
those upper limits should be (e.g., 95th percentile, 97.5th percentile, interquartile range). Despite these study design differences, the mean and median values from both our study and most other reports (4, 5, 13, 14) suggest that the commonly used cutoff of 0.45 g/L is lower than commonly encountered CSF-TP values in older patients. It is interesting to note the patients approximately 18 to 20 years of age did have 97.5th upper limits near to 0.45 g/L, likely reflecting the origin of these reference values in young healthy adults. Most studies are supportive of an age-dependent increase in CSF-TP; 1 “rule of thumb” we encountered in our literature describes CSF-TP to roughly equal the patient’s age in milligrams per deciliter (16). Future studies are aimed at addressing the clinical sensitivity and specificity of the age-dependent reference intervals for different neurological disorders. With the existing data, it can be confidently stated that the use of a 0.45-g/L cutoff overestimates the frequency of abnormal CSF-TP values, and that this overestimation increases with patient age. The use of the 0.45-g/L cutoff resulted in an overestimation of abnormally high samples ranging from 6.5% of patients aged 30 to 35 years to 35% of patients aged 80 to 85 years.

In addition to the age dependence observed for CSF-TP, we also identified significant differences between males and females. Males had a small (approximately 0.06 g/L, but statistically significantly higher, CSF-TP (P < 0.00001). Sex differences in CSF-TP have been previously reported (13, 14), although the present data appear to be the first replication in some 40 years and the first using more modern CSF-TP measurement methods (automated pyrogallol red vs Ponseau-S dye precipitation). The earlier studies reported male CSF-TP was approximately 0.04 g/L higher than that of females, somewhat similar to values observed in the present study; more recent studies do not indicate whether they examined sex differences. It is unclear why there are CSF-TP sex differences. Early reports do not discuss causes (13, 14), although we speculate that it may reflect sex-specific differences in endocrinology and muscle mass, which is common to other laboratory

Fig. 4. Comparison of the CSF-TP between analytical instruments. Individual points for each are shown in black with boxplots overlaid.
values, such as creatinine, urea, and creatinine phosphokinase. Based on the observed sex differences, we also chose to partition CSF-TP by sex and age in combination. Combined sex and age partitions showed partial overlap with some age bins, such that implementation of sex-specific intervals would have a small effect on abnormal classification (frequency of high values). Thus, although laboratories may or may not choose to implement sex partitions, they are provided for completeness and consideration. Additional studies may help refine the sex partitions and establish whether disease incidence between sexes warrants implementation of partitions, as is being investigated for other analytes, such as troponin [17].

Another practical observation from this study was the relative similarity between different analytical methods for CSF-TP. Although there were statistically significant differences between methods, there was a small effect size of approximately 0.04 g/L. This small difference across 3 manufacturers over 20 years is smaller than that
observed with proficiency testing sample data. Proficiency testing data, which rely on testing of unknown samples as part of regulatory compliance and quality assurance, show good agreement within methods and for manufacturers using pyrogallol red (e.g., Beckman and Siemens) but substantial differences between other methods. Using College of American Pathologists survey data, the benzethonium-chloride method is up to 60% lower than pyrogallol red methods for low CSF-TP concentrations (approximately 0.4 g/L). It is possible that the method differences observed with proficiency testing material may result from matrix effects, where human samples behave differently than spiked or otherwise modified samples. Despite these apparent proficiency testing differences, reference intervals partitioned by instrument/method did not show substantial differences, where 95% CIs overlapped (data not shown). Because of the relative similarity between methods, we pooled data yielding a common reference interval for the Siemens Vista (pyrogallol red), Beckman Lx20 (pyrogallol red), and Roche 917 (benzethonium-chloride) methods. Irrespective of the method, the partitioned interval defined in this study will differ substantially from the 0.45-g/L cutoff that has been used in the laboratory for >20 years. As described above, additional work is focused on determining the effect of making such a change to the reference intervals. Collectively, it is our opinion that the methodological differences observed in the present study did not support the need to use manufacturerspecific intervals.

In addition to developing CSF-TP reference intervals, we examined the correlations between other laboratory values and CSF-TP. CSF WBC and RBC counts have been widely used as exclusion criteria based on their effect on CSF-TP concentration. Here, we observed small, but statistically significant, correlation between CSF WBC and RBC counts and CSF-TP. However, because of the weak correlation, it is not possible to draw conclusions regarding a reasonable cutoff for the impact of WBC/RBC counts on CSF-TP. Existing studies have applied cutoffs for CSF WBC/RBC variability, ranging from exclusion of all samples with detected cells (13) to a maximum of $10 \times 10^6$/L for WBCs and $500 \times 10^6$/L for RBCs (4). Here we used relatively conservative cutoffs of $5 \times 10^6$/L for WBCs and $50 \times 10^6$/L for RBCs. Despite the abundance of data, it remains challenging to assert a CSF WBC or RBC cutoff above which laboratories and physicians may want to avoid reporting or using CSF-TP for diagnosis. Dedicated interference studies show the benzethonium-chloride method is relatively resistant to hemolysis as compared with the pyrogallol methods (18). The benzethonium-chloride is unaffected up to 100 mg/L hemoglobin (in hemolysate), whereas the pyrogallol methods exceeded 10% bias with 50 mg/L. Based on the linear relationship between RBC count and hemoglobin concentration, 100 mg/L hemoglobin translates to an RBC count of approximately $500 \times 10^6$/L. This would support commonly used exclusion cutoffs and would apply to benzethonium-chloride CSF-TP methods. For pyrogallol red methods, a hemoglobin interference cutoff of 50 mg/L would translate to an RBC count of $\sim 250 \times 10^6$/L. A data-derived exclusion cutoff for WBC based on interference studies is not as readily available.

We also examined laboratory correlations between CSF-TP and serum creatinine and serum total protein. These conclusively demonstrate there is effectively no association between renal function and CSF-TP. It is also reasonable to conclude that, based on the extremely low correlations (<0.001), serum total protein does not impact CSF-TP concentration. Finally, we also examined a small sample of data ($n = 50$) where serum free light chains were available along with CSF-TP (data not shown). Free light chains were examined, as they are relatively small molecules (molecular weight, free $\kappa$ approximately 22 kDa and free $\lambda$ approximately 45 kDa) that may cross the blood–brain barrier (19). Neither free $\kappa$ (range, 4.4–1468 mg/L) nor free $\lambda$ (range, 1.5–3210 mg/L) light chains showed significant correlation with CSF-TP; the $\kappa$/$\lambda$ ratio was also not found correlated with CSF-TP. Although these are preliminary results and more research is needed to confirm the observation, it seems myeloma and other diseases that cause increased light chains in serum do not impact CSF-TP.

The main strengths of this study are the large sample size (>6500 samples) and the thorough, detailed, and conservative exclusion criteria. The inclusion of multiple instruments across decades also supports a wide use for the reference intervals determined in the study. In addition, 5-year age partitions were extracted from continuous data to allow for implementation in laboratory information systems, which are typically incapable of using continuous (equation-based intervals). Limitations of the study include the lack of chart review for samples with CSF-TP <0.45 g/L; exclusion of many of these samples would increase both the upper and lower cutoffs. Despite the detailed chart review, it is possible that the quantity or quality of the data in the chart varied over time. Although the written chart notes appeared complete throughout the study, we did observe substantial changes in rates of administrative coding (International Classification of Diseases) over time. For this reason, we did not rely on the International Classification of Diseases coding for exclusion criteria of diagnostic classification.

In summary, this study provides robust age-partitioned reference intervals for CSF-TP. These intervals appear applicable to a range of instruments. Further work is needed to determine the appropriateness of these
intervals or other clinical cutoffs for diagnosis and neurological disease classification.

**Authors’ Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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