

Measurement of CSF core Alzheimer Disease biomarkers for routine clinical diagnosis: do fresh vs frozen samples differ?

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Abstract

Background: cerebrospinal fluid (CSF) amyloid-beta (A β) 42/40 ratio, threonine-181-phosphorylated-tau (p-tau) and total-tau (t-tau) represent core biomarkers of Alzheimer Disease (AD). The recent availability of automated platforms has represented a significant achievement for reducing the pre-analytical variability of these determinations in clinical setting. With respect to classical manual ELISAs, these platforms give us also the possibility to measure any single sample and to get the result within approximately 30 min. So far, reference values have been calculated from measurements obtained in frozen samples. In this work, we wanted to check if the values obtained in fresh CSF samples differ from those obtained in frozen samples, since this issue is mandatory in routine diagnostic work.

Methods: fifty-eight consecutive CSF samples have been analyzed immediately after lumbar puncture and after one-month deep freezing (-80°C). As automated platform we used Lumipulse G600-II (Fujirebio Inc.). Both the fresh and the frozen aliquots were analyzed in their storage tubes.

Results: in fresh samples, a mean increase of A β 40 (6%), A β 42 (2%), p-tau (2%) and t-tau (4%) was observed as compared to frozen samples, whereas a slight decrease was observed for A β 42/A β 40 ratio (4%), due to the higher deviation of A β 40 in fresh samples compared to A β 42. These differences are significant for A β 40, A β 42/A β 40 ratio, p-tau and t-tau. Nevertheless, A β 42/A β 40 ratio showed a lower variability (smaller standard deviation of relative differences) with respect to A β 42. With respect to the AD profile according to the A/T/(N) criteria for AD diagnosis, no significant changes in classification were observed when comparing results obtained in fresh vs frozen samples.

Conclusions: small but significant differences have been found for A β 40, A β 42/A β 40 ratio, p-tau and t-tau in fresh vs frozen samples. Importantly, these differences did not imply a modification in the A/T/(N) classification system. In order to know if different cut-offs for fresh and frozen samples are required, larger, multi-center investigations are needed.

Keywords

Biomarkers, Alzheimer's Disease, cerebrospinal fluid, automated platforms, pre-analytical variables

Background

Cerebrospinal fluid (CSF) amyloid-beta 42 (A β 42), A β 40, A β 42/A β 40 ratio, threonine-181-phosphorylated-tau (p-tau) and total-tau (t-tau) are reliable biomarkers for amyloidosis (A), tauopathy (T) and neurodegeneration (N). These biomarkers are able to identify Alzheimer's disease (AD) independent of the clinical stage, thus, including the earliest stages [1,2] and mild cognitive impairment (MCI) [3]. Also, they are reliable predictors of progression to dementia [4] up to or more than 10 years before [5]. The application of CSF biomarkers in clinical practice has been strongly encouraged by the NIA-AA (National Institute on Aging and the Alzheimer's Association) [6], in order to promote the shift of the definition of AD from a syndromic to a biological construct.

The classical way to measure AD biomarkers is through manual Enzyme-Linked Immunosorbent Assay (ELISA) [7], which suffers from user- and laboratory-dependent procedures that may cause reproducibility issues [8,9]. Manual ELISA is carried out in 96-well plates, which often requires to wait for an adequate number of samples available, in order to avoid waste of materials. Moreover, intra-assay variability also affects ELISA, due to the time required to fulfill the plate. These procedures make the use of ELISA troublesome for analyzing small numbers of samples or samples that require urgent diagnosis (i.e., suspected Creutzfeldt Jacob disease). The standardization of pre-analytical and analytical procedures (i.e., freeze/thaw cycles [11], CSF storage volumes [11], pipette-tips and tube materials [12,13], storage temperature [14] and variability due to the operator [15]) represents a crucial factor in biomarker assays [10]. In this context, automated [16–20] and semi-automated [21] platforms may play a major role in minimizing inter-laboratory differences in biomarker assays. Fully automated chemiluminescent platforms [20], showed high sensitivity and specificity for early diagnosis of AD, with an optimal concordance with manual ELISA assays [18,20] and amyloid-targeted Positron Emission Tomography (PET) [19,22]. Moreover, these platforms offer the possibility to easily and quickly analyze any single and fresh CSF samples, a feature that could be useful when a timely response is needed by clinicians. So far, reference values are represented by measurements obtained in samples aliquoted and frozen before being thawed for biomarker assays. The perspective to analyze fresh CSF samples, getting the results in half an hour, is really

of high impact in clinical setting. This new possibility made us wonder if biomarker measurements can differ between fresh and frozen samples. If yes, then new cutoffs should be defined for fresh samples. Accordingly, in this work, we evaluated the values of A β 42, A β 40, A β 42/A β 40, t-tau and p-tau, obtained in fresh and after one-month freezing CSF samples.

Methods

Lumbar puncture and general CSF handling

CSF samples were obtained from a consecutive series of out-patients referring to Center of Memory Disturbances of the University of Perugia for routine diagnostic work-up, and collected according to international guidelines [23–25]. All patients gave their informed written consent. All the procedures were performed following the Helsinki Declaration. Lumbar punctures were performed from 8:00 to 10:00, after an overnight fasting. CSF (~ 12 mL) was immediately collected in sterile polypropylene tubes (Sarstedt® tubes, codes: 62.610.210) and gently mixed to avoid possible gradient effects. All samples were centrifuged at 2000 x g for 10 min., at room temperature and then aliquoted in 0.5 mL aliquots in sterile polypropylene tubes (Sarstedt® tubes, codes: 72.730.007).

Biomarker assay on fresh and frozen CSF samples

58 (28 males and 30 females) CSF samples were consecutively collected from patients with undisclosed diagnoses, processed as described above and then divided. For each sample, one aliquot was immediately analyzed in its 0.5 mL Sarstedt® tube on the Lumipulse G600-II (Fujirebio inc.) for A β 40, A β 42, t-tau and p-tau, while the others were frozen at -80°C. We analyzed a second aliquot after 30 days of freezing at -80°C, using then same methodology. The experimental workflow of this study is summarized in Figure 1.

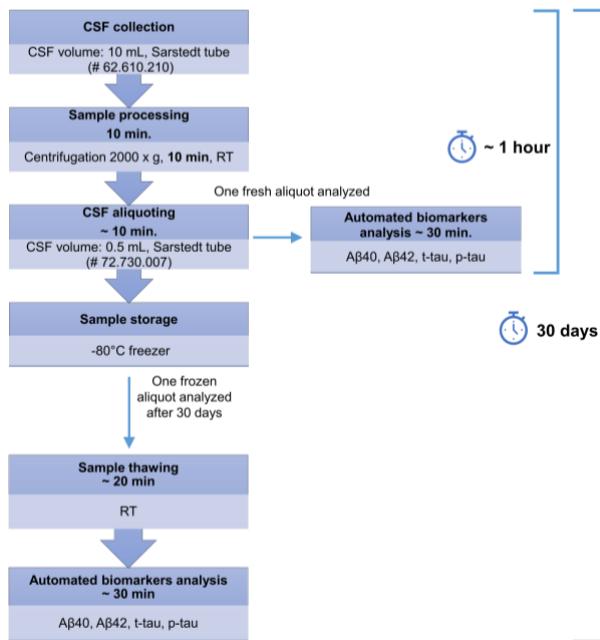


Figure 1 Scheme of the experimental workflow followed to test the impact of fresh vs frozen CSF using automated platform.

Statistical analysis

To evaluate the impact of freezing, biomarkers values measured on fresh samples were compared to the ones obtained from another aliquot of the same sample after one-month storage at -80°C. Due to the non-normality of biomarker data, Passing Bablok regressions [26] were performed instead of parametric least squares regressions. Confidence intervals (CI) for the fitted parameters were calculated with the bootstrap method [27]. Correlations between biomarkers measured in fresh and frozen samples were estimated in terms of Spearman's correlation coefficients (ρ). Bland-Altman plots [28] with trends and CI were also generated with the R-package *blandr* [29]. To assess the significance of the observed differences, we computed the relative difference ΔB between freshly acquired (B_{fresh}) and post-freezing (B_{frozen}) values of y Aβ40, Aβ42, Aβ40/Aβ42 ratio, p-tau and t-tau, from two aliquots originating from the same CSF sample.

$$\Delta B = \frac{B_{fresh} - B_{frozen}}{B_{fresh}}$$

Kolmogorov-Smirnov normality test could not reject the normality of the ΔB values for each tested biomarker, thus one-sample Student's t-test was applied to assess the significance of the relative differences in biomarkers between fresh and frozen CSF samples. A p-value below 0.05 was

considered significant to reject the null hypothesis. Cut-off values for fresh samples were calculated using both regression analysis [30] and by maximizing Youden's index with the p-ROC package in R [31]. For the regression transfer method, cut-offs CI were calculated from the ones obtained for the coefficients of the Passing Bablok regression, while for the Youden's index maximization, CI were calculated using 2000 bootstrap replicates.

Biomarker classification

In order to appreciate the variability of fresh vs frozen CSF aliquots, we compared the NIA-AA A/T/(N) classification criteria using standard cut-offs. These standards, developed in our laboratory, are also reported in the biomarker assay cartridge datasheets of the Lumipulse G600-II. Biomarker values were classified as positive (+) or negative (-) by using the cut-off values of 0.069 for A β 42/A β 40 ratio, 56.5 pg/mL for p-tau and 404 pg/mL for t-tau. Samples positive for both A β 42/A β 40 ratio and p-tau (A+/T+) have been classified as AD [6] (raw classification). A more robust classification was also applied by classifying AD or non-AD CSF profiles considering a tolerance of \pm 10% on the cut-off values of A β 42/A β 40 ratio and p-tau. This tolerance is usually considered in routine diagnostics in order to overcome inter-assay variability.

Results

The levels of A β 40, A β 42, p-tau and t-tau were measured with a fully automated chemiluminescent platform (Lumipulse G600-II, Fujirebio inc.) on two aliquots of the same CSF sample, one analyzed immediately after the sampling ("fresh") and the other after 30 days of storage at -80°C. To investigate the relation between fresh and frozen biomarker levels, Passing Bablok regressions were performed, the results of this analysis are plotted in Figure 2 (panels A-E). To assess the significance of the deviations from identity, the relative differences on A β 40, A β 42, A β 42/A β 40, t-tau and p-tau were also calculated, the results are represented in Figure 2, panel F.

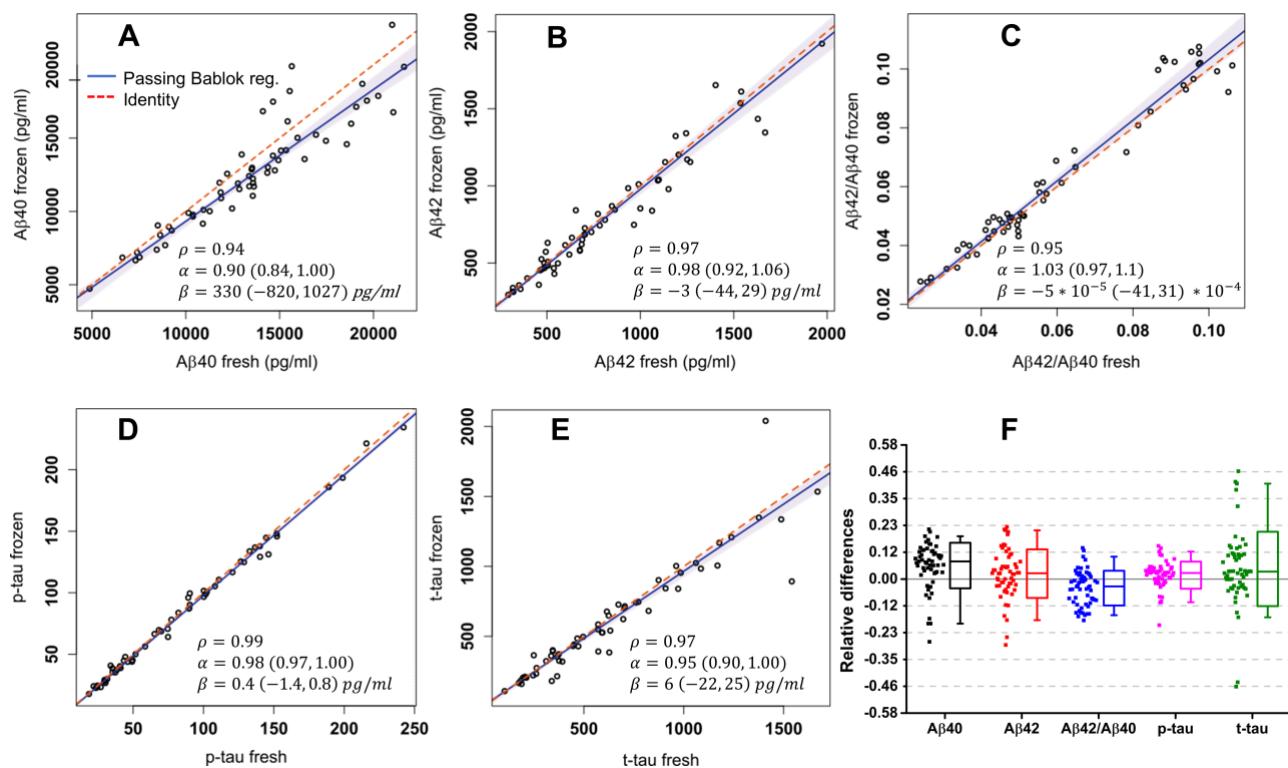


Figure 2 A-E) Passing Bablok regression analyses with 95% CI of core biomarkers measured in fresh CSF samples and after 1 month of deep-freezing. Correlations have been calculated in terms of Spearman's correlation coefficients (ρ). Fitted slopes (α) and intercepts (β) with their 95% CI are also shown. **F)** Mean relative differences of A β 40, A β 42, A β 42/A β 40, t-tau and p-tau between fresh and frozen samples are plotted together. Boxes representing data distributions are centered on the mean values, with the internal horizontal line representing the median. Boxes heights are equal to the standard deviations of the relative differences while whiskers represent the 5%-95% data range.

As shown in the panels A-E, fitted intercepts are consistently equal to zero within their CI for all the measured quantities. The largest deviation from identity was obtained for A β 40. Bland Altman plots (Supplementary Figure 1) showed higher concentration values for A β 40, A β 42, p-tau and t-tau in fresh samples and a trend of larger differences in magnitude for higher values of biomarkers. However, about 95% of the samples fell within 2 SD from the mean fresh vs frozen difference, confirming the high correlation obtained with Passing-Bablok analysis. As shown in figure 2 F and Table 1, mean relative differences greater than 0.06 have not been observed for any of the tested biomarkers. Even if small, the differences on A β 40, A β 42/A β 40 ratio, t-tau and p-tau were significant according to Student's t-test (see table 1). Although the relative differences on A β 42/A β 40 ratio were significant, this biomarker showed less variability in terms of standard deviation (SD) of relative differences with respect to A β 42 and A β 40 alone (0.075 vs 0.11). The highest SD of relative

differences were obtained for t-tau (0.19) while the smallest for p-tau (0.06), the latter showed also the smallest mean relative difference (0.017) and the highest correlation between measurements ($\rho = 0.99$).

Table 1 mean relative differences between fresh and frozen samples with their 95%CI, standard deviations of relative differences (SD) and p-values obtained by applying one-sample Student's t-test.

Biomarker	Mean relative difference (95%CI)	SD	p-values t-test
A β 40	0.059 (0.033, 0.084)	0.10	< 0.001
A β 42	0.023 (-0.004, 0.051)	0.11	0.10
A β 42/A β 40	-0.039 (-0.059, -0.020)	0.075	< 0.001
t-tau	0.044 (0.001, 0.086)	0.16	0.04
p-tau	0.017 (0.0015, 0.032)	0.059	0.03

For each aliquot, A β 42/A β 40, p-tau and t-tau have been classified according to the A/T/(N) criteria [6] by using standard cutoffs and tolerances (reported in the Methods section). The results of this classification are shown in Table 2.

Table 2 Classification of core AD biomarkers and in fresh and frozen samples by using the A/T/(N) criteria. Number of changes in classification between biomarkers measured in fresh and stored at -80°C CSF samples are also reported. Samples with both pathologic A β 42/A β 40 ratio and pathologic p-tau (A+/T+) have been classified positive for AD. For "robust" AD diagnosis and AD exclusion (non-AD) a $\pm 10\%$ tolerance was applied on the cut-offs of A β 42/A β 40 ratio and p-tau.

	+ (%)	- (%)	N° of changes (%)
A β 42/A β 40 frozen	39 (67%)	19 (33%)	1 (2%)
A β 42/A β 40 fresh	40 (69%)	18 (31%)	
p-tau frozen	34 (59%)	24 (41%)	0 (0%)
p-tau fresh	34 (59%)	24 (41%)	
t-tau frozen	34 (59%)	24 (41%)	2 (3%)
t-tau fresh	36 (62%)	22 (38%)	
AD frozen	34 (59%)	24 (41%)	0 (0%)
AD fresh	34 (59%)	24 (41%)	
robust AD frozen	32 (55%)	26 (45%)	1 (2%)
robust AD fresh	33 (57%)	25 (43%)	
robust non-AD frozen	24 (41%)	34 (59%)	1 (2%)
robust non-AD fresh	23 (40%)	35 (60%)	

Considering the classification (Table 2), in some samples, A β 42/A β 40 ratio and t-tau changed their classification between fresh and frozen samples. However, when looking at AD diagnosis, which mainly relies on the values of A β 42/A β 40 ratio and p-tau (A/T), none of the samples changed classification directly from + to - or the other way around considering the raw cut-offs. Only one sample changed from + to – by considering a + 10% tolerance on A β 42/A β 40 ratio and p-tau cut-offs (robust AD diagnosis) and another one when considering a - 10% for excluding AD.

To further investigate the impact of these differences in diagnostics, we recalculated the cut-offs for A β 42/A β 40 ratio, p-tau and t-tau for fresh samples. One way to do that is to transfer frozen-defined cut-offs by applying a linear transformation based on the parameters estimated with the Passing Bablok regression (cut-off fresh R in Table 3). Another way can be to consider the raw classification of frozen samples as a reference and maximize Youden's index for fresh samples (cut-off fresh Y in Table 3). The results of both procedures are shown in Table 3.

Table 3 Recalculated cut-offs for freshly measured CSF core AD biomarkers with their 95% CI. Cut-off fresh R: cut-off transferred by applying Passing Bablok regression. Cut-off fresh Y: cut-off calculated maximizing the Youden's index for fresh samples, by taking as reference the classification performed on frozen ones.

Biomarker	cut-off frozen	cut-off fresh R (95%CI)	cut-off fresh Y (95%CI)
A β 42/A β 40	0.069	0.067 (0.060, 0.075)	0.062 (0.061, 0.075)
p-tau	56.5	57.2 (55.7, 59.7)	55.1 (52.3, 60.9)
t-tau	404	419 (379, 473)	419 (406, 581)

As it is shown in Table 3, only for t-tau the recalculated cut-off from direct diagnostic information (Youden) does not include the cut-off defined in frozen samples within its 95% CI.

Discussion

Compared to traditional ELISAs, automated chemiluminescent platforms allow the measurement of biomarkers on CSF samples with minimal manual handling and a timely response. In particular, the analysis of single freshly collected CSF samples has become also possible. The analysis of fresh samples may still be not convenient in most cases, since quality control samples, should be used for each measurement, regardless of the number of samples analyzed. However, the non-optimization of the use of quality controls can be a reasonable cost when a very rapid diagnosis is needed for clinical reasons. For this purpose, we evaluated if core AD biomarkers, namely A β 40, A β 42,

A β 42/A β 40, p-tau and t-tau, differ from fresh to frozen CSF samples and if new cut-offs should be defined for freshly measured biomarkers. The effect of freezing on biomarker measurements was previously tested by Sjögren et al.[32]. No significant differences were measured in A β 42 and t-tau between fresh and one-time thawed samples by ELISAs. However, only 8 samples were tested and thus the significance of small variations could have been hindered by the limited sample size. Similar considerations can be done also for the more recent works of Le Bastard et al. [9] and Janelidze et al. [33], in which, together with other preanalytical factors, the impact of freezing was tested for A β 42, p-tau and t-tau and for A β 40, A β 42 and A β 42/A β 40 ratio, respectively on 10 samples by ELISAs. In our experiments, by using aliquots originating from 58 distinct patients, small but significant systematic differences were observed for A β 40, A β 42/A β 40, p-tau and t-tau between fresh and frozen samples, while a non-significant decreased value of A β 42 was also measured. The slight decrease in concentration of the tested proteins on frozen samples may have been produced by an unspecific loss caused by tube adsorption [11,34] or by a slight degradation during the freezing/thaw step cused by residual proteases. Among the tested proteins, A β 40 showed the largest decrease (6%). A β 40 is a naturally unfolded peptide, which makes it very accessible to the solvent, thus also very small amounts of proteases may produce an appreciable degradation [35]. We questioned if a release of proteases may have been produced by the lysis of residual cells in CSF but, as can be evinced from Table s1, no significant differences in variability were observed for samples containing different number of cells with the exception of A β 42 that, however, did not show significant variations upon freezing. The decrease of A β 40 altered also the value of AB42/A β 40 ratio. In routine clinical practice, A β 42/A β 40 ratio is usually preferable to A β 42 alone [36,37] and, although it showed a greater mean relative difference between fresh and frozen samples with respect to A β 42, it also showed a lower SD of relative differences between values measured in fresh and frozen CSF samples. With respect to the A/T/(N) classification, whereas for some CSF samples with borderline profiles, A β 42/A β 40 ratio and t-tau changed classification, while considering the diagnostic criteria for AD, none of the samples changed diagnosis in a raw dichotomous classification. By taking into account a tolerance of $\pm 10\%$ on cut-offs for confirming of excluding AD, only two samples (2%), one for the confirmation and one for the exclusion, changed in classification. We successively transferred

the cut-off values to fresh samples by applying two of the three methods described in the work of Barrado et al. [30]. Due to the non-normality of biomarker values we could not apply the *Bayesian two-stage cut-off transfer* method. For the *Linear-regression-based transfer* of the cut-off value we bypassed the normality assumption by using the coefficients obtained through the Passing Bablok regression, while for the *direct cut-off estimation from diagnostic information* we determined the cut-offs that maximized Youden's index in fresh samples by using the classification of frozen samples as reference. Both methods have pros and cons; the regression-based method utilizes all the data information but it may produce biased results when unequal populations of high and low biomarker values are present. Conversely, the direct estimation from diagnostic information is more robust but it may suffer from the non-optimal diagnostic representativeness of our samples. The two methods produced similar results and generally included frozen-defined cut-offs within 95% CI. The only exception is the cut-off obtained for t-tau from direct diagnostic information (Youden) that did not include the frozen-defined cut-off within its CI. However, considering the variability of biomarkers among measurements, the number of tested samples ($N = 58$) may have been insufficient to prove or disprove a real discrepancy between frozen-defined and fresh-defined cut-offs.

Conclusions

The wide availability of automated platforms represents an important achievement for reducing the pre-analytical variability of core AD biomarker assays in routine clinical setting [20]. With respect to classical manual ELISAs, automated platforms give us also the possibility to measure single samples and to obtain the results within approximately 30 minutes, allowing clinicians to provide urgent diagnoses in very short time. For this purpose, we wanted to check if the values obtained in fresh CSF differ from those obtained in frozen samples. Small but significant differences were found for A β 40, A β 42/A β 40 ratio, p-tau and t-tau in fresh vs frozen samples, although cut-offs for AD diagnosis recomputed on fresh samples did not significantly differ from those obtained in frozen samples. Larger, multi-center investigations are needed to definitely show the need of calculating specific cut-offs calculated in fresh CSF samples.

List of abbreviations

CSF: cerebrospinal fluid

A β 40: amyloid- β 1-40

A β 42: amyloid- β 1-42

AD: Alzheimer's disease

p-tau: threonine-181-phosphorylated tau

t-tau: total-tau

AD: Alzheimer's disease

MCI: mild cognitive impairment

NIA-AA: National Institute on Aging and the Alzheimer's Association

ELISA: enzyme-linked immunosorbent assay

PET: positron emission tomography

LP: lumbar puncture

PD: Parkinson's disease

CI: confidence interval

ρ : Spearman's correlation coefficient

α : linear regression slope

β : linear regression intercept

Declarations

- Ethics approval and consent to participate: All the procedures involving human subjects were performed following Helsinki Declaration. All patients and/or their legal representatives gave informed written consent for the lumbar puncture, CSF collection, assessment, analysis and the inclusion in the study, that was approved by the local Ethics Committee (CEAS n°1287/08), University of Perugia.
- Consent for publication: Not applicable.
- Availability of data and materials: The raw data used in this study are fully shown in Table S3 in the Supplementary Information.
- Competing interests: The authors declare that they have no competing interests
- Funding: We did not receive any funding for this study.

- Authors' contributions: G.B. analyzed the data and wrote the first draft, S.C. analyzed the fresh/frozen samples and revised the manuscript, S.P. analyzed the samples and revised the manuscript, D.C. contributed to the analyses and revised the manuscript, F.P.P. assisted with data management of samples, L.P. designed the study and revised the final version of the manuscript. All the authors read and approved the final manuscript.
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