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## Blood contamination in cerebrospinal fluid and its effect on biogenic amines and vitamins

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### Abstract

Cerebrospinal fluid (CSF) analysis offers the most innocuous analytical tool for assessing the cellular and biochemical environment of CNS and hence aids in studying neurometabolic conditions. The aim of this study was to assess the effect of blood contamination of CSF on the levels of selected biogenic amines and vitamins. CSF samples were spiked with increasing volumes of whole fresh blood (2.5%, 5%, 10% and 20%) under conditions: (a) spiking of pooled CSF samples followed by freezing to cause red blood cell (RBC) lysis; (b) spiking of pooled CSF samples followed by centrifugation to remove RBCs. CSF concentrations of two biogenic amines Homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA); and three vitamins Pyridoxal 5'-phosphate (PLP), 5-methyltetrahydrofolate (MTHF) and Thiamine were analysed by HPLC coupled with electrochemical detection. HVA and 5-HIAA illustrated lower values when RBC lysis was caused. However, PLP, 5-MTHF, and thiamine depicted higher concentrations on RBC lysis in contrast to RBC removal. CSF metabolomic investigations involving biogenic amines and vitamins as biomarkers is possible even when remarkable RBC CSF contamination occurs provided that CSF is centrifuged for the removal of RBC prior to freezing.

**Keywords:** Cerebrospinal fluid, blood contamination, biogenic amines, vitamins

### 1. Introduction

Cerebrospinal fluid (CSF), the biological fluid surrounding the central nervous system (CNS), is of tremendous scientific and clinical importance. CSF is primarily produced in the choroid plexus and is in close connection with the extracellular space of the brain, therefore biochemical modifications in the brain are indirectly reflected in the CSF (Jiménez-Jiménez *et al.*, 2014) [9]. The blood-brain barrier and blood-CSF barrier define the major exit and entry routes of components into the central nervous system from the circulation through these structures. These structures expedite transport of biological substrates for brain cell metabolism, and excretion of toxic and waste substances. (Batllori *et al.*, 2019; Huhmer *et al.*, 2006) [1, 7] hence control the CSF composition.

The blood-brain barrier generally hampers the influx of most metabolites, including amino acids and other molecules to a great extent. Biogenic amines like Homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), the major catabolites of dopamine and serotonin group of neurotransmitters, have compartmentalized biosynthetic pathways in the brain, and comparable concentrations may be detected in CSF and blood as no transport from blood to CSF is estimated (Mori *et al.*, 2003) [14]. In contrast, several vitamins like Pyridoxal-5'-phosphate (PLP) and Thiamine have to be transported into the brain through CNS barriers via specific transporters, and there are significant differences between vitamin concentrations in CSF and blood samples (Ortigoza-Escobar *et al.*, 2016) [17]. Pyridoxine and thiamine display lower concentrations in CSF than in the blood, however, Folic acid, a water soluble vitamin, is one of the few molecules that are more concentrated in CSF as compared to plasma (Footitt *et al.*, 2011) [5]. Interpretation of the concentration of these metabolites, particularly in cerebrospinal fluid (CSF), precisely reflects the integrity of biogenic amine and vitamin metabolism within the CNS and has been instrumental in studying the specific neurometabolic pathways and exploring transport of metabolites from the blood into the brain. Various neurogenetic disorders are the result of abnormalities in these processes. Considering these significant differences in the metabolite concentrations between CSF and blood, blood contamination of CSF may possibly cause substantial effects in the concentrations of majority of the aforementioned metabolites (Batllori *et al.*, 2019) [1].

CSF is collected by an invasive method of lumbar puncture. As blood/plasma contamination can be frequently observed due to different causes of impaired blood–brain barrier permeability, traumatic lumbar punctures, or intraventricular bleeding (Klebe *et al.*, 2019; Tan *et al.*, 2017)<sup>[11, 18]</sup>, a misinterpretation of metabolic profiles is a problem that should be curtailed to avoid diagnostic errors and repeated lumbar puncture procedures. The study has therefore been planned with the objective of evaluating the effect of blood contamination of CSF on the concentrations of biogenic amines and vitamins which act as biomarkers for the study of various neurometabolic disorders as shown in Table 1.

## 2. Materials and Methods

### 2.1 Sample collection and preparation

CSF remnant samples were procured from patients in which lumbar puncture was done to rule out meningitis, and stored at -80 °C, following the protocol reported by Hyland (2003)<sup>[8]</sup>. The 40 anonymized CSF samples' leftovers were taken and assessed for red blood cell (RBC) contamination using light microscopy (samples with less than 5 RBC/field were considered as contamination free). After thawing, the samples were pooled into 2 pools (20 samples each). The final volume of 10 ml was reached for each pool. A healthy volunteer's fresh blood sample was used to spike the CSF pools. Non-spiked samples and 4 spiking conditions were prepared in duplicates in the 2 CSF pools. The CSF pooled samples were divided into 1ml aliquots and were spiked with whole blood concentration of 2.5%, 5%, 10% and 20%, were kept under two different conditions after spiking:

- Freezing at -80 °C to cause RBC lysis.
- Centrifugation at 1500×g for 12 min at 4 °C, with the clear supernatant (to remove RBC) frozen at -80 °C.

After sample preparation, all the samples were frozen at the same time.

### 2.2 Methods and Data Analysis

5-HIAA and HVA were separated and analysed by reverse phase HPLC using an octadecylsilyl column (250 × 4.6 mm, C-18) and with electrochemical detection (Dionex ED50 detector) by following protocol reported by Batllori *et al* (2017)<sup>[2]</sup>. 5-HIAA elutes before HVA in the order of increasing hydrophobicity (Heales, 2008)<sup>[6]</sup>. Thiamine, 5-MTHF (Blau and Opladen, 2008)<sup>[3]</sup> and PLP (Batllori *et al.*, 2017)<sup>[2]</sup> were analysed by HPLC with electrochemical and fluorescence detection using ODS column (250 × 4.6 mm). Standards were obtained from Sigma, St. Louis, MO, USA and stock standards were made to a final concentration of 500 µM. The coefficient of variation (CV= [Standard deviation/average] ×100%) from 15 samples was initially calculated to test the accuracy of methods and it resulted out to be less than 10% for both biogenic amines and vitamins.

Thus, it was considered that the effect of blood contamination on CSF samples was insignificant when it was lower than 10% in comparison to the values in the non- spiked CSF samples.

### 2.3 Ethical Issues

In every case, prior the lumbar puncture and sampling, informed consent was obtained from each patient. Anonymized CSF samples from remnants were collected in our Laboratory after the diagnostic protocols, and were used for this study. All procedures performed in this study

involving human participants were in accordance with the ICMR ethical guidelines for biomedical research. The study was approved by the Ethical committee of Elite Pathological Diagnostic Centre.

## 3. Results and Discussion

CSF analysis offers the most innocuous analytical tool for assessing the cellular and biochemical environment of CNS and hence aids in studying neurometabolomic conditions (Ormazabal *et al.*, 2017)<sup>[16]</sup>. However, quantifying these metabolites in blood or urine can be misleading as they usually depict normal or inconsistent results (Wassenberg *et al.*, 2010)<sup>[20]</sup>. Table 1 depicts the list of several biogenic amines and vitamins acting as biomarkers along with the neurological diseases they are associated with.

CSF RBC contamination is frequent and has been documented as a substantial confounding factor for accurate elucidation of CSF analysis data of amino acids concentrations and other molecules (Krishnamurthy *et al.*, 2019)<sup>[12]</sup>. This blood contamination might be artifactually due to impaired permeability of the blood–brain barrier, intrathecal bleeding or traumatic lumbar punctures (Krueger *et al.*, 2019; You *et al.*, 2005)<sup>[13,21]</sup>. Therefore, having an estimation of misinterpretation of the metabolic profile due to RBC or plasma contamination is imperative, as lumbar puncture is challenging to perform due to its invasive intervention, and sample collected is sometimes low in volume in pediatric patients (Batllori *et al.*, 2019)<sup>[1]</sup>. However, literature concerning the effects of blood contamination on biogenic amines and vitamins is limited (Verbeek *et al.*, 2008)<sup>[19]</sup>.

Biogenic amines are synthesized in the periphery of some tissues but also in the brain, and no significant transport has been acknowledged between blood and brain so far. There were no significant differences in the concentrations of 5-HIAA and HVA upon blood contamination of CSF (Figure 1), as both blood and CSF have similar concentrations of these biogenic amines (Mori *et al.*, 2003)<sup>[14]</sup>. Interestingly, both HVA and 5-HIAA illustrated lower values when RBC lysis was caused. This decrease could be attributed to the autoxidation of these molecules by haemoglobin and free radicals (Kato *et al.*, 2016)<sup>[10]</sup>, and therefore, care should be taken while interpreting data if CSF has not been subjected to centrifugation before freezing, as low 5-HIAA and HVA concentrations are surrogate biomarkers of serotonin and dopamine deficiencies (Ng *et al.*, 2015)<sup>[15]</sup>. The Fig 1. clearly depicts that centrifugation and RBC removal before freezing rectified the consequences when paralleled to non-spiked CSF.

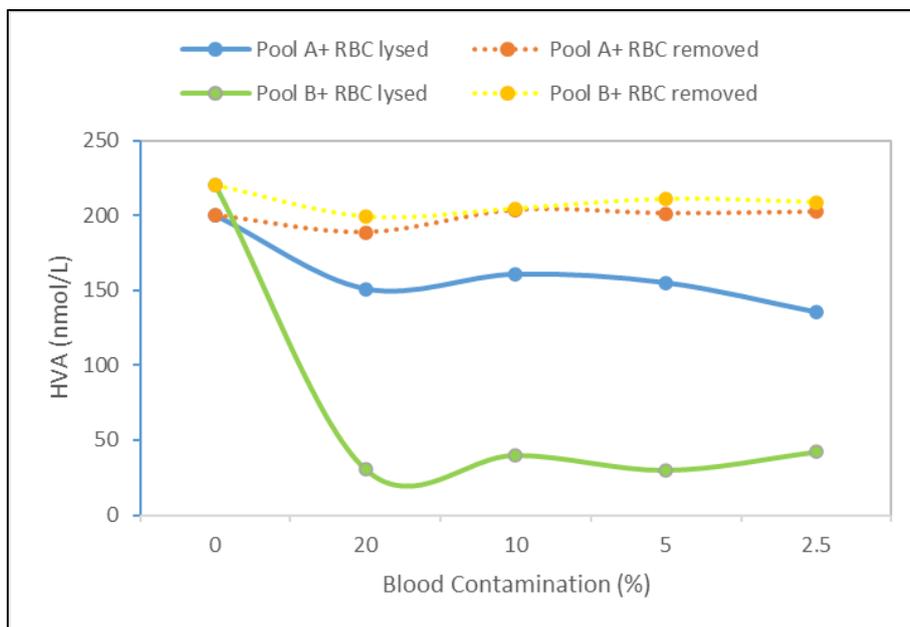
PLP, 5-MTHF, and thiamine depicted higher concentrations when RBC were lysed in contrast to RBC removal before freezing. Vitamins displayed unpredictable results, except for 5-MTHF which is highly concentrated in RBC as compared to plasma and this would elucidate the positive interference witnessed upon RBC lysis, however not in the case of RBC removal. At 2.5% spiked CSF, Thiamine (12.54 to 29.68 nmol/L) and PLP (5.03 to 12.34 nmol/L) had increased values when comparing with non-spiked samples under both the conditions, while it was less significant when RBC were removed (Fig 2.). RBCs have a high activity of thiamine phosphokinase (an enzyme that phosphorylates thiamine to form TDP), and thiamine phosphatases (an enzyme that converts TDP to TMP and thiamine (Batllori *et al.*, 2019; Coolie *et al.*, 2017)<sup>[1,4]</sup>.

This would support the plateau results observed upon RBC lysis, results that were minimized when RBCs were removed. In all the four cases, thiamine concentration was higher in blood than in CSF, and hence, care should be taken while interpreting RBC contamination (Ortigoza-Escobar *et al.*, 2016) [17]. With PLP, the observations were similar, while less significant, even upon RBCs' removal from the CSF samples, PLP depicted higher levels in the spiked CSF samples. A complex intracellular metabolic pathway synthesizes various

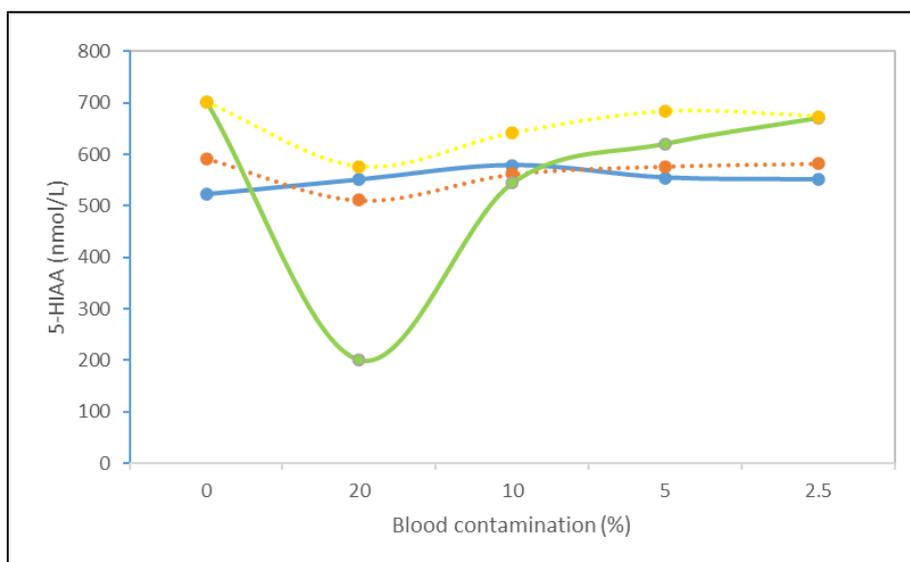
pyridoxine-related vitamers. Additionally, several of these vitamins can be degraded by oxygen-derived free-radicals and nucleophiles, since CSF is low in concentrations of other molecules reacting with these compounds (Footitt *et al.*, 2011) [5]. Therefore, results should be analyzed cautiously in case of TDP and PLP, as diagnostic hallmarks to assess related disorders are lower CSF thiamine and PLP concentrations (Batllori *et al.*, 2019; Footitt *et al.*, 2011; Ortigoza-Escobar *et al.*, 2016) [1, 5, 17].

**Table 1:** Metabolic diseases and CSF biomarkers with expected values

	Biomarkers	Disorders	Expected Value	References
Biogenic Amines	HVA	Dopamine related	High or Low	[10, 14, 15]
	5-HIAA	Serotonin related	Low	[10, 15]
Vitamins	PLP	Transport or metabolic defects	Low	[5, 8]
	5-MTHF	Transport or metabolic defects	Low	[4, 8]
	Thiamine	Transport defects	Low	[4, 17]

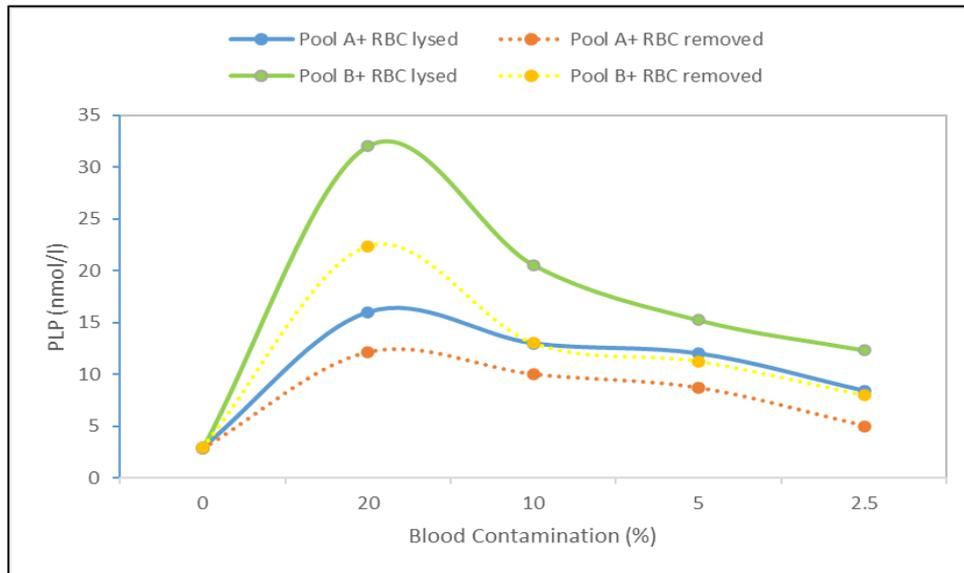


A

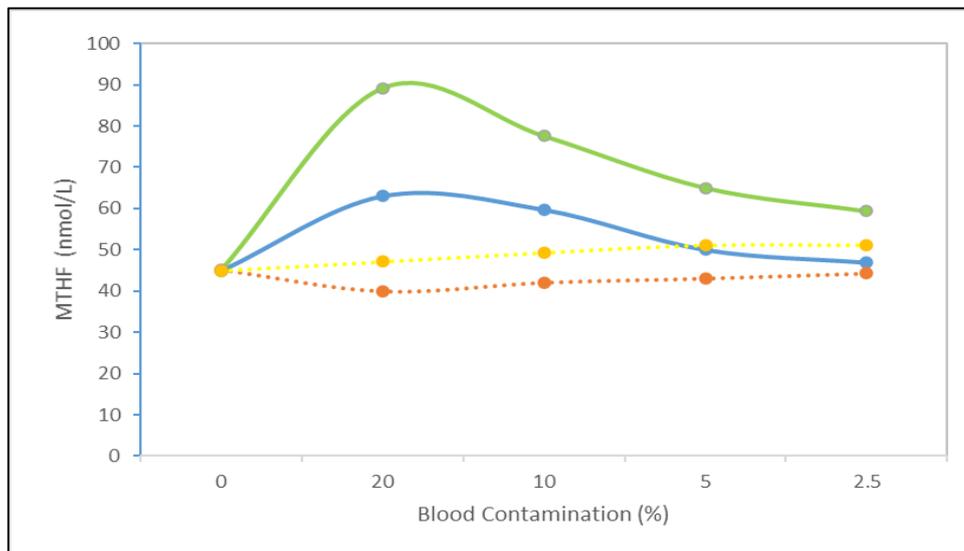


B

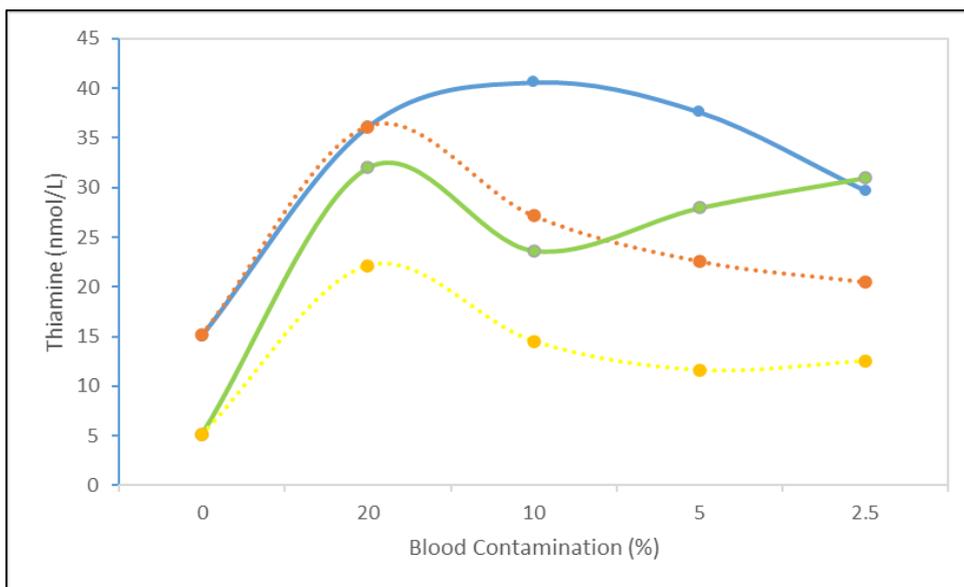
**Fig 1:** Effect of blood contamination on biogenic amines (nmol/L) (A) Homovanillic acid (HVA) (B) 5-hydroxyindoleacetic acid (5-HIAA) analyzed in non-spiked CSF samples and spiked CSF samples (20%, 10%, 5% and 2.5% of whole blood).



A



B



C

**Fig 2:** Effect of blood contamination on vitamins (nmol/L) (A) Pyridoxal 5'-phosphate (PLP) (B) 5-methyltetrahydrofolate (MTHF) (C) Thiamine analyzed in non-spiked CSF samples and spiked CSF samples (20%, 10%, 5% and 2.5% of whole blood).

#### 4. Conclusions

CSF metabolomic investigations involving biogenic amines and vitamins as biomarkers is possible even when remarkable RBC CSF contamination occurs, as CSF centrifugation for removal of RBC prior to freezing eliminates majority of the interferences witnessed. However, the data should be cautiously interpreted, especially for 5-HIAA, HVA PLP and TDP.

#### 5. Acknowledgments

A. K. contributed to the conceptualization and designing, analysis, interpretation of the biochemical data, drafted the original manuscript and approved the final manuscript. J. K. contributed to initial conception and design, analysis of data, reviewing and revising the manuscript. P. S. reviewed, supervised and approved the final draft of this manuscript as submitted. Every author has contributed adequately in this study, satisfying the authorship criteria, and each one has reviewed and approved this version of manuscript.

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