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Preanalytical, analytical, gestational and pediatric aspects of the S100B immuno-assays

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Abstract

Background: Traumatic brain injury management is a tricky issue in children and pregnant women (due to adverse effects of computer tomography). To facilitate management, we report the main analytical performances and reference ranges for blood tests for the well-established S100B biomarker in under-16 children on a DiaSorin® Liaison XL analyzer and in pregnant women on DiaSorin® Liaison XL and Roche Diagnostics® Cobas e411 analyzers.

Methods: Serum S100B concentrations were determined by chemiluminescent immunoassay on a DiaSorin® analyzer in a population of 409 healthy children aged 0–16 years and on DiaSorin®/Roche Diagnostics® instruments in a population of 50 pregnant women (one blood sample for each trimester). The analytical performances of both instruments and the influence of blood cells and skin pigmentation on the assay were also studied.

Results: For children, four age-groups emerged, i.e. 0–3 months (mean: 0.97 µg/L; standard deviation (SD): 0.36; 95th percentile: 1.55), 4–9 months (mean: 0.58 µg/L; SD: 0.30; 95th: 1.18), 10–24 months (mean: 0.31 µg/L; SD: 0.12; 95th: 0.54) and 2–16 years (mean: 0.20 µg/L; SD: 0.07; 95th: 0.32). For pregnant women, serum S100B concentrations were similar to defined ranges for adults and not significantly different between trimesters on DiaSorin® (p=0.652)/Roche Diagnostics® (p=0.877) analyzers. We also found S100B expression (protein, total mRNA) in lymphocytes, an influence of skin pigmentation, and good analytical performances for both instruments.

Conclusions: Data provided here is useful for interpreting serum S100B test results, in terms of preanalytical conditions, analytical performances, pediatric and pregnancy' environment.

Keywords: children; lymphocyte; pregnancy; reference ranges; S100B.

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Introduction

Clinical diagnosis of mild traumatic brain injury (mTBI) is difficult in children (a very exposed population) and pregnant women, as cranial computed tomography (CCT) is strongly limited due to increased tumor risk in children [1] or teratogenic effects in the embryo and fetus [2]. An alternative strategy to CCT has been developed based on blood biomarkers, with S100B protein emerging as the most sensitive and specific candidate biomarker in children and adults [3, 4]. S100B is one of a multigenic family of calcium-binding proteins and is predominantly expressed by central nervous system cells (mainly astroglial but also neuronal cells) [5]. S100B plays a trophic role in age dependent manner and correlates with parameters suggestive of growth as height growth velocity [6] or head circumference [7] in a gender dependent manner [6]. S100B

has been clearly established as a powerful diagnostic tool (compared to CCT) for mTBI management if sample collection is performed within the first 3 hours after trauma [3]. Both DiaSorin® and Roche Diagnostics® have developed systems for in vitro determination of serum S100B protein. For both techniques, it lacks certain essential data for the biological interpretation. On a pre-analytical level, we know that hemolysis does not influence the result [8]. However, the influence of leucocytes has not been clearly studied. On an analytical level, the precision study has never been published. On a post-analytical level, reference ranges in children (unrealized for DiaSorin® technique) and the study of the influence of pregnancy are very important. In addition, it is known that pigmentation of the skin influences the serum S100B concentration (study conducted with the technology DiaSorin® only) [9].

To answer to all these points, we set out to evaluate and compare the pre-analytical (influence of erythrocytes and leukocytes), analytical (imprecision, comparison) and post-analytical (reference ranges in children for the DiaSorin method, influence of pregnancy, influence of skin pigmentation) performances of both the DiaSorin® and Roche Diagnostics® S100B assays techniques.

Materials and methods

Patient inclusions

Clermont-Ferrand teaching hospital used routine consultations to recruit a pooled total of 409 healthy children (males, n=251; females, n=158) aged between 1 day and 16 years (mean=5 years, with 51% aged under 3 years old), 161 healthy adults (males, n=71, females, n=90), and 50 pregnant women. In addition, 19 healthy volunteers with white skin pigmentation and 19 healthy volunteers with black skin pigmentation were recruited. The study was led in accordance with Declaration of Helsinki principles for ethical medical research involving human subjects, and the project was approved by the institutional ethics review boards of the hospital. Written informed consent for venous blood sampling and collection was obtained from all adults or parents before enrolment. Pregnant women gave 3 blood samples, i.e. one per trimester (3rd month, 6th month and 9th month).

Pregnancies were described healthy in response to the lack of pathogenicity criteria systematically sought in the clinical and laboratory monitoring of pregnant women. This monitoring is driven by the recommendations coming from the French High Authority of Health (HAS) and the French National College of Gynecologists and Obstetricians (CNGOF) [10]. Exclusion criteria included multiple pregnancies, intra-uterine growth retardation, gestational hypertension, diabetes, infections, fetal malformations, chromosomal abnormalities and proteinuria. All of the children were delivered at term without perinatal complications, and their clinical history, from birth to the time of blood sampling, was negative for neurologic abnormalities and comorbidities. For all patients, exclusion criteria (known to increase S100B blood concentrations [11]) were: history of traumatic brain injury, central nervous system tumor, neonates with hypoxic ischemic encephalopathy, seizure in the previous 7 days, stroke, cardiopulmonary bypass, Down syndrome, extra-cerebral single or multiple fracture(s) and diagnosed melanoma. All patients presented normal kidney and liver function. Head circumference of the children was determined as previously reported by Remontet et al. [12]. For sampling, venous blood (2 mL) was drawn into a standard serum vial without additive, allowed to clot for at least 30 min, and centrifuged at 1200 *g* for 10 min at 4 °C, and the supernatants were removed and stored at –70 °C until analysis.

Study of the influence of erythrocytes and leukocytes

To study the influence of hematopoietic cellular components of circulating blood on serum S100B concentrations, venous blood samples were taken from healthy volunteers. Whole blood was collected in 10 mL heparinized syringes and 4 mL EDTA tubes. Whole blood collected in heparinized syringes was pelleted for 2 h at room temperature, and the different fractions were recovered for purification and lysis. Erythrocyte fraction was lysed in ammonium chloride solution (Stemcell Technologies®, Vancouver, Canada) for 10 min at 4 °C (Ammonium Chloride Lysing Solution 10×). Leukocyte fraction was lysed by treatment with RIPA lysis buffer (300 µL, 1×) followed by a sonication step. The plasma and the various lysed fractions were centrifuged at 3500 *g* for 15 min for S100B protein assay in each supernatants. For whole blood collected in EDTA, after a buffy coat and red cell lysis for 10 min at 4 °C (Ammonium Chloride Lysing Solution 10×), total leukocyte content was washed in PBS-1% BSA, the pellets were suspended in 500 µL PBS, and the major leukocyte subpopulations (neutrophils, lymphocytes, monocytes) were sorted by flow cytometry (BD FACS AriaSORP, BD Bioscience®, Franklin Lakes, NJ, USA) according to FSC/SSC morphology after elimination of doublets. A fraction of each recovered leukocyte subpopulation was taken to verify purity by simple analysis of morphological graphics on the flow cytometer. The leukocyte sub-populations of 10^6 cells were then centrifuged and each pellet was frozen at –80 °C. The pellets of neutrophils, lymphocytes and monocytes were then subjected to cell lysis in RIPA lysis buffer (300 µL, 1×) followed a sonication step, and centrifuged at 3500 *g* for 15 min for S100B protein assay (with DiaSorin® method) on the supernatants.

Total RNA was extracted from pellets of neutrophils, lymphocytes, monocytes and human brain (positive control) using TRIzol reagent (Invitrogen®, Carlsbad, CA, USA). RNA quantity was determined by spectrophotometry at 260 and 280 nm (protein ratios). RNA quality was studied by gel electrophoresis (2% agarose) to test for the presence of intact 28S and 18S RNA bands. The cDNA was synthesized from 3 µg of RNA using a Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen®). The primer sequences used for classic RT-PCR were originally generated by Primer 3 (Whitehead Institute for Biomedical Research/MIT) as AAGGGAGGGAGACAAGCACA for forward primer and TCCTGGAAGTCACATTCGCC for reverse primer (S100B gene Accession no. NM_006272, product size: 159 bp). RT-PCR assays counted 40 amplification cycles with an initial denaturation step of 10 min at 94 °C, final extension of 10 min at 72 °C, and for each cycle, a denaturation step of 45 s at 94 °C, a hybridization step of 45 s at 61 °C, and an elongation step of 45 s at 72 °C. A negative control

for amplimer contamination was set up using a complete PCR mix without cDNA. We ran a positive housekeeping gene (36B4/RPL0) on each extraction as technical positive control. All experiments were performed in duplicate. All PCR products were separated on a 2% agarose gel and sequenced on both strands to confirm the specificity of the reaction.

Study of precision and comparison

The repeatability (n=30 in 1 day) and reproducibility (n=30 over 15 days) of the methods were determined with two quality controls (ref #11731416190 for the Roche Diagnostics® system and ref #319112 for the DiaSorin® system) and two laboratory-made serum pools at clinically-relevant decision levels, which included pools at median and highly pathological range within the reference range. For each pool, thirty 400 µL aliquots were prepared and frozen at −80 °C. For the measurements, which were carried out twice daily over 15 days, aliquots were freshly defrosted, centrifuged, and measured using both the DiaSorin® and Roche Diagnostics® methods. For comparison of the methods, parallel measurements were performed in 161 serum samples from healthy adult volunteers.

S100B assay

Serum S100B concentrations were determined by an electrochemiluminescence immunoassay on a Cobas e411 analyzer (Roche Diagnostics®, Meylan, France) and on a Liaison XL analyzer (DiaSorin®, Saluggia, Italy). S100B calibration curve was linear up to 39 µg/L for the Roche Diagnostics® method and 30 µg/L for the DiaSorin® method. The lower limit of detection is 0.005 µg/L for the Roche Diagnostics® assay and 0.02 µg/L for the DiaSorin® assay.

Statistics

Statistical analysis was performed using SAS 9.1.3 software (SAS Institute®, Cary, NC, USA). For pregnant women, it seemed difficult to propose a priori sample size estimation in order to achieve the main objective of our study. However, 44 subjects would highlight an effect size equals 0.5 for a type I error α=0.05 (two-tailed), a statistical power of 90% and a correlation coefficient of 0.05. Considering possible missing data, we have proposed to include a minimum of 50 patients. Clinical literature and statistical works concerning sample size estimation about longitudinal study give some reasons to accept this sample size [13]. For descriptive analyses, data are presented as means (SD; range; 95th percentile). Correlations (between mean S100B and head circumference or age, between the two methods) were tested by Spearman's rank correlation coefficient, according to statistical distribution. The Mann-Whitney test (conditions of Student's t-test were not met: normality verified by the Shapiro-Wilk test and assumption of homoscedasticity studied using the Fisher-Snedecor test) was used to compare S100 between age groups (< and >24 months) and S100B concentrations between different groups (white vs black skin pigmentation). Age-dependent reference values were computed with quantile regression as 95% confidence bands as previously reported by Bouvier et al. [7]. The analyses were completed using multivariate regression to take into account adjustment on possible confounding factors as gender and age. A particular attention was focused on interaction age × gender. Concerning the statistical power, a sample of more than 400 children could be considered acceptable for this analysis according to a recent work [14]. Concerning repeated measures, S100B values were compared between first, second and third trimester of pregnancy by random-effect models considering a log-transformation to access the normality of S100B (dependent variable). These models, particularly adapted to longitudinal data, allowed to determine whether S100B concentrations varied during pregnancy taking into account between and within patient variability. Differences were considered statistically significant at $p<0.05$.

Results

Influence of erythrocytes and leukocytes

S100B protein was assayed on the Liaison XL (DiaSorin®) analyzer in plasma and supernatants of the leukocyte and erythrocyte fractions (Table 1A). Plasma S100B concentration was normal (0.071 µg/L) in a healthy volunteer. S100B

Table 1: Study of the influence of hematopoietic cellular components in serum S100B assays.

(A) S100B protein concentrations (DiaSorin method) in the different fractions of whole blood (obtained after 2 h of sedimentation) from a healthy volunteer.

	Plasma	Supernatant of the erythrocyte fraction	Supernatant of the leukocyte fraction
S100B concentration, µg/L	0.071	<0.02	6.95

(B) S100B protein concentrations (DiaSorin method) in the different leukocyte subpopulations (10^6 cell pellets) of whole blood (obtained by flow cytometry) from a healthy volunteer.

	Supernatant of the neutrophil fraction	Supernatant of the monocyte fraction	Supernatant of the lymphocyte fraction
S100B concentration, µg/L	<0.02	<0.02	2.74

concentration in the erythrocyte fraction was under the lower limit of detection (<0.02 µg/L). S100B concentration in the leukocyte fraction was 6.95 µg/L.

S100B protein was measured on the Liaison XL (DiaSorin®) in the 3 supernatants from leukocyte subpopulations sorted by flow cytometry (Table 1B). S100B concentration in the neutrophil and monocyte fractions was under the lower limit of detection (<0.02 µg/L). S100B concentration in the lymphocyte fraction was 2.74 µg/L. mRNA gene expression of S100B protein in the leukocyte subpopulations was studied by RT-PCR. Only lymphocytic cells expressed the S100B transcript (Figure 1).

Analytical performance

For L (low level) control reproducibility, 30 measurements taken over 15 days were used to calculate a coefficient of variation (CV) of 1.8% (mean 0.17 µg/L) for the Roche Diagnostics® method and 5.7% (mean 0.46 µg/L) for the DiaSorin® method. Repeatability was evaluated by 30 repeated measurements of the same controls and led to a calculated CV of 1.4% (mean 0.18 µg/L) for the Roche Diagnostics® method and 5.6% (mean 0.48 µg/L) for the DiaSorin® method (Table 2). For H (high level) control, 30 measurements taken over 15 days were used to calculate a CV of 2.1% (mean 2.33 µg/L) for the Roche Diagnostics® method and 5.3% (mean 4.48 µg/L) for the DiaSorin® method. Repeatability was evaluated by 30 repeated measurements of the same controls and led to a calculated CV of 1.1% (mean 2.27 µg/L) for the Roche Diagnostics® method and 4.2% (mean 4.42 µg/L) for the DiaSorin® method (Table 2).

For "L pool serum" reproducibility, 30 measurements taken over 15 days were used to calculate a CV of 1.9% (mean 0.12 µg/L) for the Roche Diagnostics® method and 6.1% (mean 0.15 µg/L) for the DiaSorin® method. Repeatability was evaluated by 30 repeated measurements of the same controls and led to a calculated CV of 2.0% (mean 0.12 µg/L) for the Roche Diagnostics® method and 4.4% (mean 0.12 µg/L) for the DiaSorin® method (Table 2). For "H pool serum", 30 measurements taken over 15 days were

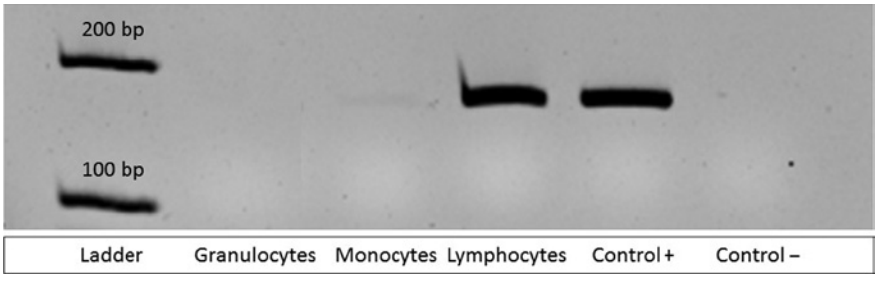

Figure 1: S100B mRNA expression in human leukocyte subpopulations.
RT-PCRs were performed on total mRNA extracted from leukocyte subpopulations (obtained by flow cytometry), i.e. granulocytes, monocytes and lymphocytes. In the absence of cDNA matrix, a negative control (Control-) was performed. Positive control is a RT-PCR product on total mRNA extracted from human brain.

Table 2: Precision study of the S100B assay on COBAS e411 (Roche Diagnostics®) and LIAISON XL (DiaSorin®) analyzers.

Method	Samples	n	Repeatability				Reproducibility			
			Mean, µg/L	SD	CV, %	Manufacturer CV, %	Mean, µg/L	SD	CV, %	Manufacturer CV, %
Roche Diagnostics®	L pool serum	30	0.12	0.002	2	1.5	0.12	0.002	1.9	4.4
	L control	30	0.18	0.003	1.4	1.0	0.17	0.003	1.8	3.1
	H pool serum	30	0.62	0.004	1.3	2.1	0.61	0.012	1.9	5.3
	H control	30	2.27	0.026	1.1	0.7	2.33	0.048	2.1	3.2
DiaSorin®	L pool serum	30	0.12	0.005	4.4	3.2	0.15	0.009	6.1	4.3
	L control	30	0.48	0.027	5.6	2.9	0.46	0.027	5.7	2.5
	H pool serum	30	0.69	0.028	4.0	2.6	0.69	0.026	3.7	3.4
	H control	30	4.42	0.188	4.2	2.5	4.48	0.239	5.3	4.2

L, low; H, high; SD, standard deviation; CV, coefficient of variation.

used to calculate a CV of 1.9% (mean 0.61 µg/L) for the Roche Diagnostics® method and 3.7% (mean 0.69 µg/L) for the DiaSorin® method. Repeatability was evaluated by 30 repeated measurements of the same controls and led to a calculated CV of 1.3% (mean 0.62 µg/L) for the Roche Diagnostics® method and 4.0% (mean 0.69 µg/L) for the DiaSorin® method (Table 2).

Correlation between DiaSorin® S100B and Roche Diagnostics® S100B (n=161) values for the entire measurement range was: r=0.92, slope 1.27, intercept 0.015 (Figure 2A). Bland-Altman plot for the entire measurement range showed lower values for Roche Diagnostics® S100B (Figure 2B).

Reference ranges for serum S100B in children with the DiaSorin® method

All 409 children presented measurable serum S100B protein concentrations. S100B concentration in the global cohort of 409 children aged 0–16 years old (with 51% under 3 years old) averaged 0.31 µg/L (SD 0.24; range 0.04–1.59), with no significant gender-related difference (p=0.19). As previously described with the Roche Diagnostics® method [7], we were able to stratify fine-grained age-groups for mean S100B concentrations (Table 3) and to identify at least 4 wider age-brackets (without statistical differences in mean S100B between the mixed subgroups), i.e. 0–3 months with mean serum S100B at 0.97 µg/L (SD 0.36; range 0.46–1.59 µg/L); 4–9 months with mean serum S100B at 0.58 µg/L (SD 0.30; range 0.15–1.36 µg/L); 10–24 months with mean serum S100B at 0.31 µg/L (SD 0.12; range 0.09–0.70 µg/L); and 2–16 years with mean serum S100B at 0.20 µg/L (SD 0.07; range 0.05–0.36). Maximum (95th percentile) serum S100B reference levels (Table 3) were also derived for these four age groups as 1.55 µg/L for age 0–3 months, 1.18 µg/L for age 4–9 months, 0.54 µg/L for age 10–24 months and 0.32 µg/L for age 2–16 years. Quantile regression analysis for the median pointed to a horizontal trend for patients older than 2 years (Figure 3). In the under-2-years-old age bracket, S100B averaged 0.46 µg/L (SD 0.30; range 0.09–1.59; 95th percentile: 1.12) and was

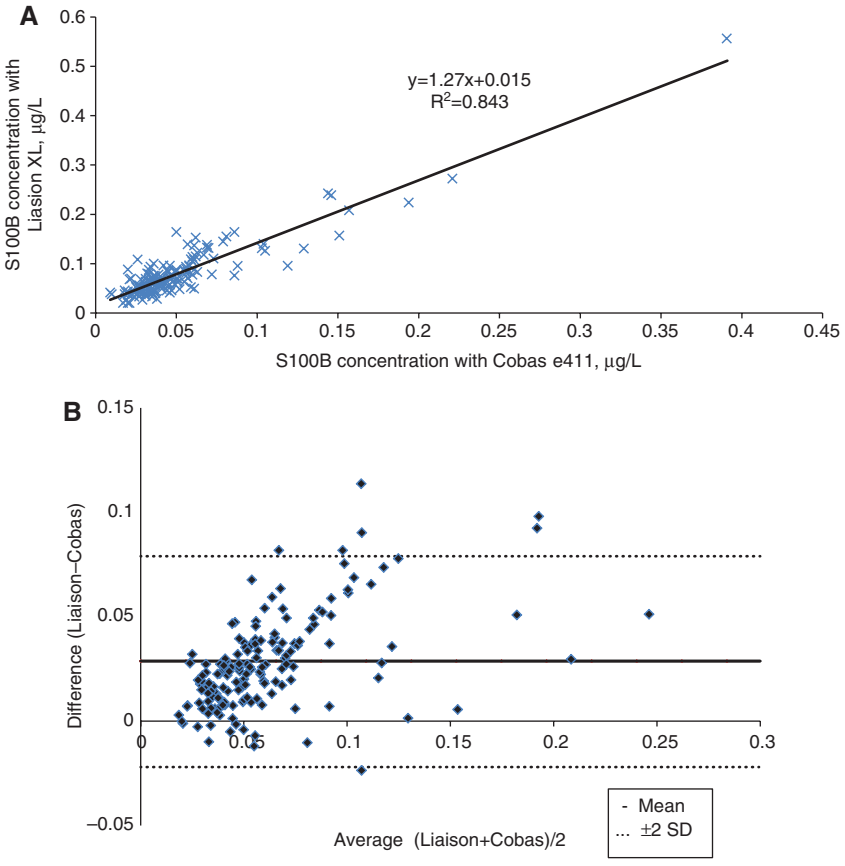

Figure 2: Correlation of COBAS e411 and LIAISON XL S100B concentrations (A) and normalized differences from mean values were calculated according to Bland-Altman plots for the whole range of values (B).

Table 3: S100B levels in children from 0 to 16 years of age using the DiaSorin® method.

Age group	n	S100B, µg/L		
		Mean	SD	95th percentile
0–3 months	19	0.97	0.36	1.55
4–6 months	26	0.69	0.30	1.19
7–9 months	23	0.46	0.27	0.76
4–9 months	49	0.58	0.30	1.18
10–12 months	24	0.37	0.12	0.55
13–15 months	22	0.32	0.14	0.56
16–18 months	24	0.27	0.09	0.44
19–21 months	18	0.34	0.13	0.54
22–24 months	21	0.27	0.09	0.39
10–24 months	109	0.31	0.12	0.54
25–36 months	32	0.22	0.07	0.34
3–4 years	25	0.22	0.06	0.28
4–6 years	33	0.22	0.05	0.31
6–8 years	29	0.21	0.05	0.29
8–10 years	29	0.21	0.05	0.31
10–12 years	33	0.21	0.05	0.29
12–14 years	25	0.20	0.09	0.32
14–16 years	26	0.11	0.06	0.24
2–16 years	232	0.20	0.07	0.32
<24 months	177	0.46	0.30	1.12
>24 months	232	0.20	0.07	0.32

Measurable serum S100B concentrations in different age brackets defined for each group defined by number of samples (n), mean, standard deviation (SD) and 95th percentile.

statistically different ($p<0.001$) from the mean obtained for the over-2-years-old age bracket. In this "<2 years" group, serum S100B concentration was found to decrease with increasing age ($r=-0.65$; $p<0.001$; Figure 3A), with a gender-related difference ($p=0.037$) (Figures 3B and 3C). We also found a significant correlation between serum S100B concentrations and head circumference, defined by the equation: serum S100B (µg/L)=−0.049 × head circumference (cm)+2.69 ($r=0.98$, $p<0.001$).

Influence of pregnancy

S100B concentrations were measured in 50 pregnant women (mean age: 24 years, minimum: 17, maximum: 38 years). Pregnant women gave 3 blood samples, i.e. one per trimester. Average serum S100B of the 50 women was 0.079 µg/L (SD 0.057; range 0.02–0.48) for the Liaison XL analyzer and 0.047 µg/L (SD 0.036; range 0.01–0.34) for the Cobas analyzer. Comparative analysis of mean S100B concentrations between the three trimesters found no significant differences with either the DiaSorin® ($p=0.46$) or Roche Diagnostics® methods ($p=0.15$) (Figure 4).

Influence of skin pigmentation

S100B concentrations were measured in 19 healthy volunteers with white skin pigmentation and 19 healthy volunteers with black skin pigmentation. Mean serum S100B of 19 healthy white-skinned volunteers aged 33–64 years old was 0.068 µg/L (SD 0.031; range 0.06–0.14) with the DiaSorin® method and 0.046 µg/L (SD 0.017; range 0.04–0.11) with the Roche Diagnostics® method. Mean serum S100B of 19 healthy black-skinned volunteers aged 24–53 years old was 0.12 µg/L (SD 0.11; range 0.04–0.2) with the DiaSorin® method and 0.091 µg/L (SD 0.082; range 0.04–0.16) with the Roche Diagnostics® method. Average S100B concentration was significantly higher in healthy black-skinned volunteers than in healthy white-skinned volunteers with both the DiaSorin® ($p=0.01$) and Roche Diagnostics® ($p=0.009$) methods.

Discussion

There are currently two companies specializing in in vitro diagnosis (Roche Diagnostics® and DiaSorin®) that offer automated analyzers able to determine S100B protein concentration in serum based on electrochemiluminescence. Here we evaluated the analytical performances of these two analyzer systems, i.e. the Roche Diagnostics® Cobas e411 and the DiaSorin® Liaison XL.

The precision study (repeatability and reproducibility) found that both methods proved very satisfactory and consistent with figures reported by both manufacturers, with a CV at <2.5% for the Roche Diagnostics® system and <6.5% for the DiaSorin® system. In addition, both methods showed good correlation ($r=0.92$ $p<0.001$) for the determination of S100B protein, as described elsewhere [15–17]. However, the two methods are not interchangeable, as mean vales were 27% higher with the DiaSorin assay. Moreover, this difference between the two techniques increase with the increasing S100B serum concentrations. The increase in concentration between the two techniques has also been reported previously [15–17]. Overall, the results of this study are in line with the literature, as the results of the two techniques are correlated and more precise with the Roche Diagnostics® method but they are not interchangeable due to consistently higher values with the DiaSorin method. Values at the recommended cut-off (95th percentile) were also different, at 0.10 µg/L for the Roche Diagnostics® method vs. 0.15 µg/L for the DiaSorin® method [16], which brings further arguments for recommending the use of a single S100B protein assay technique for patient management and follow-up.

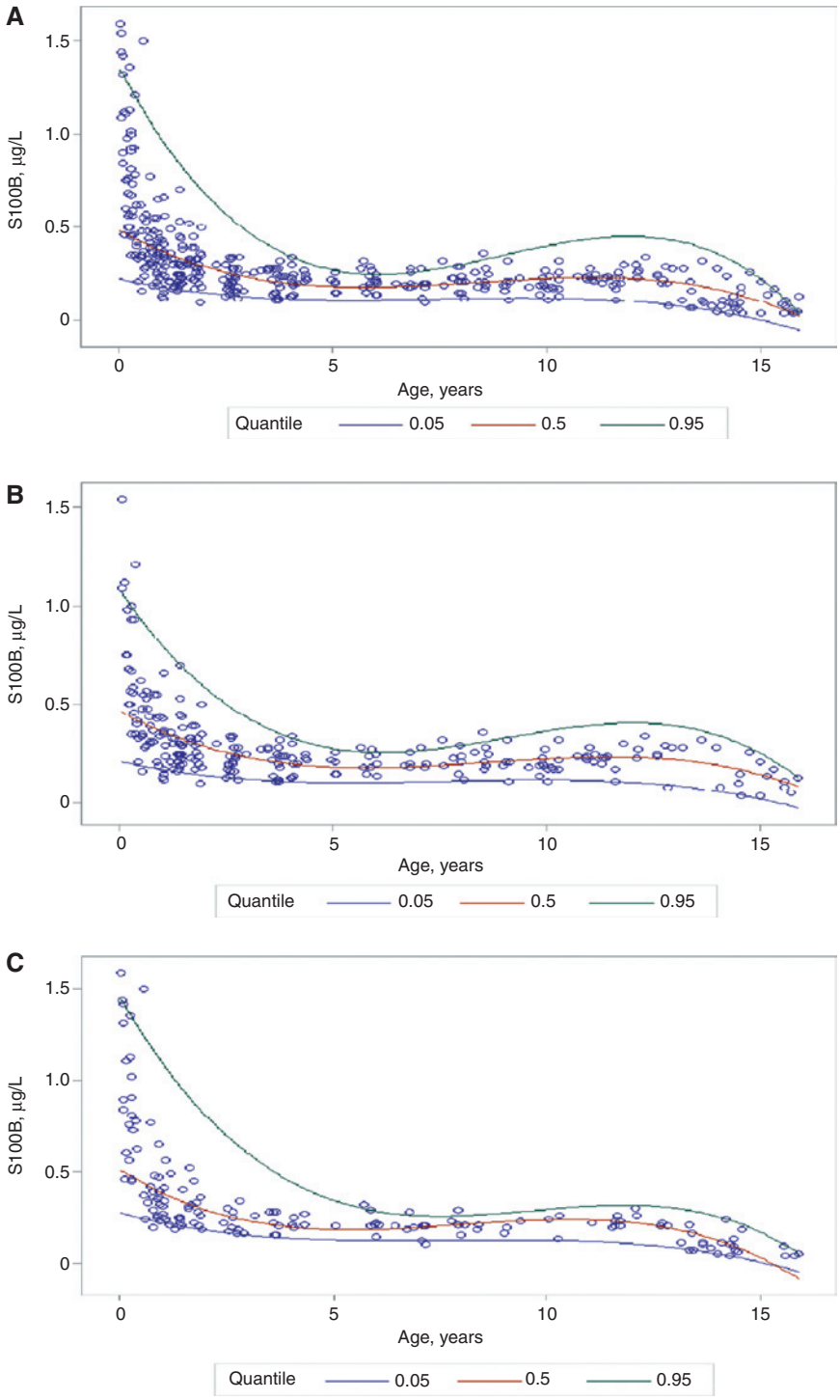

Figure 3: Quantile regression analysis of serum S100B concentrations in healthy children using the DiaSorin® method.
Serum S100B concentrations were determined in a cohort of 409 healthy children aged 0–16 years (A), separated secondly in a cohort of 251 healthy boys (B) and in a cohort of 158 healthy girls (C).

The continued absence of a standardized S100 immunoassay could clearly explain this observed difference.

This study also established that erythrocytes do not contain any S100B protein, thus confirming the absence of hemolytic interference in serum S100B protein assays [8] in contrast to other biomarkers of mTBI such as neuron specific enolase. Further analysis also found that leukocyte fractions, and specifically lymphocytes, contain

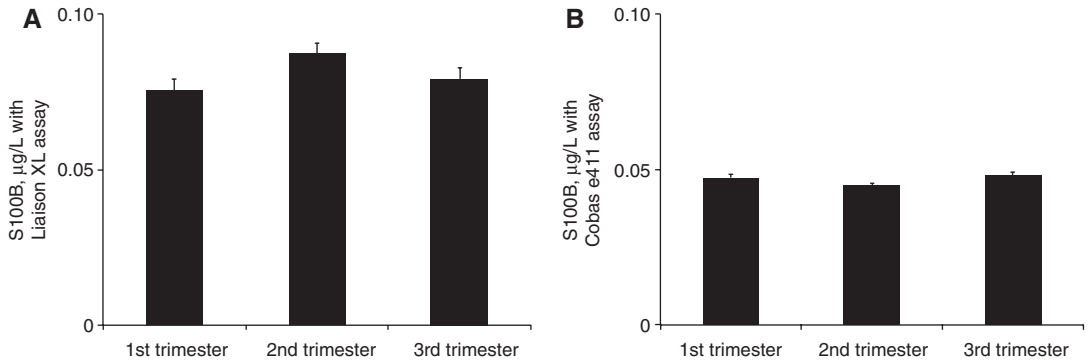

Figure 4: Influence of pregnancy on serum S100B concentrations.
S100B concentrations were measured in 50 pregnant women with DiaSorin® (A) and Roche Diagnostics® (B) methods. Pregnant women gave three blood samples, i.e. one per trimester.

significant amounts of S100B. The literature to date only carries reports using flow cytometry techniques, which have demonstrated that the cells containing protein S100B are CD8+ T lymphocytes and NK cells [18]. To differentiate lymphocyte cell secretion capture, here we showed for the first time that S100B mRNA is present in lymphocytes. The results found here indicate the importance of swift sample routing to the lab for centrifugation in order to safeguard against assay contamination by lymphocyte lysis.

This is the first study to report reference ranges for serum S100B protein with the DiaSorin® method in what, to date, is the largest published population in healthy pediatric patients aged up to 16 years old. This work aligns to two similar studies done on the Roche Diagnostics® method [7, 19] and confirms the first studies of Portela et al. [20] and Gazzolo et al. [6] done on Sangtec® method. Indeed, Gazzolo et al. showed in their study a reference curve produced from 1004 children. They demonstrate a correlation with age and height growth velocity in a gender dependent manner. The biological data reported here provides clinicians with practical pointers for the management of mTBI in pediatric patients and could help reduce the use of currently-recommended CCT procedures in this population [4]. Our analysis of the different groups identified clearly confirms a negative correlation between S100B concentrations and age, in agreement with other reports [6–7, 19–20]. Without losing sight of the differences in terms of methods, our values are also consistent with figures reported in neonates (0.43–2.7 µg/L) [21]. These high values in the beginning of life could be explained by normal vaginal delivery involving a brief brain injury [22]. Although this issue is still open to debate, high S100B concentrations in the "under-2-years-old" age group could also be linked with putative blood-brain barrier permeability and cerebral circulation, increased protein turnover in neuronal cells, and low renal excretion of S100B. Another explanation for higher concentrations of blood S100B in neonates and children than in adults could tie into the fact that S100B protein plays a role in dynamic central neurodevelopmental processes. S100B is involved in glial cell proliferation and maturation, synaptogenesis and fiber sprouting [5]. This explanation is strongly supported by previous results, as correlation with height growth velocity [6], and by our finding that head circumference correlates to blood S100 concentration – a relationship that could be used to approximate the reference values after simple clinical examination.

In adults, we and other teams have discussed the value of using S100B protein in mTBI management in an effort to cut CCT use by 30% and limit unnecessary exposure to radiation doses [3]. This use of S100B is particularly interesting in the most X-ray-sensitive populations such as pregnant women. Here, we evaluated for the first time whether the physiological changes during pregnancy, including volume expansion and relative hemodilution have an influence on serum S100B protein concentrations. A recent study demonstrated that a few parameters (out of 36 tested) were unaffected during uncomplicated pregnancy, delivery, and the early postpartum period, suggesting a need to implement gestational age-specific reference intervals [23]. S100B has already been tested in pregnancy in amniotic fluid or cord blood to evaluate the fetal brain damages or the presence of a protein's gradient between fetal and maternal bloodstreams [24, 25], but not in maternal blood during physiological pregnancy. Both the Roche Diagnostics® and DiaSorin® methods found that S100B levels in pregnant women were not significantly different between trimesters and largely comparable to the general population ($p<0.001$). By

extension, this would mean that clinical and biological interpretation of serum S100B protein would be no different for pregnant women, assuming mTBI is confirmed in this specific population.

Both the Roche Diagnostics® and DiaSorin® methods found significantly higher S100B concentrations in black-skinned individuals. This result, which confirms previous findings [9], can be explained by the fact that melanocytes are non-brain cells that synthesize small amounts of S100B protein under physiological conditions.

In conclusion, this study provides valuable new data for the management of patients particularly vulnerable to mTBI (children, pregnant women) and for the concerted interpretation of S100B assay results by biologists and clinicians.

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