Emma Calluy, Charlotte Beaudart, Majed S. Alokail, Nasser M. Al-Daghri, Olivier Bruyère, Jean-Yves Reginster, Etienne Cavalier and Aurélie Ladang*

Confounding factors of the expression of mTBI biomarkers, S100B, GFAP and UCH-L1 in an aging population

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Abstract

Objectives: To evaluate some confounding factors that influence the concentrations of S100 calcium binding protein B (S100B), glial fibrillary acidic protein (GFAP), and ubiquitin carboxyl-terminal hydrolase L-1 (UCH-L1) in older individuals. Indeed, recent guidelines have proposed the combined use of S100B and the "GFAP-UCH-L1" mTBI test to rule out mild traumatic brain injuries (mTBI). As older adults are the most at risk of mTBI, it is particularly important to understand the confounding factors of those mTBI rule-out biomarkers in aging population.

Methods: The protein S100B and the "GFAP and UCH-L1" mTBI test were measured using Liaison XL (Diasorin) and Alinity I (Abbott), respectively, in 330 and 341 individuals with non-suspected mTBI from the SarcoPhAge cohort.

Results: S100B, GFAP and UCH-L1 were all significantly correlated with renal function whereas alcohol consumption, Geriatric Depression Score (GDS), smoking habits and anticoagulant intake were not associated with any of these three biomarkers. Body mass index (BMI) and age were associated with GFAP and UCH-L1 expression while sex and mini-mental state examination (MMSE) were only associated with GFAP. According to the manufacturer's cut-offs for mTBI rule-out, only 5.5 % of participants were positive for S100B whereas 66.9 % were positive for the "GFAP-UCH-L1" mTBI test. All positive "GFAP-UCH-L1" mTBI tests were GFAP+/UCH-L1-. Among individuals with cystatin C>1.55 mg/L, 25 % were positive for S100B while 90 % were positive for the mTBI test.

Conclusions: Our data show that confounding factors have different impacts on the positivity rate of the "GFAP-UCH-L1" mTBI test compared to S100B.

Keywords: mild traumatic brain injury; blood-based biomarkers; specificity; confounding factors; normally aging non-suspected cases

***Corresponding author: Mrs. Aurélie Ladang**, PhD, Clinical Chemistry Department, CHU de Liège, University of Liège, Avenue de L'Hôpital, 1, 4000 Liège, Belgium, E-mail: aladang@chuliege.be. https://orcid.org/0000-0002-5316-3108

Emma Calluy and Etienne Cavalier, Clinical Chemistry Department, CHU de Liège, University of Liège, Liège, Belgium. https://orcid.org/0000-0003-0947-2226 (E. Cavalier)

Charlotte Beaudart, WHO Collaborating Center for Public Health Aspects of Musculoskeletal Health and Aging, Division of Public Health, Epidemiology and Health Economics, University of Liège, Liège, Belgium; and Clinical Pharmacology and Toxicology Research Unit (URPC), NARILIS, Department of Biomedical Sciences, Faculty of Medicine, University of Namur, Namur, Belgium

Majed S. Alokail, Protein Research Chair, Biochemistry Department, College of Science, KSU, Riyadh, Kingdom of Saudi Arabia

Nasser M. Al-Daghri, Chair for Biomarkers of Chronic Diseases, Biochemistry Department, College of Science, KSU, Riyadh, Kingdom of Saudi Arabia

Olivier Bruyère, WHO Collaborating Center for Public Health Aspects of Musculoskeletal Health and Aging, Division of Public Health, Epidemiology and Health Economics, University of Liège, Liège, Belgium; and Department of Sport and Rehabilitation Sciences, University of Liège, Liège, Belgium

Jean-Yves Reginster, WHO Collaborating Center for Public Health Aspects of Musculoskeletal Health and Aging, Division of Public Health, Epidemiology and Health Economics, University of Liège, Liège, Belgium; and Protein Research Chair, Biochemistry Department, College of Science, KSU, Riyadh, Kingdom of Saudi Arabia. https://orcid.org/0000-0001-6290-752X

Introduction

Annually in Europe, over 2.5 million individuals undergo the experience of traumatic brain injury (TBI) at least once [1]. Especially, TBI mostly impact children and young adults under 24 years old and older adults above 75 years old [2]. The severity of TBI is particularly serious in older people, as its repercussions are accentuated by the presence of comorbidities leading to a potential death of the patients [2, 3].

TBI can be classified in different categories which are severe, moderate, and mild TBI, according to the level of Glasgow coma score (GCS) [4]. Mild traumatic brain injury (mTBI) is further defined by a GCS of 13–15 points combined to one of the following criteria: confusion or disorientation, loss of consciousness for less than 30 min, post-traumatic amnesia for less than 24 h or transient neurological abnormalities [1]. mTBI accounts for almost 95 % of all the TBIs and is usually

confirmed by a positive CT scan [4]. Even if the CT scan has the advantage of providing an early prognostic, the tool contains some limitations that may explain why the CT scan is considered as overused compared to its efficiency in diagnosing the condition [1, 5, 6]. Actually, the Canadian CT Head Rule (CCTHR) and the French guidelines have been published to help reduce the use of CT scan.

In the French guidelines, the combined use of the measurement of biomarkers, namely S100 calcium binding protein B (S100B) and the glial fibrillary acidic protein-ubiquitin carboxyl-terminal hydrolase L-1 ("GFAP-UCH-L1") mTBI test is recommended to rule out mTBI [1]. GFAP is physiologically expressed in astrocytes [7], UCH-L1 in neurons [8] and S100B both in astrocytes and neurons [9]. The level of these three proteins is known to be low in physiological conditions but increased specific pathological conditions such as in sport-related concussion, ischemic stroke or Alzheimer disease [10–12]. In case of head trauma, the release of the three biomarkers in the bloodstream could be caused by cells rupture and a probable blood brain barrier increased permeability due to the impact during mTBI [13].

Both S100B and mTBI tests have demonstrated a good sensitivity in the general population [14, 15] and the need to combine several biomarkers is justified by the different half-lifes and time of release of the three proteins [9, 16]. Indeed, S100B has been largely characterized to rule out mTBI within 3 h post-concussion whereas the "GFAP and UCH-L1" mTBI test offers the opportunity of extending the rule-out up to 12 h [15]. However, the specificity of both S100B and the "GFAP and UCH-L1" mTBI test has been reported to be age dependent [5, 17, 18]. Although S100B has been extensively characterized [9], data on the mTBI test remain scarce and the confounding factors of GFAP and UCH-L1 are largely unreported [19]. Therefore, the aim of this study was to assess several confounding factors that could influence the concentration of S100B and the "GFAP and UCH-L1" mTBI test, in the normal aging population to further understand the impact of these confounding factors on the positivity rate of these tests in older adults.

Materials and methods

Sample collection

The SarcoPhAge study (for sarcopenia and physical impairment with advancing age) is a long-term prospective study with a 10-year ongoing follow-up [20]. The SarcoPhAge cohort is composed of 534 community-dwelling Belgian participants who were older than 65 years old at the time of the inclusion [20]. The recruitment was done by newspaper advertisement [20]. Limb amputation and BMI over 50 kg/m^2 were the only exclusion criteria in this cohort [20]. Comorbidities were self-reported through a questionnaire completed at the time of inclusion and cognition has been assessed with mini-mental state examination (MMSE). History of falls was self-reported but no data were gathered concerning recent mTBI event [20]. Of the 534 participants recruited, only 409 had a serum sample collected at time of the inclusion (June 2013–June 2014). Given that this cohort has been used in several other studies, only 330 samples had enough left-over volume to properly measure S100B and 341 samples had enough left-over volume for "GFAP-UCH-L1" measurements. The SarcoPhAge study was approved by the Ethics Committee of the CHU de Liège (2012/277).

Laboratory analysis

The level of GFAP and UCH-L1 was measured in 341 samples thanks to the "GFAP-UCH-L1" mTBI test on an Alinity I system from Abbott (Abbott, USA) following manufacturer's instructions. In our hands, the analytical coefficients of variation were 2.9 % for UCH-L1 (mean concentration: 73.61 pg/mL) and 3.69 % for GFAP (mean concentration: 16.47 pg/mL). Abbott proposes positivity cut-offs for the mTBI of 35 pg/mL for GFAP and 400 pg/mL for UCH-L1. If both biomarker concentrations are below the cut-offs, then the mTBI test is considered as negative whereas if GFAP alone, UCH-L1 alone or both are above the cut-off, then the mTBI test would be considered as positive [14]. Whenever the test is GFAP+/UCH-L1-, GFAP-/UCH-L1+ or GFAP+/UCH-L1+, the mTBI test is considered as positive without any additional information.

The protein S100B was measured in 330 samples thanks to the Liaison XL S100 kit (DiaSorin, Italy) following manufacturer's instructions. In our hands, the analytical coefficients of variation were 12.045 % for low level of control (mean concentration: 0.2225 µg/L) and 11.26 % for high level of control (mean concentration: 3.035 µg/L). DiaSorin proposed a positivity cut-off at 0.15 µg/L. If the biomarker concentration is below the cut-off, the test is considered as negative and if the biomarker concentration is above the cut-offs, the test is considered as positive [21].

Serum cystatin C was determined with the Roche Cobas turbidimetric assay (Mannheim, Germany) and the cut-off for chronic kidney disease (CKD) was set at 1.55 mg/L. The estimation of glomerular filtration rate (eGFR) was calculated with the 2012 CKD-EPI formula [22, 23].

Statistical study

Firstly, variables were assessed for normality using two techniques (Shapiro-Wilk test and histograms) but none of the continuous variable shown a normal distribution. Therefore, variables were expressed with their median and interquartile range (IQR) and all subsequent tests used were nonparametric tests. Subgroups have been created for each covariate to make descriptive statistics. When the variable was categorical, a Kruskal–Wallis test has been carried out. When the variable was continuous data, rank correlations (Spearman test) were performed to investigate their association with S100B/GFAP/UCH-L1 results. The Spearman correlation has demonstrated if two continuous variables were positively or negatively associated. To ensure that the two cohort are statistically comparable, a chi-squared test was used for categorical data and a Mann–Whitney test for continuous data.

The studied confounding factors were age, sex, BMI, self-reported neurological disorders, MMSE, Geriatric Depression Score (GDS), cystatin C, eGFR, alcohol consumption, smoking habits, anticoagulant/antiaggregant intake. Finally, for the multivariate analysis, each biomarker concentration was considered as the dependent variable and each significant respective confounding factor of the univariate analysis was added to the model as independent variables.

Reference range (2.5–97.5) were calculated using the "age-related reference interval" centile method after a single round of outliers exclusion according to Tukey method and after logarithmic transformation. The reference population was individuals with cystatin C<1.55 mg/L, MMSE≥26 and absence of self-reported neurological troubles.

For all statistical tests, the level of significance was fixed at 0.05. All statistic tests have been realized on Medcalc® (Medcalc software, Belgium).

Results

Cohort description

The SarcoPhAge cohort had a median age of 72 years old, a median BMI of 26.03 kg/cm^2 and a median eGFR of 66 mL/min/1.73 m^2 at the inclusion with a ratio of 55 % of female for 45 % of male. Less than 4 % reported neurological disorders and 8 % had cognitive troubles evaluated through MMSE≤25. We observed no statistical difference between the 341 and 330 included participants (Table 1, Supplementary Table 1).

Table 1: Description of the characteristics of the SarcoPhAge cohort (%).

	Description of the cohort, %
Median age, years (IQR)	72.00 (9)
Gender	
Male	45
Female	55
BMI	
<20 kg/m^2	5
20–24 kg/m^2	34.5
25–29 kg/m^2	39.5
≥30 kg/m^2	21
Neurological disorders	
Yes	3.5
No	96.5
MMSE	
≤25	8
>25	92
GDS	
<5	67
5–9	25
>9	8
Cystatin C	
>1.55 mg/mL	6
≤1.55 mg/mL	94
eGFR, n	
eGFR<60 mL/min/1.73 m^2	36
eGFR≥60 mL/min/1.73 m^2	64
Smoker	
Yes	8
No	92
Alcohol consumer	
Yes	52.5
No	47.5
Anticoagulant/aggregate intake	
Yes	39
No	61

MMSE, mini-mental state examination; GDS, Geriatric Depression Score.

GFAP expression in normal aging

To study the impact of age, sex, BMI, self-reported neurological disorders, MMSE, GDS, cystatin C level, eGFR, alcohol consumption, smoking habits and anticoagulant/antiaggregant intake on GFAP concentration, we performed a univariate analysis. GFAP was positively associated with age whereas GFAP was negatively associated with BMI, MMSE and renal function (Table 2). Additionally, GFAP was increased in women and in participants that reported neurological troubles (Table 2).

All these significant confounding factors of GFAP were included in a multivariate analysis except for the eGFR since its calculation encompasses age, sex, and cystatin C. In this model, age ($r_{partial}$: 0.1125; p=0.0397), BMI ($r_{partial}$: −0.2008; p=0.0002), cystatin C ($r_{partial}$: 0.1099; p=0.0444), MMSE ($r_{partial}$: −0.1586; p=0.0036), sex ($r_{partial}$: 0.1129; p=0.0388) were all significantly associated with GFAP concentration but the self-reported neurological disorders was not ($r_{partial}$: 0.0425; p=0.4383) (Table 2).

UCH-L1 expression in normal aging

To study the confounding factors of UCH-L1, the same strategy as above was applied. In the univariate analysis, UCH-L1 was positively associated with age and BMI and negatively associated with MMSE, renal function and alcohol consumption (Table 2). UCH-L1 was increased by anticoagulant/antiaggregant intake and decreased by alcohol consumption (Table 2).

All the significant confounding factors of UCH-L1 were then evaluated in a multivariate analysis except for the eGFR as explained before. In this model, age ($r_{partial}$: 0.201; p=0.0001), BMI ($r_{partial}$: 0.191; p=0.0005) and Cystatin C ($r_{partial}$: 0.237; p<0.0001) are all associated with UCH-L1 but not the alcohol ($r_{partial}$:-0.1059; p=0.0525), MMSE

Table 2: Univariate and multivariate analysis for GFAP, UCH-L1 and S100B.

Confounding factors	GFAP				UCH-L1				S100B			
	Univariate model		Multivariate model		Univariate model		Multivariate model		Univariate model		Multivariate model	
	Rho	p-Value	$r_{partial}$	p-Value	Rho	p-Value	$r_{partial}$	p-Value	Rho	p-Value	$r_{partial}$	p-Value
Age	**0.321**	**p<0.0001** [c]	**0.113**	**p=0.0397** [c]	**0.275**	**p<0.0001** [c]	**0.201**	**p=0.0001** [c]	**0.168**	**p=0.0022** [c]	−0.0123	p=0.8245
BMI	**−0.278**	**p<0.0001** [c]	**−0.201**	**p=0.0002** [c]	**0.177**	**p=0.0010** [c]	**0.191**	**p=0.0005** [c]	−0.084	p=0.1285	[b]	
MMSE	**−0.224**	**p<0.0001** [c]	**−0.159**	**p=0.0036** [c]	**−0.134**	**p=0.0135** [c]	0.278	p=0.0595	−0.021	p=0.7100	[b]	
GDS	−0.072	p=0.1853	[b]		−0.036	p=0.5084	[b]		0.070	p=0.2047	[b]	
Cystatin C	**0.262**	**p<0.0001** [c]	**0.110**	**p=0.0444** [c]	**0.351**	**p<0.0001** [c]	**0.237**	**p<0.0001** [c]	**0.192**	**p=0.0005** [c]	**0.1807**	**p=0.001** [c]
eGFR	**−0.321**	**p<0.0001** [c]	[b]		**−0.357**	**p<0.0001** [c]	[b]		**−0.222**	**p<0.0001** [c]	[b]	
Sex (female=1)	[a]	**p<0.0001** [c]	**0.113**	**p=0.0388** [c]	[a]	p=0.1729	[b]		[a]	p=0.0856	[b]	
Anticoagulant/aggregant intake (yes=1)	[a]	p=0.4404	[b]		[a]	**p=0.0108** [c]	0.0845	p=0.1127	[a]	p=0.1020	[b]	
Alcohol consumer (yes=1)	[a]	p=0.1533	[b]		[a]	**p=0.0103** [c]	−0.111	p=0.0525	[a]	p=0.3744	[b]	
Smoker (yes=1)	[a]	p=0.3557	[b]		[a]	p=0.2795	[b]		[a]	p=0.6505	[b]	
Self-reported neurological disorders (yes=1)	[a]	**p=0.0113** [c]	0.043	0.4383	[a]	p=0.0862	[b]		[a]	**p=0.0273** [c]	0.0188	p=0.7355

[a]For categorial confounding factors, Kruskal-Wallis test was performed. Multivariate model: Model was generated from statistically significant covariates of the univariate analysis. [b]Variables not included in the model. [c]Significant confounding factors are in bold. S100B, S100 calcium binding protein B; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin carboxyl-terminal hydrolase L-1; MMSE, mini-mental state examination; GDS, Geriatric Depression Score.

($r_{partial}$: 0.278; p=0.0595) and anticoagulant and/or anti-aggregant intake ($r_{partial}$: 0.08679; p=0.1123) (Table 2).

S100B expression in normal aging

In the univariate analysis, S100B was positively associated with age and negatively associated with renal function (Table 2). Furthermore, S100B was increased in participants self-reporting neurological troubles (Table 2).

When all the significant confounding factors of S100B (except eGFR) were included in a multivariate analysis, only the Cystatin C ($r_{partial}$: 0.1807; p=0.001) was significantly associated with S100B concentration whereas the self-reported neurological disorders and age were not ($r_{partial}$: 0.01875, −0.01231; p=0.7355, 0.8245, respectively) (Table 2).

Reference ranges in aging population

After the evaluation of confounding factors, we decided to calculate 2.5–97.5 reference ranges. To do so, we selected patient with normal MMSE (MMSE≥26) and without CKD (cystatin C<1.55 mg/L) or self-reported neurological troubles. Reference ranges are reported in Table 3.

Impact of confounding factors on the "GFAP-UCH-L1" mTBI and S100B test positivity rate

According to manufacturers' cut-offs, only 18 participants (5.5 %) were positive for S100B, suggesting that almost all aging participants are negative for S100B under physiological conditions. On the opposite, 66.9 % of the participants were positive for the "GFAP-UCH-L1" mTBI test under physiological conditions. All mTBI positive tests were GFAP+/UCH-L1- meaning that "GFAP-UCH-L1" mTBI test

Table 3: Reference ranges for GFAP (pg/mL), UCH-L1 (pg/mL) and S100B (µg/L).

Age, years	GFAP		UCH-L1		S100B	
	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
65	13.6	74.8	68.3	165.3	0.020	0.132
70	15.9	79.0	60.3	194.8	0.020	0.150
75	18.5	84.5	59.6	214.1	0.022	0.161
80	21.6	91.7	66.1	220.0	0.025	0.163
85	25.2	100.8	82.4	211.2	0.029	0.155

S100B, S100 calcium binding protein B; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin carboxyl-terminal hydrolase L-1.

specificity is solely driven by GFAP. Based on those results, the general positivity rate of the "GFAP-UCH-L1" mTBI test in the normal aging population was statistically higher compared to the positivity rate of S100B (Chi-squared= 177.565, p-value<0.0001) (Table 4).

To better understand the link between confounding factors and the positivity rate for S100B and mTBI test, the confounding factors were divided into subgroups. Regarding the impact of age, the median GFAP for the youngest group is at the cut-off level for mTBI positivity (<70 years, median GFAP: 36.9 pg/mL) while the median GFAP for the oldest group is almost double (≥80 years, median GFAP 56.00 pg/mL), meaning that almost all participants older than 80 years old are positive (87.04 %) without suspected mTBI. On the opposite, for S100B, the median in the youngest group is three times lower than the cut-off level for positivity (<70 years, median S100B: 0.056 µg/L) while the median S100B in the oldest group is half of the cut-off level for positivity (≥80 years, median S100B 0.076 µg/L), meaning that although age is associated with S100B expression level in univariate models, it does not impact positivity rate for mTBI detection (Table 5, Supplementary Table 2).

When looking at cystatin C level, renal function is impacting the positivity rates of GFAP and S100B but the effect is more limited for S100B. Indeed, the median GFAP in the group with normal renal function is slightly above cut-off level for mTBI positivity (cystatin C≤1.55 mg/L, median GFAP: 40.0 pg/mL) while the median GFAP in the group with abnormal renal function (cystatin C>1.55 mg/L) is 49.7 pg/mL, meaning that 90 % of participants from this group are positive for mTBI. For S100B, the median of the group with normal renal function is three times lower than the cut-off level for positivity (cystatin C≤1.55, median S100B: 0.058 µg/L) but the median S100B in individuals with cystatin C>1.55 µg/L increased up to 0.10 µg/L which is still below the cut-off level but 25 % of participants were positive (Table 5, Supplementary Table 2).

Finally, for the "GFAP-UCH-L1" mTBI test, 89.3 % of people with an MMSE≤25, 88.9 % of people with a BMI<20 and 83.3 % of people that have reported neurological disorders were positive without any suspicion of mTBI (Table 5). For S100B, 9.1 % of participants who reported having neurological disorders and 7.4 % of participants older than 80 years old had a positive S100B measurement (Table 5).

Table 4: Results of the "GFAP-UCH-L1" mTBI and S100B tests on the SarcoPhAge cohort.

	S100B	GFAP	UCH-L1	"GFAP-UCH-L1"
Positive	18 (5.45 %)	228 (66.86 %)	0	228 (66.86 %)
Negative	312 (94.55 %)	113 (33.14 %)	341 (100 %)	113 (33.14 %)
Total	330	341	341	341

S100B, S100 calcium binding protein B; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin carboxyl-terminal hydrolase L-1.

Table 5: Positivity rates of the "GFAP-UCH-L1" mTBI test and S100B in the SarcoPhAge cohort.

Cofounding factor	"GFAP-UCH-L1" mTBI		S100B	
	n	Positivity rate, n (%)	n	Positivity rate, n (%)
Age				
<70 years	118	69 (58.47 %)	117	4 (3.42 %)
70–74 years	94	55 (58.51 %)	90	3 (3.33 %)
75–79 years	75	57 (76.00 %)	72	7 (9.72 %)
≥80 years	54	47 (87.04 %)	51	4 (7.84 %)
Sex				
Male	153	87 (56.86 %)	150	8 (5.33 %)
Female	188	141 (75 %)	180	10 (5.56 %)
BMI				
<20	18	16 (88.89 %)	16	0
20–24	118	83 (70.34 %)	114	8 (7.02 %)
25–29	134	95 (70.90 %)	131	7 (5.34 %)
≥30	71	34 (47.89 %)	69	3 (4.35 %)
Self-reported neurological disorders				
Yes	12	10 (83.33 %)	11	1 (9.09 %)
No	329	218 (66.26 %)	319	17 (5.33 %)
MMSE				
≤25	28	25 (89.29 %)	27	2 (7.41 %)
>25	313	203 (64.86 %)	303	16 (5.28 %)
GDS				
<5	227	59 (25.99 %)	220	7 (3.18 %)
5–9	85	53 (62.35 %)	82	10 (12.20 %)
>9	26	14 (53.85 %)	26	1 (3.85 %)
Renal function				
Cystatin C≤1.55	320	209 (65.31 %)	309	13 (4.21 %)
Cystatin C>1.55	20	18 (90 %)	20	5 (25 %)
eGFR				
eGFR<60	123	100 (81.3 %)	119	9 (7.56 %)
eGFR≥60	217	127 (58.53 %)	210	9 (4.29 %)
Anticoagulant/antiaggregant				
Yes	133	87 (65.41 %)	130	13 (10 %)
No	208	141 (67.79 %)	200	5 (2.5 %)
Alcohol consumer				
Yes	179	118 (65.92 %)	174	9 (5.17 %)
No	162	110 (67.90 %)	156	9 (5.77 %)
Smoker				
Yes	28	18 (64.29 %)	26	2 (7.69 %)
No	313	210 (67.09 %)	304	16 (5.26 %)

S100B, S100 calcium binding protein B; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin carboxyl-terminal hydrolase L-1; MMSE, mini-mental state examination; GDS, Geriatric Depression Score.

Discussion

This study focuses on confounding factors of S100B, GFAP and UCH-L1 in normal aging and evaluates the impact

of these confounding factors on the positivity rate of the protein S100B and "GFAP-UCH-L1" mTBI test in non-suspected cases of mTBI. In a multivariate model, we showed that GFAP confounding factors are age, BMI, sex as well as cognitive function assessed by MMSE and renal function evaluated through cystatin C measurement. For UCH-L1, these confounding factors were age, BMI and renal function while, for S100B, only renal function was identified.

GFAP confounding factors were already evaluated in a cohort dedicated to Alzheimer's disease (the Bio-FINDER cohort) with similar results regarding creatinine and BMI [12]. GFAP being a known biomarker of cognitive impairment and Alzheimer's disease, its association with MMSE was therefore expected [19]. Yet, the association with sex is more controversial as sex was reported to be associated with GFAP in some but not all cohorts [5, 14]. Knowing that GFAP is independent of anticoagulant/anti-aggregant medication is of particular interest since those drugs are a risk factor for mTBI [24].

Although GFAP confounding factors were reported, UCH-L1 confounding factors are relatively undescribed. Still as a neurological biomarker, its association with age and neurological disease is expected but nothing was reported regarding renal function or BMI [13]. Our data show that although UCH-L1 is associated with age, BMI and cystatin C, the impact of these confounding factors is low compared to the expression levels expected in mTBI.

Regarding S100B, its efficacy for the rule-out of mTBI has been largely studied and most of the confounding factors described [9]. Age is often cited as a confounding factor for S100B [17] and in a recent systematic review, S100B specificity was shown to be age-dependent highlighting the limited clinical value of S100B in the oldest patients [18]. In our study, S100B was associated with age in the univariate analysis but not in the multivariate one. It might be due to the SarcoPhAge cohort itself that only covers older people and not the entire adulthood, therefore masking some age dependent effects. It might also be because a part of the age-dependent effects is related to age-dependent decline of renal function. Unfortunately, chronic kidney disease was not studied in Santing et al. recent systematic review [18] to confirm this hypothesis. Another confounding factor for S100B is skin pigmentation. Unfortunately, we could not evaluate the importance of skin pigmentation since our cohort is a Belgian cohort mostly composed of white skinned individuals. Still, this study highlights that S100B is independent of anticoagulant/antiaggregant intake.

All these confounding factors do not have the same impact on the positivity rate of the protein S100B or the "GFAP-UCH-L1" mTBI test. Indeed, all participants that were positive for the mTBI test were GFAP+/UCH-L1- meaning that the confounding factors of UCH-L1 expression have no impact on the positivity rate of the mTBI test. Still, GFAP confounding factors have a major impact on the positivity rate of this test. Indeed, 66.9 % of our cohort was positive for GFAP alone without any suspicion of recent traumatic event. Unfortunately, although a decrease in specificity of the "GFAP-UCH-L1" mTBI test was reported in the older population by Ward and colleagues [5], the efficacy of age or renal-function dependent cut-offs has not been assessed yet.

On the other hand, only 5.5 % of our cohort was positive for S100B (mostly participants with renal insufficiency) showing that these confounding factors have a reduced impact on S100B positivity rate. However, S100B specificity is expected to be at 24.6 % [21] highlighting that S100B specificity is driven by the traumatic event. Indeed, S100B is known to be increased upon fractures and extracranial traumas [25–27]. Still, by applying age-dependent cut-offs, Oris et al. could increase their specificity in older patients [17] but the efficiency of renal function dependent cut-offs remains to be evaluated. Of note, the study of Oris and al. being made with Roche method, we could not apply the cut-offs of this study in our cohort as a bias between the two methods has been reported [21]. In this study, a moderate agreement between methods was also reported which might also impact S100B test specificity. Taken together, this suggest that age-dependent cut-offs might improve both the specificity of S100B and the "GFAP-UCH-L1" mTBI test.

Importantly, these biomarkers are not specific to mTBI, and part of their blood concentrations might be explained by other physiological pathways or neurological diseases [9, 19, 28]. Physiologically, both GFAP and S100B are produced by astrocytes while UCH-L1 is a neuron protein involved in the ubiquitinylation pathway. Still, S100B is also produced by melanocytes explaining the importance of skin pigmentation in S100B protein levels [29]. UCH-L1 seems to be also expressed in the gastrointestinal tract (colon and rectum), in the kidneys and urinary bladder (distal tubules and collecting ducts), in the male tissues (testis) and the connective and soft tissues in addition to the brain [30]. About the expression of GFAP in neurological disorders, GFAP is often mentioned as a potential blood-based biomarker for Alzheimer disease and a possible predictor of moderate cognitive impairment [31] as GFAP seems to be an early marker of amyloid beta [32, 33]. GFAP is also cited for various brain and spinal cord disorder as it might be an indicator of brain metastases or a prognosis biomarker after acute ischemic stroke [34, 35]. GFAP, UCH-L1 and S100B have been reported to be increased in contact sports such as rugby or American football [36–38].

The major limitation of this study is the lack of CT-scan that would objectify the absence of mTBI. We cannot totally

exclude the risk of recent falls even if these participants are community dwelling participants, autonomous and cognitively healthy. Yet, participants of the SarcoPhAge cohort are not consulting for head trauma and are non-suspected mTBI cases. Therefore, we cannot properly talk about specificity but only about positivity rate in non-suspected cases. The second limitation is the low number of participants with high cystatin C level, low BMI, and self-reported neurological disorders. Further studies should be dedicated to extend the findings to younger populations and to the study of these confounding factors in a larger cohort.

A strength of this study is that the SarcoPhAge cohort is representative of the population at higher risk of mTBI but also with higher risk to undergo a CT scan when suffering from mild traumatic brain injury [20]. Indeed, the CCTHR guidelines that rule out mTBI based on clinical decision criteria (without biomarkers) has defined being 65 years old or older to be a decision criterion for CT-scan [6]. Additionally, although cognitive function was only assessed through the MMSE and the self-report of neurological disorders, participants are considered as cognitively healthy as they have sufficient cognitive function to understand newspapers [20, 39]. Therefore, the impact of neurodegenerative diseases expression should be limited to very few individuals [20].

To conclude, this study has identified several confounding factors of S100B, GFAP and UCH-L1 expression and their impact on the "GFAP-UCH-L1" mTBI test and the protein S100B positivity. Given the high positivity rate of the "GFAP-UCH-L1" mTBI test, it is important to understand that these tests should only be used for the rule-out of mTBI and that mTBI must be objectified by CT-scan. Given the association between these biomarkers and their confounding factors, it is conceivable that the specificity of the "GFAP-UCH-L1" mTBI test or S100B might be improved by confounding factors dependent cut-offs.

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Research ethics: The SarcoPhage study was approved by the Ethical Committee of the CHU de Liège (2012/277). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Informed consent: Written and signed informed consent was obtained from all individual participants included in this study.

Author contributions: Design of the study: EmC, EC, AL. Experiments: EmC. Statistical analysis: EmC, AL. Cohort design and sampling: CB, OB, JYR. Writting and reviewing: EmC, CB, MA, NAD, OB, JYR, EC, AL. The authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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