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Biomarkers of traumatic brain injury in vitreous humor: A pilot study

Luisa Lanzilao [a], Ilenia Bianchi [b,c], Simone Grassi [b,c,*], Beatrice Defraia [b,c], Marco Brogi [a], Martina Da Ros [a], Tiziana Biagioli [a], Alessandra Fanelli [a], Vilma Pinchi [b,c], Martina Focardi [b,c]

[a] Biochemestry laboratory, Department of Diagnostics, Careggi Hospital, Largo Brambilla 3, 50134 Florence, Italy
[b] Forensic Medical Sciences, Department of Health Science, University of Florence, Largo Brambilla 3, 50134 Florence, Italy
[c] Laboratory of Personal Identification and Forensic Morphology, Department of Health Sciences, University of Florence, Largo Brambilla 3, 50134 Florence, Italy

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ABSTRACT

Background: Traumatic brain injury (TBI) is one of the major causes of morbidity and mortality worldwide. The patients' and injuries' heterogeneity associated with TBI, alongside with its variable clinical manifestations, make it challenging to make diagnosis and predict prognosis. Therefore, the identification of reliable prognostic markers would be relevant both to support clinical decision-making and forensic evaluation of polytraumatic deaths and cases of medical malpractice. This pilot study aimed to evaluate some of the main biomarkers specific for brain damage in sTBI and mmTBI deaths in samples of vitreous humor (VH) in order to verify whether predictors of prognosis in TBI can be found in this matrix.
Methods: VH were obtained from both eyes (right and left) of 30 cadavers (20 sTBI and 10 mmTBI) and analysed. These factors were evaluated: NSE (neuron-specific enolase), S100 calcium-binding protein (S100), glial fibrillary acidic protein (GFAP), Brain-derived neurotrophic factor (BDNF), Copeptin, Interleukin 6 (IL-6), Ferritin, Lactate dehydrogenase (LDH), C-Reactive Protein (CRP), Procalcitonin (PCT), Glucose and Neutrophil gelatinase-associated lipocalin (N-Gal).
Results: Four of the analysed proteins (LDH, ferritin, S100 and NSE) proved to be particularly promising. In particular, logistic regression analysis found a good discriminatory power.
Conclusions: Given the peculiarity of the matrix and the poor standardization of the sampling, such promising results need to be furtherly investigated in serum before being implemented in the forensic practice.

1. Introduction

Traumatic brain injury (TBI) is one of the major causes of morbidity and mortality worldwide, leading to significant direct and indirect costs for its acute and chronic treatment.

The patients' and injuries' heterogeneity associated with TBI, alongside with its variable clinical manifestations, can make it challenging to reliably make diagnosis and predict prognosis [1]. Therefore, the identification of reliable prognostic markers to support clinical decision-making would be a critical step in the treatment of brain damage [2].

The heterogeneity of these cases is also due to the fact that the physical insult starts complex pathophysiological cascades, and different insults may lead to different kind of clinical manifestations: physical penetration of the skull may cause a direct damage of the brain, while closed head injuries are associated with blunt, overpressure, or accelerative forces. Moreover, as a consequence of the insult, initial features of injury do not always relate to long-term consequences [1].

TBIs can be classified into different categories depending on the operative context: while traditional clinical classification follows the Glasgow Coma Scale (GCS) score, in the forensic field they are usually classified on the basis of the kind of means and forces involved. Finally, from a neuro-pathological point of view TBIs are classified as primary or secondary depending on when it had manifested [3–5]. Primary TBI occurs at the time of the injury as a direct result of traumatic event, while secondary TBI can occur after hours or even days and is characterized by complex biochemical cascade of events (often due to cerebral edema and elevated intracranial pressure) [3,6,7]. Besides purely mechanical damage, the injury can be due to the activation of complex pathways, namely: metabolic, necrotic and inflammatory cascades due to cells and tissue disruption that bring further neurodegenerative processes [8].

* Corresponding author at: Forensic Medical Sciences, Department of Health Science, University of Florence, Largo Brambilla 3, 50134 Florence, Italy.
E-mail address: simone.grassi@unifi.it (S. Grassi).

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That being said, despite the diagnostic/prognostic value of some proteins has been reported (Table 1), currently in clinical practice there are no reliable biomarkers helping in the classification and management of TBI and in particular in crucial steps like its early identification at hospital admission and its monitoring [9].

The lack of reliable biomarkers is a significant issue because the diagnosis of TBIs, especially of mild TBIs (that account for the 81% of the reported TBIs), is frequently missed [6,7,24]. This issue should be considered a public health problem, since the annual incidence of the sole severe TBI is about 73 per 100,000 people and TBIs account for about the half of the traumatic deaths in the world [25].

The current study aimed to evaluate in post-mortem samples of vitreous humor the levels of some biomarkers specific for brain damage: neuron-specific enolase (NSE), S100 calcium-binding protein (S100), glial fibrillary acidic protein (GFAP) [21,26], Brain-derived neurotrophic factor (BDNF) [22], Copeptin [27,28], Interleukin 6 (IL-6), Ferritin, Lactate dehydrogenase (LDH), C-Reactive Protein (CRP), Procalcitonin (PCT) [29], Glucose [30] and Neutrophil gelatinase-associated lipocalin (N-Gal) [31].

The primary endpoint was to verify whether there were statistically significant differences in these values between those died of TBI and those died with TBI.

2. Materials and Methods

2.1. Case Selection

Cadavers of patients died of/with TBI were considered. Those who died of TBI were classified as sTBI cases (severe TBI) while those with mild-to-moderate TBI who did not die of TBI were classified as mmTBI cases. All the cases underwent full forensic autopsy between 2018 and 2020 at the Section of Forensic Medical Sciences, Department of Health Science, University of Florence, Florence, Italy. Vitreous humor (VH) samples were obtained from both eyes using a sterilized 20-gauge hypodermic needle near the center of the eyeball. Samples were stored at − 80 °C.

In detail, the inclusion criteria were:

death occurred within 6 h from the trauma;.

full autopsy performed within 48 h from the death with following histological analysis.

As exclusion criteria we considered:

unknown cause of death;.

pre-existing pathological conditions that could have altered the concentration of the biomarkers in VH (kidney failure, drug overdose, metabolic disorders; neurodegenerative, neoplastic pathologies or brain acute vascular pathologies, axonal diffuse damage).

Table 1
Brief presentation of the diagnostic/prognostic significance of current TBI biomarkers.

IL-6	It is reported to increase following damage (due to post-traumatic inflammatory processes) and tends to relate to the severity of TBI and increased Intracranial pressure[10,11]
Ferritin	It shows higher concentration in patients with severe TBI[12]
LDH	It relates to the degree of brain damage[13]
CRP	In TBI patients, it has been reported to be significantly elevated in subjects with death/severe disability compared to those with moderate disability/good recovery[14]
PCT	In response to inflammation due to toxic molecules from bacteria or in response to pro-inflammatory cascade, its serum concentration is reported to increase[14,15]
Glucose	Both increase and decrease in blood glucose level are associated with poor prognosis[16]
N-Gal	Enhanced NGAL level is revealed after acute spontaneous intracerebral hemorrhage, in association with inflammatory degree and hemorrhagic severity, and it is intimately correlated with a worse prognosis[17]
NSE	It is the only marker that reflects neuronal damage and is detectable in the blood after TBI of all severities[18]
S100	Increased S100b levels can be found after severe TBI in both cerebrospinal fluid and serum (due to structural damage and cell death) [19]
Copeptin	Copeptin reflects the individual stress response at a hypothalamic level and has a controversial role as marker of TBI[20]
GFAP	Serum GFAP is considered a possible marker for moderate-to-severe TBI [21]
BDNF	BDNF is reported to increase in the acute phase of TBI[22,23]

2.2. Biochemical Markers determination

Pre-analitical preparation of samples was performed as described by Blana et al.: inflammation biomarkers (IL-6, Ferritin, LDH, CRP, PCT, Glucose and N-Gal) were analyzed on the automated system Cobas C8000 platform; NSE and S100 on Cobas C6000 system, Copeptin on Kryptor immunoanalyzer and BDNF and GFAP by Enzyme-Linked Immunosorbent assay (ELISA) from Human BDNF ELISA Kit (Thermo Fisher) and Human GFAP ELISA Kit (MyBiosource) [32]. Pre-analitical preparation of samples was performed as described by Blana et al.: inflammation biomarkers (IL-6, Ferritin, LDH, CRP, PCT, Glucose and N-Gal) were analyzed on the automated system Cobas C8000 platform; NSE and S100 were measurements on Cobas C6000 system, Copeptin on Kryptor immunoanalyzer and BDNF and GFAP by Enzyme-Linked Immunosorbent assay (ELISA) from Human BDNF ELISA Kit (Thermo Fisher) and Human GFAP ELISA Kit (MyBiosource) respectively. All the described methods are tested and validated for serum samples [32].

2.3. Statistical analysis

Statistical analysis was performed through SPSS v.24 software. Mann-Whitney tests were applied to test differences between the two groups for each variable. Each biomarker was evaluated both in its square root and logaritmic transformations. According to significative (p-value < 0.05) covariates, a logistic regression model was performed.

3. Results

Applying the inclusion/exclusion criteria, a total of 30 cadavers were selected for the study: 23 were males and 7 females, with ages ranging from 25 to 64 years (mean age: 45.6 years).

Among the 30 cadavers, 20 died of sTBI, while 10 were classified as mmTBI.

Of the evaluated inflammatory biomarkers, only ferritin and LDH showed a statistically significant difference between the two groups, with values higher in the sTBI cases.

Among the markers involved in the neurological damage, only the S100 showed a statistically significant increase in the TBI patients in comparison with mmTBI group. Finally, NSE showed to be much higher in traumatic patients but not statistically significant ($p = 0.052$) (Fig. 1).

For these four parameters, we evaluated the receiver operating characteristic (ROC) (Table 2) curves, as shown in Table 1.

NSE was then added as a covariate in the logistic regression model, which showed a ROC curve of 0.929 with a sensitivity $= 0.89$ and a specificity $= 0.714$ (Fig. 2).

4. Discussion

TBI is one of the leading causes of death and disability, potentially leading to neurological impairment and psychiatric disorders (post-traumatic stress disorders) [25].

In this study we evaluated the potential use of biomarkers in TBI cases. The clinical interest for tools of this kind is linked to the need for fast, reliable and objective parameters that can be of some help in the diagnosis and in the prognosis, that are currently based only on imaging techniques (computed tomography or MRI) and clinical assessment [26–31]. Biomarkers are of interest also for forensic purposes, helping in the causal inference process in challenging cases as polytraumatic

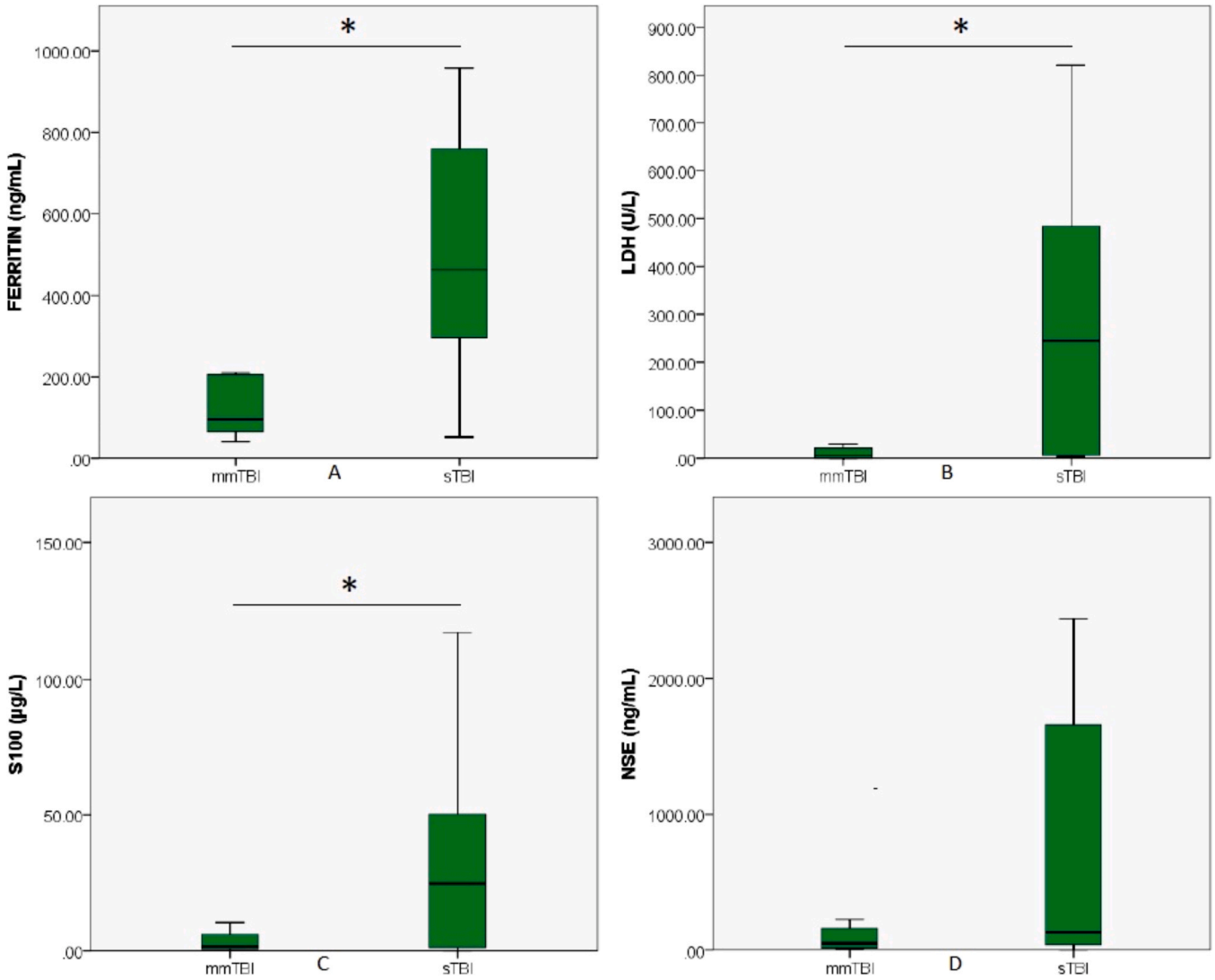

Fig. 1. Differences in serum concentration of Ferritin (a), LDH (b), S100 (c) and NSE (d) between sTBI vs mmTBI patients. Ferritin, LDH and S100 demonstrated to be statistically significant (* $p < 0.05$), while NSE showed $p = 0.052$.

Table 2
ROC curves for each of the analytes considered.

Biomarker	ROC
Ferritin	0.825
LDH	0.792
S100	0.680
NSE	0.585

deaths (e.g., to assess the involvement in the causation of death of different traumas of different organs) and deaths allegedly related to medical malpractice (e.g., cases of missed diagnosis or incorrect treatment in which it is important to evaluate how likely the patient would have survived in case of correct diagnosis/treatment) [24].

According to recent evidence, serum values of proteins NSE and S100 can be used effectively as prognostic indicators, as circulating values of copeptin for monitoring of TBI patients has been evaluated for the last years [26–28].

In the forensic context, serum can be used as a matrix also for post-mortem evaluation of these cases in association with immunohistochemical techniques [33]. However, these techniques are strongly limited, respectively, by the difficulty to obtain valid cadaveric serum in forensic contexts and the technical complexity of immunohistochemical techniques. To overcome the first limitation, we decided to evaluate the VH as matrix, since VH is protected inside the posterior chamber of the eye, can be easily sampled, stored and analyzed and contains values of proteins that tend to mirror those in the serum, as reported by many authors [34]. To the best of our knowledge, this is the first study to report the evaluation of biomarkers of TMI in this matrix.

Our results showed that these biomarkers can help to differentiate those died of TBI from those merely died with (mild-to-moderate) TBI, if death occurred few (6) hours after the trauma.

Moreover, we were able to build a logistic regression model that could discriminate leading-to-death TBI, an information of significant forensic interest (especially in cases of suspected medical malpractice in which reliable causal inference is crucial).

In detail, ferritin, LDH, and S100 showed to have a statistically significant difference among the two groups, while NSE, albeit being associated with a p-value slightly above the cut-off, proved to improve the logistic regression model.

In particular, we found an increase in values of S100. This finding is interesting because the time interval we evaluated (first six hours after death) is considerably higher than its half-life (1–2 h) and is consistent with previous (clinical) evidence, that showed the maximal concentration of S100 in TBI reached by the 6th hour after the trauma [35–37].

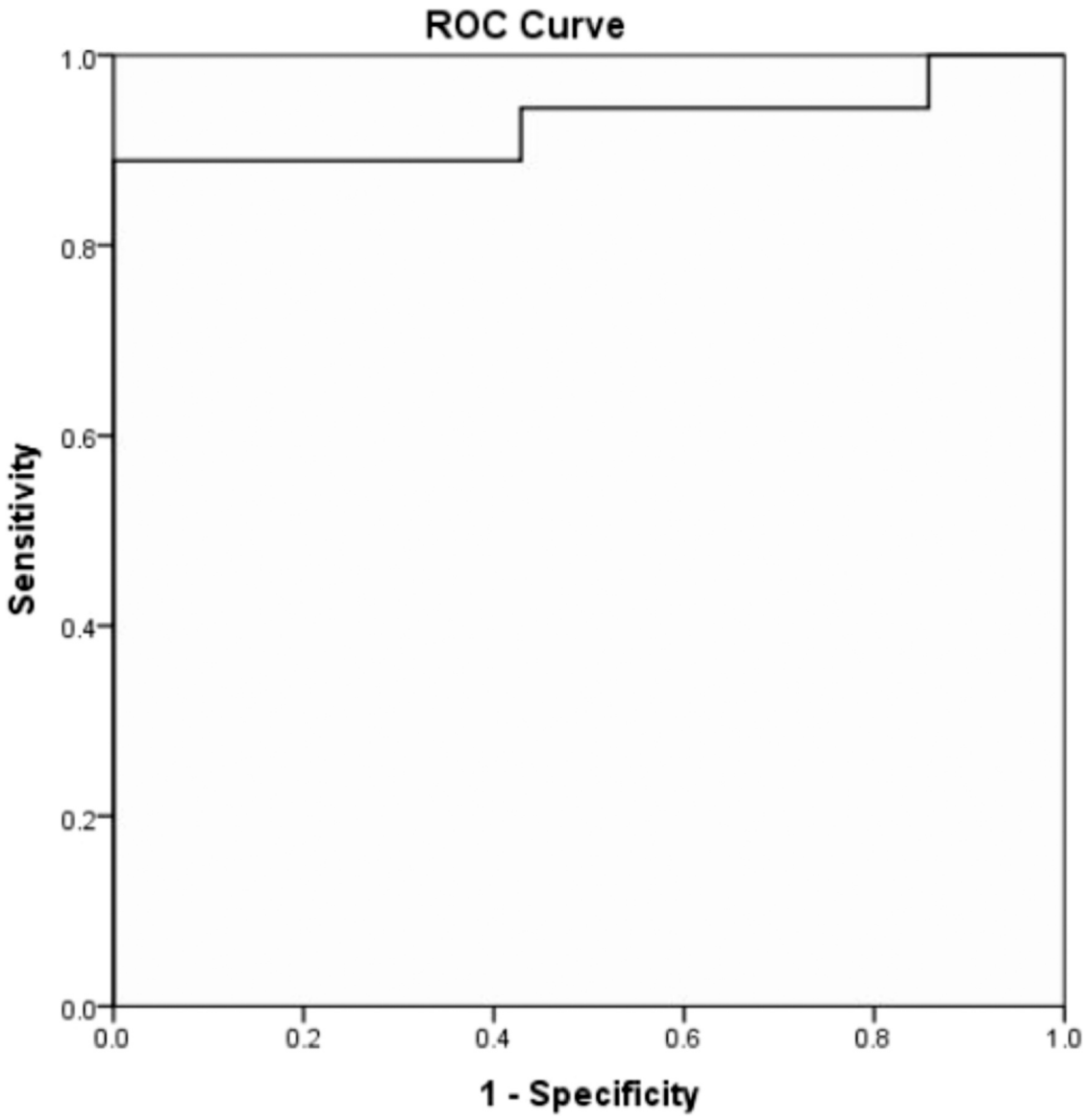

Fig. 2. ROC curve for the logistic regression model considering four covariates: ferritin, LDH, S100 and NSE.

We found very low concentrations of copeptin, close to zero in both sTBI and mmTBI patients. This might be due to the fact that, although in literature is described an increase after 6 h from the TBI, the peak is reached after 24 h [38].

The same evolution is reported for the biochemical marker GFAP, that in our case showed similarly high concentrations in both groups (sTBI and mmTBI) in the first 6 h.

Regarding NSE, it increased particularly in the sTBI group. It is important to stress that, according to current evidence, this increase in NSE occurs within the first hours from the trauma, while a downward trend can be usually observed afterwards [39].

Regarding the other biomarkers (BDNF, IL-6, ferritin, LDH, CRP, PCT, Glucose and N-Gal) no critical comparison between our data and serum levels can be made because the serum variations of their levels is currently unknown.

This research has some limitations. First, this is a pilot study based on a limited sample. The small number of cases was due to the peculiarity of the specimen (VH from forensic cadavers) and the inclusion/exclusion criteria.

Second, the half-lives of most of the examined biomarkers must be considered: only a few studies have examined this aspect.

Moreover, we do not precisely know the diffusion dynamics of proteins into the vitreous compartment in cadavers, but we may suppose several factors may influence it, as the molecular weight of the proteins and the post-mortem disruption of the blood retinal barrier or the release of such proteins in certain brain compartments closer to the eye.

Despite the abovementioned limitations, the findings of the present study revealed meaningful differences in some biomarkers, that, through a regression model, proved to have an inferential value helping to infer on the severity of the TBI, a parameter of significant forensic interest (for instance, when a delay in its treatment is claimed). However, found results must be validated on larger samples before the implementation of this testing in forensic routine.

5. Conclusions

Proper classification of TBI using objective, fast and reliable fluid biomarker would significantly empower clinicians and forensic pathologists, helping them – respectively- to manage these patients and infer on the cause of the death.

In our study we aimed to determine severity (leading-to-death) of the trauma analysing the concentration of already reported in literature

biochemical markers in VH instead of serum; this allowed for a logistic regression model that has shown promising results that will need to be confirmed on a larger sample and expanding the timing of the TBI (> 6 h).

This pilot study has limitations, due to the small sample size and to the non-standardized timing of draining, but we believe that it may serve as a proof of concept to deeper investigation in serum which may support clinicians in classifying and monitoring TBI patients and in implementing the forensic practice.

Institutional Review Board Statement

Ethical approval was obtained by Regional Ethics Committee for Clinical Trials in Tuscany – section Center Area.

Informed Consent Statement

Consent for participation and publication of anonymized data was waived in compliance with national law (d.lgs. 10 agosto 2018, n. 101).

CRediT authorship contribution statement

Luisa Lanzilao: Conceptualization, Writing – original draft, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. **Ilenia Bianchi:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. **Grassi Simone**: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. **Beatrice Defraia**: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. **Marco Brogi:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. **Martina Da Ros:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. **Tiziana Biagioli**: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. **Alessandra Fanelli**: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision. **Vilma Pinchi:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Martina Focardi:** Conceptualization, Writing – original draft, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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