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ORIGINAL PAPER

Investigation by top-down high-performance liquid chromatography–mass spectrometry of glutathionylation and cysteinylation of salivary S100A9 and cystatin B in preterm newborns

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Glutathionylation and cysteinylation can be involved in the protection of critical cysteines from irreversible oxidative damages. S100A9 long and cystatin B, proteins highly represented in the saliva of preterm and at-term newborns, can undergo these modifications. Levels of S100A9 long and cystatin B and their glutathionylated and cysteinylated derivatives have been determined by a top-down platform based on high-performance liquid chromatography–electrospray ionization–mass spectrometry in 100 salivary samples serially collected from 17 preterm newborns with different postconceptional age at birth (178-226 days) and in 90 salivary samples collected from at-term newborns and babies. Results showed that: (1) S100A9 long and cystatin B were mainly present as unmodified

Abbreviations: NICU, neonatal intensive care unit; PCA, postconceptional age; PTMs, posttranslational modifications; SPRR3, small proline rich proteins 3

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forms in extremely preterm newborn; (2) the percentage of the S-thiolated derivatives of both proteins increased with increasing the postconceptional age; (3) the greatest variation occurred up to about 280 days of postconceptional age. Interestingly, differences in the levels of the S-thiolated derivatives only depended on the postconceptional age and not on whether the infant was born preterm or at-term. Inadequate levels of cysteine and glutathione might be responsible for the low level of S-thiolated derivatives measured in preterm newborns. Data are available via ProteomeXchange with identifier PXD025517.

KEYWORDS
cystatin B, cysteinylation, glutathionylation, preterm human saliva, S100A9

1 | INTRODUCTION

The salivary proteome of preterm newborns undergoes major changes in qualitative and quantitative composition from the last periods of fetal life to adult [1]. Previous HPLC-ESI-MS analysis on the acid-soluble fraction of saliva from preterm newborns allowed evidencing high levels of more than 40 proteins at birth with potential biological function mainly involved in fetal growth [1] and poorly represented in adult saliva. The concentration of these proteins dropped after birth to become almost undetectable or detected at low concentration when the postconceptional age (PCA) of preterm newborns correspond to that of infants born at the normal term of delivery [1]. Concurrently, proteins typically present in adult saliva became detectable, even though with different time courses [2]. The recent technological advances have permitted the high-throughput analysis of saliva [3]. In particular, the availability of new high-resolution mass instruments (Orbitrap-Elite) allowed determining, by a top-down platform, the structure of some proteins and their proteoforms originated by posttranslational modifications (PTMs), that were found in preterm and at term newborns. For instance, S100A9 was very represented in the acid-soluble fraction of preterm newborn saliva, and it was revealed even after birth [1]. S100A9 can exist in two forms differing for the length, the so-called "short form" generated by proteolytic cleavage at Met_5 residue, releasing the N-terminal MTCKM pentapeptide and the "long form" generated by the classical cleavage on N-terminal methionine. It has been demonstrated that the long form can undergo glutathionylation at the level of Cys_3 via GSSG or GSNO intermediates [4]. Higher amounts of glutathionylated and cysteinylated forms of S100A9 were detected at first in saliva from patients affected by Wilson's disease with respect to the healthy control group [5].

Cystatin B was also detectable in saliva from preterm newborns, mainly as unmodified form, but also as glutathionylated and cysteinylated derivatives at Cys_3 [6]. Interestingly, cystatin B was detected in adult human whole saliva mostly as S-modified derivatives, being about 30% of the protein present as the S–S covalent dimer, about 55% as S-glutathionylated, and the remaining 15% as S-cysteinylated [7]. The S-unmodified cystatin B derivative was generally not detectable in adult human whole saliva [7]. A more detailed description of S100A9 and Cystatin B functions is reported in the Supporting Information.

Reversible cysteine oxidation, such as glutathionylation and cysteinylation, can be involved in a variety of redox signaling/regulation events, and in the protection of critical cysteines from irreversible oxidative damages [8–10]. In this respect, Vento et al. measured lower superoxide dismutase and catalase activities, as well as lower ability to synthesize glutathione, in very premature infants [11]. This condition prevents the premature infant to face adequately the oxidative stress conditions intrinsic to the fetal-to-neonatal transition [12].

On these premises, as an extension of our previous study based on the characterization of the salivary proteome of preterm newborn, we have investigated, by top-down HPLC-MS approach, the variations of glutathionylation and cysteinylation levels of S100A9 long and cystatin B. Salivary samples were longitudinally collected from 17 preterm newborns with different PCA at birth, as well as from 90 at-term newborns, and babies. The aim of this work is to highlight possible differences as a clue of the ability to protect cysteines from irreversible oxidative damage, and to establish whether any differences persist over time.

2 | MATERIALS AND METHODS

2.1 | Reagents

All general chemicals and reagents of LC−MS grade were purchased from J. T. Baker (Deventer, The Netherlands), and Merck (Darmstadt, Germany).

2.2 | Subjects enrolled, sample collection, and treatment

The study protocol and the written consent form were approved by the Ethical Committee of the Neonatal Intensive Care Unit (NICU) of the Institute of Clinical Pediatric of the Catholic University of Rome, and it has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All rules were observed, and written consent forms were signed by at least one parent of each child. For ethical reasons, saliva was collected only when sample collection caused no stress. One hundred ninety samples of resting whole saliva were gathered and analyzed from the following subjects:

1. Seventeen preterm newborns with a birth weight between 470 and 1340 g and gestational age between 178 and 226 days, admitted to the NICU were enrolled for this study. Infants with major congenital malformations or prenatal infections were excluded from the study. All premature newborns were given intravenous ibuprofen as lysine salt (Arfen, Lysafarma, Erba-Como, Italy) as prophylaxis for patent ductus arteriosus: 10 mg/kg within the first 2 h of life, and afterward two further 5 mg/kg administrations after 24 and 48 h from the first dose. Caffeine citrate (caffeine citrate 10 mg/mL; Monico, Venice, Italy) was administered intravenously at the dosage of 5 mg/kg per day after a loading dose of 20 mg/kg, from birth to the 33rd week of PCA to prevent apnoeic spell. A dose of 200 mg/kg surfactant (pig-derived natural surfactant, Curosurf, Chiesi Farmaceutici, Parma, Italy) was administered to nine newborns with a diagnosis of respiratory distress syndrome, which needed intubation and mechanical ventilation. Other eight newborns only required continuous positive airways pressure by short nasal prongs during the hospital stay. All infants were given daily parenteral nutrition during the first 36 h of life, according to a standard protocol of NICU. Thank to this regimen, fluid intake progressively increased from 60 to 150 (mL/kg)/day with serum sodium levels of 130–150 mmol/L, daily weight loss between 2% and 5%, and urine osmolarity < 350 mOsm/L. A glucose and fat emulsion preparation (Intralipid 20%-Pharmacia) was used to reach an optimal target of nonprotein calories of 85 (kcal/kg)/day, and an amino acid solution (TPH 0.6-Baxter, IL) was used to reach 3.5 (g/kg)/day of protein intake by parenteral route. Enteral feed was started when the vital signs of the newborn reached stability, usually within the first week of life, while parenteral nutrition was gradually reduced as the volume of milk feeding increased (Preaptamil, preterm infant formula-Milupa, Germany, distributed in Italy by Nutricia Italia, MI, Italy), and was stopped when 100 kcal enterally introduced could be easily tolerated. A total of 100 salivary samples were longitudinally collected from the 17 preterm newborns.
2. Ninety healthy at-term newborn donors with an age between around 0 and 5 years of age.

Collection time was carried out usually between 10:00 and 12:00 a.m. The donors were under healthy clinical conditions. Except for preterm newborns, donors did not eat 1 h before the collection. Whole saliva was collected with a soft plastic aspirator as it flowed into the anterior floor of the mouth. After collection, each salivary sample was immediately diluted 1:1 (v/v) with 0.2% aqueous 2,2,2-trifluoroacetic acid (TFA) in ice bath. The solution was then centrifuged at 8000 × *g* for 5 min (4°C). Finally, the acidic supernatant was separated from the pellet and either immediately analyzed with the HPLC−ESI−MS apparatus or stored at −80°C until the analysis.

2.3 | RP−HPLC low-resolution ESI–MS analysis of intact protein

RP–HPLC low-resolution ESI–MS experiments (6000 at 400 m/z) were performed using a Surveyor HPLC systems (ThermoFisher Scientific) connected by a T splitter to a photo diode-array detector and to an LCQ Deca XP Plus ion trap mass spectrometer (ThermoFisher Scientific). The chromatographic column was a 150 × 2.1 mm Vydac (Hesperia, CA) C8 column with 5 μm particle diameter. The following solutions were utilized for the RP−HPLC separation: (eluent A) 0.056% aqueous TFA and (eluent B) 0.05% TFA in acetonitrile:water 80/20, v/v. The gradient applied for the analysis was linear from 0 to 55% of B in 40 min and from 55 to 100% of B in 10 min at a flow rate of 0.30 mL/min. The T splitter permitted 0.20 mL/min to flow toward the diode array detector and 0.10 mL/min to flow toward the ESI source. The UV diode array detector was set at 214 and 276 nm. The MS spray voltage was 4.0 kV, and the capillary temperature was 220°C. Detailed instrumental operative conditions are reported in a previous paper [13]. The injection volume was 100 μL, corresponding to 50 μL of saliva.

2.4 | MS data analysis and statistics

Deconvolution of averaged ESI−MS spectra was automatically performed by MagTran 1.0 software [14] or by the HPLC−MS apparatus management software (Xcalibur 2.0.7 SP1, ThermoFisher Scientific). Pearson r coefficient was used to evaluate linear correlations of the percentage of the s-derivatives of cystatin B and S100A9 long proteoforms as a function of PCA.

2.5 | Intact protein relative quantification

Quantification of the proteins of interest was performed by measuring the area of the eXtracted Ion Current (XIC) peak (signal/noise ratio > 5) revealed in low-resolution HPLC−ESI−MS profiles. The m/z values used for the XIC procedure were carefully selected to exclude values in common with other co-eluting peptides or proteins and are reported in Table 1. The area of the XIC peak is proportional to protein/peptide concentration; therefore, under constant analytical conditions, it can be used for quantitative analysis and comparative studies [15–16]. For each sample, the percentages of the different proteoforms were determined by calculating the ratio between the XIC peak area of the proteoform of interest and the sum of the XIC peak areas of all the proteoforms of reference as described in detail in the results. The estimated percentage error of the XIC procedure was <10%.

2.6 | RP–HPLC high-resolution ESI–MS/MS analysis

S100A9 short, S100A9 short P, S100A9 long, S100A9 long cysteinyl, S100A9 long glutathionyl, S100A9 long P, cystatin B, cystatin B cysteinyl, and cystatin B glutathionyl proteoforms have been characterized by a top-down approach by HPLC-high-resolution ESI–MS/MS with an LTQ-Orbitrap Elite apparatus (Table 1). The chromatographic separation was carried out using eluent A: 0.1% (v/v) aqueous formic acid (FA) and eluent B: 0.1% (v/v) FA in ACN–water 80/20. The gradient was as follows: 0–2 min 5% B, 2–10 min from 5 to 25% B (linear), 10–25 min from 25 to 34% B, 25–45 min from 34 to 70% B, and 45–55 min from 70 to 90% B at a flow rate of 50 μL/min. The injection volume was 19 μL. Full MS experiments were performed in positive ion mode with a mass range from 400 to 2000 m/z at a resolution of 120,000 (at 400 m/z). The capillary temperature was 275°C, the source voltage was 4.0 kV, and the S-Lens RF level was 69%. In data-dependent acquisition mode, the five most abundant ions were acquired and fragmented by using collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) with a 35% normalized collision energy for 10 ms, isolation width of 5 m/z, and activation q of 0.25. HPLC–ESI–MS and MS/MS data were generated by Xcalibur 2.2 SP1.48 (ThermoFisher Scientific, CA) using default parameters of the Xtract program for the deconvolution. The structural information derives from manual inspections of the MS/MS spectra, obtained by both CID and HCD fragmentation, against the theoretical ones generated by MS-Product software available at the Protein Prospector website (http://prospector.ucsf.edu/prospector/mshome.htm).

Identification of the phosphorylated proteoforms of S100A9 long cysteinyl and S100A9 long glutathionyl was based on the correspondence between the experimental and the theoretical monoisotopic monocharged mass values. Cystatin B dimer proteoform has been characterized by a bottom-up approach as previously described [7].

The mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium (http://www.ebi.ac.uk/pride) via the PRIDE partner repository [17] with the dataset identifier PXD025517.

3 | RESULTS

Figure 1 shows the typical HPLC–ESI–IT–MS total ion current (TIC) profile of saliva from a preterm newborns in comparison to that of an adult.

From Figure 1, it is evident that the major salivary proteins in preterm and at-term newborns are represented by small proline-rich proteins 3 (SPRR3), cystatins A and B, and S100 proteins. Salivary proteins typically present in adults are almost undetectable.

In human saliva, cystatin B has been detected not only unmodified, but also as cysteinylated, glutathionylated, carboxymethyl, and dimeric derivatives at Cys_3 [7, 18].

Glutathionylation and cysteinylation of Cys_3 in S100A9 long, and phosphorylation of the threonine residue located at position 108 in the short form, and 112 in the long form, are responsible for the existence of the following eight proteoforms of S100A9, namely S100A9 short, S100A9 long, cysteinylated and glutathionylated S100A9 long, in their phosphorylated or non-phosphorylated forms. The chromatographic window where the different proteoforms of cystatin B and S100A9 elute are shown in Figure 2. Experiments by HPLC-high-resolution ESI–MS/MS with an LTQ-Orbitrap Elite apparatus allowed confirming the structure of cystatin B and S100A9 proteoforms (Table 1).

The m/z ions selected to extract the ion current (XIC) plot of these proteoforms for peak area determination, and

TABLE 1 S100A9 and Cystatin B experimental (exp.) and theoretical (th.) average mass values (Mav) and monoisotopic monocharged ($[M+H]^+$) mass values, multiply charged ions used for XIC quantification and for top-down high-resolution MS/MS structural characterization

Protein	Exp. (th) Mav. (Da)	multiply charged ions selected for the XIC procedure ($m/z_{(charge)}$)	Exp. (th.) $[M+H]^+$	$m/z_{(charge)}$ selected for MS/MS
S100A9 short	12689 ± 2 (12689.23)	$1058.4_{(+12)}$ $1154.6_{(+11)}$ $1269.9_{(+10)}$ $1410.9_{(+9)}$	12682.3±0.2 (12682.29)	$705.85_{(+18)}$ $846.76_{(+15)}$ $977.22_{(+13)}$ $1058.20_{(+12)}$ $1269.93_{(+10)}$ $1410.67_{(+9)}$
S100A9 short P*	12769 ± 2 (12769.21)	$1061.1_{(+12)}$ $1161.8_{(+11)}$ $1277.9_{(+10)}$ $1419.8_{(+9)}$	12762.3±0.2 (12762.26)	$798.96_{(+16)}$ $852.29_{(+15)}$ $912.96_{(+14)}$
S100A9 long	13153 ± 2 (13152.84)	$940.5_{(+14)}$ $1012.8_{(+13)}$ $1097.1_{(+12)}$ $1196.7_{(+11)}$ $1316.3_{(+10)}$	13145.4±0.2 (13145.48)	$877.70_{(+15)}$ $940.33_{(+14)}$ $1012.59_{(+13)}$
S100A9 long P*	13233 ± 2 (13232.82)	$946.2_{(+14)}$ $1018.9_{(+13)}$ $1103.7_{(+12)}$ $1204.0_{(+11)}$ $1324.3_{(+10)}$	13226.5 ±0.2 (13225.45)	$877.85_{(+15)}$ $1012.74_{(+13)}$
S100A9 long cysteinyl	13272 ± 2 (13271.98)	$949.0_{(+14)}$ $1021.9_{(+13)}$ $1107.0_{(+12)}$ $1207.6_{(+11)}$ $1328.2_{(+10)}$	13264.5 ±0.2 (13264.49)	$885.17_{(+15)}$ $948.98_{(+14)}$ $1021.82_{(+13)}$
S100A9 long cysteinyl P*[a]	13352 ± 2 (13351.96)	$954.7_{(+14)}$ $1028.1_{(+13)}$ $1113.7_{(+12)}$ $1214.8_{(+11)}$ $1336.2_{(+10)}$	13344.7 ±0.2 (13344.45)	
S100A9 long glutathionyl	13458 ± 2 (13458.15)	$962.3_{(+14)}$ $1036.3_{(+13)}$ $1122.5_{(+12)}$ $1224.5_{(+11)}$ $1346.8_{(+10)}$	13450.6 ±0.2 (13450.55)	$748.65_{(+18)}$ $792.57_{(+17)}$ $842.04_{(+16)}$ $962.19_{(+14)}$ $1036.21_{(+13)}$
S100A9 long glutathionyl P*[a]	13538 ± 2 (13538.13)	$968.0_{(+14)}$ $1042.4_{(+13)}$ $1129.2_{(+12)}$ $1231.8_{(+11)}$ $1354.8_{(+10)}$	13530.5 ±0.2 (13530.69)	
Cystatin B	11182 ± 1 (11181.63)	$1119.2_{(+10)}$ $1243.4_{(+9)}$ $1398.7_{(+8)}$ $1598.4_{(+7)}$ $1864.6_{(+6)}$	1175.6 ±0.1 11175.609)	799.77 (+14)
Cystatin B dimer[b]	22361 ± 3 (22361.25)	$1017.4_{(+22)}$ $1065.8_{(+21)}$ $1190.0_{(+20)}$ $1177.9_{(+19)}$ $1243.3_{(+18)}$ $1316.4_{(+17)}$ $1398.6_{(+16)}$ $1491.8_{(+15)}$ $1598.2_{(+14)}$ $1721.1_{(+13)}$ $864.4_{(+12)}$		
Cystatin B cysteinyl	11301 ± 1 (11300.77)	$1028.4_{(+11)}$ $1131.1_{(+10)}$ $1256.7_{(+9)}$ $1413.6_{(+8)}$ $1615.4_{(+7)}$ $1884.5_{(+6)}$	11294.6 ±0.1 (11294.6129)	766.78 (+15) 884.45 (+13) 1045.25 (+11)
Cystatin B glutathionyl	11487 ± 1 11486.94	$1045.3_{(+11)}$ $1149.7_{(+10)}$ $1277.3_{(+9)}$ $1436.9_{(+8)}$ $1642.0_{(+7)}$ $1915.5_{(+6)}$	11480.7 ± 0.1 (11480.677)	$718.79_{(+16)}$ $766.76_{(+15)}$ $1149.48_{(+10)}$ $1045.25_{(+11)}$ $1277.31_{(+9)}$ $1436.72_{(+8)}$

*P, monophosphorylated form.

[a]Attribution based on the correspondence between the experimental and the theoretical monoisotopic monocharged mass values.

[b]Characterized by a bottom-up approach [6].

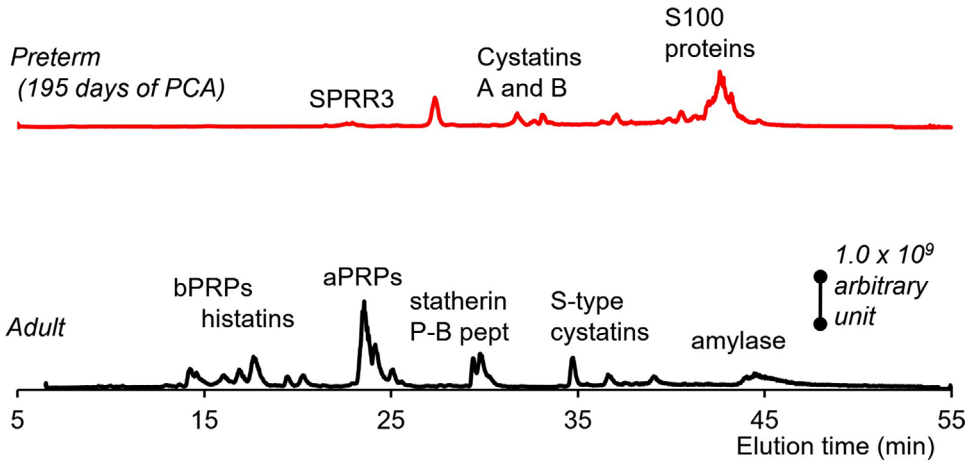

FIGURE 1 Typical age-dependent HPLC-ESI-MS TIC profiles of the acid-soluble fraction of human saliva. On top, the profile from a preterm newborn with 195 days of PCA and on the bottom the profile from an adult. Salivary amylase was mainly detectable in adult saliva. Elution ranges of the principal proteins are evidenced

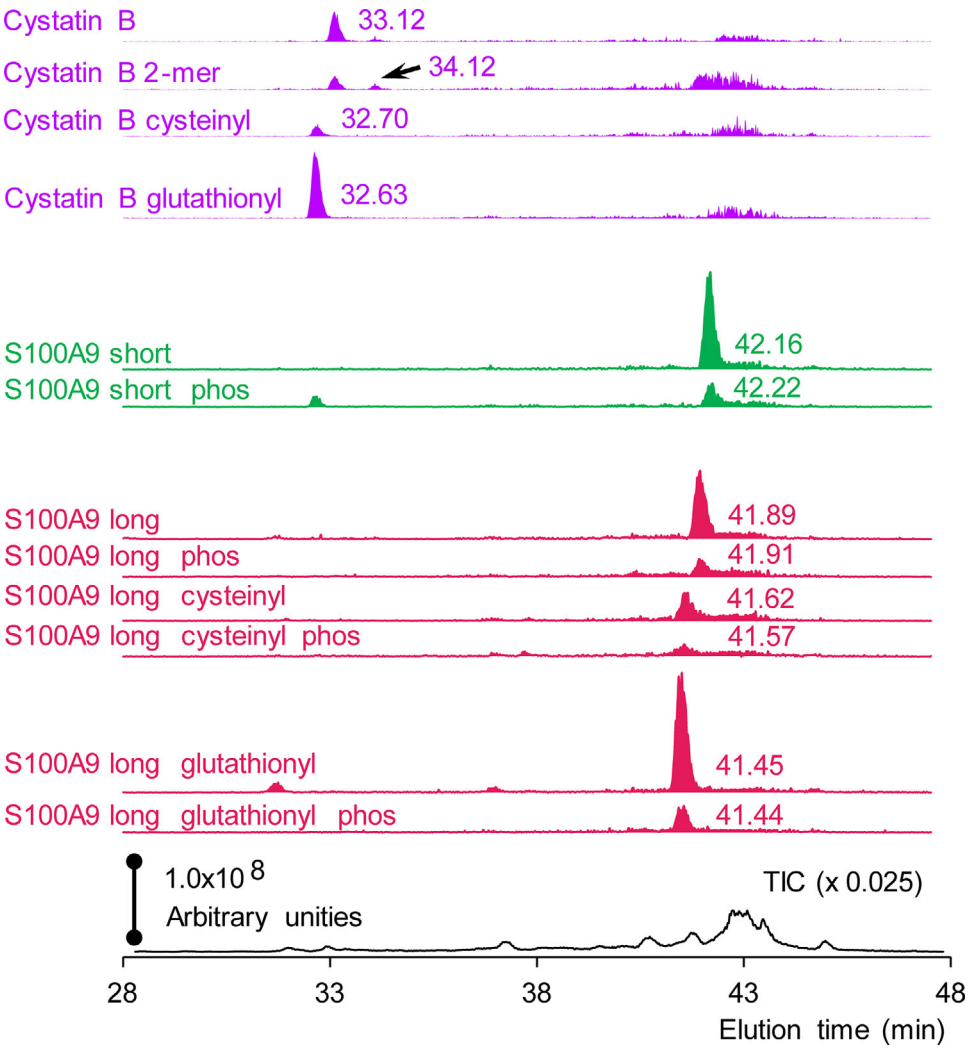

FIGURE 2 From the top to bottom. Extracted ion plots of cystatin B and its dimeric, cysteinylated, and glutathionylated derivatives at Cys_3. Extracted ion plots of the eight proteoforms of S100A9, namely S100A9 short, S100A9 long, cysteinylated, and glutathionylated S100A9 long in their phosphorylated or non-phosphorylated forms. Enlargement of the TIC profile between 28 and 48 min with the intensity reduced 40 times

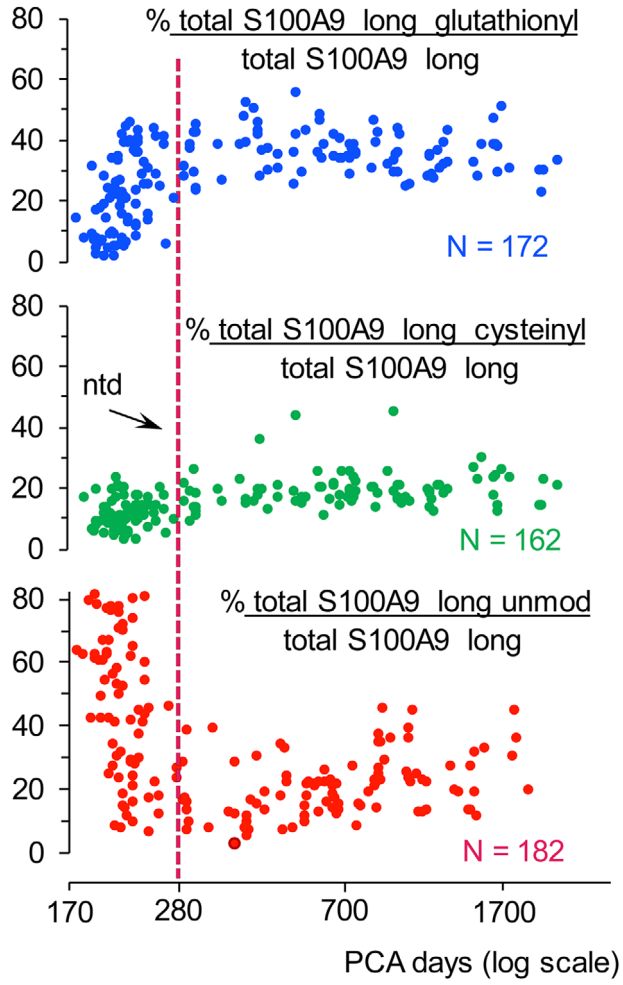

FIGURE 3 From the top, percentage of the glutathionylated, cysteinylated, and unmodified proteoforms of S100A9 long as a function of PCA. The percentage of each specific proteoform was calculated as follows: sum the XIC peak area of the differently phosphorylated forms/sum of the XIC peak areas of all six derivatives of S100A9 long. Ntd: Normal term of delivery

the relative experimental and theoretical Mav values are reported in Table 1.

Figure 3 shows the percentages of the glutathionylated, cysteinylated, and unmodified forms of S100A9 long as a function of PCA. Since it has been demonstrated that the phosphorylation level of S100A9 is not depending on age [2], the XIC peak areas of the differently phosphorylated forms were not considered separately to study the relative level of the three S100A9 long proteoforms with respect to age.

From Figure 3, it is evident that in extremely preterm newborns, at the time of birth, the major proteoform of S100A9 long (considering both P and not P) was represented by the unmodified protein (up to 80%). After birth, the level of this proteoform showed a rapid decrease with increasing PCA, and when preterm newborns reached PCA greater than 280 days, roughly corresponding to the PCA of full-term newborns, the percentage of the unmodified S100A9 long did not exceed the value of about 40%.

It should be emphasized that after birth the percentage of the unmodified form had similar values for newborns with the same PCA, regardless of whether they were born premature or full term.

In correspondence with the decrease of the unmodified S100A9 long levels, we observed a parallel increase of the level of the glutathionylated and cysteinylated derivatives (P and not P). The glutathionylated derivative became the most representative Cys3 oxidized form of S100A9 long (P and not P) after 280 days of PCA in both preterm and full-term newborns, with a percentage ranging from about 30 to 50%.

Analysis of saliva samples collected over time from preterm and at-term newborns with different PCA allowed also to extend the study previously performed by our group and to confirm the results obtained on cystatin B [6].

Similarly to S100A9 long, cystatin B was mainly present as unmodified form at Cys_3 in premature newborns with lower PCA, and the levels of the oxidized derivatives, represented in this case also by the dimeric form in addition to the glutathionylated and cysteinylated ones, increased with increasing PCA, though to a lesser extent as regards the cysteinylated form (Figure 4).

After birth, the percentage of the unmodified cystatin B had similar values for newborns with the same PCA regardless of whether they were born premature or full term, as observed for long S100A9.

A good correlation was observed between the percentages of the different proteoforms of S100A9 long and cystatin B measured in newborns with different PCA (Figure 5), suggesting a common pathway of S-thiolation for the two proteins.

The percentage of the glutathionylated derivative of both S100A9 long and cystatin B, calculated with respect to the sum of the glutathionylated and cysteinylated derivatives, is shown in Figure 6. From the Figure, it is evident that in some very premature newborns the glutathionylated derivative compared to the cysteinylated is less represented with respect to newborns with higher PCA and full-term newborns.

4 | DISCUSSION

S-Glutathionylation is a well-known oxidative post-translational modification that occurs on cysteine residues under basal conditions for some proteins, and as transient modification during oxidative stress for others [19]. It has been demonstrated that it can serve to regulate a variety of cellular processes by modulating protein function, and

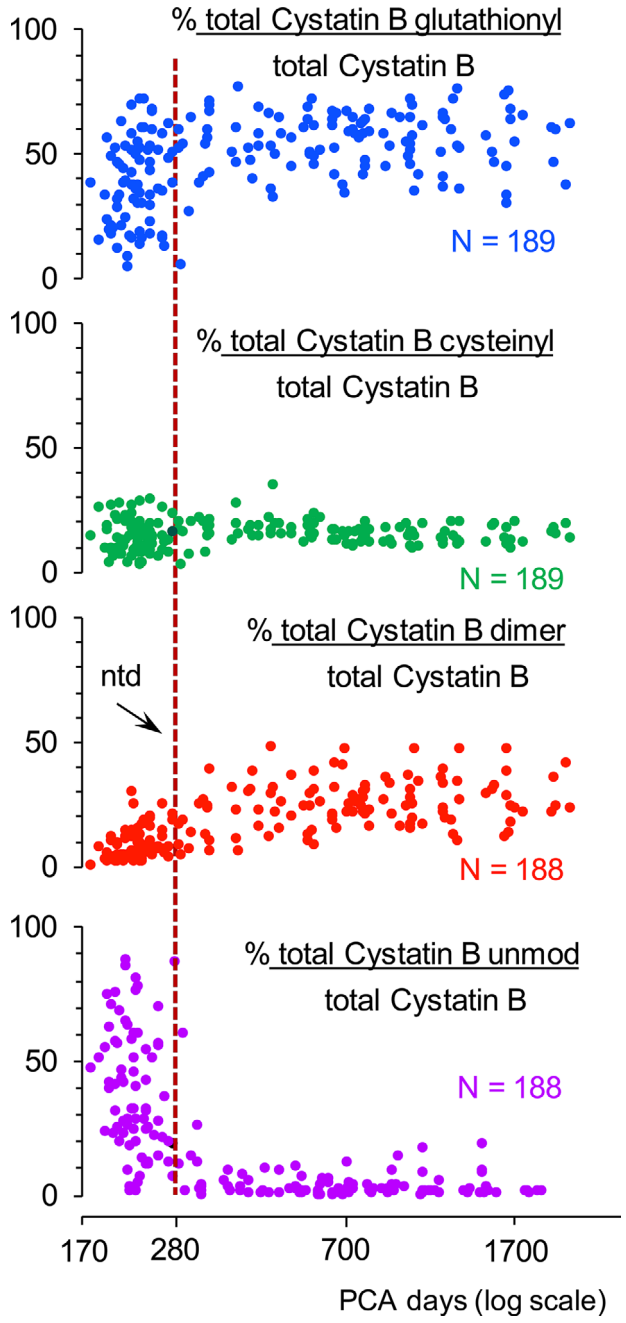

FIGURE 4 From the top, percentage of the glutathionylated, cysteinylated, dimeric, and unmodified proteoforms of cystatin B as a function of PCA. The percentage was calculated as follows: XIC peak area of each specific proteoform/sum of the XIC peak areas of all four derivatives of cystatin B. Ntd: Normal term of delivery

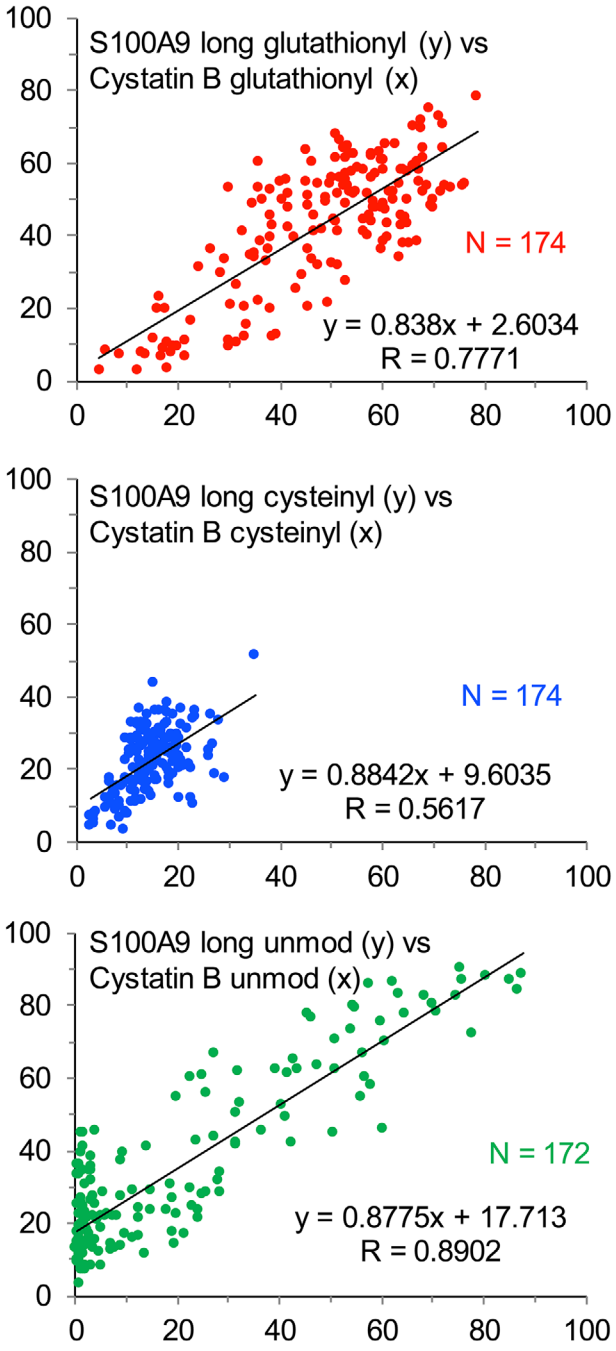

FIGURE 5 From the top, correlation between the percentages of the glutathionylated forms of S100A9 long and cystatin B, the cysteinylated forms of S100A9 long and cystatin B, and the unmodified forms of S100A9 long and cystatin B measured in newborns with different PCA

to prevent irreversible oxidation of protein thiols [20]. The long proteoform of S100A9 and cystatin B can undergo S-thiolation, and in this study we detected and determined the levels of S-glutathionylated and S-cysteinylated derivatives of both proteins in preterm and at-term newborns, to evaluate possible differences as a clue of the ability to protect cysteines from irreversible oxidative damage in preterm newborns. Salivary samples were from preterm and full-term newborns even after birth, and the study included children up to 5 years of age.

Due to the small volume of salivary samples collected from preterm and at-term newborns, it was impossible to determine the total protein concentration, and therefore to reduce the bias due to individual variability of such parameter, we decided to analyze data by calculating the

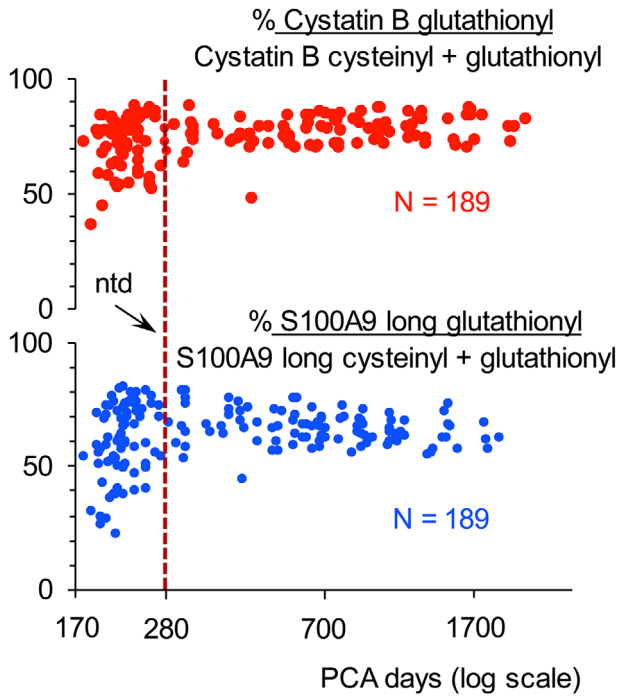

FIGURE 6 From the top, percentage of the glutathionylated forms of cystatin B, and the glutathionylated forms of S100A9 long (phosphorylated and non-phosphorylated) as a function of PCA. The percentage was calculated as follows: XIC peak area of the specific glutathionylated proteoform/sum of the XIC peak areas of the glutathionylated and cysteinylated derivatives. Ntd: Normal term of delivery

percentage of the different proteoforms. Despite the great intervariability, data clearly showed that the lower level of glutathionylation and cysteinylation for both S100A9 and cystatin B proteins was present in very preterm newborns, indicating that the ability to synthesize those derivatives was more reduced the more the newborn was premature. Glutaredoxins are thiol oxidoreductases involved in S-glutathionylation of proteins and they act as glutathionylating enzyme under an oxidative stimulus and as a deglutathionylase when the oxidative stress drops [19]. The rapid increase in oxygen availability occurring in the first minutes after birth is responsible for oxidative stress and the generation of reactive oxygen species [12]. It has been demonstrated that preterm infants are generally characterized by a less effective antioxidant defense system than full-term newborns [11], and this condition is relevant if we consider the pro-oxidant aggression due to oxygen supplementation to which they may be subject at birth [21]. However, from our data, it is not possible to establish whether the lower level of glutathionylation found in very preterm newborns was due to the inappropriate regulation or synthesis of the glutathionylating enzymes, or in glutathione synthesis. Glutathione, a tripeptide formed by γ-glutamine, L-cysteine, and glycine, is the most abundant low-molecular-mass thiol within most cell types, and its synthesis has been studied also in tissues of newborns [22]. In particular, the study performed in leukocytes from tracheal aspirates of oxygen-dependent newborns concluded that biosynthesis of glutathione was active in leukocytes from preterm infants [22]. However, Viña et al. suggested that γ-cystathionase activity may be the limiting step in cysteine synthesis from methionine, as they observed an impaired glutathione synthesis in erythrocytes of premature newborns with less than 32 weeks of gestation, when methionine was used as cysteine precursor [23]. Indeed, they measured cysteine plasma concentration ca. 20 times lower in preterm newborns with less than 32 weeks of gestation, and 8 times lower in those with 33–36 weeks of gestation than in at-term newborns. Conversely, the rate of glutathione synthesis from methionine, resulted not significantly different in preterm than in at-term newborns when N-acetyl cysteine was utilized as precursor [23]. Thus, the very low level of glutathionylated S100A9 long and cystatin B in preterm newborns must be ascribed to an inadequate synthesis of glutathione due to low cysteine concentration, even though we cannot rule out a possible immaturity of the enzymatic machinery involved in the process of S-thiolation. Furthermore, it should be outlined that red blood cells of at-term and preterm newborns exposed to oxidative stress showed higher levels of reduced and oxidized glutathione, but lower GSH/GSSG ratio and higher GSH-recycling rates than those of adults [24].

Interestingly, the percentage of the glutathionylated derivatives with respect to the sum of the cysteinylated and glutathionylated proteoforms was found particularly low in some preterm newborns. This result suggests that the enzymes responsible for glutathione synthesis are less active in some preterm newborns at birth.

Our study evidenced that the greatest variation of S-thiolated derivatives levels occurred up to about 280 days of PCA, which corresponds to the age of full-term newborns. Interestingly, data highlighted that differences in S-thiolation processes observed in preterm with respect to full-term babies did not persist over time, and that ability to synthesize S-thiolated proteoforms only depended on the PCA and not on whether the infant was born full term or preterm. Indeed, after birth, no differences between preterm and full-term infants, with the same PCA, were observed.

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AUTHOR CONTRIBUTIONS

I.M. and M.C. were associated with conceptualization; M.B. was associated with data curation; M.B., S.S., C.C., B.M., G.F., and I.M. were associated with formal analysis; T.C., A.O., and M.C. were associated with funding acquisition; B.M., T.C., F.I., A.O., L.P., M.T.S., and C.D. were associated with investigation; G.V., C.T., and A.F. were associated with acquiring resources; I.M. and M.C. supervised the study; M.B., I.M., and M.C. wrote the original draft; B.M., S.S., F.I., A.O., T.C., L.P., C.D., C.C., I.M., and M.C. reviewed and edited the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in at ProteomeXchange repository at http://proteomecentral.proteomexchange.org/cgi/GetDatas, reference number PXD025517.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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