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Homocysteine metabolism in children and adolescents with epidermolysis bullosa

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Abstract

Background: Epidermolysis bullosa (EB) belongs to a family of rare heterogeneous, genetic disorders characterized by blistering of the skin and mucous membranes in response to minor mechanical trauma. The involvement of the oral mucosa and oesophagus stenosis is suggested to be responsible for severe nutritional deficiencies, but few studies have till now considered this aspect. This observational study aimed to evaluate homocysteine status in children and adolescents with EB by assessing total plasma homocysteine (tHcy) and metabolically related vitamins (B_6, B_{12}, folate) concentrations.

Methods: Twenty EB patients (12 M; age range 0.5–19 years) were evaluated for: plasma tHcy, serum B_{12} and holotranscobalamin (HoloTC, the active fraction of B_{12}), serum and erythrocyte folate (s-F and Ery-F, respectively), plasma B_6 and serum high sensitive C-reactive-protein (hsCRP) levels. Clinical severity was also evaluated through the Birmingham Epidermolysis Bullosa Severity (BEBS) score. A sex and age well-matched population was also enrolled.

Results: EB patients showed tHcy levels higher ($p = 0.04$) and B_6 levels lower ($p = 0.03$) than controls. B_{12}, HoloTC, s-F and ery-F concentrations did not differ between patients and controls. Multiple linear regression analysis showed that tHcy levels were independent of the metabolically related vitamins levels. In addition, serum hsCRP levels were higher in EB patients than in controls ($p = 0.003$) and correlated negatively with B_6 concentrations ($r = -0.6$; $p = 0.009$). BEBS score correlated negatively with HoloTC ($p = 0.022$) and B_6 ($p = 0.005$) levels and positively with age ($p = 0.031$) and hsCRP levels ($p < 0.001$).

Conclusions: The assessment of tHcy and metabolically related vitamin levels describes an important aspect of EB patients' nutritional *status* which can result essential for their long term care. Monitoring B_6 levels in EB patients could be particularly important in order to prevent several complications associated with B_6 deficiency and to avoid a B_6 excess which sustains an inflammatory condition.

Keywords: Epidermolysis Bullosa, Homocysteine, Vitamin B_6, Holotranscobalamin

Background

Epidermolysis bullosa (EB) belongs to a family of inherited autosomal (dominant or recessive) skin disorders. It is characterized by recurrent blistering formation of the skin and mucous membranes in response to minor mechanical trauma. The blisters easily rupture, and the resulting erosions and ulcerations are prone to infection [1, 2].

EB is classified into four main types, depending on the level of epidermal separation from the underlying basal lamina: EB Simplex (EBS), Junctional EB (JEB), Dystrophic EB (DEB), *Kindler* Syndrome (KS) [3]. Because of the involvement of the oral mucosa and oesophagus stenosis, EB patients, especially JEB and DEB types, are at risk of severe nutritional deficiencies, such as B vitamins group deficiencies (vitamin B_6, vitamin B_9 or folate, vitamin B_{12}). This may be due to oral, oesophageal, and oropharyngeal problems (oral blistering and ulcerations, abnormal oesophageal motility,

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oesophageal strictures, dysphagia, and dental problems); digestion and absorption problems; anal erosions; fissures, and rectal strictures resulting in chronic constipation; loss of blood and protein through open skin blisters; and hyper-metabolism resulting in increased heat loss and protein turnover, especially in the setting of skin infections. These complications affect all infants, children, and adolescents with EB due to higher nutritional needs required for growth [2].

Hyperhomocysteinemia (HHcy) may be a result of one or more B vitamins group depletion and has been associated with several diseases, e.g. cardiovascular disease, Alzheimer disease and other dementias, peripheral neuropathy, renal failure and hypothyroidism [4–6]. Moreover, recent studies have demonstrated the involvement of homocysteine (Hcy) both in the enhancement of inflammatory activation and in autoimmunity triggering mechanisms, thus suggesting a possible role for Hcy not only in the development of cardiovascular disease but also in the pathogenesis of autoimmune diseases [7]. Since Hcy metabolism is catalysed by enzymes requiring B vitamins as cofactors, high total Hcy levels (tHcy) can indicate undernourishment due to a lack of metabolically related vitamins: in particular vitamin $B_{12,}$ holotranscobalamin (HoloTC, the biologically active form of B_{12}) [8] and/or folate, cofactors for the re-methylation pathway, and B_6 for the transulfuration pathway [4].

Up to now few studies have taken into consideration EB patients' nutritional deficiencies. To the best of our knowledge, this is the first study aimed at evaluating Hcy *status* by assessing tHcy and metabolically related vitamins levels in EB patients.

Methods

Subjects

Twenty consecutive EB children and adolescents (12 M/8 F; age range 0.5–19 years) were enrolled at the Pediatric Highly Intensive Care Unit of Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico, Milan, Italy.

EB types were classified based on genetic, immunofluorescence mapping results, and clinical features, according with Fine JD. et al. [3].

Type analysis identified 6 cases of EBS (30 %), 1 case of JEB (5 %), 11 cases of DEB (55 %) and 2 cases of KS (10 %). Among DEB patients, 3 cases (27.3 %) were dominant dystrophic epidermolysis bullosa (DDEB) and 8 cases (72.7 %) were recessive dystrophic epidermolysis bullosa (RDEB).

In all patients, disease severity was also evaluated through the Birmingham Epidermolysis Bullosa Severity (BEBS) score. This score takes into account: area of damaged skin; involvement of nails, mouth, eyes, larynx and oesophagus; scarring of hands; skin cancer; chronic wounds; alopecia; nutritional compromise. Area was allocated 50 points, and the 10 other items 5 points each, giving a maximum score of 100 [9]. The BEBS score was performed by the paediatrician who regularly followed the patients (SG) at blood sample collection.

EB patients were compared with a healthy and well-matched age and gender control population (12 M/8 F; age range 1–19 years).

The study protocol was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy (Registration number: 2014–359), and conducted in accordance with the standards of Good Clinical Practice for trials of medicinal products in humans. The informed written consent of a parent or legal guardian was required for subjects aged <18, and the subjects aged ≥8 were asked to give their written assent. Patients' parents and patients >8 years gave their written consent to data publication. Ethics Committee also approved data publication.

Nutritional status

Children with severe EB tend to be short and underweight for their age. Nutritional status assessment was conducted by anthropometry. Patients and controls' height and weight were measured under standard conditions by the same nurse, always using the same measuring equipment; Body Mass Index (BMI) was calculated. Weight, height and BMI for age were evaluated using Cacciari E. et al. growth curves [10].

Biochemical analysis

Plasma tHcy and B_6, serum folate (s-F), B_{12} and HoloTC, erythrocyte (ery-F) levels were measured in order to evaluate homocysteine metabolism. Serum high sensitive C reactive protein (hsCRP) was also assessed.

Blood samples were drawn in the morning, after an overnight fast. Two blood specimens from each patient were collected in light protected tubes, either with no additive (for serum B_{12}, HoloTC, s-F and hsCRP) or containing ethylenediaminetetracetic acid (EDTA) to prevent coagulation, for ery-F, tHcy and B_6 concentration assays. EDTA specimens were immediately put on ice and, after collecting whole blood aliquots for complete blood count and ery-F determination, were centrifuged within 30 min in order to obtain plasma samples for tHcy and B_6 determination. Serum and plasma samples were frozen and stored at -80 ° C until analysed.

Plasma tHcy and serum B_{12} concentrations were determined by immunoenzymatic assay using the relevant commercial kits on AIA 600II analyser (Tosoh Bioscience, Tokyo, Japan), while HoloTC, s-F and ery-F by means of immunoenzymatic assay using the relevant commercial kits on Architect analyser i2000SR (Abbott

Diagnostics, Abbott Park, IL, USA). Plasma B_6 concentrations were evaluated by HPLC method using the relevant commercial kit (Chromsystems Instruments & Chemicals, Munich, Germany). HsCRP levels were assessed by immunoenzymatic assay using the relevant commercial kit on Modular P analyzer (Roche, Switzerland).

Because of the continuous physiological changes that occur throughout childhood, appropriately partitioned pediatric reference values are not readily available. For this reason, tHcy levels and metabolically related vitamins of EB patients were compared to those of a healthy population well-matched age and gender.

In addition, in our study, hyperhomocysteinemia and B_{12} deficiency were defined according to the reference intervals and *cut-off* values described by Bailey D. et al. in the Canadian Laboratory Initiative for Pediatric Reference Intervals (CALIPER) program [11] (Table 3), while B_6, s-F and ery-F levels were compared to the data obtained from a meta-analysis which described biochemical vitamin-status data in different groups of the Spanish population [12] (Table 3). Particularly, assuming that B_6, s-F and ery-F were normally distributed, we considered as reference interval the mean vitamins levels obtained in the Spanish Population, taking two standard deviations either side of the mean [12].

HoloTC levels were classified by using the relevant *cut-off* value for adult population [8] as any study has not yet performed in children and adolescent.

Statistical analysis

Results, reported as the median value with InterQuartile Range (IQR; 25–75 percentiles), were analyzed using the Mann-Whitney test to assess any difference between EB patients and controls. In addition, because of the small number of JEB and KS subjects, only EBS and DEB groups' Hcy metabolically related vitamins and hsCRP levels were compared.

Multiple linear regression analysis was used to find predictors of homocysteine values. Pearson's coefficient was used to test the correlation between continuous variables. The statistical analysis was performed by using Stata 13 (StataCorp. 2013, Stata: Release 13 Statistical Software. College Station, TX: StataCorp LP).

Results

Demographic characteristics and growth *outcomes* of patients and controls are reported in Table 1.

With regard to weight, 35 % ($n = 7$) and 10 % ($n = 2$) of patients and controls, respectively, were below the 3rd percentile ($p = 0.13$); particularly, among these patients, 57 % were DEB ($n = 4$; RDEB), 29 % were EBS ($n = 2$) and 14 % were JEB ($n = 1$).

In regard to height, only 2 subjects were under the 3rd percentile both in patients and in controls. Forty percent of patients ($n = 8$) and 20 % of controls ($n = 4$) had a BMI below the 3rd percentile ($p = 0.30$); among these patients, 50 % were DEB ($n = 4$; RDEB), 37.5 % were EBS ($n = 3$) and 12.5 % were JEB ($n = 1$).

In addition, 1 DDEB patient (BEBS score = 2) showed both weight between the 90th-97th percentile and BMI >95th percentile; 1 EBS patient (BEBS score = 1) reported BMI > 95th percentile.

Controls showed both weight and BMI under the 90th percentile.

Among DEB patients, 5 of them (45.5 %) had oesophagus stenosis. Only one patient (JEB) was celiac while any other patients did not present clinical signs and symptoms of malabsorption.

BEBS score ranged from 2 to 67 points (Table 2).

Although we did not reported daily caloric and nutrient intake of our patients, 25 % of EB subjects ($n = 4$; 1 KS patient and 3 DEB patients) were taking calorically dense liquid supplements fortified with vitamins and minerals orally, containing vitamin B_{12}, B_6 and folic acid. Another 25 % of them ($n = 4$; 1 EBS patients, 3 DEB patients and 1 JEB patient) were supplemented only with vitamin B_{12}. However the supplemented vitamin doses were less than the Italian Official Recommendation (IOR) vitamin intake [13].

Both EB patients and controls had normal renal functions (data not shown). The presence of microcytic anaemia was highlighted in 45 % of EB patients while controls' hematologic parameters were within the relevant reference intervals (data not shown).

Biochemical parameters of patients and controls are listed in Table 3; results were reported as median value;interquartile range (median;IQR).

Plasma tHcy levels were significantly higher in EB patients than in controls (9.9;7.5–10.5 μmol/L *vs* 6.5;5.8–8.4 μmol/L; $p = 0.04$) (Fig. 1a) and 55 % of patients showed tHcy levels above the *cut-off* value (10.4;10.1-10.9 μmol/L), according to CALIPER program's reference interval [11]. Particularly, DEB patients showed significantly higher tHcy concentrations than controls (10.0;8.3-10.6 μmol/L *vs* 6.5;5.8-8.4 μmol/L; $p = 0.03$), while EBS patients' tHcy levels did not differ significantly from controls' levels (9.4;6.5-10.4 μmol/L *vs* 6.5;5.8-8.4 μmol/L; $p = 0.25$).

The evaluation of the Hcy metabolically related vitamins highlighted altered plasma B_6 levels in 70 % of EB patients [12] and, interestingly, B_6 levels were significantly lower in EB patients than in controls (6.9;4.2-12.3 μg/L *vs* 11.3;9.2-14.3 μg/L; $p = 0.03$) (Fig. 1b). Moreover, B_6 levels were significantly lower both in patients with weight <3rd percentile compared to the other patients (3.6;1.8-6.3 μg/L *vs* 9.1;6.9-18.3 μg/L; $p = 0.03$)

Table 1 Demographic characteristics and growth outcomes of patients and controls

	EB patients ($n = 20$)	Controls ($n = 20$)	p^*
Sex (M/F)	12/8	12/8	n.a.
age (years)	8.0;6.5-12.5	6.1;5.5-13	0.89
Growth outcomes			
Weight for age[a]	*n = 20*	*n = 20*	
< 3 p	35 % (7)	10 % (2)	0.13
3-10 p	10 % (2)	15 % (3)	0.99
10-25 p	15 % (3)	10 % (2)	0.99
25-50 p	10 % (2)	35 % (7)	0.13
50-75 p	20 % (4)	15 % (3)	0.99
75-90 p	5 % (1)	15 % (3)	0.60
90-97 p	5 % (1)	0 % (0)	0.99
Height for age[a]	*n = 20*	*n = 20*	
< 3 p	10 % (2)	10 % (2)	0.60
3-10 p	20 % (4)	20 % (4)	0.60
10-25 p	15 % (3)	10 % (2)	0.99
25-50 p	25 % (5)	20 % (4)	0.99
50-75 p	15 % (3)	5 % (1)	0.60
75-90 p	10 % (2)	15 % (3)	0.99
90-97 p	5 % (1)	20 % (4)	0.34
BMI (Kg/m^2)[a]	*n = 20*	*n = 20*	
< 3 p	40 % (8)	20 % (4)	0.30
3-10 p	5 % (1)	15 % (3)	0.60
10-25 p	20 % (4)	10 % (2)	0.66
25-50 p	20 % (4)	20 % (4)	0.69
50-75 p	5 % (1)	25 % (5)	0.18
75-90 p	0 % (0)	10 % (2)	0.47
90-95 p	0 % (0)	0 % (0)	n.a.
95-97 p	5 % (1)	0 % (0)	0.99
> 97 p	5 % (1)	0 % (0)	0.99

Demographic characteristics are reported as median;IQR while growth *outcomes* are reported as percentage values and (number of subjects). * Mann-Whitney test for age and chi-square test for growth *outcomes*; *n.a* not applicable; [a] Cacciari E. et al. [10]

and in patients with a BMI <3rd percentile (4.6;2.3-6.2 μg/L *vs* 9.6;7.5-20.0 μg/L, $p = 0.01$) compared to the other patients.

S-F levels were altered in 50 % of EB subjects [12], and were significantly lower both in patients with weight <3rd percentile compared to the other patients (8.6;6.1-10.4 nmol/L *vs* 13.8;10.4-22.4 nmol/L; $p = 0.05$) and in patients with BMI <3rd percentile (7.6;5.8-9.9 nmol/L *vs* 15.9;11.0-23.3 nmol/L; $p = 0.01$) compared to the other patients. However median s-F concentrations between patients and controls did not show any difference ($p = 0.64$).

Table 2 The Birmingham Epidermolysis Bullosa Severity (BEBS) score of EB patients

Patient	Sex (F: female; M: male)	Age (years)	EB classification	BEBS score
1	F	6	DDEB	2
2	M	8	KS	15
3	M	11	KS	17
4	M	19	RDEB	60
5	F	13	RDEB	50
6	M	10	EBS	8
7	F	7	RDEB	40
8	M	13	RDEB	65
9	M	3	RDEB	27
10	M	3	EBS	3
11	M	18	JEB	34
12	F	7	RDEB	32
13	M	8	EBS	1
14	F	3	EBS	25
15	F	13	EBS	17
16	M	0.5	EBS	3
17	M	7	DDEB	2
18	M	12	DDEB	1
19	F	7	RDEB	34
20	F	10	RDEB	67

EBS Epidermolysis Bullosa Simplex, *JEB* Junctional Epidermolysis Bullosa, *DDEB* Dominant Dystrophic Epidermolysis Bullosa, *RDEB* Recessive Dystrophic Epidermolysis Bullosa, *KS* Kindler Syndrome

Serum B_{12} and HoloTC levels were normal both in most of EB patients and controls, according to Bailey D. et al. [11] and Bamonti F. et al studies [8], respectively.

No differences between patients and controls were observed in B_{12}, HoloTC and ery-F levels.

However, interestingly, HoloTC concentrations were lower both in patients whose weight was under the 3rd percentile, compared to the other EB patients (83.0;71.6-99.7 pmol/L *vs* 126.0;93.9-128.0 pmol/L; $p = 0.09$) and in patients with BMI <3rd percentile (90.5;74.6-99.2 pmol/L *vs* 128.0;92.8-128.0 pmol/L; $p = 0.08$), compared to the other EB patients.

Despite the altered levels of B_6 and s-F, multiple linear regression analysis showed that tHcy levels were independent of the metabolically related vitamins levels.

Serum hsCRP levels were significantly higher in EB patients than in controls ($p = 0.003$), altered in 50 % of patients, (Fig. 1c) and higher depending on the severity of EB type. In fact, hsCRP median levels were significantly higher both in DEB patients than controls (3.0;0.2-5.4 mg/dL *vs* 0.04;0.03-0.1 mg/dL; $p = 0.000$) and in DEB than in EBS patients (3.0;0.2-5.4 mg/dL *vs* 0.2;0.03-1.0, mg/dL; $p = 0.04$).

Table 3 Biochemical parameters of patients and controls

Analyte (reference interval or cut-off)	EB patients ($n = 20$)	Controls ($n = 20$)	p^*
tHcy[a] (1 < 7 years; 2.7-7.6 μmol/L 7 < 12 years; 3.4 -8.4 μmol/L 12 < 15 years; M: 4.7-10.4 μmol/L; F: 4.1-10.4 μmol/L 15 < 19 years; M: 5.5-13.4 μmol/L; F: 4.9-11.9 μmol/L)	9.9;7.5-10.5 (55 %)	6.5;5.8-8.4 (15 %)	0.04 [0.02]
B^a_{12} (1 < 9 years; 208.8- 1190 pmol/L 9 < 14 years; 185.9-830.2 pmol/L 14 < 17 years; 180.1-655.3 pmol/L 17 < 19 years; 149.8-598.5 pmol/L)	583.4;402.2-822.3 (0 %)	468.8;360.2-652.9 (5 %)	0.30 [1.00]
HoloTC (>40 pmol/L)	99.2;79.7-128 (10 %)	84.9;51.1-128 (10 %)	0.42 [0.60]
s-F[b] (0-15 years; 11.6-33.2 nmol/L 15-65 years; 5.8-28.6 nmol/L)	11.1;8.4-21.3 (50 %)	13.1;11.1-15.7 (20 %)	0.64 [0.10]
ery-F[b] (2-15 years; 409.9-1162 nmol/L)	843.3;591.0-1559.7 (10 %)	608.4;556.3-742.5 (10 %)	0.10 [0.60]
B^b_6 (2-5 years; 10.0-21.6 μg/L 9-13 years; 9.6-24.4 μg/L)	6.9;4.2-12.3 (70 %)	11.3;9.2-14.3 (25 %)	0.03 [0.01]
hsCRP (<0.5 mg/dL)	0.55;0.01-3.1 (50 %)	0.05;0.03-0.16 (0 %)	0.003 [0.00]

Data are reported as median;IQR. Percentage of altered values are reported in parentheses (%). *Mann-Whitney test [chi-square test]
[a] Bailey D. et al. [11]; [b] Ortega RM. et al. [12]. tHcy: total Homocysteine; B_{12}: vitamin B_{12}; HoloTC: Holotranscobalamin; s-F: serum Folate; ery-F: intraerythrocyte; B_6: vitamin B_6; hsCRP: high sensitive C Reactive Protein

A negative correlation was found between hsCRP levels and B_6 levels (r = -0.6; $p = 0.009$) (Fig. 1d).

Finally, BEBS score correlated negatively with HoloTC (r = -0.5; $p = 0.022$) and B_6 (r = -0.6; $p = 0.005$) levels and positively with age (r = 0.5; $p = 0.031$) and hsCRP levels (r = 0.8; $p < 0.001$).

Discussion

Hyperhomocysteinemia, a marker for folate, B_{12} and B_6 deficiency, has been associated with enhancement of inflammatory activation and with autoimmunity triggering mechanisms [7]. Patients with EB suffer from acute and chronic malnutrition. In fact, in these patients, oesophagus stenosis and oral mucosa erosions are responsible for restricted ingestion, and protein-calorie malnutrition is worsened by losses from cutaneous blisters and by chronic inflammatory syndrome secondary to chronic skin infection [2]. Therefore, nutritional management is an essential aspect of the long term care of patients with EB because protein, vitamin and trace element intakes are essential for growth and wound healing and for the host resistance against bacterial infections.

Nowadays few studies have described vitamin status in EB. A previous study by Ingen-Housz-Oro S. et al. [14], described vitamin and trace metal status of 14 recessive DEB (RDEB) patients. The authors reported iron, vitamin D, C, B_6, PP, zinc, and selenium deficiencies in 36–70 % of the patients, while vitamin B_1, B_{12}, B_2, A, retinol binding protein and carnitine levels were within the relevant reference interval. For the first time, on the basis of these considerations, we evaluated, homocysteine status by assessing tHcy levels and metabolically related vitamins in 20 EB children and adolescents and compared them to those of a healthy population well-matched for age and gender. Our results showed tHcy levels above the *cut-off* value in more than 50 % of EB patients. Particularly, even if tHcy levels did no differ between EBS subjects and controls, DEB subjects' tHcy levels were higher than in controls.

Analysing Hcy related vitamins our results concerning B_6 were in agreement with previous findings [14, 15]; in fact, plasma B_6 levels were under the lower limit of the reference interval in most of our patients [12] and lower in EB patients than in controls. As regards this vitamin deficiency, B_6 levels in EB patients with weight under the 3rd percentile, differed from B_6 levels of patients with weight above the 10th percentile. A plausible explanation of this deficiency was due to the EB patients' undernourishment.

Shen J et al. [16] reported that plasma pyridoxal 5'-phosphate (PLP), the biologically active form of vitamin B_6, was adversely associated with inflammatory markers, such as CRP, fibrinogen, and blood cell count. In addition, an inverse relationship between CRP and PLP has also been found among participants in the Framingham Heart Study and the National Health and Nutrition Examination Survey (NHANES) [17]. Current evidence

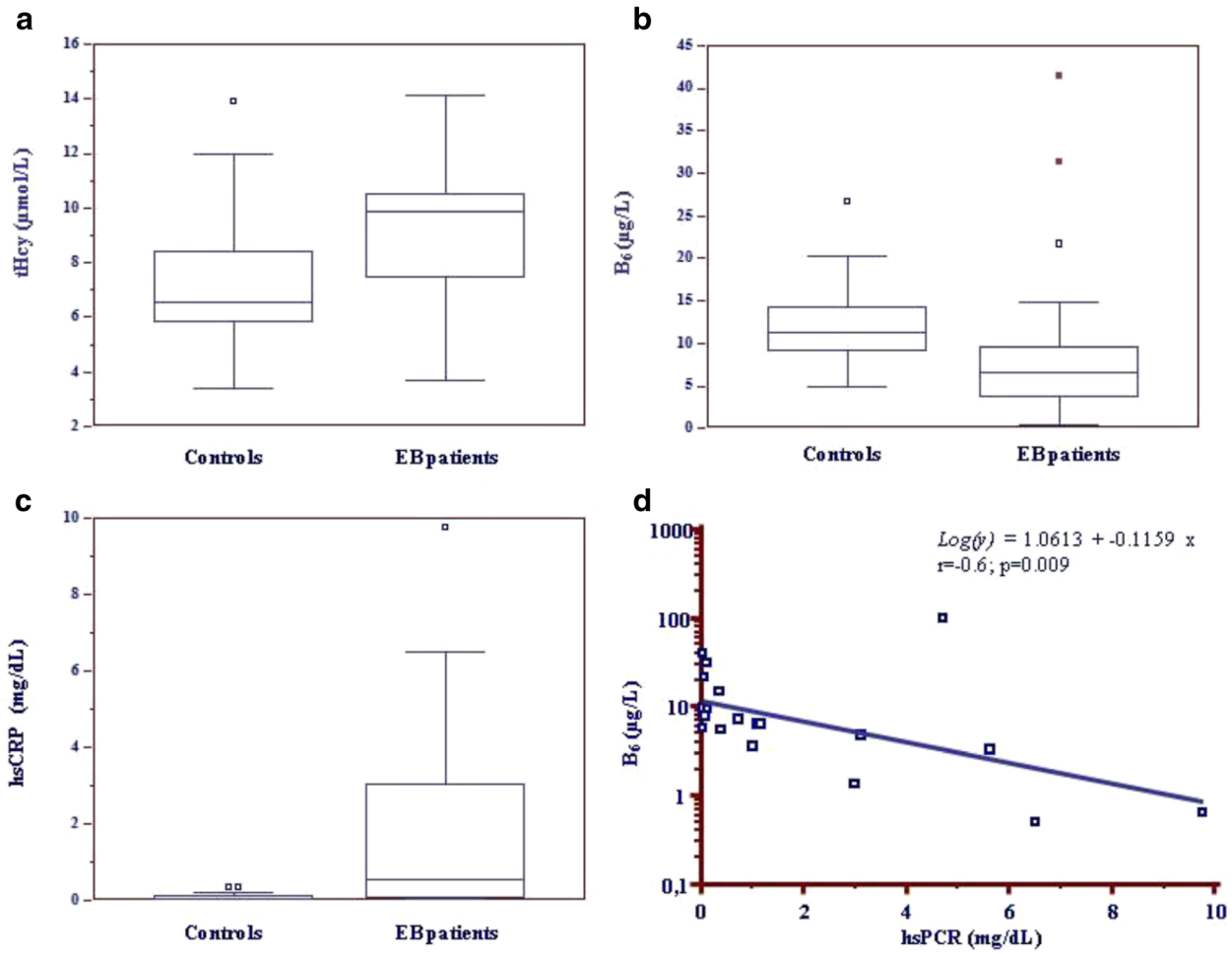

Fig. 1 Biochemical parameters analysis in EB patients and controls. **a** Box-and-Whisker plot of tHcy levels between EB patients and controls ($p = 0.04$); **b** Box-and-Whisker plot of vitamin B_6 levels between EB patients and controls ($p = 0.03$); **c** Box-and-Whisker plot of hsCRP levels between EB patients and controls ($p = 0.003$). Data are reported as median;Interquartile range (IQR); **d** Correlation between vitamin B_6 and hsCRP levels in EB patients ($p = 0.009$)

suggests that the inverse association between plasma PLP and inflammation may be the result of this coenzyme used by the PLP-dependent enzymes for the kynurenine pathway of tryptophan degradation, for the metabolism of the immunomodulatory sphingolipids, ceramide and sphingosine 1-phosphate, and for serine hydroxymethylase for immune cell proliferation [17, 18]. According to the previous findings [17, 18], in the present study an inverse correlation was found between B_6 levels and hsCRP in EB patients. In fact, our EB patients showed hsCRP levels higher than controls and, interestingly, higher in DEB than in EBS. No difference was found between EBS and controls. This was probably due to the extent of blistering and ulcerations, underlying a possible correlation between the severity disease and the inflammatory degree. Therefore, we hypothesized that B_6 deficiency was not only a consequence of undernourishment but was also related to the systemic inflammation which characterized these patients.

Ery-F concentration is a reliable indicator of long-term folate *status* and general dietary intake, and s-F of more recent intake; a more comprehensive estimate of total folate *status* is generally obtained by assessing both parameters, as reported in this and in our previous studies [19–21]. In the present study, 50 % of the patients had altered s-F values due to undernourishment; in fact, in patients whose weight was under the 3rd percentile, s-F levels were lower than in the other patients. However, s-Fol levels did not differ between the two groups, underlying only a mild deficiency in EB subjects. This was also confirmed by assessing ery-F levels which were within the relevant reference interval both in most patients and in controls and similar to those obtained by Ortega RM et al. [12].

The determination of cobalamin *status*, by measuring also HoloTC concentrations, represents an approach for diagnosing subtle cobalamin deficiency. HoloTC, the transcobalamin (TC)-cobalamin complex representing the biologically active form of the vitamin and consisting of 10 %–30 % of total serum B_{12}, is recognized by ubiquitous specific membrane receptors and could have high diagnostic value as a marker of storage [8]. It has been demonstrated that HoloTC is a more sensitive marker of vitamin B_{12} *status* compared with total serum cobalamin [8], and it could be the earliest and most sensitive marker for vitamin B_{12} deficiency [8]. According to Ingen-Housz-Oro S. et al's study [14], B_{12} levels were within the relevant reference interval in most patients and controls; this was probably due to the enterohepatic recycling preventing an early onset of cobalamin deficiency [22]. Although HoloTC levels did not differ significantly between the two groups, were altered in 10 % of patients and controls. Additionally, undernourished EB subjects weight <3rd percentile) showed decreased HoloTC concentrations but vitamin B_{12} levels within the relevant reference interval when compared to the other well-nourished patients. Hence the importance of HoloTC determination, as an early marker of B_{12} deficiency, in order to monitor cobalamin *status*.

However, in the present study it was considered the HoloTC *cut-off* value of an adult population (*cut-off* value >40 pmol/L) [8] and this possible limitation could suggest that further studies are necessary in order to assess HoloTC levels in a larger paediatric population and create an appropriate *cut-off* value.

Finally, considering Hcy metabolism, despite the presence of B_6 and s-F deficiency in some patients, the multiple linear regression analysis showed that tHcy levels did not depend on the metabolically related vitamins. A possible explanation is that it could be related to the limited number of studied patients; further studies are required to extend these preliminary results by expanding EB population.

A well-known fact is that even among EB patients within the same EB type, clinical symptoms could vary significantly as regards the severity of the disease. The nutritional *status* of these patients does not always correlate with their genetic diagnosis but is rather related to the clinical symptoms and could also be influenced by other socio-economic factors [23]. Therefore, in the present study, we scored disease severity for each patient using the BEBS [9] which might help to characterize patients with unexpectedly mild or severe disease, and contributing to genotype phenotype correlation [9]. As previously reported by other authors [9], BEBS score correlated positively with age, suggesting that changes in BEBS score reflect clinical observations, particularly disease progression with age. Additionally, BEBS score confirmed the B_6 deficiency due to undernourishment and severe inflammation and the usefulness of HoloTC as an early marker of B_{12} deficiency.

The findings of our study are in agreement with the aforementioned consideration, particularly as regards BMI and B_6 levels; in fact, in our population, 1 DDEB patient, despite the genetic diagnosis, showed good clinical symptoms (BEBS score = 2), BMI >95th percentile and normal B_6 levels. On the contrary, 3 EBS patients (BEBS score: 8; 17; 25) showed BMI <3rd percentile and B_6 levels under the lower limit of the reference interval [12].

Conclusions

EB patients are at risk of severe nutritional deficiencies; to the best of our knowledge, this is the first study that took into consideration the possible nutritional deficiencies (B vitamins group) of young EB patients by evaluating Hcy *status*.

Most of EB patients showed tHcy levels above the *cut-off* value, probably due to the S-F and B_6 levels deficiency. In fact, S-F levels decreased in half of the patients and even more so in the undernourished ones. However, by comparing EB patients's-F levels with controls', we noticed only a mild deficiency in EB patients. The consistent B_6 deficiency was due not only to the undernourishment but also to the severe inflammation which in turn was underlined by an increase in EB patients' hsCRP levels.

Finally, tHcy levels were not correlated significantly with metabolically related vitamins deficiency (particularly S-F levels and B_6), because the limited number of studied cases.

In addition, we also reported the importance of HoloTC evaluation as an early marker of cobalamin deficiency.

In conclusion, monitoring tHcy and metabolically related vitamin levels enable us to describe the EB patients' nutritional *status* could represent an essential aspect of their long term care. Monitoring B_6 levels is particularly important in order to avoid both a number of complications associated with B_6 deficiency and an excess of B_6 which sustains an inflammatory condition.

Abbreviations

BEBS: Birmingham epidermolysis bullosa severity; BMI: Body mass index; DDEB: Dominant dystrophic epidermolysis bullosa; DEB: Dystrophic epidermolysis bullosa; EB: Epidermolysis bullosa; EBS: Epidermolysis bullosa simplex; EDTA: Ethylenediaminetetracetic acid; ery-F: erythrocyte; Hcy: Homocysteine; HHcy: Hyperhomocysteinemia; HoloTC: Holotranscobalamin; hsCRP: high sensitive C reactive protein; JEB: Junctional epidermolysis bullosa; KS: Kindler syndrome; RDEB: Recessive dystrophic epidermolysis bullosa; s-F: serum folate; tHcy: total Hcy levels

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Availability of data and material
All the data and materials used are included in the manuscript.

Authors' contributions
RDG designed the study and the experiments, carried out the experiments, analysed and interpreted data as well as wrote the first draft of the manuscript; GV carried out the experiments, analysed and interpreted data; SG and SS enrolled patients and interpreted data; GC and FM participated in patients' enrollment; CDV, CD and RM carried out the experiments; DC carried out statistical analysis; FB and SE interpreted data and critically revised the text. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Patients' parents and patients >8 years gave their written consent to data publication. Ethics Committee also approved data publication.

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, (Registration number: 2014-359) and conducted in accordance with the standards of Good Clinical Practice for trials of medicinal products in humans. The informed written consent of a parent or legal guardian was required for subjects aged <18, and the subjects aged ≥8 were asked to give their written assent.

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