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Original Article

Oxidative stress status during the acute phase of haemolytic uraemic syndrome

Veronica Ferraris[1], Andrea Acquier[3], Jorge R. Ferraris[1], Graciela Vallejo[2], Cristina Paz[3] and Carlos F. Mendez[3,4]

[1]Pediatric Nephrology Service, Hospital Italiano de Buenos Aires, Argentina, [2]Nephrology Unit, Hospital de Niños Ricardo Gutierrez, Argentina, [3]Institute of Molecular Research in Hormonal, Neurodegenerative and Oncological Diseases, Department of Biochemistry, School of Medicine, University of Buenos Aires, Argentina and [4]Pharmacology Unit, School of Dentistry, University of Buenos Aires, Buenos Aires, Argentina

Correspondence and offprint requests to: Carlos F. Mendez; E-mail: cfmendez@fmed.uba.ar

Abstract

Background. Haemolytic uraemic syndrome (HUS) is characterized by haemolytic anaemia, thrombocytopaenia and acute renal failure. The aim of this study was to investigate the levels of oxidative stress (OS) during the acute phase of HUS.

Methods. This prospective study included 18 patients diagnosed with D+HUS, 6 age-matched healthy controls and 29 children with end-stage renal disease (ESRD) not caused by HUS under regular haemodialysis. Plasma lipid peroxidation and non-enzymatic antioxidant defences were measured as thiobarbituric acid-reactive substances (TBARs) and total reactive antioxidant potential (TRAP), respectively, during hospitalization and in control individuals.

Results. TBARs were significantly higher in both oliguric and non-oliguric patients at admission (1.8 ± 0.1; 1.7 ± 0.2 μM) and discharge (1.5 ± 0.1; 1.0 ± 0.1 μM) vs controls (0.5 ± 0.1 μM, $P < 0.01$) following disease progression. Maximal TBARs values differed significantly between oliguric and non-oliguric groups (4.5 ± 0.9 vs 2.4 ± 0.3 μM, $P < 0.01$) and were significantly higher ($P < 0.05$) than those found in ESRD patients (1.63 ± 0.1). TRAP values were significantly higher at admission and when the disease was fully established (measured here as highest TBARs record) vs controls (675 ± 51, 657 ± 60 and 317 ± 30 μM Trolox, $P < 0.01$), and were similar to control values at discharge (325 ± 33 μM Trolox).

Conclusions. We demonstrate here increased levels of OS during the acute phase of HUS, with peak plasma lipid peroxidation values well above those registered in ESRD individuals, and suggest a connection between OS and the clinical course of HUS.

Keywords: acute renal failure; haemolytic uraemic syndrome; oxidative stress

Introduction

Haemolytic uraemic syndrome (HUS) is a disease characterized by thrombotic microangiopathic haemolytic anaemia, thrombocytopaenia and variable organ impairment with a predominant feature of acute renal failure (ARF) in children [1,2]. Over 95% of affected children recover from the disease during the acute phase, and overall mortality rate is currently under 5%. Chronic renal failure correlates well with the duration of oligoanuria during the acute phase. According to a local study, in Argentina, chances of evolution to chronic renal failure are as high as 90% with 15 days or over of oligoanuria, a probability that is reduced to 30 or <2% when oligoanuria lasts between 7 and 14 days or less than a week, respectively [3].

In its classical form, HUS develops as a consequence of the interaction of the Shiga toxin (Stx), produced by enterohaemorrhagic strains of *Escherichia coli*, with the vascular endothelium [4]. Strong epidemiologic evidence links Stx-producing *E. coli* of different serotypes to sporadic cases and outbreaks of HUS. However, although less frequent, HUS is associated with gastrointestinal infections caused by other bacteria [5].

Although important evidence exists pointing to vascular endothelial injury as the initiating event after the infection [6–8], the pathogenesis of HUS is not completely clear. There are various factors implicated in the aetiology of the syndrome, including reactive oxygen species (ROS), but neither the source nor the role that ROS play have been determined [9,10]. It has also been reported that there is an increase in the plasma production of an inhibitor of the vessel-wall enzyme prostacyclin synthase during the course of the disease. This could result in a deficiency of prostacyclin and, therefore, increased platelet aggregation with thrombosis. The inhibitor was initially identified as a lipid peroxide, probably a metabolite of arachidonic acid [11].

Table 1. Demographic features of the population studied

		Patients (*n*)	Gender		Median age months (range)
			Male	Female	
Non-oliguric		5	4	1	23 (8–57)
Oliguric	Haemodialysis	6	1	5	31 (11–92)
	Peritoneal	7	3	4	26 (7–52)
Total		18	8	10	25 (7–92)

Subjects are divided according to the degree of renal impairment in either a non-oliguric or an oliguric group. Patient age is expressed in median months and range.

Oxidative stress is a condition that defines the steady-state level of oxidants and antioxidants in a cell, tissue, or organ [12]. The level of oxidative stress is determined by the balance between the rate at which ROS are produced and the rate at which they are inactivated by endogenous antioxidant defences [13]. Previous reports have incriminated free oxygen radicals as causative agents of renal damage, particularly during episodes of ischaemia–reperfusion but also during acute and chronic renal failure [14–17]. We therefore conducted an observational, prospective and longitudinal study aimed at exploring plasma oxidative stress levels during the acute phase of HUS.

Materials and methods

Subjects

A total of 18 patients diagnosed with HUS upon admission to the Pediatric Nephrology Service of the Hospital Italiano de Buenos Aires or to the Nephrology Unit of the Hospital de Niños Ricardo Gutierrez between November 2006 and April 2007 were included in the present study. All patients were diagnosed with the typical form of the disease and presented with a diarrhoeal prodrome. The demographic features of the population studied are summarized in Table 1.

Samples for establishing a reference value were taken from six age-matched healthy volunteers. The inclusion criteria for healthy volunteers were no history of or active renal or haemolytic disease, no active infective diseases, no medication, standard diet and no ingestion of antioxidants of any type.

For comparison, 29 children with end-stage renal disease (ESRD) not caused by HUS and under regular haemodialysis were also included in the study. The inclusion criteria for this group were no active diseases and no intravenous iron administration.

The study protocol, which adhered to the Declaration of Helsinki, was approved by the ethics committees of the respective hospitals, and all participants and their parents were informed of the study and signed a written consent to participation.

Biological samples and collection

Blood was collected daily at 6 am during the hospital stay of patients. For patients receiving dialysis, blood was drawn always prior to the procedure. For healthy controls, a single sample was derived from the blood obtained after an extraction indicated during the course of a medical examination of subjects meeting the inclusion criteria. In both cases, blood was collected by venipuncture into polypropylene tubes containing EDTA. After centrifugation at 4°C for 10 min at 1500 × g, obtained plasma was carefully removed and frozen at −75°C until used.

Reagents

2-Thiobarbituric acid (TBA), glycine, luminol and 1,1,3,3-tetraethoxypropane (TTP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2,2'-Azo-bis-(2-methylpropionamidine) dihydrochloride (ABAP) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich. 1-Butanol and acetic acid were from Cicarelli (San Lorenzo, Argentina), and hydrochloric acid and absolute ethanol were purchased from Merck (Darmstadt, Germany). All other reagents were of highest quality available.

Determination of thiobarbituric acid-reactive substances (TBARs)

TBARs were determined fluorometrically on a daily basis in the plasma of patients and control subjects as described by Wasowicz *et al.* [18]. Briefly, 50 µL of plasma or an adequate volume of malondialdehyde (MDA) working standard solution was pippeted into 10-mL glass tubes containing distilled water to a final volume of 1 mL. After addition of 1 mL of 29 mM TBA in 8.75 M acetic acid and mixing, the samples were placed in a water bath and heated for 1 h at 95–100°C. After cooling of the samples, 25 µL of 5 mM HCl was added, and the reaction mixture was extracted by a brief vortexing with 3.5 mL of 1-butanol. The butanolic phase was then separated by centrifugation at 1500 × g for 10 min, and its fluorescence measured with a Jasco FP-770 spectrofluorometer at wavelengths of 525 and 547 nm for excitation and emission, respectively.

The calibration curve was prepared with MDA generated by hydrolysis of TTP. The stock standard solution of MDA was prepared by dissolving TTP in ethanol. Just before use, the solution was diluted in distilled water to yield a 1-µM MDA working standard.

Determination of total reactive antioxidant potential (TRAP)

TRAP, representing the non-enzymatic antioxidant defences of the plasma, was measured in the plasma of patients and control subjects as described by Lissi *et al.* and Vargas *et al.* [19,20]. The reaction medium consisted of 50 µM ABAP and 40 µM luminol. ABAP is a source of free radicals that react with luminol yielding chemiluminescence. The resulting chemiluminescence was measured in a scintillation counter in the out-of-coincidence mode. The addition of a plasma aliquot decreased the chemiluminescence to basal levels, for a period proportional to the amount of antioxidants present in the sample, until luminol radicals were regenerated (induction time). Different concentrations of Trolox (vitamin E water-soluble analogue) were used for calibration. A comparison between the induction time of known concentrations of Trolox and of plasma allows calculation of TRAP as the equivalent of Trolox concentration necessary to produce the same induction time. Results are thus expressed as micromolar Trolox.

Clinical determinations

The following laboratory determinations were performed throughout hospital stay as part of the routine control: haemogram, platelet count, plasma urea, plasma creatinine, lactate dehydrogenase (LDH), ionogram and acid–base status. Determinations were performed at the Hospital Clinical Laboratory according to standardized laboratory practice.

Statistics

Unless otherwise stated, data are expressed as the mean ± standard error of the mean (SEM). Significance of differences was determined either by one-way analysis of variance (ANOVA) followed by Bonferroni or one-tailed Dunnett's *t*-test as post hoc tests. Non-normally distributed variables

Table 2. Disease course

	Hospital stay	Dialysis	Transfusions			Neurological signs	Minimum platelet count
			Plasma	RBC	Platelet		
Non-oliguric	7 (5–8)	0	0	2 (1–4)	0	0/5	48.8 ± 14.5
Oliguric	15 (10–44)**	7 (1–109)**	2 (0–8)*	5 (3–12)**	1 (0–6)*	4/13	48.9 ± 55.8

Summary of disease evolution. Hospital stay is expressed in median days whereas dialysis and transfusions in median events; in all cases, the range of observations is given. The variable 'Neurological signs' is shown as number of patients/total cases in the respective group. Minimum platelet count is expressed as number × 10^3/mm^3 platelets ± SD. Cases were 100% diarrhoea positive (D+HUS) with no mortality. RBC, red blood cells.
*P < 0.05.
**P < 0.01 vs non-oliguric group by Kruskal–Wallis *H*-test.

Table 3. Laboratory findings

	Admission		Hospital discharge	
	Non-oliguric	Oliguric	Non-oliguric	Oliguric
Haematocrit (%)	25.5 ± 4.9	27.2 ± 3.9	27.3 ± 2.3	28.1 ± 2.7[a]
Haemoglobin (g/L)	6.9 ± 1.8	8.8 ± 1.1	9.36 ± 1.0[b]	9.5 ± 1.0[c]
Urea (mg/dL)	93.6 ± 38.9	167.2 ± 78.9[d]	43.6 ± 23.5[e]	96.1 ± 62.7[f]
Creatinine (mg/dL)	0.9 ± 0.4	4.5 ± 2.4[g]	0.6 ± 0.3	2.1 ± 1.8[h]
Platelets/mm^3	48 840 ± 14 494	44 380 ± 55 761[i]	187 980 ± 92 136[j]	296 792 ± 137 716[k,l]

Main laboratory findings at hospital admission and discharge. Subjects are divided according to the degree of renal impairment in either a non-oliguric or an oliguric group. Values shown represent mean ± SD of individual determinations performed at the hospital clinical laboratory according to standardized laboratory practice.
[a]P < 0.05 vs respective admission group.
[b]P < 0.05 vs respective admission group.
[c]P < 0.01 vs respective admission group.
[d]P < 0.01 vs non-oliguric group.
[e]P < 0.05 vs respective admission group.
[f]P < 0.01 vs respective admission group.
[g]P < 0.01 vs non-oliguric group.
[h]P < 0.01 vs respective admission group.
[i]P < 0.05 vs non-oliguric group.
[j]P < 0.01 vs respective admission group.
[k]P < 0.01 vs non-oliguric group.
[l]P < 0.01 vs respective admission group by Kruskal–Wallis *H*-test.

were analysed by Kruskal–Wallis *H*-test. Differences were deemed significant when P was <0.05.

Results

All patients enrolled in this study were diagnosed with the typical form of HUS and presented with a diarrhoeal prodrome. According to the degree of renal impairment upon presentation, they can be grouped as oliguric or non-oliguric.

Of the 18 patients studied, 5 presented with conserved diuresis, while the other 13 showed oligoanuria at admission or developed the condition thereafter. Expectedly, oliguric patients required longer hospitalization and several dialysis interventions. Seven subjects of this last group received peritoneal dialysis, while the other six were subjected to haemodialysis. All four patients who developed neurological signs belonged to the haemodialysis subgroup. A summary of salient disease course data is presented in Table 2. Cases were 100% diarrhoea positive (D+HUS) with no mortality.

Main laboratory findings at hospital admission and discharge of the population studied are provided in Table 3. None of the patients developed ESRD during follow-up; however, three patients regained renal function only 3 months after hospital discharge.

The levels of plasma lipid peroxidation are shown in Figure 1A. Plasma TBARs levels at admission were significantly higher than those of healthy controls. Values registered at hospital discharge were also significantly different when compared with controls, although not significantly different from the mean admission registry. Mean maximal TBARs was significantly higher in the oliguric group when compared with non-oliguric patients (Figure 1). Plasma TBARs were also determined in a group of ESRD patients undergoing regular haemodialysis treatment, and values included for comparison. Expectedly, TBARs resulted significantly higher as compared with healthy controls, while no significant differences were observed when compared with admission and hospital discharge values of HUS-affected patients. Maximum plasma TBARs of patients were significantly higher (both for oliguric and non-oliguric groups) than those registered for ESRD patients (Figure 1A).

The status of plasma non-enzymatic antioxidant defences evaluated as TRAP is shown in Figure 1B. TRAP values were augmented already at the onset of the disease,

and patients exhibited plasma values significantly higher as compared with those obtained for healthy controls. Patients within the oliguric group exhibited also significantly higher TRAP values than those of the ESRD control group. TRAP was also evaluated when TBARs reached its highest plasma value. Although TRAP levels were significantly higher ($P < 0.01$) than those of healthy controls, no significant differences were found when comparing with the respective values of non-oliguric and oliguric groups registered at hospital admission. TRAP values at hospital discharge returned to healthy control levels (Figure 1B).

Also, the relationship between TRAP and TBARs values was analysed by means of a TRAP/TBARs ratio obtained for each patient at admission, when TBARs reached its highest value in plasma and upon hospital discharge. Also, TRAP/TBARs values of healthy controls were used for comparison. As observed in Figure 1C, the TRAP/TBARs ratio obtained at admission was significantly lower in non-oliguric patients as compared with controls, whereas values for the oliguric group were lower but not statistically different than those of healthy controls. When calculated using the values obtained either at disease peak or at hospital discharge, the TRAP/TBARs ratio resulted significantly lower (for both non-oliguric and oliguric patients) than that obtained with admission values (Figure 1C).

Individual TBARs and TRAP values were also pooled and plotted vs time from admission to Day 20 (Figure 2A and B, respectively). As shown, TBARs values raised up to Day 7 and decreased thereafter. Differences reached statistical significance ($P < 0.05$ by ANOVA) for the oliguric group. No significant differences were observed for TBARs values obtained in patients requiring haemodialysis as compared with those receiving peritoneal dialysis (data not shown).

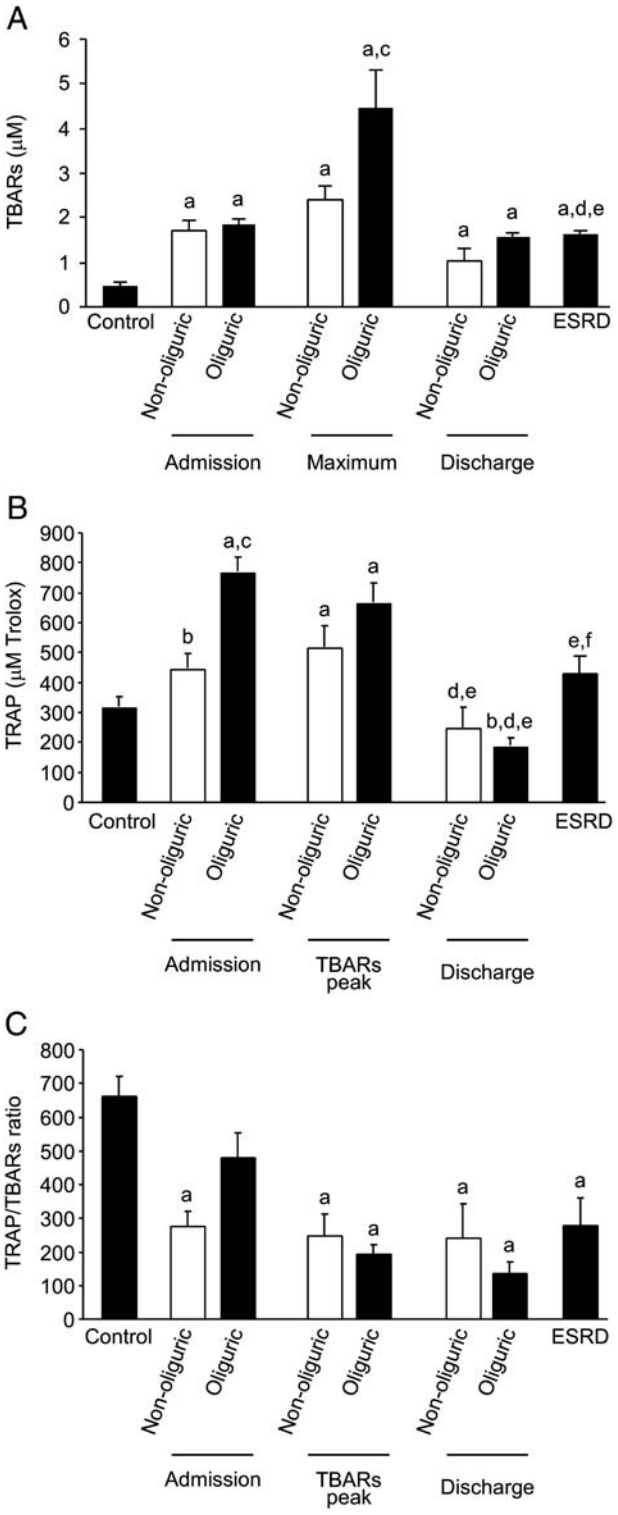

Fig. 1. Plasma oxidative status of HUS-affected children. Plasmatic oxidative status was determined in both non-oliguric and oliguric groups of HUS-affected children, as well as in healthy controls (Control) and in end-stage renal disease patients (ESRD). **A**. Lipid peroxidation was measured as TBARs at hospital admission (Admission) and discharge (Discharge). Maximal obtained values (Maximum) are also shown. Results are expressed as mean TBARs (micromolar) ± SEM. **B**. The state of non-enzymatic antioxidant defences was evaluated as TRAP at admission, the same day in which the highest TBARs value was observed (TBARs peak) and at discharge from the hospital. Results are expressed as mean TRAP (micromolar Trolox) ± SEM. **C**. Relationship between non-enzymatic antioxidant defences and lipid peroxidation values. A ratio between TRAP and TBARs was calculated from the respective values obtained for each patient at admission, when TBARs reached its peak values in plasma (TBARs peak) and upon hospital discharge. Data are expressed as the mean ± SEM of the different observations. a, $P < 0.01$ vs healthy controls; b, $P < 0.05$ vs healthy controls; c, $P < 0.01$ vs respective non-oliguric group; d, $P < 0.05$ vs TBARs peak non-oliguric group; e, $P < 0.01$ vs TBARs peak oliguric group; f, $P < 0.05$ vs Admission oliguric group by ANOVA and Bonferroni post hoc test.

Discussion

We sought here to investigate the oxidative stress status during the acute phase of HUS.

Oxidative injury was measured here by means of ROS-induced damage to lipids. Lipid molecules are rapidly and extensively oxidized in the presence of ROS, particularly polyunsaturated lipids [21]. The TBARs assay used here provides an indication of the current status of fatty acid peroxide formation and decomposition, and it is commonly used as a screening parameter of oxidative injury [22]. The employed fluorometric determination offers also acceptable levels of precision and accuracy in a simple and

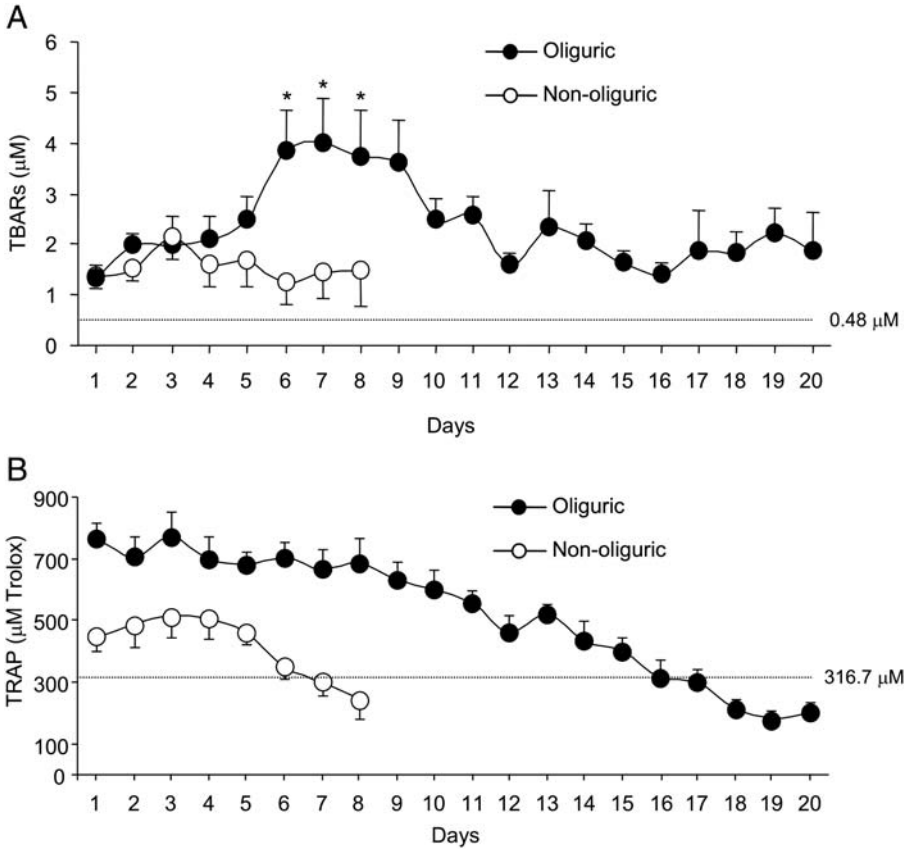

Fig. 2. Daily profile of plasma lipid peroxidation and of non-enzymatic antioxidant defences in HUS-affected children. **A**. Lipid peroxidation was determined from admission and up to Day 20 of hospitalization as TBARs in the plasma of HUS-affected children. **B**. The state of non-enzymatic antioxidant defences was determined as TRAP also from admission and up to Day 20 of hospitalization. Obtained values for the different patients were pooled according to the day of evolution from disease start. Results are expressed as mean TBARs (micromolar) ± SEM or mean TRAP (micromolar Trolox). Filled dots, oliguric group; open dots: non-oliguric group. Dotted line indicates healthy control values for comparison. $P < 0.05$ by ANOVA for the oliguric group. *$P < 0.05$ vs Day 1 by one-tailed Dunnett's *t*-test.

inexpensive procedure that can be used in a clinical setting or even for population studies.

Different mechanisms operate to defend the organism from oxidative insults. Those mechanisms can be grouped into enzymatic and non-enzymatic. Enzymatic defences are mainly comprised by catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, while non-enzymatic protectors include a series of organic molecules that can scavenge or inactivate reactive species [23]. Erythrocytes represent an important component of the antioxidant capacity of blood comprising, in particular, intracellular enzymes, e.g. SOD and CAT, but also the glutathione system. The extensive destruction of red blood cells registered during the acute phase of HUS results in the release of cellular enzymes to the blood stream. The determination of blood enzymatic antioxidants could then be affected by haemolysis and lead to abnormally high values. Furthermore, the need for red blood cell transfusions in certain patients could complicate the interpretation of results. Thus, we determined here the levels of non-enzymatic defence mechanisms as a more reliable estimation of the antioxidant status of the patient. The major contributors of TRAP values in plasma are urate, plasmatic proteins, ascorbic acid, vitamin E and glutathione.

Although established reference values for both TBARs and TRAP are lacking for the age group included in this study, our reference interval is in accordance and lies within an expected range of that reported for plasma determinations in humans [19,24–26].

It follows from our studies that oxidative stress is increased during the acute phase of HUS. Earlier reports have suggested a link between HUS and oxidative stress. O'Regan *et al.* reported abnormalities in red blood cell membrane phospholipids and subnormal plasma tocopherol levels that the investigators found suggestive of peroxidation damage to erythrocytes [27]. Oxidation-induced damage to erythrocytes was later confirmed and further studied by others [28,29] who found increased basal levels of lipid peroxidation products and altered membrane fluidity. Powell *et al.* adhered to the oxidative stress connection by studying the effect of vitamin E in a pilot study conducted in a small group of patients [9]. Our results complement and extend those observations by demonstrating an elevation of oxidative stress during the acute phase of HUS arising from an increment of oxidative injury that is not accompanied by a similar increase in the levels of non-enzymatic antioxidant defences of the plasma. In our study, we detected that non-enzymatic antioxidant de-

fences were incremented as compared with healthy controls already at the onset of the disease, indicating a body response to the oxidative insult. Although the levels of non-enzymatic antioxidant defences remained elevated, they reached a plateau during disease progression and did not follow further increments in lipid peroxidation.

The relationship between non-enzymatic antioxidant defences and lipid peroxidation was also examined here by means of a TRAP/TBARs ratio obtained for healthy controls and each patient at admission, when plasma TBARs reached its highest value and upon hospital discharge. The TRAP/TBARs ratio was higher in controls as compared with HUS-affected patients because of the low levels of lipid peroxidation found in the plasma of healthy infants. Expectedly, that value was significantly lower in patients already at admission, as a result of the marked increase in TBARs (nearly 3-fold) registered, which exceeded that observed for TRAP (2-fold). When calculated using the values registered when TBARs were as highest, the ratio was reduced as compared with that at admission due to further increments in TBARs that were not accompanied by increments in TRAP values. Thus far, a possible interpretation of our results could be that the clinical course of the syndrome reflects a situation in which ROS production outweighs bodily antioxidant capacity. However, an even lower TRAP/TBARs ratio was calculated from the values registered upon hospital discharge of the patients. This late result originates from the status we registered at the end of the acute phase of the disease with lipid peroxidation still elevated over control values but normalized levels of non-enzymatic antioxidant defences, a result that challenges this interpretation.

Alternatively, the oxidative stress condition we report here could be explained in view of the redox state of the antioxidant species involved. This explanation would predict that sustained levels of oxidants could shift the redox state of antioxidants towards an oxidized form, thereby limiting their protective capacity [30].

Experimental animal models and *in vitro* studies have established a role for reactive oxygen species and the therapeutic potential for free radical scavengers in acute renal failure of different aetiologies (reviewed in [31]). A limited number of studies have explored and confirmed that hypothesis in humans. Our results are in agreement with those of Himmelfarb *et al.* [32], Mishra *et al.* [33] and Balakrishnan *et al.* [34] who demonstrated increased levels of oxidants and antioxidants in ARF of different causes. Taken together, the different studies show that ARF can be triggered by ROS but also that established ARF may, by itself, contribute to oxidant generation and further compromise antioxidant defences. Thus, our observations may also be explained in the context of the oxidant/antioxidant dynamics of ARF, where regaining renal function during recovery would allow for a more efficient clearance of oxidants from the blood together with the restoration of the antioxidant defence capacity of erythrocytes expected when the haemolysis that occurs in HUS decreases or stops.

In conclusion, our results indicate increased oxidative stress during the acute phase of HUS. The oxidative stress situation we observed in our patients could play a part in the development of the renal failure and haemolysis that characterize the syndrome. However, current evidence presented in this paper does not allow us to distinguish between causal or associative relationships of oxidative stress and the disease. Further studies are required to explore if oxidative stress plays a role in the aetiopathology of HUS or if it is an epiphenomenon arising during the course of the disease.

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Conflict of interest statement. None declared.

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