



## ORIGINAL ARTICLE

# Oxidative Stress and Antioxidant Vitamin Profile in Term Neonates with Indirect Hyperbilirubinaemia: A Case–Control Study

Enas Jabbar Hasan<sup>\*1</sup> , Abeer J. Hassan<sup>2</sup> , Ammar Adil Jasim<sup>3</sup>

1. Department of Science, College of Basic Education, University of Al-Mustansiriya, Iraq

2. Institute of Technical Medical Baghdad, Middle Technical University, Iraq

3. Department of Biochemistry, College of Medicine, University of Baghdad, Iraq

## ARTICLE INFO

**Received:** 2026/06/29

**Revised:** 2026/07/20

**Accepted:** 2026/07/22

## ABSTRACT

Neonatal indirect hyperbilirubinaemia has been associated with altered redox homeostasis, but the combined pattern of oxidative-stress markers and antioxidant-related vitamins remains uncertain. This study compared these biomarkers between jaundiced and healthy term neonates. This single-centre case–control study included 60 term neonates aged  $\leq 7$  days with indirect hyperbilirubinaemia classified according to the 2022 American Academy of Pediatrics guideline and 60 healthy term controls. Case samples were collected before phototherapy. Serum total bilirubin, reduced glutathione (GSH), and vitamins C, E, and D were measured. Lipid peroxidation was assessed using a spectrophotometric thiobarbituric acid-reactive substances assay and reported as malondialdehyde equivalents. Compared with controls, jaundiced neonates had higher total bilirubin (median 18.60 vs 2.17 mg/dL), TBARS (6.23 vs 4.26 nmol MDA equivalents/mL), and vitamin C (0.94 vs 0.63 mg/dL), and lower GSH (228 vs 370  $\mu$ mol/L), vitamin E (7.75 vs 10.60 mg/L), and vitamin D (8.66 vs 18.55 ng/mL; 59 cases). All differences remained significant after false-discovery-rate correction and adjustment for head circumference and mode of delivery. No within-group correlation remained significant after correction for multiple testing. Indirect hyperbilirubinaemia was associated with higher TBARS, lower GSH, vitamin E, and vitamin D, and higher vitamin C. These findings indicate an altered redox and vitamin profile but do not establish causality or diagnostic utility.

**Keywords:** Hyperbilirubinaemia, Neonatal; Oxidative Stress; Malondialdehyde; Glutathione; Vitamin D; Vitamin E; Ascorbic Acid

## \*Corresponding:

Enas Jabbar Hasan

## Address:

Department of Science,  
College of Basic Education, University  
of Al-Mustansiriya, Iraq

## E-mail:

[enasjabbar.edu@uomustansiriyah.edu.iq](mailto:enasjabbar.edu@uomustansiriyah.edu.iq)

**Cite this article:** Jabbar Hasan E, et al. Oxidative Stress and Antioxidant Vitamin Profile in Term Neonates with Indirect Hyperbilirubinaemia: A Case–Control Study. International Journal of Molecular and Cellular Medicine. 2026; 15(2):1335-1344. DOI: 0



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Publisher: Babol University of Medical Sciences

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## Introduction

Neonatal hyperbilirubinaemia is common during the first days of life (1,2). It is usually mild and resolves without complications. However, high concentrations of unconjugated bilirubin can cross the blood–brain barrier and cause acute bilirubin encephalopathy or kernicterus, with permanent neurological damage (2).

Jaundice develops when bilirubin production exceeds the capacity of the liver for uptake, conjugation, and excretion. Contributing factors include increased erythrocyte breakdown, immature hepatic conjugation, haemolysis, infection, prematurity, poor feeding, bruising, and inherited erythrocyte disorders such as glucose-6-phosphate dehydrogenase deficiency (G6PD) deficiency(3). Nevertheless, many cases of indirect hyperbilirubinaemia have no clear cause.

Oxidative stress occurs when the production of reactive oxygen and nitrogen species exceeds antioxidant capacity. Neonates are susceptible because birth involves a rapid transition to a higher-oxygen environment while their antioxidant systems remain immature. Their erythrocyte membranes are also rich in polyunsaturated fatty acids and are vulnerable to oxidative damage. This damage may promote haemolysis and increase bilirubin production (4,5).

The relationship between bilirubin and oxidative stress is complex. At low concentrations, bilirubin can act as an antioxidant. At high concentrations, free unconjugated bilirubin may damage cell membranes, impair mitochondrial function, and increase reactive oxygen species (6). It may also activate Nrf2-dependent antioxidant responses, including glutathione-related pathways (7). Clinical studies have reported inconsistent changes in oxidative stress and antioxidant capacity among jaundiced neonates (8,9).

Malondialdehyde (MDA) is an indirect marker of lipid peroxidation, while reduced glutathione (GSH) is an important intracellular antioxidant (8). Vitamin E protects membrane lipids, and vitamin C supports antioxidant defence and helps regenerate vitamin E. Differences in gestational age, bilirubin concentration, haemolysis, phototherapy, sampling time, and laboratory methods may explain the inconsistent findings for these biomarkers (7,10,11).

Vitamin D may also contribute to antioxidant defence through the Nrf2–Keap1 pathway and regulation of GSH metabolism. Several studies have

reported lower vitamin D concentrations in jaundiced neonates, and a meta-analysis of six case–control studies supported this association (12–16). However, findings remain inconsistent and may be affected by maternal vitamin D status, season, feeding pattern, and assay method (17).

This study aimed to compare serum total bilirubin, MDA, GSH, and vitamins C, E, and D between term neonates with indirect hyperbilirubinaemia and healthy term controls. The secondary aim was to examine associations among these biomarkers within the hyperbilirubinaemia groups.

## Methods

### Study design and setting

This case–control study was conducted at Al-Elwiya Maternity Teaching Hospital in Baghdad, Iraq, between January and March 2026. The study was approved by the Institutional Review Board of the Middle Technical University: Ethics Committee Reference Number: MEC: 234; June 2025 and conducted in accordance with the Declaration of Helsinki. The study purpose and procedures were explained to the parents, and informed consent was obtained before blood sampling. Participant data were de-identified and recorded using unique study codes.

### Participants

The minimum sample size was calculated using serum vitamin E concentrations reported by Abdul-Razzak et al. (18), Mean concentrations were  $7.5 \pm 2.0$   $\mu\text{mol/L}$  in neonates with significant hyperbilirubinaemia and  $10.4 \pm 5.0$   $\mu\text{mol/L}$  in controls, corresponding to a standardized mean difference of 0.76. With a two-sided  $\alpha$  of 0.05, 80% power, and equal group allocation, at least 29 neonates were required per group. After allowing 10% for incomplete data or unusable samples, the target was increased to 33 per group. Sixty neonates were enrolled in each group to improve the precision of the estimates. A total of 120 term neonates aged  $\leq 7$  days were recruited by convenience sampling. The case group included 60 neonates with indirect hyperbilirubinaemia admitted to the neonatal unit. Hyperbilirubinaemia was classified according to the 2022 American Academy of Pediatrics guideline (1). Cases had total serum bilirubin at or above the phototherapy threshold for postnatal age, gestational age, and neurotoxicity risk factors. Direct

serum bilirubin was  $\leq 1.0$  mg/dL ( $17.1 \mu\text{mol/L}$ ). Blood samples were collected before phototherapy. The control group included 60 term neonates of comparable postnatal age recruited from the postnatal maternity unit during the same period. Controls had no clinical jaundice, normal findings on paediatric examination, total serum bilirubin below the corresponding phototherapy threshold, and direct serum bilirubin  $\leq 1.0$  mg/dL. Exclusion criteria were gestational age  $< 37$  weeks, major congenital anomalies, sepsis or other serious infections, birth asphyxia, documented ABO or Rh incompatibility, and previous phototherapy. Sex, postnatal age, birth weight, head circumference, gestational age, and mode of delivery were recorded to assess baseline comparability.

### Sample collection and handling

A 5 mL sample of venous blood was drawn from each newborn into a plain tube. The blood was left to clot and was then centrifuged at 3000 rpm for 10 min to separate the serum. Serum was transferred into clean tubes and stored at  $-20^\circ\text{C}$  until analysis. All samples were handled in the same way to keep conditions equal across the two groups.

### Laboratory measurements

Serum total 25-hydroxyvitamin D [ $25(\text{OH})\text{D}_2 + 25(\text{OH})\text{D}_3$ ] was measured using the competitive Vit D Direct AccuBind ELISA kit (Monobind Inc., Lake Forest, CA, USA; product code 9425-300). The reported analytical sensitivity was  $1.14 \text{ ng/mL}$ . Serum vitamin C and vitamin E were measured using colorimetric assay kits (Elabsience Biotechnology Inc., Wuhan, China; vitamin C, catalogue no. E-BC-K034-M; vitamin E, catalogue no. E-BC-K033-M). Absorbance was measured at 536 nm for vitamin C and 533 nm for vitamin E. All assays were performed according to the manufacturers' instructions. Lipid peroxidation was assessed using the thiobarbituric acid-reactive substances assay (19). The coloured reaction products were measured spectrophotometrically at 535 nm without HPLC purification of the MDA-TBA<sub>2</sub> adduct. Results were expressed as MDA equivalents (nmol/mL). Absorbance was measured at 535 nm without high-performance liquid chromatography purification of the MDA-TBA<sub>2</sub> adduct. The results were expressed as MDA equivalents in nmol/mL. Serum GSH was measured using Ellman's method with 5,5'-dithiobis-

(2-nitrobenzoic acid), and absorbance was read at 412 nm (20). Total serum bilirubin was measured by commercial kit. Vitamin D is reported in ng/mL, vitamins E in mg/L, vitamin C in mg/dL, MDA in nmol/mL, GSH in  $\mu\text{mol/L}$ , and total bilirubin in mg/dL.

### Assay batching and blinding

For each analyte, case and control samples were assayed together within the same run, in mixed order, and laboratory staff were blinded to group assignment. All blood samples in the patient group were drawn before phototherapy was started.

### Statistical analysis

Data were analyzed using RStudio 4.6.0 (2026-04-24 ucrt). Before analysis, the data were screened for recording errors. One vitamin D value in the case group ( $0.78 \text{ ng/mL}$ ) was more than five standard deviations from the group median and was considered a probable decimal-entry error. Because the original value could not be verified, it was excluded according to the predefined rule. Vitamin D analyses therefore included 59 cases. A sensitivity analysis retaining this value produced similar results. Continuous variables were assessed using the Shapiro–Wilk test for normality and Levene's test for homogeneity of variance. Non-normally distributed variables were presented as median and interquartile range and compared using the Mann–Whitney U test. Normally distributed variables were compared using Welch's t-test.

Categorical variables were presented as numbers and percentages and compared using the chi-square test. Effect sizes were expressed as Cohen's *d* with 95% confidence intervals. The Benjamini–Hochberg method was used to control the false-discovery rate across the biomarker comparisons. Both unadjusted and adjusted *p* values were reported. Robustness was examined by repeating the analyses after retaining the excluded vitamin D value and after excluding the lowest and highest 5% of observations for each biomarker. Because the groups differed in head circumference and mode of delivery, these variables were included as covariates in analysis of covariance models. A change of less than 10% in the group effect estimate after adjustment was considered to indicate little influence from these imbalances. Associations among biomarkers within the case group were assessed using Spearman's rank correlation. Partial correlations

controlling for study group were also calculated for the full sample to reduce the effect of between-group differences. All tests were two-sided, and  $p < 0.05$  was considered statistically significant.

## Results

### Study population and baseline characteristics

In total, 120 neonates were analyzed: 60 with jaundice (40 females, 20 males) and 60 healthy controls (37 females, 23 males). The groups were similar in postnatal age ( $3.00 \pm 1.01$  vs  $3.03 \pm 0.55$  days), birth weight ( $3.06 \pm 0.30$  vs  $3.09 \pm 0.11$  kg), gestational age ( $37.02 \pm 0.75$  vs  $37.02 \pm 0.79$  weeks), and sex (all  $p > 0.05$ ). They differed in two respects. Head circumference was larger in jaundiced neonates ( $33.91 \pm 0.99$  vs  $32.89 \pm 0.19$  cm;  $p < 0.001$ ), and

Cesarean delivery was less common among cases (16/60, 26.7%) than controls (35/60, 58.3%;  $\chi^2 = 12.3$ ,  $p < 0.001$ ). Both variables were carried into the adjusted analyses (Section 3.4). Table 1 gives the baseline data.

The distribution of each marker was checked first (Supplementary Table S1). Total bilirubin and vitamin C departed from normality in both groups, GSH in the jaundice group, and vitamin E in the control group (all  $p < 0.05$ ), while MDA and vitamin D were consistent with a normal distribution. Levene's test showed unequal variances for total bilirubin, MDA, vitamin C, and vitamin D. Group differences were therefore tested with the Mann–Whitney U test for the non-normal variables and with Welch's t-test for the rest, and the results are given as median [IQR].

**Table 1. Baseline characteristics of the two groups**

| Variable                    | Jaundice (n = 60) | Control (n = 60) | Test           | P       |
|-----------------------------|-------------------|------------------|----------------|---------|
| Age (days)                  | $3.00 \pm 1.01$   | $3.03 \pm 0.55$  | Mann–Whitney U | 0.22    |
| Birth weight (kg)           | $3.06 \pm 0.30$   | $3.09 \pm 0.11$  | Welch t        | 0.39    |
| Gestational age (weeks)     | $37.02 \pm 0.75$  | $37.02 \pm 0.79$ | Mann–Whitney U | 0.99    |
| Head circumference (cm)     | $33.91 \pm 0.99$  | $32.89 \pm 0.19$ | Mann–Whitney U | < 0.001 |
| Gender (female/male)        | 40 / 20           | 37 / 23          | Chi-square     | 0.57    |
| Mode of delivery (CS / NVD) | 16 / 44           | 35 / 25          | Chi-square     | < 0.001 |

Continuous data are mean  $\pm$  SD. CS, Cesarean section; NVD, normal vaginal delivery. Head circumference and mode of delivery differed between groups and were adjusted for in later analyses.

### Oxidative-stress biomarkers and vitamins

All six biomarkers differed between the groups, and all differences remained statistically significant after Benjamini–Hochberg correction ( $q < 0.001$ ; Table 2). Compared with controls, jaundiced neonates had higher total bilirubin (difference 16.18 mg/dL, 95% CI 15.92–16.44), MDA (2.03 nmol/mL, 95% CI 1.84–2.21), and vitamin C (0.33 mg/dL, 95% CI 0.28–0.39). Conversely, GSH (difference  $-142.1$   $\mu$ mol/L, 95% CI  $-145.7$  to  $-138.6$ ), vitamin E ( $-2.86$  mg/L, 95% CI  $-3.14$  to  $-2.58$ ), and vitamin D ( $-9.94$  ng/mL, 95% CI  $-10.18$  to  $-9.69$ ) were lower in the jaundice group. The

distributions showed limited overlap, particularly for total bilirubin, GSH, and vitamin D. This pronounced separation is considered further in the discussion. The distributions of total bilirubin, MDA, GSH, and vitamins C, E, and D in the jaundice and control groups are presented in Figure 1.

### Associations among markers within the jaundice group

Within jaundiced neonates, associations among the markers were examined by Spearman correlation (Table 3). The strongest raw associations were between

MDA and GSH ( $\rho = 0.28$ ,  $p = 0.033$ ) and between GSH and vitamin C ( $\rho = 0.27$ ,  $p = 0.041$ ); all other coefficients were small ( $|\rho| \leq 0.22$ ). After correction for the 15 comparisons, none stayed significant (all  $q > 0.30$ ). Correlations across the whole sample were large in size, but they reflected the gap between groups rather than any link within individuals: when group was held constant by partial correlation, the associations fell

towards zero (for example, vitamin D with MDA, partial  $r = 0.21$ ; vitamin D with vitamin C, partial  $r = -0.19$ ; all others  $|r| \leq 0.11$ ). Within the jaundice group, then, the markers were not strongly tied to one another. Pairwise Spearman correlations among the measured biomarkers within the jaundice group are presented in Table 3 and Figure 2.

**Table 2. Oxidative-stress markers and vitamins in jaundiced neonates and controls**

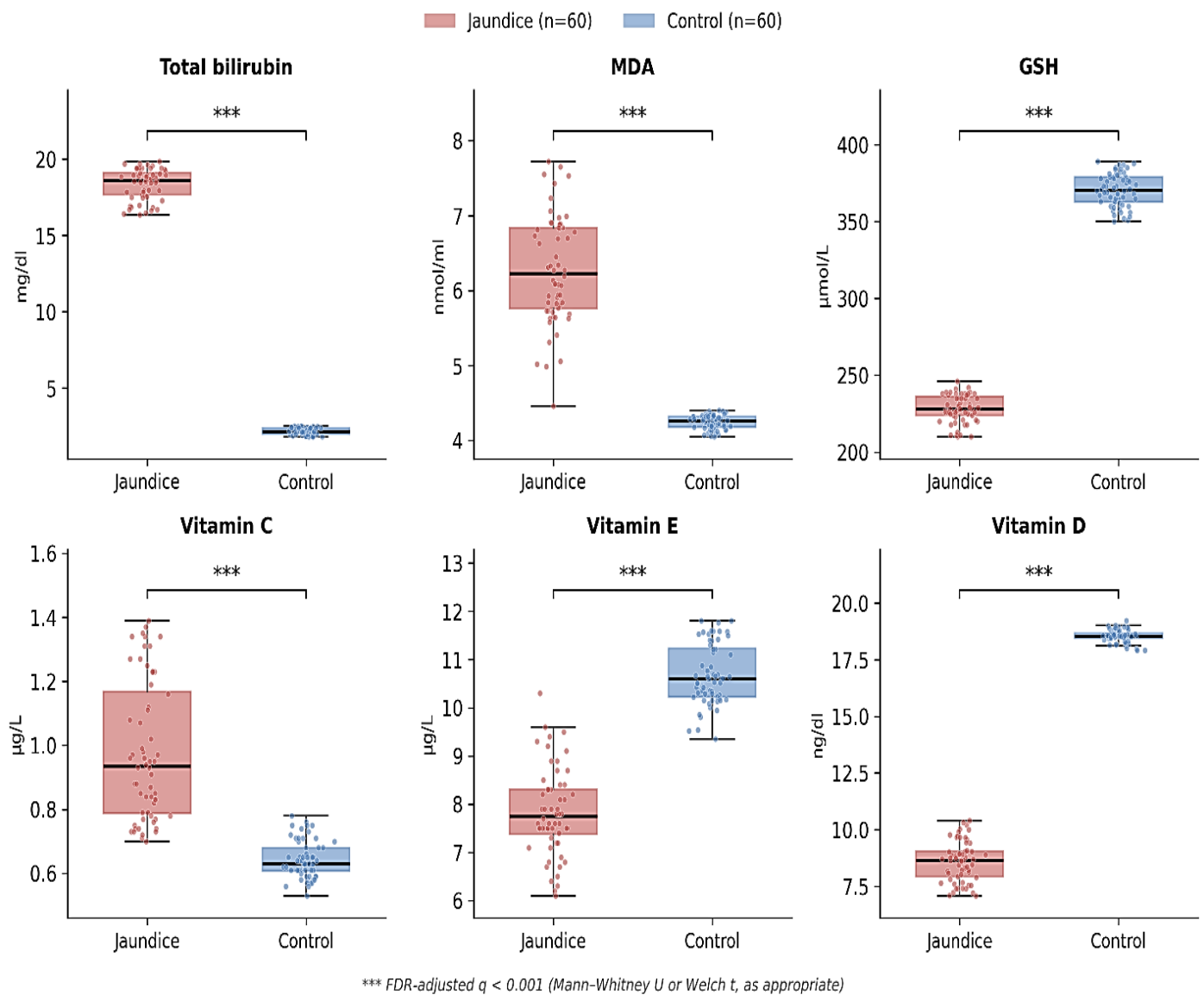
| Parameter               | Jaundice, median [IQR] | Control, median [IQR] | Test                   | <i>p</i> | Difference [95% CI]       | <i>Q</i> |
|-------------------------|------------------------|-----------------------|------------------------|----------|---------------------------|----------|
| Total bilirubin (mg/dL) | 18.60 [17.67–19.10]    | 2.17 [1.98–2.38]      | MWU                    | <0.001   | 16.18 [15.92–16.44]       | <0.001   |
| MDA (nmol/mL)           | 6.23 [5.76–6.84]       | 4.26 [4.18–4.32]      | Welch's <i>t</i> -test | <0.001   | 2.03 [1.84–2.21]          | <0.001   |
| GSH ( $\mu$ mol/L)      | 228 [224–236]          | 370 [363–379]         | MWU                    | <0.001   | –142.1 [–145.7 to –138.6] | <0.001   |
| Vitamin C (mg/dL)       | 0.94 [0.79–1.17]       | 0.63 [0.61–0.68]      | MWU                    | <0.001   | 0.33 [0.28–0.39]          | <0.001   |
| Vitamin E (mg/L)        | 7.75 [7.38–8.30]       | 10.60 [10.23–11.23]   | MWU                    | <0.001   | –2.86 [–3.14 to –2.58]    | <0.001   |
| Vitamin D (ng/mL)       | 8.66 [7.92–9.06]       | 18.55 [18.44–18.68]   | Welch's <i>t</i> -test | <0.001   | –9.94 [–10.18 to –9.69]   | <0.001   |

CI, confidence interval; GSH, reduced glutathione; IQR, interquartile range; MDA, malondialdehyde; MWU, Mann–Whitney U test; *q*, Benjamini–Hochberg-adjusted *p* value. Differences are expressed in the original measurement units and calculated as the jaundice group minus the control group; therefore, negative values indicate lower concentrations in the jaundice group. Vitamin D analyses included 59 cases. Vitamin D analysis included 59 cases.

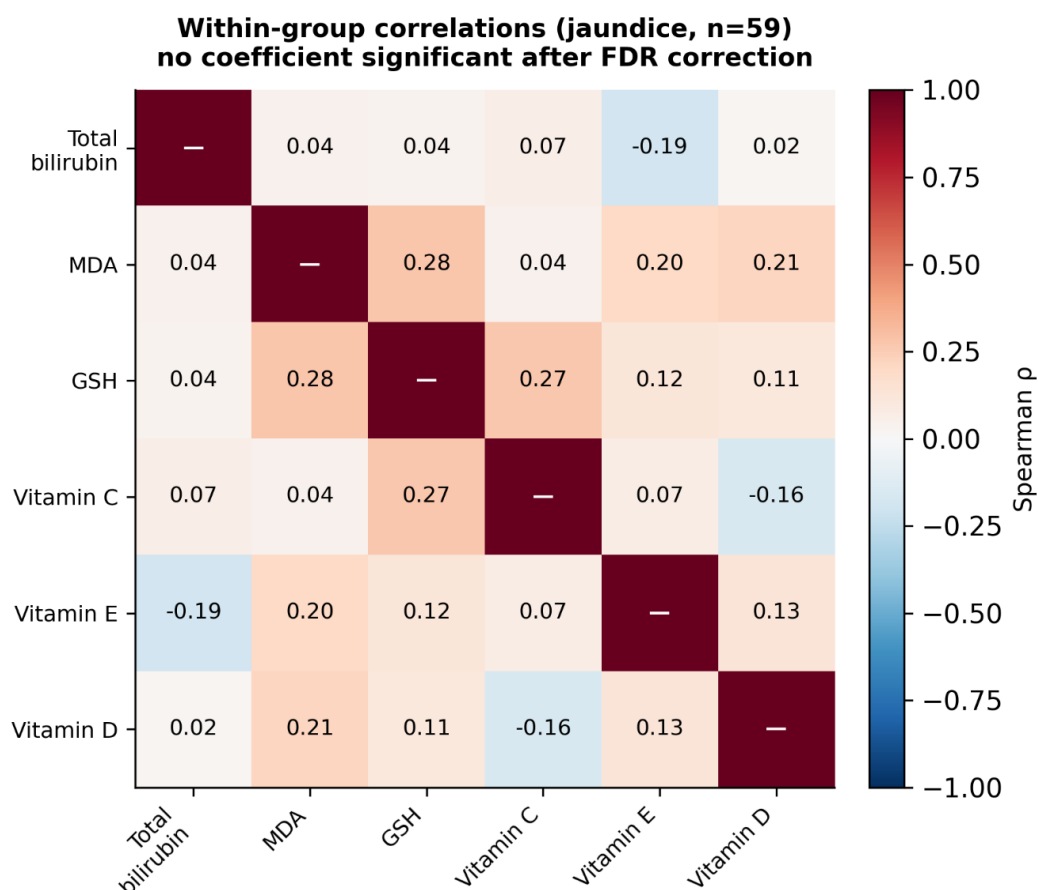
**Table 3. Spearman correlations ( $\rho$ ) among markers within the jaundice group ( $n = 59$ )**

|                        | TB    | MDA  | GSH  | Vit C | Vit E | Vit D |
|------------------------|-------|------|------|-------|-------|-------|
| <b>Total bilirubin</b> | —     | 0.04 | 0.04 | 0.07  | –0.19 | 0.02  |
| <b>MDA</b>             | 0.04  | —    | 0.28 | 0.04  | 0.20  | 0.21  |
| <b>GSH</b>             | 0.04  | 0.28 | —    | 0.27  | 0.12  | 0.11  |
| <b>Vitamin C</b>       | 0.07  | 0.04 | 0.27 | —     | 0.07  | –0.16 |
| <b>Vitamin E</b>       | –0.19 | 0.20 | 0.12 | 0.07  | —     | 0.13  |
| <b>Vitamin D</b>       | 0.02  | 0.21 | 0.11 | –0.16 | 0.13  | —     |

No coefficient remained significant after false-discovery-rate correction (all  $q > 0.30$ ). TB, total bilirubin.



**Figure 1. Oxidative-stress biomarkers and vitamin concentrations in jaundiced neonates versus healthy controls.** Box-and-whisker plots compare six biomarkers between neonates with jaundice (n = 60; red) and healthy controls (n = 60; blue). In each panel the central horizontal line denotes the median, the box spans the interquartile range (IQR, 25th–75th percentile), and the whiskers extend to the most extreme observations within  $1.5 \times \text{IQR}$ ; superimposed points represent individual neonates. Vitamin D values for the jaundice group are based on 59 neonates after exclusion of one implausible value (0.78 ng/mL). Between-group differences were assessed with the Mann–Whitney U test (total bilirubin, GSH, vitamin C, vitamin E) or Welch's t-test (MDA, vitamin D), selected according to Shapiro–Wilk normality and Levene's variance testing; \*\*\* denotes a Benjamini–Hochberg false-discovery-rate-adjusted  $q < 0.001$  for all six comparisons. MDA, malondialdehyde; GSH, reduced glutathione. Vitamin D values are based on 59 cases and 60 controls.



**Figure 2. Intercorrelations among oxidative-stress biomarkers and vitamins within the jaundice group. Heatmap of pairwise Spearman rank-correlation coefficients ( $\rho$ ) among the six biomarkers in neonates with jaundice ( $n = 59$ ). Cell colour encodes the direction and strength of each correlation (red, positive; blue, negative) according to the scale at right, and the coefficient is printed within each cell. None of the 15 pairwise correlations reached statistical significance after Benjamini–Hochberg false-discovery-rate correction (all  $q > 0.30$ ), indicating that the measured oxidative and antioxidant markers were not meaningfully inter-correlated within jaundiced neonates. MDA, malondialdehyde; GSH, reduced glutathione.**

#### Adjustment for baseline differences and sensitivity analyses

Because head circumference and mode of delivery differed at entry, both were added as covariates and the change in each group difference was measured (Supplementary Table S2). For every marker the adjusted difference was close to the unadjusted one, the largest shift being 4.6% (MDA) well under the 10% mark usually taken as a sign of meaningful confounding. In an analysis of covariance for vitamin D, the adjusted patient–control difference was  $-9.69$  ng/mL ( $p < 0.001$ ); head circumference made a small independent contribution ( $\beta = -0.24$ ,  $p = 0.005$ ), while mode of delivery did not ( $\beta = -0.004$ ,  $p = 0.98$ ). The findings also held after the single vitamin D recording error was removed ( $p < 0.001$  with and without it) and

after the most extreme 5% of values were trimmed (all  $p < 0.001$ ). The group differences were therefore not explained by the two measured imbalances and did not depend on a few extreme readings.

#### Exploratory subgroup analyses

Exploratory analyses within the jaundice group showed that vitamin C was higher in girls than in boys (median  $0.97$  [IQR  $0.86$ – $1.23$ ] vs  $0.80$  [ $0.75$ – $0.92$ ] mg/dL;  $p=0.002$ ). GSH showed a nonsignificant difference according to mode of delivery ( $225$  vs  $230$   $\mu\text{mol/L}$ ;  $p=0.097$ ). No other marker differed significantly according to sex or mode of delivery. These analyses were considered hypothesis-generating because of the small subgroup sizes.

## Discussion

In this case-control study, term neonates with indirect hyperbilirubinaemia had higher MDA and vitamin C concentrations and lower GSH, vitamin E, and vitamin D concentrations than healthy controls. These differences remained significant after correction for multiple comparisons and adjustment for head circumference and mode of delivery. However, none of the correlations among bilirubin, redox markers, and vitamins remained significant after multiplicity correction. The findings indicate an altered circulating redox profile in jaundiced neonates but do not establish causality.

The higher MDA concentration is consistent with several previous studies of term neonates with indirect hyperbilirubinaemia (8,9). However, other studies have reported lower MDA and higher antioxidant-enzyme activity in jaundiced neonates. A previous review also found inconsistent results among studies of term infants(5). These differences may be related to bilirubin severity, haemolysis, gestational age, sampling time, treatment status, and laboratory methods. In the present study, higher MDA may reflect increased lipid peroxidation, erythrocyte turnover, unrecognized haemolysis, or immature antioxidant defence. It cannot be concluded that bilirubin itself caused the increase.

The lower GSH concentration may indicate increased antioxidant use or reduced regeneration. GSH is required for the removal of reactive species and maintenance of erythrocyte membrane integrity (4). A previous study reported lower GSH in jaundiced neonates after phototherapy, although the effects of jaundice and treatment could not be separated (10). GSH regeneration also depends on NADPH produced through the G6PD pathway. Reduced G6PD activity may therefore affect both antioxidant defence and bilirubin production (21). Studies from Iraq have reported G6PD deficiency in approximately 8% of neonates, including those with hyperbilirubinaemia (22,23). G6PD activity, haemoglobin, reticulocyte count, glutathione S-transferase, and the direct antiglobulin test were not measured in our study. Unrecognized haemolysis or G6PD deficiency may therefore have influenced the GSH and MDA findings.

Vitamin E was also lower in jaundiced neonates. This vitamin protects erythrocyte and other cell membranes by interrupting lipid peroxidation. It can

then be regenerated by vitamin C (24). Lower vitamin E may reflect increased use during membrane protection, but maternal supply, nutritional status, maturity, and lipoprotein transport may also contribute (25). Previous studies have linked vitamin E deficiency with neonatal haemolysis and jaundice and have reported lower vitamin E concentrations in affected neonates (11,18,26). However, intervention studies have produced inconsistent results. Oral vitamin E was associated with a delayed reduction in bilirubin in one study of late-preterm infants, whereas a placebo-controlled trial in term infants receiving phototherapy found no benefit (27,28). Our data give no support for routine vitamin E supplementation.

Unlike GSH and vitamin E, vitamin C was higher in the jaundice group. This finding differs from studies that reported lower vitamin C concentrations in jaundiced neonates (11,18). Vitamin C functions in aqueous compartments and helps regenerate oxidized vitamin E. GSH-dependent reactions also contribute to the recycling of oxidized vitamin C. Thus, a higher serum vitamin C concentration does not necessarily indicate greater intracellular antioxidant capacity. It could reflect altered distribution, cellular uptake, renal handling, maternal intake, feeding, or haemolysis. Vitamin C is also sensitive to light, temperature, processing delay, and storage conditions. Since measurements were obtained at one time point, the higher concentration cannot be identified as a compensatory response and should be considered exploratory (29).

The lower vitamin D concentration agrees with much of the available observational evidence. A meta-analysis of six case-control studies found lower vitamin D concentrations in jaundiced neonates (12). Individual studies have reported lower vitamin D with either a negative or no significant correlation with bilirubin (18,19). Vitamin D is not a direct radical-scavenging antioxidant. Its possible role is regulatory. Vitamin D receptor signaling may activate the Nrf2-Keap1 pathway, support GSH synthesis, and reduce reactive oxygen species (13,16). However, neonatal 25(OH)D is strongly influenced by maternal vitamin D status, placental transfer, supplementation, season, and feeding. These factors were not measured and may explain part of the observed difference.

No correlation within the jaundice group remained significant after false-discovery-rate correction. Therefore, the differences between cases and controls

should not be considered evidence of linear relationships among bilirubin, oxidative stress, and vitamin concentrations within jaundiced neonates. The higher MDA and lower GSH and vitamin E concentrations may indicate increased lipid peroxidation and lower antioxidant availability. However, the higher vitamin C concentration does not support uniform depletion of antioxidants.

The separation between the groups should be interpreted in relation to the study design. The difference in total bilirubin was expected because bilirubin concentration was used to define case status. Differences in the other biomarkers may partly reflect the comparison of neonates who met phototherapy thresholds with healthy controls. The study groups did not cover the full clinical range of neonatal jaundice, including physiological jaundice, subthreshold hyperbilirubinaemia, and jaundice with different causes. Therefore, the findings should not be considered evidence of diagnostic accuracy or used to define clinical cut-off values.

Other limitations should also be considered. The case-control design and single-time-point measurements did not allow assessment of the temporal relationships between jaundice and changes in redox status. Participants were recruited from a single centre using convenience sampling, which may limit the application of the findings to other populations. Some possible confounding factors were not measured, including G6PD status, markers of haemolysis, albumin, breastfeeding adequacy, and maternal vitamin status, nutrition, and supplementation. Serum GSH may not reflect intracellular GSH or the GSH/GSSG ratio. Vitamin D was measured by ELISA rather than LC-MS/MS, and the spectrophotometric TBARS assay is not specific for MDA. Possible preanalytical, calibration, or batch effects cannot be completely excluded. However, blood samples from jaundiced neonates were collected before phototherapy, which reduced the possible effect of treatment on the measured biomarkers.

In this group of term neonates, jaundice was accompanied by higher MDA and vitamin C and lower GSH, vitamin D, and vitamin E. These findings indicate an altered balance between oxidants and antioxidants in indirect hyperbilirubinaemia. Further evidence is needed before these biomarkers can be considered for clinical use. Prospective multicentre studies with representative comparison groups and

serial sampling could clarify the temporal changes and the effects of relevant clinical factors.

## Funding

The study was approved by the Medical Ethics Committee of the Middle Technical University (approval number MEC 234) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from a parent or legal guardian of every newborn before enrollment and sample collection.

## Acknowledgements

The authors thank the medical and nursing staff of the neonatal and maternity units at Al-Elwiya Maternity Teaching Hospital for their help during sample collection, and the laboratory staff who assisted with the biochemical measurements. The authors are grateful to the parents who allowed their newborns to take part in the study.

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