Biochemical signs of impaired cobalamin function do not affect hematological parameters in young infants: results from a double-blind randomized controlled trial

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BACKGROUND: Whereas iron deficiency is considered the leading cause of anemia in infants, cobalamin deficiency is foremost characterized by developmental delay, and the typical macrocytic anemia is confined to severe and longstanding cobalamin deficiency in this age group. Hematological parameters were investigated in 4-mo-old infants with biochemical signs of impaired cobalamin function who participated in a randomized controlled cobalamin intervention study at 6 wk.

METHODS: One hundred and seven infants were randomly assigned to receive either an intramuscular injection with 400 µg cobalamin or no intervention at 6 wk. Hematological parameters, and cobalamin and folate status were determined at inclusion and 4 mo.

RESULTS: Cobalamin supplementation improved all markers of impaired cobalamin function but had no effect on hematological cell counts at 4 mo ($P > 0.18$). Signs indicative of an iron-restricted erythropoiesis were observed at 6 wk and 4 mo. At 4 mo, the strongest predictors of low iron status were male gender and a high percentage weight increase from birth.

CONCLUSION: In infants with biochemical signs of impaired cobalamin function, supplementation does not improve hematological cell counts. Variations in erythrocyte parameters seem to be foremost associated with iron status in this age group.

Infancy is a period characterized by rapid growth with increased demands of several micronutrients and a subsequent risk of nutritional deficiency (1). In mainly breastfed infants, both iron and cobalamin deficiency are reported to be prevalent (1,2). Whereas iron deficiency is considered the leading cause of anemia in infants (3), cobalamin deficiency is foremost characterized by developmental delay in this age group (2,4). Infants with marginal cobalamin deficiency are reported to have normal hematological values (5,6), and pancytopenia and macrocytic anemia are mainly observed in severe and longstanding cobalamin deficiency (2).

Nutritional evaluation in infants may be difficult due to the extensive physiological changes occurring during the first months of life, complicating the interpretation of ordinary laboratory parameters (2,7). Serum cobalamin decreases substantially during the first weeks and remains low the first 6 mo, whereas serum folate increases and remains high throughout the first year of life (8). These changes are accompanied by an increase in the metabolic markers total homocysteine (tHcy) and methylmalonic acid (8). Cobalamin supplementation changes all markers of impaired cobalamin status toward a profile observed in cobalamin-replete older children and adults, implying that the high tHcy and methylmalonic acid levels reported in a large fraction of mainly breastfed infants do not reflect immature metabolism but rather insufficient cobalamin to fully sustain cobalamin-dependent reactions (9).

The rate of hemoglobin synthesis and erythrocyte production decreases dramatically during the first days after birth and reaches a minimum during the second week of life. Due to a suppressed erythropoiesis and a reduced life span of fetal erythrocytes (60–70 d), the hemoglobin (Hb) level and the erythrocyte count reach their lowest values ~2 mo after birth (10,11). The size of the erythrocyte, determined by mean cellular volume (MCV), decreases and is smallest at 6 mo of age, thereafter gradually increasing (10). Whereas ordinary red cell parameters, such as Hb level and MCV, reflect mean values of an erythrocyte population with an average life span of 120 d, reticulocyte hemoglobin content (CHr) provides a real-time evaluation of the erythropoiesis in the preceding 24–48 h (12). This advantage makes CHr useful during periods with rapid physiological changes, and it has been suggested that CHr is the best predictor of iron deficiency in infants and is superior to traditional iron parameters such as serum ferritin and transferrin saturation (7).

We have previously conducted a randomized controlled cobalamin intervention trial in mainly breastfed infants aged 6 wk with biochemical signs of impaired cobalamin function (9). In this article, we report on the effect of cobalamin supplementation on hematological parameters in the infants at 4 mo. The purpose of this study was to examine whether the metabolic profile indicating impaired cobalamin function was

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Table 1. Demographic data of infants and mothers at 6 wk and 4 mo

Infant data	6 wk	4 mo
Number	107	102
Weight, g, mean (SD)	4,946 (681)	6,936 (821)
Nutrition, *n* (%)		
Exclusively breastfed	86 (80%)	71 (70%)
Weaning to food	0	14 (14%)
Vitamin supplementation	1 (1%)	2 (2%)
Maternal data		
Number	104	99
BMI, mean (SD)	25.2 (3.9)	24.6 (4.0)
Daily use of vitamin supplementation, *n* (%)	36 (34%)	37 (37%)
Daily use of iron supplementation, *n* (%)	28 (27%)	15 (15%)
Daily smoking, *n* (%)	9 (9%)	9 (9%)

Table 2. Hematological parameters according to intervention at 6 wk and 4 mo

		Cobalamin group	Control group	*P* value[a]
N	6 wk	54	53	
	4 mo	52	50	
Hemoglobin, g/dl	6 wk	13.1 (2.1)	12.9 (1.6)	0.67
	4 mo	12.0 (0.8)	12.1 (1.0)	0.33
	P value[b]	0.001	0.004	
Erythrocytes, $\times 10^{12}$/l	6 wk	4.00 (0.60)	4.00 (0.42)	0.89
	4 mo	4.33 (0.32)	4.42 (0.39)	0.24
	P value[b]	<0.001	<0.001	
Mean cellular volume, fl	6 wk	96.5 (4.6)	95.9 (4.5)	0.52
	4 mo	82.7 (3.4)	82.3 (3.6)	0.58
	P value[b]	<0.001	<0.001	
Reticulocytes, $\times 10^{12}$/l[c]	6 wk	0.041 (0.019)	0.042 (0.020)	0.78
	4 mo	0.060 (0.013)	0.057 (0.013)	0.99
	P value[b]	0.001	<0.001	
Reticulocyte hemoglobin content, pg[c]	6 wk	31.9 (1.5)	32.1 (1.6)	0.61
	4 mo	29.6 (1.4)	29.6 (1.4)	0.53
	P value[b]	<0.001	<0.001	
Leukocytes, $\times 10^{9}$/l	6 wk	8.8 (1.9)	9.0 (2.3)	0.54
	4 mo	9.5 (2.7)	9.5 (2.8)	0.59
	P value[b]	0.020	0.28	
Platelets, $\times 10^{9}$/l	6 wk	438 (137)	482 (123)	0.08
	4 mo	519 (127)	516 (107)	0.93
	P value[b]	0.001	0.15	

[a]Student's *t*-test, group statistics. [b]Student's *t*-test; paired samples. [c]Reticulocyte data were available for 63 infants at 6 wk (29 girls and 34 boys) and 74 infants at 4 mo (34 girls and 40 boys).

associated with hematological cell line production during the first months of life.

RESULTS

Demographics and Nutrition

The study group included 107 healthy, 6-wk-old infants, 56 boys (52%), born at term (mean gestational age 39.9 (SD 1.3) wk), with an appropriate-for-gestational-age weight (mean 3,578 (SD 506) g). There were no significant differences between the cobalamin and control group according to demographic characteristics of infants and mothers at inclusion and follow-up (*P* > 0.05), and data for the total group are presented in **Table 1**. Two different kinds of milk formula were used, both enriched with iron (0.7–0.8 mg per 100 ml of prepared milk formula) and cobalamin (0.13–0.2 µg per 100 ml of prepared milk formula). Various kinds of porridge were used, all enriched with iron (4.8–11.1 mg per 100 mg powder). No infant received iron supplementation.

Mean percentage weight increase from birth was 39% (SD 14%) at 6 wk and 95% (SD 26%) at 4 mo. By Spearman correlation percentage weight increase at 6 wk (*r* = −0.44, *P* < 0.001) and at 4 mo (*r* = −0.57, *P* < 0.001) were inversely correlated to birth weight. No significant differences in percentage weight increase or in feeding pattern were observed between girls and boys at 6 wk and 4 mo (*P* > 0.06).

Mean maternal age was 31.5 (SD 4.8) y, and 63 of the 104 mothers were multiparous, and had a mean number of 1.4 (range: 1–4) prior children. Daily use of iron supplementation for shorter or longer periods during pregnancy was reported by 38 of 104 mothers. Two of the mothers were vegetarians, and the others reported having an omnivorous diet, although nine mothers reported that they rarely or never ate meat (*n* = 6) or fish (*n* = 3).

Infant Cobalamin and Folate Status

A detailed description of cobalamin and folate status has been published (9). At 6 wk, the median serum cobalamin level was 170 (134–255) pmol/l and median plasma tHcy was 7.59 (6.21–9.28) µmol/l; no significant differences in vitamin and metabolite levels were observed between the cobalamin and control group. At 4 mo, the cobalamin group had a 75% higher median serum cobalamin level as compared with the control group, and their median tHcy level was reduced by 39% but remained unchanged in the controls, indicating a metabolic response to cobalamin supplementation.

Infant Hematological Parameters

Mean Hb level, MCV, and CHr decreased from 6 wk to 4 mo, whereas the mean number of all three cell lines increased; we observed no significant differences between the cobalamin and the control group (**Table 2**). Neither did we see any effect of cobalamin supplementation on hematological parameters (*P* > 0.18 for all parameters, data not shown) when using a multiple linear regression model, which in addition included gender, percentage weight gain, nutrition, and maternal use of iron supplements (**Table 3**). At 4 mo, high postnatal percentage weight gain and male gender were the main determinants of hematological parameters and associated with a lower iron status, as determined by MCV and CHr (**Table 3**).

No significant predictors were observed for Hb level at 4 mo. Generalized additive model illustrations of the dose–response changes in CHr and MCV with percentage weight gain are shown in **Figure 1**. Males had significantly smaller erythrocytes and lower CHr as compared with girls both at 6 wk and 4 mo (**Table 4**).

At 6 wk, reticulocytosis was inversely associated with Hb level ($r = -0.62$, $P < 0.001$, by Spearman correlation), MCV ($r = -0.63$, $P < 0.001$), and CHr ($r = -0.54$, $P < 0.001$) in the combined group, as illustrated by the generalized additive model in **Figure 2**. Reticulocytosis at 6 wk was also inversely associated with MCV and CHr at 4 mo ($r = -0.55$ and $r = -0.33$, $P \leq 0.01$), whereas no significant associations were seen between reticulocytosis at 4 mo and red cell parameters ($P > 0.06$).

DISCUSSION

In predominantly breastfed, term infants with a biochemical profile indicative of impaired cobalamin function, cobalamin supplementation at 6 wk had a profound effect on metabolic markers of cobalamin status (9) but no effect on hematological parameters at 4 mo. A high postnatal percentage weight gain and male gender were the main determinants of hematological parameters and were associated with a lower iron status. Reticulocytosis was associated with a low MCV and CHr at both 6 wk and 4 mo, demonstrating the existence of an iron-restricted erythropoiesis during the first months of life in mainly breastfed infants.

Formal limitations to the study design included lack of blinding of the investigators and the mothers to the cobalamin injection and a lack of placebo injection in the control group. However, we do not consider these factors important for hematological outcome in the infants at 4 mo.

Microbiological assays were used for the determination of both serum folate and cobalamin. In recent years, competitive-binding luminescence assays have been more commonly used, but the microbiologic assay is still considered the gold standard for serum folate (13). Whereas the microbiological assay determines biologically active cobalamin, the competitive-binding luminescence assays also include functionally inactive cobalamin analogs. Recent studies have suggested that the competitive-binding luminescence assays may have widespread malfunction, especially by giving false-normal values in cobalamin deficiency (14).

Cobalamin supplementation normalized markers of cobalamin status and was associated with lower serum folate levels, possibly due to a reduced inhibition of the cobalamin-dependent enzyme methionine synthase, i.e., so-called methyl-folate trapping (15,16). Cobalamin deficiency in infants has been associated with tHcy levels above 12 µmol/l (5). In the cobalamin-supplemented infants at 4 mo, the 97.5 percentile for plasma tHcy was 6.50 µmol/l, and at 6 wk, 68% of the infants had a plasma tHcy above this level (median plasma tHcy 7.59 (interquartile range: 6.21–9.28) µmol/l), and a median serum cobalamin level of 170 (interquartile range: 134–255) pmol/l, i.e., a biomarker profile indicating impaired cobalamin function (9). However, cobalamin supplementation did not improve hematological parameters in these infants. The

Table 3. Determinants of erythrocyte parameters in infants at 4 mo by multiple linear regression

Independent variable	Mean cellular volume (fl)		Reticulocyte hemoglobin content (pg)		Hemoglobin (g/dl)	
	B	P	B	P	B	P
Gender (girl, boy)	−2.4	<0.001	−0.55	0.09	0.21	0.28
Percentage weight increase, quartiles[a]	−0.6	0.05	−0.48	0.002	−0.07	0.40
Cobalamin intervention (no, yes)	0.4	0.50	0.04	0.90	−0.21	0.27

The regression model contained infant nutrition (exclusively breastfed vs. nonexclusively breastfed/formula-fed) and maternal use of iron supplements from 6 wk to 4 mo (no, yes) as independent variables in addition to the parameters listed in the table. *B*: regression coefficient.

[a]Percentage weight increase from birth to 4 mo, quartiles: <79%, 79–93%, 93–109%, >109%.
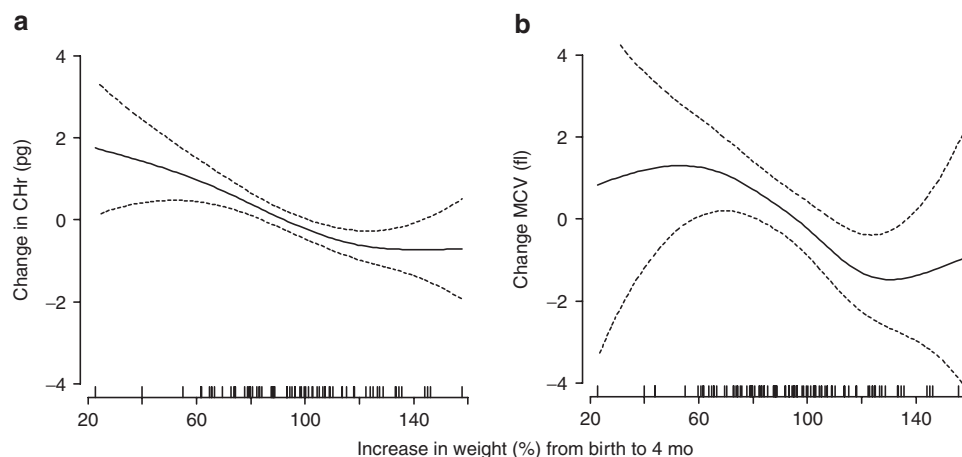
Figure 1. Dose–response relationship between percentage weight gain from birth to 4 mo with (**a**) reticulocyte hemoglobin content (CHr) and (**b**) mean cellular volume (MCV) at 4 mo by generalized additive models. The dose–response curves are shown as solid lines, with 95% confidence intervals as stippled lines.

numbers of all three hematological cell lines increased equally in both groups despite a persistent cobalamin biomarker profile in the controls. This finding may indicate that cell replication is prioritized in a growing infant and that the cobalamin needed for this function is provided, despite a biomarker profile of moderate cobalamin deficiency. In support of this hypothesis

**Table 4.** Hematological parameters according to gender at 6 wk and 4 mo

		Girls	Boys	P value[a]
N	6 wk	51	56	
	4 mo	47	55	
Hemoglobin, g/dl	6 wk	13.3 (1.8)	12.7 (1.9)	0.09
	4 mo	12.0 (0.9)	12.1 (0.9)	0.45
	P value[b]	<0.001	0.02	
Erythrocytes, $\times 10^{12}$/l	6 wk	4.08 (0.49)	3.93 (0.54)	0.13
	4 mo	4.27 (0.36)	4.46 (0.34)	0.009
	P value[b]	0.03	<0.001	
Mean cellular volume, fl	6 wk	97.5 (4.6)	95.0 (4.1)	0.005
	4 mo	84.0 (3.3)	82.2 (3.1)	<0.001
	P value[b]	<0.001	<0.001	
Reticulocytes, $\times 10^{12}$/l[c]	6 wk	0.034 (0.017)	0.048 (0.019)	0.007
	4 mo	0.059 (0.014)	0.058 (0.012)	0.27
	P value[b]	<0.001	0.01	
Reticulocyte hemoglobin content, pg[c]	6 wk	32.5 (1.4)	31.7 (1.5)	0.04
	4 mo	30.0 (1.1)	29.2 (1.5)	0.07
	P value[b]	<0.001	<0.001	
Leukocytes, $\times 10^{9}$/l	6 wk	8.9 (1.9)	8.9 (2.4)	0.92
	4 mo	9.6 (2.3)	9.4 (3.0)	0.28
	P value[b]	0.11	0.10	
Platelets, $\times 10^{9}$/l	6 wk	468 (138)	452 (126)	0.54
	4 mo	543 (125)	496 (106)	0.04
	P value[b]	0.01	0.01	

[a]Student's *t*-test; group statistics. [b]Student's *t*-test; paired samples. [c]Reticulocyte data were available for 63 infants at 6 wk (29 girls and 34 boys) and 74 infants at 4 mo (34 girls and 40 boys).

is a study of 84 Egyptian infants born to mothers with a low median serum cobalamin level (152 pmol/l) in which neither maternal nor infant cobalamin levels had any effect on growth velocity during the first 6 mo of life (17). Although severe cobalamin deficiency has been associated with growth retardation, affecting weight, length, and head circumference (4,18), case reports on moderate cobalamin deficiency in infants often state that the child has a normal weight and may even be obese, the main symptoms being subtle neurological signs and a delayed psychomotor development (19).

At 4 mo, high postnatal weight gain and male gender were the main determinants of hematological parameters, and both were associated with a lower iron status. A rapid growth with subsequent higher nutritional needs may cause problems if the diet is nonoptimal. The majority (70%) of these infants were exclusively breastfed from birth, and although breast milk is energy rich, it is low in some nutrients, such as iron and vitamins D and K (20). Declining concentrations of iron and cobalamin in breast milk have been reported during the lactation period (21,22), and prolonged exclusive breastfeeding is associated with an increased risk of both cobalamin and iron deficiency (23). Male gender is also a recognized risk factor for iron deficiency in young infants (24,25). Boys have been reported to have significantly lower MCV and Hb and ferritin levels as compared with the girls during the first 9 mo of life (24), but the mechanism behind this gender difference is unknown. Gender difference may be partly explained by a higher weight increase in boys, which could, however, not be confirmed in our study; male gender and percentage weight increase appeared to be independent risk factors for low iron status.

In these young infants, the number of reticulocytes was inversely correlated to Hb level and associated with low MCV and CHr, indicators of an iron-restricted erythropoiesis (26). Contrary to adults, in whom a low Hb level due to iron deficiency is usually associated with a normal or decreased number of erythrocytes (27), a high erythrocyte count was seen in infants aged 6–48 mo with iron deficiency anemia (28). The reticulocytosis observed in this age group was more pronounced in mild vs. severe anemia, explained by the fact that
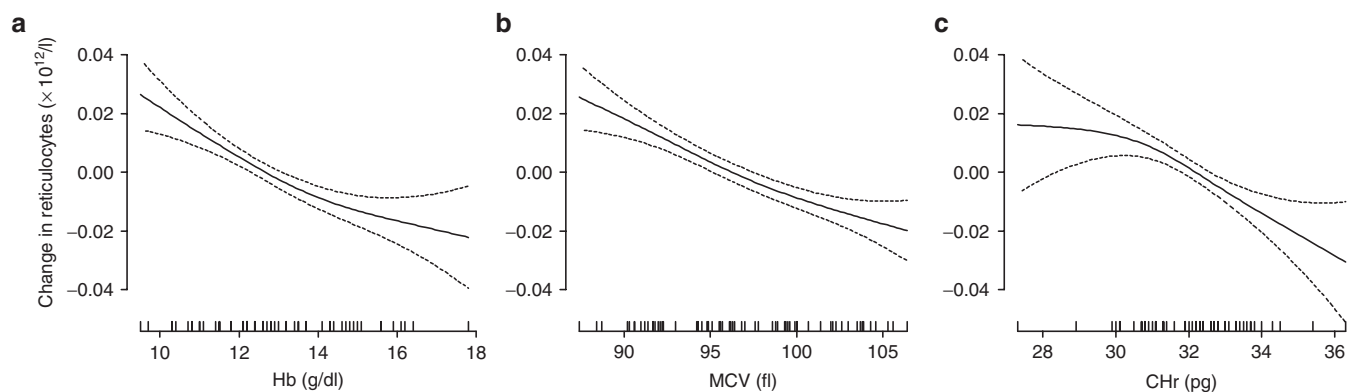
Figure 2. Dose–response relationship between (**a**) hemoglobin level (Hb), (**b**) mean cellular volume (MCV), and (**c**) reticulocyte hemoglobin content (CHr) with reticulocyte count at 6 wk by generalized additive models. The dose–response curves are shown as solid lines, with 95% confidence intervals as stippled lines.

severe iron deficiency is associated with dyserythropoiesis, which may reduce the number of surviving erythrocytes (28).

Conclusion

In infants with biochemical signs of impaired cobalamin function, cobalamin supplementation at 6 wk had a pronounced effect on intracellular metabolic markers of cobalamin status but had no effects on hematological parameters at 4 mo. In these mainly breastfed infants, a high percentage weight gain and male gender were the main determinants of hematological parameters and were associated with a lower iron status at 4 mo. Further studies on micronutrient status in young, mainly breastfed infants are warranted.

METHODS

Study Population and Design

Between December 2004 and April 2006, 107 healthy 6-wk-old infants, including three pairs of twins, and their 104 mothers were recruited from a local health service in Bergen, Norway, and enrolled in a cobalamin intervention study. A detailed description of study methods and results have been published (9). Hematological data were available for 107 infants at 6 wk and 102 infants at 4 mo, and these children were included in this analysis.

A questionnaire on infant and maternal nutrition, vitamin supplementation, growth parameters, parity, and maternal use of tobacco was completed at 6 wk and 4 mo. Because all the various types of formula and porridge used contained iron, infant nutrition was defined as exclusively breastfed vs. nonexclusively breastfed, which included combined breastfeeding and formula feeding, exclusive formula feeding, and either of these combined with solid food.

Infants were assigned by block randomization to receive an intramuscular injection of 400 μg hydroxycobalamin (Vitamin B12 Depot; Nycomed Pharma, Asker, Norway) ($n = 54$, cobalamin group) or no treatment ($n = 53$, control group). The laboratory personnel responsible for blood sampling and analyses were blinded to group assignment.

Ethical approval of the protocol was granted by the regional committee on medical research ethics, and the mothers gave written, informed consent. This trial is registered with ClinicalTrials.gov, number NCT00479479.

Blood Sample Collection and Biochemical Analyses

Blood samples were obtained at 6 wk and 4 mo by antecubital venipuncture and collected into EDTA Vacutainer Tubes (Becton Dickinson, Franklin Lakes, NJ). Hematological parameters (Hb, MCV, erythrocytes, red blood cell distribution width, CHr, leukocytes, and platelets) were analyzed within 4 h with an automated hematology analyzer (ADVIA 120; Bayer Diagnostics, Tarrytown, NY). Reticulocyte indexes (reticulocytes and CHr) were available for a subpopulation of the study group ($n = 62$ of 107 at 6 wk, $n = 72$ of 102 at 4 mo, and $n = 58$ of 102 paired data for 6 wk and 4 mo).

The blood samples used for the preparation of EDTA-plasma were placed in ice water, and plasma was separated within 4 h. Plasma levels of tHcy and methylmalonic acid were assayed using a gas chromatography–mass spectrometry method based on methylchloroformate derivatization (29). Serum was obtained by collecting blood into Vacutainer tubes with no additive (Becton Dickinson). Serum cobalamin was determined by a *Lactobacillus leichmannii* microbiological assay (30) and serum folate by a *Lactobacillus casei* microbiological assay (31).

Statistical Analysis

Results are presented as median and interquartile range and mean and SD. Medians were compared by Wilcoxon's signed-rank test and Mann–Whitney U-test, and means with Student's t-test. Differences in categorical variables were tested with the χ^2 test.

Spearman's correlation was used to assess the relation between percentage weight gain and birth weight and the relations between hematological parameters at 6 wk and 4 mo. Multiple linear regression models were used to assess the relations of infant hematological parameters (Hb, MCV, and CHr) at 4 mo with cobalamin intervention, gender, percentage weight increase from birth to 4 mo, infant nutrition, and maternal use of iron supplements from 6 wk to 4 mo.

Graphical illustrations of the dose–response relationships of Hb level, MCV, and CHr with reticulocyte count at 6 wk, and of percentage weight gain with CHr and MCV at 4 mo were obtained by generalized additive models.

Generalized additive models were computed using the software mgcv-package (version 1.4–1) in R (The R Foundation for Statistical Computing, version 2.8.1; Vienna, Austria), and the SPSS statistical package (version 18) was used for the remaining statistical analyses. Two-sided P values <0.05 were considered statistically significant.

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