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Wait a minute? An observational study assessing iron stores in four months old children after 60 s umbilical cord clamping compared to <10 s and 180 s

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Abbreviations: ANOVA- analysis of variance, CI – confidence interval, CRP – C reactive protein, ID - iron deficiency, MCV - mean cell volume, NS – non significant, RCT - randomized controlled trial, s – seconds, SD – standard deviation, UC – umbilical cord, UCB – umbilical cord blood.

Abstract

BACKGROUND AND OBJECTIVE: Umbilical cord blood (UCB) is a valuable stem cell source used for transplantation. Immediate umbilical cord (UC) clamping is normally practiced in UCB collection but delayed UC clamping is increasingly advocated to reduce possible infant anaemia. This policy obstructs collection of UCB for banking. The objective was to evaluate an UC clamping method that enables collection of altruistically donated UCB, without adverse effects for the donating infant. We investigated the consequences of different UC clamping times with regard to infant's iron status at the age of four months.

DESIGN: Prospective observational study with two historical controls.

SETTING: A university hospital in Stockholm, Sweden and a county hospital in Halland, Sweden.

METHOD: Iron status was assessed at four months in 200 prospectively recruited term infants whose UC was clamped 60 seconds (s) after birth in connection with UCB donation. The results were compared to data from a previously conducted randomized controlled trial including infants subjected to UC clamping at ≤10 s (n=200) or ≥180 s (n=200) after delivery.

RESULTS: After adjustment for age differences at the time of follow-up, serum ferritin concentrations were 77 µg/L, 103 µg/L and 114 µg/L in the 10 s, 60 s and 180 s groups, respectively. The adjusted ferritin concentration was significantly higher in the 60 s group compared to the 10 s group (P=0.002) while the difference between the 60 s and 180 s groups was not significant (P=0.29).

CONCLUSION: Delaying UC clamping for 60 s, compared to 10 s, results in higher serum ferritin concentrations at four months in term infants. This study suggests that it is possible to obtain the iron preserving benefits from delayed UC clamping also in connection with altruistic UCB donation.

Strengths and limitations of the study

- This study compares iron- and blood status at the age of four months in children whose UC were clamped after 60 s due to donation of UCB, with children whose UC were clamped ≤10 s or ≥180 s.
- In this study we focus on potential consequences for the UCB donors caused by different UC clamping times, and not the quality of the collected UCB.
- This prospective observational study uses historical controls and results must be interpreted with caution because of potential confounding bias.
- We did not use a non-inferiority approach for this study since we aimed to compare results after 60 s UC clamping with both 10 s- and 180 s UC clamping.

INTRODUCTION

Umbilical cord blood (UCB) is a valuable stem cell source for the 30-50% of patients that need a hematopoietic stem cell transplantation, but who lack a suitable family or public registry donor.[1 2] More than 35.000 transplantations with UCB have been performed worldwide.[1]

The number of hematopoietic stem cells in the UCB unit highly affects the outcome of transplantation.[3 4] A small cell dose in a transplanted unit is directly correlated with a delay in recovery of the immune system and lower incidence of sustained donor engraftment.[5 6] Large cell numbers are essential and closely correlated with the collected volume of UCB. Volume is dependent on the time of umbilical cord (UC) clamping.[7] The earlier the UC is clamped, the more blood is left to collect from the placenta and for this reason immediate UC clamping is normally practiced in UCB collection.

During the last decades, immediate UC clamping has been part of active management of labour with the aim to reduce maternal haemorrhage at birth.[8 9] Hence, the placenta and UCB, have been regarded as waste products following birth. However, immediate UC clamping may deprive infants of up to one third of the total fetoplacental blood volume[10 11] and increase the risk of iron deficiency (ID) in the first 3-6 months of life.[12-16] Iron deficiency is associated with impaired neurodevelopment affecting cognitive, motor, and behavioural skills in young children.[17-19] For this reason the World Health Organization recommends delayed UC clamping for 1-3 minutes after birth.[20]

We hypothesized that if the UC is clamped at 60 seconds (s) after birth, a sufficient amount of blood will have passed from the placenta to the infant to reduce the risk of ID and anaemia while still allowing collection of UCB for banking[21]. The aim of the current study was to evaluate iron status and complete blood counts at the age of four months in infants who had the UC clamped after 60s in connection with UCB donation and compare the results to immediate (≤10s) and late (≥180s) UC clamping. In this study we focused on potential consequences for the donors only.

MATERIALS AND METHODS

We performed an observational cohort study using one prospective clinical cohort and data from a previously performed randomized controlled trial (RCT).[13] The prospectively recruited infants were born at Karolinska University Hospital Huddinge, Sweden between April 2012 and May 2015, to parents who agreed to donate UCB to the Swedish National Umbilical Cord Blood Bank. To enable collection of UCB, the UC was clamped after 60 ±10s. The historical cohorts consisted of data from infants who were randomized to immediate (within 10s after birth) or late (after 180s) UC clamping, respectively, in a trial conducted by three of the co-authors at the Hospital of Halland in Halmstad, Sweden between April 2008 and September 2009.[13]

Outcome measures

The primary outcome variable was ferritin as a measure of iron stores, at the age of four months and secondary outcomes included haemoglobin, transferrin saturation, soluble transferrin receptors, mean cell volume (MCV) as well as ID (defined as ≥2 iron indicators outside reference range with the following cut-off values: ferritin <20 µg/L,[22] MCV <73 fL,[22] transferrin saturation <10%[23] and transferrin receptor >7 mg/L[13]). Since ferritin is an acute phase reactant, C reactive protein (CRP) was analysed in order to exclude falsely elevated ferritin measurements. The infant's dietary habits at four months were also considered as well as the relevance of infant sex for iron and blood status at that age.

Sample size

Based on results from the original RCT (the historical cohorts)[13], demonstrating a 45% difference in ferritin concentration between 10s and 180s UC clamping, we decided to include an equal number of infants in the prospective cohort as in the two historical groups. Hence, 200 children in each group were included in the study without any formal power analysis.

Study population and inclusion criteria

The same inclusion criteria applied for the prospective and historical cohorts: healthy mother, normal, singleton pregnancy, gestational age 37+0 to 41+6 weeks and planned vaginal delivery with

cephalic presentation.[13] The mother also had to understand Swedish well enough to consent participation in the study. In the prospectively recruited cohort, parents also had to agree to altruistic donation of UCB, and to the modified UC clamping strategy. Exclusion criteria were also the same in the three groups: smoking mother, serious congenital malformations, syndromes, or other congenial diseases that could affect the outcome measures. For this study, we only included data from the RCT that were handled according to per protocol, as compared to the analysis by intention to treat in the original RCT.[13] Moreover, like the RCT, we only included children with CRP<10 in the analysis. All parents donating UCB at Karolinska University Hospital Huddinge that met the inclusion criteria were offered to participate in the study upon arrival at the delivery unit. Midwives were instructed to clamp the UC 60s after birth in deliveries where parents had consented to participate in the study. Staff in charge of collection of UCB measured the exact timing of UC clamping using a digital timer. Only children whose UC were clamped at 60 ±10s were included in the study. Oral and written informed consent was obtained from one or both of the parents. The study was approved by the regional ethical review board in Stockholm (2011/2142-31/3) and the RCT was approved by the regional ethical review board at Lund University (41/2008).

Data collection and blood samples

Midwives were instructed to hold the new-born baby below the uterine level for the first 30s before placing the infant on the mother's abdomen. The UC clamping technique used in the prospectively recruited cohort was similar to the historical groups except for the timing. Venous blood samples were analysed at birth from the UC, at two to three days of age in connection with metabolic screening and at four months (±21 days) at a scheduled follow-up visit. At birth and at four months, serum ferritin, haemoglobin, haematocrit, MCV, reticulocyte count, transferrin saturation, transferrin receptor and CRP were analysed and at two to three days of age we analysed haemoglobin, haematocrit and bilirubin. EDTA tubes were used for blood count and serum separator tubes for iron status, bilirubin and CRP (Microvette, Sarstedt AG & Co, Nümbrecht, Germany). Blood status was analysed using equal methods and equipment in both the laboratory at Karolinska University Hospital

(Sysmex XE5000, Sysmex, Kobe, Japan) and at the Hospital of Halland (Sysmex XE2100, Sysmex, Kobe, Japan). Analysis of iron status and CRP could be performed at the same laboratory as in the RCT[13], and therefore the blood samples collected at Karolinska University Hospital were centrifuged and the serum kept at -70 degrees Celsius before sent for analysis at the Clinical Chemistry Laboratory at the Hospital of Halland. The samples were then thawed and analysed using a Cobas 6000 (Roche Diagnostics, Basel, Switzerland), the same method as in the RCT.[13]

Statistical analysis

Analyses on the two historical cohorts, 10s and 180s, were calculated only on infants receiving intervention per protocol in order to obtain clearly defined groups according to time of UC clamping. Baseline characteristics were compared across groups using analysis of variance (ANOVA). Means of outcome measures in the three cohorts were compared using the Bonferroni method. We also performed pairwise comparisons (60s vs 10s and 60s vs 180s) using Student's t-test for variables with normal distribution that were statistically significant in the Bonferroni analyses. Ferritin concentration was $\log_{10}$ transformed for analysis because of skewed distribution and the results were presented as geometric means. Categorical variables were compared between groups using Fisher's Exact test for pairwise comparisons. Adjusted group means were compared using ANOVA and a binary logistic regression model was used to adjust for sex and age in the evaluation of ID at four months. A P-value less than 0.05 were considered significant. IBM SPSS for Windows, version 22 was used for the analyses.

RESULTS

Of the 200 prospectively recruited infants, 191 were included in the analysis as compared to 166 in the 10s and 168 in the 180s groups. Infants lost to follow up or excluded due to not fulfilling inclusion criteria are shown in Fig 1.

Baseline data

There were no significant differences in the 60s group as compared to the 10s and 180s groups with regard to maternal or neonatal characteristics. The 10s group had higher UC haemoglobin (163 vs 153 g/L, P<0.001) and higher UC haematocrit (48.7 vs 47.0%, P=0.005) than the 60s group. There were no significant differences between the 60s and the 180s groups (Table 1).

Two to three day-follow-up

At two to three days, blood samples were obtained from 140 infants in the 10s group, 107 in the 60s group, and 141 in the 180s group (Figure 1). The prevalence of anaemia was higher in the 10s compared to the 60s and 180s groups (9 (6.4%), 2 (1.9%) and 2 (1.4%)) respectively (P=0.04). Haemoglobin was lower in the 10s and 60s groups compared to the 180s group; 175, 180 and 189 g/L respectively (P=0.001 for 60s vs 180s). Bilirubin did not differ between the groups. Anaemic children were referred for further diagnostics but none needed treatment.

Primary outcome

In total 468 (78%) infants had follow up at four months (children excluded or lost to follow up are shown in Figure 1). In the 60s group, the infants (n=147) were four days older than the historical groups from the RCT (126±7 days vs 122±6 days, P<0.001). The geometric mean ferritin levels were 80, 96 and 117 µg/L respectively in the 10s, 60s and 180s groups (P=0.052 for 10s vs 60s; P=0.02 for 60s vs 180s) (Table 2). After adjustment for the age difference, the geometric means were 77, 103 and 114 µg/L respectively (P=0.002 for 10s vs 60s; P=0.29 for 60s vs 180s) (Table 3).

Secondary outcomes

All secondary outcome variables are shown in Table 2. Haemoglobin levels at four months were higher in the 60s group compared to 10s and 180s, but the difference did not remain in the age adjusted model (Table 3). There were no statistically significant differences in the prevalence of anaemia in the 60s group as compared to the other two groups (Table 4). After adjustment for age at the time of follow-up, there were no differences between the 10s and 60s groups in haematocrit,

MCV, reticulocyte count, transferrin receptor or transferrin saturation. The 60s group had significantly lower adjusted MCV and higher adjusted transferrin receptor compared to the 180s group, but there were no differences in haematocrit, reticulocyte count or transferrin saturation (Table 3). The prevalence of ID was 8 (5.2%), 7 (4.8%) and 1 (0.7%) respectively in the 10s, 60s and 180s groups (P>0.99 for 10s vs 60s and P=0.04 for 60s vs 180s) (Table 4). After statistical adjustments for sex and age at the time of follow-up, the prevalence of ID did not differ between the 60s group and the two other groups. The proportion of infants that were exclusively breast fed at four months did not differ between the groups (79 (52%), 92 (58%) and 89 (56%) respectively in the 10s, 60s and 180s groups. The impact of exclusive breast feeding on ferritin concentration and ID were further assessed in an adjusted model but no correlation was found (data not shown).

Sex differences

Iron and blood status at four months were further assessed by analysing differences according to infant sex. When data from the three cohorts were analysed together, geometric mean ferritin was lower in boys than in girls and ID was more prevalent amongst boys (Fig 2A and 2B). Haemoglobin concentrations did not differ significantly between boys and girls and neither did the prevalence of anaemia (data not shown).

DISCUSSION

We compared infant's iron and blood status at the age of four months after three different UC clamping time intervals (60s versus ≤10s, and ≥180s, respectively). Previous studies have compared immediate and late UC clamping but the definition of "late" in these studies varies from 60s to 180s or after birth of the placenta.[12 14 15] The current study evaluates an intermediate UC clamping strategy that can be used in connection with altruistic donation of UCB for clinical banking. When asking parents to donate their child's UCB for the potential good of others, it is essential to inform of possible consequences for the child. We demonstrated that after adjustment for age difference at the time of follow-up, there were no statistically significant differences in ferritin concentration

between the 60s and 180s groups but the 60s group had significantly higher ferritin concentrations compared to 10s. The main reason for the effect of age on ferritin during infancy is rapid growth which leads to a rapidly expanding blood volume and depletion of iron stores.[16]

Results from the present study are in accordance with a Brazilian RCT that demonstrated higher ferritin concentration at the age of three months in children whose UC were clamped after 60s compared to immediate UC clamping.[24] However, our findings are in contrast to a study by Ceriani Cernadas et al, who reported no differences in ferritin six months after birth comparing 15s and 60s UC clamping. The difference in ferritin between the 60s and 180s groups were not presented.[25]

The present study indicates that a 60s delay before UC clamping results in a mean serum ferritin at 4 months of age corresponding to 90% of the mean ferritin after 180s UC clamping

In the current study, children in the 60s group had the lowest haemoglobin of all three groups at birth (10s 163 g/L, 60s 153 g/L, 180s 158 g/L) but at four months, there were no longer any differences between the groups in our adjusted model. The original RCT as well as a Cochrane report found higher concentrations of haemoglobin after immediate than late UC clamping at birth.[13 15] As speculated in a previous publication, this difference may not reflect true difference in haemoglobin but could be a result of trans endothelial plasma leakage in the UC after UC clamping.[13]

The prevalence of ID was higher in the 10s and 60s groups compared to the 180s group. However, when adjusting for sex and age at the time of follow-up, there were no longer any statistically significant differences between the three groups. In accordance with previous studies on ID in infancy, we found that at four months, boys had significantly lower geometric mean ferritin and more often ID than girls.[26 27] It has been suggested that the differences in iron status between the two sexes may be hormone-related but it is also influenced by genetic factors, which may differ in populations with different ethnicity.[27]

Limitations of the study

A limitation of this study is that we have no record of the parents' ethnic or socio-economic background, and we were therefore unable to draw any conclusions on possible impact of these factors. When comparing different interventions, RCTs are regarded as the golden standard. However, randomization into 10s UC clamping for this study was considered unethical since there is evidence that children benefit from prolonged placental transfusion for at least 60s, and the clinical routine in Sweden is to wait 180s before UC clamping in normal deliveries. We used the historical cohorts from the RCT in Halmstad, Sweden as controls in this study. The prospectively recruited cohort (60s) is from a large birth clinic in Stockholm, Sweden while the controls are from a smaller town where the participants were recruited two to five years earlier. Although the inclusion criteria were the same and pregnant mothers in Sweden follow the same well-controlled antenatal program and the baseline data showed no significant differences in maternal or infant characteristics, it is difficult to know if the study populations are homogenous. We did not use a non-inferiority approach for this study since we aimed to compare results after 60s UC clamping with10s and 180s. We have taken measures to avoid systematic biases caused by differences in laboratory routines between the two hospitals by using the same laboratory for analysing the iron status variables as well as CRP. Collection of UCB for transplantation is a growing and life-saving practice. Tens of thousands UCB units are altruistically donated and stored for public use each year, and as a result new-borns are exposed to limited UC clamping time. The idea that UCB left in the placenta after birth is a mere waste that is thrown away if not collected is obsolete. Optimizing UC clamping time and investigating the clinical significance to minimize risks for the infant while still enabling collection of UCB for transplantation is a great, but necessary, challenge.

CONCLUSION

This observational study indicates that in a healthy Swedish population, UC clamping after 60s reduces the risk for low iron stores at four months of life compared to UC clamping at 10s. After adjustment for age and sex, there were no statistically significant differences in ferritin concentration between 60 and 180s UC clamping. 60s UC clamping is therefore preferable to immediate UC clamping and enables collection of UCB for transplantation. Delaying UC clamping may be more important in boys than in girls to avoid ID in early childhood. Larger studies with long term follow-up is needed to establish the clinical relevance of different UC clamping strategies.

CONTRIBUTORS

Dr Götherström conceptualized and designed the study, analyzed data, critically revised and approved the final manuscript as submitted. Mrs Askelöf conceptualized and designed the study, carried out all data collection in the prospective cohort, carried out all analyses, drafted the initial manuscript, and approved the final manuscript as submitted. Dr Andersson designed the data collection instruments, contributed to the study design and provided data for the historical cohorts. He also carried out the initial analyses, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. Profs Domellöf, Fasth, Hellström-Westas, Westgren, Associate Profs Pettersson and Wiklund and Dr Hallberg participated in the study design, reviewed and revised the manuscript and approved the final manuscript as submitted.

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TABLE 1. Baseline characteristics of mothers and infants according to time for umbilical cord clamping

	Umbilical cord clamping time			P
	10s n=166 (HC)	60s n=191	180s n=168 (HC)	
Maternal characteristics				
Age (years)	31.7 (4.2)	30.7 (4.7)	30.8 (4.9)	NS
Parity (including study child)	1.81 (0.87)	1.86 (0.76)	1.76 (0.74)	NS
Weight at first antenatal visit (kg)	66.4 (12.0)	67.3 (11.5)	67.3 (12.1)	NS
Body mass index	23.6 (3.8)	24.5 (3.9)	23.9 (3.6)	NS
Haemoglobin at first antenatal visit (g/L)	128 (9)	128 (10)	128 (11)	NS
No (%) of vaginal deliveries:				
Non-instrumental	157 (95)	184 (96)	157 (94)	NS
Vacuum extraction	8 (5)	7 (4)	11 (7)	NS
Forceps	1 (0.6)	0 (0)	0 (0)	NS
Infant characteristics				
Gestational age (weeks)	40.0 (1.1)	40.0 (1.0)	40.0 (1.1)	NS
No (%) of males	83 (50)	98 (51)	73 (43)	NS
Birth weight (g)	3523 (483)	3603 (454)	3633 (464)	NS
Birth length (cm)	50.7 (1.9)	50.6 (1.8)	50.9 (1.9)	NS
Head circumference (cm)	34.7 (1.3)	35.1 (1.3)	35.0 (1.4)	NS
Umbilical cord haemoglobin (g/L)	163 (15)	153 (20)	158 (18)	<0.001*
Umbilical cord haematocrit (%)	48.7 (4.3)	47.0 (5.5)	47.1 (5.0)	0.005**

Values are means (SD) unless stated otherwise. P<0.05 was considered significant.
NS=non-significant; HC= Historical cohort.
*Comparison of 10s and 60s. For 180s and 60s, P=0.063.
**Comparison of 10s and 60s. For 180s and 60s, P>0.99.

TABLE 2. Outcome measures at the age of four months in infants whose umbilical cord were clamped after 10s, 60s or 180s

	Umbilical cord clamping time			60s vs 10s		60s vs 180s	
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	Mean difference (95% CI)	P	Mean difference (95% CI)	P
Infant age (days)	122 (6)	126 (7)	122 (6)	5 (3 to 6)	<0.001	4 (3 to 6)	<0.001
Main outcome measures							
Geometric mean (range) ferritin (µg/L)	80 (33 to 191)	96 (44 to 208)	117 (58 to 232)	0.83 (0.69 to 1.45)*	0.052	1.21 (1.03 to 1.44)*	0.02
Haemoglobin (g/L)	113 (7)	115 (8)	113 (8)	2 (0 to 4)	0.047	2 (0 to 4)	0.03
Blood count							
Haematocrit (%)	33.0 (2.0)	33.3 (2.2)	32.7 (2.1)	0.3 (-0.2 to 0.8)	0.19	1 (0 to 1)	0.02
Mean cell volume (fL)	77.9 (3.1)	77.7 (3.7)	79.2 (3.0)	-0.2 (-1 to 0.6)	0.67	-1.5 (-2.3 to -0.7)	<0.001
Reticulocyte count ($x10^9$/L)	37 (11)	36 (11)	40 (11)	-1 (-4 to 1)	0.39	-4 (-6 to -1)	0.01
Iron status							
Transferrin receptor (mg/L)	4.00 (0.80)	4.07 (0.92)	3.71 (0.69)	0.07 (-0.13 to 0.26)	0.51	0.35 (0.17 to 0.54)	<0.001
Transferrin saturation (%)	16 (6)	16 (8)	18 (6)	1 (-1 to 2)	0.40	-2 (-3 to 0)	0.03

Values are means (SD) unless stated otherwise. HC= Historical cohort.
*Geometric mean ratio (95% CI for geometric mean).
Successful analyses in the 60s and 180s groups: haemoglobin (n=140, 144), haematocrit, mean cell volume, reticulocyte count (n=135, 144), ferritin (n=147, 148), transferrin receptor (n=147, 148), transferrin saturation (n=145, 148).

TABLE 3. Outcome measures at the age of four months adjusted for infant age

	Umbilical cord clamping time Adjusted means (95% CI)			P	P
	10s n=152 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Main outcome measures					
Geometric mean (range) ferritin (µg/L)	77 (68 to 87)	103 (90 to 117)	114 (100 to 129)	0.002	0.29
Haemoglobin (g/L)	113 (112 to 115)	114 (113 to 116)	113 (112 to 114)	0.30	0.17
Blood count					
Haematocrit (%)	33.1 (32.7 to 33.4)	33.1 (32.8 to 33.5)	32.7 (32.4 to 33.1)	0.30	0.12
Mean cell volume (fL)	77.8 (77.3 to 78.3)	77.9 (77.3 to 78.4)	79.1 (78.6 to 79.6)	0.30	0.003
Reticulocyte count ($x10^9$/L)	36.9 (35.2 to 38.7)	37.0 (35.0 to 38.9)	39.4 (37.6 to 41.2)	0.99	0.07
Iron status					
Transferrin receptor (mg/L)	4.01 (3.87 to 4.14)	4.05 (3.92 to 4.19)	3.72 (3.59 to 3.85)	0.62	0.001
Transferrin saturation (%)	15.7 (14.7 to 16.7)	16.6 (15.5 to 17.7)	18.1 (17 to 19.2)	0.25	0.06

Values are means (95% CI) adjusted for infant age (days from birth) unless stated otherwise.
HC= Historical cohort. Successful analyses for the 60s group were: ferritin (n=147), haemoglobin (n=140), haematocrit, mean cell volume, reticulocyte count (n=135), transferrin receptor, and transferrin saturation (n=145). For the 180s group; blood status (n=144).

TABLE 4. Infants with iron status indicators outside reference limits at the age of 4 months according to umbilical cord clamping time

	Umbilical cord clamping time			P	P
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Ferritin <20 µg/L	11 (7.2)	4 (2.7)	0 (0)	0.11	0.06
Anaemia (Hb<105 g/L)	20 (13.1)	16 (11.4)	20 (13.9)	0.72	0.60
Mean cell volume <73 fL	8 (5.2)	14 (10.4)	3 (2.1)	0.12	0.01
Transferrin saturation <10%	22 (14.4)	15 (10.3)	8 (5.4)	0.38	0.13
Transferrin receptor >7 mg/L	0 (0)	2 (1.4)	0 (0)	0.24	0.25
Iron deficiency*	8 (5.2)	7 (4.8)	1 (0.7)	1.00	0.04

Values are unadjusted numbers (%). HC= Historical cohort.
Numbers of successful analyses: mean cell volume (n=153, 135, 144), transferrin saturation (n=153, 145, 148), anaemia (n=153, 140, 144).

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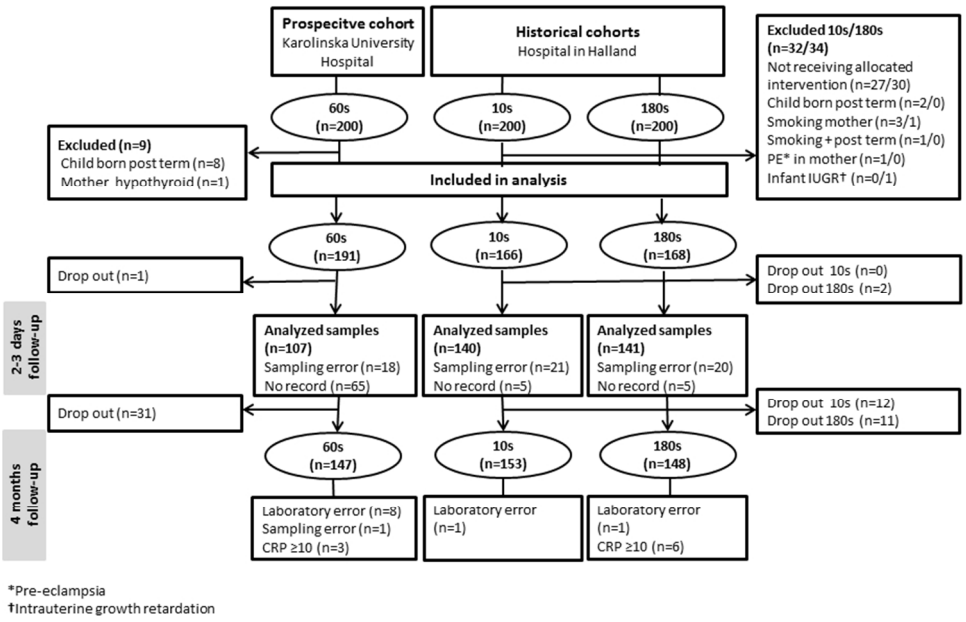

FIGURE 1. Flow chart of the study populations.

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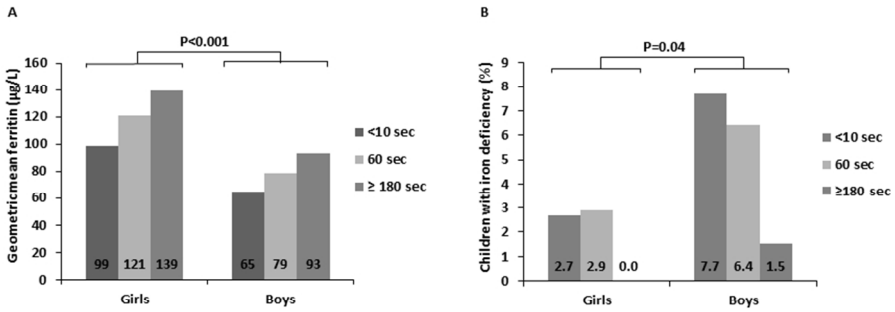

FIGURE 2. Differences in infants' iron status at the age of 4 months according to sex in three groups divided according to umbilical cord clamping time. A: Geometric mean ferritin according to sex and time for umbilical cord clamping, B: Proportion of children with iron deficiency according to time for umbilical cord clamping and sex.

254x190mm (96 x 96 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	
Bias	9	Describe any efforts to address potential sources of bias	6, 10
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Fig 1
		(b) Give reasons for non-participation at each stage	Fig 1
		(c) Consider use of a flow diagram	Fig 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	Tables 1-4
		(c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Fig 2
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9, 11
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Wait a minute? Effect of umbilical cord clamping time on iron stores in four months old children: An observational cohort study of healthy Swedish infants

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Wait a minute? Effect of umbilical cord clamping time on iron stores in four months old children: An observational cohort study of healthy Swedish infants

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Clinical Trial Registration: Clinicaltrial.gov NCT01245296.

Ethics Approval: By the Regional Ethical Review Board in Stockholm (2011/2142-31/3) and the Regional Ethical Review Board at Lund University (41/2008).

Abbreviations: ANOVA- analysis of variance, CI – confidence interval, CRP – C reactive protein, ID - iron deficiency, MCV - mean cell volume, NS – non significant, RCT - randomized controlled trial, s – seconds, SD – standard deviation, UC – umbilical cord, UCB – umbilical cord blood.

Abstract

BACKGROUND AND OBJECTIVE: Umbilical cord blood (UCB) is a valuable stem cell source used for transplantation. Immediate umbilical cord (UC) clamping is normally practiced in UCB collection but delayed UC clamping is increasingly advocated to reduce possible infant anaemia. This policy obstructs collection of UCB for banking. The objective was to evaluate an UC clamping method that enables collection of altruistically donated UCB, without adverse effects for the donating infant. We investigated the consequences of different UC clamping times with regard to infant's iron status at the age of four months.

DESIGN: Prospective observational study with two historical controls.

SETTING: A university hospital in Stockholm, Sweden and a county hospital in Halland, Sweden.

METHOD: Iron status was assessed at four months in 200 prospectively recruited term infants whose UC was clamped 60 seconds (s) after birth in connection with UCB donation. The results were compared to data from a previously conducted randomized controlled trial including infants subjected to UC clamping at ≤10 s (n=200) or ≥180 s (n=200) after delivery.

RESULTS: After adjustment for age differences at the time of follow-up, serum ferritin concentrations were 77 µg/L, 103 µg/L and 114 µg/L in the 10 s, 60 s and 180 s groups, respectively. The adjusted ferritin concentration was significantly higher in the 60 s group compared to the 10 s group (P=0.002) while the difference between the 60 s and 180 s groups was not significant (P=0.29).

CONCLUSION: Delaying UC clamping for 60 s, compared to 10 s, results in higher serum ferritin concentrations at four months in term infants. This study suggests that it is possible to obtain the iron preserving benefits from delayed UC clamping also in connection with altruistic UCB donation.

Strengths and limitations of the study

- This study compares iron- and blood status at the age of four months in children whose UC were clamped after 60 s due to donation of UCB, with children whose UC were clamped ≤10 s or ≥180 s.
- In this study we focus on potential consequences for the UCB donors caused by different UC clamping times, and not the quality of the collected UCB.
- This prospective observational study uses historical controls and results should be interpreted with caution because of potential confounding bias.
- We did not use a non-inferiority approach for this study since we aimed to compare results after 60 s UC clamping with both 10 s- and 180 s UC clamping.

INTRODUCTION

Umbilical cord blood (UCB) is a valuable stem cell source for the 30-50% of patients that need a hematopoietic stem cell transplantation, but who lack a suitable family or public registry donor [1 2]. More than 35.000 transplantations with UCB have been performed worldwide [1].

The number of hematopoietic stem cells in the UCB unit highly affects the outcome of transplantation [3 4]. A small cell dose in a transplanted unit is directly correlated with a delay in recovery of the immune system and lower incidence of sustained donor engraftment [5 6]. Large cell numbers are essential and closely correlated with the collected volume of UCB. Volume is dependent on the time of umbilical cord (UC) clamping [7]. The earlier the UC is clamped, the more blood is left to collect from the placenta and for this reason immediate UC clamping is normally practiced in UCB collection.

During the last decades, immediate UC clamping has been part of active management of labour with the aim to reduce maternal haemorrhage at birth [8 9]. Hence, the placenta and UCB, have been regarded as waste products following birth. However, immediate UC clamping may deprive infants of up to one third of the total fetoplacental blood volume [10 11] and increase the risk of iron deficiency (ID) in the first 3-6 months of life [12-16]. Iron deficiency is associated with impaired neurodevelopment affecting cognitive, motor, and behavioural skills in young children [17-19]. For this reason the World Health Organization recommends delayed UC clamping for 1-3 minutes after birth [20].

We hypothesized that if the UC is clamped at 60 seconds (s) after birth, a sufficient amount of blood will have passed from the placenta to the infant to reduce the risk of ID and anaemia while still allowing collection of UCB for banking [21]. The aim of the current study was to evaluate iron status and complete blood counts at the age of four months in infants who had the UC clamped after 60s in connection with UCB donation and compare the results to immediate (≤10s) and late (≥180s) UC clamping. In this study we focused on potential consequences for the donors only.

MATERIALS AND METHODS

We performed an observational cohort study using one prospective clinical cohort and data from a previously performed randomized controlled trial (RCT)[13]. The prospectively recruited infants were born at Karolinska University Hospital Huddinge, Sweden between April 2012 and May 2015, to parents who agreed to donate UCB to the Swedish National Umbilical Cord Blood Bank, a public bank without profit interest. To enable collection of UCB, the UC was clamped after 60 ±10s. The historical cohorts consisted of data from infants who were randomized to immediate (within 10s after birth) or late (after 180s) UC clamping, respectively, in a trial conducted by three of the co-authors at the Hospital of Halland in Halmstad, Sweden between April 2008 and September 2009 [13].

Outcome measures

The primary outcome variable was ferritin as a measure of iron stores, at the age of four months and secondary outcomes included haemoglobin, transferrin saturation, soluble transferrin receptors, mean cell volume (MCV) as well as ID (defined as ≥2 iron indicators outside reference range with the following cut-off values: ferritin <20 µg/L,[22] MCV <73 fL,[22] transferrin saturation <10% [23] and transferrin receptor >7 mg/L [13]). Since ferritin is an acute phase reactant, C reactive protein (CRP) was analysed in order to exclude falsely elevated ferritin measurements. The infant's dietary habits at four months were also considered as well as the relevance of infant sex for iron and blood status at that age.

Sample size

A formal power calculation was not performed for the current study but the sample size was based on the group size in the original RCT by Andersson *et al*. 2011 (the historical cohorts). The power calculation by Andersson *et al.* showed that a group size of 200 would enable detection of a 29% difference in geometric mean serum ferritin between 10s and 180s UC clamping with a power of 80% and a significance level of 0.05, assuming a mean serum ferritin concentration of 110 µg/L in the180s-group while allowing an attrition of 25% [13]. Based on the results from Andersson *et al.*,

demonstrating a 45% difference in ferritin concentration between 10s and 180s UC clamping [13], we decided to include 200 children also in the prospective group.

Study population and inclusion criteria

The same inclusion criteria applied for the prospective and historical cohorts: healthy mother, normal, singleton pregnancy, gestational age 37+0 to 41+6 weeks and planned vaginal delivery with cephalic presentation [13]. The mother also had to understand Swedish well enough to consent participation in the study. In the prospectively recruited cohort, parents also had to agree to altruistic donation of UCB, and to the modified UC clamping strategy. Exclusion criteria were also the same in the three groups: smoking mother, serious congenital malformations, syndromes, or other congenial diseases that could affect the outcome measures. For this study, we only included data from the RCT that were handled according to per protocol, as compared to the analysis by intention to treat in the original RCT [13]. Moreover, like the RCT, we only included children with CRP<10 in the analysis. All parents donating UCB at Karolinska University Hospital Huddinge that met the inclusion criteria were offered to participate in the study upon arrival at the delivery unit. Midwives were instructed to clamp the UC 60s after birth in deliveries where parents had consented to participate in the study. Staff in charge of collection of UCB measured the exact timing of UC clamping using a digital timer. Only children whose UC were clamped at 60 ±10s were included in the study. Oral and written informed consent was obtained from one or both of the parents. The study was approved by the regional ethical review board in Stockholm (2011/2142-31/3) and the RCT was approved by the regional ethical review board at Lund University (41/2008).

Data collection and blood samples

Midwives were instructed to hold the new-born baby below the uterine level for the first 30s before placing the infant on the mother's abdomen, in accordance with the study by Andersson et al 2011 [13]. The UC clamping technique used in the prospectively recruited cohort was similar to the historical groups except for the timing. Venous blood samples were analysed at birth from the UC, at two to three days of age in connection with metabolic screening and at four months (±21 days) at a

scheduled follow-up visit. At birth and at four months, serum ferritin, haemoglobin, haematocrit, MCV, reticulocyte count, transferrin saturation, transferrin receptor and CRP were analysed and at two to three days of age we analysed haemoglobin, haematocrit and bilirubin. EDTA tubes were used for blood count and serum separator tubes for iron status, bilirubin and CRP (Microvette, Sarstedt AG & Co, Nümbrecht, Germany). Blood status was analysed using equal methods and equipment in both the laboratory at Karolinska University Hospital (Sysmex XE5000, Sysmex, Kobe, Japan) and at the Hospital of Halland (Sysmex XE2100, Sysmex, Kobe, Japan). Analysis of iron status and CRP could be performed at the same laboratory as in the RCT [13], and therefore the blood samples collected at Karolinska University Hospital were centrifuged and the serum kept at -70 degrees Celsius before sent for analysis at the Clinical Chemistry Laboratory at the Hospital of Halland. The samples were then thawed and analysed using a Cobas 6000 (Roche Diagnostics, Basel, Switzerland), the same method as in the RCT [13].

Statistical analysis

Analyses on the two historical cohorts, 10s and 180s, were calculated only on infants receiving intervention per protocol in order to obtain clearly defined groups according to time of UC clamping. Baseline characteristics were compared across groups using analysis of variance (ANOVA). Means of outcome measures in the three cohorts were compared using the Bonferroni method. We also performed pairwise comparisons (60s vs 10s and 60s vs 180s) using Student's t-test for variables with normal distribution that were statistically significant in the Bonferroni analyses. Ferritin concentration was log10 transformed for analysis because of skewed distribution and the results were presented as geometric means. Categorical variables were compared between groups using Fisher's Exact test for pairwise comparisons. Adjusted group means were compared using ANOVA and a binary logistic regression model was used to adjust for sex and age in the evaluation of ID at four months. A P-value less than 0.05 were considered significant. No imputation of missing data was performed. Loss to follow-up was registered, but not examined further. IBM SPSS for Windows, version 22 was used for the analyses.

RESULTS

Of the 200 prospectively recruited infants, 191 were included in the analysis as compared to 166 in the 10s and 168 in the 180s groups. Infants lost to follow up or excluded due to not fulfilling inclusion criteria are shown in Fig 1.

Baseline data

There were no significant differences in the 60s group as compared to the 10s and 180s groups with regard to maternal or neonatal characteristics. The 10s group had higher UC haemoglobin (163 vs 153 g/L, P<0.001) and higher UC haematocrit (48.7 vs 47.0%, P=0.005) than the 60s group. There were no significant differences between the 60s and the 180s groups (Table 1).

Two to three day-follow-up

At two to three days, blood samples were obtained from 140 infants in the 10s group, 107 in the 60s group, and 141 in the 180s group (Figure 1). The prevalence of anaemia was higher in the 10s compared to the 60s and 180s groups (9 (6.4%), 2 (1.9%) and 2 (1.4%)) respectively (P=0.04). Haemoglobin was lower in the 10s and 60s groups compared to the 180s group; 175, 180 and 189 g/L respectively (P=0.001 for 60s vs 180s). Bilirubin did not differ between the groups. Anaemic children were referred for further diagnostics but none needed treatment.

Primary outcome

In total 468 (78%) infants had follow up at four months (children excluded or lost to follow up are shown in Figure 1). In the 60s group, the infants (n=147) were four days older than the historical groups from the RCT (126±7 days vs 122±6 days, P<0.001). The geometric mean ferritin levels were 80, 96 and 117 µg/L respectively in the 10s, 60s and 180s groups (P=0.052 for 10s vs 60s; P=0.02 for 60s vs 180s) (Table 2). After adjustment for the age difference, the geometric means were 77, 103 and 114 µg/L respectively (P=0.002 for 10s vs 60s; P=0.29 for 60s vs 180s) (Table 3).

Secondary outcomes

All secondary outcome variables are shown in Table 2. Haemoglobin levels at four months were higher in the 60s group compared to 10s and 180s, but the difference did not remain in the age

adjusted model (Table 3). There were no statistically significant differences in the prevalence of anaemia in the 60s group as compared to the other two groups (Table 4). After adjustment for age at the time of follow-up, there were no differences between the 10s and 60s groups in haematocrit, MCV, reticulocyte count, transferrin receptor or transferrin saturation. The 60s group had significantly lower adjusted MCV and higher adjusted transferrin receptor compared to the 180s group, but there were no differences in haematocrit, reticulocyte count or transferrin saturation (Table 3). The prevalence of ID was 8 (5.2%), 7 (4.8%) and 1 (0.7%) respectively in the 10s, 60s and 180s groups (P>0.99 for 10s vs 60s and P=0.04 for 60s vs 180s) (Table 4). After statistical adjustments for sex and age at the time of follow-up, the prevalence of ID did not differ between the 60s group and the two other groups. The proportion of infants that were exclusively breast fed at four months did not differ between the groups (79 (52%), 92 (58%) and 89 (56%) respectively in the 10s, 60s and 180s groups. The impact of exclusive breast feeding on ferritin concentration and ID were further assessed in an adjusted model but no correlation was found (data not shown).

Sex differences

Iron and blood status at four months were further assessed by analysing differences according to infant sex. When data from the three cohorts were analysed together, geometric mean ferritin was lower in boys than in girls and ID was more prevalent amongst boys (Fig 2A and 2B). Haemoglobin concentrations did not differ significantly between boys and girls and neither did the prevalence of anaemia (data not shown).

DISCUSSION

We compared infants' iron and blood status at the age of four months after three different UC clamping time intervals (60s versus ≤10s, and ≥180s, respectively). Previous studies have compared immediate and late UC clamping but the definition of "late" in these studies varies from 60s to 180s or after birth of the placenta [12 14 15]. The current study evaluates an intermediate UC clamping strategy that can be used in connection with altruistic donation of UCB for clinical banking. When asking parents to donate their child's UCB for the potential good of others, it is essential to inform of

possible consequences for the child. We demonstrated that after adjustment for age difference at the time of follow-up, there were no statistically significant differences in ferritin concentration between the 60s and 180s groups but the 60s group had significantly higher ferritin concentrations compared to 10s. The main reason for the effect of age on ferritin during infancy is rapid growth which leads to a rapidly expanding blood volume and depletion of iron stores [16].

Results from the present study are in accordance with a Brazilian RCT that demonstrated higher ferritin concentration at the age of three months in children whose UC were clamped after 60s compared to immediate UC clamping [24]. However, our findings are in contrast to a study by Cernadas et al, who reported no differences in ferritin six months after birth comparing 15s and 60s UC clamping. The difference in ferritin between the 60s and 180s groups were not presented [25].

The present study indicates that a 60s delay before UC clamping results in a mean serum ferritin at 4 months of age corresponding to 90% of the mean ferritin after 180s UC clamping

In the current study, children in the 60s group had the lowest haemoglobin of all three groups at birth (10s 163 g/L, 60s 153 g/L, 180s 158 g/L) but at four months, there were no longer any differences between the groups in our adjusted model. The original RCT as well as a Cochrane report found higher concentrations of haemoglobin after immediate than late UC clamping at birth [13 15]. As speculated in a previous publication, this difference may not reflect true difference in haemoglobin but could be a result of trans endothelial plasma leakage in the UC after UC clamping [13].

The prevalence of ID was higher in the 10s and 60s groups compared to the 180s group. However, when adjusting for sex and age at the time of follow-up, there were no longer any statistically significant differences between the three groups. In accordance with previous studies on ID in infancy, we found that at four months, boys had significantly lower geometric mean ferritin and more often ID than girls [26 27]. Nevertheless, the aim of the study was not to compare differences according to sex, and the groups were therefor too small to draw any reliable conclusions of sex and ID. It has been suggested that the differences in iron status between the two sexes may be hormone-

related but it is also influenced by genetic factors, which may differ in populations with different ethnicity [27]. Also, when considering the generalisability of this study, readers should bear in mind that all children were placed below the uterine level for 30s directly after birth. This may be in contrast to the practice in other delivery settings, where the children are often placed directly on the woman's abdomen, which may affect the speed of the placental transfusion.

Limitations of the study

A limitation of this study is that we have no record of the parents' ethnic or socio-economic background, and we were therefore unable to draw any conclusions on possible impact of these factors. When comparing different interventions, RCTs are regarded as the golden standard. However, randomization into 10s UC clamping for this study was considered unethical since there is evidence that children benefit from prolonged placental transfusion for at least 60s, and the clinical routine in Sweden is to wait 180s before UC clamping in normal deliveries. We used the historical cohorts from the RCT in Halmstad, Sweden as controls in this study. The prospectively recruited cohort (60s) is from a large birth clinic in Stockholm, Sweden while the controls are from a smaller town where the participants were recruited two to five years earlier. Although the inclusion criteria were the same and pregnant mothers in Sweden follow the same well-controlled antenatal program and the baseline data showed no significant differences in maternal or infant characteristics, it is difficult to know if the study populations are homogenous. We did not use a non-inferiority approach for this study since we aimed to compare results after 60s UC clamping with10s and 180s. Nor did we perform a power calculation when designing the study, but included an equally large group as the two historical groups. This is a weakness which, should be taken into consideration. We have taken measures to avoid systematic biases caused by differences in laboratory routines between the two hospitals by using the same laboratory for analysing the iron status variables as well as CRP.

Collection of UCB for transplantation is a growing and life-saving practice. Tens of thousands UCB units are altruistically donated and stored for public use each year, and as a result new-borns are exposed to limited UC clamping time. The idea that UCB left in the placenta after birth is a mere

waste that is thrown away if not collected is obsolete. Optimizing UC clamping time and investigating the clinical significance to minimize risks for the infant while still enabling collection of UCB for transplantation is a great, but necessary, challenge. Furthermore, parents have the right to know that not all collected UCB can be used for transplantation. If the amount of blood in a collected UCB unit is too small, it will not be processed and banked for practical and economic reasons.

CONCLUSION

This observational study indicates that in a healthy Swedish population, UC clamping after 60s reduces the risk for low iron stores at four months of life compared to UC clamping at 10s. After adjustment for age and sex, there were no statistically significant differences in ferritin concentration between 60 and 180s UC clamping. 60s UC clamping is therefore preferable to immediate UC clamping and enables collection of UCB for transplantation. Delaying UC clamping may be more important in boys than in girls to avoid ID in early childhood. Larger studies with long term follow-up is needed to establish the clinical relevance of different UC clamping strategies.

CONTRIBUTORS

Dr C. Götherström conceptualized and designed the study, analyzed data, critically revised and approved the final manuscript as submitted. Mrs U. Askelöf conceptualized and designed the study, carried out all data collection in the prospective cohort, carried out all analyses, drafted the initial manuscript, and approved the final manuscript as submitted. Dr O. Andersson designed the data collection instruments, contributed to the study design and provided data for the historical cohorts. He also carried out the initial analyses, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. Profs M. Domellöf, A. Fasth, L. Hellström-Westas, M. Westgren, Associate Profs K. Pettersson and I. Wiklund and Dr B. Hallberg participated in the study design, reviewed and revised the manuscript and approved the final manuscript as submitted.

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TABLE 1. Baseline characteristics of mothers and infants according to time for umbilical cord clamping

	Umbilical cord clamping time			P
	10s n=166 (HC)	60s n=191	180s n=168 (HC)	
Maternal characteristics				
Age (years)	31.7 (4.2)	30.7 (4.7)	30.8 (4.9)	NS
Parity (including study child)	1.81 (0.87)	1.86 (0.76)	1.76 (0.74)	NS
Weight at first antenatal visit (kg)	66.4 (12.0)	67.3 (11.5)	67.3 (12.1)	NS
Body mass index	23.6 (3.8)	24.5 (3.9)	23.9 (3.6)	NS
Haemoglobin at first antenatal visit (g/L)	128 (9)	128 (10)	128 (11)	NS
No (%) of vaginal deliveries:				
Non-instrumental	157 (95)	184 (96)	157 (94)	NS
Vacuum extraction	8 (5)	7 (4)	11 (7)	NS
Forceps	1 (0.6)	0 (0)	0 (0)	NS
Infant characteristics				
Gestational age (weeks)	40.0 (1.1)	40.0 (1.0)	40.0 (1.1)	NS
No (%) of males	83 (50)	98 (51)	73 (43)	NS
Birth weight (g)	3523 (483)	3603 (454)	3633 (464)	NS
Birth length (cm)	50.7 (1.9)	50.6 (1.8)	50.9 (1.9)	NS
Head circumference (cm)	34.7 (1.3)	35.1 (1.3)	35.0 (1.4)	NS
Umbilical cord haemoglobin (g/L)	163 (15)	153 (20)	158 (18)	<0.001*
Umbilical cord haematocrit (%)	48.7 (4.3)	47.0 (5.5)	47.1 (5.0)	0.005**

Values are means (SD) unless stated otherwise. P<0.05 was considered significant.
NS=non-significant; HC= Historical cohort.
*Comparison of 10s and 60s. For 180s and 60s, P=0.063.
**Comparison of 10s and 60s. For 180s and 60s, P>0.99.

TABLE 2. Outcome measures at the age of four months in infants whose umbilical cord were clamped after 10s, 60s or 180s

	Umbilical cord clamping time			60s vs 10s		60s vs 180s	
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	Mean difference (95% CI)	P	Mean difference (95% CI)	P
Infant age (days)	122 (6)	126 (7)	122 (6)	5 (3 to 6)	<0.001	4 (3 to 6)	<0.001
Main outcome measures							
Geometric mean (range) ferritin (µg/L)	80 (33 to 191)	96 (44 to 208)	117 (58 to 232)	0.83 (0.69 to 1.45)*	0.052	1.21 (1.03 to 1.44)*	0.02
Haemoglobin (g/L)	113 (7)	115 (8)	113 (8)	2 (0 to 4)	0.047	2 (0 to 4)	0.03
Blood count							
Haematocrit (%)	33.0 (2.0)	33.3 (2.2)	32.7 (2.1)	0.3 (-0.2 to 0.8)	0.19	1 (0 to 1)	0.02
Mean cell volume (fL)	77.9 (3.1)	77.7 (3.7)	79.2 (3.0)	-0.2 (-1 to 0.6)	0.67	-1.5 (-2.3 to -0.7)	<0.001
Reticulocyte count ($x10^9$/L)	37 (11)	36 (11)	40 (11)	-1 (-4 to 1)	0.39	-4 (-6 to -1)	0.01
Iron status							
Transferrin receptor (mg/L)	4.00 (0.80)	4.07 (0.92)	3.71 (0.69)	0.07 (-0.13 to 0.26)	0.51	0.35 (0.17 to 0.54)	<0.001
Transferrin saturation (%)	16 (6)	16 (8)	18 (6)	1 (-1 to 2)	0.40	-2 (-3 to 0)	0.03

Values are means (SD) unless stated otherwise. HC= Historical cohort.
*Geometric mean ratio (95% CI for geometric mean).
Successful analyses in the 60s and 180s groups: haemoglobin (n=140, 144), haematocrit, mean cell volume, reticulocyte count (n=135, 144), ferritin (n=147, 148), transferrin receptor (n=147, 148), transferrin saturation (n=145, 148).

TABLE 3. Outcome measures at the age of four months adjusted for infant age

	Umbilical cord clamping time Adjusted means (95% CI)			P	P
	10s n=152 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Main outcome measures					
Geometric mean (range) ferritin (µg/L)	77 (68 to 87)	103 (90 to 117)	114 (100 to 129)	0.002	0.29
Haemoglobin (g/L)	113 (112 to 115)	114 (113 to 116)	113 (112 to 114)	0.30	0.17
Blood count					
Haematocrit (%)	33.1 (32.7 to 33.4)	33.1 (32.8 to 33.5)	32.7 (32.4 to 33.1)	0.30	0.12
Mean cell volume (fL)	77.8 (77.3 to 78.3)	77.9 (77.3 to 78.4)	79.1 (78.6 to 79.6)	0.30	0.003
Reticulocyte count ($x10^9$/L)	36.9 (35.2 to 38.7)	37.0 (35.0 to 38.9)	39.4 (37.6 to 41.2)	0.99	0.07
Iron status					
Transferrin receptor (mg/L)	4.01 (3.87 to 4.14)	4.05 (3.92 to 4.19)	3.72 (3.59 to 3.85)	0.62	0.001
Transferrin saturation (%)	15.7 (14.7 to 16.7)	16.6 (15.5 to 17.7)	18.1 (17 to 19.2)	0.25	0.06

Values are means (95% CI) adjusted for infant age (days from birth) unless stated otherwise.
HC= Historical cohort. Successful analyses for the 60s group were: ferritin (n=147), haemoglobin (n=140), haematocrit, mean cell volume, reticulocyte count (n=135), transferrin receptor, and transferrin saturation (n=145). For the 180s group; blood status (n=144).

TABLE 4. Infants with iron status indicators outside reference limits at the age of 4 months according to umbilical cord clamping time

	Umbilical cord clamping time			P	P
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Ferritin <20 µg/L	11 (7.2)	4 (2.7)	0 (0)	0.11	0.06
Anaemia (Hb<105 g/L)	20 (13.1)	16 (11.4)	20 (13.9)	0.72	0.60
Mean cell volume <73 fL	8 (5.2)	14 (10.4)	3 (2.1)	0.12	0.01
Transferrin saturation <10%	22 (14.4)	15 (10.3)	8 (5.4)	0.38	0.13
Transferrin receptor >7 mg/L	0 (0)	2 (1.4)	0 (0)	0.24	0.25
Iron deficiency*	8 (5.2)	7 (4.8)	1 (0.7)	1.00	0.04

Values are unadjusted numbers (%). HC= Historical cohort.
Numbers of successful analyses: mean cell volume (n=153, 135, 144), transferrin saturation (n=153, 145, 148), anaemia (n=153, 140, 144).

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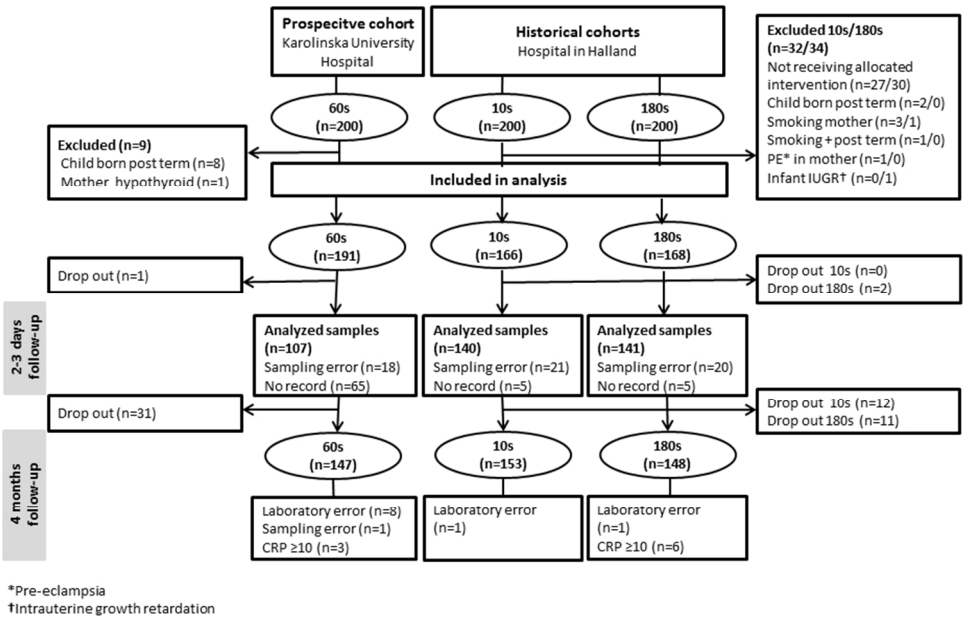
FIGURE LEGENDS

FIGURE 1. Flow chart of the study populations.

FIGURE 2. Differences in infants' iron status at the age of 4 months according to sex in three groups divided according to umbilical cord clamping time. **A:** Geometric mean ferritin according to sex and time for umbilical cord clamping, **B**: Proportion of children with iron deficiency according to time for umbilical cord clamping and sex.



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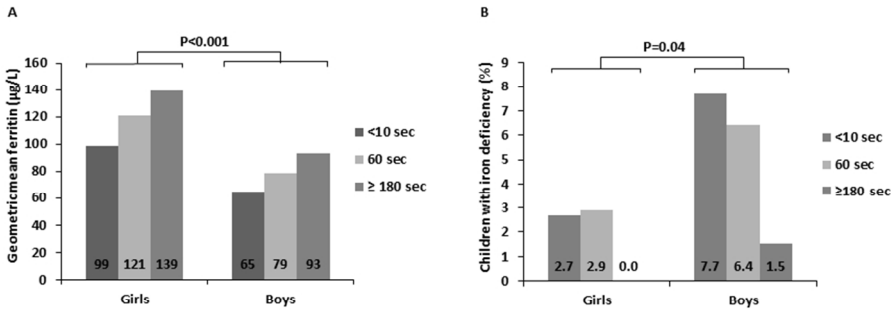

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254x190mm (96 x 96 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4, 5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	Not applicable
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4, 5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5, 6
Bias	9	Describe any efforts to address potential sources of bias	6, 10
Study size	10	Explain how the study size was arrived at	4, 5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	Not applicable
		(*c*) Explain how missing data were addressed	6, 7
		(*d*) If applicable, explain how loss to follow-up was addressed	6, 7
		(*e*) Describe any sensitivity analyses	Not applicable
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Fig 1, 6, 7
		(b) Give reasons for non-participation at each stage	Fig 1
		(c) Consider use of a flow diagram	Fig 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4, 5
		(b) Indicate number of participants with missing data for each variable of interest	Tables 1-4
		(c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	Not applicable
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7
		(*b*) Report category boundaries when continuous variables were categorized	Not applicable
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Fig 2
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9, 11
Generalisability	21	Discuss the generalisability (external validity) of the study results	9, 10
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Wait a minute? An observational cohort study comparing iron stores in healthy Swedish infants at four months of age after 10 s, 60 s and 180 s umbilical cord clamping

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Secondary Subject Heading:	Paediatrics, Haematology (incl blood transfusion)
Keywords:	PAEDIATRICS, UMBILICAL CORD CLAMPING, ALTRUISTIC UMBILICAL CORD BLOOD DONATION, IRON DEFICIENCY, HAEMATOPOIETIC STEM CELL TRANSPLANTATION, Fetal medicine < OBSTETRICS

Wait a minute? An observational cohort study comparing iron stores in healthy Swedish infants at four months of age after 10 s, 60 s and 180 s umbilical cord clamping

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Abbreviations: ANOVA- analysis of variance, CI – confidence interval, CRP – C reactive protein, ID - iron deficiency, MCV - mean cell volume, NS – non significant, RCT - randomized controlled trial, s – seconds, SD – standard deviation, UC – umbilical cord, UCB – umbilical cord blood.

Abstract

BACKGROUND AND OBJECTIVE: Umbilical cord blood (UCB) is a valuable stem cell source used for transplantation. Immediate umbilical cord (UC) clamping is normally practiced in UCB collection but delayed UC clamping is increasingly advocated to reduce possible infant anaemia. This policy obstructs collection of UCB for banking. The aim was to investigate an UC clamping time point that enables collection of altruistically donated UCB, without adverse effects for the donating newborn. The objective of the current study was to evaluate iron status at the age of four months in infants who had the UC clamped after 60 seconds (s) in connection with UCB donation and compare the results to immediate and late UC clamping.

DESIGN: Prospective observational study with two historical controls.

SETTING: A university hospital in Stockholm, Sweden and a county hospital in Halland, Sweden.

METHOD: Iron status was assessed at four months in 200 prospectively recruited term infants whose UC was clamped 60s after birth in connection with UCB donation. The results were compared to data from a previously conducted randomized controlled trial including infants subjected to UC clamping at ≤10 s (n=200) or ≥180 s (n=200) after delivery.

RESULTS: After adjustment for age differences at the time of follow-up, serum ferritin concentrations were 77 µg/L, 103 µg/L and 114 µg/L in the 10s, 60s and 180s groups, respectively. The adjusted ferritin concentration was significantly higher in the 60s group compared to the 10s group (P=0.002) while the difference between the 60s and 180s groups was not significant (P=0.29).

CONCLUSION: In this study of healthy term infants, 60s UC clamping, resulted in higher serum ferritin concentrations at four months compared to 10s UC clamping. The results suggest that delaying the UC clamping for 60s reduces the risk for iron deficiency in connection with altruistic UCB donation.

Strengths and limitations of the study

- This study compares iron- and blood status at the age of four months in children whose UC were clamped after 60 s due to donation of UCB, with children whose UC were clamped ≤10 s or ≥180 s.
- In this study we focus on potential consequences for the UCB donors caused by different UC clamping times, and not the quality of the collected UCB.
- This prospective observational study uses historical controls and results should be interpreted with caution because of potential confounding bias.
- We did not use a non-inferiority approach for this study since we aimed to compare results after 60 s UC clamping with both 10 s- and 180 s UC clamping.

INTRODUCTION

Umbilical cord blood (UCB) is a valuable stem cell source for the 30-50% of patients that need a hematopoietic stem cell transplantation, but who lack a suitable family or public registry donor [1 2]. More than 35.000 transplantations with UCB have been performed worldwide [1].

The number of hematopoietic stem cells in the UCB unit highly affects the outcome of transplantation [3 4]. A small cell dose in a transplanted unit is directly correlated with a delay in recovery of the immune system and lower incidence of sustained donor engraftment [5 6]. Large cell numbers are essential and closely correlated with the collected volume of UCB. Volume is dependent on the time of umbilical cord (UC) clamping [7]. The earlier the UC is clamped, the more blood is left to collect from the placenta and for this reason immediate UC clamping is normally practiced in UCB collection.

During the last decades, immediate UC clamping has been part of active management of labour with the aim to reduce maternal haemorrhage at birth [8 9]. Hence, the placenta and UCB, have been regarded as waste products following birth. However, immediate UC clamping may deprive infants of up to one third of the total fetoplacental blood volume [10 11] and increase the risk of iron deficiency (ID) in the first 3-6 months of life [12-16]. Iron deficiency is associated with impaired neurodevelopment affecting cognitive, motor, and behavioural skills in young children [17-19]. For this reason the World Health Organization recommends delayed UC clamping for 1-3 minutes after birth [20].

We hypothesized that if the UC is clamped at 60 seconds (s) after birth, a sufficient amount of blood will have passed from the placenta to the infant to reduce the risk of ID and anaemia while still allowing collection of UCB for banking [21]. The objective of the current study was to evaluate iron status and complete blood counts at the age of four months in infants who had the UC clamped after 60s in connection with UCB donation and compare the results to immediate (≤10s) and late (≥180s) UC clamping. In this study we focused on potential consequences for the donors only.

MATERIALS AND METHODS

We performed an observational cohort study using one prospective clinical cohort and data from a previously performed randomized controlled trial (RCT)[13]. The prospectively recruited infants were born at Karolinska University Hospital Huddinge, Sweden between April 2012 and May 2015, to parents who agreed to donate UCB to the Swedish National Umbilical Cord Blood Bank, a public bank without profit interest. To enable collection of UCB, the UC was clamped after 60 ±10s. The historical cohorts consisted of data from infants who were randomized to immediate (within 10s after birth) or late (after 180s) UC clamping, respectively, in a trial conducted by three of the co-authors at the Hospital of Halland in Halmstad, Sweden between April 2008 and September 2009 [13].

Outcome measures

The primary outcome variable was ferritin as a measure of iron stores, at the age of four months and secondary outcomes included haemoglobin, transferrin saturation, soluble transferrin receptors, mean cell volume (MCV) as well as ID (defined as ≥2 iron indicators outside reference range with the following cut-off values: ferritin <20 µg/L,[22] MCV <73 fL,[22] transferrin saturation <10% [23] and transferrin receptor >7 mg/L [13]). Since ferritin is an acute phase reactant, C reactive protein (CRP) was analysed in order to exclude falsely elevated ferritin measurements. The infant's dietary habits at four months were also considered as well as the relevance of infant sex for iron and blood status at that age.

Sample size

A formal power calculation was not performed for the current study but the sample size was based on the group size in the original RCT by Andersson *et al*. 2011 (the historical cohorts). The power calculation by Andersson *et al.* showed that a group size of 200 would enable detection of a 29% difference in geometric mean serum ferritin between 10s and 180s UC clamping with a power of 80% and a significance level of 0.05, assuming a mean serum ferritin concentration of 110 µg/L in the180s-group while allowing an attrition of 25% [13]. Based on the results from Andersson *et al.*,

demonstrating a 45% difference in ferritin concentration between 10s and 180s UC clamping [13], we decided to include 200 children also in the prospective group.

Study population and inclusion criteria

The same inclusion criteria applied for the prospective and historical cohorts: healthy mother, normal, singleton pregnancy, gestational age 37+0 to 41+6 weeks and planned vaginal delivery with cephalic presentation [13]. The mother also had to understand Swedish well enough to consent participation in the study. In the prospectively recruited cohort, parents also had to agree to altruistic donation of UCB, and to the modified UC clamping strategy. Exclusion criteria were also the same in the three groups: smoking mother, serious congenital malformations, syndromes, or other congenial diseases that could affect the outcome measures. For this study, we only included data from the RCT that were handled according to per protocol, as compared to the analysis by intention to treat in the original RCT [13]. Moreover, like the RCT, we only included children with CRP<10 in the analysis. All parents donating UCB at Karolinska University Hospital Huddinge that met the inclusion criteria were offered to participate in the study upon arrival at the delivery unit. Midwives were instructed to clamp the UC 60s after birth in deliveries where parents had consented to participate in the study. Staff in charge of collection of UCB measured the exact timing of UC clamping using a digital timer. Only children whose UC were clamped at 60 ±10s were included in the study. Oral and written informed consent was obtained from one or both of the parents. The study was approved by the regional ethical review board in Stockholm (2011/2142-31/3) and the RCT was approved by the regional ethical review board at Lund University (41/2008).

Data collection and blood samples

Midwives were instructed to hold the new-born baby below the uterine level for the first 30s before placing the infant on the mother's abdomen, in accordance with the study by Andersson *et al.* 2011 [13]. The UC clamping technique used in the prospectively recruited cohort was similar to the historical groups except for the timing. Venous blood samples were analysed at birth from the UC, at two to three days of age in connection with metabolic screening and at four months (±21 days) at a

scheduled follow-up visit. At birth and at four months, serum ferritin, haemoglobin, haematocrit, MCV, reticulocyte count, transferrin saturation, transferrin receptor and CRP were analysed and at two to three days of age we analysed haemoglobin, haematocrit and bilirubin. EDTA tubes were used for blood count and serum separator tubes for iron status, bilirubin and CRP (Microvette, Sarstedt AG & Co, Nümbrecht, Germany). Blood status was analysed using equal methods and equipment in both the laboratory at Karolinska University Hospital (Sysmex XE5000, Sysmex, Kobe, Japan) and at the Hospital of Halland (Sysmex XE2100, Sysmex, Kobe, Japan). Analysis of iron status and CRP could be performed at the same laboratory as in the RCT [13], and therefore the blood samples collected at Karolinska University Hospital were centrifuged and the serum kept at -70 degrees Celsius before sent for analysis at the Clinical Chemistry Laboratory at the Hospital of Halland. The samples were then thawed and analysed using a Cobas 6000 (Roche Diagnostics, Basel, Switzerland), the same method as in the RCT [13].

Statistical analysis

Analyses on the two historical cohorts, 10s and 180s, were calculated only on infants receiving intervention per protocol in order to obtain clearly defined groups according to time of UC clamping. Baseline characteristics were compared across groups using analysis of variance (ANOVA). Means of outcome measures in the three cohorts were compared using the Bonferroni method. We also performed pairwise comparisons (60s vs 10s and 60s vs 180s) using Student's t-test for variables with normal distribution that were statistically significant in the Bonferroni analyses. Ferritin concentration was log10 transformed for analysis because of skewed distribution and the results were presented as geometric means. Categorical variables were compared between groups using Fisher's Exact test for pairwise comparisons. Adjusted group means were compared using ANOVA and a binary logistic regression model was used to adjust for sex and age in the evaluation of ID at four months. A P-value less than 0.05 were considered significant. No imputation of missing data was performed. Loss to follow-up was registered, but not examined further. IBM SPSS for Windows, version 22 was used for the analyses.

RESULTS

Of the 200 prospectively recruited infants, 191 were included in the analysis as compared to 166 in the 10s and 168 in the 180s groups. Infants lost to follow up or excluded due to not fulfilling inclusion criteria are shown in Fig 1.

Baseline data

There were no significant differences in the 60s group as compared to the 10s and 180s groups with regard to maternal or neonatal characteristics. The 10s group had higher UC haemoglobin (163 vs 153 g/L, P<0.001) and higher UC haematocrit (48.7 vs 47.0%, P=0.005) than the 60s group. There were no significant differences between the 60s and the 180s groups (Table 1).

Two to three day-follow-up

At two to three days, blood samples were obtained from 140 infants in the 10s group, 107 in the 60s group, and 141 in the 180s group (Figure 1). The prevalence of anaemia was higher in the 10s compared to the 60s and 180s groups (9 (6.4%), 2 (1.9%) and 2 (1.4%)) respectively (P=0.04). Haemoglobin was lower in the 10s and 60s groups compared to the 180s group; 175, 180 and 189 g/L respectively (P=0.001 for 60s vs 180s). Bilirubin did not differ between the groups. Anaemic children were referred for further diagnostics but none needed treatment.

Primary outcome

In total 468 (78%) infants had follow up at four months (children excluded or lost to follow up are shown in Figure 1). In the 60s group, the infants (n=147) were four days older than the historical groups from the RCT (126±7 days vs 122±6 days, P<0.001). The geometric mean ferritin levels were 80, 96 and 117 µg/L respectively in the 10s, 60s and 180s groups (P=0.052 for 10s vs 60s; P=0.02 for 60s vs 180s) (Table 2). After adjustment for the age difference, the geometric means were 77, 103 and 114 µg/L respectively (P=0.002 for 10s vs 60s; P=0.29 for 60s vs 180s) (Table 3).

Secondary outcomes

All secondary outcome variables are shown in Table 2. Haemoglobin levels at four months were higher in the 60s group compared to 10s and 180s, but the difference did not remain in the age

adjusted model (Table 3). There were no statistically significant differences in the prevalence of anaemia in the 60s group as compared to the other two groups (Table 4). After adjustment for age at the time of follow-up, there were no differences between the 10s and 60s groups in haematocrit, MCV, reticulocyte count, transferrin receptor or transferrin saturation. The 60s group had significantly lower adjusted MCV and higher adjusted transferrin receptor compared to the 180s group, but there were no differences in haematocrit, reticulocyte count or transferrin saturation (Table 3). The prevalence of ID was 8 (5.2%), 7 (4.8%) and 1 (0.7%) respectively in the 10s, 60s and 180s groups (P>0.99 for 10s vs 60s and P=0.04 for 60s vs 180s) (Table 4). After statistical adjustments for sex and age at the time of follow-up, the prevalence of ID did not differ between the 60s group and the two other groups. The proportion of infants that were exclusively breast fed at four months did not differ between the groups (79 (52%), 92 (58%) and 89 (56%) respectively in the 10s, 60s and 180s groups. The impact of exclusive breast feeding on ferritin concentration and ID were further assessed in an adjusted model but no correlation was found (data not shown).

Sex differences

Iron and blood status at four months were further assessed by analysing differences according to infant sex. When data from the three cohorts were analysed together, geometric mean ferritin was lower in boys than in girls and ID was more prevalent amongst boys (Fig 2A and 2B). Haemoglobin concentrations did not differ significantly between boys and girls and neither did the prevalence of anaemia (data not shown).

DISCUSSION

We compared infants' iron and blood status at the age of four months after three different UC clamping time intervals (60s versus ≤10s, and ≥180s, respectively). Previous studies have compared immediate and late UC clamping but the definition of "late" in these studies varies from 60s to 180s or after birth of the placenta [12 14 15]. The current study evaluates an intermediate UC clamping strategy that can be used in connection with altruistic donation of UCB for clinical banking. When asking parents to donate their child's UCB for the potential good of others, it is essential to inform of

possible consequences for the child. We demonstrated that after adjustment for age difference at the time of follow-up, there were no statistically significant differences in ferritin concentration between the 60s and 180s groups but the 60s group had significantly higher ferritin concentrations compared to 10s. The main reason for the effect of age on ferritin during infancy is rapid growth which leads to a rapidly expanding blood volume and depletion of iron stores [16].

Results from the present study are in accordance with a Brazilian RCT that demonstrated higher ferritin concentration at the age of three months in children whose UC were clamped after 60s compared to immediate UC clamping [24]. However, our findings are in contrast to a study by Cernadas *et al.*, who reported no differences in ferritin six months after birth comparing 15s and 60s UC clamping. The difference in ferritin between the 60s and 180s groups were not presented [25].

The present study indicates that a 60s delay before UC clamping results in a mean serum ferritin at 4 months of age corresponding to 90% of the mean ferritin after 180s UC clamping.

In the current study, children in the 60s group had the lowest haemoglobin of all three groups at birth (10s 163 g/L, 60s 153 g/L, 180s 158 g/L) but at four months, there were no longer any differences between the groups in our adjusted model. The original RCT as well as a Cochrane report found higher concentrations of haemoglobin after immediate than late UC clamping at birth [13 15]. As speculated in a previous publication, this difference may not reflect true difference in haemoglobin but could be a result of trans endothelial plasma leakage in the UC after UC clamping [13].

The prevalence of ID was higher in the 10s and 60s groups compared to the 180s group. However, when adjusting for sex and age at the time of follow-up, there were no longer any statistically significant differences between the three groups. In accordance with previous studies on ID in infancy, we found that at four months, boys had significantly lower geometric mean ferritin and more often ID than girls [26 27]. Nevertheless, the aim of the study was not to compare differences according to sex, and the groups were therefor too small to draw any reliable conclusions of sex and ID. It has been suggested that the differences in iron status between the two sexes may be hormone-

related but it is also influenced by genetic factors, which may differ in populations with different ethnicity [27]. Also, when considering the generalisability of this study, readers should bear in mind that all children were placed below the uterine level for 30s directly after birth. This may be in contrast to the practice in other delivery settings, where the children are often placed directly on the woman's abdomen, which may affect the speed of the placental transfusion.

Limitations of the study

A limitation of this study is that we have no record of the parents' ethnic or socio-economic background, and we were therefore unable to draw any conclusions on possible impact of these factors. When comparing different interventions, RCTs are regarded as the golden standard. However, randomization into 10s UC clamping for this study was considered unethical since there is evidence that children benefit from prolonged placental transfusion for at least 60s, and the clinical routine in Sweden is to wait 180s before UC clamping in normal deliveries. We used the historical cohorts from the RCT in Halmstad, Sweden as controls in this study. The prospectively recruited cohort (60s) is from a large birth clinic in Stockholm, Sweden while the controls are from a smaller town where the participants were recruited two to five years earlier. Although the inclusion criteria were the same and pregnant mothers in Sweden follow the same well-controlled antenatal program and the baseline data showed no significant differences in maternal or infant characteristics, it is difficult to know if the study populations are homogenous. We did not use a non-inferiority approach for this study since we aimed to compare results after 60s UC clamping with10s and 180s. Nor did we perform a power calculation when designing the study, but included an equally large group as the two historical groups. This is a weakness which, should be taken into consideration. We have taken measures to avoid systematic biases caused by differences in laboratory routines between the two hospitals by using the same laboratory for analysing the iron status variables as well as CRP.

Collection of UCB for transplantation is a growing and life-saving practice. Tens of thousands UCB units are altruistically donated and stored for public use each year, and as a result new-borns are exposed to limited UC clamping time. The idea that UCB left in the placenta after birth is a mere

waste that is thrown away if not collected is obsolete. Optimizing UC clamping time and investigating the clinical significance to minimize risks for the infant while still enabling collection of UCB for transplantation is a great, but necessary, challenge. A study evaluating volume and TNC in UCB units collected at the Swedish National Umbilical Cord Blood Bank showed that 37 % of units collected after 60s UC clamping met the banking criteria compared to 47 % after immediate UC clamping [21].

CONCLUSION

In this observational study, significantly more children in the 10s UC clamping group had low iron stores at four months compared to children in the 60s and 180s UC clamping groups. After adjustment for age and sex, there were no statistically significant differences in ferritin concentration between 60 and 180s UC clamping. 60s UC clamping is therefore preferable to immediate UC clamping and enables collection of UCB for transplantation since it reduces the risk for low iron stores. Delaying UC clamping may be more important in boys than in girls to avoid ID in early childhood. Larger studies with long term follow-up is needed to establish the clinical relevance of different UC clamping strategies.

CONTRIBUTORS

Dr C. Götherström conceptualized and designed the study, analyzed data, critically revised and approved the final manuscript as submitted. Mrs U. Askelöf conceptualized and designed the study, carried out all data collection in the prospective cohort, carried out all analyses, drafted the initial manuscript, and approved the final manuscript as submitted. Dr O. Andersson designed the data collection instruments, contributed to the study design and provided data for the historical cohorts. He also carried out the initial analyses, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. Profs M. Domellöf, A. Fasth, L. Hellström-Westas, M. Westgren, Associate Profs K. Pettersson and I. Wiklund and Dr B. Hallberg participated in the study design, reviewed and revised the manuscript and approved the final manuscript as submitted.

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TABLE 1. Baseline characteristics of mothers and infants according to time for umbilical cord clamping

	Umbilical cord clamping time			P
	10s n=166 (HC)	60s n=191	180s n=168 (HC)	
Maternal characteristics				
Age (years)	31.7 (4.2)	30.7 (4.7)	30.8 (4.9)	NS
Parity (including study child)	1.81 (0.87)	1.86 (0.76)	1.76 (0.74)	NS
Weight at first antenatal visit (kg)	66.4 (12.0)	67.3 (11.5)	67.3 (12.1)	NS
Body mass index	23.6 (3.8)	24.5 (3.9)	23.9 (3.6)	NS
Haemoglobin at first antenatal visit (g/L)	128 (9)	128 (10)	128 (11)	NS
No (%) of vaginal deliveries:				
Non-instrumental	157 (95)	184 (96)	157 (94)	NS
Vacuum extraction	8 (5)	7 (4)	11 (7)	NS
Forceps	1 (0.6)	0 (0)	0 (0)	NS
Infant characteristics				
Gestational age (weeks)	40.0 (1.1)	40.0 (1.0)	40.0 (1.1)	NS
No (%) of males	83 (50)	98 (51)	73 (43)	NS
Birth weight (g)	3523 (483)	3603 (454)	3633 (464)	NS
Birth length (cm)	50.7 (1.9)	50.6 (1.8)	50.9 (1.9)	NS
Head circumference (cm)	34.7 (1.3)	35.1 (1.3)	35.0 (1.4)	NS
Umbilical cord haemoglobin (g/L)	163 (15)	153 (20)	158 (18)	<0.001*
Umbilical cord haematocrit (%)	48.7 (4.3)	47.0 (5.5)	47.1 (5.0)	0.005**

Values are means (SD) unless stated otherwise. P<0.05 was considered significant.
NS=non-significant; HC= Historical cohort.
*Comparison of 10s and 60s. For 180s and 60s, P=0.063.
**Comparison of 10s and 60s. For 180s and 60s, P>0.99.

TABLE 2. Outcome measures at the age of four months in infants whose umbilical cord were clamped after 10s, 60s or 180s

	Umbilical cord clamping time			60s vs 10s		60s vs 180s	
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	Mean difference (95% CI)	P	Mean difference (95% CI)	P
Infant age (days)	122 (6)	126 (7)	122 (6)	5 (3 to 6)	<0.001	4 (3 to 6)	<0.001
Main outcome measures							
Geometric mean (range) ferritin (µg/L)	80 (33 to 191)	96 (44 to 208)	117 (58 to 232)	0.83 (0.69 to 1.45)*	0.052	1.21 (1.03 to 1.44)*	0.02
Haemoglobin (g/L)	113 (7)	115 (8)	113 (8)	2 (0 to 4)	0.047	2 (0 to 4)	0.03
Blood count							
Haematocrit (%)	33.0 (2.0)	33.3 (2.2)	32.7 (2.1)	0.3 (-0.2 to 0.8)	0.19	1 (0 to 1)	0.02
Mean cell volume (fL)	77.9 (3.1)	77.7 (3.7)	79.2 (3.0)	-0.2 (-1 to 0.6)	0.67	-1.5 (-2.3 to -0.7)	<0.001
Reticulocyte count ($x10^9$/L)	37 (11)	36 (11)	40 (11)	-1 (-4 to 1)	0.39	-4 (-6 to -1)	0.01
Iron status							
Transferrin receptor (mg/L)	4.00 (0.80)	4.07 (0.92)	3.71 (0.69)	0.07 (-0.13 to 0.26)	0.51	0.35 (0.17 to 0.54)	<0.001
Transferrin saturation (%)	16 (6)	16 (8)	18 (6)	1 (-1 to 2)	0.40	-2 (-3 to 0)	0.03

Values are means (SD) unless stated otherwise. HC= Historical cohort.
*Geometric mean ratio (95% CI for geometric mean).
Successful analyses in the 60s and 180s groups: haemoglobin (n=140, 144), haematocrit, mean cell volume, reticulocyte count (n=135, 144), ferritin (n=147, 148), transferrin receptor (n=147, 148), transferrin saturation (n=145, 148).

TABLE 3. Outcome measures at the age of four months adjusted for infant age

	Umbilical cord clamping time Adjusted means (95% CI)			P	P
	10s n=152 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Main outcome measures					
Geometric mean (range) ferritin (µg/L)	77 (68 to 87)	103 (90 to 117)	114 (100 to 129)	0.002	0.29
Haemoglobin (g/L)	113 (112 to 115)	114 (113 to 116)	113 (112 to 114)	0.30	0.17
Blood count					
Haematocrit (%)	33.1 (32.7 to 33.4)	33.1 (32.8 to 33.5)	32.7 (32.4 to 33.1)	0.30	0.12
Mean cell volume (fL)	77.8 (77.3 to 78.3)	77.9 (77.3 to 78.4)	79.1 (78.6 to 79.6)	0.30	0.003
Reticulocyte count ($x10^9$/L)	36.9 (35.2 to 38.7)	37.0 (35.0 to 38.9)	39.4 (37.6 to 41.2)	0.99	0.07
Iron status					
Transferrin receptor (mg/L)	4.01 (3.87 to 4.14)	4.05 (3.92 to 4.19)	3.72 (3.59 to 3.85)	0.62	0.001
Transferrin saturation (%)	15.7 (14.7 to 16.7)	16.6 (15.5 to 17.7)	18.1 (17 to 19.2)	0.25	0.06

Values are means (95% CI) adjusted for infant age (days from birth) unless stated otherwise.
HC= Historical cohort. Successful analyses for the 60s group were: ferritin (n=147), haemoglobin (n=140), haematocrit, mean cell volume, reticulocyte count (n=135), transferrin receptor, and transferrin saturation (n=145). For the 180s group; blood status (n=144).

TABLE 4. Infants with iron status indicators outside reference limits at the age of 4 months according to umbilical cord clamping time

	Umbilical cord clamping time			P	P
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Ferritin <20 µg/L	11 (7.2)	4 (2.7)	0 (0)	0.11	0.06
Anaemia (Hb<105 g/L)	20 (13.1)	16 (11.4)	20 (13.9)	0.72	0.60
Mean cell volume <73 fL	8 (5.2)	14 (10.4)	3 (2.1)	0.12	0.01
Transferrin saturation <10%	22 (14.4)	15 (10.3)	8 (5.4)	0.38	0.13
Transferrin receptor >7 mg/L	0 (0)	2 (1.4)	0 (0)	0.24	0.25
Iron deficiency*	8 (5.2)	7 (4.8)	1 (0.7)	1.00	0.04

Values are unadjusted numbers (%). HC= Historical cohort.
Numbers of successful analyses: mean cell volume (n=153, 135, 144), transferrin saturation (n=153, 145, 148), anaemia (n=153, 140, 144).

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FIGURE LEGENDS

FIGURE 1. Flow chart of the study populations.

FIGURE 2. Differences in infants' iron status at the age of 4 months according to sex in three groups divided according to umbilical cord clamping time. **A:** Geometric mean ferritin according to sex and time for umbilical cord clamping, **B**: Proportion of children with iron deficiency according to time for umbilical cord clamping and sex.

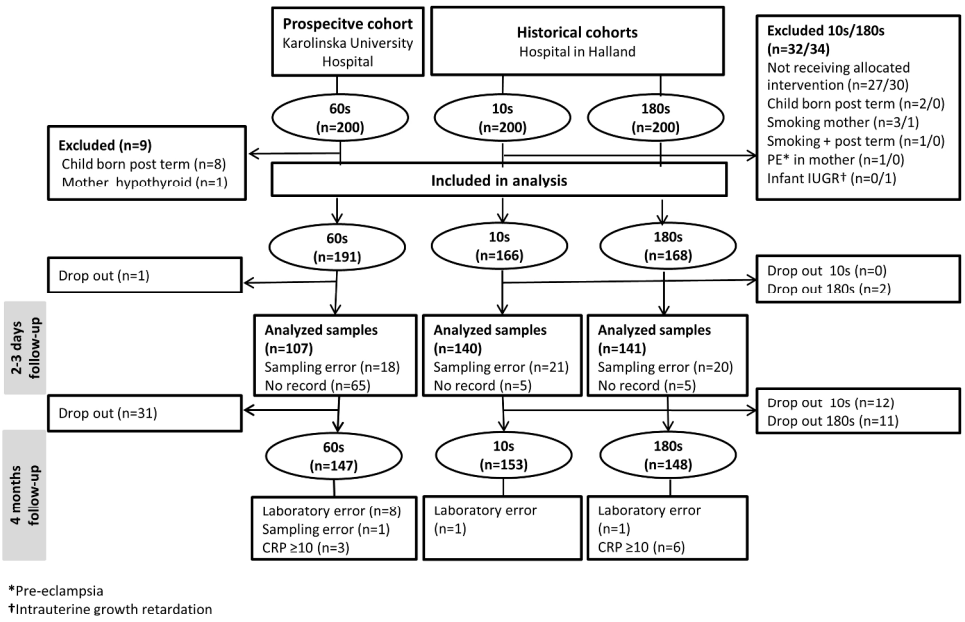

FIGURE 1. Flow chart of the study populations.

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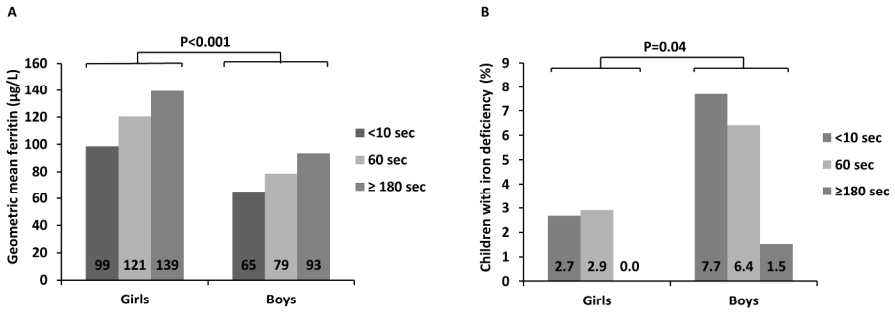

FIGURE 2. Differences in infants' iron status at the age of 4 months according to sex in three groups divided according to umbilical cord clamping time. A: Geometric mean ferritin according to sex and time for umbilical cord clamping, B: Proportion of children with iron deficiency according to time for umbilical cord clamping and sex.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4, 5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	Not applicable
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4, 5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5, 6
Bias	9	Describe any efforts to address potential sources of bias	6, 10
Study size	10	Explain how the study size was arrived at	4, 5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	Not applicable
		(*c*) Explain how missing data were addressed	6, 7
		(*d*) If applicable, explain how loss to follow-up was addressed	6, 7
		(*e*) Describe any sensitivity analyses	Not applicable
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Fig 1, 6, 7
		(b) Give reasons for non-participation at each stage	Fig 1
		(c) Consider use of a flow diagram	Fig 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4, 5
		(b) Indicate number of participants with missing data for each variable of interest	Tables 1-4
		(c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	Not applicable
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7
		(*b*) Report category boundaries when continuous variables were categorized	Not applicable
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Fig 2
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9, 11
Generalisability	21	Discuss the generalisability (external validity) of the study results	9, 10
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Wait a minute? An observational cohort study comparing iron stores in healthy Swedish infants at four months of age after 10, 60 and 180 seconds' umbilical cord clamping

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Wait a minute? An observational cohort study comparing iron stores in healthy Swedish infants at four months of age after 10, 60 and 180 seconds' umbilical cord clamping

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Data Sharing Statement: There are no other unpublished data available.

Clinical Trial Registration: Clinicaltrial.gov NCT01245296.

Ethics Approval: By the Regional Ethical Review Board in Stockholm (2011/2142-31/3) and the Regional Ethical Review Board at Lund University (41/2008).

Abbreviations: ANOVA- analysis of variance, CI – confidence interval, CRP – C reactive protein, ID - iron deficiency, MCV - mean cell volume, NS – non significant, RCT - randomized controlled trial, s – seconds, SD – standard deviation, UC – umbilical cord, UCB – umbilical cord blood.

Abstract

BACKGROUND AND OBJECTIVE: Umbilical cord blood (UCB) is a valuable stem cell source used for transplantation. Immediate umbilical cord (UC) clamping is widely practiced but delayed UC clamping is increasingly advocated to reduce possible infant anaemia. The aim of this study was to investigate an intermediate UC clamping time point and to evaluate iron status at the age of four months in infants who had the UC clamped after 60 seconds (s) and compare the results to immediate and late UC clamping.

DESIGN: Prospective observational study with two historical controls.

SETTING: A university hospital in Stockholm, Sweden and a county hospital in Halland, Sweden.

METHOD: Iron status was assessed at four months in 200 prospectively recruited term infants whose UC was clamped 60s after birth. The results were compared to data from a previously conducted randomized controlled trial including infants subjected to UC clamping at ≤10 s (n=200) or ≥180 s (n=200) after delivery.

RESULTS: After adjustment for age differences at the time of follow-up, serum ferritin concentrations were 77 µg/L, 103 µg/L and 114 µg/L in the 10s, 60s and 180s groups, respectively. The adjusted ferritin concentration was significantly higher in the 60s group compared to the 10s group (P=0.002) while the difference between the 60s and 180s groups was not significant (P=0.29).

CONCLUSION: In this study of healthy term infants, 60s UC clamping, resulted in higher serum ferritin concentrations at four months compared to 10s UC clamping. The results suggest that delaying the UC clamping for 60s reduces the risk for iron deficiency.

Strengths and limitations of the study

- This study compares iron- and blood status at the age of four months in children whose UC were clamped after 60s with children whose UC were clamped ≤10s or ≥180s.
- In this study we focus on potential consequences for the child caused by different UC clamping times, and not the quality of the collected UCB.
- This prospective observational study uses historical controls and results should be interpreted with caution because of potential confounding bias.
- We did not use a non-inferiority approach for this study since we aimed to compare results after 60s UC clamping with both 10s- and 180s UC clamping.

INTRODUCTION

During the last decades, immediate the umbilical cord (UC) clamping has been part of active management of labour with the aim to reduce maternal haemorrhage at birth [1, 2]. Hence, the placenta and umbilical cord blood (UCB), have been regarded as waste products following birth. However, immediate UC clamping may deprive infants of up to one third of the total fetoplacental blood volume [3, 4], and increase the risk of iron deficiency (ID) in the first 3-6 months of life [5-9]. Iron deficiency is associated with impaired neurodevelopment affecting cognitive, motor, and behavioural skills in young children [10-12]. For this reason the World Health Organization recommends delayed UC clamping for 1-3 minutes after birth [13].

On the other side, UCB is a valuable stem cell source for the 30-50% of patients that need a hematopoietic stem cell transplantation, but who lack a suitable family or public registry donor [14, 15]. Therefore, UCB is collected and stored in altruistic and private UCB banks. The earlier the UC is clamped, the more blood is left to collect from the placenta and for this reason immediate UC clamping is normally practiced in UCB collection. More than 35.000 transplantations with UCB have been performed worldwide [14].

We hypothesized that if the UC is clamped at 60 seconds (s) after birth, a sufficient amount of blood will have passed from the placenta to the infant to reduce the risk of ID and anaemia while still allowing collection of UCB for banking [16]. The objective of the current study was to evaluate iron status and complete blood counts at the age of four months in infants who had the UC clamped after 60s and compare the results to immediate (≤10s) and late (≥180s) UC clamping. In this study we focused on potential consequences for the donors only.

MATERIALS AND METHODS

We performed an observational cohort study using one prospective clinical cohort and data from a previously performed randomized controlled trial (RCT) [6]. The prospectively recruited infants were born at Karolinska University Hospital Huddinge, Sweden between April 2012 and May 2015, to parents who agreed to donate UCB to the Swedish National Umbilical Cord Blood Bank, a public bank without profit interest. The UC was clamped after 60 ±10s. The historical cohorts consisted of data from infants who were randomized to immediate (within 10s after birth) or late (after 180s) UC clamping, respectively, in a trial conducted by three of the co-authors at the Hospital of Halland in Halmstad, Sweden between April 2008 and September 2009 [6].

Outcome measures

The primary outcome variable was ferritin as a measure of iron stores, at the age of four months and secondary outcomes included haemoglobin, transferrin saturation, soluble transferrin receptors, mean cell volume (MCV) as well as ID (defined as ≥2 iron indicators outside reference range with the following cut-off values: ferritin <20 µg/L, [17] MCV <73 fL, [17] transferrin saturation <10% [18] and transferrin receptor >7 mg/L [6]). Since ferritin is an acute phase reactant, C reactive protein (CRP) was analysed in order to exclude falsely elevated ferritin measurements. The infant's dietary habits at four months were also considered as well as the relevance of infant sex for iron and blood status at that age.

Sample size

A formal power calculation was not performed for the current study but the sample size was based on the group size in the original RCT by Andersson *et al*. 2011 (the historical cohorts) [6]. The power calculation by Andersson *et al.* showed that a group size of 200 would enable detection of a 29% difference in geometric mean serum ferritin between 10s and 180s UC clamping with a power of 80% and a significance level of 0.05, assuming a mean serum ferritin concentration of 110 µg/L in the180s-group while allowing an attrition of 25% [6]. Based on the results from Andersson *et al.*,

demonstrating a 45% difference in ferritin concentration between 10s and 180s UC clamping, we decided to include 200 children also in the prospective group.

Study population and inclusion criteria

The same inclusion criteria applied for the prospective and historical cohorts: healthy mother, normal, singleton pregnancy, gestational age 37+0 to 41+6 weeks and planned vaginal delivery with cephalic presentation [6]. The mother also had to understand Swedish well enough to consent participation in the study. In the prospectively recruited cohort, parents also had to agree to altruistic donation of UCB, and to the modified UC clamping strategy. Exclusion criteria were also the same in the three groups: smoking mother, serious congenital malformations, syndromes, or other congenial diseases that could affect the outcome measures. For this study, we only included data from the RCT that were handled according to per protocol, as compared to the analysis by intention to treat in the original RCT [6]. Moreover, like the RCT, we only included children with CRP<10 in the analysis. All parents donating UCB at Karolinska University Hospital Huddinge that met the inclusion criteria were offered to participate in the study upon arrival at the delivery unit. Midwives were instructed to clamp the UC 60s after birth in deliveries where parents had consented to participate in the study. Staff in charge of collection of UCB measured the exact timing of UC clamping using a digital timer. Only children whose UC were clamped at 60 ±10s were included in the study. Oral and written informed consent was obtained from one or both of the parents. The study was approved by the regional ethical review board in Stockholm (2011/2142-31/3) and the RCT was approved by the regional ethical review board at Lund University (41/2008).

Data collection and blood samples

Midwives were instructed to hold the new-born baby below the uterine level for the first 30s before placing the infant on the mother's abdomen, in accordance with the study by Andersson *et al.* 2011 [6]. The UC clamping technique used in the prospectively recruited cohort was similar to the historical groups except for the timing. Venous blood samples were analysed at birth from the UC, at two to three days of age in connection with metabolic screening and at four months (±21 days) at a

scheduled follow-up visit. At birth and at four months, serum ferritin, haemoglobin, haematocrit, MCV, reticulocyte count, transferrin saturation, transferrin receptor and CRP were analysed and at two to three days of age we analysed haemoglobin, haematocrit and bilirubin. EDTA tubes were used for blood count and serum separator tubes for iron status, bilirubin and CRP (Microvette, Sarstedt AG & Co, Nümbrecht, Germany). Blood status was analysed using equal methods and equipment in both the laboratory at Karolinska University Hospital (Sysmex XE5000, Sysmex, Kobe, Japan) and at the Hospital of Halland (Sysmex XE2100, Sysmex, Kobe, Japan). Analysis of iron status and CRP could be performed at the same laboratory as in the RCT [6], and therefore the blood samples collected at Karolinska University Hospital were centrifuged and the serum kept at -70 degrees Celsius before sent for analysis at the Clinical Chemistry Laboratory at the Hospital of Halland. The samples were then thawed and analysed using a Cobas 6000 (Roche Diagnostics, Basel, Switzerland), the same method as in the RCT [6].

Statistical analysis

Analyses on the two historical cohorts, 10s and 180s, were calculated only on infants receiving intervention per protocol in order to obtain clearly defined groups according to time of UC clamping. Baseline characteristics were compared across groups using analysis of variance (ANOVA). Means of outcome measures in the three cohorts were compared using the Bonferroni method. We also performed pairwise comparisons (60s vs 10s and 60s vs 180s) using Student's t-test for variables with normal distribution that were statistically significant in the Bonferroni analyses. Ferritin concentration was log10 transformed for analysis because of skewed distribution and the results were presented as geometric means. Categorical variables were compared between groups using Fisher's Exact test for pairwise comparisons. Adjusted group means were compared using ANOVA and a binary logistic regression model was used to adjust for sex and age in the evaluation of ID at four months. A P-value less than 0.05 were considered significant. No imputation of missing data was performed. Loss to follow-up was registered, but not examined further. IBM SPSS for Windows, version 22 was used for the analyses.

RESULTS

Of the 200 prospectively recruited infants, 191 were included in the analysis as compared to 166 in the 10s and 168 in the 180s groups. Infants lost to follow up or excluded due to not fulfilling inclusion criteria are shown in Fig 1.

Baseline data

There were no significant differences in the 60s group as compared to the 10s and 180s groups with regard to maternal or neonatal characteristics. The 10s group had higher UC haemoglobin (163 vs 153 g/L, P<0.001) and higher UC haematocrit (48.7 vs 47.0%, P=0.005) than the 60s group. There were no significant differences between the 60s and the 180s groups (Table 1).

Two to three day-follow-up

At two to three days, blood samples were obtained from 140 infants in the 10s group, 107 in the 60s group, and 141 in the 180s group (Figure 1). The prevalence of anaemia was higher in the 10s compared to the 60s and 180s groups (9 (6.4%), 2 (1.9%) and 2 (1.4%)) respectively (P=0.04). Haemoglobin was lower in the 10s and 60s groups compared to the 180s group; 175, 180 and 189 g/L respectively (P=0.001 for 60s vs 180s). Bilirubin did not differ between the groups. Anaemic children were referred for further diagnostics but none needed treatment.

Primary outcome

In total 468 (78%) infants had follow up at four months (children excluded or lost to follow up are shown in Figure 1). In the 60s group, the infants (n=147) were four days older than the historical groups from the RCT (126±7 days vs 122±6 days, P<0.001). The geometric mean ferritin levels were 80, 96 and 117 µg/L respectively in the 10s, 60s and 180s groups (P=0.052 for 10s vs 60s; P=0.02 for 60s vs 180s) (Table 2). After adjustment for the age difference, the geometric means were 77, 103 and 114 µg/L respectively (P=0.002 for 10s vs 60s; P=0.29 for 60s vs 180s) (Table 3).

Secondary outcomes

All secondary outcome variables are shown in Table 2. Haemoglobin levels at four months were higher in the 60s group compared to 10s and 180s, but the difference did not remain in the age

adjusted model (Table 3). There were no statistically significant differences in the prevalence of anaemia in the 60s group as compared to the other two groups (Table 4). After adjustment for age at the time of follow-up, there were no differences between the 10s and 60s groups in haematocrit, MCV, reticulocyte count, transferrin receptor or transferrin saturation. The 60s group had significantly lower adjusted MCV and higher adjusted transferrin receptor compared to the 180s group, but there were no differences in haematocrit, reticulocyte count or transferrin saturation (Table 3). The prevalence of ID was 8 (5.2%), 7 (4.8%) and 1 (0.7%) respectively in the 10s, 60s and 180s groups (P>0.99 for 10s vs 60s and P=0.04 for 60s vs 180s) (Table 4). After statistical adjustments for sex and age at the time of follow-up, the prevalence of ID did not differ between the 60s group and the two other groups. The proportion of infants that were exclusively breast fed at four months did not differ between the groups (79 (52%), 92 (58%) and 89 (56%) respectively in the 10s, 60s and 180s groups. The impact of exclusive breast feeding on ferritin concentration and ID were further assessed in an adjusted model but no correlation was found (data not shown).

Sex differences

Iron and blood status at four months were further assessed by analysing differences according to infant sex. When data from the three cohorts were analysed together, geometric mean ferritin was lower in boys than in girls and ID was more prevalent amongst boys (Fig 2A and 2B). Haemoglobin concentrations did not differ significantly between boys and girls and neither did the prevalence of anaemia (data not shown).

DISCUSSION

We compared infants' iron and blood status at the age of four months after three different UC clamping time intervals (60s versus ≤10s, and ≥180s, respectively). Previous studies have compared immediate and late UC clamping but the definition of "late" in these studies varies from 60s to 180s or after birth of the placenta [5, 7, 8]. The current study evaluates an intermediate UC clamping strategy that can be used in connection with altruistic donation of UCB for clinical banking. When asking parents to donate their child's UCB for the potential good of others, it is essential to inform of

possible consequences for the child. We demonstrated that after adjustment for age difference at the time of follow-up, there were no statistically significant differences in ferritin concentration between the 60s and 180s groups but the 60s group had significantly higher ferritin concentrations compared to 10s. The main reason for the effect of age on ferritin during infancy is rapid growth which leads to a rapidly expanding blood volume and depletion of iron stores [9].

Results from the present study are in accordance with a Brazilian RCT that demonstrated higher ferritin concentration at the age of three months in children whose UC were clamped after 60s compared to immediate UC clamping [19]. However, our findings are in contrast to a study by Cernadas *et al.*, who reported no differences in ferritin six months after birth comparing 15s and 60s UC clamping. The difference in ferritin between the 60s and 180s groups were not presented [20]. The present study indicates that a 60s delay before UC clamping results in a mean serum ferritin at 4 months of age corresponding to 90% of the mean ferritin after 180s UC clamping.

In the current study, children in the 60s group had the lowest haemoglobin of all three groups at birth (10s 163 g/L, 60s 153 g/L, 180s 158 g/L) but at four months, there were no longer any differences between the groups in our adjusted model. The original RCT as well as a Cochrane report found higher concentrations of haemoglobin after immediate than late UC clamping at birth [6, 8]. As speculated in a previous publication, this difference may not reflect true difference in haemoglobin but could be a result of trans endothelial plasma leakage in the UC after UC clamping [6].

The prevalence of ID was higher in the 10s and 60s groups compared to the 180s group. However, when adjusting for sex and age at the time of follow-up, there were no longer any statistically significant differences between the three groups. In accordance with previous studies on ID in infancy, we found that at four months, boys had significantly lower geometric mean ferritin and more often ID than girls [21, 22]. Nevertheless, the aim of the study was not to compare differences according to sex, and the groups were therefor too small to draw any reliable conclusions of sex and ID. It has been suggested that the differences in iron status between the two sexes may be hormone-related but it is also influenced by genetic factors, which may differ in populations with different

ethnicity [22]. Also, when considering the generalisability of this study, readers should bear in mind that all children were placed below the uterine level for 30s directly after birth. This may be in contrast to the practice in other delivery settings, where the children are often placed directly on the woman's abdomen, which may affect the speed of the placental transfusion.

Limitations of the study

A limitation of this study is that we have no record of the parents' ethnic or socio-economic background, and we were therefore unable to draw any conclusions on possible impact of these factors. When comparing different interventions, RCTs are regarded as the golden standard. However, randomization into 10s UC clamping for this study was considered unethical since there is evidence that children benefit from prolonged placental transfusion for at least 60s, and the clinical routine in Sweden is to wait 180s before UC clamping in normal deliveries. We used the historical cohorts from the RCT in Halmstad, Sweden as controls in this study. The prospectively recruited cohort (60s) is from a large birth clinic in Stockholm, Sweden while the controls are from a smaller town where the participants were recruited two to five years earlier. Although the inclusion criteria were the same and pregnant mothers in Sweden follow the same well-controlled antenatal program and the baseline data showed no significant differences in maternal or infant characteristics, it is difficult to know if the study populations are homogenous. We did not use a non-inferiority approach for this study since we aimed to compare results after 60s UC clamping with10s and 180s. Nor did we perform a power calculation when designing the study, but included an equally large group as the two historical groups. This is a weakness which, should be taken into consideration. We have taken measures to avoid systematic biases caused by differences in laboratory routines between the two hospitals by using the same laboratory for analysing the iron status variables as well as CRP. Collection of UCB for transplantation is a growing and life-saving practice. Tens of thousands UCB units are altruistically donated and stored for public use each year, and as a result new-borns are exposed to limited UC clamping time. The idea that UCB left in the placenta after birth is a mere waste that is thrown away if not collected is obsolete. Optimizing UC clamping time and investigating

the clinical significance to minimize risks for the infant while still enabling collection of UCB for transplantation is a great, but necessary, challenge. A study evaluating volume and TNC in UCB units collected at the Swedish National Umbilical Cord Blood Bank showed that 37 % of units collected after 60s UC clamping met the banking criteria compared to 47 % after immediate UC clamping [16]. This is information prospective parents considering donating UCB should receive.

CONCLUSION

In this observational study, significantly more children in the 10s UC clamping group had low iron stores at four months compared to children in the 60s and 180s UC clamping groups. After adjustment for age and sex, there were no statistically significant differences in ferritin concentration between 60 and 180s UC clamping. 60s UC clamping is therefore preferable to immediate UC clamping. Delaying UC clamping may be more important in boys than in girls to avoid ID in early childhood. Larger studies with long term follow-up is needed to establish the clinical relevance of different UC clamping strategies.

CONTRIBUTORS

Dr C. Götherström conceptualized and designed the study, analyzed data, critically revised and approved the final manuscript as submitted. Mrs U. Askelöf conceptualized and designed the study, carried out all data collection in the prospective cohort, carried out all analyses, drafted the initial manuscript, and approved the final manuscript as submitted. Dr O. Andersson designed the data collection instruments, contributed to the study design and provided data for the historical cohorts. He also carried out the initial analyses, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. Profs M. Domellöf, A. Fasth, L. Hellström-Westas, M. Westgren, Associate Profs K. Pettersson and I. Wiklund and Dr B. Hallberg participated in the study design, reviewed and revised the manuscript and approved the final manuscript as submitted.

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TABLE 1. Baseline characteristics of mothers and infants according to time for umbilical cord clamping

	Umbilical cord clamping time			P
	10s n=166 (HC)	60s n=191	180s n=168 (HC)	
Maternal characteristics				
Age (years)	31.7 (4.2)	30.7 (4.7)	30.8 (4.9)	NS
Parity (including study child)	1.81 (0.87)	1.86 (0.76)	1.76 (0.74)	NS
Weight at first antenatal visit (kg)	66.4 (12.0)	67.3 (11.5)	67.3 (12.1)	NS
Body mass index	23.6 (3.8)	24.5 (3.9)	23.9 (3.6)	NS
Haemoglobin at first antenatal visit (g/L)	128 (9)	128 (10)	128 (11)	NS
No (%) of vaginal deliveries:				
Non-instrumental	157 (95)	184 (96)	157 (94)	NS
Vacuum extraction	8 (5)	7 (4)	11 (7)	NS
Forceps	1 (0.6)	0 (0)	0 (0)	NS
Infant characteristics				
Gestational age (weeks)	40.0 (1.1)	40.0 (1.0)	40.0 (1.1)	NS
No (%) of males	83 (50)	98 (51)	73 (43)	NS
Birth weight (g)	3523 (483)	3603 (454)	3633 (464)	NS
Birth length (cm)	50.7 (1.9)	50.6 (1.8)	50.9 (1.9)	NS
Head circumference (cm)	34.7 (1.3)	35.1 (1.3)	35.0 (1.4)	NS
Umbilical cord haemoglobin (g/L)	163 (15)	153 (20)	158 (18)	<0.001*
Umbilical cord haematocrit (%)	48.7 (4.3)	47.0 (5.5)	47.1 (5.0)	0.005**

Values are means (SD) unless stated otherwise. P<0.05 was considered significant.
NS=non-significant; HC= Historical cohort.
*Comparison of 10s and 60s. For 180s and 60s, P=0.063.
**Comparison of 10s and 60s. For 180s and 60s, P>0.99.

TABLE 2. Outcome measures at the age of four months in infants whose umbilical cord were clamped after 10s, 60s or 180s

	Umbilical cord clamping time			60s vs 10s		60s vs 180s	
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	Mean difference (95% CI)	P	Mean difference (95% CI)	P
Infant age (days)	122 (6)	126 (7)	122 (6)	5 (3 to 6)	<0.001	4 (3 to 6)	<0.001
Main outcome measures							
Geometric mean (range) ferritin (µg/L)	80 (33 to 191)	96 (44 to 208)	117 (58 to 232)	0.83 (0.69 to 1.45)*	0.052	1.21 (1.03 to 1.44)*	0.02
Haemoglobin (g/L)	113 (7)	115 (8)	113 (8)	2 (0 to 4)	0.047	2 (0 to 4)	0.03
Blood count							
Haematocrit (%)	33.0 (2.0)	33.3 (2.2)	32.7 (2.1)	0.3 (-0.2 to 0.8)	0.19	1 (0 to 1)	0.02
Mean cell volume (fL)	77.9 (3.1)	77.7 (3.7)	79.2 (3.0)	-0.2 (-1 to 0.6)	0.67	-1.5 (-2.3 to -0.7)	<0.001
Reticulocyte count ($x10^9$/L)	37 (11)	36 (11)	40 (11)	-1 (-4 to 1)	0.39	-4 (-6 to -1)	0.01
Iron status							
Transferrin receptor (mg/L)	4.00 (0.80)	4.07 (0.92)	3.71 (0.69)	0.07 (-0.13 to 0.26)	0.51	0.35 (0.17 to 0.54)	<0.001
Transferrin saturation (%)	16 (6)	16 (8)	18 (6)	1 (-1 to 2)	0.40	-2 (-3 to 0)	0.03

Values are means (SD) unless stated otherwise. HC= Historical cohort.
*Geometric mean ratio (95% CI for geometric mean).
Successful analyses in the 60s and 180s groups: haemoglobin (n=140, 144), haematocrit, mean cell volume, reticulocyte count (n=135, 144), ferritin (n=147, 148), transferrin receptor (n=147, 148), transferrin saturation (n=145, 148).

TABLE 3. Outcome measures at the age of four months adjusted for infant age

	Umbilical cord clamping time Adjusted means (95% CI)			P	P
	10s n=152 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Main outcome measures					
Geometric mean (range) ferritin (µg/L)	77 (68 to 87)	103 (90 to 117)	114 (100 to 129)	0.002	0.29
Haemoglobin (g/L)	113 (112 to 115)	114 (113 to 116)	113 (112 to 114)	0.30	0.17
Blood count					
Haematocrit (%)	33.1 (32.7 to 33.4)	33.1 (32.8 to 33.5)	32.7 (32.4 to 33.1)	0.30	0.12
Mean cell volume (fL)	77.8 (77.3 to 78.3)	77.9 (77.3 to 78.4)	79.1 (78.6 to 79.6)	0.30	0.003
Reticulocyte count ($x10^9$/L)	36.9 (35.2 to 38.7)	37.0 (35.0 to 38.9)	39.4 (37.6 to 41.2)	0.99	0.07
Iron status					
Transferrin receptor (mg/L)	4.01 (3.87 to 4.14)	4.05 (3.92 to 4.19)	3.72 (3.59 to 3.85)	0.62	0.001
Transferrin saturation (%)	15.7 (14.7 to 16.7)	16.6 (15.5 to 17.7)	18.1 (17 to 19.2)	0.25	0.06

Values are means (95% CI) adjusted for infant age (days from birth) unless stated otherwise.
HC= Historical cohort. Successful analyses for the 60s group were: ferritin (n=147), haemoglobin (n=140), haematocrit, mean cell volume, reticulocyte count (n=135), transferrin receptor, and transferrin saturation (n=145). For the 180s group; blood status (n=144).

TABLE 4. Infants with iron status indicators outside reference limits at the age of 4 months according to umbilical cord clamping time

	Umbilical cord clamping time			P	P
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Ferritin <20 µg/L	11 (7.2)	4 (2.7)	0 (0)	0.11	0.06
Anaemia (Hb<105 g/L)	20 (13.1)	16 (11.4)	20 (13.9)	0.72	0.60
Mean cell volume <73 fL	8 (5.2)	14 (10.4)	3 (2.1)	0.12	0.01
Transferrin saturation <10%	22 (14.4)	15 (10.3)	8 (5.4)	0.38	0.13
Transferrin receptor >7 mg/L	0 (0)	2 (1.4)	0 (0)	0.24	0.25
Iron deficiency*	8 (5.2)	7 (4.8)	1 (0.7)	1.00	0.04

Values are unadjusted numbers (%). HC= Historical cohort.
Numbers of successful analyses: mean cell volume (n=153, 135, 144), transferrin saturation (n=153, 145, 148), anaemia (n=153, 140, 144).

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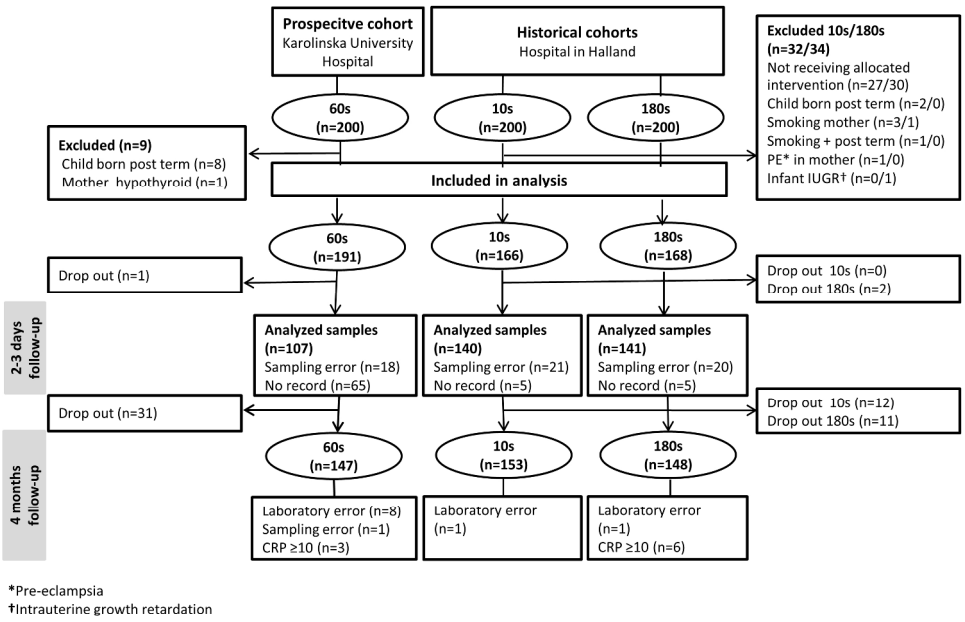
FIGURE LEGENDS

FIGURE 1. Flow chart of the study populations.

FIGURE 2. Differences in infants' iron status at the age of 4 months according to sex in three groups divided according to umbilical cord clamping time. **A:** Geometric mean ferritin according to sex and time for umbilical cord clamping, **B**: Proportion of children with iron deficiency according to time for umbilical cord clamping and sex.



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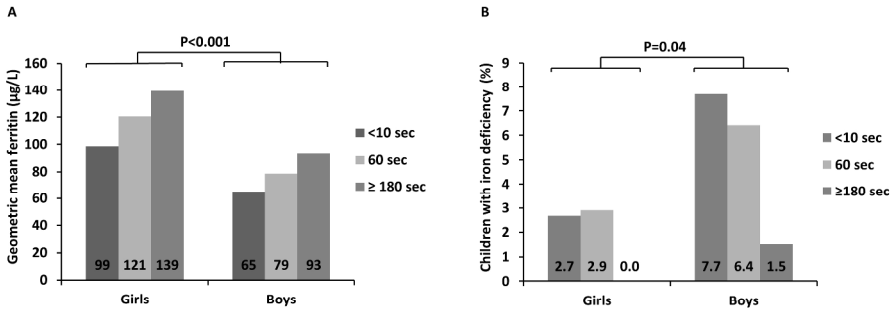

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254x190mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4, 5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	Not applicable
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4, 5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5, 6
Bias	9	Describe any efforts to address potential sources of bias	6, 10
Study size	10	Explain how the study size was arrived at	4, 5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	Not applicable
		(*c*) Explain how missing data were addressed	6, 7
		(*d*) If applicable, explain how loss to follow-up was addressed	6, 7
		(*e*) Describe any sensitivity analyses	Not applicable
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Fig 1, 6, 7
		(b) Give reasons for non-participation at each stage	Fig 1
		(c) Consider use of a flow diagram	Fig 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4, 5
		(b) Indicate number of participants with missing data for each variable of interest	Tables 1-4
		(c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	Not applicable
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7
		(*b*) Report category boundaries when continuous variables were categorized	Not applicable
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Fig 2
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9, 11
Generalisability	21	Discuss the generalisability (external validity) of the study results	9, 10
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Wait a minute? An observational cohort study comparing iron stores in healthy Swedish infants at four months of age after 10, 60 and 180 seconds' umbilical cord clamping

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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Paediatrics, Haematology (incl blood transfusion)
Keywords:	PAEDIATRICS, UMBILICAL CORD CLAMPING, IRON DEFICIENCY, Fetal medicine < OBSTETRICS

Wait a minute? An observational cohort study comparing iron stores in healthy Swedish infants at four months of age after 10, 60 and 180 seconds' umbilical cord clamping

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Conflict of Interest: The authors have no conflicts of interest relevant to this article to disclose.

Data Sharing Statement: There are no other unpublished data available.

Clinical Trial Registration: Clinicaltrial.gov NCT01245296.

Ethics Approval: By the Regional Ethical Review Board in Stockholm (2011/2142-31/3) and the Regional Ethical Review Board at Lund University (41/2008).

Abbreviations: ANOVA- analysis of variance, CI – confidence interval, CRP – C reactive protein, ID - iron deficiency, MCV - mean cell volume, NS – non significant, RCT - randomized controlled trial, s – seconds, SD – standard deviation, UC – umbilical cord, UCB – umbilical cord blood.

Abstract

BACKGROUND AND OBJECTIVE: Umbilical cord blood (UCB) is a valuable stem cell source used for transplantation. Immediate umbilical cord (UC) clamping is widely practiced but delayed UC clamping is increasingly advocated to reduce possible infant anaemia. The aim of this study was to investigate an intermediate UC clamping time point and to evaluate iron status at the age of four months in infants who had the UC clamped after 60 seconds (s) and compare the results to immediate and late UC clamping.

DESIGN: Prospective observational study with two historical controls.

SETTING: A university hospital in Stockholm, Sweden and a county hospital in Halland, Sweden.

METHOD: Iron status was assessed at four months in 200 prospectively recruited term infants whose UC was clamped 60s after birth. The new-born baby was held below the uterine level for the first 30s before placing the infant on the mother's abdomen for additional 30s. The results were compared to data from a previously conducted randomized controlled trial including infants subjected to UC clamping at ≤10s (n=200) or ≥180s (n=200) after delivery.

RESULTS: After adjustment for age differences at the time of follow-up, serum ferritin concentrations were 77 µg/L, 103 µg/L and 114 µg/L in the 10s, 60s and 180s groups, respectively. The adjusted ferritin concentration was significantly higher in the 60s group compared to the 10s group (P=0.002) while the difference between the 60s and 180s groups was not significant (P=0.29).

CONCLUSION: In this study of healthy term infants, 60s UC clamping with 30s lowering of the baby below the uterine level, resulted in higher serum ferritin concentrations at four months compared to 10s UC clamping. The results suggest that delaying the UC clamping for 60s reduces the risk for iron deficiency.

Strengths and limitations of the study

- This study compares iron- and blood status at the age of four months in children whose UC were clamped after 60s with children whose UC were clamped ≤10s or ≥180s.
- In this study we focus on potential consequences for the child caused by different UC clamping times, and not the quality of the collected UCB.
- This prospective observational study uses historical controls and results should be interpreted with caution because of potential confounding bias.
- We did not use a non-inferiority approach for this study since we aimed to compare results after 60s UC clamping with both 10s- and 180s UC clamping.

INTRODUCTION

During the last decades, immediate the umbilical cord (UC) clamping has been part of active management of labour with the aim to reduce maternal haemorrhage at birth [1, 2]. Hence, the placenta and umbilical cord blood (UCB), have been regarded as waste products following birth. However, immediate UC clamping may deprive infants of up to one third of the total fetoplacental blood volume [3, 4], and increase the risk of iron deficiency (ID) in the first 3-6 months of life [5-9]. Iron deficiency is associated with impaired neurodevelopment affecting cognitive, motor, and behavioural skills in young children [10-12]. For this reason the World Health Organization recommends delayed UC clamping for 1-3 minutes after birth [13].

On the other side, UCB is a valuable stem cell source for the 30-50% of patients that need a hematopoietic stem cell transplantation, but who lack a suitable family or public registry donor [14, 15]. Therefore, UCB is collected and stored in altruistic and private UCB banks. The earlier the UC is clamped, the more blood is left to collect from the placenta and for this reason immediate UC clamping is normally practiced in UCB collection. More than 35.000 transplantations with UCB have been performed worldwide [14].

We hypothesized that if the UC is clamped at 60 seconds (s) after birth, a sufficient amount of blood will have passed from the placenta to the infant to reduce the risk of ID and anaemia while still allowing collection of UCB for banking [16]. The objective of the current study was to evaluate iron status and complete blood counts at the age of four months in infants who had the UC clamped after 60s and compare the results to immediate (≤10s) and late (≥180s) UC clamping. In this study we focused on potential consequences for the donors only.

MATERIALS AND METHODS

We performed an observational cohort study using one prospective clinical cohort and data from a previously performed randomized controlled trial (RCT) [6]. The prospectively recruited infants were born at Karolinska University Hospital Huddinge, Sweden between April 2012 and May 2015, to parents who agreed to donate UCB to the Swedish National Umbilical Cord Blood Bank, a public bank without profit interest. The UC was clamped after 60 ±10s. The new-born baby was held below the uterine level for the first 30s before placing the infant on the mother's abdomen for additional 30s. The historical cohorts consisted of data from infants who were randomized to immediate (within 10s after birth) or late (after 180s) UC clamping, respectively, in a trial conducted by three of the co-authors at the Hospital of Halland in Halmstad, Sweden between April 2008 and September 2009 [6].

Outcome measures

The primary outcome variable was ferritin as a measure of iron stores, at the age of four months and secondary outcomes included haemoglobin, transferrin saturation, soluble transferrin receptors, mean cell volume (MCV) as well as ID (defined as ≥2 iron indicators outside reference range with the following cut-off values: ferritin <20 µg/L, [17] MCV <73 fL, [17] transferrin saturation <10% [18] and transferrin receptor >7 mg/L [6]). Since ferritin is an acute phase reactant, C reactive protein (CRP) was analysed in order to exclude falsely elevated ferritin measurements. The infant's dietary habits at four months were also considered as well as the relevance of infant sex for iron and blood status at that age.

Sample size

A formal power calculation was not performed for the current study but the sample size was based on the group size in the original RCT by Andersson *et al*. 2011 (the historical cohorts) [6]. The power calculation by Andersson *et al.* showed that a group size of 200 would enable detection of a 29% difference in geometric mean serum ferritin between 10s and 180s UC clamping with a power of 80% and a significance level of 0.05, assuming a mean serum ferritin concentration of 110 µg/L in the180s-group while allowing an attrition of 25% [6]. Based on the results from Andersson *et al.*,

demonstrating a 45% difference in ferritin concentration between 10s and 180s UC clamping, we decided to include 200 children also in the prospective group.

Study population and inclusion criteria

The same inclusion criteria applied for the prospective and historical cohorts: healthy mother, normal, singleton pregnancy, gestational age 37+0 to 41+6 weeks and planned vaginal delivery with cephalic presentation [6]. The mother also had to understand Swedish well enough to consent participation in the study. In the prospectively recruited cohort, parents also had to agree to altruistic donation of UCB, and to the modified UC clamping strategy. Exclusion criteria were also the same in the three groups: smoking mother, serious congenital malformations, syndromes, or other congenial diseases that could affect the outcome measures. For this study, we only included data from the RCT that were handled according to per protocol, as compared to the analysis by intention to treat in the original RCT [6]. Moreover, like the RCT, we only included children with CRP<10 in the analysis. All parents donating UCB at Karolinska University Hospital Huddinge that met the inclusion criteria were offered to participate in the study upon arrival at the delivery unit. Midwives were instructed to clamp the UC 60s after birth in deliveries where parents had consented to participate in the study. Staff in charge of collection of UCB measured the exact timing of UC clamping using a digital timer. Only children whose UC were clamped at 60 ±10s were included in the study. Oral and written informed consent was obtained from one or both of the parents. The study was approved by the regional ethical review board in Stockholm (2011/2142-31/3) and the RCT was approved by the regional ethical review board at Lund University (41/2008).

Data collection and blood samples

Midwives were instructed to hold the new-born baby below the uterine level for the first 30s before placing the infant on the mother's abdomen, in accordance with the study by Andersson *et al.* 2011 [6]. The UC clamping technique used in the prospectively recruited cohort was similar to the historical groups except for the timing. Venous blood samples were analysed at birth from the UC, at two to three days of age in connection with metabolic screening and at four months (±21 days) at a

scheduled follow-up visit. At birth and at four months, serum ferritin, haemoglobin, haematocrit, MCV, reticulocyte count, transferrin saturation, transferrin receptor and CRP were analysed and at two to three days of age we analysed haemoglobin, haematocrit and bilirubin. EDTA tubes were used for blood count and serum separator tubes for iron status, bilirubin and CRP (Microvette, Sarstedt AG & Co, Nümbrecht, Germany). Blood status was analysed using equal methods and equipment in both the laboratory at Karolinska University Hospital (Sysmex XE5000, Sysmex, Kobe, Japan) and at the Hospital of Halland (Sysmex XE2100, Sysmex, Kobe, Japan). Analysis of iron status and CRP could be performed at the same laboratory as in the RCT [6], and therefore the blood samples collected at Karolinska University Hospital were centrifuged and the serum kept at -70 degrees Celsius before sent for analysis at the Clinical Chemistry Laboratory at the Hospital of Halland. The samples were then thawed and analysed using a Cobas 6000 (Roche Diagnostics, Basel, Switzerland), the same method as in the RCT [6].

Statistical analysis

Analyses on the two historical cohorts, 10s and 180s, were calculated only on infants receiving intervention per protocol in order to obtain clearly defined groups according to time of UC clamping. Baseline characteristics were compared across groups using analysis of variance (ANOVA). Means of outcome measures in the three cohorts were compared using the Bonferroni method. We also performed pairwise comparisons (60s vs 10s and 60s vs 180s) using Student's t-test for variables with normal distribution that were statistically significant in the Bonferroni analyses. Ferritin concentration was log10 transformed for analysis because of skewed distribution and the results were presented as geometric means. Categorical variables were compared between groups using Fisher's Exact test for pairwise comparisons. Adjusted group means were compared using ANOVA and a binary logistic regression model was used to adjust for sex and age in the evaluation of ID at four months. A P-value less than 0.05 were considered significant. No imputation of missing data was performed. Loss to follow-up was registered, but not examined further. IBM SPSS for Windows, version 22 was used for the analyses.

RESULTS

Of the 200 prospectively recruited infants, 191 were included in the analysis as compared to 166 in the 10s and 168 in the 180s groups. Infants lost to follow up or excluded due to not fulfilling inclusion criteria are shown in Fig 1.

Baseline data

There were no significant differences in the 60s group as compared to the 10s and 180s groups with regard to maternal or neonatal characteristics. The 10s group had higher UC haemoglobin (163 vs 153 g/L, P<0.001) and higher UC haematocrit (48.7 vs 47.0%, P=0.005) than the 60s group. There were no significant differences between the 60s and the 180s groups (Table 1).

Two to three day-follow-up

At two to three days, blood samples were obtained from 140 infants in the 10s group, 107 in the 60s group, and 141 in the 180s group (Figure 1). The prevalence of anaemia was higher in the 10s compared to the 60s and 180s groups (9 (6.4%), 2 (1.9%) and 2 (1.4%)) respectively (P=0.04). Haemoglobin was lower in the 10s and 60s groups compared to the 180s group; 175, 180 and 189 g/L respectively (P=0.001 for 60s vs 180s). Bilirubin did not differ between the groups. Anaemic children were referred for further diagnostics but none needed treatment.

Primary outcome

In total 468 (78%) infants had follow up at four months (children excluded or lost to follow up are shown in Figure 1). In the 60s group, the infants (n=147) were four days older than the historical groups from the RCT (126±7 days vs 122±6 days, P<0.001). The geometric mean ferritin levels were 80, 96 and 117 µg/L respectively in the 10s, 60s and 180s groups (P=0.052 for 10s vs 60s; P=0.02 for 60s vs 180s) (Table 2). After adjustment for the age difference, the geometric means were 77, 103 and 114 µg/L respectively (P=0.002 for 10s vs 60s; P=0.29 for 60s vs 180s) (Table 3).

Secondary outcomes

All secondary outcome variables are shown in Table 2. Haemoglobin levels at four months were higher in the 60s group compared to 10s and 180s, but the difference did not remain in the age

adjusted model (Table 3). There were no statistically significant differences in the prevalence of anaemia in the 60s group as compared to the other two groups (Table 4). After adjustment for age at the time of follow-up, there were no differences between the 10s and 60s groups in haematocrit, MCV, reticulocyte count, transferrin receptor or transferrin saturation. The 60s group had significantly lower adjusted MCV and higher adjusted transferrin receptor compared to the 180s group, but there were no differences in haematocrit, reticulocyte count or transferrin saturation (Table 3). The prevalence of ID was 8 (5.2%), 7 (4.8%) and 1 (0.7%) respectively in the 10s, 60s and 180s groups (P>0.99 for 10s vs 60s and P=0.04 for 60s vs 180s) (Table 4). After statistical adjustments for sex and age at the time of follow-up, the prevalence of ID did not differ between the 60s group and the two other groups. The proportion of infants that were exclusively breast fed at four months did not differ between the groups (79 (52%), 92 (58%) and 89 (56%) respectively in the 10s, 60s and 180s groups. The impact of exclusive breast feeding on ferritin concentration and ID were further assessed in an adjusted model but no correlation was found (data not shown).

Sex differences

Iron and blood status at four months were further assessed by analysing differences according to infant sex. When data from the three cohorts were analysed together, geometric mean ferritin was lower in boys than in girls and ID was more prevalent amongst boys (Fig 2A and 2B). Haemoglobin concentrations did not differ significantly between boys and girls and neither did the prevalence of anaemia (data not shown).

DISCUSSION

We compared infants' iron and blood status at the age of four months after three different UC clamping time intervals (60s versus ≤10s, and ≥180s, respectively). Previous studies have compared immediate and late UC clamping but the definition of "late" in these studies varies from 60s to 180s or after birth of the placenta [5, 7, 8]. The current study evaluates an intermediate UC clamping strategy that can be used in connection with altruistic donation of UCB for clinical banking. When asking parents to donate their child's UCB for the potential good of others, it is essential to inform of

possible consequences for the child. We demonstrated that after adjustment for age difference at the time of follow-up, there were no statistically significant differences in ferritin concentration between the 60s and 180s groups but the 60s group had significantly higher ferritin concentrations compared to 10s. The main reason for the effect of age on ferritin during infancy is rapid growth which leads to a rapidly expanding blood volume and depletion of iron stores [9].

Results from the present study are in accordance with a Brazilian RCT that demonstrated higher ferritin concentration at the age of three months in children whose UC were clamped after 60s compared to immediate UC clamping [19]. However, our findings are in contrast to a study by Cernadas *et al.*, who reported no differences in ferritin six months after birth comparing 15s and 60s UC clamping. The difference in ferritin between the 60s and 180s groups were not presented [20]. The present study indicates that a 60s delay before UC clamping results in a mean serum ferritin at 4 months of age corresponding to 90% of the mean ferritin after 180s UC clamping.

In the current study, children in the 60s group had the lowest haemoglobin of all three groups at birth (10s 163 g/L, 60s 153 g/L, 180s 158 g/L) but at four months, there were no longer any differences between the groups in our adjusted model. The original RCT as well as a Cochrane report found higher concentrations of haemoglobin after immediate than late UC clamping at birth [6, 8]. As speculated in a previous publication, this difference may not reflect true difference in haemoglobin but could be a result of trans endothelial plasma leakage in the UC after UC clamping [6].

The prevalence of ID was higher in the 10s and 60s groups compared to the 180s group. However, when adjusting for sex and age at the time of follow-up, there were no longer any statistically significant differences between the three groups. In accordance with previous studies on ID in infancy, we found that at four months, boys had significantly lower geometric mean ferritin and more often ID than girls [21, 22]. Nevertheless, the aim of the study was not to compare differences according to sex, and the groups were therefor too small to draw any reliable conclusions of sex and ID. It has been suggested that the differences in iron status between the two sexes may be hormone-related but it is also influenced by genetic factors, which may differ in populations with different

ethnicity [22]. Also, when considering the generalisability of this study, readers should bear in mind that all children were placed below the uterine level for 30s directly after birth. This may be in contrast to the practice in other delivery settings, where the children are often placed directly on the woman's abdomen, which may affect the speed of the placental transfusion.

Limitations of the study

A limitation of this study is that we have no record of the parents' ethnic or socio-economic background, or no measure of adequacy of samples for donation, and we were therefore unable to draw any conclusions on possible impact of these factors. When comparing different interventions, RCTs are regarded as the golden standard. However, randomization into 10s UC clamping for this study was considered unethical since there is evidence that children benefit from prolonged placental transfusion for at least 60s, and the clinical routine in Sweden is to wait 180s before UC clamping in normal deliveries. We used the historical cohorts from the RCT in Halmstad, Sweden as controls in this study. The prospectively recruited cohort (60s) is from a large birth clinic in Stockholm, Sweden while the controls are from a smaller town where the participants were recruited two to five years earlier. Although the inclusion criteria were the same and pregnant mothers in Sweden follow the same well-controlled antenatal program and the baseline data showed no significant differences in maternal or infant characteristics, it is difficult to know if the study populations are homogenous. We did not use a non-inferiority approach for this study since we aimed to compare results after 60s UC clamping with10s and 180s. Nor did we perform a power calculation when designing the study, but included an equally large group as the two historical groups. This is a weakness which, should be taken into consideration. We have taken measures to avoid systematic biases caused by differences in laboratory routines between the two hospitals by using the same laboratory for analysing the iron status variables as well as CRP.

Collection of UCB for transplantation is a growing and life-saving practice. Tens of thousands UCB units are altruistically donated and stored for public use each year, and as a result new-borns are exposed to limited UC clamping time. The idea that UCB left in the placenta after birth is a mere

waste that is thrown away if not collected is obsolete. Optimizing UC clamping time and investigating the clinical significance to minimize risks for the infant while still enabling collection of UCB for transplantation is a great, but necessary, challenge. A study evaluating volume and TNC in UCB units collected at the Swedish National Umbilical Cord Blood Bank showed that 37 % of units collected after 60s UC clamping met the banking criteria compared to 47 % after immediate UC clamping [16]. This is information prospective parents considering donating UCB should receive.

CONCLUSION

In this observational study, significantly more children in the 10s UC clamping group had low iron stores at four months compared to children in the 60s and 180s UC clamping groups. After adjustment for age and sex, there were no statistically significant differences in ferritin concentration between 60 and 180s UC clamping. 60s UC clamping is therefore preferable to immediate UC clamping. Delaying UC clamping may be more important in boys than in girls to avoid ID in early childhood. Larger studies with long term follow-up is needed to establish the clinical relevance of different UC clamping strategies.

CONTRIBUTORS

Dr C. Götherström conceptualized and designed the study, analyzed data, critically revised and approved the final manuscript as submitted. Mrs U. Askelöf conceptualized and designed the study, carried out all data collection in the prospective cohort, carried out all analyses, drafted the initial manuscript, and approved the final manuscript as submitted. Dr O. Andersson designed the data collection instruments, contributed to the study design and provided data for the historical cohorts. He also carried out the initial analyses, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. Profs M. Domellöf, A. Fasth, L. Hellström-Westas, M. Westgren, Associate Profs K. Pettersson and I. Wiklund and Dr B. Hallberg participated in the study design, reviewed and revised the manuscript and approved the final manuscript as submitted.

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TABLE 1. Baseline characteristics of mothers and infants according to time for umbilical cord clamping

	Umbilical cord clamping time			P
	10s n=166 (HC)	60s n=191	180s n=168 (HC)	
Maternal characteristics				
Age (years)	31.7 (4.2)	30.7 (4.7)	30.8 (4.9)	NS
Parity (including study child)	1.81 (0.87)	1.86 (0.76)	1.76 (0.74)	NS
Weight at first antenatal visit (kg)	66.4 (12.0)	67.3 (11.5)	67.3 (12.1)	NS
Body mass index	23.6 (3.8)	24.5 (3.9)	23.9 (3.6)	NS
Haemoglobin at first antenatal visit (g/L)	128 (9)	128 (10)	128 (11)	NS
No (%) of vaginal deliveries:				
Non-instrumental	157 (95)	184 (96)	157 (94)	NS
Vacuum extraction	8 (5)	7 (4)	11 (7)	NS
Forceps	1 (0.6)	0 (0)	0 (0)	NS
Infant characteristics				
Gestational age (weeks)	40.0 (1.1)	40.0 (1.0)	40.0 (1.1)	NS
No (%) of males	83 (50)	98 (51)	73 (43)	NS
Birth weight (g)	3523 (483)	3603 (454)	3633 (464)	NS
Birth length (cm)	50.7 (1.9)	50.6 (1.8)	50.9 (1.9)	NS
Head circumference (cm)	34.7 (1.3)	35.1 (1.3)	35.0 (1.4)	NS
Umbilical cord haemoglobin (g/L)	163 (15)	153 (20)	158 (18)	<0.001*
Umbilical cord haematocrit (%)	48.7 (4.3)	47.0 (5.5)	47.1 (5.0)	0.005**

Values are means (SD) unless stated otherwise. P<0.05 was considered significant.
NS=non-significant; HC= Historical cohort.
*Comparison of 10s and 60s. For 180s and 60s, P=0.063.
**Comparison of 10s and 60s. For 180s and 60s, P>0.99.

TABLE 2. Outcome measures at the age of four months in infants whose umbilical cord were clamped after 10s, 60s or 180s

	Umbilical cord clamping time			60s vs 10s		60s vs 180s	
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	Mean difference (95% CI)	P	Mean difference (95% CI)	P
Infant age (days)	122 (6)	126 (7)	122 (6)	5 (3 to 6)	<0.001	4 (3 to 6)	<0.001
Main outcome measures							
Geometric mean (range) ferritin (µg/L)	80 (33 to 191)	96 (44 to 208)	117 (58 to 232)	0.83 (0.69 to 1.45)*	0.052	1.21 (1.03 to 1.44)*	0.02
Haemoglobin (g/L)	113 (7)	115 (8)	113 (8)	2 (0 to 4)	0.047	2 (0 to 4)	0.03
Blood count							
Haematocrit (%)	33.0 (2.0)	33.3 (2.2)	32.7 (2.1)	0.3 (-0.2 to 0.8)	0.19	1 (0 to 1)	0.02
Mean cell volume (fL)	77.9 (3.1)	77.7 (3.7)	79.2 (3.0)	-0.2 (-1 to 0.6)	0.67	-1.5 (-2.3 to -0.7)	<0.001
Reticulocyte count ($x10^9$/L)	37 (11)	36 (11)	40 (11)	-1 (-4 to 1)	0.39	-4 (-6 to -1)	0.01
Iron status							
Transferrin receptor (mg/L)	4.00 (0.80)	4.07 (0.92)	3.71 (0.69)	0.07 (-0.13 to 0.26)	0.51	0.35 (0.17 to 0.54)	<0.001
Transferrin saturation (%)	16 (6)	16 (8)	18 (6)	1 (-1 to 2)	0.40	-2 (-3 to 0)	0.03

Values are means (SD) unless stated otherwise. HC= Historical cohort.
*Geometric mean ratio (95% CI for geometric mean).
Successful analyses in the 60s and 180s groups: haemoglobin (n=140, 144), haematocrit, mean cell volume, reticulocyte count (n=135, 144), ferritin (n=147, 148), transferrin receptor (n=147, 148), transferrin saturation (n=145, 148).

TABLE 3. Outcome measures at the age of four months adjusted for infant age

	Umbilical cord clamping time Adjusted means (95% CI)			P	P
	10s n=152 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Main outcome measures					
Geometric mean (range) ferritin (µg/L)	77 (68 to 87)	103 (90 to 117)	114 (100 to 129)	0.002	0.29
Haemoglobin (g/L)	113 (112 to 115)	114 (113 to 116)	113 (112 to 114)	0.30	0.17
Blood count					
Haematocrit (%)	33.1 (32.7 to 33.4)	33.1 (32.8 to 33.5)	32.7 (32.4 to 33.1)	0.30	0.12
Mean cell volume (fL)	77.8 (77.3 to 78.3)	77.9 (77.3 to 78.4)	79.1 (78.6 to 79.6)	0.30	0.003
Reticulocyte count ($x10^9$/L)	36.9 (35.2 to 38.7)	37.0 (35.0 to 38.9)	39.4 (37.6 to 41.2)	0.99	0.07
Iron status					
Transferrin receptor (mg/L)	4.01 (3.87 to 4.14)	4.05 (3.92 to 4.19)	3.72 (3.59 to 3.85)	0.62	0.001
Transferrin saturation (%)	15.7 (14.7 to 16.7)	16.6 (15.5 to 17.7)	18.1 (17 to 19.2)	0.25	0.06

Values are means (95% CI) adjusted for infant age (days from birth) unless stated otherwise.
HC= Historical cohort. Successful analyses for the 60s group were: ferritin (n=147), haemoglobin (n=140), haematocrit, mean cell volume, reticulocyte count (n=135), transferrin receptor, and transferrin saturation (n=145). For the 180s group; blood status (n=144).

TABLE 4. Infants with iron status indicators outside reference limits at the age of 4 months according to umbilical cord clamping time

	Umbilical cord clamping time			P	P
	10s n=153 (HC)	60s n=147	180s n=148 (HC)	60s vs 10s	60s vs 180s
Ferritin <20 µg/L	11 (7.2)	4 (2.7)	0 (0)	0.11	0.06
Anaemia (Hb<105 g/L)	20 (13.1)	16 (11.4)	20 (13.9)	0.72	0.60
Mean cell volume <73 fL	8 (5.2)	14 (10.4)	3 (2.1)	0.12	0.01
Transferrin saturation <10%	22 (14.4)	15 (10.3)	8 (5.4)	0.38	0.13
Transferrin receptor >7 mg/L	0 (0)	2 (1.4)	0 (0)	0.24	0.25
Iron deficiency*	8 (5.2)	7 (4.8)	1 (0.7)	1.00	0.04

Values are unadjusted numbers (%). HC= Historical cohort.
Numbers of successful analyses: mean cell volume (n=153, 135, 144), transferrin saturation (n=153, 145, 148), anaemia (n=153, 140, 144).

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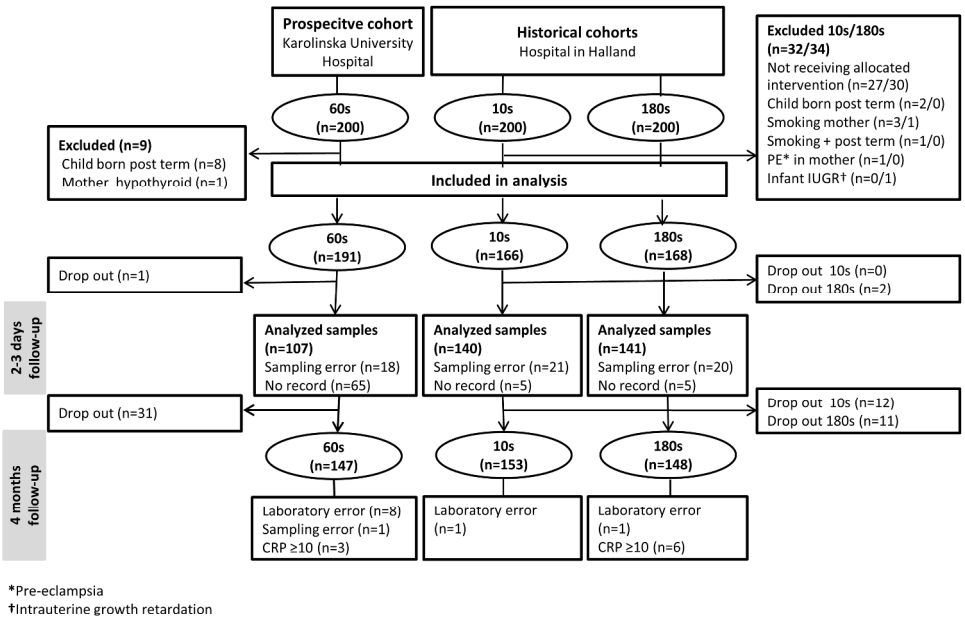
FIGURE LEGENDS

FIGURE 1. Flow chart of the study populations.

FIGURE 2. Differences in infants' iron status at the age of 4 months according to sex in three groups divided according to umbilical cord clamping time. **A:** Geometric mean ferritin according to sex and time for umbilical cord clamping, **B**: Proportion of children with iron deficiency according to time for umbilical cord clamping and sex.



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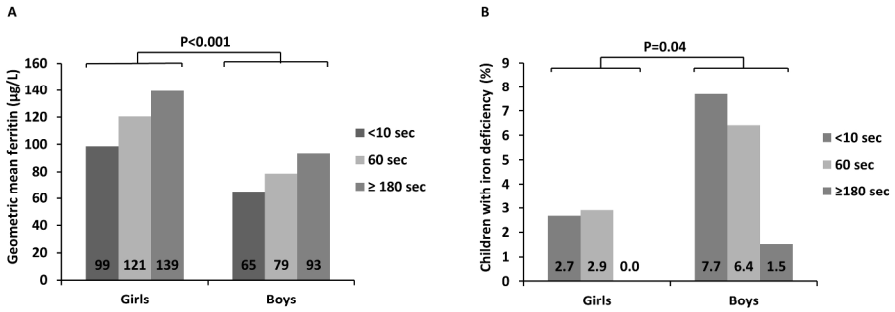

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4, 5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	Not applicable
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4, 5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5, 6
Bias	9	Describe any efforts to address potential sources of bias	6, 10
Study size	10	Explain how the study size was arrived at	4, 5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	Not applicable
		(*c*) Explain how missing data were addressed	6, 7
		(*d*) If applicable, explain how loss to follow-up was addressed	6, 7
		(*e*) Describe any sensitivity analyses	Not applicable
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Fig 1, 6, 7
		(b) Give reasons for non-participation at each stage	Fig 1
		(c) Consider use of a flow diagram	Fig 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4, 5
		(b) Indicate number of participants with missing data for each variable of interest	Tables 1-4
		(c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	Not applicable
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7
		(*b*) Report category boundaries when continuous variables were categorized	Not applicable
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Fig 2
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9, 11
Generalisability	21	Discuss the generalisability (external validity) of the study results	9, 10
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.