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1 **Fetal haemoglobin, blood transfusion, and retinopathy of prematurity in very preterm infants: A**
2 **pilot prospective cohort study**

3 Chris Stutchfield,^{1,2} Anoo Jain,¹ David Odd,² Cathy Williams,^{3,4} Richard Markham³

4 ¹ Neonatal Intensive Care Unit, St. Michael's Hospital Bristol, UK. ² Neonatal Intensive Care Unit,
5 Southmead Hospital, Bristol, UK. ³ Bristol Eye Hospital, University Hospitals Bristol NHS Foundation
6 Trust, UK, ⁴ School of Social and Community Medicine, University of Bristol, UK.

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8

9 Corresponding author:

10 Christopher James Stutchfield

11 Work address: Care of Dr. Anoo Jain, Neonatal Intensive Care Unit, St. Michael's Hospital Bristol,
12 Southwell Street, Bristol, UK, BS2 8EG

13 Email: cstutch@gmail.com

14 Department Tel: 0117 342 5276

15

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18

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35 **Abstract**

36 **Purpose:** To identify if there is an association between fetal haemoglobin (HbF) concentration and
37 retinopathy of prematurity (ROP) in very preterm infants.

38 **Methods:** Prospective cohort study. Infants born <32 weeks' gestational age or <1501g in two tertiary
39 neonatal units between January 2012 and May 2013 (n=42) were enrolled. Fetal haemoglobin (HbF)
40 and adult haemoglobin (HbA) concentrations were measured using High Pressure Liquid
41 Chromatography from blood samples sent as part of routine neonatal care once routinely requested
42 laboratory tests had been performed. Clinical data were obtained from case notes. We calculated
43 Odds Ratios (95% CIs) to quantify the relationship between initial and mean %HbF with ROP severity
44 (none, Stages 1,2,3).

45 **Results:** 42 infants were recruited: mean gestation 28.0w (SD 1.91); mean birthweight 1042g (SD
46 264). 6 infants died before ROP screening; 14/36 developed ROP (39%) and 22/36 (61%) did not.
47 Infants who developed ROP had similar initial %HbF (83.3% vs. 92.3%, p=0.06) but significantly lower
48 mean %HbF (61.75% vs. 91.9%, p=0.0001) during their inpatient stay than those who did not develop
49 ROP. In Ordinal Logistic regression models adjusted for birthweight, gestation and transfusion volume,
50 mean postnatal %HbF was negatively associated with ROP severity: adjusted OR 0.94 (0.90-0.99)
51 whilst initial %HbF at birth was not: adjusted OR 1.05 (0.97 – 1.16).

52 **Conclusion:** Replacing HbF by HbA during transfusion may promote ROP development by rapidly
53 increasing oxygen availability to the retina. Conversely, maintaining a higher %HbF may be a protective
54 factor against ROP.

55 Word limit= 239/250

56 Keywords: Retinopathy, prematurity, transfusion, fetal haemoglobin

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59 **Introduction**

60 Studies in preterm neonates have highlighted that a balance must be found between the toxic effects
61 of high oxygen saturations and the increased morbidity and mortality associated with targeting lower
62 saturations.¹⁻⁴ Birth weight and gestation have also been shown to be consistently independently
63 associated with ROP development. A number of other factors appear to have an association with ROP,
64 including genetics, poor nutrition and poor weight gain, sepsis and necrotising enterocolitis.⁵⁻⁷ Various
65 studies have identified an association between ROP and blood transfusion.⁸⁻¹⁰

66 Neonates have a predominance of fetal haemoglobin (HbF) at birth. Approximately 85% of total
67 haemoglobin is HbF in infants born at 35 weeks' gestation which gradually declines until it has
68 disappeared by the age of 1-2 years.^{11, 12} HbF has a greater affinity for oxygen compared to HbA,
69 shifting the haemoglobin-oxygen dissociation curve to the left, causing preferential fetal oxygen
70 binding in utero. However, HbF preponderance postnatally in very preterm infants leads to greater
71 difficulty unloading oxygen to the tissues. This is partly offset by a steeper oxygen-haemoglobin
72 dissociation curve than that for HbA, but exacerbated as the levels of 2,3-diphosphoglycerate (a
73 product of glycolysis that promotes oxygen release from oxy-haemoglobin) are low in preterm
74 neonates.¹³

75 Anaemia of prematurity, in part thought to be related to reduced red cell life span and low
76 erythropoietin levels, is exacerbated by clinical blood sampling, leading to the need for blood
77 transfusion in many very preterm infants.¹⁴ De Halleux V *et al*, 2002,¹⁵ demonstrated clinically that the
78 oxygen-haemoglobin dissociation curve is shifted to the right in preterm infants after blood
79 transfusion. We hypothesised that as the HbF:HbA ratio decreases with blood transfusion, more
80 oxygen is made available to the developing retina for any given arterial partial pressure of oxygen
81 (PaO_2), possibly contributing to ROP development by increasing the oxygen availability in the
82 developing retina and reducing angiogenic drive. We aimed to explore whether there might be an

83 association between either initial %HbF (on admission after birth) and/or the mean inpatient HbF%
84 (during their hospital admission), with the development of ROP in very preterm infants.

85

86 **Methods**

87 **Study design**

88 We conducted a prospective cohort study across two tertiary neonatal intensive care units in Bristol,
89 UK (St. Michael's and Southmead Hospital). All inborn infants and those retrieved from neighbouring
90 hospitals within 24 hours of birth born <32 weeks' gestation or <1501 grams were eligible for inclusion.
91 Parents were counselled as to the nature of the study and provided with an information leaflet. With
92 informed parental consent all routine EDTA samples taken during the baby's admission were analysed
93 for HbF%, HbA% and HbF:A ratio. No additional blood samples were taken for the purposes of the
94 study. Analysis of HbF and HbA were performed at a single site (Southmead Hospital) using high
95 performance liquid chromatography (BioRad Variant II HPLC analyser; daily calibration; 5 µl blood
96 sample volume). Recruited patients received routine neonatal care and the existing local protocol was
97 followed for blood transfusion thresholds. Infants received on site retinopathy screening by a single
98 consultant ophthalmologist according to national guidelines.¹⁶

99 **Data collection**

100 Patient case notes were reviewed after discharge from the neonatal unit (discharge home or transfer
101 to other neonatal unit) and demographic and clinical information were extracted in addition to
102 reviewing computerised laboratory reporting systems as follows: gestational age, birth weight, sex,
103 ethnicity, multiple pregnancy, days on respiratory support, days in supplementary oxygen, duration
104 of stay, corrected gestational age at discharge. Haematological data was extracted as follows: Hb,
105 %HbF, %HbA, HbF:HbA ratio, and blood transfusions (date, time and volume as ml/kg). Episodes of
106 infection were identified as definite (blood culture positive, CRP rise >10 and ≥5 days antibiotics),

107 probable (CRP rise >10 or positive blood culture, and ≥ 5 days antibiotics), or not present. ROP
108 screening data was recorded from patient notes for the duration of their ROP screening, according to
109 established nomenclature.¹⁷ If there was no ROP at any time, the ROP outcome was recorded as stage
110 0, whilst for any infant that did develop ROP, the worst stage in the worst eye was used as their
111 outcome. The presence or absence of “Plus” (or “Pre-plus”) disease was also noted. For those infants
112 discharged to other neonatal units before retinopathy screening had been completed, data was
113 extracted from BadgerNet, an electronic patient data management system.

114 **Data analysis**

115 Initial %HbF (on admission after birth) and demographics on the cohort were extracted from the notes
116 along with the results from their ROP screening examinations. Mean %HbF (during in-patient stay)
117 was derived using time-weighted averaging (multiplying each %HbF value by the length of time
118 between samples, and then averaging the results for the whole period of an infant’s in-patient stay).
119 Initial univariate comparisons were carried out between those infants who developed any ROP versus
120 those who did not, with respect to their HbF values. %HbF from each full blood count was plotted
121 against corrected gestational age. An ordered logistic regression model was used to calculate Odds
122 Ratios (ORs) and 95% Confidence Intervals to estimate the association between the value of the %HbF
123 (either initial, or mean of inpatient stay) with the most severe stage of ROP recorded for each baby (0,
124 1, 2, 3). Corresponding adjusted ORs were derived after including gestation, birthweight, and total
125 volume of red blood cell transfusions in the logistic regression model.

126 Analysis was performed using STATA 10 (Stata Corp) and results are presented as number (%), mean
127 (standard deviation), median (inter-quartile range) or OR (95% confidence interval) as appropriate.
128 Ethical approval was obtained from The NRES Committee North East Newcastle and North Tyne 2.

129

130 **Results**

131 42 infants were recruited between January 2012 and November 2013. No infants were excluded from
132 the study. All parents approached for recruitment during the study agreed to be enrolled except the
133 parents of one set of twins. Demographic data for the cohort, split by ROP status are displayed in Table
134 1.

135 **Table 1**

136 37 neonates were inborn and 5 infants were outborn. All outborn infants were born in a local district
137 general hospital, retrieved by the tertiary neonatal team and arrived in the tertiary neonatal unit
138 before 10 hours of age. 6 infants died before ROP screening (3 male, 3 female) of which 4 were inborn
139 and 2 were outborn. Infants who developed ROP were more likely than infants who did not develop
140 ROP to be from multiple births ($p=0.027$), more preterm ($p<0.001$), of lower birthweight ($p=0.007$), to
141 have had more transfusions ($p<0.001$), larger volumes infused ($p<0.001$), and to have spent longer on
142 ventilators ($p<0.001$) and Continuous Positive Airway Pressure (CPAP) ($p=0.028$).

143 24 infants received a transfusion of red blood cells (RBC) during their admission (57%). Of the 36
144 infants who survived to ROP screening all survived to discharge from the tertiary unit. 22 of these
145 infants did not develop ROP (52% of the initial cohort). 14 infants developed ROP (33% of the initial
146 cohort): stage 1 ($n=5$), stage 2 ($n=4$), stage 3 ($n=5$, 4 of these infants also had plus disease, 4 of these
147 infants received laser treatment and one received intravitreal bevacizumab), grade ≥ 4 ($n=0$). No infant
148 developed retinal detachment (Stage 4 or worse) during the study.

149 Those infants who did not develop ROP had higher initial haemoglobin levels (on admission) than
150 infants who did develop ROP ($p=0.009$), as shown in Table 2 and there was weak evidence that they
151 have a lower initial %HbF (83.3% vs. 92.3%, $p=0.06$). Infants who developed ROP had significantly
152 lower ($p=0.0001$) mean %HbF during their admission compared to those infants who did not develop
153 ROP (Table 2).

154 **Table 2**

155

156 When plotting HbF% from each full blood count against corrected gestational age there appears to be
157 two populations. Those infants who developed ROP have noticeably lower HbF% when compared to
158 those infants without ROP (Figure 1).

159 **Figure 1**

160

161 The ordinal regression model produced similar results to the univariate associations above (Table
162 3). There was only weak evidence that initial HbF% was associated with increasing risk of ROP grade
163 ($p=0.070$) and this association disappeared after adjusting for birthweight, gestation at birth and
164 volume of transfusion ($p=0.261$). In contrast there was strong evidence for an association between
165 Mean %HbF and increasing risk of ROP grade in both the unadjusted ($p<0.001$) and the adjusted
166 analyses ($p=0.034$).

167 **Table 3**

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174 **Discussion**

175 Whilst very preterm infants who developed ROP had similar initial %HbF when compared to those
176 who did not develop ROP, we observed that as hypothesized, mean %HbF was significantly lower in
177 the ROP group during their inpatient stay than in the babies who did not develop ROP. A lower %HbF
178 may be a proxy or surrogate marker for sickness, as neonates who are more unwell will be more likely
179 to require and receive blood transfusions. We therefore included volume of blood transfused as an
180 approximate proxy marker of illness severity in the logistic regression model and an association still
181 seemed to hold between lower %HbF and ROP development. There is physiological evidence

182 supporting left shift of the oxy-haemoglobin dissociation curve with increasing %HbF. It is therefore
183 biologically plausible that a lower HbF concentration (and higher HbA concentration) provides greater
184 oxygen delivery to the developing retina. Evidence of this effect (ROP) has been found in our study. It
185 may therefore be that maintaining higher HbF levels for longer confers some protection against ROP
186 development.

187 We recognise limitations with our study, notably a small sample size. Identifying causal factors in ROP
188 development is not possible in this small cohort study due to the large number of variables and
189 confounding factors over this time period as well as a relatively heterogeneous patient population.
190 We considered supplemental oxygen therapy to be part of the causal pathway leading to ROP
191 development, rather than as a confounder and did not therefore adjust for it in these models. In a
192 larger study, it would be useful to explore in more detail the relationship between supplemental
193 oxygen, intercurrent illness and blood transfusions on %HbF and on the development of ROP. There
194 is a need for further research to establish evidence for a potential causal relationship between HbF%
195 and ROP risk.

196 Various interventions including laser therapy and angiogenesis inhibitors are utilised to manage
197 established ROP and much research is ongoing in this area. However, if high risk infants can be
198 recognised and there is a possibility of enhancing intrinsic protective factors, then ROP development
199 could potentially be minimised or prevented. Delayed cord clamping has been shown to deliver
200 additional blood to the newborn from the placental bed.^{18, 19} This could potentially reduce or delay
201 the need for subsequent transfusion, in turn facilitating maintenance of HbF. There is already
202 reluctance to transfuse liberally in preterm neonates due to NEC risk, exposure to donors and risk of
203 transfusion reaction, but risk of ROP development might also need to be considered if further research
204 supports the hypothesis that early loss of HbF predisposes an infant to developing ROP.

205

206 **Conclusions**

207 To our knowledge this is the first study to investigate and find an association between HbF
208 concentration, blood transfusion and ROP development. It is possible that HbF is a protective factor
209 against ROP and that transfusion of adult (HbA) blood may play a part in ROP development by suddenly
210 making more oxygen available to the developing retina and downregulating VEGF, resulting in arrest
211 of the advancing front of retinal vasculature. Subsequent reduction of oxygen supply leads to
212 ischaemia of the unvascularised peripheral retina and marked upregulation of VEGF with the
213 development of fundoscopic signs of ROP. Larger studies are required to investigate these associations
214 further.

215

216 **Summary**

217 **What was known before**

- 218 • Retinopathy of prematurity (ROP) is a major cause of morbidity in the preterm population
- 219 • Oxygen is known to be the predominant causal factor in ROP development
- 220 • ROP has been shown to be associated with blood transfusion

221 **What this study adds**

- 222 • Blood transfusion dramatically reduces the fetal haemoglobin (HbF) concentration
- 223 • Those infants who develop ROP have significantly lower mean fetal haemoglobin (HbF) levels

224 Maintaining higher HbF levels may be protective against ROP

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230 forwarding to analysis laboratory.

231

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233

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235

236 **Contributors**

237 C Stutchfield: Contributed to study design. Recruited patients, designed data extraction proforma and
238 extracted all case note and laboratory data. Assisted with data analysis. Drafted scientific paper.

239 A Jain: Contributed to study design. Recruited patients, assisted with data analysis, edited scientific
240 paper.

241 D Odd: Contributed to study design. Recruited patients, statistical analysis, edited scientific paper.

242 C Williams: Contributed to study design, performed all ROP screening, assisted with data analysis,
243 edited scientific paper.

244 R Markham: Principal Investigator. Study inception and design. Secured charitable funding and edited
245 scientific paper.

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321 **Tables**

322 **Table 1: Demographic and ROP* outcome data**

Variable	No ROP	ROP	P
Male	9 (40.9%)	5 (35.7%)	0.441
Non-white ethnicity	2 (9.1%)	0 (0%)	0.411
Multiple birth	11 (50.0%)	1 (7.1%)	0.027
RBC* transfusions	0 (0-1)	3 (1-5)	<0.001
Total RBC transfusion (ml/kg)	0 (0-20)	53 (20-103)	<0.001
Culture positive sepsis	2 (9.1%)	3 (21.4%)	0.297
Gestation (weeks)	29.2 (1.1)	26.6 (1.6)	<0.001
Birth weight (grams)	1160 (261)	924 (205)	0.007
Days on ventilator	1 (0-2)	6 (2-20)	<0.001
Days on CPAP	10 (6-27)	30 (14-39)	0.028
Days on supplementary O2	28 (7-51)	50 (21-73)	0.051

323 Values are mean (SD), median (IQR) or n(%) as appropriate. *Retinopathy of Prematurity; † Red blood
324 cell

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328 **Table 2: Comparison of haematological values between those infants who developed ROP and those**
329 **that did not**

	No ROP (95% CI) (n=22)	ROP (95% CI) (n=14)	p value
Initial Hb (g/L)	162.5 (153.2, 171.8)	143.6 (133.3, 153.9)	0.009
Mean Hb (g/L)	134.9 (116.0, 153.7)	112.4 (102.8, 122.1)	0.06
Initial %HbF	92.3 (89.9, 94.7)	83.3 (71.1, 95.5)	0.06
Mean %HbF	91.87 (87.2, 96.5)	61.75 (44.5, 79.0)	0.0001

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333 **Table 3: Association between haematological values and ROP**

Variable	OR (95% CI)	Adjusted* OR (95% CI)
Initial HbF%	0.96 (0.93-1.00)	0.97 (0.91-1.03)
Mean HbF%	0.94 (0.90-0.97)	0.94 (0.90-0.99)

334 * Adjusted for birthweight, gestation and total transfusion volume. OR= odds ratio. CI= confidence interval.