Original Article

The Use of HPLC as a Tool for Neonatal Cord Blood Screening of haemoglobinopathy: A Validation Study

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Abstract. *Background:* Newborn cord blood screening identifies infants with underlying haemoglobinopathies before they develop the characteristic symptoms or sequelae.
Aims: This study was performed to validate the interpretation high-performance chromatography (HPLC) along with complete blood count (CBC) results as a tool for universal neonatal screening of hemoglobin disorders in Oman.
Methods: HPLC and CBC data on subjects who participated in the National Neonatal screening program at birth were obtained from archival records. The results recorded at birth were compared with a second study performed on the same subjects, after approval from the local medical research and ethics committee.
Results: Only 290 subjects from amongst the original cohort of 3740 newborns could be recalled between April 2010 to March 2011, to repeat HPLC and CBC, as well as perform confirmatory DNA studies, wherever necessary. All these subjects had been documented to show an initial abnormal result. 31 cases who had no HbA at birth on HPLC were confirmed as either homozygous β-thalassaemia major (n=5 subjects) or homozygous sickle cell anemia (n=26 subjects) by appropriate DNA analysis. Additionally, amongst 151 subjects, 72 subjects were studied in the initial study by Hb Bart's quantitation using the alpha thalassaemia short program at birth. In this cohort, 42 subjects with Hb Bart's >1% at birth could be confirmed as having either deletional or non-deletional thalassaemia by GAP PCR studies. No case of HbH was detected in this cohort. Further, carrier status for structural hemoglobin variants (HbS, HbC, HbD, HbE) (n=67) and beta thalassaemia allele with low HbA at birth (n=29 out of 41) were confirmed by relevant molecular studies.
Conclusions: The study validated the earlier observation by 100% concordance with the results of CBC and HPLC. Presence of Hb Bart's at birth does not always mean the presence of alpha thalassemia, as subjects with Hb Bart's below 1% by quantitation, were shown to be normal by molecular studies.

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Introduction. The mortality rate in sickle cell disease (SCD) is highest in the first five years of life and the greatest risk period is the second 6 month of life, so early diagnosis of SCD by the newborn screening can have a huge impact on the mortality and morbidity.[1-6] Early detection of SCD in this period allows for the introduction of penicillin, administration of recommended vaccinations along with counseling & education of affected families, before the onset of symptoms.

Oral penicillin prophylaxis in children with SCD provided an impressive 85% reduction in the incidence of infections and led to the reduction in morbidity and mortality of this disease in childhood as there was a 30% fatality rate observed amongst children with SCD who developed sepsis.[7,8] Vichinsky[9] in 1988 had shown that the mortality rate was 1.8% for those diagnosed in the newborn period, as compared to 8% amongst those patients diagnosed after three months of age. Similarly, the Cooperative Study of Sickle Cell Disease observed that by instituting prophylactic penicillin, the incidence of sepsis was reduced to 8% and the mortality rate to 25%.[1] Further evidence can be seen from the Jamaican newborn cohort study, which showed a mortality rate of 25% with only 30% of infants experiencing acute sequestration crisis.[10]

Health education and genetic counseling are the two pillars of any genetic screening program. A good example of a preventive program is when it is coupled with health education as has been reported in a study by Riddington C and Owusu-Ofori S.[7] This study showed that 70% of parents were able to determine spleen size when proper training was given to them, and it was also found that 21% of acute sequestration crisis were diagnosed as a result of the mother's examination. Furthermore, newborn screening programs in which a strong parent education component was not incorporated have been unsuccessful in reducing mortality. Thus, mortality rates of 30% and 14% have been reported when no comprehensive medical follow-up was provided.[9]

The early identification of haemoglobinopathies and initial referral of those infants ensures prompt delivery of health care and allows screening for markers of disease severity as well as the initiation of prophylactic interventions before the development of clinical complications. The parents can also be counselled about their plans for the current and future children. Further, early detection of thalassaemia major can allow for the monitoring of the development of signs and symptoms of anemia and institute blood transfusions and chelation promptly.

Different laboratory techniques are employed for neonatal screening including isoelectric focusing (IEF), and HPLC. IEF has the disadvantage of being a labor-intensive manual technique, whereas the Bio-Rad's Variant HPLC system (Bio-Rad Laboratories, Hercules, CA, USA) is a rapid semi-automated system that is widely used and is the backbone for screening hemoglobin variants at our institution. There are several programs available for this system including sickle cell short program, which is a rapid 3-min assay capable of using either filter paper blood spots or whole blood samples. This program is specifically designed to provide a qualitative result for hemoglobins A, F, S, C, D, and E in the neonate. A second program is the β-Thalassemia Short Program, which is a 6.5-min assay designed to quantify HbA2 and HbF, however confirmation of some hemoglobin variants at birth can be difficult, in particular, carriers of β thalassemia until adequate HbA2 has developed.

Our study is aimed at validating the results obtained at the initial testing of the neonates enrolled in the national neonatal screening program[11] with a simple, cost-effective HPLC and CBC. We were able to follow up 290 such subjects who were initially tested in the national neonatal screening program at birth and then recalled now for repeat testing, and use the opportunity to validate the initial results of HPLC and CBC by confirmatory molecular studies to document and ascertain the final diagnosis.

Methods. This prospective follow-up study was conducted at Sultan Qaboos University Hospital throughout one year between April 2010 to March 2011. In this study, 290 babies from the original cohort of 3740 newborns were studied.[11] All children had an abnormal cord blood screening test. The study was approved by the local Medical Research & Ethics committee of the hospital.

5 ml of venous blood was collected in vacutainer tube with K2EDTA anticoagulant. HPLC and CBC were performed using this blood sample, and 2 ml blood was used for obtaining genomic DNA according to the manufacturer's instructions using the QIAamp DNA Blood mini kit (Qiagen, Inc., Valencia, CA, USA).

HPLC was performed within 12-24 hours of collection of a blood sample using the Bio-Rad VARIANT™ instrument (Bio-Rad Laboratories, Hercules, CA, USA) and the "β-thalassemia short program. A CBC was performed on Cell Dyn 4000 automated blood cell counter (Abbott Diagnostics, Santa Clara, CA, USA).

Hb Bart's quantitation was performed using α-thalassaemia short program (Biorad Variant II) at birth (within 12-24 hours of collection).[11] In this follow-up study, Hb Bart's positive cases were initially screened by Genescan technique to determine the next approach.[12] Multiplex Gap PCR procedure was used to detect the seven common deletions occurring in the α-globin gene cluster.[12] Additionally, in subjects with no deletional defect (n=1), automated direct nucleotide sequencing (ABI 3130; Applied Biosystems, Foster City, CA, USA) of the selectively amplified alpha1 and

alpha2 globin genes was performed to characterize non-deletional α-thalassaemia determinants using appropriate primers to ascertain the nature of the underlying molecular defect.[13]

Statistical Analysis. The data were archived on a Microsoft Excel Database on a dedicated computer. All analysis was carried out by using the SPSS software (IBM SPSS Inc., USA. Ver. 23). Normally distributed results were expressed as mean value $\pm$ SD, whereas, the non–parametric data as median (interquartile range). Students' t-test was used to compare the statistical significance between the means of various groups. A p-value <0.05 was considered as significant. HPLC sensitivity of Hb Bart's quantitation at birth was reported using the manufacturer's values (alpha thalassaemia short program, Variant II) and tested by appropriate molecular techniques.

Results. Only 290 subjects from amongst the original cohort of 3740 newborns could be recalled between April 2010 to March 2011, to repeat HPLC and CBC, as well as perform confirmatory DNA studies. This cohort could be categorized into three groups according to the cord blood results at birth.[11] Group A: Subjects with no haemoglobin A by HPLC at birth (n=31); Group B: Subjects with Hb Bart's at birth on HPLC using the "β-thalassemia short program (n=151); Group C: Subjects with abnormal qualitative or quantitative beta chain variants based on HPLC at birth (n=108).

Group A comprised of 31 children with no adult haemoglobin detected at birth by HPLC. These babies were all re-tested between 3 to 6 months of age according to the current guidelines. Repeat HPLC was consistent with homozygous β-thalassaemia major in 5 babies with a significantly high HbF and HbA2, whereas the remaining 26 babies HPLC showed an HbS values between 92-94% consistent with sickle cell anaemia. Repeat red cell indices in comparison to cord blood results (**Table 1a**) showed a significant drop in Hemoglobin (p < 0.001), MCV (p < 0.0001) and MCH (p < 0.001) with increase in RBC count (p < 0.01). In this group, the earlier diagnosis by HPLC was re-confirmed in all 31 cases by appropriate DNA analysis to document the underlying mutation. Sequence analysis of the entire β-globin gene and the β-globin gene promoter region was amplified by polymerase chain reaction (PCR) using appropriate primers.[12]

In Group B, there were 151 babies who were suspected to have α Thalassaemia at birth based on the presence of Hb Bart's on HPLC.[11] Repeat red cell indices in this follow-up study showed that the hemoglobin, RBC counts, and MCHC were with-in the age-related normal ranges. However as expected, in comparison to cord blood results, there was a significant drop in hemoglobin (p < 0.001), MCV (p < 0.001) and MCH (p < 0.001) with increase in RBC count (p < 0.05).

There were no significant differences in MCHC (**Table 1b).** On repeat HPLC, no abnormal haemoglobin variant was detected. Further, the fetal hemoglobin and adult haemoglobin were different but appropriate for age. **Table 1c** & **d** show comparison of red cell indices at birth and at follow-up study in group B cohort subjects with one alpha gene deletion (n=20) and two alpha gene deletions (n=22) with similar observations as above respectively.

Table 1a. Comparative analysis of red cell indices in the group A cohort at birth (Mean$\pm$SD) and at the follow-up study (n=31).

	At birth	**At follow-up**	**P value**
Hb (g/dl)	15.1$\pm$2.0	11.0$\pm$1.1	<0.001[#]
RBC (x10^{12}/L)	4.8$\pm$0.8	5.3$\pm$0.8	<0.01[#]
MCV (fl)	98.3$\pm$9.3	66.1$\pm$8.6	<0.0001[#]
MCH (pg)	31.5$\pm$3.5	21.8$\pm$3.2	<0.001[#]
MCHC (g/dl)	32.0$\pm$1.2	32.9$\pm$1.7	<0.005[#]
RDW (%)	16.5$\pm$1.9	15.4$\pm$2.3	<0.008[#]

[#]Students t test

Table 1b. Comparative analysis of red cell indices in the group B cohort at birth (Mean$\pm$SD) and at the follow-up study (n=151).

	At birth	**At follow-up**	**P value**
Hb (g/dl)	15.14+1.7	11.3+1.2	<0.001[#]
RBC (x10^{12}/L)	4.8$\pm$0.7	5.2$\pm$0.5	<0.05[#]
MCV (fl)	98.3+7.9	67.9+7.0	<0.0001[#]
MCH (pg)	31.7+3.3	21.8+2.4	<0.001[#]
MCHC (g/dl)	32.35+1.3	32.1+1.4	0.96[#]
RDW (%)	16.4+1.6	14.2+2.1	<0.001[#]

[#]Students t test

Table 1c. Comparative analysis of red cell indices in the group B cohort at birth (Mean$\pm$SD) and at the follow-up study with one alpha gene deletion (n=20).

	At birth	**At follow-up**	**P value**
Hb (g/dl)	13.61+1.55	11. 3+1.17	0.006[#]
RBC (x10^{12}/L)	4.07$\pm$0.95	4.96$\pm$0.72	0.07[#]
MCV (fl)	100.1+8.2	69.04+3.6	<0.001[#]
MCH (pg)	32.4+3.3	22.5+1.62	<0.001[#]
MCHC (g/dl)	32.3+1.54	32.77+1.86	0.63[#]
RDW (%)	15.7+1.29	14.29+1.14	0.05[#]

[#]Students t test

Table 1d. Comparative analysis of red cell indices in group B cohort at birth (Mean$\pm$SD) and at the follow-up study with two alpha gene deletions (n=22).

	At birth	**At follow-up**	**P value**
Hb (g/dl)	15.21+1.4	10.83+1.26	<0.001[#]
RBC (x10^{12}/L)	5.35$\pm$0.45	5.37$\pm$0.33	0.91[#]
MCV (fl)	91.84+4.1	63.12+5.8	<0.001[#]
MCH (pg)	28.46+1.4	20.1+1.9	<0.001[#]
MCHC (g/dl)	31+1.23	31.92+1.2	0.11[#]
RDW (%)	17.29+1.7	14.64+3.1	<0.03[#]

[#]Students t test

Amongst these 151 subjects who had Hb Bart's at birth, only 72 subjects had been earlier studied by Hb Bart's quantitation using α alpha thalassaemia short program (Biorad Variant II) at birth. Hb Bart's was ($\leq$ 1%) in 30 babies; between >1 to <3% in 20 babies, and $\geq$3% in twenty-two babies (**Table 2a** and **2b**) (**Figures 1** and **2**). **Table 2a** shows the comparative analysis of the various red cell indices at birth and follow-up. Most of the parameters were significantly altered except MCHC. MCV followed by MCH were the most important discriminators reflecting the microcytic hypochromic red cell maturation. Multiplex GAP PCR in the recalled subjects correctly identified the presence of alpha thalassaemia in subjects that had more than 1% Hb Bart's at birth (n=42).

Table 2b shows a correlation between Hb Bart's at birth and results from molecular DNA studies. All subjects with two equal peaks on Genescan showed normal genotype (αα/αα; n=30) and had Hb Bart's less than 1%. However, in subjects with two unequal peaks

(n=20), GAP PCR confirmed single deletional alpha thalassaemia (αα/-$\alpha^{3.7}$) in all the cases. In one subject with Hb Bart's of 4.2%, an additional non-deletional mutation was found by complete alpha gene sequencing explaining the higher than expected Hb Bart's. Lastly, in subjects with one peak on Genescan (n=22), GAP PCR confirmed two gene deletional alpha thalassaemia (-$\alpha^{3.7}$/-$\alpha^{3.7}$) in all the cases.

Table 3 shows the comparative analysis of red cell indices and hemoglobin values on electrophoresis in the Group C with heterozygous structural β-globin gene defect (HbS, HbD, HbE & HbC) at birth and at the follow-up study (n=67). The earlier diagnosis by HPLC was re-confirmed in all 67 cases by a repeat HPLC and appropriate DNA studies to confirm the underlying β-hemoglobin variant mutation.

Table 4 shows the comparative analysis of red cell indices and hemoglobin values on electrophoresis in Group C (n=41) with low HbA at birth (<10%) and at the follow-up study (n=29). These babies with Hb

Table 2a. Red cell indices at follow up (Mean$\pm$SD) in the Hb Bart's quantitation cohort at birth (n=72).

Hb Bart's at birth	Hb (g/dl)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)
$\leq$1% [n=30]	11.75$\pm$1.0	5.2$\pm$0.5	70.87$\pm$7.5	22.6$\pm$2.4	31.9$\pm$1.5	13.7$\pm$1.5
>1-<3% [n=20]	11.13$\pm$1.2	5.16$\pm$0.7	69.0$\pm$3.6	21.5$\pm$1.6	32.7$\pm$1.8	14.3$\pm$1.1
$\geq$3-10% [n=22]	10.8$\pm$1.2*	5.3$\pm$0.3	63.1$\pm$5.8*	20.1$\pm$1.9*	31.9$\pm$1.2	14.6$\pm$3.1

* p <0.05, Students t test

Table 2b. Correlation of Hb Bart's, Genescan peaks with DNA studies in the Hb Bart's quantitation cohort at birth (n=72).

α genotype (GAP PCR)	GeneScan Peaks (Figure 2)	Hb Barts at birth	Hb Barts Mean	Hb Barts Median	Hb Barts IQ25	Hb Barts IQ75
αα/αα	Two equal peaks	$\leq$1% [n=30]	0.98	0.8	0.6	1.0
αα/-$\alpha^{3.7}$	Two unequal peaks	>1-<3% [n=20]	1.48*	1.4	1.2	1.7
-$\alpha^{3.7}$/-$\alpha^{3.7}$	One peak	$\geq$3-10% [n=22]	5.6	5.9	4.25	7.2

* One subject who had a Hb Bart's 4.2%, also had additional non-deletional alpha thalassaemia mutation shown by complete alpha gene sequencing study in this patient. IQ25- 25th Centile, IQ75-75th Centile.
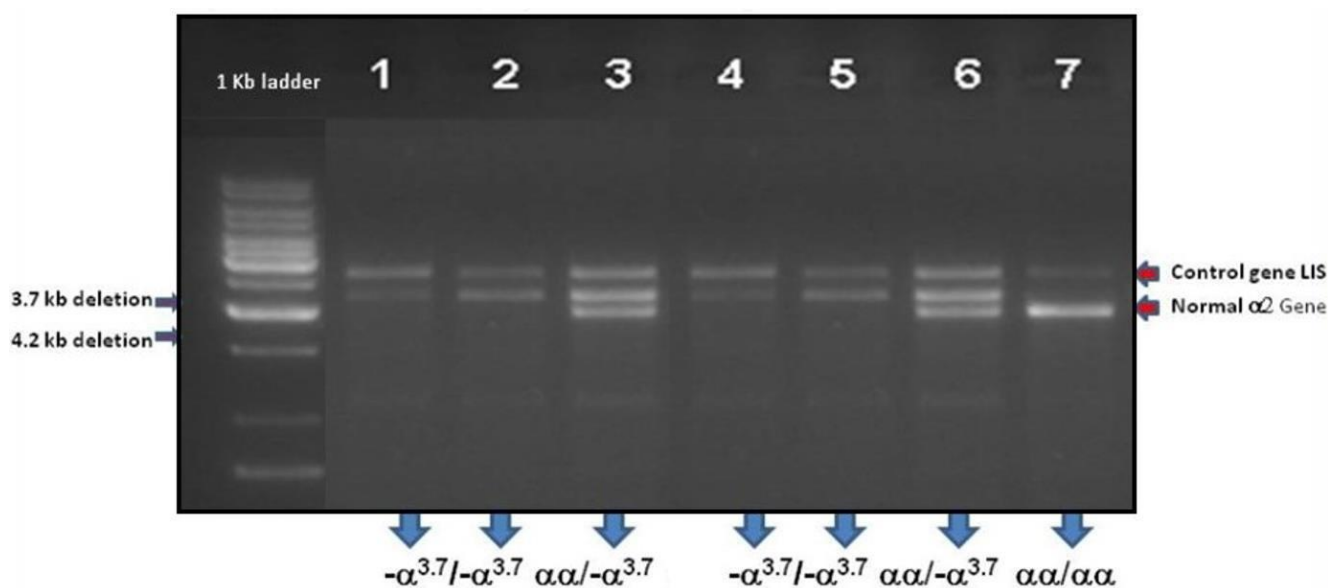

Figure 1. Ethidium Bromide stained Agarose gel with PCR products of Alpha gene using control gene LIS. Lanes 1,2,4,5 shows one alpha gene deletion, lanes 3 & 6 show 2 alpha gene deletion whereas lane 7 shows normal alpha genes [No alpha gene deletion].
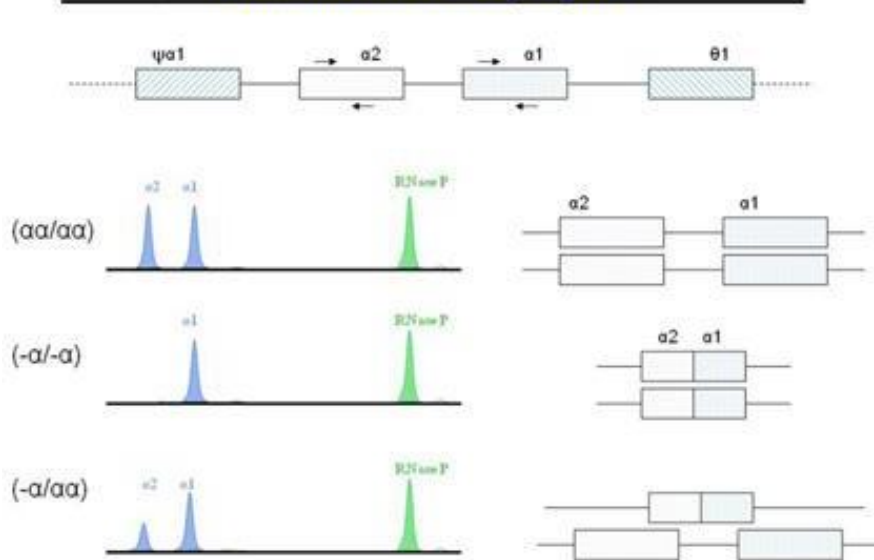
Figure 2. Schematic diagram of Genescan studies for Alpha genes using control gene RNaseP.

Table 3. Comparative analysis of red cell indices, HbF, HbA, HbS and HbA2 (Mean+SD) in the cohort with heterozygous structural β-globin gene defect [HbS, HbD, HbE & HbC] at birth and follow-up (n=67).

CBC	HbS [n=38]			HbD [n=18]			HbE [n=10]			HbC [n=1]	
	At birth	Follow- up	P value	At birth	Follow-up	P value	At birth	Follow-up	P value	At birth	Follow-up
Hb (g/dl)	15.2+2.0	11.0+1.0	<0.001*	15.0+1.3	10.9+1.3	<0.001*	16.7+1.7	11.6+0.5	<0.001*	13.9	12
RBC (x10^{12}/L)	5.0+0.8	5.2+0.8	NS*	4.5+0.4	4.9+0.6	NS*	5.4+0.8	4.7+0.3	<0.05*	4.15	5.3
MCV (fl)	95.7+8.4	64.3+6.2	<0.001*	101.8+7.8	65.1+4.1	<0.001*	98.1+7.3	71.9+5.8	<0.001*	98.5	64.7
MCH (pg)	30.5+3.2	21.3+2.5	<0.001*	33.6+3.5	22.3+2.2	<0.001*	31.6+3.1	24.7+2.2	<0.001*	33.5	22.6
MCHC (g/dl)	31.9+1.3	33.0+1.5	<0.001*	33.0+1.7	34.0+1.3	<0.001*	32.1+1.7	34.4+0.3	<0.001*	34	35
RDW	16.5+1.7	15.2+2.1	NS*	16.6+2.0	15.9+2.5	NS*	17.6+2.2	15.0+3.1	<0.05*	16.5	19.3
HPLC											
HbF%	91.0+8.0	7.8+11.0	<0.001*	91.9+4.3	2.2+0.7	<0.001*	87.7+9.2	3.8+1.2	<0.001*	96.9	5.4
HbA%	13.0+6.5	50.5+18.5	<0.001*	13.8+9.9	54.6+5.6	<0.001*	23.1+7.9	60.0+3.4	<0.001*	8.8	60
HbS%	7.1+3.2	29.6+5.4	<0.001*	8.2+3.4	33.2+6.2	<0.001*	8.3+2.7	24.7+3.4	<0.001*	6	34.6
HbA2 %		3.2+1.6	--		3.1+1.2	--	87.7+9.2	3.8+1.2	<0.001*	--	3.2

*p-value, Students t-test; NS- p>0.05

Table 4. Comparative analysis of red cell indices, HbA, HbF, and HbA2 (Mean+SD) in the cohort with Low HbA (<10%) at birth and follow-up (n=29).

	At birth	At follow-up	p-value
Hb (g/dl)	14.7+2.0	10.8+1.1	<0.01*
RBC (x10^{12}/L)	4.3+0.5	5.2+0.9	<0.01*
MCV (fl)	105.7+7.6	71.6+11.6	<0.0001*
MCH (pg)	34.4+2.6	23.5+4.3	<0.0001*
MCHC (g/dl)	32.5+0.9	32.7+2.3	0.74*
RDW (%)	16.3+2.5	16.0+2.7	0.77*
HPLC			
HbF (%)	106.9+4.6	3.8+1.4	<0.0001*
HbA (%)	7.5+1.5	85.3+5.4	<0.0001*
HbA2 (%)	--	5.0+0.9	--

*p-value, Students t-test

A < 10% on HPLC had no other abnormalities on the HPLC at birth. However, repeat HPLC in the follow-up study showed that amongst those 41 cases only 29 had an elevated HbA2 with the mean HbA2 5.0 (range 3.6-6.2). Sequence analysis of the entire β-globin gene and the β-globin gene promoter region using appropriate primers showed that these 29 subjects were carriers for an underlying beta thalassaemia mutation as described in the earlier study.[11] Repeat red cell indices at follow-up (n=29) showed that the hemoglobin, RBC counts with MCHC were normal for the age of the subjects. However, in comparison to cord blood results, there was a significant drop in hemoglobin ($p < 0.01$), MCV ($p < 0.0001$) and MCH ($p < 0.0001$) and a rise in the RBC counts ($p < 0.01$). There was no significant alteration in MCHC. HbA2 in all these 29 subjects was >3.5% consistent with a diagnosis of beta thalassaemia trait.

Discussion. Hemoglobinopathies are quite common in ethnically diverse Omani subjects and represent a major public health concern.[11] In this context, disease-oriented specific prevention and control programs are essential and particularly relevant in the context of high consanguinity rate in this population.[14] In developed countries, newborn screening accompanied by a continuous, comprehensive care program (CCCP) has significantly reduced the morbidity and mortality rate of SCD.[6] Alkindi et al.[11,15] found that 48.5% subjects showed the presence of Hb Bart's, and 9.5% of the same subjects showed the presence of one of the β-hemoglobin variants namely HbS, HbD, HbE, HbC and beta thalassaemia, although no case of HbH was detected.[11]

In this follow-up study, 290 cases from the original neonatal screening study cohort could be recalled to perform validation using HPLC and molecular confirmation. Amongst the 31 neonates who on the HPLC at birth, did not show any HbA (Group A), five were confirmed as beta thalassaemia major by subsequent HPLC as well as molecular studies. The remaining twenty-six were confirmed as homozygous sickle cell disease subjects by molecular studies. The carrier status in the parents of these 31 subjects was also confirmed, and they were given appropriate counseling. All these children with SCD were referred to the paediatric unit for the implementation of CCCP as the most critical aspect of CCCP is optimizing management by early identification of affected patients, before the onset of signs and symptoms of disease. CCCP caregivers also provide extensive parental education including the prescription of prophylactic penicillin, leading to a substantial reduction in morbidity and mortality in early childhood. Oral penicillin prophylaxis in children with sickle cell disease provides an impressive 85% reduction in the incidence of infection.[16] However, of concern is that there is a 30% fatality rate observed among children with SCD who develop sepsis.[6,16] Because infants with sickle cell disease may develop sepsis as young as four months of age, it is imperative that newborn screening is universally implemented. The five homozygous thalassaemia children are currently receiving regular monthly blood transfusions and chelation therapy.

α-Thalassaemia, the most common genetic disorder occurs widely throughout Africa, the Mediterranean countries, the Middle East and the Southeast Asia.[17-19] It is reported that about 45-65% of the ethnic population in the Sultanate of Oman have α-thalassaemia.[11,20] Type 1 and 2 α-thalassaemia are the commonest α-thalassaemias seen. They are caused by partial (type-2;-α) or total (type-1;--) α-gene deletion, which can give rise to various degrees of impaired ($-\alpha/\alpha\alpha, --/\alpha\alpha, --/-\alpha$) or even completely absent (--/--) hemoglobin α-chain synthesis as well as abnormally low red cell indices (MCV, MCH, MCHC) as seen in our cohort of subjects (**Tables 1** and **2**).

Amongst the other 259 subjects that we were able to recall, 151 subjects at birth had shown the presence of Hb Bart's by the β-thalassaemia short program (Biorad Variant II) (Group B). The presence of Hb Bart's in at birth should lead to investigate the possibility of an underlying alpha thalassaemia. The mean hemoglobin in this cohort was 11.3 g/dl (Range 7.8-13.4), mean MCV was 67.9 (Range 52.3-77.7). MCV followed by MCH were the most important discriminators reflecting the microcytic hypochromic red cell maturation. MCV was the most significant discriminator ($p < 0.89^{E-21}$; **Table 1b**).

We had data on Hb Bart's quantitation at birth in only 72 subjects of the 151 subjects from group B in whom Hb Bart's was detected at birth by Hb Bart's quantitation using α alpha thalassaemia short program (Biorad Variant II) (**Tables 2a** and **2b**). These 72 subjects were further subdivided using Hb Bart's quantitation and Genescan. In 22 subjects Genescan showed a single peak implying an underlying deletion of two alpha genes (**Figure 2**). Using a cut-off of Hb Bart's ≥3% and ≥2% at birth yielded a sensitivity of 90.9% and 95.45% respectively. Further, there was a good correlation with the Hb Bart's detected at birth with molecular validation by GAP PCR performed in the follow-up study. In the remaining 50 subjects who showed two peaks on Genescan, 30 had the two peaks of equal heights implying the presence of 4 normal alpha genes which was confirmed by GAP PCR (**Figure 1**). These patients had Hb Bart's at birth, but it was below 1% by Hb Bart's quantitation. Thus, saying that Hb Bart's is pathognomic of an underlying alpha thalassaemia is not correct. The remaining 20 subjects showed two peaks of unequal heights on Genescan implying one alpha gene deletion (**Figure 2**). Using a cut-off of >1% Hb Bart's at birth yielded a sensitivity of 95%. The highest Hb Bart's levels were seen in subjects with two alpha gene

deletions (**Table 2b**). Further, the hemoglobin, MCV, and MCH also showed an inverse correlation with Hb Bart's quantitation at birth (**Table 2a**). Unfortunately, the alpha thalassaemia short program kits have been currently discontinued by Biorad.

Amongst the remaining hundred and eight subjects, 67 subjects were shown to have the presence of a beta gene structural variants namely HbS, HbD, HbC and HbE (**Table 3**). The mean abnormal hemoglobin at birth respectively for HbS, HbD, HbE was 7.1%, 8.2 and 8.3% which respectively rose to 29.6% (range 22.4-44.1), 33.2% (range 28.9-37.4) and 24.7% (range 22.3-27.1) (**Table 3**). In the single patient with HbC the abnormal hemoglobin rose from 6% to 34.6% in the follow-up study. Thus, in the follow-up study, there was a 100% concordance and validation (including molecular confirmation) with the observations made at birth with regards to beta-globin structural variants.

In the remaining 41 subjects with HbA levels below 10% at birth, it is likely that beta thalassaemia minor was a possibility, as in the absence of HbA2 HPLC cannot show diagnostic discrimination in such cases at birth. Repeat HPLC in the follow-up study showed that amongst these 41 cases only 29 had an elevated HbA2 with the mean HbA2 5.0 (range 3.6-6.2) (**Table 4**), and which was confirmed in all these 29 cases by sequencing the β globin gene including the promoter region, all exons and introns. The mutation found were consistent with the earlier report.[11] Using a cut-off of HbA <10 at birth yielded a sensitivity of only 67%, that improved to 90% with a cutoff of HbA <9 at birth and to 100% with a cut-off of HbA <8.0 at birth.

The study has certain limitations, especially regarding the small sample size. Although the original study cohort included 3740 newborn babies, we were able to recruit only 290 subjects (7.75%) mainly due to logistical reasons. Although there were no cases of HbH (3 genes abnormalities;(—–/−α)) or Hydrops fetalis (4 genes abnormalities; (--/--)) in this study, cases with HbH have been reported in other studies from Oman.[12] Furthermore, we also did not encounter the single gene deletional defect (αα/-α[4.2]) in the follow-up subjects although it had been reported in the initial study.[11]

Conclusions. Although the study population is small, what needs to be highlighted is that in this small cohort of subjects, HPLC and CBC were instrumental in the initial approach to identifying an underlying haemoglobinopathy. This follow-up study found 100% concordance with the results of initial HPLC and CBC results and was this was confirmed with appropriate molecular studies. Hb Bart's ≥ 2% yielded 95.45% sensitivity to identify two α-globin gene deletions, whereas Hb Bart's ≥ 1% yielded 95% sensitivity to identify one α-globin gene deletions. A cut-off of HbA <10 at birth yielded a sensitivity of only 67%, which improved to 90% and 100% with a cutoff of HbA <9 and <8.0 at birth respectively.

Furthermore, the hemoglobin, MCV, and MCH, correlated well with the alpha genotype by GAP PCR and the Hb Bart's level by the quantitation method. Also, MCV ≤ 95 fl and MCH ≤ 30 pg yielded 100% sensitivity to identify two α-globin gene deletions. Considering the simplicity, consistency and rapid results, HPLC can be a major tool in our neonatal screening program.

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