FRONTIERS IN HEMOGLOBINOPATHIES: NEW INSIGHTS AND METHODS

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FRONTIERS IN HEMOGLOBINOPATHIES: NEW INSIGHTS AND METHODS

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Editorial: Frontiers in Hemoglobinopathies: New Insights and Methods

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Editorial on the Research Topic

Frontiers in Hemoglobinopathies: New Insights and Methods

Inherited hemoglobin disorders are estimated to be the most common monogenic diseases worldwide. Deletions or point mutations in α- or β-globin genes cause abnormalities in the synthesis or in the structure of hemoglobin, leading to α and β thalassaemia syndromes or structural hemoglobin variants, respectively. The highest prevalence of both diseases has been recorded in the Mediterranean area, Middle East, Indian subcontinent, Southeast Asia and north coast of Africa, moreover, as a result of migration of ethnic minority groups with high frequency of these mutations, the hemoglobin disorders are also present in northern European and American countries.

Sickle cell anemia (SCA) is by far the most common hemoglobin disorder (Ware et al., 2017), followed by the serious forms of thalassemia syndromes including also the co-inheritance of β thalassaemia with hemoglobin E and hemoglobin S (β-thalassemia major, HbE/β-thalassemia, HbS/ thalassemia, Hb Bart hydrops, HbH disease) (Weatherall, 2010). Considerable research has been carried out in the last decades to broaden our knowledge both on clinical and therapeutic aspects of haemoglobinopathies, to improve the laboratory diagnosis and to introduce diagnostic tools useful for screening programs in areas with a high incidence of these diseases. The aim of this Research Topic is to provide an update on hemoglobinopathies and this ebook collects important and significant reports covering several areas. Advances in laboratory diagnosis are proposed by new diagnostic methods and screening tools for prevention programs and differential diagnosis of anemia. Advances in clinical management and treatment of thalassemia major, thalassemia intermedia and sickle cell anemia are also reported. Diagnosis of hemoglobinopathies requires several laboratory tests (Green et al., 2015) and in this book is reported a review (Munkongdee et al.) that highlights the primary biochemical and molecular techniques commonly used in the clinical lab today, including red blood cell indices (Risoluti et al., 2018), hemoglobin analysis by HPLC and capillary electrophoresis and several molecular technologies for analysis of α and β globin gene mutations.(Nadkarni et al., 2019). The progress in molecular diagnostics has also provided new methods to define the globin gene mutations and correlate genotype to phenotype. A recently applied molecular analysis for the screening of thalassemias is the Next Generation Sequencing (NGS) that has provided interesting preliminary data of an increase in accurate diagnoses and new understanding of these diseases. The main limitation of using NGS techniques is the very high cost for screening purposes. The development of effective and inexpensive techniques for thalassemia and hemoglobin variant screening is extremely important, especially in countries that have populations with a high percentage of these disorders (Risoluti et al., 2016; Colah et al., 2018). The application of thermogravimetry (De Angelis Curtis et al., 2008; Materazzi and Risoluti, 2014; Risoluti et al., 2017) coupled to chemometrics (Massart et al., 1998; Materazzi et al., 2017) as a new screening method to perform an early diagnosis of thalassemia and sickle cell disease has been

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proposed (Risoluti et al.)., providing an innovative multilevel test for Hemoglobinopathies that simultaneously identifies and classifies Sickle Cell Disease from Thalassemia. The novel test is able to simultaneously perform a simple and fast diagnosis of sickle cell anemia or thalassemia, in a single analysis of few microliters of non-pretreated whole blood at low cost, and with high accuracy. This innovative multilevel test has been applied for diagnosis of a case of congenital hemolytic anemia (Risoluti et al.) for which the common first level diagnostic tests were not able to find the erythrocyte congenital defect. The test evidenced the presence of a hemoglobinopathy and the molecular analysis confirmed the presence of a rare hemoglobin variant (Risoluti et al.). National haemoglobinopaties screening programmes are performed in the areas with high frequencies of these diseases and a "one-stop" screening protocol for hemoglobinopathy traits and iron deficiency has been proposed for the detection of carriers of HbE- β-thalassemia and iron deficiency in Sri Lanka (Allen et al.). Infections are major complications and a common cause of mortality and morbidity in thalassemia, being severe anemia, splenectomy, iron overload, and immune abnormalities (Sabbieti et al., 2005) predisposing factors to infections. Moreover, transfusion associated viral infections, especially hepatitis C increase the risk of liver disease leading to liver fibrosis, cirrhosis, and hepatocellular carcinoma. The development of the new effective Direct-acting Antiviral Agents (DAAS) toward the HCV infection is discussed (Maffei et al.) and the HCV eradication followed to DAAS treatment seems to improve the iron overload demonstrating a synergic action between DAAS therapy and iron chelation. Other complications of iron overload are the endocrine comorbidities including hypogonadism, hypothyroidism, diabetes mellitus, and bone diseases. Studies regarding adrenal impairment are heterogeneous in terms of methods used, incidence and populations studied. Hematological and hormonal data of a large group of thalassemia patients associated with their clinical history have been evaluated and showed a prevalence of adrenal insufficiency in thalassemia patients, particularly in male subjects (Poggi et al.). The authors pointed out the limit of the methods usually used to investigate adrenal insufficiency in adult thalassemia patients and highlighted the importance of specific, accurate and validated methods to achieve a real diagnosis. The increase in life expectancy of thalassemia patients has also changed their perspective including their desire to have children. A retrospective study (Sorrentino et al.) describes the experience of pregnancies in women affected by thalassemia major, thalassemia intermedia and sickle cell anemia by applying well-defined protocols that cover all the critical aspects of pregnancy in these diseases. The pregnancies have been followed by a multidisciplinary team from the preconception phase until the post-partum period, with a close monitoring of the maternal and fetal conditions in order to reduce complications and improve their outcomes. A particular focus was placed on SCA and related vascular complications. The hemorheological profile of SCA subjects have been studied (Caprari et al.) and high blood viscosity, increased RBC aggregation, and decreased RBC deformability have been demonstrated. These impaired flow properties were associated with RBC membrane protein oxidation, with degradation of spectrin and increased membrane-bound globin. The comparison between SCA patients with and without transfusion dependence showed metabolic and structural RBC oxidative damage which was significantly different. The hypothesis that gender could play a role in determining the clinical course of SCA was investigated (Ceglie et al.) by analyzing the clinical records of 39 pediatric patients with a diagnosis of SCD (hemoglobin SS genotype) and focusing on gender differences in various aspects of the disease comprising both acute symptoms and late complications. Gender-related differences were found in pain crisis frequency and severe infectious and cardiovascular complications which were significantly increased in the male population. The development and treatment of pain, in particular, neuropathic pain in sickle cell disease patients is poorly understood and impedes our progress toward the development of novel therapies to treat pain associated with sickle cell disease. The orexin/hypocretin system offers a novel approach to treat chronic pain and hyperalgesia (Richardson et al.). Neuronal activation differences in the orexin system as a result of neuropathic pain testing have been studied in a mouse model of sickle cell disease. Identifying specific orexin neuronal populations that are integral in neuropathic pain processing will allow us to elucidate mechanisms that provide a more selective, targeted approach in treating of neuropathic pain in sickle cell disease. Microfluidic technology in sickle cell research has been reviewed by Aich et al. as a valuable tool for a biophysical characterization of sickle red cells, to measure their deformability under defined oxygen gradient and shear, and to test *in vitro* models of intercellular interaction on endothelialized or adhesion molecule-functionalized channels. These studies will allow to understand adhesion in sickle microenvironment, to characterize biomechanics and microrheology, and to develop diagnostic technologies. In conclusion the papers in this e-book collect contributions of different expertises in the field of hematology and analytical methods and will address different specific problems such as novel diagnostic methods, prevention programmes, screening tools and prediction of biomolecules for diagnosis, adding new insights and perspectives in the Hemoglobinopathies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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A "One-Stop" Screening Protocol for Haemoglobinopathy Traits and Iron Deficiency in Sri Lanka

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Introduction: The high frequencies of carriers of severe haemoglobinopathies and of iron deficiency in Southeast Asia require reliable and affordable tests to improve on current screening procedures.

Objectives: We evaluate a "one stop" approach using the THALCON dichlorophenolindophenol (DCIP) and one-tube osmotic fragility (OF) tests and measurement of Zinc Protoporphyrin (ZPP) to detect and distinguish HbE and β-thalassaemia traits from iron deficiency. We compare findings with current screening practice in Sri Lanka that relies on the identification of low mean red cell volume and/or mean red cell hemoglobin for this purpose.

Methods: Between November 2017 and May 2018, we undertook a cross-sectional survey of secondary school students in Gampaha district, Sri Lanka. The THALCON–DCIP and OF tests were compared to capillary electrophoresis (CE), used as a gold standard to detect haemoglobinopathies. ZPP was measured in whole blood. Plasma ferritin and C-reactive protein (CRP) were measured in students with a raised ZPP concentration.

Results: We collected venous blood samples from 1,324/1,332 (99.4%) students. The median age of the students was 17 (IQR 16–18) years, all were Sinhalese and 814/1,324 (61.5%) were female. CE identified 3 students with HbE trait and 26 students with β-thalassaemia trait. The THALCON–DCIP test was positive only in the 3 students with HbE (sensitivity 100%, 95% CI 29.2–100.0; specificity 100%, 95% CI 99.7–100.0). The THALCON–OF test identified all 26 students with β-thalassaemia trait (sensitivity = 100%, 95% CI 86.8–100.0) and 287 students with a normal CE result (specificity = 77.9%; 95% CI 75.5–80.1). It was also positive in 2/3 (66.7%) students with HbE trait. Iron deficiency (ZPP > 70 μmol/mol heme) was present in 118/1,240 (9.5%) students with a normal hemoglobin genotype, all of whom had plasma ferritin <15 ng/ml and CRP <5 mg/L.

Conclusion: This one–stop approach offers reliable and affordable population screening for both haemoglobinopathy traits and iron deficiency in resource-limited settings where these conditions are common and ensures that iron supplements are targeted only to those who require them. Further work is warranted to refine the OF test to reduce the number of false positive results.

Keywords: haemoglobinopathy traits, iron deficiency, osmotic fragility, dichlorophenolindophenol, red cell indices

INTRODUCTION

It is estimated that >7% of the world's population carry a hemoglobin variant, resulting in 300,000–500,000 babies born each year with a significant hemoglobin disorder. Ninety percent of these births occur in low or middle-income countries (Weatherall, 2010). Hemoglobin disorders are estimated to account for at least 3.4% of under-five deaths (Modell and Darlison, 2008).

A recent survey of 23 thalassaemia treatment centers in Sri Lanka identified 1,219 patients with β-thalassaemia major, 360 with HbE/β-thalassaemia, and 50 with sickle β-thalassemia (A Premawardhena, personal communication). Patients with β-thalassaemia major require life-long intensive clinical management and monthly blood transfusion (now referred to as transfusion-dependent thalassaemia TDT). The clinical course of HbE/β-thalassaemia is more variable, ranging from mild (non-transfusion dependent thalassaemia NTDT) to severe anemia. In both conditions, in the absence of early and effective iron-chelation therapy, transfusional iron overload may result in liver and cardiac dysfunction, and endocrine abnormalities including growth disturbances and glucose intolerance. Often, patients experience a severely reduced quality of life, suffer from social stigmatization, and may have difficulties in securing employment. In higher-resource settings, survival now extends to the fifth or sixth decade, but this is not true in lower income settings where the costs and complexities of care mean that survival is curtailed (Cunningham et al., 2004). The cost of care for thalassaemia in Sri Lanka is estimated to be more than 5% of the national health budget each year (de Silva et al., 2000). In a survey of 7,526 school children in Sri Lanka, the frequency of β-thalassaemia and HbE traits varied from 0.0 to 8.1% and 0.0 to 1.9%, respectively, according to district (Premawardhena et al., 2017).

Iron deficiency is the most common micronutrient disorder and causes anemia in up to 30% of individuals throughout the world. In adolescents, anemia impairs school performance and resistance to infection (WHO/UNICEF/UNU Iron deficiency anaemia: Assessment, Prevention and Control, 2001; Black, 2003; Zimmerman and Hurrell, 2007). In the above survey of Sri Lankan school children and adolescents, the frequency of iron deficiency was 19.2% (Allen et al., 2017).

A national haemoglobinopathy screening programme began in Sri Lanka in 2005 with the goal of reducing births of severely affected children. The screening programme targets school children, adolescents and young people, to allow individuals to make informed choices regarding future marriage partners and child-bearing. In view of the high cost of the definitive tests for haemoglobinopathy detection (Capillary Electrophoresis (CE), high-performance liquid chromatography and DNA analysis), in Sri Lanka, as in many Asian countries, population screening relies on detecting low mean red cell volume and/or mean red cell hemoglobin (hereafter "low red blood cell indices") to identify possible carriers of haemoglobinopathies who require further investigation. However, this approach has several limitations. Reliable measurement of red cell indices requires automated hematology analysers that are maintained and calibrated regularly, with quality controls run daily. Accuracy may vary according to the instrument used. Low red cell indices also occur in iron deficiency, complicating differentiation from haemoglobinopathy traits. Finally, red cell indices may be normal in carriers of unusually mild β-thalassaemia alleles and in HbE trait (Weatherall, 2001; Fucharoen et al., 2004; Sanchaisuriya et al., 2005; Galanelo, 2012; Singha et al., 2019) and in people who inherit more than one haemoglobinopathy trait (e.g., α-thalassaemia together with β-thalassaemia (Penman et al., 2015).

The DCIP and OF tests have been proposed as simple visual screening tests for the detection of carriers of HbE and β-thalassaemia, respectively (Sanchaisuriya et al., 2005). The DCIP test, first described in 1976 (Kulapongs et al., 1976), is based on the principle that HbE is an unstable hemoglobin that when added to the blue dye dichlorophenolindophenol, at neutral pH, is oxidized, forming a precipitate (Kulapongs et al., 1976; Old, 2012). In the OF test, thalassaemic red blood cells are more resistant to haemolysis than normal red blood cells because of their small size and low cellular hemoglobin content, and when added to hypotonic buffered saline, typically of 0.36% (w/v) concentration, produce a turbid solution (Parpart et al., 1947; Winichagoon et al., 2002; Old, 2012; Ansari et al., 2014). Both the DCIP and OF tests have been successfully used as screening tests in Thailand since 1990, and reagents available in kit form have been developed and extensively validated. In previous studies, the sensitivity and specificity of the tests when used in combination for the detection of HbE and β-thalassaemia traits ranged between 99.2 and 100% and 79.3–97.1%, respectively (Sangkitporn et al., 2005; Savongsy et al., 2008; Viprakasit and Ekwattanakit, 2018).

We have evaluated the THALCON-DCIP and THALCON-OF tests in 60 parents of patients with either β thalassaemia or HbE/β- thalassaemia attending the Thalassemia Care Unit, North Colombo Teaching Hospital, Ragama, Sri Lanka. The DCIP test identified all 15 parents with HbE-trait. The OF test correctly

identified all 45 parents with β-thalassaemia trait and was also positive in 5/15 HbE traits. There were no false negatives in either test.

The ZPP test is based on the principle that during the final stage of heme synthesis in the bone marrow, if the iron supply is limited or unavailable, zinc, instead of iron, is incorporated into the protoporphyrin ring of the hemoglobin molecule and accumulates in red blood cells (WHO, 2007). The World Health Organization recognizes the usefulness of ZPP as a screening test for iron deficiency in population surveys in low–resource settings (WHO, 2007), and ZPP was used to identify iron deficiency in one of the largest randomized control studies of iron supplements in children in Zanzibar (Sazawal et al., 2006).

We took the opportunity to join the national haemoglobinopathy screening program during its annual survey of secondary schools in Gampaha district, Sri Lanka to evaluate the THALCON-DCIP and THALCON–OF test kits and the measurement of ZPP as a "one-stop" screening procedure for the detection of haemoglobinopathy traits and iron deficiency. We compared this approach to the measurement of low red cell indices, the test currently used by the national screening program.

METHODS

School Surveys

Between November 2017 and May 2018 we recruited students attending 9/172 secondary schools with Advanced Level classes from Gampaha district, Sri Lanka. Schools were purposefully selected so that they were geographically spaced within the Gampaha district. Study personnel visited each school to explain the purpose of the study to students and teachers. All students in grades 10–13 (>14 years of age) were invited to partake. Signed, informed consent was obtained from parents/guardians; all students also provided verbal consent. Any student who reported feeling un-well on the day of the survey was not enrolled into the study. Approximately 150 students, drawn from across the four school years were enrolled from each school. Age, sex, ethnicity, and place of residence were recorded. In students with mixed ethnicity, that of the father was recorded.

Laboratory Procedures

A 2.5 ml venous blood sample was collected into EDTA anticoagulant from each student. Samples were stored in a cool box and transferred to the laboratory within 3 h of collection for measurement of hemoglobin concentration, red cell indices (Beckman Coulter Ac.T diff analyser, Luton, UK), detection of hemoglobin variants by CE (Capillarys 2 Flex Piercing Instrument, Sebia, Lisses, France), and the THALCON- DCIP and THALCON-OF tests (Surathin International Company Ltd., Thailand), in accordance with the manufacturer's guidelines. Briefly, for the DCIP test, 20 μl whole blood was added to 2 ml DCIP solution, gently mixed by inversion and incubated at 37°C for 15 min. 20 μl of clearing solution was then added to each reaction and the results interpreted immediately. For the THALCON-OF test, 20 μl whole blood was added to 2 ml of a 0.36% buffered saline solution, gently mixed by inversion and incubated at room temperature for 5 min before the results were read. In both tests, a turbid pink solution indicated a positive result and a clear pink solution indicated a negative/normal result (see **Figure 1**). Positive and negative control blood samples were included for each set of tests.

ZPP was measured in whole blood using a front-faced haematofluorimeter and Protofluor reagent system (Helena BioSciences, South Shields, UK). The remaining samples were centrifuged, the buffy coats and plasma removed and stored at −20°C for further analyses. Plasma CRP and ferritin were measured by Enzyme Linked Immunosorbent assay (DCRP00, Biotechne, UK and DB59111, Tecan, UK, respectively) in samples with a raised ZPP to confirm iron deficiency and to explore whether inflammation may have resulted in a raised ZPP result.

To investigate possible causes of a positive OF test in samples with a normal CE result, DNA was extracted from buffy coat samples using QIAGEN DNA mini-kit (QIAGEN, UK) and α- globin genotype determined by Multiplex polymerase chain reaction (Tan et al., 2001; Shaji et al., 2003).

Statistical Methods

Categorical variables were summarized using counts and percentages. Continuous variables tended to have skewed distributions; they were described using median and interquartile

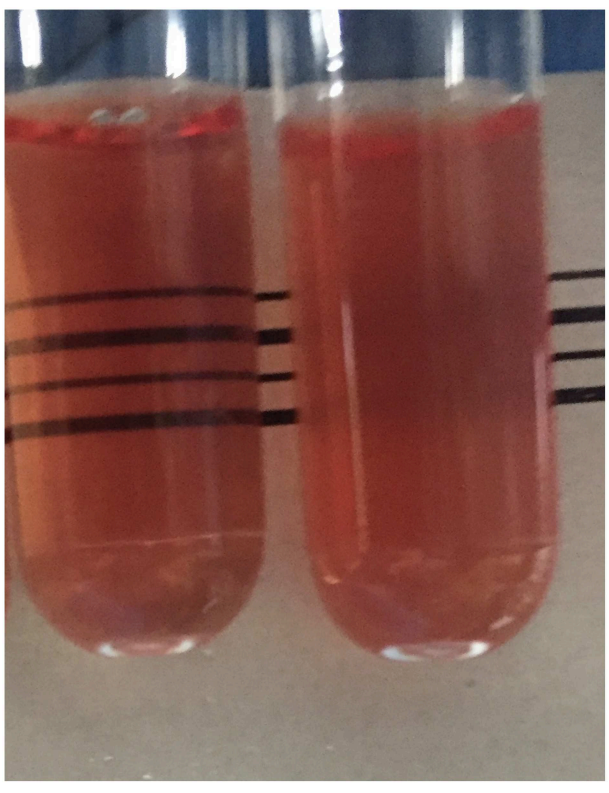

FIGURE 1 | The clear pink solution observed in the tube on the left indicates a normal test result, whereas the turbid pink appearance of the tube on the right indicates a positive test result.

range and compared using the Mann-Whitney *U* test. Sensitivity and specificity and the corresponding 95% confidence intervals (CI) of the DCIP and OF tests were calculated with capillary electrophoresis (CE) as the gold standard. Considering the small number of positive results for the DCIP test, the exact 95% CIs were obtained. All the estimates whose corresponding 95% CI did not span 50% (i.e., proportion = 0.5) were considered significant and all tests were performed at 5% significance level. All data analysis was performed using SPSS statistical software version 25.

Ethical Approval

The study and the consent procedures were approved by the Ethics Committee, University of Kelaniya, Sri Lanka and Oxford University Tropical Research Ethics Committee, Oxford, UK. The study was conducted in accordance with the declaration of World Medical Association (2008).

RESULTS

A total of 1,332 students were enrolled and venous blood samples were collected from 1,324 (99.4%). The 8 students for whom it was not possible to obtain a blood sample have been excluded from the analysis. The median age of the students was 17 (IQR 16–18) years; all were Sinhalese and 814/1,324 (61.5%) were female.

The sensitivity and specificity for the screening tests are shown in **Table 1**. Laboratory findings according to hemoglobin genotype and sex are shown in **Supplementary Table S1**.

Haemoglobinopathy Traits

CE identified hemoglobin variants in 29 (2.2%) students. Three students were HbE trait and the DCIP test was positive only in these 3 samples (sensitivity = 100%, 95% confidence interval (CI) 29.2–100.0; specificity = 100%, 95% CI 99.72–100.0). Twenty-six students had raised HbA_2, indicative of β-thalassaemia trait. The OF test correctly identified all 26 students with β-thalassaemia trait but was also positive in 287 samples with normal CE results (sensitivity = 100%, 95% CI 86.8–100.0; specificity = 77.9%, 95% CI 75.5–80.1). The OF test was also positive in 2 of 3 students with HbE trait. Overall, in the detection of either β-thalassaemia trait or HbE trait, the combination of the DCIP and OF tests had a sensitivity of 100% (95% CI 88.1–100.0) and specificity of 77.8% (95% CI 75.5–80.1).

Low Red Cell Indices

Low red cell indices (mean cell volume (MCV) <80 fl or mean cell hemoglobin (MCH) < 27 pg) were present in all 26 students with β-thalassaemia trait and the 3 students with HbE trait but were also present in 409/1,293 (31.6%) students with a normal CE result (sensitivity = 100%; 95% CI 88.1–100.0; specificity = 68.4%, 95% CI 65.8–70.9).

Iron Deficiency

ZPP was raised (>70 μmol/mol heme) in 141/1,314 (10.7%) students including 14/26 (53.8%) with β-thalassaemia trait and 9 students with α-thalassaemia trait. In 118/1,240 (9.5%) students with a normal hemoglobin genotype and raised ZPP, all had normal plasma CRP (<5 mg/L) and low plasma ferritin concentrations (<15 ng/ml), confirming iron deficiency.

In the 14 students with β-thalassaemia trait and raised ZPP, plasma CRP was normal in 12/14 (85.7%) and ferritin in 13/14 (92.9%); only one student had iron deficiency.

Sufficient plasma was available to measure CRP and ferritin in 8/9 students with α-thalassaemia trait and a raised ZPP. Iron deficiency was confirmed 7/8 (87.5%) students; one student had a normal plasma ferritin and a raised CRP.

TABLE 1 | Sensitivity and specificity of screening tests for haemoglobinopathy traits compared to capillary electrophoresis as the gold standard.

Test		Capillary electrophoresis		Sensitivity (95% CI)	Specificity (95% CI)
		Pos	Neg		
Capillary electrophoresis					
HbE trait		3	1,321		
β-thalassaemia trait		26	1,298	-	-
HbE trait or β-thalassaemia trait		29	1,295		
Thalcon DCIP test (HbE)	**Pos**	3	0	100.0	100.0
	Neg	0	1,321	(29.2–100.0)	(99.7–100.0)
Thalcon OF test (β-thalassaemia trait)*	**Pos**	26	287	100.0	77.9
	Neg	0	1,009	(86.8–100.0)	(75.5–80.1)
Thalcon DCIP and Thalcon OF	**Pos**	29	287	100.0	77.8
	Neg	0	1,008	(88.1–100.0)	(75.5–80.1)
Low red cell indices (MCV<80 fl or MCH < 27 pg)	**Pos**	29	409	100.0	68.4
	Neg	0	884	(88.1–100.0)	(65.8–70.9)

**The two students with HbE trait and positive OF test are excluded.*

ANALYSIS OF UNEXPLAINED-POSITIVE OF TESTS

α-globin Genotype

Amongst the 287 students with an unexplained positive OF result, sufficient DNA was available to determine α-globin genotype in 286/287 (99.7%). Forty-five students had α-thalassaemia; 35 with $-\alpha^{3.7}/\ \alpha\alpha$, 4 with $-\alpha^{3.7}/-\alpha^{3.7}$ and 6 with $-\alpha^{4.2}/\ \alpha\alpha$. The less common α-globin gene mutations $--^{SEA}$, $--^{FIL}$, $--^{MED}$, $--^{THAI}$, $-\alpha^{-20.5}$ were not present in any sample.

Low Red Cell Indices and Anemia

Mean cell volume, mean cell hemoglobin and hemoglobin concentration were measured in 235/242 (97.1%) of the remaining students with an unexplained positive OF test, and median values were similar to those of 1,005 students with a true negative OF result (**Figures 2A–D**). However, the frequency of low red cell indices and of anemia in the two groups were significantly different (85/235 (36.2%) and 280/1,005 (27.9%; $p = 0.0137$), respectively for low red cell indices and 75/235 (31.9%) and 202/1005 (20.1%; $p = 0.0002$) for anemia.

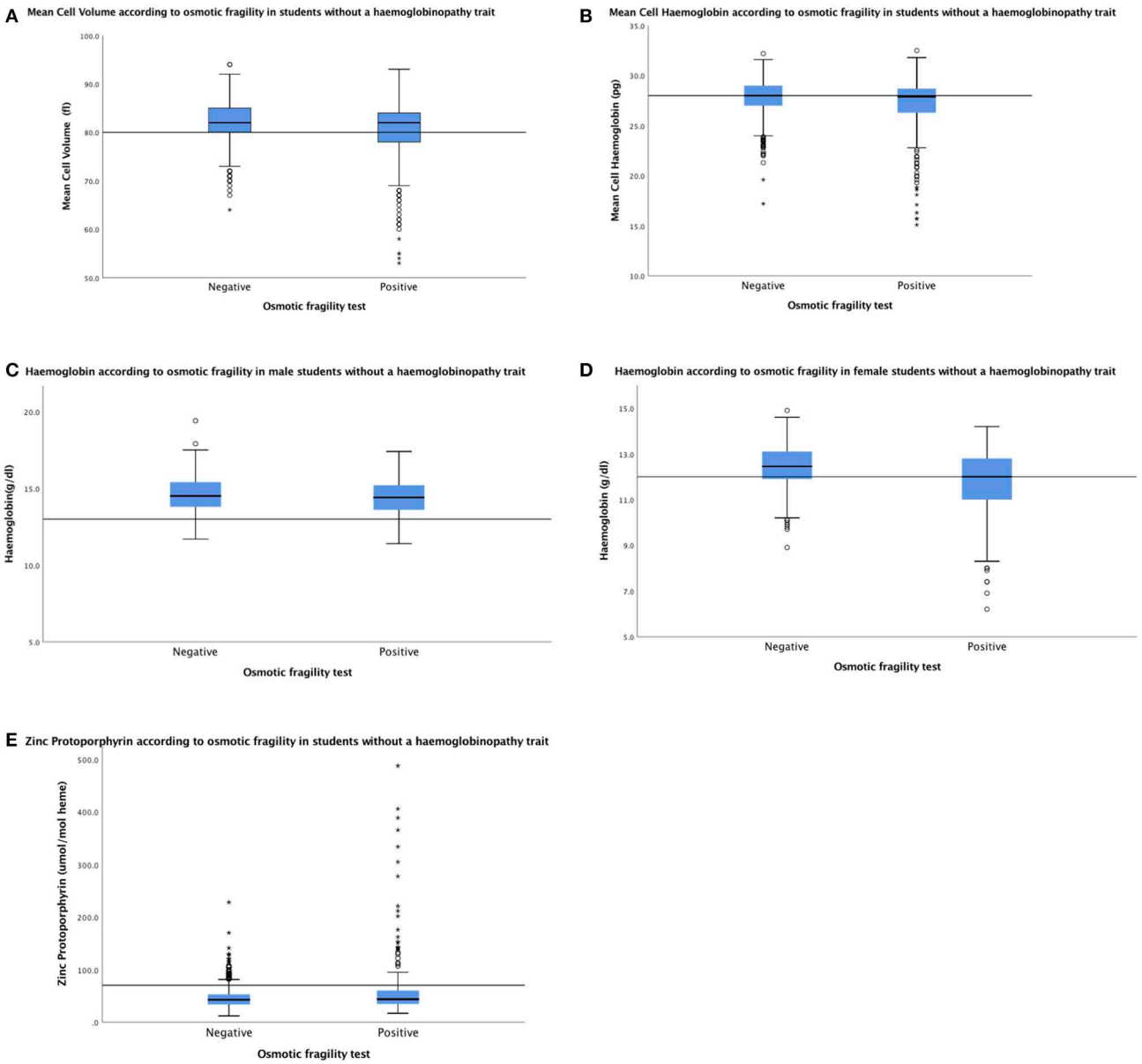

FIGURE 2 | Median (IQR) MCV **(A)** and MCH **(B)** were similar in 235 students with an unexplained positive OF result and 1,005 students with a true negative OF result: 82.0 fl (77.0–84.0) and 27.8 pg (25.7–28.6) vs. 82.0fl (80.0–85.0) and 28.0 (26.9–29.0), respectively. Median (IQR) Hb was 14.4 g/dl (13.6–15.2) and 12.0 g/dl (10.9–12.8) in 96 males **(C)** and 139 females **(D)**, respectively, with an unexplained positive OF result vs. 14.5 g/dl (13.8–15.4) and 12.5 g/dl (11.9–13.1) in 383 males and 622 females with a true negative OF result. Median (IQR) ZPP was similar in 235 students with an unexplained positive OF result and 1,005 with a true negative OF result; 44 μmol/mol heme (add IQR) and 43 μmol/mol heme (add IQR), respectively **(E)**. Horizontal lines inside the box show the median value, box length is the interquartile range and whiskers show the range, excluding outliers. Outlying values 1.5–3, or > 3 box lengths from the upper and lower edge of the box are shown as open circles and stars, respectively. Reference lines shows the lower limit of the normal range for MCV, MCH, and Hb and the upper limit of the normal range for ZPP.

Iron Deficiency

Median ZPP was similar in 235 students with an unexplained positive OF result and 1,005 with a true negative OF result; 44 μmol/mol heme and 43 μmol/mol heme, respectively (**Figure 2E**). However, the frequency of raised ZPP was greater in students with an unexplained positive OF test (47/235; 20.0%) than those with a true negative result (77/1,005; 7.7%; $p < 0.0001$).

DISCUSSION

The THALCON-DCIP and THALCON-OF tests performed well in our hands, were quick and easy to use and required minimal laboratory equipment. The inclusion of a clearing agent in the DCIP kit, used to decolorize the blue DCIP dye at the end of the incubation period, allowed the tests to be read easily.

Although the THALCON–DCIP test had 100% sensitivity and specificity for carriers of HbE, the low number of cases in our study resulted in a wide 95% confidence interval for sensitivity (29.2–100.0). Low red cell indices, the current screening criteria used in Sri Lanka, also identified all HbE carriers in this study. However, low red cell indices are not always a feature of HbE trait, and we have reported previously that in a larger survey of 7,526 secondary school students in Sri Lanka, red cell indices were normal in 3/38 (7.9%) of students with HbE trait (Premawardhena et al., 2017). Similarly, in a study of pregnant women in Thailand, 14/93 (15.5%) HbE traits had normal red cell indices (Sanchaisuriya et al., 2005). Further evaluation of the THALCON–DCIP test in populations with a low frequency of HbE carriage will require large numbers. For example, In Sri Lanka where the overall prevalence of HbE trait is 0.23%, with a lower 95% confidence limit for sensitivity to be >90% and 0.80 probability, a sample size of 18,261 participants would be required and 53,479 participants would be required for the lower 95% confidence limit for sensitivity to be >95%.

The THALCON-OF test identified all carriers of β-thalassaemia, and the greater prevalence of this haemoglobinopathy in Sri Lanka allowed a more reliable assessment of sensitivity (95% CI 86.8–100.0). Use of low red cell indices had a similar sensitivity. Although the THALCON-OF test identified many false positives (specificity 77.9%; 95% CI 75.5–80.1), specificity was better than when using low red cell indices (specificity = 68.4%; 95% CI 65.8–70.9). Using low red cell indices would have resulted in 438/1,322 (33.1%) samples requiring expensive confirmatory tests compared to 315/1,324 (23.7%) with the OF test (representing approximately 30% fewer tests).

Although ZPP can be raised in conditions other than iron deficiency, such as inflammation and some haemoglobinopathies (Thomas et al., 1977; Labbé, 1992; WHO, 2007; Parischa and Drakesmith, 2016), we found that the inclusion of ZPP measurement in this study was helpful in identifying students who were truly iron deficient as confirmed by reduced plasma ferritin and normal CRP concentrations, excluding inflammation. However, we did not measure CRP and ferritin in students with a normal ZPP and it is possible that some students with earlier stages of iron deficiency may have been missed. Raised ZPP is also useful in idenitfying people with lead poisoning (Thomas et al., 1977; Labbé, 1992; WHO, 2007; Parischa and Drakesmith, 2016); however, this is unlikely to be common in Sri Lanka following the introduction of unleaded petroleum in 2002 (Senanayake et al., 2004).

Almost one third of this study population had low red cell indices. Because low indices are a feature of both iron deficiency and some haemoglobinopathy traits, current screening practice in Sri Lanka means that staff are often faced with a dilemma when deciding on further investigations to identify the cause of the low indices. In addition, we have reported that low red cell indices not attributable to either iron deficiency or haemoglobinopathy traits are also common in this population (Allen et al., 2017; Rodrigo et al., 2018). Because the definitive tests for haemoglobinopathy traits are expensive and iron deficiency is more common in Sri Lanka, screening staff recommend that individuals with low indices repeat their full blood count after a 3–6 month course of oral iron, to exclude iron deficiency as the cause. If at follow-up the red cell indices remain low, the definitive test is then performed. Based on these current screening procedures, in this study, iron supplements would have been recommended to 438 (33.1%) students with low red cell indices. However, by measuring ZPP in combination with the DCIP and OF tests in a one-stop procedure this number would be reduced to 77/1,005 (7.7%) students, representing a considerable saving. Moreover, the risk of loss to follow-up that occurs with current screening practice would be limited. Avoiding the unnecessary use of iron supplements is important given that tolerance of oral iron is poor and iron increases the risk of infection (Murray et al., 1978; Drakesmith and Prentice, 2012; Kortman et al., 2012). Furthermore, the high frequency of the H63D variant of the haemochromatosis gene and the presence of haemoglobinopathy traits in Sri Lanka (Premawardhena et al., 2017; Allen et al., 2019) mean that it is important to ensure that iron supplements are targeted to only those who require them, to avoid the possible deleterious effects of increased iron availability in iron replete individuals.

Although our numbers were small, ZPP appeared to be a reliable marker of iron deficiency in α-thalassaemia but not in β-thalassaemia traits. Our findings in β-thalassaemia trait are concordant with other studies but are discordant with studies for α-thalassaemia (Tillyer and Tillyer, 1994; Graham et al., 1996). Additonal confirmatory tests for iron deficiency are required for students with haemoglobinopathy traits before recommending iron supplements.

Consistent with other studies of OF (Kattamis et al., 1981; Jopang et al., 2009), we observed a significant number of false positive results with the THALCON-OF test. DNA analysis confirmed that α-thalassaemia accounted for the positive OF result in about 20%, and low red cell indices, anemia or iron deficiency may also have contributed. It is possible that the concentration of saline used in the test (0.36%) was too high for the complete lysis of normal red blood cells, and using a weaker saline solution may have reduced the number of false positives. Supporting this hypothesis is a study of haemoglobinopathy traits in a rural population of Thai Khmer, in which a buffered saline solution of 0.34% reduced OF false positivity without compromising the sensitivity of the test (Fucharoen et al., 2004).

We consider that the combination of the OF, DCIP, and ZPP assays is a useful approach to screening for haemoglobinopathy traits and iron deficiency in population surveys in Sri Lanka, and has several advantages over the use of low red cell indices alone. These findings are directly relevant to populations in other low and middle-income countries in which haemoglobinopathies and iron deficiency are common. The study was conducted under research conditions by trained laboratory staff, and now needs to repeated in routine and remote settings, where laboratory expertise may be variable.

Regardless of the screening methods used, there is a pressing need to establish external quality assessment (EQA) programmes to monitor how well the tests are performed and interpreted by local staff, to ensure the accuracy and success of all haemoglobinopathy screening programmes. Indeed, an

EQA programme implemented in Thailand has been shown to improve the performance of many screening laboratories (Prommetta et al., 2017).

CONCLUSION

A combination of OF, DCIP, and ZPP assays improves upon current screening procedures for haemoglobinopathy traits that are currently based on identification of low red cell indices, and also identifies iron deficiency in a one-stop procedure. Further work should be undertaken to refine the OF assay in an effort to reduce the number of false positives. Further research should evaluate the performance and cost effectiveness of this one-stop approach in routine and remote laboratory settings.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AA, DW, NO, SA, and AP designed the study. AA, SP, LP, RR, and CF performed the laboratory work. SP, LP, SM, IS, and NH performed the field work. SP performed the data entry. AA, AM, and SA performed the statistical analysis. AA and SA wrote the manuscript and all authors reviewed and approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2019.00066/full#supplementary-material

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BRIEF RESEARCH REPORT
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TGA/Chemometric Test Is Able to Detect the Presence of a Rare Hemoglobin Variant Hb Bibba

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In this study the TGA/Chemometric test was applied for diagnosis of a case of congenital hemolytic anemia for which the common first level diagnostic tests were not able to find the erythrocyte congenital defect. A 6 years old girl presented chronic hemolytic anemia characterized by hyperbilirubinemia, increased spleen, negative Coombs tests, normal hemoglobin values, decreased mean corpuscular volume (MCV), increased red cell distribution width (RDW), reticulocytes and lactate dehydrogenase (LDH), and altered erythrocyte morphology (ovalocytes, spherocytes, and rare schizocytes). The diagnostic protocols for differential diagnosis of hereditary hemolytic anemia were carried out by the investigation of the congenital hemolytic anemias due to defects of membrane proteins and the most common erythrocyte enzymes, but no defect was found. The TGA/Chemometric test was applied and the PLS-DA model of prediction was used to process results. The thermogravimetric profile of the patient was very distinct from those of healthy subjects and comparable with those of thalassemia patients. The classification model applied to the patient identified a chronic hemolytic anemia due to a hemoglobin defect and the molecular characterization confirmed the TGA/Chemometrics results, demonstrating the presence of a very rare hemoglobin variant Hb Bibba ($\alpha_2$136(H19)Leu $\rightarrow$ Proβ_2). In conclusion the TGA/Chemometric test proved to be a promising tool for the screening of the hemoglobin defects, in a short time and at low cost, of this case of congenital hemolytic anemia of difficult diagnosis. This method results particularly suitable in pediatric patients as it requires small sample volumes and is able to characterize patients subjected to transfusion.

Keywords: thermogravimetric analysis, chemometrics, Hb Bibba, hemoglobin defect, screening

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INTRODUCTION

The differential diagnosis of hereditary hemolytic anemias (Haley, 2017) is generally carried out by applying different diagnostic protocols depending on the specific congenital erythrocyte defects such as hereditary erythrocyte enzyme deficiencies (Grace and Glader, 2018), RBC membrane proteins defects (King and Zanella, 2013), or hemoglobinopathies (Cao and Galanello, 2010).

Hemoglobin disorders are characterized by pathologic defects on globin chain synthesis: quantitative defects that give rise to thalassemia (mainly α and β thalassemia); qualitative defects,

namely hemoglobinopathies, that are due to structural hemoglobin variants; hereditary persistence of fetal hemoglobin. These globin defects determine a wide array of heterogeneous thalassemia syndromes and related diseases. The diagnosis of hemoglobinopathy could be particularly difficult in neonatal period, especially when transfusions are needed to compensate anemia. Generally, the protocol for hemoglobinopathy screening includes the whole blood count followed by the determination of hemoglobin A_2 (HbA_2) and hemoglobin F (HbF) levels, as well as the identification of any rare variant present by Hb electrophoresis or high performance liquid chromatography. Nevertheless, there do exist silent β thalassemic mutations with normal HbA_2 levels or borderline elevated HbA_2 values of difficult interpretation (Weatherall and Clegg, 2001). These methods need equipment, time and personnel with expertise in the interpretation of the screening results. A positive screening test still needs to be confirmed by molecular analysis of the globin genes (Cao et al., 2002; Giardine et al., 2014).

Thermoanalytical techniques such as thermogravimetry, proved to be versatile tools able to investigate different materials (Di Donna et al., 2004; De Angelis Curtis et al., 2008; Skreiberg et al., 2011; Fonseca et al., 2012; Materazzi and Risoluti, 2014; Materazzi et al., 2014c; Aiello et al., 2015; Shan-Yang et al., 2015; Papadopoulos et al., 2016; Marcilla et al., 2017; Risoluti et al., 2017). In addition, hyphenated techniques based on thermogravimetry demonstrated the improvement in the ability of this approach to further characterize samples for different applications (Materazzi et al., 2014a,b, 2015; Risoluti et al., 2016c).

Recently, the feasibility of thermogravimetry coupled to chemometrics in processing complex matrices is emerging as rapid and effective tool allowing the multiparametric analysis of different samples (Khanmohammadi et al., 2012; Caramés-Alfaya et al., 2013; Strzemieck et al., 2014). The main advantage of this approach consists of the possibility to identify significant and diagnostic differences in the data and to simultaneously correlate results from different measurements. Therefore, thermoanalytical data obtained from the TGA may be dependent on more than one variable simultaneously (corresponding to the different increase in temperature) or may be a results of specific experimental design where variables such as the concentration, pH, polymer, or metal ligands are randomizing modified to assess the most performing results. In both cases, a chemometric approach based on multivariate analysis becomes very useful as the nature of data is multivariate.

Regardless to clinical application, thermogravimetry coupled with chemometrics demonstrated to be an effective diagnostic tool for β-thalassemia screening requiring short times and low costs of analysis (Risoluti et al., 2016b). This model, consisting of Partial Least Square-Discriminant Analysis (PLS-DA), permitted the discrimination of thalassemic patients and healthy individuals, using the thermogravimetric curves of blood samples. In addition, the TGA screening test allowed differentiating thalassemia patients according to disease clinical severity and was not influenced by drug therapies, such as aspirin, commonly used to prevent thromboembolic events in thalassemia patients after splenectomy (Romolo et al., 2015; Catauro et al., 2018; Risoluti et al., 2018a,b, 2019).

The objective of this study was the application of the TGA/Chemometric test, previously used for thalassemia diagnosis, to investigate a rare case of chronic hemolytic anemia of difficult interpretation, for which the first level tests of the conventional diagnostic protocols were not able to find the erythrocyte congenital defect.

MATERIALS AND METHODS

Blood Samples

In this study we have analyzed blood samples from β-thalassemia patients (65 subjects) and healthy individuals (120 subjects) that were obtained according to guidelines established by the Ethical Committee for human subject studies, in accordance with the 1975 Helsinki Declaration, revised in 2008. All the participants provided their written informed consent to participate in this study and copies of the informed consent are available on request. In the case of the child, the written informed consent was obtained from the parents.

Patient

A 6-year-old Italian girl, followed at the U.O. Hematology of the Bambino Gesù Pediatric Hospital of Rome (Italy), was suffering from chronic hemolytic anemia of a nature not determined. The clinical picture was characterized by anemia, hyperbilirubinemia, and an increased spleen volume. Laboratory tests demonstrated negative direct and indirect Coombs tests, and increased LDH value that suggested the presence of an erythrocyte congenital defect as cause of the hemolytic anemia. Therefore, investigations have been carried out for the screening of congenital hemolytic anemias from hemoglobin disorders, defects of membrane proteins and the most common red blood cell enzymopathies.

Hematological Analyses

Blood samples were collected in K_2EDTA and the hematological parameters red blood cell counts (RBC), Hb, MCV, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and RDW, reticulocyte counts (Ret) were measured by an automated hematology analyzer (ADVIA 120; Siemens, USA). Laboratory examinations also included the evaluation of red cell morphology on peripheral blood smears, the determination of bilirubin, serum ferritin, haptoglobin, transferrin, hemoglobin fractions Hb A_2, Hb F, Hb S, and Hb C, osmotic fragility test, acidified glycerol lysis time ($AGLT_{50}$) (King and Zanella, 2013) and the activities of erythrocyte enzymes glucose-6-phospate dehydrogenase (G6PD), pyruvate kinase (PK), esokinase (HK), 6-phosphogluconate dehydrogenase (6PGD) (Caprari et al., 1991). The membrane proteins analysis was performed by SDS-PAGE of erythrocyte membranes (Caprari et al., 1999).

Genetic Analysis for Globin Mutations

Molecular analysis of DNA sequences of β and α globin genes were carried out, at the Microcitemie Center in Rome, by direct sequencing on the Beckman Coulter CEQTM8000 Genetic

Analysis System (Beckman Coulter Inc., Fullerton, CA, USA) as previously described (Amato et al., 2012).

Thermogravimetric Analysis (TGA)

A Perkin Elmer TGA7 Thermobalance (Massachusetts, USA) was used to acquire the thermogravimetric curves. About 30 μl of whole blood was placed into the crucible with no pretreatment. Temperature was measured using a thermocouple directly attached to the crucible and was raised from 20 to 800°C, with a 10°C/min heating rate, as the best resolution rate. The atmosphere was air as carrier gas at 100 ml/min flow rate. Calibration of the thermobalance was performed using the Curie-point transition of standard metals, as specified by the equipment recommendations and a number of three replicates for each sample were acquired to ensure reproducibility. Derivative Thermogravimetric data (DTG) were also calculated to compare samples and represent the derivative of the function TG(T) with respect T.

Analytical Strategy and Chemometrics

Chemometrics was used to process the thermogravimetric curve of the anemic patient and to compare results of the mass changes as a function of temperature with those of healthy and thalassemia subjects from the collected dataset.

Multivariate statistical analysis based on Principal Component Analysis (PCA; Risoluti et al., 2016a; Materazzi et al., 2017c) was used as exploratory method, while Partial Least Square Linear Discriminant Analysis (PLS-DA; Savitzky and Golay, 1964; Barker and Rayens, 2003; Materazzi et al., 2017a,b) was applied as the classification model of prediction. Each blood sample was analyzed three times and data was reported as means ± standard deviations. The Pyris software (Thermo Fisher Scientific Inc., Waltham, MA, USA) was utilized for diagnostics and acquisition of the thermogravimetric curves and data were exported as ASCII files. The Unscrambler package by Camo was used to perform statistical analysis.

RESULTS

Screening Tests for Congenital Hemolytic Anemias

The diagnostic protocols for differential diagnosis of congenital hemolytic anemia were applied and the results are reported in **Table 1**. The hematological data showed the presence of hemolytic anemia as demonstrated by the increase in total and indirect bilirubin, and LDH values and the low haptoglobin level. The full blood count revealed a decrease in the RBC, Hb, MCV, and MCH values, an increase in the RDW value and reticulocytes count, while the peripheral blood smear showed anisopoikilocytosis and the presence of ovalocytes, spherocytes, schistocytes, and dacryocytes suggesting congenital alterations of erythrocyte morphology. The application of the screening tests for spherocytic hemolytic anemia did not evidenced osmotic fragility of the erythrocytes (**Table 1**) and the study of erythrocyte membrane proteins did not detect qualitative and quantitative abnormalities of the membrane proteins (data not showed). The screening tests for thalassemia and hemoglobinopathies demonstrated normal HbA_2 and HbF values, no presence of other hemoglobin variants such as Hb C or Hb S, therefore the presence of hemoglobinopathy was excluded. The study of the enzymatic activities of red blood cell metabolism did not show enzyme defects, while the erythrocyte activities were increased consistent with a chronic hemolytic state and reticulocytosis (**Table 1**).

TABLE 1 | Hematological data of the patient.

	Value	Reference intervals
RBC (10^6/μL)	3.92	3.8–4.8
Hb (g/dL)	8.8	10.5–15.5
Hct (%)	30.4	33–40
MCV (fL)	73.5	75–95
MCH (pg)	22.4	25–35
MCHC (g/dL)	29.0	31–36
RDW (%)	21.8	12–15
Reticulocyte (%)	9.9	0.2–2.0
Total bilirubin (mg/dL)	2.75	0.25–1.00
Direct bilirubin (mg/dL)	1.08	0.08–1.00
Haptoglobin (mg/dL)	1	30–200
LDH (IU/L)	688	230–470
Transferrin (mg/dL)	241	200–365
Ferritin (mg/dL)	56	10–290
Hb A_2 (%)	2.5	2.0–3.2
HB F (%)	0.9	0–2.0
Hb S (%)	Absent	Absent
Hb C (%)	Absent	Absent
$AGLT_{50}$ (s)	>1800	>1800
G6PD (IU/g Hb)	16.0	7.0–9.6
6PGD (IU/g Hb)	16.4	7.7–9.6
PK (IU/g Hb)	35	12.0–16.3
HK (IU/g Hb)	3.0	0.8–1.4

TGA/Chemometric Test

In order to search for an explanation of the hemolytic anemia and to verify the diagnostic power of the TGA/chemometric screening test, the thermal behavior of the unknown blood sample was estimated by thermogravimetry (**Figure 1A**, solid line) and the characteristic thermally induced decomposition processes under combustive conditions were investigated by calculating the derivative thermogravimetric curve (DTG) of the TG (**Figure 1A**, dashed line).

In accordance with previously findings (Risoluti et al., 2016b), three main releasing steps can be observed in **Figure 1A**, corresponding to the loss of water (occurring between 50 and 200°C) and the decomposition of the corpuscular fraction of blood (around 350 and 550°C). Two different processes may be described during water release: the first, at lower temperatures (20–130°C) is related to the bulk water release (56.3%) while the second peak (130–180°C) may be attributed to the bound

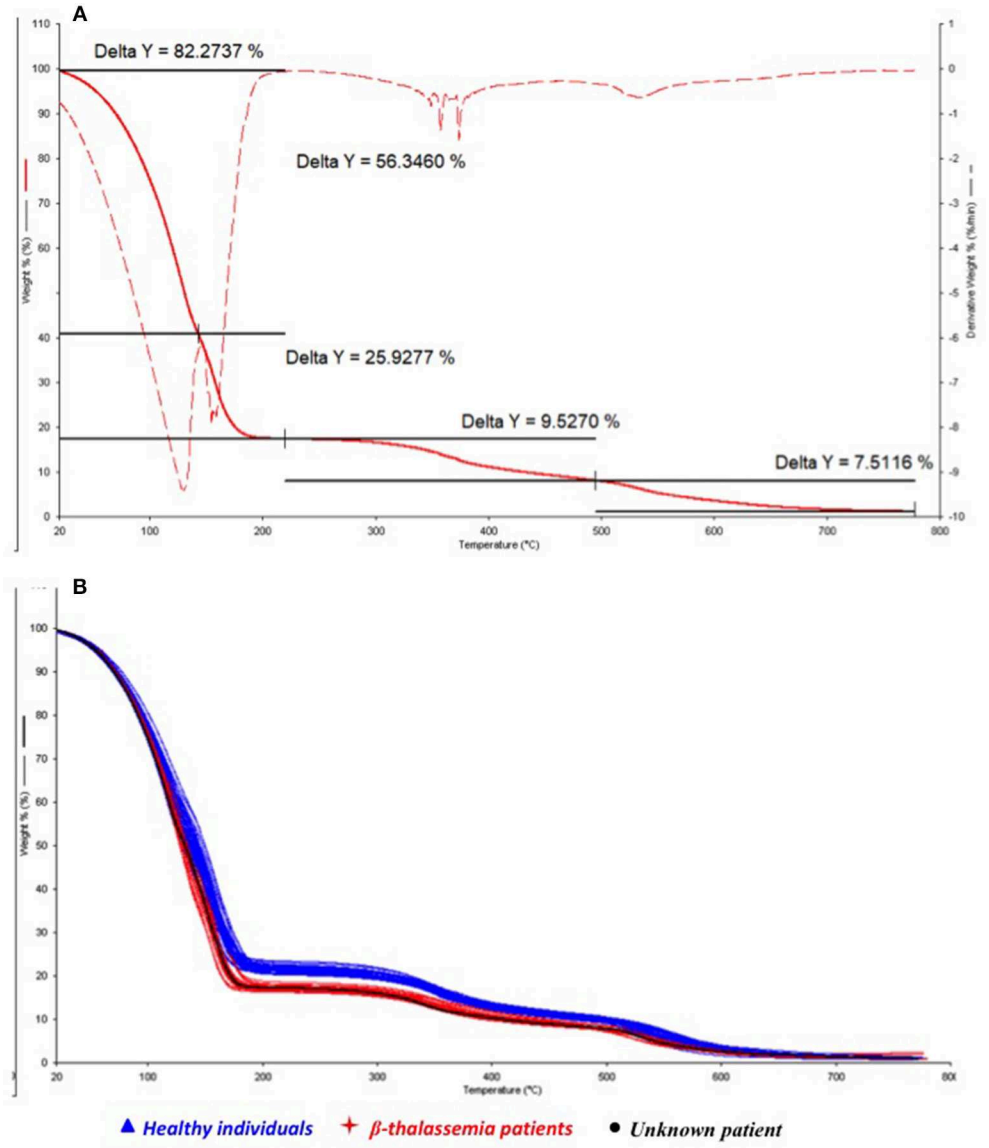

FIGURE 1 | Overlapped thermogravimetric (TG) and Derivative Thermogravimetric (DTG) curves of the hemolytic anemia patient **(A)** and comparison **(B)** of the TG profile of the hemolytic anemia patient (black) with healthy subjects (blue) and thalassemic patients (red).

water release (25.9%). The remaining processes lead to a percentage weight losses of 9.5 and 7.5% with a final residue close to zero.

The thermal profile of the patient's samples (black line) was consequently compared to those observed for the healthy and thalassemia subjects, respectively, reported in blue and red in **Figure 1B**, where the TG curves are overlapped. Results of the integration of the decomposition processes for all the investigated samples are reported in **Table 2**.

A significant lower amount of water content (p-value of 1.5 E-17) was observed in healthy subject with respect to thalassemic ones, and a consequent lower value of the bulk/bound water ratio. On the contrary, the corpuscular fraction of blood was found to be higher in healthy subjects than thalassemia patients. The thermogravimetric profile of the patient's blood (**Figure 1**) was very distinct from those of healthy subjects and comparable with that of thalassemia subjects. In particular, the water amount of the patient was found to be within the group of thalassemics, as well as the decomposition processes of the corpuscular fraction.

The acquired TG curves were processed by the novel test TGA/Chemometrics and Principal Component Analysis algorithm was used to display results. In **Figure 2A**, the resulting scores plot exhibits a significant separation of the samples according to the presence or the absence of the anemic traits, resulting in two clusters of samples, the healthy subjects (blue)

TABLE 2 | Thermogravimetric features of the hemolytic anemia subject (patient) in comparison with β-Thalassemia patients (T) and healthy subjects (CTR) groups.

	Water content (%)	Bulk water (%)	Bound water (%)	Bulk/bound water ratio	2nd weight loss (%)	3rd weight loss (%)
CTR	77.7 ± 0.9	48.4 ± 5.3	29.3 ± 4.0	1.7 ± 0.6	11.4 ± 0.7	9.2 ± 0.6
T	82.5 ± 1.2	57.3 ± 6.5	25.2 ± 5.9	2.5 ± 1.0	9.0 ± 0.7	6.9 ± 0.7
p-value	1.5E–17	2.3E–05	2.0E–02	2.2E–02	4.7E–12	2.6E–15
Patient	82.5 ± 0.1	51.9 ± 2.6	30.9 ± 2.8	1.8 ± 0.4	8.6 ± 0.1	6.5 ± 0.2

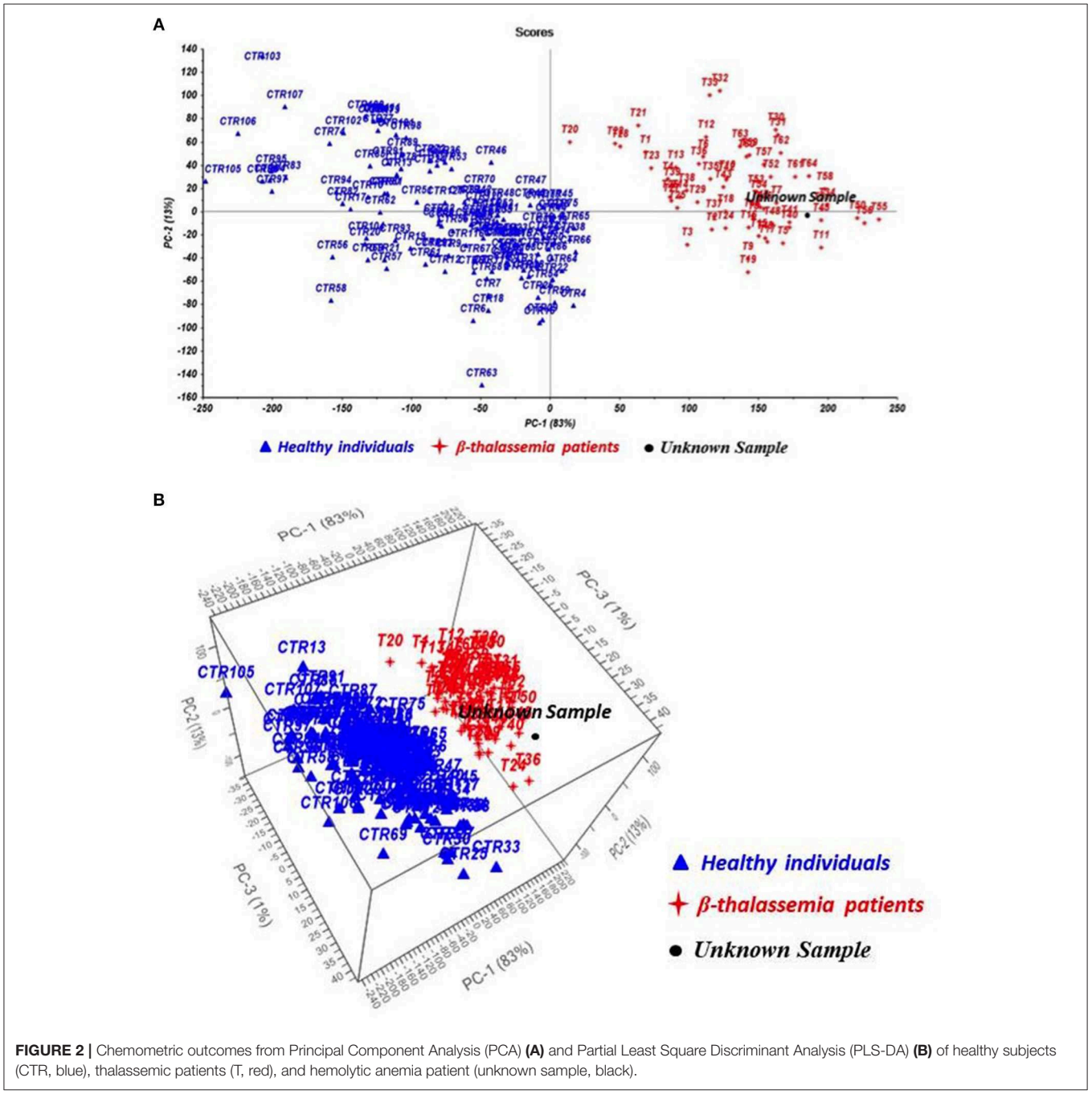

FIGURE 2 | Chemometric outcomes from Principal Component Analysis (PCA) **(A)** and Partial Least Square Discriminant Analysis (PLS-DA) **(B)** of healthy subjects (CTR, blue), thalassemic patients (T, red), and hemolytic anemia patient (unknown sample, black).

and the thalassemia patients (red), located in different side of the plot.

The classification model applied to the TG curve of the patient identified a chronic hemolytic anemia and in particular, the sample was located in the group of thalassemia patients suggesting the presence of a hemoglobin defect (**Figure 2B**).

Molecular Analysis of the Globin Genes

To confirm the results obtained by TGA/chemometrics test the presence of a hemoglobin defect was investigated by molecular analysis of the globin genes. The molecular characterization of α and β globin genes, demonstrated the presence of a very rare hemoglobin variant Hb Bibba ($\alpha_2$136(H19)Leu → Proβ_2) due to the substitution of a leucine residue in position 136 of the alpha chain by a proline residue. This α chain abnormal hemoglobin is an unstable hemoglobin with an autosomal dominant inheritance and was the cause of the chronic hemolytic anemia. Hb Bibba is an uncommon unstable hemoglobin variant which was not detected by the common protocols for the diagnosis of hemoglobinopathies. Therefore, the molecular analysis confirmed the results of the TGA/chemometrics test and demonstrated the capability of this method to discriminate healthy subjects and patients with a rare hemoglobin variant Hb Bibba not diagnosed by the common screening tests.

DISCUSSION

The TGA/Chemometric approach, previously used for diagnosis of thalassemia (Risoluti et al., 2016b, 2018b), was applied for the first time to investigate a rare case of chronic hemolytic anemia of difficult interpretation, for which the first level tests of the conventional diagnostic protocols were not able to find the erythrocyte congenital defect. The TGA/chemometric screening test allowed to make diagnosis of hemoglobinopathy, which was confirmed by the second level tests. The molecular analysis of the globin genes demonstrated the presence of a rare hemoglobin variant Hb Bibba ($\alpha_2$136(H19)Leu → Proβ_2) which was the cause of the chronic hemolytic anemia.

In this study, a new method to obtain an early detection of hemoglobinopathy by a TGA/chemometric screening test is proposed, a method that requires few microliters of blood sample that are directly analyzed without any pre-treatment. This method results particularly suitable in pediatric patients as it requires small sample volumes and is able to detect hemoglobinopathies also in transfused patients (Risoluti et al., 2016b). Our results demonstrate that this diagnostic approach permits the screening of hemoglobinopathies in patients with heterogeneous clinical phenotype, as in this case of chronic hemolytic anemia characterized by microcytosis, hypochromia, normal HbA_2, and HbF that was not diagnosed by the common first level protocols of investigation for congenital hemolytic anemias.

TGA/chemometric screening test is able to detect hemoglobinopathies determined by both quantitative defects as thalassemias and qualitative defects due to structural hemoglobin variants and not only in presence of hypochromic and microcytic anemia, but also in macrocytic anemia, and conditions that need molecular analysis for diagnosis such as δβ-thalassemia and β-thalassemia combined with Hb Lepore (Risoluti et al., 2016b, 2018a,b, 2019).

The effectiveness of this approach mainly consists of the multiparametric evaluation of the blood samples during the thermally induced decomposition under controlled temperature scanning. In fact, chemometric tools permit to correlate the entire TG curve of the anemic patient, to the collected dataset and to recognize a fingerprint profile of anemia.

CONCLUSIONS

The TGA/Chemometrics test is a new screening method for an early detection not only of thalassemia but also hemoglobin defects. The ability of the TGA/Chemometrics method to early detect an anemic status due to an unstable hemoglobin variant is very attractive, so this new method could provide a new approach for diagnosis of congenital hemolytic anemias. The positive outcome to this test would permit to immediately address patients to confirmatory analyses for hemoglobinopathies with a consequent save in time and costs and to quickly identify the correct therapeutic protocol for the patient.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Roma 2 of the S. Eugenio Hospital, Rome. Written informed consent for participating to the study and publishing clinical data in an anonymized manner are collected and copies of the informed consent are available on request. In the case of child, written informed consent for participating to the study and publishing clinical data in an anonymized manner was obtained from the parents.

AUTHOR CONTRIBUTIONS

RR, SM, and PC conceived the study and wrote the manuscript. Data analysis was performed by contributions of all authors. All authors have approved the final version of the manuscript.

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Hemorheological Alterations and Oxidative Damage in Sickle Cell Anemia

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Sickle cell anemia (SCA) is the most common hereditary disorder of hemoglobin (Hb) characterized by a mutation in the β globin gene, which leads to synthesis of HbS a hemoglobin which, under hypoxic conditions, gels and leading to the sickling of the red blood cells (RBC). The dehydration of the RBC increases the concentration of the intracellular Hb with an increase in the internal viscosity and consequently a decrease in the erythrocyte deformability. Sickle red blood cells due to their difficulty to flow through the microcirculation cause frequent vaso-occlusive episodes, tissue ischemia, and infarctions. Moreover, the reduced RBC deformability causes cell fragility leading to hemolysis and recently a key role of hemolysis and oxidative stress in the development of vascular dysfunction has been demonstrated. The aim of this study was to evaluate the hemorheological profiles of patients with SCA in order to point out new indices of vascular impairment, and to characterize the membrane oxidative damage of sickled RBC. Blood viscosities, erythrocyte aggregation, and viscoelastic profiles of SCA patients were determined, and the RBC oxidative damage was investigated by comparing metabolic capability and RBC membrane proteins from SCA patients with and without transfusion dependence. The hemorheological profile of SCA subjects demonstrated high blood viscosity, increased RBC aggregation, and decreased RBC deformability. These impaired flow properties were associated with RBC membrane protein oxidation, with degradation of spectrin and increased membrane-bound globin. The comparison between SCA patients with and without transfusion dependence showed metabolic and structural RBC oxidative damage significantly different.

Keywords: sickle cell anemia, hemorheology, oxidative damage, erythrocyte deformability, erythrocyte membrane

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INTRODUCTION

Sickle cell anemia (SCA) is the most common hereditary disorder of hemoglobin synthesis characterized by a mutation in the β globin gene, which leads to the replacement of glutamic acid with valine at the sixth codon and synthesis of Hb S a hemoglobin which, under hypoxic conditions, gels leading to the sickening of the red blood cells (RBC). The dehydration of the RBC increases the concentration of the intracellular Hb with an increase in the internal viscosity and consequently a decrease in the erythrocyte deformability (Ballas and Mohandas, 2004; Rees et al., 2010; Azar and Wong, 2017; Ware et al., 2017).

Blood viscosity and erythrocyte deformability are the main determinants the maintenance and regulation of microcirculation. Hemorheological changes produced by alterations both of blood cells and plasma components induce a rise in blood viscosity, which may slow blood flow and cause occlusions through the erythrocyte rouleaux formation and the platelet aggregation. Sickle red blood cells due to their difficulty to flow through the microcirculation, cause frequent vaso-occlusive episodes, poor microvascular blood flow, tissue ischemia, and infarction (Bowers et al., 2013, 2018; Connes et al., 2016; Azar and Wong, 2017; Risoluti et al., 2017; Ware et al., 2017). Moreover, the reduced RBC deformability causes an increased cell fragility leading to enhanced hemolysis (Grau et al., 2013) and a key role of hemolysis and oxidative stress in the development of vascular dysfunction has been demonstrated (Barodka et al., 2014; Connes et al., 2014; Hierso et al., 2014; Hermann et al., 2016; Mockesch et al., 2017; Renoux et al., 2018). A reduction in RBC deformability associated with an increase in oxidative stress has been observed in several pathological conditions such as hypertension and diabetes (Lee et al., 2017; Diederich et al., 2018). In patients with retinal vein occlusion, hemorheological alterations associated with the erythrocyte oxidative stress and consisting in increased blood viscosity and decreased RBC deformability have also been described (Becatti et al., 2016). These studies have been confirmed by *in vitro* experiments demonstrating that reactive oxygen species modify the fluidity of the erythrocyte membrane (Becatti et al., 2017; Diederich et al., 2018).

Sickle RBCs and their membranes are sensitive to auto-oxidation due to endogenous activated oxygen species (Lux et al., 1976; Rice-Evans and Omorphos, 1983; Platt et al., 1985; Rice-Evans et al., 1986; Amer et al., 2006). *In vitro* studies on the exposure of sickle cells to oxidative stress with different oxidants (t-butylhydroperoxide, hydrazine, diamide, hydrogen peroxide) have demonstrated lipid peroxidation and irreversible hemoglobin denaturation more pronounced in sickle RBCs than normal ones (Snyder et al., 1981; Hebbel et al., 1982; Rice-Evans and Baysal, 1987). Moreover, in sickle cell disease a reduced capability to counteract the oxidation was described, and the effects of antioxidants, free radical scavengers, and also iron chelators on sickle RBCs and their membranes have been evaluated (Chiu and Lubin, 1979; Das and Nair, 1980; Rice-Evans et al., 1986; Rice-Evans and Baysal, 1987; Amer et al., 2006; Gizi et al., 2011; Voskou et al., 2015).

The aim of this study was to evaluate the hemorheological profiles of patients with SCA in order to point out new indices of vascular impairment, and to characterize the membrane oxidative damage of sickled RBC from patients with and without transfusion therapy dependence. The whole blood viscosity, plasma viscosity, erythrocyte aggregation index, and blood viscoelastic profiles of SCA patients, in comparison with that of healthy subjects, have been determined. Furthermore, the oxidative membrane damage of RBC in SCA have been investigated by comparing metabolic capability and membrane structure integrity of RBC from transfusion dependent (TD-SCA) and non-transfusion-dependent (NTD-SCA) patients. The hemorheological profile of SCA subjects demonstrated high blood viscosity at both low and high shear rates, increased RBC aggregation, and decreased RBC deformability. These impaired flow properties were associated with metabolic and structural RBC oxidative damage significantly different in TD-SCA and NTD-SCA patients.

METHODS

Subjects

In this study the analysis of blood samples from subjects affected by SCA and healthy individuals was performed. Blood collecting protocol and all experimental procedures were approved by the local ethics committee and the patients gave their written informed consentto participate in this study, which conforms to the International Compilation of Human Research Standards (Department of Health and Human Services, 2011). The study was done in accordance with the principles of Good Clinical Practice, the Declaration of Helsinki, and all the local regulations.

Hematological and Biochemical Analyses

Blood samples collected on K2-EDTA were analized within 2–3 h. Red blood cell indices were determined by ADVIA 120 (Siemens, USA). Hemoglobins quantification was performed by high-pressure liquid chromatography (HPLC-Variant, Bio-Rad). Erythrocyte enzyme activities pyruvate kinase (PK) and glucose-6-phosphate dehydrogenase were assayed according to the International Committee for Standardization in Hematology (Beutler et al., 1977).

The reduced glutathione (GSH) content was determined by assessing the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by sulfhydryl compounds at λ 412 nm (Beutler, 1971). The GSH content was expressed as μmol/gHb.

Adenosine triphosphate (ATP) concentration was determined on whole blood perchloric acid extract by an enzyme assay at 340 nm at 37°C according to International Committee for Standardization in Haematology (ICSH) (1989) methods. The ATP concentration was expressed as μmol/gHb.

Membrane Protein Analysis

Leukocyte and platelet free red cells were obtained by filtration through microcrystalline cellulose-α-cellulose (1:1) column equilibrated with a phosphate-buffered saline (5 mM Na/Na_2HPO_4, pH 7.4, 0.15 M NaCl, 0.1 mM PMSF). The erythrocytes were washed twice with the same buffer, and lysed with hypotonic buffer (5 mM Na_2HpO_4 pH 8.0, 0.1 mM PMSF) in 1:30 (v/v) ratio to obtain erythrocyte membrane. The ghosts were washed three times with the same buffer (Caprari et al., 1995). RBC membrane proteins were analyzed by 7.5% polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) according to Laemmli (1970) with slight modifications (Caprari et al., 1991). RBC ghosts were dissolved in SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, 63 mM Tri-HCl, pH 6.8) to a concentration of 1 mg/ml, and, after an incubation for 2 min at 95°C, loaded on the gel. Staining of protein bands was performed with Coomassie Blue and a laser beam densitometer (Gel Doc

XR+) equipped with a Lab Image Software Package (BIORAD) was use to quantify the percentage of the membrane proteins.

Hemorheological Assays

Hemorheological profile analysis was carried out by Rheo-Microscope (Anton Paar, Germany) that is a glass parallel platerheometer, Physica MCR301, with a Peltier system for temperature control (37 ± 0.5°C). Whole Blood Viscosity (WBV) (η) was determined at shear rates 1 s^{-1} (η1) and 200 s^{-1} (η200), low and high shear rates, respectively, according to the Recommendation of the International Committee for Standardization in Haematology (ICSH) (1986) and the International Expert Panel for Standardization of Hemorheological (Baskurt et al., 2009). Plasma viscosity (ηpl) testing was performed at shear rates 200 s^{-1}. Erythrocyte Aggregation Index (EAI) was determined as η1 and η200 ratio (η1/η200). Since blood viscosity increases with the rise of hematocrit, these hemorheological parameters were determined in conditions of native and normalized hematocrit (i.e., adjusted to 40 or 45%, for females and males, respectively). RBCs viscoelastic properties were evaluated by determining elastic modulus G′, viscous modulus G″, and the tangent of phase shift angle ($Tg\delta = G''/G'$) as a function of strain rate at a constant value of deformation amplitude in the range from 0.1 to 10 Hz ($f = \omega/2\pi$). The values of the modules G′ and G″, and Tgδ, expressed in Pa, were determined by oscillatory measurements in the range of viscoelastic linearity (10% deformation), as previously described (Martorana et al., 2007).

TABLE 1 | Clinical and hematological characterization of SCA patients: comparison between transfusion-dependent (TD) and non-transfusion-dependent (NTD) subjects.

Patients	TD	NTD
N	14	10
Age	35 ± 14	30 ± 10
Gender	7 M/7 F	6 M/4 F
Origin country (*n*)	Italy (11) Africa (2) Albania (1)	Italy (8) Africa (2)
Genotype (*n*)	Hb S/Hb S (4) Hb S/Hb D (1) HbS/β Thal (7) HbS/β Thal/α-3.7 (2)	Hb S/Hb S (2) HbS/β Thal (7) HbS/β Thal/α-3.7 (1)
Clinical phenotype	n. 6 severe n. 8 mild	n. 2 severe n. 8 mild
Complications	Pretibial ulcers, bone pains, bone infarcts Aseptic necrosis of femora Transient Ischemic Attack (TIA)	Aseptic necrosis of femora Portal carvenoma Bone infarcts
Hb S (%)	29 ± 8	70 ± 5
Hb F (%)	5.0 ± 3.2	14.1 ± 12.5
Hb (g/dL)	10.9 ± 1.5	10.6 ± 2.0
Hct (%)	33.0 ± 4.4	32.8 ± 5.9
RDW (%)	19.0 ± 2.6	19.5 ± 3.1
PLT (10^9/L)	399 ± 142	308 ± 165
Ret (10^9/L)	268 ± 124	237 ± 97

The values are expressed as mean ± standard deviation.

RESULTS

Patients

Twenty-four patients affected by SCA and followed by the Thalassemia Unit of S. Eugenio Hospital of Rome were enrolled for this study between 2016 and 2018. The protocol for diagnosis of SCA included an assessment of the patient's clinical presentation together with screening tests, and molecular characterization of globin genes mutations (Materazzi et al., 2014, 2017a; Green et al., 2015; Risoluti et al., 2016, 2018; Catauro et al., 2018).

In **Table 1** are shown clinical and demographic characterization of the patients. Fourteen SCA patients (age 35 ± 14 years, mean ± standard deviation) were transfusion dependent (TD-SCA) and the age of SCA diagnosis ranged was from 1 to 18 years. In this group (7 males/7 females) two women were of African origin, a man was from Albania and the others patients had Italian origin. There were n. 4 subjects with HbS homozygosity, n. 1 with double heterozygosity for HbS/HbD, n. 7 with heterozygosity for HbS/β-thalassemia (HbS/β-thal), and n. 2 subjects with HbS/β-thal/α 3.7-thal.

Ten SCA patients (age 30 ± 10 years, mean ± standard deviation) were non-trans fusion dependent (NTD-SCA), and the age of SCA diagnosis ranged was from 2 to 32 years. In the NTD group (6 males/4 females) there were n. 2 women of African origin with genotype HbSS, and the other eight patients of Italian origin with genotypes heterozygotes for HbS/β-thal (7 subjects), and a heterozygote for HbS/β-thal/α 3.7-thal. All the patients did not had been splenectomized, and the TD patients had a median free time to transfusion of 30 days, and have been analyzed far from transfusion.

Thirty healthy subjects (18 males/12 females) of Italian origin with age 30 ± 10 years (mean ± standard deviation) were used as controls (CTR).

Clinical Data

The SCA patients showed a wide heterogeneity concerning the severity of clinical symptoms and complications (**Table 1**). Generally, TD patients showed a severe clinical phenotype associated with typical symptoms and signs of SCA: bone infarcts, pretibial ulcers, aseptic necrosis of femora, and transient ischemic attach (TIA), also recurrent. The clinical phenotype of the NTD patients was milder with the exception of two patients (**Table 1**) and the complications observed were avascular necrosis of the femoral head, portal cavernoma, and in few cases bone infarcts.

Hematological Data

The hematological data (**Table 1**) showed reduced contents of HbS (29 ± 8%) and Hb F (5.0 ± 3.2%) in TD patients as the effect of transfusion, while the NTD patients showed significantly high values of HbS (70 ± 5%) ($p < 0.0001$) and variable values of HbF (14.1 ± 12.5%). Comparable values of Hb and hematocrit (Hct), and high values of red cell distribution width (RDW) and reticulocyte counts were determined in TD and NTD SCA patients.

Hemorheological Profile

The hemorheological profile of SCA patients was compared with that of healthy subjects. We have analyzed the whole blood viscosities (η) determined at low (η1) and high (η200) shear rates, and the erythrocyte aggregation index in conditions of both native and normalized hematocrit (**Figure 1**).

The values η200n, and η1n of SCA patients obtained at normalized hematocrit (Hct 40–45%) were significantly higher than control ones (**Figure 1A**), particularly the values of η1n, which are indicative of a high viscosity at low shear rates, which correspond to the microcirculation. In agreement with this observation the values EAI of SCA patients (**Figure 1B**) were higher than that of healthy subjects. Plasma viscosity resulted lower in SCA patients than in control subjects (**Figure 1C**).

Viscoelastic properties were analyzed by determining the storage modulus G′ (elastic modulus), the loss modulus G″ (viscous modulus), and the tangent δ from SCA patients and healthy subjects.

In **Figure 2** the comparison between the G′, G″, and Tgδ mean curves are showed. High values of G′ are found in SCA patients in comparison with the control ones demonstrating high rigidity of the RBC and decreased deformability, associated with a constant increase in G″ which represents an increase in viscous modulus, in agreement with the previously reported blood viscosity results. The tangent δ curve, which represents the combination of the two components G′ and G″, shows a highly altered viscoelastic profile in patients with SCA compared to that of healthy subjects. The tangent δ curve is far below the normal curve to indicate a severe reduction of erythrocyte deformability with increase rigidity of the RBC membrane.

Oxidative Damage of RBC Membrane

The oxidative damage of RBC membrane was investigated by comparing both membrane structure integrity and metabolic capability of RBC from healthy subjects and SCA patients divided in TD and NTD, since periodic transfusion of normal red blood cells may be an additional factor influencing oxidative stress in SCA. As markers of the RBC metabolic and reduction capabilities we have chosen the metabolic intermediate GSH and ATP and the related enzyme activities G6PD and PK (**Table 2**).

Significantly high values of G6PD and PK were found in both TD and NTD patients as compared with control ones, and significantly differences were observed also between the two groups of patients with the highest values in NTD patients. These high values of G6PD and PK are closely related to the high reticulocyte counts in SCA patients, confirm the presence of a hemolytic state, and are indicative of a greater request of metabolic and reducing capacities for erythrocyte survival to which the RBCs respond with production of ATP and GSH. Indeed, ATP values were higher in SCA patients in comparison with the controls' ones and this difference was significant ($P < 0.01$) between TD and CTR. Erythrocyte GSH values were within

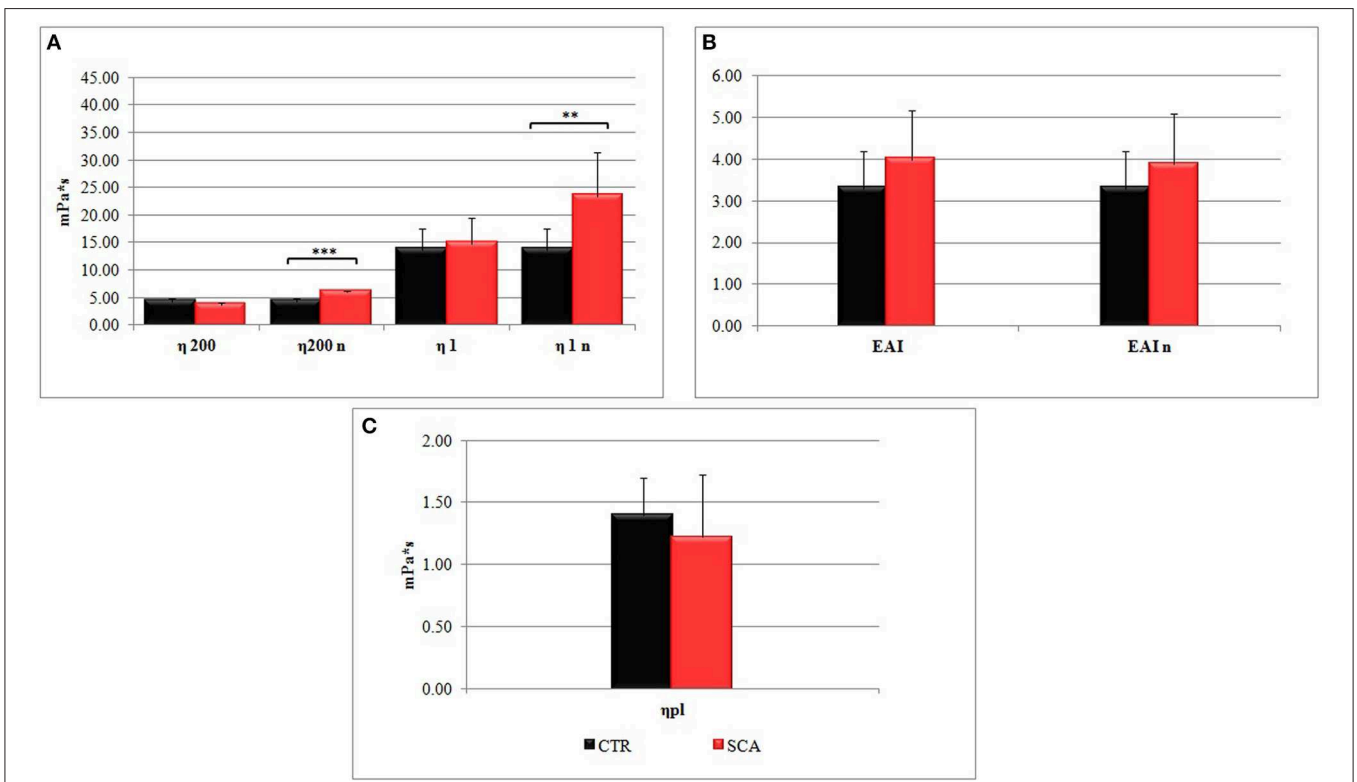

FIGURE 1 | Hemorheological profiles of sickle cell anemia (SCA) patients and healthy subjects (SCA): **(A)** whole blood viscosities η200 and η1 determined at low shear rates (1 s^{-1}) and high shear rates (200 s^{-1}), and **(B)** the erythrocyte aggregation index (EIA) (η1/η200) in conditions of native hematocrit. The values η200n, η1n, and EIAn were obtained at normalized hematocrit (Hct 40–45%). **(C)** Plasma viscosity (ηpl) is determined at shear rates 200 s^{-1}.

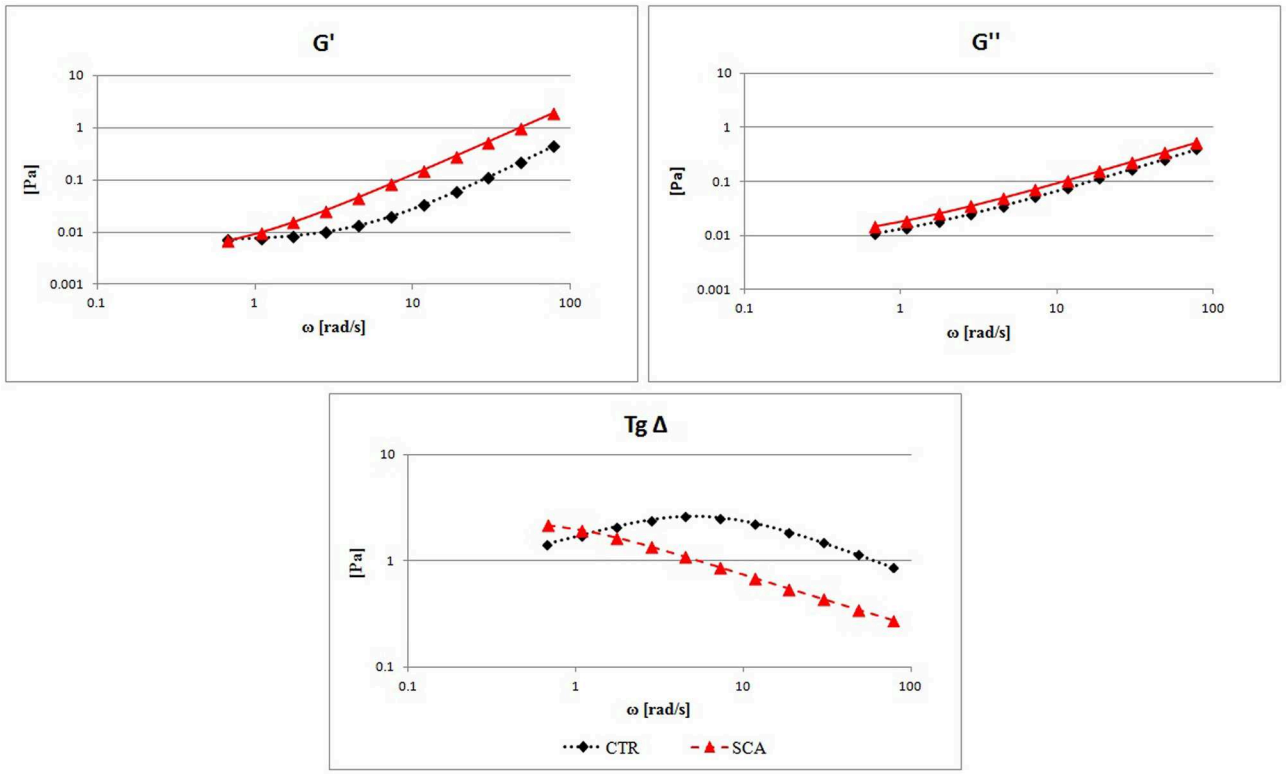

FIGURE 2 | Evaluation of RBCs viscoelastic properties from sickle cell anemia (SCA) patients and healthy subjects (CTR): elastic modulus G′, viscous modulus G″, and the tangent of phase shift angle (Tgδ = G″/G′) as a function of strain rate at a constant value of deformation amplitude, selected on the linear viscoelastic range by strain test.

TABLE 2 | Oxidative damage of RBCs from transfusion-dependent SCA patients (TD-SCA), not transfusion-dependent SCA patients (NTD-SCA), and healthy subjects (CTR).

	TD	NTD	CTR	TD vs. NTD	CTR vs. TD	CTR vs. NTD
GSH (μmol/gHb)	7.4 ± 0.4	6.7 ± 0.8	7.2 ± 0.6	$p = 0.01$		
G6PD (IU/gHb)	16.3 ± 5.1	21.3 ± 4.8	11.5 ± 2.6	$p < 0.05$	$p < 0.05$	$p < 0.0001$
ATP (μmol/gHb)	5.0 ± 0.7	4.8 ± 0.9	4.1 ± 0.4		$p < 0.01$	
PK (IU/gHb)	25.9 ± 7.5	34.2 ± 3.6	15.2 ± 2.7	$p < 0.05$	$p = 0.001$	$p < 0.0001$
Spectrin (%)	24.5 ± 2.8	30.3 ± 5.4	30.9 ± 3.3	$p < 0.05$	$p < 0.05$	
4.1 b (%)	1.6 ± 0.5	1.8 ± 0.5	1.3 ± 0.3			$p < 0.05$
Globin (%)	9.1 ± 3.4	4.1 ± 3.3	1.8 ± 2.0	$p < 0.01$	$p < 0.01$	

The oxidative membrane damage was investigated by comparing metabolic capability and membrane structure integrity of RBC from SCA patients with and without transfusion dependence. RBCs, red blood cells; GSH, reduced glutathione; ATP, adenosine triphosphate; G6PD, glucose-6-phosphate dehydrogenase activity; PK, pyruvate kinase activity. The values are expressed as mean ± standard deviation. The statistical evaluation between the groups was performed by t Student test.

normal values, but significant differences were observed between TD and NTD patients ($p = 0.001$) with GSH mean value of NTD-SCA subjects lower in comparison with the values of TD-SCA and controls.

The analysis of the erythrocyte membrane proteins demonstrated an impairment of the eryhtrocyte membrane of TD-SCA patients since spectrin content was significantly lower than spectrin contents of both NTD-SCA patients and healthy subjects ($p < 0.05$). NTD-SCA patients had a normal value of membrane spectrin. Moreover, the electrophoretic patterns of membrane showed an evident band of globin bound to the membrane in SCA patients in comparison with controls. The membrane globin content was significantly higher in TD-SCA in comparison with values of NTD-SCA and healthy subjects. The concentrations of the membrane proteins ankyrin, protein B and 3, protein 4.1, and protein 4.2 were within the normal values and comparable between SCA patients and healthy subjects. It is also noteworthy an increase in the protein 4.1b content of the membrane in the RBCs of SCA patients, this increase reaches significant differences in NTD patients compared to healthy subjects.

DISCUSSION

The clinical manifestations of SCA are extremely heterogeneous from asymptomatic individuals to patients with severe and recurrent pain crisis, ischemia, inflammatory state, and chronic complications. In the homozygous (HbSS) and in double heterozygous (HbS/β thalassemia) conditions four major morbidities are frequently observed: chronic hemolytic anemia, systemic manifestations with susceptibility to infections, painful vaso-occlusive crises (VOC) that can vary from patient to patient in intensity and frequency, and multi-organ damage.

Generally patients are anemic and from the hemorheological point of view (Connes et al., 2016) the low hematocrit value can in part compensate the sickle cell rigidity and determine a blood viscosity in the normal ranges, as we observed in the patients analyzed in this study. However, when the hematocrit increases, even toward values considered in the norm, an increase in blood viscosity may extremely relevant and determine, in conditions of slow flow as in the microcirculation, an increased erythrocyte aggregation. The results of this study show alterations in blood viscosity in hematocrit conditions at 40–45% that concern both the viscosity at low shear rates and the erythrocyte aggregation index, and the viscosity at high shear rates representing the macrocirculation. Therefore, the evaluation of blood viscosity and erythrocyte aggregation should be carried out both with native hematocrit and normalized hematocrit to be able to evaluate the effect in conditions of constrictions and vaso-occlusions in the microcirculation.

The study of the viscoelastic profile of SCA subjects demonstrated high rigidity of the RBC and decreased deformability, associated with a constant increase in G'' which represents an increase in viscous modulus, in agreement with the blood viscosity results. The tangent δ curve of RBC from SCA patients is far below the normal curve to indicate a severe reduction of erythrocyte deformability with increase rigidity of the RBC membrane. Overall these results confirm that red blood cells of SCA patients have impaired flow properties with enhanced aggregability, and reduced deformability that induce micro-circulatory disorders. These hemorheological alterations can derive from the structural alterations of the red blood cell induced by oxidative stress. Several recent studies have investigated the role of oxidative stress in SCA and in the progression of complications (Agas et al., 2008; Barodka et al., 2014; Grau et al., 2015; Materazzi et al., 2017b; Mockesch et al., 2017; Biswal et al., 2018; Renoux et al., 2018). The studies have described several causes of oxidative stress in SCA, including NADPH-oxidase-derived superoxide from endothelial cells, from activated poly-morphonuclear neutrophils, as well as from RBCs. Moreover, the free hemoglobin and hem into plasma are known to induce the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), with a decrease in nitric oxide. ROS and RNS are also produced inside the sickle RBCs for the HbS auto-oxidation in the cycles of sickling/unsickling (Hierso et al., 2014).

The important role that membrane proteins play in the ability to deform red blood cells is known and congenital alterations of these proteins can cause hemolysis of erythrocytes and anemia such as hereditary spherocytosis, and hereditary elliptocytosis. Several studies on the effect of *in vitro* oxidative stress on RBC demonstrated alteration of the skeletal network at the horizontal junction sites involving spectrin, actin, and protein 4.1 thus to modify the cytoskeletal assembly, and to play a role in the hemolytic process (Lux et al., 1976; Platt et al., 1985; Rice-Evans et al., 1986; Caprari et al., 1995; Rees et al., 2010; Gizi et al., 2011). The studies reported in the literature do not demonstrate quantitative defects of the main membrane and cytoskeletal proteins in SCA (Lux et al., 1976; Schwartz et al., 1987), but alterations in the interactions between the main components: spectrin, ankyrin, and protein 4.1. It should be noted, that the patients analyzed are very heterogeneous, some authors have only studied patients with HbS homozygosity, while others have studied patients with both homozygous and heterozygous SCA. To our knowledge, there are no studies that have described the oxidative damage of membrane proteins by comparing SCA patients with and without transfusion dependence. Blood transfusions are life-saving therapy for many patients with SCA, mainly patients with severe SCA forms. In this study we have demonstrated that a degradation of spectrin in addition to the increase in membrane-bound globin are the relevant alterations of membrane in SCA producing reduced RBC viscoelastic properties leading both to a reduced RBC deformability and probably clustering of Band 3, as previously reported (Hierso et al., 2014). The increased content of protein 4.1b, the high values of PK and G6PD activities and the high reticolocyte counts in SCA are indicative of a reduced mean age of RBC population caused by to hemolysis. To a greater request of antioxidant capacity the RBCs respond with production of ATP and GSH, both in TD and in NTD patients, with ATP values higher in SCA patients in comparison with the controls' ones. The comparison between SCA patients with and without transfusion dependence showed metabolic and structural oxidative damage of RBC significantly different, that could be attributable in part to the RBCs for transfusion. Some studies reported GSH values lower in SCA RBCs as compared to healthy individuals, as well as impaired catalase activity and other proteins involved in antioxidant protection (Amer et al., 2006; Voskou et al., 2015). On the contrary, as concerns the concentrations of ATP and GSH, and G6PD activity, our results are in agreement with the study of Rice-Evans et al. (1986) on homozygous SCA patients in which they found that the oxidative damage correlated with the proportion of irreversibly sickled cells. Comparable GSH content, increased G6PD activity, and similar ATP content were determined in SCA patients with low sickle cells, and a decrease in ATP values only in SCA patients with 5–25% sickle cells was observed. This observation highlights the importance of transfusion therapy to balance the oxidative damage.

In conclusion RBC physiology is severely impaired in SCA, and this contributes to the chronic vascular dysfunction. Many factors affect both clinical severity and frequency of VOC episodes. In addition to the globin genes genotype, and HbS and HbF content, also the alterations of the hemorhelogical profile and oxidation of the RBC membrane proteins with degradation of spectrin and membrane-bound globin are relevant. The determination of the hemorheological parameters and the

evaluation of the oxidative damage of the membrane may result important for a better understanding the heterogeneity of clinical signs and the pathophysiology of SCA. The introduction of these new parameters of evaluation could give useful information to carry out personalized therapeutic and care protocols in SCA patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Roma 2 of the S. Eugenio Hospital, Rome. Written informed consent for participation in the study and publication of clinical data in an anonymized manner were collected and copies of the informed consent are available on request.

AUTHOR CONTRIBUTIONS

PC, RR, and SMat conceived and designed the study, wrote the manuscript, and evaluated data for statistics. FS and LM enrolled the patients, performed the clinical evaluation, and management of subjects. SMas and LD performed experiments. All the authors have revised and approved the final version of the manuscript.

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Gender-Related Differences in Sickle Cell Disease in a Pediatric Cohort: A Single-Center Retrospective Study

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Sickle cell disease (SCD) is one of the most common monogenic disease worldwide. The incidence of SCD is not strictly gender-related as it is transmitted as an autosomal recessive disorder. In particular, the gender-related differences in pediatric SCD are not well-characterized. To address this matter, we retrospectively analyzed the clinical records of 39 pediatric patients with a diagnosis of SCD (hemoglobin SS genotype) focusing on gender differences analyzing various aspects of the disease and comprising both acute symptoms and late complications. We found various gender-related differences in our pediatric population. In particular, pain crisis frequency per year was significantly increased in the male population with a mean number of crisis per year of 1.6 vs. 0.6 in the female population ($p = 0.04$). Also, severe complications (both infectious and cardiovascular) were mostly found in the male population. SCD-related late cardiac complications were observed mainly in the male population ($p = 0.04$). Our data support the hypothesis that gender could play a role in determining the clinical course of SCD, even though further studies are needed to assess the exact weight of its influence over the course of the disease. The higher morbidity in males is a well-known feature of SCD in adults and these findings have been only partially studied in the pediatric population. These differences have, in adults, been attributed to hormonal variations found in the two sexes after puberty. In a pediatric population, other factors must be responsible for these discrepancies. These findings suggest that gender could be a valuable factor in the risk stratification of these patients at diagnosis, and possibly guide therapeutic decisions, with the final aim of personalizing the therapy.

Keywords: sickle-cell disease, gender medicine, pediatric anemia, hemoglobinopathies, drepanocytosis

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INTRODUCTION

Sickle cell disease (SCD) is a systemic disease, associated with episodes of acute illness and progressive organ damage, and is one of the most common and severe, monogenic disorder worldwide.

The disease is caused by an aminoacidic substitution in the beta-globin gene that leads to the production of an abnormal hemoglobin called HbS. The formation of abnormal HbS is caused by

a mutation in the β-globin gene in which the 17th nucleotide is changed from thymine to adenine leading to the substitution of the sixth amino-acid in the β-globin chain, glutamine acid in normal Hb becomes valine in HbS. This structural variation changes the surface of hemoglobin promoting the polymerization of hemoglobin in deoxygenation conditions. This leads to important alterations of red blood cells stability culminating in the formation of sickle-shaped erythrocytes.

Although the variation here described is the most common, the term sickle-cell disease is used to refer to different genotypes that share a similar clinical phenotype: homozygosity for the βS allele (HbS/S), heterozygosis for the βS allele and β-thalassemia (HbS/β), heterozygosity for the βS allele and some hemoglobin variants: HbS/C, HbS/D, HbS/lepore-Boston (Ware et al., 2017).

The disease mainly affects tropical regions, particularly sub-Saharan Africa, India and the Middle East, but it is also found in the Mediterranean area. The distribution of the disease in these regions is thought to be due to the so-called "Malaria hypothesis." The hypothesis is that there is a partial resistance of HbS carriers to all forms of Plasmodium falciparum malaria so that individuals heterozygous for HbS might have had a selective advantage during malaria epidemies, thus perpetuating the mutated allele (Aidoo et al., 2002). Currently the SCD prevalence is increasing in Europe and other Western countries, mainly because of globalization and human migration. The frequency of SCD carriers in Europe is 1 subject on 150 in the general population (Piel et al., 2013).

Painful crises are the most common symptom in SCD and they are caused by recurrent acute vaso-occlusion, they usually occur whenever partially or totally deoxygenated Hb molecules polymerize, thus distorting red blood cells normal disk shape, producing stiff, sticky, sickle-shaped cells that obstruct small blood vessels and produce occlusion and consequently the disruption of oxygen to body tissues.

The incidence of sickle cell disease is not gender-related since it is transmitted as an autosomal recessive disorder. However, there have been reports of sex related differences in SCD mortality and morbidity in adult patients. For example, one study of Platt et al. showed a greater mortality in males with a mean death age 42 years for men and 48 for woman (Mortality in sickle cell disease, 2018).

Another gender dependent factor that could play a role is Nitric oxide production. Nitric oxide is thought to be important in maintaining vasomotor tone, limiting platelet aggregation, inhibiting ischemia-reperfusion injury, and modulating endothelial adhesion molecule expression (Kim-Shapiro and Gladwin, 2018). Sickle cell-related vascular phenomena of increased shear stress and compensatory responses to chronic vascular injury normally promote increased endothelial nitric oxide production, but this system is impaired in males. Estrogens facilitate nitric oxide production and limit its consumption. Since nitric oxide is linked to transcriptional control of fetal hemoglobin, it could contribute to gender differences in fetal hemoglobin expression, known to be higher in the female population. Therapies that restore nitric oxide bioactivity or reduce its consumption (or enhance non-nitric oxide induced vasodilatation) could be particularly beneficial in patients with sickle cell anemia, especially males (Gladwin et al., 2003).

Despite the presence of reports of sex related differences in SCD mortality and morbidity in adult patients, few studies (Rosenberg and Hutcheson, 2011; Kumar et al., 2018; Alexandre-Heymann et al., 2019; Amilon et al., 2019; Arigliani et al., 2019) are currently available about gender heterogeneity in the pediatric population. The objective of the present retrospective study is to find gender-related differences in the clinical course of SCD in a pediatric population.

METHODS AND MATERIALS

We conducted a retrospective review of medical records of SCD pediatric patients followed at the Bambino Gesù Childrens' Hospital in the last 12 years (2006–2018). We considered pediatric patients all subjects with <18 years of age. Patients of both sexes were diagnosed between 3 months and 11 years of age, and the data were extracted to find subjects who had a diagnosis of SCD assessed by quantitative electrophoresis and with HbS levels >45%. We excluded patients with a follow up <6 months; this decision was based on the fact that in those cases we would not have enough anamnestical and laboratory informations. The examiners conducted a retrospective chart analysis of 65 consecutively treated patients. Thirty-nine patients were identified according to the afore-mentioned inclusion and exclusion criteria.

The following parameters were analyzed:

1. Painful annual crises: Painful crises per year were recorded. Painful crises treated in other hospitals and those not needing hospitalization were excluded. Painful crises were stratified according to severity. Pain was evaluated through the visual analog scale (VAS) and divided into three category: mild (VAS: 1–3), moderate (VAS: 4–5), and severe (VAS: 6–10). Crises severity was also classified according to the therapeutical management required.
2. Treatment of painful crises:
   - Non-steroidal anti-inflammatory drugs (NSAIDs).
   - Minor opioids (tramadol or codeine).
   - Major opioids (morphine).

We considered morphine treatment in painful crises not sufficiently controlled by the previous administration of NSAIDs or minor opioids.

3. Complications:
   - Splenomegaly (and eventual splenectomy) assessed by ultrasound and defined as a spleen long axis in cm beyond the 90th percentile adjusted for age.
   - Biliary lithiasis assessed by ultrasound.
   - Heart disease assessed by echocardiographic evaluation. The main cardiac alteration correlated with SCD is ventricular eccentric hypertrophy, therefore, we considered this condition as the main cardiac complication of SCD.
   - Vascular events (Ischemic Stroke, Deep Thrombosis, Transient Ischemic Attack) evaluated by CT and MRI in symptomatic patients.

- Osteomyelitis and septic arthritic assessed by laboratory and radiology evaluations (MRI and bone scan).
- Number of transfusions per year.

4. Age at diagnosis: the age of the patients at the time of SCD diagnosis was recorded.
5. Laboratory evaluation of HbS, HbF.

Patient data were compiled from the records of the eligible patients using an Excel spreadsheet (Microsoft) that reflected the parameters in the patient records. Descriptive analysis was performed for numeric parameters using means and standard deviations. Parameters comparison between females and males was made by T-student, χ^2 or Fisher test depending on the type of data analyzed and on sample size. All statistical comparisons were two-tailed and conducted at the 0.05 level of significance.

RESULTS

Data from 39 patients of both sexes (23 males and 16 females), with a mean age of 10.3 years (range, 3–26 years; males mean age 10.3 years and females mean age 10.5 years) followed-up for a minimum period 6 months, were collected and analyzed in the statistical analysis. Median follow-up time was 6 years (range 6 months−12 years).

A total of 89 painful crises were recorded (29 in females, 60 in males). The number of painful crises per year was calculated for each patient and means were calculated considering the sex groups (males 1.6 crisis/year, females, 0.6 crisis/year). A statistically significant difference was found between the two groups ($p = 0.04$).

As for the severity of the crisis assessed through VAS evaluation, we did not find a difference in these two groups, with 20 severe crises on 60 total crises in the male population vs. 10 severe crises on a total of 29 in the females.

In this context, we considered pain a dynamic parameter and, to evaluate it more precisely, we decided to classify the crisis according to the analgesic treatment required. The percentages related to the treatment of crises are described in the table below (**Table 2**) and a greater use of morphine was observed in males than females (p-value = 0.008).

Cardiopathy: 31 out of the 39 patients underwent cardiac assessment. Cardiac anomalies were detected in 13 patients, including 10 males (77%) and 3 females (23%). A statistically significant difference was found between the two groups ($p = 0.04$).

Splenectomy: Splenectomy interventions were performed in two patients of the group of males (9%) and in two patients of the group of females (19%).

Splenomegaly: Patients who performed at least one abdominal ultrasound were 38 (22 males and 16 females). Splenomegaly was found in 12 patients in the males group (55%) and in 10 patients in the females group (63%).

Biliary lithiasis: Cholelithiasis was found in two male children (14%) and six females (38%) of the group. Cholecystectomy interventions were recorded in two female patients (13%).

Vascular events: A case of transient ischemic attack (TIA) and an episode of venous portal vein thrombosis have been observed, both of which in male patients (8.7%).

Osteomyelitis and septic arthritis: Four cases of osteomyelitis have been reported, of which three were found in male (14%) and one in a female (6%).

Transfusions: The analysis of the median number of transfusions per year was almost identical in the two groups: a mean of 2.3 transfusions/year was reported for males and 1.9 transfusions/year for females.

The age at diagnosis was assessed for all patients followed by the OPBG Center, from 2006 onwards. Patients whose SCD diagnosis was made elsewhere then OPBG were not included in this analysis. The group included 30 patients (15 males, 15 females). The results of this comparison showed that the average age at diagnosis in the group of males was 1.7 years, while in the group of females 4.3 years (**Table 3**) (see **Tables 1** and **3** for a summary of the parameters evaluated).

As for HbS level, we found a median value of 62.47% in the males vs. a 65.94% in the females. This difference was not statistically relevant: $p = 0.6$. Fetal hemoglobin values were found to be slightly more elevated in the females group. The median value for the males was in fact 10.5% while in the females it was 12.8%, $p = 0.25$ No alpha globin mutations were found in our population.

DISCUSSION

Sickle cell anemia is a monogenic disease but presents a very complex phenotype and very variable clinical manifestations between subjects. In this study, conducted on 39 patients followed at Bambino Gesù Childrens' Hospital, we analyzed the main factors characterizing the pathology to assess if gender

TABLE 1 | Descriptive analysis of the complications recorded.

	Males	Females
Splenomegaly	12 (55%)	10 (63%)
Splenectomy	2 (9%)	3 (19%)
Biliary lithiasis	3 (14%)	6 (38%)
Cholecystectomy	0	2 (13%)
Cardiopathy	10 (77%)	3 (23%)
Vascular events	2 (9%)	0
Osteomyelitis	3 (14%)	1 (6%)
Transfusions/year	2.3	1.9

TABLE 2 | Analysis of the different pain treatment in males and females.

	Males	Females	*p*-value
Paracetamol	50%	55%	0.79
Ibuprofen	2%	3%	0.6
Toradol	33%	23%	0.37
Tramadol	37%	38%	0.93
Morphine	33%	7%	0.008

TABLE 3 | Descriptive analysis of the age at diagnosis laboratory evaluation of HbS, HbF, and hemolytic state (LDH and bilirubin).

	Males	Females	*p*-value
Age at diagnosis	1.7 years	4.3 years	0.88
HbS	64.27%	65.94%	0.6
HbF	10.46%	12.78%	0.25
LDH	948.7 UI/L	939.4 UI/L	0.92
Bilirubin	2.32 mg/dl	2.61 mg/dl	0.64

could have an influence in the clinical and therapeutic course of the disease.

To the best of our knowledge scarce data are available in the literature about SCD sex differences in pediatric population. Sex hormones were recognized as responsible for gender differences in adult patients with SCD, but in the pre-puberty setting of childhood their role could be less relevant in the pathogenesis of gender differences in the pediatric population (Gladwin et al., 2003; Kato et al., 2007; Nebor et al., 2011; Jit et al., 2019).

The aim of this study was to find gender-related differences in the clinical course of SCD in a pediatric population.

Our first focus concerns the number of painful crises per year; painful crises, in fact, in addition to being very heterogeneous, are also highly unpredictable and the few studies in literature addressing this issue speculate that the reduction of fetal hemoglobin, the increase of hematocrit and leukocyte counts, are, among the parameters studied, the only ones associated with a higher rate of crisis (Niscola et al., 2009). Moreover, an annual average of crisis between the 0.4 and the 0.8 per patient is reported, regardless of the sex of patients (Kato et al., 2007; Niscola et al., 2009; Nebor et al., 2011; Jit et al., 2019).

Our results showed that males had more episodes of painful crises per year than females ($p = 0.04$), with an average higher than that reported in the literature. The reason of that difference could be attributed to the different bioavailability of nitric oxide, higher in females, as suggested in various studies (Gladwin et al., 2003; Ilesanmi, 2010).

Concerning SCD clinical complications, the literature shows a greater severity of clinical manifestations in males (Lamarre et al., 2013).

In the present study a high variability in the results was noticed: the comparison between the incidence of splenomegaly in the two groups showed no gender specificity, whereas the incidence of cholelithiasis tended to be more frequent in females, as extensively documented in literature (Currò et al., 2007).

In the category of cardiopathies, eccentric left ventricular hypertrophy is the most frequently reported in SCD patients. The present study showed that males are most frequently affected by this complication ($p = 0.04$). Same results were already obtained by Morrison et al. at the Lady's Children's Hospital Crumlin (Morrison et al., 2018).

Concerning the incidence of vascular events, a total of two episodes was recorded (5%), both cases in male patients. Osteomyelitis also manifested mainly in males (three episodes in males and 1 in females) confirming the tendency that males are more prone to complications than females.

Transfusions number was also analyzed. The initial hypothesis was that males needed more transfusions, as transfusions improve oxygenation and disrupt the intravascular mowing process by dilution (in the case of simple transfusion) of pathological red blood cells containing HbS. However, the results did not support this hypothesis, showing an identical average between males and females.

In our sample males were more represented than females. The sample size of male patients and the fact that they had more episodes of painful/annual crises, has led us to analyze the average age to the diagnosis of the two groups: males would have an earlier diagnosis, as they have a worse clinical course than females and they need a greater number of hospitalizations and investigations. This could be a possible explanation to the higher number of males in our pediatric sample compared to adult populations, in which the sample is more gender balanced. The results of this analysis confirmed our theory, for males the average age is 2 years, for females 4 years.

Regarding vaso-occlusive crises an analysis of the treatment necessary to control the pain during painful crises was performed. The pain, in the crises, is classified according to the visual analog scale (VAS) scale in mild, moderate and severe and the most recent AIEOP (Italian Association of Hematology Pediatric Oncology) guidelines recommend the use of morphine in the pain control of moderate-severe crises. The analysis of our data showed that, the percentage of severe painful crises (VAS > 6) is very similar in the two groups and, nevertheless, almost exclusively male children have needed a treatment with morphine, with "p" < 0.05. Also, this data confirms our hypothesis: males seem to have a worse clinical course than females, thus requiring more important pain therapy than females. Many studies in literature described gender differences in frequency and intensity of pain. In these studies, women often report lower pain thresholds, higher pain ratings, and lower tolerance for pain (Sorge and Totsch, 2017). Nevertheless, these assumptions have not been reported in SCD patients both in adult (McClish et al., 2006) and in pediatric populations (Fosdal, 2015). Since these patients experience both chronic and acute pain, there might be a long-term modulation of pain sensitivity. Any differences in gender in this regard should be more thoroughly assessed in further studies.

As for laboratory results, we didn't find any statistically relevant difference in the two groups, the slightly higher median value for HbF in females has already been described in adults (Amid and Odame, 2014).

In conclusion, our results confirmed that gender plays a role in the pathogenesis and in the course of the disease, in particular, male gender seems to represent an indicator of a more aggressive disease course. Thus, our results showed that there is more morbidity in the male sex. This data had not yet been directly addressed in any study in the pediatric age, although it has already been shown in the adult population. The gender-specific differences observed, partly already known in the adult, have always been attributed to the hormonal changes that are physiologically present in the two sexes after

puberty. However, in the pediatric population, other factors must be implicated in determining the described differences. Further studies are encouraged to highlight possible risk factors connected to gender in the SCD pediatric population. Therefore, taking these preliminary data, and their possible confirmation in wider studies, male gender could be taken into account in the initial assessment of the patients. This could, in fact, represent a simple and intuitive risk factor that could be implemented in the prognostic stratification since diagnosis, thus leading to personalized therapeutic decisions for the two sexes and the implementation of major prevention and surveillance programs for males.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

GC, MD, and GP designed the study. GC, MD, MQ, FG, and CB cured the collection of the data. GC, MD, IT, FG, AV, and GP interpreted and analyzed the data. GC and MD drafted the manuscript. AV and GP critically revised the manuscript for intellectual content.

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New Insights and Methods in the Approach to Thalassemia Major: The Lesson From the Case of Adrenal Insufficiency

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Background: Thalassemia Major (TM) is a complex pathology that needs a highly skilled approach. Endocrine comorbidities are nowadays the most important complications, including hypogonadism, hypothyroidism, diabetes mellitus, and bone diseases. Recent works stated that there could be a relevant prevalence of adrenal insufficiency (AI) present in TM, and this fact may become crucial, especially in case of major stressful events.

Aim: Test the reliability of the standard test to diagnose AI in a group of TM and correlate it with clinical, hematological, and radiological data.

Methods: We evaluated endocrine damages and the efficacy of iron chelation therapy in 102 patients affected by TM. AI was assessed by tetracosactide (Synacthen) 1 mcg iv (low-dose test, LDT) stimulation test. Patients with a subnormal response (peak cortisol < 500 nmol/L) were followed up to 5 years to check the symptoms and signs of AI.

Results: We found AI in 13.7% of the population studied. We did not find any correlation between AI and all data evaluated. Only female gender seems to be a protective factor. A follow up of the patients affected by AI showed no signs of adrenal crisis, in spite of no replacement therapy.

Conclusions: Our study shows a relevant prevalence of AI in TM, especially in males. The absence of an adrenal crisis, in spite of no replacement therapy, during the long-term follow up, seems to underline that current methods to evaluate AI, in TM, should consider a different and specific diagnostic test or different cut off for diagnosis.

Keywords: Thalassemia Major, adrenal insufficiency, synachten, endocrine comorbidities, cortisol peak

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INTRODUCTION

β-Thalassemia Major (TM) is a hereditary disease characterized by an impaired production of hemoglobin chains which can be due to over 200 mutations of the β chains gene. These mutations induce an abnormal production of the same hemoglobin and cause a severe haemolytic anemia (Rund and Rachmilewitz, 2005). The main therapy of the disease consists of a regular blood transfusion regimen that, frequently, hesitates in iron deposition and excessive storage. The

possibility of an adequate chelation therapy has dramatically improved the quality and the life expectancy of these patients (Borgna-Pignatti et al., 2005). Nowadays, these patients have the chance to survive until adult age, resulting in a clinical picture of this disease that is deeply different in comparison with the past (Angelucci et al., 2008; Pinto et al., 2019).

The iron deposition, related to a transfusion treatment, causes a cytotoxic effect in many organs between which are the endocrine glands. Endocrine complications represent one of the most relevant problems and, specifically, alterations of pituitary, thyroid, pancreatic and bone status are the most important (De Sanctis et al., 2018; Pinto et al., 2019). Less is known about the chance of suffering from adrenal impairment (AI). Since the pituitary-adrenal axis is necessary to face stresses, evaluation of adrenal function in all adult TM patients could be crucial.

Previous studies in children and adolescents affected by TM have reported variable prevalence of endocrine impaired functions (De Sanctis et al., 1995, 2011; Scacchi et al., 2010). Results regarding adrenal impairment are heterogeneous in terms of methods used, incidence and population studied (adults, adolescents, and children; Nakavachara and Viprakasit, 2013; Ambrogio et al., 2018).

In order to clarify the incidence of AI in TM patients, we decided to assess the pituitary/adrenal axis in a large group of adult patients, focusing not only on hematological and hormonal data but also on clinical history, thanks to a long follow up evaluation (up to 5 years).

MATERIALS AND METHODS

This study was performed at our Endocrine Unit dedicated to patients affected by hemoglobinopathies. We collected data from 102 adult polytransfused β-Thalassemic Major patients, 19–50 years old, including both males and females (47 and 55, respectively). All of them were regularly blood transfused and were treated with chelation therapy. Patients who were taking glucocorticoids or other drugs known to affect adrenal function were excluded. This is a retrospective analysis of patient records of routine care. Therefore, ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Assessment included general data, medical history collection, physical examination, blood samples at baseline and after stimulation test and T2* sequences magnetic resonance imaging.

We collected personal data like age and gender. Clinical evaluation included height, weight, and blood pressure. In all patients, we evaluated the presence of other endocrine damage, in particular, the presence of hypogonadism, hypothyroidism, diabetes mellitus, hypoparathyroidism and bone disease, and evaluated serological positivity to HBV, HCV, and HIV. The adequacy of chelation treatment through medium serum ferritin levels and by liver T2* magnetic resonance imaging (T2*MRI) was investigated. Finally, we collected hematological data to evaluate liver function and electrolyte serum levels. At baseline, blood samples for biochemical (ferritin, hemoglobin, sodium, potassium, hepatic transaminases, albumin) and hormonal assay (adrenocorticotropic hormone—ACTH, thyroid stimulating hormone—TSH, free thyroxine—fT4, dehydroepiandrosterone sulfate—DHEAS) were collected fasting, between 8:00 and 9:00 a.m. Soon after baseline, blood samples of all patients underwent a 1 μg cosyntropin test. Blood samples for total cortisol measurements were collected at baseline and at 30 and 60 min after iv injection of 1 μg cosyntropin. One vial of 0.25 mg cosyntropin ($ACTH_{1-24}$, tetracosactide) was diluted in sterile normal saline solution to a concentration of 5 μg/ml. One microgram cosyntropin (0,2 ml) was injected through a short iv catheter (Cross et al., 2018).

Patients with a subnormal response of cortisol (cortisol post-dose < 500 nmol/L, in according to most recent recommendation) were strictly followed up for a period of 5 years to check clinical and hematological signs of adrenal failure, in order to promptly start a replacement therapy. The check included clinical evaluation such as blood pressure measurement and the research of other signs and symptoms of adrenal insufficiency, hematochemical, and hormonal evaluation.

All hormones were analyzed using a chemiluminescence immunoassay (CLIA). Sensitivity of assays was 0.25 pmol/L for ACTH and 0.5 nmol/L for serum cortisol; intra-assay and inter-assay coefficient of variations were 4.9 and 8.9% for ACTH and 4.3 and 5.5% for serum cortisol (LIASON XL Analyzer).

Statistical Analysis

A descriptive analysis of all the sample parameters collected was carried out. The normality of the distribution of the continuous quantitative variables was evaluated through the Shapiro-Wilk test; the variables with Gaussian distribution were reported as mean and standard deviation (SD), while the variables with Non-Gaussian distribution were reported as median and interquartile range (IQR). The qualitative variables were presented as absolute frequencies and percentages. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression analysis; univariate and multi-variable analyses were carried out to evaluate the role of variables as risk factors determining the response of cortisol. A $p < 0.05$ was considered as statistically significant and all tests were two-sided. All statistical analyses were performed with the software R version 3.5.1.

RESULTS

A total of 102 polytransfused β-TM adult patients without previous diagnosis of adrenal insufficiency were identified. Herein we present all the data we collected the day the LDT was performed. Demographic, biochemical and hormonal data are reported in **Table 1**. Forty-seven patients (46.1%) were male, 55 were female with median age of 37 years old (range 19–50 years old).

Blood pressure (BP) was in the normal range with median systolic BP 110 mmHg (range 80–135 mmHg) and median diastolic BP 70 mmHg (range 40–90 mmHg). Fasting glycaemia

was normal in the majority of patients (median values of 83 mg/dl; range 41–270). Liver function tests showed median alanine transaminase of 33 U/L (range 7–261) and median aspartate transaminase of 29 U/L (range 8–253), also albumin levels were normal (median 4.1 g/dl, range 3.4–5.2). Only 19 patients were serological negative for HBV, HCV, and HIV, for the remaining, 77 patients (75.49%) were HCV positive, 17 patients (16.67%) were HBV positive and 3 (2.94%) were HIV positive.

TABLE 1 | Patient's characteristics.

Sex	47M/55 F (46.1% male)*
Age	37 years (19–50)*
BMI	22.6 Kg/m^2 (15.5–34.5)*
Hemoglobin	10.5 g/dl (8–12.3)*
Ferritin	42.2 ng/ml (7.5–492.7)*
¥ Liver T2* MRI	14 ms (0.9–46)*
Albumin	4.1 g/dl (3.4–5.2)*
AST	29 U/L (8–253)*
ALT	33 U/L (7–261)*
Sodium	139 mmol/l (129–144)*
Potassium	4.4 mEq/l (3.6–8.8)*
PAS	110 mmHg (80–135)*
PAD	70 mmHg (40–90)*
DHEAS	81.6 μg/dl (3.7–487.7)*
ACTH	20.2 pg/ml (5–144)*
HCV positivity	75.49%
HBV positivity	16.67%
HIV positivity	2.94%
Endocrinopathies	
Hypoparathyroidism	4.9%
Osteoporosis	65.69%
Diabetes mellitus	11.76%
Hypothyroidism	45.1%
Growth hormone deficiency	15.67%
Hypogonadism	64.71%

**All values are median. In brackets are expressed range values. ¥ Available on 68 patients. BMI, body mass index; ALT, Alanine transaminase; AST, Aspartate transaminase; DHEAS, Dehydroepiandrosterone sulfate; ACTH, Adrenocorticotropic hormone; HCV, hepatitis C virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; MRI, magnetic resonance imaging.*

The entire group showed good levels of ferritin (median 42.2 ng/ml, interquartile range 24.7–88.5, range 7.5–492.7) and T2* MRI (median 14 ms, range 0.9–46), signs of the efficacy transfusion and chelation therapy, as expected in a population treated in an Italian tertiary dedicated center.

Despite these results, we observed a high prevalence of global endocrine damage. In particular, our population showed 64.7% of hypogonadism, 45.1% of hypothyroidism, 11.7% of diabetes mellitus, 15.6% of growth hormone deficiency, 4.9% of hypoparathyroidism, and 65.6% of osteoporosis. The univariate logistic regression didn't show any correlation between adrenal insufficiency and any other endocrinopathy.

Regarding the assessment of adrenal function, the main target of our work, 14 patients (13.7% of population) failed to reach the value of 500 nmol/L on LDT. Moreover, median cortisol peak value in the 14 patients with adrenal impairment (peak <500 nmol/L) was 461 nmol/L (range 372–480 nmol/L) **Figure 1**.

When considering the failure to pass the LDT, the only significant factor was gender, in particular female gender resulted to be a protective factor against the failure to the LDT (OR 0.110; IC 0.023–0.522; *p*: 0.005).

Any statistical correlation was found with data of efficacy of chelation therapy (ferritin and T2*MRI) neither regarding liver function, assessed by transaminases and albumin serum levels.

None of the patients who did not achieve the cut-off during the LDT complains of any symptom potentially related with adrenal insufficiency. Therefore, we decided not to start daily replacement steroid treatment but to advice the treatment only when needed (in case of major stress).

In the follow up period of up to 5 years, nobody experienced any AI symptoms nor adrenal crises. Moreover, none of

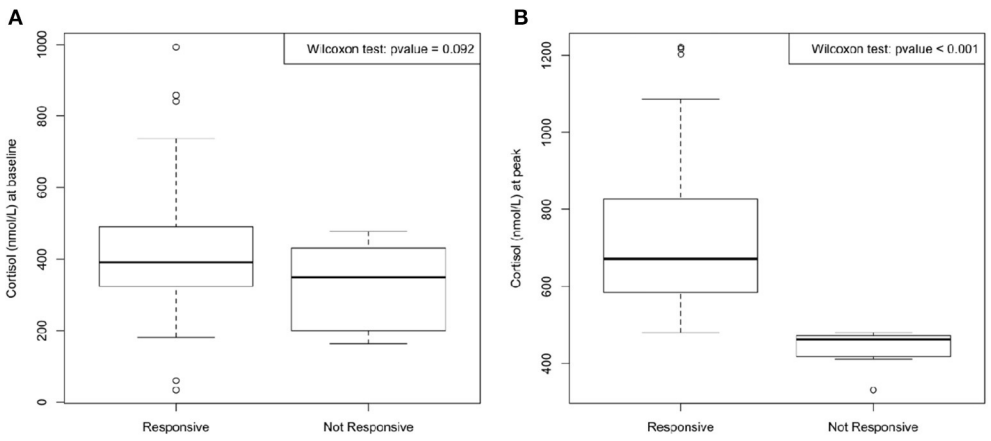

FIGURE 1 | Median cortisol at baseline **(A)** and at peak (time 30 and 60 min) after 1 μg Cosyntropin test **(B)**. No significant difference between cortisol at baseline in responsive and in not responsive group was found; on the contrary, cortisol at peak reached a significant difference ($p < 0.001$) between the two groups.

these patients needed to start steroid replacement therapy also in a more stressed clinical context such as fever, or intercurrent illnesses.

DISCUSSION

Nowadays patients affected by TM live longer thanks to better transfusion regimens and more efficient chelation therapies. This is the reason why physicians, especially endocrinologists, need to be faced with problems never experienced before. It is also important if we consider the high economic impact of the endocrine therapies (Ehrnborg et al., 2000). Over the past decade we have tried to extensively study different aspects of endocrine comorbidities (Poggi et al., 2010, 2011, 2016) but still much has to be done in this field. Endocrinopathies are one of the most important and challenging problems for the disease team involved in the cure approach. Studies performed in high skilled centers showed that endocrine failure could reach up to 60–70% for hypogonadism and bone disease and up to 30–40% for thyroid disease and diabetes mellitus. All these endocrine comorbidities considerably impair quality of life (De Sanctis et al., 2018).

Between endocrinopathies, it is crucial to diagnose adrenal impairment in this clinical setting, especially if we consider the possibility for these patients to be faced with acute stress or intercurrent illness. Moreover, TM patients frequently need splenectomy which is consider a major surgery that could precipitate adrenal function and hesitate in a fatal event, also because of an increased risk of post-splenectomy sepsis and thromboembolic events (Galanello and Origa, 2010). In accordance with this possibility, Matin et al. recommend adrenal function test prior to surgery (Matin et al., 2015).

Another relevant aspect is to consider the possibility of misdiagnosed AI in this clinical setting because of the overlap between typical symptoms of patients affected by anemia (Cascio and DeLoughery, 2017) and the non-specific pivotal signs and symptoms of adrenal failure, such as asthenia, fatigue, hypotension, abdominal pain (Borgna-Pignatti and Gamberini, 2011).

For all these considerations, we retain it is crucial that adequate and well-defined studies are conducted in order to fully evaluate and focus adrenal status in TM.

Recently several other authors published studies regarding the impairment of adrenal function in Thalassaemic patients (Poomthavorn et al., 2010; Scacchi et al., 2010; Uçar et al., 2016; Ambrogio et al., 2018). These studies are heterogeneous in term of population (TM or intermedia, children or adult, number of patients involved) and methods (insulin tolerance test, ACTH test with 1 mcg or 250 mcg, salivary cortisol) with different outcome in term of AI prevalence.

To explore this aspect, we conducted a retrospective study to define the real prevalence of AI in our population of TM.

To our knowledge, this study is the largest adult TM patients series tested for adrenal function to date. Moreover, differently from other authors (Matin et al., 2015; Ambrogio et al., 2018), we selected only patients affected by major thalassemia and excluded those affected by thalassemia intermedia and we evaluated only adult patient. All patients were followed by the same investigator (M.P.) during the follow up time.

To diagnose AI, we focused not only on biochemical data and on the LDT results but also on the real clinical significance of these data following the entire group in a long-term follow up to 5 years. Interestingly, despite the fact that 14 patients did not normally achieve the cut off during the SST, similar to data available in literature, none of our patients required steroid treatment. Moreover, we could not find any correlation with other factors, except with gender. This difference could be due to the different population studied (number of patient, median age, history of iron overload).

The initial result of our work, like others authors (Ambrogio et al., 2018; De Sanctis et al., 2018), was that biochemical adrenal impairment is not a negligible event which affects 14 patient (13.7% of our population). As suggested by the most recent consensus and guidelines (Husebye et al., 2014; Fleseriu et al., 2016), we used the cortisol cut-off of 500 nmol/L during LDT to determine normal adrenal function. In our cohort, patients who did not achieve this cut-off had median cortisol peak of 461 nmol/L (range 372–480 nmol/L).

Despite the fact that the insulin intolerance test is the gold standard in this setting, SST is widely used (Cross et al., 2018) with good accuracy, but probably with the need to adopt new and different cut off also in the general population (Karaca et al., 2011; Simsek et al., 2015; Burgos et al., 2019).

In agreement with this, Cho et al. (2014) tried to re-assess the cut-off for the different test (ITT, high and low dose SST). In their population they identify the threshold of 16 μg/dl (≃441.4 nmol/l) for the LDT: despite this result they underline the need to individualize the cut-off on the basis of the tested population.

Some authors described the presence of a chronic hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis in thalassaemic subjects, probably due to physical stress such as anemia or oxidative stress (Poomthavorn et al., 2010). They speculated the possibility that the 1-mcg-Cosytropin test could be a too low stimulus in an hyperfunctioning axis and it could not be a suitable diagnostic test for this condition.

The necessity to identify different, new and more stringent cut-off could be even more crucial in some clinical settings like thalassemia and hemoglobinopathies. Moreover, considering the clinical complexity of the disease and the presence of different comorbidities we need to focus and to avoid starting unnecessary steroid therapy. This is particularly important if we consider the impact that such therapy could have especially on bone disease.

Looking for differences between patients affected by lower cortisol peak and those with a good response to LDT, we noted that only female gender reaches a significant difference between subgroups and it could be considered a protective factor, as underlined recently by other groups (Ambrogio et al., 2018).

Notably, we did not find any correlation between cortisol peak and the major parameters of efficacy of chelating therapy (ferritin and T2*MRI) which were previously identified as predictive factors of endocrine impairment progression (Chirico et al., 2015). The lack of this correlation agrees to our previous work

regarding other endocrine damage (Poggi et al., 2010). We could speculate that iron deposition is not the only actor in the induction and progression of endocrine damage but other factors such as chronic hypoxia or different tissue sensibility to iron overload must be taken into consideration. In agreement with other authors (Singh et al., 2018), we underline the importance of checking liver function to fully understand the LDT results. Nevertheless, we did not find any correlation between liver function parameters and LDT adequate responders. We can speculate that other factors as low serum protein, but not albumin which was in the normal range in our cohort, could alter the response to LDT. The other relevant aspect that we considered, which was never studied in the past, is the long term follow up (up to 5 year) that allows us to shed light on this endocrine complication.

During the follow up period we never collected any medical data that could be related to a progressive or an acute failure of adrenal function (even during major stressful clinical situations like fever or surgery). This result could suggest that the impairment of adrenal disease, as stated by the result to dynamical hormonal evaluation, is not a clear clinical entity but only a laboratory data, probably related to the fact that, in the clinical context of thalassemia, we cannot adopt the same tools as in different condition of hypoadrenalism.

Our experience, for first time to our knowledge, shows the limit of the current methods to investigate adrenal insufficiency in adult TM. Once more, the lesson from the case of adrenal insufficiency, as the title of this paper states, remind us how of the importance of using specific and accurate diagnostic methods for different clinical conditions and this could be particularly true in a very complex pathology like TM that needs dedicated and highly skilled resources. We think that by only using specific, accurate and validated methods can we do a real diagnosis.

In conclusion, we have reported a large series of 102 adult TM patients on adequate transfusion and chelation treatment who underwent LDT to assess adrenal function. Fourteen patients (13.7%) did not achieve the threshold of cortisol value of 500 nmol/L, which is considered a normal response to the test. Female gender is the only statistically significant parameter being associated to normal response to LDT. None of our patients presented signs or symptoms of adrenal insufficiency at the time of the test and during the long-term follow up. We speculate that the threshold of 500 nmol/L is not adequate for this population and a lower limit should be used. In this work, the median cortisol peak of non-responders was 461 nmol/L, therefore we suggest that a lower value should be adopted, considering the absence of suggestive clinical signs of AI reported among these cases, but more studies are needed to support this hypothesis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MP, IS, LM, CI, SM, GP, and VT: conceptualization, investigation, methodology, and writing-original draft. AD: data curation and formal analysis. MP: project administration, supervision, and validation.

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HCV Infection in Thalassemia Syndromes and Hemoglobinopathies: New Perspectives

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Hepatitis C virus (HCV) infection is one of the most serious complications of transfusion therapy in the thalassemia and sickle cell disease (SCD) population before 1990; in fact, since 1990 serological tests were made available to detect infection in blood donors. The iron chelation therapy has improved the life expectancy of these patients and, consequently, a decrease in death due to heart disease may be observed, as well as an increase in liver disease due to the iron overload and HCV infection that lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Until few years ago, the recommended therapy for HCV treatment consisted of pegylated-interferon alpha plus ribavirin, a therapy with important side effects. This treatment has been severely limited to thalassemic and SCD patients due to the hemolytic anemia induced by ribavirin causing an increase in the number of blood transfusions. The development of highly effective Direct-acting Antiviral Agents toward different viral genotypes has led to a real HCV eradication with negative viremia and sustained viral response between 90 and 98%. At the beginning some indications of Direct-acting Antiviral Agents administration were available for those patients exhibiting advanced cirrhosis or needing liver transplantation over time for the high costs of the new drugs. Recently, all treatment regimens can be used for patients with various HCV genotypes, different stages of liver disease, and comorbidities. The HCV eradication has also led to a marked improvement in the parameters of martial accumulation, demonstrating a synergic action also between the effect of antiviral therapy and iron chelation.

Keywords: hepatitis C, direct acting antivirals, thalassemia major, sickle cell disease, iron overload, transfusion, liver disease

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most serious complications of transfusion therapy in the thalassemia and Sickle Cell Disease (SCD) population receiving transfusions before 1990; in fact, since the year 1990 serological tests were made available for detect infections in blood donors.

The iron chelation therapy has modified the life expectancy of thalassemia patients; mortality data show a decrease in causes of death due to heart disease and an increase in liver disease (Prati et al., 1998, 2004; Borgna-Pignatti et al., 2004; Agas et al., 2008). Iron overload and HCV infection have been established to be risk factors for thalassemia patients to develop liver fibrosis, cirrhosis,

and hepatocellular carcinoma (HCC) (Di Marco et al., 2008; Voskaridou et al., 2012; Borgna-Pignatti et al., 2014; Chung et al., 2015; Moukhadder et al., 2017; Hodroj et al., 2019). Only 15–25% of the infected subjects can eliminate the infection alone. The remaining 75–85% of them make the infection chronic, developing cirrhosis that can be complicated by liver failure and hepatocellular carcinoma. In several studies from different population of thalassemic patients (United States, Italy and Greece) the prevalence of cirrhosis ranged from 10 to 20% and the incidence of HCC in thalassemia patients was progressively increasing (Voskaridou et al., 2012; Borgna-Pignatti et al., 2014; Chung et al., 2015; Moukhadder et al., 2017; Rumi et al., 2018; Hodroj et al., 2019). These data have made an intervention to eradicate HCV infection more urgent.

Regarding Sickle Cell Anemia, about 10–20% of patients have chronic HCV infection. Moreover, due to the need of blood transfusions to manage the sickling crisis and anemia, patients affected by HCV infection frequently are exposed to higher risk of iron overload and hemosiderosis, leading to liver-related morbidity and mortality (Moon et al., 2017). The hepatic involvement ranges in severity from liver dysfunction to liver failure, and occurs as the result of many factors: (a) sickling process (with acute hepatic vaso occlusion, hepatic sickle cell cholestasis, liver ischemia, and reperfusion injury, hemolysis, and cholelithiasis); (b) chronic viral hepatitis; (c) transfusion related hemosiderosis; and (d) autoimmune liver disease (Porter and Garbowski, 2013; Jitraruch et al., 2017; Theocharidou and Suddle, 2019).

HCV GENOTYPES

HCV is a heterogeneous virus with at least seven genotypes up to now identified. Each genotype includes multiple subtypes which differ to one another from 31 to 33% over the whole viral genome (Petruzziello et al., 2019). This genetic heterogeneity influences the efficacy of the antiviral therapy as response rate, since it must be specific for different viral strains as concerns type and duration of treatment. Detailed knowledge of HCV genotype has a great clinical relevance since the efficacy of antiviral therapies is greatly influenced by genotypes and subtypes distribution. HCV genotypes are geographically heterogeneous and are characterized by "epidemic subtypes," such as 1a, 1b, 2a, and 3a in high income countries and the "endemic" strains in restricted areas, such as West Africa, Southern Asia, Central Africa, and Southeastern Asia (Petruzziello et al., 2019). Regardless to Europe, genotype 1 is the most frequent and seems to be equally distributed among European countries (between 50 and 70%) followed by the genotype 3 (between 20 and 29%). About 8.9% of genotype 2 is more frequent in Western Europe, while about 4.9 and 5.8% of genotype 4 may be observed in Central and Western Europe, respectively (Petruzziello et al., 2019).

THE STUDY DESIGN

In the present study, we report the experience of patients with thalassemia and sickle cell disease treated with DAAs therapy at the Thalassemia Unit of S. Eugenio Hospital of Rome. The study was conducted in accordance with the principles of Good Clinical Practice, the Declaration of Helsinki and all the local regulations. The treatments with DAAs were applied according to criteria defined by national guidelines and were authorized by Italian Medicines Agency. All patients provided their written informed consent to participate in this study.

Effectiveness of the treatment according to the clinical case, was compared to HCV therapy by interferon and DAAs, commonly used to improve the life expectancy of the patients.

HCV THERAPY: INTERFERON

The treatment of chronic HCV infection began with the administration of interferon (INF) mono therapy, and until a few years ago the recommended therapy available for HCV treatment was pegylated-interferon (PEG-IFN) alpha, plus ribavirin (RBV) with an administration of about 24 weeks (genotypes 2–3) or 48 weeks (genotype 1) (Reddy et al., 2001; Li et al., 2002; Inati et al., 2005; Ricchi et al., 2011; Aminizadeh et al., 2016; Risoluti et al., 2016a,b, 2017, 2018; Catauro et al., 2018). This treatment produced important side effects, such as irritation at the injection site, febrile influenza-like manifestations, mental disorders, thyropathy, neutropenia, and hemolytic anemia. Eradication of the infection did not reach the expected results, due to the presence of subjects who did not respond to therapy or developed recurrences. Infection is assumed eradicated when there is a sustained virological response (SVR), defined as the lack of HCV RNA in serum by a sensitive test performed 24 weeks after completion of antiviral therapy. PEG-IFN plus RBV obtained SVR rates of 25–64% in patients with thalassemia and HCV infection (Inati et al., 2005; Harmatz et al., 2008; Di Marco et al., 2016), and demonstrated an improvement of patient survival due to a reduction in the risk of cirrhosis and hepatocellular carcinoma (Ray and Thomas, 2015). However, this treatment was not well-tolerated by thalassemic and SCD patients and the use has been severely reduced because ribavirin (RBV) proved to cause hemolytic anemia and to increase blood transfusions, in addition to interferon side effects, such as influenza-like symptoms, depression and cytopenias (Materazzi et al., 2014a; Origa et al., 2015; Di Marco et al., 2016; Moon et al., 2017). More recently, the use of Direct-acting Antiviral Agents (DAAs) demonstrated to be suitable in HCV management in patients with thalassemia and sickle cell disease for whom previous regimens gave restrictions (Materazzi et al., 2015, 2017a; Di Marco et al., 2016; Moon et al., 2017; Origa et al., 2017; Premkumar et al., 2017; Mehta et al., 2018).

HCV THERAPY: DIRECT-ACTING ANTIVIRAL AGENTS (DAAS)

In 2011 the development of DAAs gave a breakthrough to the treatment of patients with chronic HCV. The therapy with DAAs was initially performed together with PEG-INF plus RBV for patients with genotype 1 and this led to an increase in SVR to about 70%. The limitation of these treatments consisted of the

combination with PEG-INF plus RBV. In 2013, simeprevir and sofosbuvir were introduced. These two drugs were proposed to treat genotype 1 infections with no interferon, and in October 2014 the FDA approved the combination ledipasvir/sofosbuvir to manage HCV genotype 1. Several clinical trials demonstrated a SVR ranging from 94 to 97% after 12 weeks in previously treated and untreated patients, respectively (Afdhal et al., 2014). Subsequently, with the introduction of new types of DAAs, this therapy became available for all HCV genotypes.

At first the indication of DAAs administration were for patients with advanced cirrhosis or needing liver transplantation over time, while recently all regimens have been approved to treat patients with various HCV genotypes, stages of liver disease, and comorbidities. Recent outcomes from clinical trials showed a SVR higher than 95% when LDV/SOF (treatment from 8 to 12 weeks) was used to treat HCV genotypes 1 and 4, including patients with human immunodeficiency virus (HIV) coinfection and cirrhosis (Moon et al., 2017). Several trials have been performed on thalassemia patients, while few studies focused on safety, tolerability and efficacy of LDV/SOF treatment for patients with SCD (Moon et al., 2017). The development in recent years of highly effective and increasingly selective DAAs toward different viral genotypes has led to a real revolution in the HCV eradication for patients with thalassemia and hemoglobinopathies, leading to negative viremia and SVR between 90 and 98% (Materazzi et al., 2014b, 2017b,c; Nagral et al., 2017; Origa et al., 2017; Mehta et al., 2018; Premkumar and Dhiman, 2018; Mangia et al., 2019; Ponti et al., 2019).

A problem on which there has been extensive discussion on the use of DAAs was the cost of such therapies and this has led to give the indication to apply these treatments only to patients with extensive liver disease (F3 or F4). The high costs of the new drugs led to some prescriptive restrictions in the initial phase which unfortunately did not take into account the increase in the neoplastic risk linked to the iron accumulation for the transfusion dependent and hemolytic patients (Chung et al., 2015; Hodroj et al., 2019).

The eradication of the virus determined by the new drugs has also led to a marked improvement in the parameters of martial accumulation, demonstrating a synergic action also between the effect of antiviral therapy and iron chelation (Dharamsi et al., 2017; Nagral et al., 2017; Origa et al., 2017; Premkumar et al., 2017; Mehta et al., 2018; Mangia et al., 2019; Ponti et al., 2019).

THE EXPERIENCE OF THE DAAS THERAPY OF AN ITALIAN THALASSEMIA CENTER

Thalassemia major, thalassemia intermedia, and sickle cell disease patients with chronic HCV infection and fibrosis stage of F3–F4 were enrolled and then treated with DAAs according to the criteria defined by Italian Medicines Agency and the access to these treatments included a Fibroscan of at least 10 KPa.

Thirty-four patients with a confirmed diagnosis of hemoglobinopathy and HCV infection were treated with DAAs in the period 2015–2018. The characteristics of the patients are described in **Table 1**. In particular, 28 subjects (16 females and 12 males, mean age 44 ± 7 years) affected by Thalassemia Major, 3 subjects (2 females and 1 males) affected by Thalassemia Intermedia, and 3 subjects (1 female and 2 males)

TABLE 1 | Characteristics of the patients treated with DAAs and comparison of serum alanine aminotransferase (ALT), liver iron concentration (LIC), and serum ferritin values between baseline and after treatment at the SVR evaluation.

Patients, *n*	**34**
Age, years	
Mean ± SD	44 ± 7
Range	32–59
Gender, *n* (%)	
Male (M)	15 (44.1%)
Female (F)	19 (55.9%)
Hemoglobinopathy, *n* (%) (M/F)	
Thalassemia major (TM)	28 (82.4%) 16F/12M
Thalassemia intermedia (TI)	3 (8.8%) 2F/1M
Sickle cell disease (SCD)	3 (8.8%) 1F/2M
HCV genotype, *n* (%)	
G1a	3 (8.8%)
G1b	17 (50%)
G2a	5 (14.7%)
G2a/2b	1 (2.9%)
G2a/2c	2 (5.8%)
G3	2 (5.8%)
G4	4 (11.7%)
Liver stiffness, KPa (range)	10.0–34.8
Iron chelation, mg/Kg/day (days)	
DFO	30–40 (5–7 days)
DFX	25–35 (7 days)
DFP	60–75 (7 days)
DFO + DFP	25–40 (3–4 days) + 75 (7 days)
DFO + DFX	25–30 (3–6 days) + 20–35 (1–4 days)
Comorbidities, *n* (%)	
Heart disease	5 TM (14.7%)
Essential thrombocytopenia	1 TM (2.9%)
Kidney disease	2 SCD (5.9%)
Pulmonary embolism	1 TM (2.9%)
Crioglobulinemia and neuropathy	1TM (2.9%)
Hepato cellular carcinoma	1TM (2.9%)
Serum ALT, IU/L (mean ± SD, range)	
Before therapy	44.0 ± 30.1 (9–124)
After therapy $p = 0.018$*	24.1 ± 21.8 (8–99)
LIC, mg Fe/g liver d.w. (mean ± SD, range)	
Before therapy	1.85 ± 1.22 (0.9–5.7)
After therapy $p = 0.626$*	1.66 ± 0.72 (0.9–3.2)
Serum Ferritin, ng/mL (mean ± SD, range)	
Before therapy	429 ± 355 (111–1594)
After therapy $p = 0.150$*	536 ± 528 (87–2199)

*Students' t-test for paired data.

with Sickle Cell Disease (two with HbS/β Thalassemia and one with HbS/HbS). All subjects were transfusion dependent, and because of the iron overload an iron chelation therapy was administered: deferoxamine (DFO) in 29% of patients, deferiprone (DFP) in 13% of patients, deferasirox (DFX) in 25% of patients, DFO + DFP in 23% of patients, and DFO + DFX in 10% of patients. The iron chelation therapies were different and personalized for the patients, the dosages of iron chelators are reported in **Table 1**.

All the patients had previously treated with Interferon to eradicate HCV infection but were not responsive to the therapy. The HCV genotypes were characterized in order to treat the patients with the specific DAAs (**Table 1**). Among the treated subjects, 50% were genotype 1b (17 subjects), 14.7% were genotype 2a (5 subjects), 11.7% were genotype 4 (4 subjects), 8.8% were genotype 1a (3 subjects), 5.8% were genotype 2a/2c (2 subjects), 5.8% were genotype 3 (2 subjects), and 2.9% were genotype 2a/2b (1 subjects). The liver stiffness, as determined by Fibroscan, showed values between 10.0 and 34.8 KPa.

Some comorbidities were present before treatment in the group of patients. Five thalassemia major subjects suffered with heart disease, and two HbS/β thalassemia cases with kidney disease. Moreover, a case of hepatocellular carcinoma, a cryoglobulinemia with neuropathy, a subject with essential thrombocytopenia, and a case with a pulmonary embolism event were present in thalassemia major patients (**Table 1**).

The specific DAAs therapies were applied to the patients according to HCV genotype and are reported in **Table 2**.

All patients were treated with a cycle (12 weeks) of DAAs, only two Thalassemia major patients were treated for other 12 weeks because they were non-responder to the therapy. No patient required dose reduction or termination of antiviral treatment, and 32/34 patients (94%) reached SVR12 with negative follow up. Two thalassemia major patients with severe comorbidities and treated for 24 weeks, were not responder with infection relapse after 1 month. One of them had hepatocellular carcinoma, and the other had severe dilatation and restrictive cardiomyopathy with liver from stasi. Moreover, a SCD patient died of cholestasis and severe liver failure at 6 months from the end of 12 weeks DAAs therapy without viral recurrence. Already before the treatment the patient had significant hepatic impairment with a high degree of fibrosis (F4), a high fibroscan value 34.8 KPa, liver T2* 13 ms, and normal serum ferritin value (187 ng/mL). To our knowledge the patient did not have a primary sclerosing cholangitis.

All treatment regimens were well-tolerated, and no adverse events were reported. No patient has modified the ongoing iron-chelation treatment and the number of blood transfusions remained unchanged.

The evaluation of some parameters before and after treatment (**Table 1**) suggested a significant reduction ($p = 0.018$) in alanine aminotransferase levels from 44.0 ± 30.1 IU/L at baseline to 24.1 ± 21.8 IU/L at the SVR evaluation. On the other hand, the liver iron concentration (LIC) resulted decreased after treatment from 1.85 ± 1.22 to 1.66 ± 0.72 mgFe/g liver, but the difference was not significant. Serum ferritin values did not show differences before and after DAAs therapy.

TABLE 2 | DAAs therapies and virological response after treatment regimens.

DAAs therapy, *n* (%)	
Sofosbivir + Ledipasvir	14 (41%)
Sofosbivir + Daclatasvir	4 (12%)
Sofosbuvir + Velpatasvir	9 (26%)
Simeprevir + Sofosbivir	2 (6%)
Sofosbivir	2 (6%)
Elbasvir + Grazoprevir	2 (6%)
Glecaprevir + Pibrentasvir	1 (3%)
Effectiveness of DAAs therapy, *n* (%)	
Sustained Virological Response (SVR)	32/34 (94%)
Not responder with infection relapse	2/34 (6%)

DISCUSSION

The data currently available in the literature suggest that the eradication of HVC infection using the new DAAs is very close. In addition, the results obtained on the patients with thalassemia and sickle cell disease have given high values of SVR, always higher than 90%. There are still a small percentage of non-responder subjects to DAAs therapy and one of the causes could be that some of them were already very compromised with serious comorbidities when were treated, as in our non-responder subjects with infection relapse after 1 month. Moreover, at first the treatment of patients did not include new types of DAAs currently available. Therefore, now those not responding to the therapy could be treated with other DAAs of more recent generation or with different modalities.

As for the follow-up of patients, the application of DAAs therapies is so recent that there are still not many long-term trial data available to evaluate whether DAAs improve the morbidity and mortality of SVR patients. The patients should be periodically monitored and the follow-up should include the confirm of viral negativity, and the overall assessment of liver disease, particularly in thalassemia and sickle cell disease patients who have an additional risk factor caused by the iron overload. More studies should be performed to evaluate whether the risk of carcinoma is reduced and progressively declined after SVR, but it is still early to make an assessment.

It remains the big problem of the costs of DAAs therapies. These treatment regimens are very expensive, the costs can vary according to the different countries of the world, and this can certainly limit their application, and lengthen the time for a global eradication of HCV.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was performed in accordance with the principles of Good Clinical Practice, the Declaration of Helsinki, and all the local regulations. The treatments with Direct-acting Antiviral Agents are applied according to criteria defined by national guidelines and are authorized by Italian Medicines Agency (AIFA). All patients provided their written informed consent to participate in this study. The study was approved by Comitato Etico Roma 2, S. Eugenio Hospital, Rome.

AUTHOR CONTRIBUTIONS

FS, LM, PC, RR, and SMat contributed to the conception of the study. FS, LM, and GT enrolled the patients, performed the clinical and laboratory evaluation, and management of subjects. FS, LM, PC, SMat, RR, and SMas contributed to the acquisition and evaluation of data for statistics. FS, PC, RR, and SMat wrote the manuscript. All authors have revised and approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Significant Quantitative Differences in Orexin Neuronal Activation After Pain Assessments in an Animal Model of Sickle Cell Disease

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Sickle cell disease is a hemoglobinopathy that causes sickling of red blood cells, resulting in vessel blockage, stroke, anemia, inflammation, and extreme pain. The development and treatment of pain, in particular, neuropathic pain in sickle cell disease patients is poorly understood and impedes our progress toward the development of novel therapies to treat pain associated with sickle cell disease. The orexin/hypocretin system offers a novel approach to treat chronic pain and hyperalgesia. These neuropeptides are synthesized in three regions: perifornical area (PFA), lateral hypothalamus (LH), and dorsomedial hypothalamus (DMH). Data suggest that orexin–A neuropeptide has an analgesic effect on inflammatory pain and may affect mechanisms underlying the maintenance of neuropathic pain. The purpose of this study was to determine whether there are neuronal activation differences in the orexin system as a result of neuropathic pain testing in a mouse model of sickle cell disease. Female transgenic sickle mice that express exclusively (99%) human sickle hemoglobin (HbSS-BERK) and age-/gender-matched controls (HbAA-BERK mice; $n = 10$/group, 20–30 g) expressing normal human hemoglobin A were habituated to each test protocol and environment before collecting baseline measurements and testing. Four measures were used to assess pain-related behaviors: thermal/heat hyperalgesia, cold hyperalgesia, mechanical hyperalgesia, and deep-tissue hyperalgesia. Hypothalamic brain sections from HbAA-BERK and HbSS-BERK mice were processed to visualize orexin and c-Fos immunoreactivity and quantified. The percentage of double labeled neurons in the PFA was significantly higher than the percentage of double labeled neurons in the LH orexin field of HbAA-BERK mice ($^*p < 0.05$). The percentages of double labeled neurons in PFA and DMH orexin fields are significantly higher than those neurons in the LH of HbSS-BERK mice ($^*p < 0.05$). These data suggest that DMH orexin neurons were preferentially recruited during neuropathic pain testing and a more diverse distribution of orexin neurons may be required to produce

analgesia in response to pain in the HbSS-BERK mice. Identifying specific orexin neuronal populations that are integral in neuropathic pain processing will allow us to elucidate mechanisms that provide a more selective, targeted approach in treating of neuropathic pain in sickle cell disease.

Keywords: orexin, hyperalgesia, sickle cell, pain, hypocretin

INTRODUCTION

Sickle cell disease (SCD) is characterized as a hemoglobinopathy that causes red blood cells to sickle, and pain experienced by those individuals who suffer with SCD is associated with significant morbidity and increased death. In the United States, SCD accounts for over $450 million in healthcare costs each year (Steiner and Miller, 2006; Hassell, 2010) and there is a lack of knowledge related to the development and treatment of neuropathic pain associated with SCD. According to the International Association for the Study of Pain, neuropathic pain is defined as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system either at the peripheral or central level" (Haanpaa et al., 2011; Molokie et al., 2011). It is possible that altered processing within the nervous system may be the cause for persistent and sometimes unrelieved neuropathic pain in SCD.

Neuropathic pain has not been well-studied in patients with SCD to date. It is estimated that the incidence of neuropathic pain in the SCD population may be twice as what is found in other chronic pain populations other than SCD (Brandow et al., 2014). The defining characteristics of neuropathic pain are allodynia and hyperalgesia (Ballas and Darbari, 2013). Classical components of neuropathic pain are pain from a non-painful stimulus (i.e., extreme sensitivity to cool stimuli) and increased pain from a painful stimulus and pain caused by a stimulus that what would normally not be characterized as painful (Treede et al., 1992; Sethna et al., 2007).

Optimal management of neuropathic pain is yet to be delineated and opioid and non-steroidal anti-inflammatory drugs (NSAIDs) have not provided treatments that effectively alleviate neuropathic pain. While this improvement in treatment options for neuropathic pain research have been observed, pain is not always properly managed (Brandow et al., 2014). In order to develop better treatment strategies, it is important to identify neurochemical processes that may be involved in mediating neuropathic pain and use this info to develop better treatment regimens. One possible system to explore is the orexin system since it has been reported to mediate pain. The orexin system offers a novel approach to treat chronic pain and hyperalgesia. This system has been linked to the mediation of neuropathic pain and inflammatory processes (Yamamoto et al., 2002; Razavi and Hosseinzadeh, 2017); however, no published studies have investigated its possible role in SCD.

This current study utilizes transgenic sickle mice that express human sickle hemoglobin (HbSS) to explore the possibility of the orexin system as a target region in mediating neuropathic pain in SCD. Orexins are a family of hypothalamic peptides that play a role in the regulation of feeding behavior, energy metabolism, reward, and the sleep-wake cycle (de Lecea et al., 1998; Sakurai et al., 1998; Aston-Jones et al., 2009; de Lecea, 2012). Orexin neurons are expressed in the dorsomedial hypothalamus (DMH), perifornical area (PFA), and lateral hypothalamus and send their projections into other brain regions (Peyron et al., 1998, Chen et al., 1999; Nambu et al., 1999). Some of these regions are involved in analgesia and play a role in descending pain inhibition (Ossipov et al., 2010). There are two orexinergic receptors and orexin 1 receptor has a greater affinity for orexin A vs. orexin B peptide (Trivedi et al., 1998; Lu et al., 2000; Marcus et al., 2001). It has been demonstrated that orexinergic projections from the hypothalamus project to the spinal cord (lamina I) (van den Pol, 1999), lamina X, and laminae II–VII in the dorsal horn (Date et al., 2000; Bingham et al., 2001). Data suggest that orexin-A has an analgesic effect on inflammatory pain (Yamamoto et al., 2002), but it is not clear if the same mechanisms underly the maintenance of neuropathic pain and inflammatory pain. It is also not known whether the same analgesic effect with orexin-A on inflammatory pain will be similar in a neuropathic pain model. Neuropathic pain can be difficult to manage with standard analgesics such as opioids (Arner and Meyerson, 1988). Hence, the orexin system may offer a novel approach to treat chronic pain and hyperalgesia.

Enhanced pain-related behaviors have been observed in adult mice after temporally-controlled ablation of orexin neurons (Inutsuka et al., 2016). The mechanism by which orexin system modulate neuropathic pain is not well-established in the literature. Before it can be determined how the orexin system is involved in the mediation of neuropathic pain in a model of SCD, it is important to determine whether factors associated with neuropathic pain (i.e., hyperalgesia) differentially influence orexin neuronal activity. Therefore, the purpose of this study was to identify whether there were activational and topographical changes in the various subpopulations of orexin neurons as a result of various pain assessments in a mouse model of neuropathic pain in sickle cell disease. Identifying and understanding the activity of this neuronal circuitry will allow us to gain better perspective on differential patterns of activity in orexin neurons in the DMH, PFA, and LH after pain testing. The data from these experiments can lay the foundation for a more in-depth investigation on alternative pharmacological therapies to treat neuropathic pain in the SCD population by directly targeting the subpopulations that can influence nociceptive processing and reduce hyperalgesia. These studies can move the field forward by identifying whether there are selective subpopulations of orexin neurons that may be preferentially recruited during neuropathic pain. In this study,

we established baseline measurements for pain responses and assessed orexin neuronal activation in the DMH, PFA, and LH of transgenic mice expressing human sickle hemoglobin (HbSS-BERK) and control mice expressing normal human hemoglobin A (HbAA-BERK).

MATERIALS AND METHODS

Animals

Female transgenic HbSS-BERK sickle mice and age-/gender-matched controls (HbAA-BERK) were used in this study (n = 10/group, ∼4–6 months old, 20–30 g). The HbSS-BERK express human (99%) sickle hemoglobin and HbAA-BERK control mice express normal human hemoglobin A (HbAA). Females more commonly express neuropathic pain in pain populations, including SCD (Torrance et al., 2006; Butler et al., 2013; Brandow et al., 2014). The mice were bred and characterized by phenotype in a pathogen-free facility under a 12 h light-dark cycle at the University of Minnesota. The HbSS-BERK mice display similar pathological features of human SCD such as hematologic disease, organ damage and tonic hyperalgesia (Paszty et al., 1997; Kohli et al., 2010; Giuseppe Cataldo et al., 2015). All animal care and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Behavioral Assessments

All behavioral tests were performed in a quiet room at a constant temperature (23–25°C). All mice were habituated to each test protocol and environment. Before performing baseline measurements and testing, four parameters were used to assess behaviors in the following order of testing: mechanical hyperalgesia, thermal hyperalgesia, grip force, and cold hyperalgesia (Kohli et al., 2010).

Mechanical Hyperalgesia

To assess mechanical hyperalgesia, each mouse was put on a wire mesh apparatus under a glass container, allowed to acclimate, and a von Frey filament was applied to the hind paw for 1–2 s. A 1.0 g (4.08 mN) von Frey (Semmes-Weinstein) monofilament (Stoelting) was applied to the plantar surface of the hind paw of each mouse with enough force to bend the filament. Paw withdrawal frequency was determined by the number of time paw lifting was observed per 10 applications.

Thermal/Heat Hyperalgesia

Thermal hyperalgesia was determined via measurement of heat sensitivity in the HbAA-BERK- and HbSS-BERK mice. Thermal hyperalgesia was assessed using the Hargreave's apparatus with a radiant heat stimulus. As previously described (Kohli et al., 2010; Lei et al., 2016; Tran et al., 2019), a radiant heat stimulus was applied under the hind paws of each mouse following acclimation to the floor of the Hargreave's apparatus. The radiant heat stimulus was located under the glass floor and administered using an infrared heat source. The paw withdrawal latency was recorded as the time when the mouse withdraws its paw from the heat stimulus (to the nearest 0.1 s).

Grip Force

To assess deep tissue hyperalgesia, a digital grip force meter (Chatillon) was used to measure peak forepaw grip force. The force was measured by gently holding each mouse by its tail and pulling it across a wire mesh gauge. The grip force was recorded as the force (in g) exerted at the time of grip release by each mouse.

Cold Hyperalgesia

Cold hyperalgesia was determined via measurement of cold sensitivity of the mice to a cold plate set at 4°C. Cold withdrawal latency was determined by the time it took each mouse to initially lift either forepaw. Cold withdrawal frequency was determined by the number of times that mouse lifted and rubbed the forepaws over a period of 2 min.

Immunohistochemical Processing for c-Fos and Orexin in Hypothalamic Sections

In preparation for double label immunohistochemistry, brains from each mouse were extracted 90 min after behavioral testings and immersed in 10% formalin for fixation for at 1–2 weeks. Following cryoprotection in 30% sucrose solution, coronal brain sections were cut and processed for c-Fos and orexin-A as previously described (Richardson and Aston-Jones, 2012). Sections were incubated overnight at room temperature in primary antibody against Fos-related antigens (1:1,500, SC-52, Santa Cruz), then rinsed and incubated for 2 h with secondary antibody (biotinylated donkey anti-rabbit 1:500, Jackson Immunoresearch Laboratories). Sections were transferred to avidin–biotin complex (ABC, 1:500, Vector Laboratories) for 1.5 h and then Fos neurons were visualized by placing the sections in SIGMAFAST 3,3′-diaminobenzidine (DAB, D8552, Sigma) with cobalt chloride metal enhancer. Following a 45 min incubation in PBS-azide, the sections were placed in primary antibody for orexin-A (1:1,000, SC8070, Santa Cruz) overnight. Sections were incubated in secondary antibody (biotinylated donkey anti-goat 1:500, Jackson Immunoresearch) the next day, incubated in ABC and then orexin neurons are visualized using DAB (D5637, Sigma, no metal enhancer) with 0.0002% H_2O_2. The sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped with Permount. Orexin-positive neurons exhibited brown cytoplasmic staining and Fos-positive nuclei (cobalt chloride intensified) were stained black.

Quantification of Neurons and Statistical Analysis

The number of neurons with Fos positive nuclei, orexin-A positive cytoplasmic staining, and double labeled Orexin-Fos neurons was counted in the DMH, PFA, and LH for the HbAA-BERK and HbSS-BERK mice. The area located medial to the fornix was defined as the DMH region, the region located around the fornix was defined as the PFA region and the region lateral to the fornix was defined as the LH region (similar to other studies (Harris et al., 2005; Richardson and Aston-Jones, 2012). Quantification of the labeled neurons was conducted using a

unique number code for each animal so that the investigator was blinded to the treatment groups.

Hypothalamic sections at two different levels, rostral (Bregma −1.34 mm) and caudal (Bregma −1.94 mm) (Paxinos and Franklin, 2001) level from each animal were used to count orexin- and Fos- positive neurons. A representative section from the rostral and caudal orexin fields of each animal was used to ensure that there was a good representation of the hypothalamic field, as described in Richardson and Aston-Jones (2012). A color image of the orexin field was acquired from a digital camera at 10×-20× magnification using brightfield illumination from a light microscope (Zeiss) connected to a computer station that capture images. The labeled neurons were marked using a pointer tool in Zen Pro software, preventing a cell from being counted more than once in an image. Neurons were counted bilaterally for each region and at each level (Zen Pro software [Carl Zeiss Microscopy, LLC, White Plains, NY]). The data are expressed as average counts of Fos positive, orexin positive, and percentage of double-labeled neurons (total number of double labeled neurons divided by total number of orexin positive neurons).

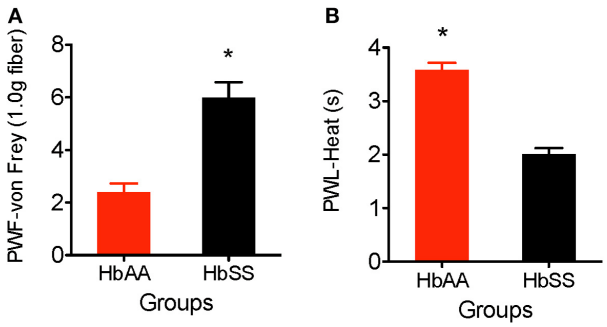

FIGURE 1 | Comparative differences in behavioral assessments for mechanical and heat hyperalgesia in Female HbAA-BERK and HbSS-BERK mice. All data are reflected as mean ± SE, $n = 9$–10/group. **(A)** Mechanical hyperalgesia was measured by paw withdrawal frequency (PWF) in HbAA-BERK and HbSS-BERK mice. HbSS-BERK mice display significantly more PWF than HbAA-BERK mice (*$p < 0.0001$). **(B)** Heat hyperalgesia was measured by paw withdrawal latency (PWL) in response to a heat stimulus in age and sex-matched HbAA-BERK and HbSS-BERK mice. HbAA-BERK mice display significantly greater PWL than HbSS-BERK mice (*$p < 0.0001$).

Statistical Analysis

Behavioral assessments and neuron counts for each hypothalamic region were quantified. Data were compared using a one-way analysis of variance (ANOVA) to determine regional/topographical differences (DMH vs. PFA vs. LH) for HbAA-BERK and HbSS-BERK mice. This analysis was followed by a Kruskal Wallis *post-hoc* test with significant levels set at $p < 0.05$). We used independent *t*-tests to determine whether there were statistical differences between the means of HbAA-BERK and HbSS-BERK mice (activational differences) for: weight, mechanical hyperalgesia, heat hyperalgesia, cold hyperalgesia, grip force and observed immunoreactive cells. All data are represented as mean ± SE, $p < 0.05$.

RESULTS

Behavioral and immunohistochemical approaches were used to determine pain-related behaviors and investigate whether activational and topographical differences in the subpopulations of orexin in HbAA-BERK and HbSS-BERK mice. We used c-Fos as a marker for neuronal activation in this study. All data reflect observations in female mice since this group expresses higher hyperalgesia than male mice (Kohli et al., 2010; Lei et al., 2016).

Mechanical Hyperalgesia: Assess Sensitivity to Mechanical Stimulus

The von Frey filament (1.0 g, 4.08 mN) was applied for 1–2 s (with enough force to bend the filament) to the plantar surface of each hind paw of HbAA-BERK and HbSS-BERK mice. This stimulus is not characterized as normally painful. However, in animals that have greater tactile sensitivity (HbSS mice), there is a greater response to the filament application. The paw withdrawal frequency evoked when using the von Frey monofilament was significantly higher in HbSS-BERK mice vs. HbAA-BERK control mice (**Figure 1A**, $p < 0.0001$, 5.99 ± 0.6 vs. 2.4 ± 0.3). This observation in higher paw withdrawal frequency indicated increased hyperalgesia in HbSS-BERK mice.

Heat Hyperalgesia: Test for Heat Sensitivity

Paw withdrawal latency was measured as the duration of time recorded after the plantar surface of a single hind paw was exposed to a radiant heat stimulus (50 W projector lamp bulb). HbAA-BERK mice display significantly higher paw withdrawal latency vs. HbSS-BERK mice (**Figure 1B**, $p < 0.0001$, 3.59 ± 0.13 vs. 2.01 ± 0.11). The shorter paw withdrawal latency observed in the HbSS-BERK mice (**Figure 1B**) indicated increased sensitivity to heat. This heat sensitivity may indicate cutaneous hyperalgesia in HbSS-BERK mice.

Deep Tissue Hyperalgesia

One of the major consequences of SCD is chronic musculoskeletal pain which can be evidenced by muscle soreness and joint tenderness. Deep tissue hyperalgesia indicates the existence of inherent pain due to activation of visceral, joint, and musculoskeletal nociceptors. In this study, we utilized the grip force test to evaluate musculoskeletal pain in HbAA-BERK and HbSS-BERK mice. Deep tissue hyperalgesia was defined as a decrease in the grip force, which indicates increased nociception. Grip force significantly decreased in HbSS-BERK mice vs. HbAA-BERK control mice (**Figure 2A**). It was observed that HbAA-BERK mice exerted significantly more grip strength vs. HbSS-BERK mice (**Figure 2A**, $p < 0.005$, 132.9 ± 3.9 vs. 118.2 ± 1.4, respectively) since a higher force (in g) exerted at the gauge at the time of grip release by the HbAA-BERK mice was recorded.

Differences in grip force/body weight

Typically, musculoskeletal strength is greater as weight and muscle development increase. However, it is possible for grip force to not significantly change when expressed per gram body weight if there are underlying physiological factors (i.e.,

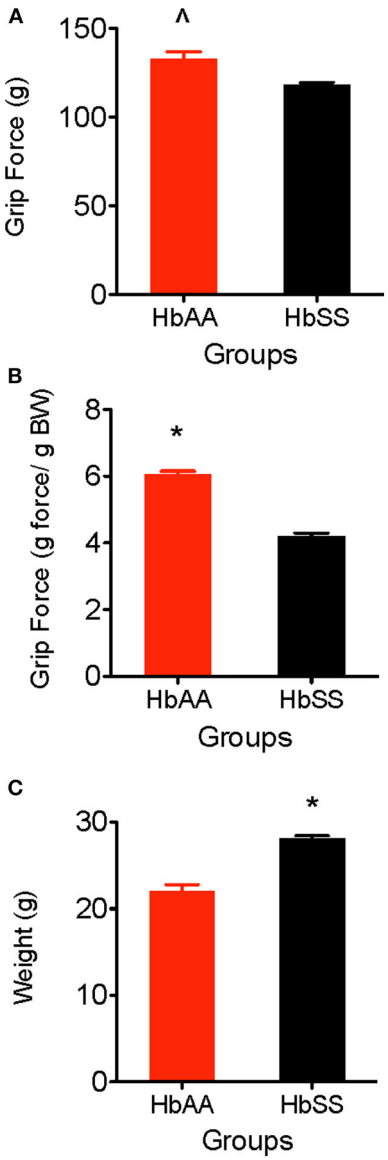

FIGURE 2 | Comparative Differences in deep tissue hyperalgesia and body weight for HbAA-BERK and HbSS-BERK mice. All data are reflected as mean ± SE, $n = 9$–10/group. **(A)** Differences in deep tissue hyperalgesia was assessed by grip force for HbAA-BERK and HbSS-BERK mice. HbAA-BERK mice display significantly more peak forepaw grip strength vs. HbSS-BERK mice (^$p < 0.005$). **(B)** When corrected for weight, HbAA-BERK mice still displayed significantly more grip strength vs. HbSS-BERK mice (*$p < 0.0001$). **(C)** The HbSS-BERK mice in this study were significantly heavier in body weight than HbAA-BERK mice (*$p < 0.0001$).

decreased muscle strength, inflammation, increased nociception) that contribute to muscle weakness and pain. When corrected for weight in this study, HbAA-BERK mice still display significantly more grip strength vs. HbSS-BERK mice (**Figure 2B**, $p < 0.0001$, 6.05 ± 0.10 vs. 4.21 ± 0.09) even though the HbSS-BERK mice were significantly heavier in body weight than the HbAA-BERK mice (**Figure 2C**, 28.81 ± 0.25 g vs. 22.02 ± 0.77 g, respectively).

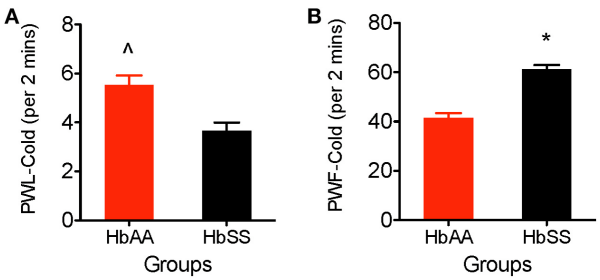

FIGURE 3 | Comparative Differences in behavioral assessments for cold hyperalgesia in female HbAA-BERK and HbSS-BERK mice. All data are reflected as mean ± SE, $n = 9$–10/group. **(A)** HbAA-BERK mice have significantly more PWL vs. HbSS-BERK mice (^$p < 0.005$), therefore, HbSS-BERK mice display more cold hyperalgesia after exposure to a 4°C cold plate, **(B)** HbSS-BERK mice display significantly more behavioral responses to the cold plate temperature of 4°C (*$p < 0.0001$).

Cold Hyperalgesia: Test for Cold Sensitivity and Behavioral Responses

We observed a higher sensitivity to the cold stimulus (aluminum plate) in HbSS-BERK vs. HbAA-BERK mice. The HbAA-BERK mice display significantly higher paw withdrawal latency vs. HbSS-BERK mice (**Figure 3A**, $p < 0.005$, 5.53 ± 0.38 vs. 3.65 ± 0.34). HbAA-BERK mice demonstrated a lower response in lifting either paw and were less likely than HbSS-BERK mice to respond to cold temperatures. HbAA mice spent more time walking around the platform on all four paws before the initial lifting of either paw vs. HbSS-BERK mice. The shorter paw withdrawal latency observed in the HbSS-BERK mice (**Figure 3A**) indicated increased sensitivity to cold temperature. This cold sensitivity may also indicate cutaneous hyperalgesia in HbSS-BERK mice. In addition, to measuring paw withdrawal latency, behavioral responses due to the exposure to the cold environment were recorded over a 2 min period. Observations were recorded for shivering/body shakes, paw flutter, and consistently lifting paws from the cold plate. HbSS-BERK mice display significantly more paw withdrawal frequency and behavioral responses vs. HbAA-BERK mice (**Figure 3B**, $p < 0.0001$, 61.18 ± 1.8 vs. 41.53 ± 1.87).

Quantification for Immunohistochemical Detection of Fos, Orexin, and Orexin-Fos Neurons in the DMH, PFA, and LH

To examine whether there were topographical and activational changes in the various subpopulations of orexin neurons located in the DMH, PFA and LH of HbAA-BERK and HbSS-BERK mice, hypothalamic sections were processed for double label immunohistochemistry for c-Fos and orexin-A peptide (**Figures 4A,B** at −1.94 mm Bregma). There were double labeled neurons (orexin-Fos positive neurons), single labeled orexin neurons, and single labeled c-Fos neurons throughout the DMH, PFA, and LH of both mouse groups (**Figure 5**).

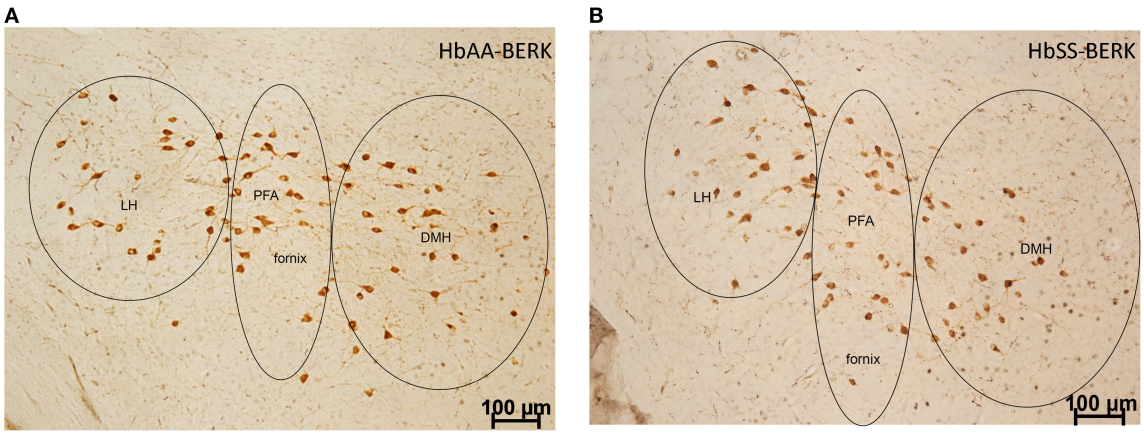

FIGURE 4 | Representation of the pattern of labeling in each hypothalamic subregion. Specific cell types were observed in HbAA-BERK and HbSS-BERK mice following c-Fos and orexin immunohistochemical procedures. **(A)** represents a photo from an HbAA-BERK animal. **(B)** represents a photo from an HbSS-BERK animal. The representative pictures reflect the pattern of labeling for each group and show the three hypothalamic subregions: DMH, PFA, and LH. Objective, 10×.

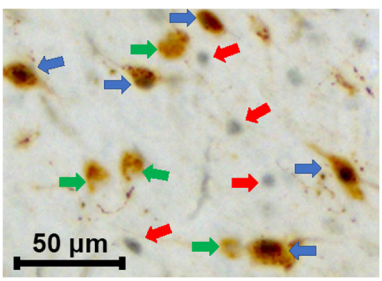

FIGURE 5 | Characterization of various cells types in HbAA-BERK and HbSS-BERK mice after behavioral assessments. The photograph shows the immunoreactivity for single labeled orexin cells (green arrows), single labeled c-Fos (red arrows), and double labeled c-Fos-activated, orexin cells (blue arrows). Black nuclear staining is indicative of c-Fos immunoreactivity and brown cytoplasmic staining is indicative of orexin-A peptide immunoreactivity. Objective, 20×.

Quantification of Double Labeled Neurons in the LH, PFA, and DMH of HbAA-BERK and HbSS-BERK Mice After Behavioral Tests

The percentages of orexin-Fos, double labeled neurons were quantified in the LH, PFA and DMH for HbAA-BERK (**Figure 6A**, 13.2 ± 2.1, 29.4 ± 4.7, 21.6 ± 2.5, respectively) and in the LH, PFA and DMH for HbSS-BERK mice (**Figure 6A**, 13.9 ± 2.1, 24.94 ± 3.3, 23.6 ± 2.6, respectively). In HbAA-BERK mice, the percentage of orexin-Fos neurons in the PFA was significantly higher than those observed in the LH orexin field (**Figure 6A**, $^{*}p < 0.05$). In HbSS-BERK mice, there was a different finding. While the percentage of orexin-Fos neurons was higher in the PFA vs. LH, the percentage of orexin-Fos neurons in the DMH were also significantly higher than those observed in the LH (**Figure 6A**, $^{*}p < 0.05$). This difference in topographical activation indicates that a greater number of orexin neurons are recruited/activated in two hypothalamic subregions (DMH and PFA) in the HbSS-BERK mice after behavioral testings.

Quantification of Single Labeled Neurons in the DMH, PFA, and LH of HbAA-BERK and HbSS-BERK Mice After Behavioral Tests

Single labeled c-Fos and orexin immunoreactive neurons were observed (**Figures 6B,C**) and quantified in all three orexin hypothalamic subregions of HbAA-BERK and HbSS-BERK mice.

Topographical differences in the number of c-fos neurons between the 3 hypothalamic regions

A one-way ANOVA was used to determine any significant differences in the means of c-Fos neurons quantified in the DMH, PFA and LH regions. In HbAA-BERK mice, there was a significant difference in the number of c-Fos neurons in the LH vs. DMH (77.6 ± 6.1 vs. 132.4 ± 12.2, respectively, $^{*}p < 0.05$). The number of c-Fos neurons quantified in the DMH was significantly higher than those observed in the PFA (132.4 ± 12.2 vs. 68.2 ± 7.6, $^{p} < 0.005$) in HbAA-BERK mice. *Post-hoc* analysis revealed that there was a significant difference in the number of c-Fos neurons in HbSS-BERK mice, when comparing LH vs. DMH (**Figure 6B**, 58.5 ± 7.3 vs. 106 ± 10.2, $^{*}p < 0.05$). Additionally, there was a significant difference in the number of Fos neurons in the PFA vs. DMH (**Figure 6B**, 41.6 ± 5.3 vs. 106 ± 10.2, respectively, $^{\#}p < 0.0005$) in HbSS-BERK mice.

Topographical differences in the number of orexin neurons between the 3 hypothalamic regions

A one-way ANOVA was used to determine any significant differences in the means of orexin neurons quantified in the DMH vs. PFA vs. LH regions. In HbAA-BERK mice, there was a significant difference in the number of orexin neurons quantified in the LH vs. PFA (**Figure 6C**, $^{*}p < 0.05$). However, there were no other significant differences observed between the regions. There was no significant difference in the number of orexin neurons in HbSS-BERK mice when comparing the three regions

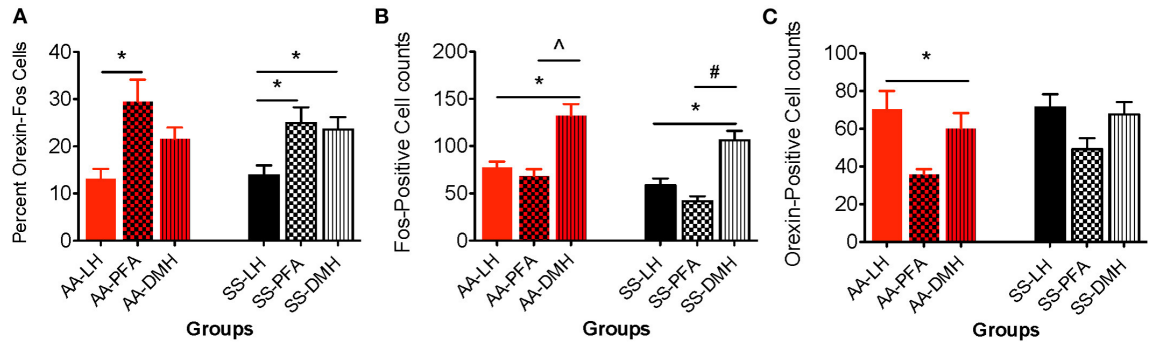

FIGURE 6 | Topographical differences in the immunohistochemical detection and quantification of c-Fos-positive, orexin-positive, and orexin-Fos cells in HbAA- and HbSS-BERK mice after behavioral assessments. All data are reflected as mean ± SE, $n = 9$–10/group. **(A)** The percentage of double labeled cells in the PFA was significantly higher than the percentage of double labeled cells in the LH orexin field of HbAA-BERK and HbSS-BERK mice ($^*p < 0.05$). The percentage of double labeled cells in the DMH orexin field was significantly higher than the percentage of double labeled cells in the LH of HbSS-BERK mice ($^*p < 0.05$). **(B)** In HbAA-BERK mice, there was a significant difference in the number of single labeled, c-Fos neurons in the LH vs. DMH ($^*p < 0.05$). Also, in HbAA-BERK mice, the number of c-Fos cells quantified in the DMH was significantly higher than those observed in the PFA ($^\wedge p < 0.005$). In HbSS-BERK mice, there was a significant difference in the number of c-Fos cells in HbSS-BERK mice, when comparing LH vs. DMH ($^*p < 0.05$). Additionally, there was a significant difference in the number of c-Fos cells in the PFA vs. DMH ($^\# p < 0.0005$) in HbSS-BERK mice. **(C)** Single-labeled, orexin positive cells were observed in the DMH, PFA, and LH of HbAA and HbSS mice. In HbAA mice, the number of orexin positive neurons was greater in the LH vs. PFA ($^*p < 0.05$).

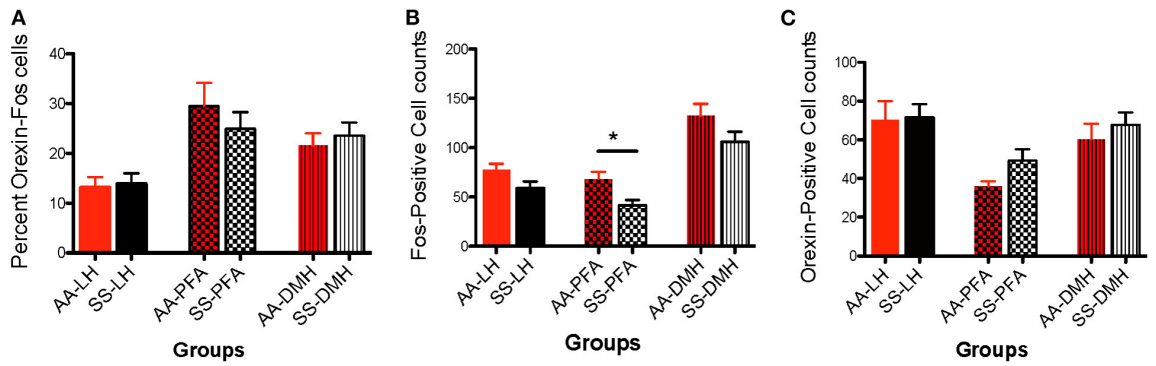

FIGURE 7 | Activational differences in the immunohistochemical detection and quantification of c-Fos-positive, orexin-positive, and orexin-Fos cells in HbAA-BERK and HbSS-BERK mice after behavioral assessments. All data are reflected as mean ± SE, $n = 9$–10/group. **(A)** There was no significant difference in the percentage of double labeled cells in the LH, PFA, and DMH of HbAA-BERK vs. HbSS-BERK mice. **(B)** Single labeled, c-Fos positive cells were observed in the DMH, PFA, and LH of HbAA-BERK and HbSS-BERK mice. HbAA-BERK mice displayed a greater number of c-Fos positive cells in the PFA vs. HbSS-BERK mice ($^*p < 0.05$). **(C)** Single-labeled, orexin positive cells were observed in the DMH, PFA, and LH of HbAA-BERK and HbSS-BERK mice, but there was no significant difference in the number of orexin positive cells in HbAA-BERK vs. HbSS-BERK mice.

Figure 6C (DMH 67.7 ± 6.5, PFA 49.2 ± 5.9, LH 71.3 ± 7.1, $p = 0.060$).

Activational differences for c-fos-activated orexin neurons in HbAA-BERK vs. HbSS-BERK mice for each hypothalamic region

Data analysis revealed that there was no significant difference in the percentage of LH-located, c-Fos activated orexin neurons from HbAA-BERK vs. HbSS-BERK mice (**Figure 7A**, 13.2 ± 2.1 vs. 13.9 ± 2.1, $p = 0.810$). There was also no significant difference in the percentage of c-Fos-activated orexin neurons in the PFA from HbAA-BERK vs. HbSS-BERK mice (25.4 ± 2.4 vs. 24.9 ± 3.3, $p = 0.923$). There also was no significant difference in the percentage of DMH-located, c-Fos-activated orexin neurons in HbAA-BERK vs. HbSS-BERK mice (**Figure 7A**, 21.5 ± 2.5 vs. 23.6 ± 2.6, $p = 0.575$).

Activational differences for c-fos neurons in HbAA-BERK vs. HbSS-BERK mice for each hypothalamic region

Data analysis revealed that there was a trend toward significance in the number of c-Fos neurons in the LH from HbAA-BERK vs. HbSS-BERK mice (**Figure 7B**, 77.6 ± 6.1 vs. 58.5 ± 7.3, $p = 0.06$). There was a significant increase in the number of c-Fos neurons in the PFA in HbAA-BERK vs. HbSS mice (**Figure 7B**, 68.2 ± 7.6 vs. 41.6 ± 5.3, $^*p < 0.05$). There was no significant difference in the number of DMH-located, c-Fos neurons from HbAA-BERK vs. HbSS-BERK mice (**Figure 7B**, 132.4 ± 12.2 vs. 106 ± 10.2, $p = 0.119$).

Activational differences for orexin neurons in HbAA-BERK vs. HbSS-BERK mice for each hypothalamic region

The presence of single labeled orexin neurons indicated that not all of the orexin neurons within the different subregions were engaged or activated after pain testing (**Figure 7C**) in HbAA-BERK and HbSS-BERK mice. There was no significant difference in the number of LH-located orexin neurons in HbAA-BERK vs. HbSS-BERK mice (70.3 ± 9.7 vs. 71.3 ± 7.0, $p = 0.934$). This means that the total orexin immunoreactive neuron counts in those subregions were similar in HbAA-BERK vs. HbSS-BERK mice. Similarly, there was no significant difference in the number of PFA-located, orexin neurons from HbAA-BERK vs. HbSS-BERK mice (35.9 ± 2.7 vs. 49.2 ± 5.9, $p = 0.08$). nor a statistical significance in the number of DMH-located, orexin neurons from HbAA-BERK vs. HbSS-BERK mice (**Figure 7C**, 60.1 ± 8.2 vs. 67.7 ± 6.5, $p = 0.475$).

DISCUSSION

In the present investigation, we sought to determine whether there were quantitative differences in the activation of orexin neurons after pain testing in a mouse model of SCD. This current study assessed the degree of hyperalgesia expressed in transgenic sickle mice (that express human sickle hemoglobin) vs. control mice (that express normal human hemoglobin) using various pain testing modules and then quantified the immunoreactivity for c-Fos, orexin, and double labeled, c-Fos activated, orexin neurons in the DMH, PFA and LH of these two groups of mice. The behavioral results showed that HbSS-BERK mice display a higher degree of hyperalgesia than HbAA-BERK mice and that while there were no significant activational differences in AA vs. SS mice for the three subregions, topographical differences were observed in HbAA-BERK and HbSS-BERK mice. Overall, the data indicate that the state of the mice (sickle hemoglobin vs. normal hemoglobin) and their sensitivity to painful stimuli may influence activation of orexin neurons within specific hypothalamic subregions.

Our behavioral findings showed that HbSS-BERK mice display significantly greater sensitivity to heat and cold hyperalgesia vs. HbAA mice. The HbSS-BERK mice showed a decreased paw withdrawal latency vs. HbAA mice to the heat stimulus as evidenced by the shorter time interval required to move the forepaw from the floor of the apparatus after being exposed to the heat. Similarly, HbSS-BERK mice displayed decreased paw withdrawal latency vs. HbAA-BERK mice when exposed to the surface of the cold plate. The HbSS-BERK mice lifted their forepaw in a shorter time and displayed a greater number of behavioral responses while exposed to the cold environment. Specifically, there was an increased number of observations for shivering/body shakes, paw flutter, and consistently lifting paws from the cold plate in the HbSS-BERK vs. HbAA-BERK mice. This increase in physical responses to the cold environment indicates that SS mice have more cold sensitivity and may also indicate cutaneous hyperalgesia in HbSS-BERK mice. It is thought that temperature changes and extremes may precipitate painful crises in patients with SCD (Smith et al., 2003) and our observations and others (Lei et al., 2016) support this claim in the HbSS-BERK mice model.

Similarly, HbSS-BERK mice displayed an increase in mechanical hyperalgesia and deep tissue hyperalgesia vs. HbAA-BERK mice with an increased sensitivity to the Von Frey filament and decreased grip force, respectively. The paw withdrawal frequency evoked when using the von Frey monofilament was significantly higher in HbSS mice vs. HbAA-BERK control mice. This observation in higher paw withdrawal frequency indicated increased hyperalgesia in HbSS-BERK mice. The measurement of deep tissue hyperalgesia in the mice was done to model the chronic musculoskeletal pain reported by SCD patients. Information gained from measuring deep tissue hyperalgesia may indicate inherent pain in the mice. Deep tissue hyperalgesia is associated with the activation of visceral, joint, and musculoskeletal pain receptors. The behavioral responses may reflect the muscle and joint tenderness that is often observed during a painful crises.

Our current findings for HbAA-BERK and HbSS-BERK mice during pain testing are consistent with that found in past studies (Kohli et al., 2010; Lei et al., 2016) and support the validity of this model to study neuropathic pain. In agreement with their findings, HbSS mice (with sickle human hemoglobin) display more responses to pain testing indicating increased hyperalgesia vs. HbAA-BERK control mice. Animal models have become increasingly important in understanding neuropathic pain in SCD patient. Transgenic sickle mice that express sickle hemoglobin are one of the best models to date. These mice experience pain episodes similar to those observed in humans. It is more common in females, therefore, we only used female mice in our study. It is estimated that the incidence of neuropathic pain in the SCD population may be twice what is found in other chronic pain populations other than SCD (Brandow et al., 2014). It is believed that neuropathic pain cases occur during painful sickle crises and resolve after the crises ends.

The data from our immunohistochemical studies identified three distinct groups of neurons within the hypothalamic regions of HbSS and HbAA-BERK mice: Fos only-single labeled, orexin only-single labeled, and c-Fos activated, orexin neurons. Differential activation of orexin subpopulations after pain testing in HbSS and HbAA-BERK mice were observed. There was a significant increase in the percentage of double labeled (c-Fos-orexin) neurons in the PFA when compared to those located in the LH of HbSS mice and this same relationship was also observed in HbAA-BERK mice. These patterns in activation of orexin cells reveal subregion, differential activation. This observation has also been reported in the literature for orexin neurons after a myriad of behavioral and pharmacological studies, including those to measure the c-Fos, activation of orexin neurons after behavioral testing for reward, reinstatement, feeding, stress and arousal (Boutrel et al., 2005; Harris et al., 2005; Winsky-Sommerer et al., 2005; Smith et al., 2009; Mahler et al., 2012; Moorman et al., 2017). Our data suggest that hyperalgesia-induced behavioral responses are associated with activation of orexin neurons and highlight anatomically and functionally distinct populations of orexin neurons.

A dichotomy in orexin function was previously proposed (Estabrooke et al., 2001; Harris and Aston-Jones, 2006; Yoshida et al., 2006), indicating that orexin neurons that are located in the DMH and PFA are preferentially associated with homeostasis and arousal/shock. Studies have shown that footshock, restraint or cold-exposure all increase c-Fos immunoreactivity in orexin neurons located in the PFA (Sakamoto et al., 2004; Plaza-Zabala et al., 2010; James et al., 2014). The orexin neurons in the LH were preferentially innervated by brainstem and areas involved in autonomic and visceral processing. These LH-located orexin neurons were activated during reward processing for both food and drugs of abuse and directly correlated with behavioral preference (Harris and Aston-Jones, 2006; Mahler et al., 2014). In another study, orexin neurons in the DMH and PFA were affected by diurnal changes; however the same did not occur for LH orexin neurons (Estabrooke et al., 2001). Additionally, activation of LH orexin neurons correlates with weight gain after the administration of anti-psychotic drugs in male rats, but not in DMH orexin neurons (Fadel et al., 2002).

Our current findings extend this hypothesis by proposing that the association between the orexin system and pain may also affected by functional dichotomy. It is possible that selective topographical activation of specific orexin neuron subpopulations were recruited during hyperalgesia. This may explain the significant differences in the percentage of c-Fos activated orexin neurons in the PFA vs. LH in HbSS and HbAA-BERK mice. Interestingly in HbSS-BERK mice only, the percentage of DMH c-Fos-orexin neurons activated after pain testing was also higher than those observed in the LH. This additional recruitment of activated DMH neurons may be as a result of increased hyperalgesia observed in the HbSS-BERK group. The increased hypersensitivity to the stimuli during the series of pain assessments in HbSS-BERK mice may be due to the afferent and efferent projections to and from the subregions.

Although there was no significant difference in activation of orexin neurons in the HbAA-BERK vs. HbSS-BERK mice (activational differences) nor in the absolute orexin neuron counts, there was a difference in the number of Fos neurons activated in HbAA-BERK vs. HbSS-BERK mice. This last finding was unexpected and may result from the sampling methodology. There was sampling from a subset of sections which may have caused some differences in the c-Fos population counts related to the measured hyperalgesia. However, further studies would need to be conducted to show that these Fos neurons were directly correlated with behavior. In our hands, this increase in Fos was not directly correlated with behaviors after pain testings. Another caveat is that the cell types for the single labeled c-Fos neurons that were recruited or activated after pain testing were not identified in this study. There are a number of other neuropeptides and neurotransmitters in these regions; however, identification of those specific neuronal cell types was beyond the scope of this investigation.

Previous studies have implicated the orexin system in the modulation of pain. In a neuropathic pain model using the partial sciatic nerve ligation in the rat, intrathecal and intracerebroventricular orexin-A administration produced a significant analgesic effect (Yamamoto et al., 2002). In another study, Orexin-A peptide reduced heat evoked hyperalgesia in a rat model of chronic constriction injury of the sciatic nerve, but the same result was not observed with orexin-B peptide (Suyama et al., 2004). This antinociceptive effect from orexin-A may be mediated partly via orexin-1 receptors (OX1R) in the dorsal horn of the spinal cord (Jeong and Holden, 2009; Wardach et al., 2016). Orexin-A produced an analgesic effect mediated by the activation of OX1R using a hot plate test (Bingham et al., 2001). While all of these studies suggest that orexin-A has an analgesic effect on pain, and specifically neuropathic pain, these data do not provide information about the entrained patterns of activation within the subregions. The lateral hypothalamus may facilitate antinociception through spinally descending orexins neurons. It is thought that directly stimulating the lateral hypothalamus produces antinociception mediated by OX1R in the dorsal horn (Wardach et al., 2016). However, it is difficult to interpret these results from (Wardach et al., 2016) as being specific to LH or at least the region that we have categorized as the LH in our current study, since it is possible that stimulation of that hypothalamic region may have also engaged neurons within the PFA and possibly DMH. In past studies, the categorization of orexin subregion boundaries has differed across studies. For this reason, the data from our study are so critical to contributing to understanding the difference in the profiling of orexin neurons. The immunohistochemical data in our study support a distinct sampling of all of the orexin neuron regions. These data provide more understanding and identification for which orexin subpopulations may be involved in pain processing. This is the first published paper to show the topography associated with the activation and/or engagement of the orexin system in a model of hyperalgesia associated with SCD.

In future studies, we seek to elucidate mechanisms to improve the management of neuropathic pain and apply them to develop appropriate interventions. Previous studies have supported the idea that hyperalgesia is reduced by mechanisms that engage spinally descending orexin-A neurons (Wardach et al., 2016). This neuropeptide system offers a novel approach to treat chronic pain and hyperalgesia. In spite of recent evidence for its effect in reducing hyperalgesia in nerve constriction models, there have been no studies to investigate the system as potential target for neuropathic pain specifically in a model of SCD. This current study is the first to show that there is regionally specific activation of orexin neurons as a result of various pain assessments for hyperalgesia (component of neuropathic pain) demonstrated in a mouse model of SCD. We believe that data from these experiments will lay the foundation for a more in-depth investigation for alternative pharmacological therapies to treat neuropathic pain in the SCD population.

In order to develop strategies to treat and even prevent neuropathic pain in SCD, an initial step in this process was to identify whether there were differences in the activation of orexin neurons in sickle mice vs. control mice and to compare the topography of activated orexin neurons. This information provides the knowledge needed to specifically delineate whether specific subpopulations are selectively recruited in sickle mice after pain assessments for hyperalgesia. These findings confirm the activation of the orexin system after pain challenge in

sickle mice vs. control mice and provide an initial map for which subpopulations are activated and can be pharmacologically targeted to treat neuropathic pain.

Final Thoughts

Despite pain being the most common complication of SCD, there is a lack of novel treatments for pain. Advancement in treatment options for neuropathic pain are needed and drugs commonly used to alleviate pain (i.e., opioids, NSAIDs) have not been reliable. The management of neuropathic pain remains challenging because this type of pain does not respond consistently. Although there has been some improvement in treatment options for neuropathic pain research, patients report that their pain is not managed effectively. Opioid compounds have continued to be the primary option to treat pain for several decades. However, chronic opioids use in SCD may adversely affect the peripheral systems, and the development of opioid tolerance or opioid-induced hyperalgesia. There is minimal use of neuropathic pain drugs (gabapentin and hydroxyurea) in the SCD population and this may be due to minimal systemic screening of this type of pain.

A large proportion of SCD patients use opioids to provide limited relief when experiencing chronic pain. However, long term opioid use may produce severe side effects and do not provide a permanent resolution of the pain. Sociocultural factors also provide a barrier to effective pain management in SCD. Ineffective pain assessment and unfounded concerns by health providers regarding addiction have hindered pain management in the SCD population (Brown et al., 2015). The socio-cultural disparity between patients and providers may contribute to the reluctance of health care providers to prescribe narcotics (Shapiro et al., 1997; Elander et al., 2006). More SCD research and changing attitudes concerning care can help to eliminate the barriers that exist. One option to begin to address this disparity is to identify a non-addictive drug that can be used to alleviate pain in the SCD population. We contend that treatments that pharmacologically target the orexin system could be a promising alternative option to reduce pain in SCD and reduce the requirement of opioid analgesics.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota, protocol: KG.

AUTHOR CONTRIBUTIONS

KR: planning and conducting experiments, collection of all data, data processing, data analyses and interpretation, figure making, and writing of this manuscript. NS: data processing and writing of this manuscript. HT: conducting experiments and figure making. VA: data analyses. SU: data processing. RT: planning experiments and writing of this manuscript. KG: planning experiments, bred and phenotyped all the mice, interpreting data, and writing of this manuscript.

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Pregnancy in Thalassemia and Sickle Cell Disease: The Experience of an Italian Thalassemia Center

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The life expectancy of thalassemia patients has increased significantly in recent years being the most "elderly" patients approaching or are over 50 years old. Consequently, patients' perspectives have changed, leading them to longer-term planning with a consequent increase in their reproductive potential and desire to have children. Crucial points in the management of pregnancy in thalassemia are the iron chelation therapy before and during pregnancy, the antithrombotic prophylaxis, the management of transfusion therapy according to the modified transfusion requirement, a cardiologic monitoring for hemodynamic changes that expose an increased risk of heart failure. Pregnancy in women with sickle cell disease is still associated with increased rates of maternal and fetal mortality and adverse outcomes. Maternal morbidity may be due to acute sickling crises, thromboembolism, infection, and chronic end-organ dysfunction, while neonatal outcomes may be intrauterine growth retardation, preterm delivery, small infants for gestational age, stillbirth, and neonatal death. The management of pregnancy in thalassemia and sickle cell disease requires to be approached by a multidisciplinary team and followed from the pre-conception phase until the post-partum period with a close monitoring of the maternal and fetal conditions, in order to ensure optimal outcome. This approach requires the application of well-defined protocols that cover all the critical aspects of pregnancies in women affected by these pathologies. We describe our experience of spontaneous and non-spontaneous pregnancies in patients with thalassemia major and intermedia and sickle cell disease followed between 1992 and 2018 at the Thalassemia Unit of S. Eugenio Hospital of Rome.

Keywords: pregnancy, thalassemia major, thalassemia intermedia, sickle cell disease, pregnancy management

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INTRODUCTION

The natural history of thalassemia has drastically changed in recent years thanks to the improvement of transfusion and iron chelation therapy and to the treatment of complications derived from them. The patients' life expectancy has increased significantly, being the most "elderly" patients approaching or are over 50 years old. Younger patients are those who benefit most from improved therapy, presenting fewer complications related to martial overload. The increase

in life expectancy has consequently changed patients' perspectives, leading them to longer-term planning with a consequent increase in their reproductive potential and desire to have children. Hypogonadism still represents an obstacle for some patients, but the progress in assisted reproduction techniques has helped many of them to fulfill this wish (Leung and Lao, 2012; Lao, 2017; Materazzi et al., 2017a; Carlberg et al., 2018; Origa and Comitini, 2019).

Pregnancies in women with sickle cell disease (SCD) are associated with increased rates of maternal and fetal mortality and adverse outcomes (Eissa et al., 2015; Kuo and Caughey, 2016; Ware et al., 2017; Jain et al., 2019; Patel et al., 2019). The physiological changes of pregnancy (increased metabolic demand, rise of blood viscosity, hypercoagulability) are aggravated in SCD women leading to increased incidence of complications. Maternal morbidity may be due to acute sickling crises, thromboembolism, infection and chronic end-organ dysfunction, while neonatal outcomes may be intrauterine growth retardation, preterm delivery, small infants for gestational age, stillbirth, and neonatal death. Early reports on the outcome of pregnancy in SCD depicted an almost universal adverse outcome for mother and child, but the medical care improvements, like the introduction of pre-conceptional care, have reduced the risk of complications.

In this retrospective study, we report the experience of pregnancies in patients with thalassemia major and intermedia and sickle cell disease followed at the Thalassemia Unit of S. Eugenio Hospital of Rome. Moreover we report the maternal and fetal outcomes of the patients comparing with the experiences previously reported in literature (Tuck, 2005; Origa et al., 2010; Leung and Lao, 2012; Voskaridou et al., 2014; Lao, 2017; Carlberg et al., 2018; Jain et al., 2019).

METHODS

We included in the study pregnant women with diagnosis of Thalassemia and SCA obtained through a comprehensive assessment of clinical presentation, screening tests, and molecular characterization of globin genes mutations (Greene et al., 2014; Aiello et al., 2015; Risoluti et al., 2016, 2018; Materazzi et al., 2017b,c; Catauro et al., 2018). The study was approved by Comitato Etico of the S. Eugenio Hospital and all the patients provided their written informed consent to participate in this study.

We have followed between 1992 and 2018 the course and outcome of spontaneous and non-spontaneous pregnancies from 31 women, 21 of them were patients of the Thalassemia Unit of S. Eugenio Hospital of Rome, while 10 were patients from other hospitals and were followed at the Thalassemia Unit during the pregnancy for the complications.

The evaluation was performed by analyzing the data of all the patients and also divided in to the following groups: thalassemia major, thalassemia intermedia transfusion-dependent, thalassemia intermedia non-transfusion dependent, and sickle cell disease.

The following parameters were analyzed:

1. Clinical and demographic characteristics: age of the patients at the pregnancy, country of origin, transfusion frequency, hypogonadism, splenectomy, iron chelation therapy.
2. Pregnancy: conception, fertilization *in vitro*, intrauterine insemination after induced ovulation.
3. Treatment during pregnancy: transfusion, iron chelation, complications.
4. Delivery: gestational age at delivery, preterm delivery, abortion, cesarean delivery.
5. Neonatal outcomes: birth weight, fetal anomalies, complications.

RESULTS

Clinical and Demographic Characteristics

The course and outcome of 33 pregnancies from 31 patients, 25 thalassemic patients and 6 women with SCD have been analyzed retrospectively, and the characteristics of the patients are described in **Table 1**.

The thalassemic patients' group included: 14 women with thalassemia major (TM) (mean age 33.2 years, range 25–46 years) regularly transfused; 11 women with thalassemia intermedia of which 7 were transfusion dependent (mean age 34.0 years, range 25–40 years) and 4 were non-transfusion dependent (mean age 34.5 years, range 29–42 years). The SCD patients (mean age 32.1 years, range 25–43 years) were two transfusion dependent (n. 1 with double heterozygosis for HbS/HbC, and n. 1 with homozygosity HbS/HbS), and four non-transfusion dependent (n. 1 HbS/HbC and n. 3 HbS/ β-thal). All thalassemia patients were of Italian origin, while in SCD patients' group three women were of African origin, one was from Albania, and two patients had Italian origin.

Pregnancy

In 21 pregnancies (64%) (**Table 1**), conception was achieved by spontaneous ovulation (seven thalassemia major, three transfusion-dependent thalassemia intermedia, five non-transfusion-dependent thalassemia intermedia, and all sickle cell disease patients), and the remaining 12 pregnancies (36%) were induced by fertilization *in vitro* in nine cases. In 3 cases, intrauterine insemination after gonadotrophin-induced ovulation was performed. The medically assisted procreations were seven thalassemia major patients affected by hypogonadotropic hypogonadism and five transfusion-dependent thalassemia intermedia. In the non-transfusion dependent thalassemia intermedia group 3 patients reported a previously pregnancy that ended with spontaneous abortion, while two have been followed for two pregnancies. Moreover, in three cases of thalassemia patients (9%) the pregnancies were twins.

Treatment During Pregnancy

During pregnancy two cases of SCD patients (HbS/HbC) needed the transfusion of one unit of red blood cells during pregnancy for vaso-occlusive crisis (VOC), and only a case of cardiac failure occurred at 6 months of pregnancy in a thalassemia major patient with previous history of poor

TABLE 1 | Pregnancy in thalassemia and sickle cell disease: the experience of an Italian Thalassemia Center.

Patients		All ($n = 31$)	TM ($n = 14$)	TD-TI ($n = 7$)	NTD-TI ($n = 4$)	SCD ($n = 6$)
Age of patient at the pregnancy, years	Mean	38.9	33.2	34.0	34.5	32.1
	Range	25–46	25–46	25–40	29–42	25–43
Origin, *n* (%, country)		27 (87%, Italy) 3 (10%, Africa) 1 (3%, Albania)	14/Italy	7/Italy	4/Italy	2/Italy 3/Africa 1/Albania
Regular transfusion, *n* (%)		23 (74%)	14	7		2
Hypogonadism, *n* (%)		8 (26%)	7	1		
Splenectomy, *n* (%)		16 (52%)	11	4		1
Pregnancies, *n* (%)	All	33	14	8	5	6
	Spontaneous	21 (64%)	7	3	5	6
	Induced	12 (36%)	7	5		
Twin pregnancy, *n* (%)		3 (9%)	2	1		
Iron chelation (DFO) (2nd−3rd trimester of pregnancy), *n* (%)		14 (42%)	10	3		1
Complications of patients, *n* (%)	Heart failure	1 (3%)	1			
	VOC	2 (6%)				2
Mean gestational age at delivery (weeks)		37.1 ± 2.2*	36.3	37.1	38.4	37.3
Preterm delivery, *n* (%)		7 (22%)	5	1		1
Abortion, *n* (%)		1 (3%)				1
Cesarean delivery, *n* (%)		31 (94%)	14	8	4	5
Birth weight, g	Mean	2,579	2,436	2,392	3,002	2,633
	Range	1,100–3,320	1,200–3,250	1,100–2,900	2,460–3,320	2,100–3,100
Fetal anomalies, *n* (%)		1 (3%)		1		

*TM, Thalassemia major; TD-TI, transfusion–dependent Thalassemia intermedia; NTD-TI, non-transfusion-dependent Thalassemia intermedia; SCD, sickle cell disease; DFO, desferrioxamine; VOC, vaso-occlusive crisis; *mean ± standard deviation.*

compliance to chelation therapy. As for iron chelation therapy during pregnancy, in the case of suspected pregnancy the chelation therapy needs to be immediately suspended, but may be resumed after the 1st trimester. In 13 thalassemic patients (10 thalassemia major and 3 transfusion-dependent thalassemia intermedia) and one SCD patient, chelation with desferrioxamine (DFO) was started again at 2nd−3rd trimester of pregnancy without complications.

Delivery

A case of abortion for fetus death occurred in a SCD patients (HbS/HbC) after a severe vaso-occlusive crisis. This patient had already previously had a pregnancy that has resulted in abortion at 6 month due to VOC crisis and eclampsia. From the 33 pregnancies 35 children have been born, only a case of abortion (3%), with seven preterm deliveries (22%), three of which were in twin pregnancies. The cesarean delivery was applied in 31/33 pregnancies (94%), only a case of natural delivery (**Table 1**). The mean gestational age at delivery of all patients was 37.1 ± 2.2 weeks, with a lower mean value in TM patients (36.3 weeks) and the higher mean value in non-transfusion dependent thalassemia patients (38.4 weeks; **Table 1**).

Neonatal Outcomes

The mean birth weight of the 35 children was 2,579 g, with a range from 1,100 to 3,320 g, taking into account the presence of three twin pregnancies in which the newborns weighed between 1,100 and 1,400 g and a child born at 7 month of pregnancy with birth weight of 1,700 g. These babies remained in the incubator for 15–45 days. Twenty children had breastfeeding (57%). Neonatal complications were four cases of jaundice, a case of cerebral hemorrhage, and fetal anomalies (3%) in a child of a medically assisted procreation pregnancy from ovodonation bearing gastroenteric tube multiple malformations that required several surgical interventions.

DISCUSSION AND CONCLUSIONS

Management of Pregnancy in Thalassemia and Sickle Cell Disease

Pregnancy of women with thalassemia major, thalassemia intermedia, and sickle cell disease should be approached by a multidisciplinary team and followed from the preconception phase until the post-partum period with a close monitoring of the maternal and fetal conditions, and planned and time delivery, in order to ensure optimal outcome. This approach requires the

application of well-defined protocols that cover all the critical aspects of pregnancies in women affected by these pathologies.

Pregnancy and delivery complications are different for women with thalassemia and sickle cell disease. As regards thalassemia patients, despite the physiologic decline in fertility, and follicle aging depending on the iron toxicity, the ovarian function is preserved in transfusion-dependent thalassemia. Data on the frequency of failure in ovulation induction or timeline to a successful pregnancy are limited (Ansari et al., 2006; Voskaridou et al., 2014; Origa and Comitini, 2019). Crucial points in the management of pregnancies are the iron chelation therapy before and during pregnancy, the antithrombotic prophylaxis, the management of transfusion therapy according to the modified transfusion requirement, paying particular attention to immune-hematological examinations, and a careful cardiologic monitoring for hemodynamic changes that expose an increased risk of heart failure. The moment of the delivery must be managed in close collaboration between the Hematological Center, Obstetrics Department and Transfusion Center, taking into account the particular needs of thalassemia patients i.e., chronic anemia, cardiac siderosis, osteoporosis, splenomegaly, and patients' anatomy (fetal-pelvic discrepancy).

In sickle cell disease patients' crucial points in pregnancy management are the vaso-occlusive crisis, thromboembolic events, acute chest syndrome, anemia, and infections. Early studies recommended prophylactic transfusion during pregnancy as there was a decrease in maternal morbidity and perinatal mortality among transfused women (Malinowski et al., 2015; Sharif et al., 2018), but transfusions increase the risk of alloimmunization, iron overload and introduce the iron chelation therapy. Transfusion increases oxygen-carrying capacity and prevent complications such as stroke by correcting anemia and reducing HbS content in the circulation; therefore, a single transfusion is given. On the contrary, UK guidelines did not recommend the prophylactic transfusion during pregnancy (Sharif et al., 2018), but only in women with an history of complications such as pre-eclampsia, acute chest syndrome, stroke, and severe anemia, taking into account the previous clinical data of the sickled patients. In our SCD patients' group two cases with genotype HbS/HbC needed the transfusion of one unit of red blood cells during pregnancy for vaso-occlusive crisis.

Iron Chelation Therapy

In the preconception period, the iron chelation therapy is strictly recommended when dealing with pregnancy forecast, in order to avoid complications due to heart diseases (heart failure and arrhythmias) or liver disease (gravid hepatosis). It is important for women to start pregnancy with low ferritin levels in order to avoid a decrease in their systolic function indices.

In the case of spontaneous pregnancy or suspected pregnancy, the chelation therapy needs to be immediately suspended, while in cases of medically assisted procreation, the iron chelation therapy should be suspended at the time of intrauterine injection of the seminal fluid or the embryonic implant, according to the involved medical procedure. Iron chelation therapy can be resumed after the first trimester of pregnancy but only with desferrioxamine (DFO), to avoid major increases in serum ferritin. DFO is the iron chelator of choice during pregnancy because its large molecular size prevents the passage through the placenta. In our patients' group, 13 thalassemia women (10 thalassemia major and 3 transfusion-dependent thalassemia intermedia) and one SCD patient started again the iron chelation therapy at 2nd−3rd trimester of pregnancy without complications, in agreement with the data reported by Tsironi et al. (2005).

Cardiological Evaluation

Changes in cardiac function and dimension occur during pregnancy particularly in these patients (Origa et al., 2010; Lao, 2017). A close monitoring of the cardiac functions should be applied by echocardiograms and laboratory test obtained once each trimester and also after the partum in order to avoid cardiac failure in the post-partum period in women with cardiac symptoms. Myocardial complications have been reported by several authors in thalassemic pregnant women (Tuck, 2005; Ansari et al., 2006; Voskaridou et al., 2014). In our patients' group only a case of cardiac failure occurred at 6 months of pregnancy in a thalassemia major patient with previous history of poor compliance to chelation therapy.

Immunohematological Monitoring

All transfusion-dependent patients must receive irregular antibodies search at each transfusion cycle, while non-transfusion-dependent patients search for irregular antibodies as per pregnancy calendar. It is desirable to know the extended erythrocyte phenotype of the patients' partners or of the oocyte or sperm donors in order to avoid possible alloimmunizations, where present, and possibly to prevent the onset of new alloimmunizations. The immunohematological monitoring is an essential point of the protocol of our Thalassemia Unit.

Transfusions and Antithrombotic Prophylaxis

Anemia is common during pregnancy, and transfusion dependent patients need transfusion regime adopted on the basis of individual needs in relation to hemoglobin values which should be maintained at hemoglobin level 10 g/dL to optimize fetal growth. In addition, non-transfusion dependent thalassemia intermedia patients can need transfusion for the first time in pregnancy due to the dilution of the hemoglobin. In some cases, it may be appropriate to reduce the transfusion interval and to administer only one unit of red blood cells at a time to support the fetal oxygenation requirements (Origa and Comitini, 2019). Two cases of SCD patients (HbS/HbC) needed the transfusion of one unit of red blood cells during pregnancy for vaso-occlusive crisis.

All splenectomised patients undergo antithrombotic prophylaxis with cardioaspirin or low molecular weight heparin (LMWH), and in these cases LMWH is administered from the beginning of pregnancy. The cardioaspirin prophylaxis is replaced with LMWH at the 32nd week. Thromboprophylaxis with LMWH is be also indicated during the post-partum period to avoid thromboembolism.

Delivery and Neonatal Outcomes

Reported rates of cesarean delivery are high, varying between 24 and 100%. The indications for this delivery are high rate of feto-pelvic disproportion and maternal short stature, osteopenia and osteoporosis, and maternal HIV infection (Leung and Lao, 2012). In our patients' group, the cesarean delivery was applied in 31/33 pregnancies (94%), only a case of natural delivery (9%) and a case of abortion for fetus death after a severe vaso-occlusive crisis occurred in a SCD patients.

Careful evaluation of obstetrics for possible fetal-pelvic discrepancy that can expose patients to pathological fractures, given the frequency of small constitution of thalassemia patients, the frequent osteopenia/osteoporosis even in young women. This evaluation is also aimed at giving an indication to cesarean delivery due to the presence of siderosis, chronic anemia, pre-existing cardiac pathologies. As a consequence, in coordination with the obstetric section and the transfusion center, blood components dedicated to transfused patients should be prepared especially in presence of specific blood components needs. Chronic anemia in these pregnant women may result in fetal hypoxia, which predisposes to premature labor, intrauterine growth retardation, and death.

In our study the observed incidence of complications was smaller compared to similar studies (Toumba et al., 2008; Kuo and Caughey, 2016; Lao, 2017; Jain et al., 2019; Origa and Comitini, 2019). Thirty two pregnancies (97%) resulted in successful deliveries, only a case of abortion (3%) in a SCD patient, no abortion in thalassemic women, only seven preterm deliveries (21%), of which three were in twin pregnancy, and severe neonatal complications only in 5.7% of children.

In conclusion, this study confirms that the progress in the multidisciplinary management of the women affected by thalassemia and sickle cell disease from the preconception phase until neonatal period, with a close monitoring of the maternal and fetal conditions. The application of well-defined protocols have been and will be in perspective the way to increase for the future the rates of pregnancies, improve the maternal and fetal outcomes, and reduce complications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study was approved by Comitato Etico of the S. Eugenio Hospital and the patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FS, LM, RC and DD'A enrolled the patients, performed the clinical evaluation, and management of subjects. FS, PC, RR, and SM wrote the manuscript and evaluated data for statistics. All the authors have revised and approved the final version of the manuscript.

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REVIEW
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Update in Laboratory Diagnosis of Thalassemia

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Alpha- and β-thalassemias and abnormal hemoglobin (Hb) are common in tropical countries. These abnormal globin genes in different combinations lead to many thalassemic diseases including three severe thalassemia diseases, i.e., homozygous β-thalassemia, β-thalassemia/Hb E, and Hb Bart's hydrops fetalis. Laboratory diagnosis of thalassemia requires a number of tests including red blood cell indices and Hb and DNA analyses. Thalassemic red blood cell analysis with an automated hematology analyzer is a primary screening for thalassemia since microcytosis and decreased Hb content of red blood cells are hallmarks of all thalassemic red blood cells. However, these two red blood cell indices cannot discriminate between thalassemia trait and iron deficiency or between α- and β-thalassemic conditions. Today, Hb analysis may be carried out by either automatic high-performance liquid chromatography (HPLC) or capillary zone electrophoresis (CE) system. These two systems give both qualitative and quantitative analysis of Hb components and help to do thalassemia prenatal and postnatal diagnoses within a short period. Both systems have a good correlation, but the interpretation under the CE system should be done with caution because Hb A2 is clearly separated from Hb E. In case of α-thalassemia gene interaction, it can affect the amount of Hb A2/E. Thalassemia genotypes can be characterized by the intensities between alpha-/beta-globin chains or alpha-/beta-mRNA ratios. However, those are presumptive diagnoses. Only DNA analysis can be made for specific thalassemia mutation diagnosis. Various molecular techniques have been used for point mutation detection in β-thalassemia and large-deletion detection in α-thalassemia. All of these techniques have some advantages and disadvantages. Recently, screening for both α- and β-thalassemia genes by next-generation sequencing (NGS) has been introduced. This technique gives an accurate diagnosis of thalassemia that may be misdiagnosed by other conventional techniques. The major limitation for using NGS in the screening of thalassemia is its cost which is still expensive. All service labs highly recommend to select the technique(s) they are most familiar and most economic one for their routine use.

Keywords: thalassemia, hemoglobinopathies, diagnosis, hemoglobin analysis, DNA analysis

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INTRODUCTION

Hemoglobinopathies may be roughly divided into two groups, the structural hemoglobin (Hb) variants (abnormal Hb) and the thalassemias. These structural Hb variants are commonly caused by single amino acid substitutions in the α or β globin chains. Most of these abnormal Hb do not have clinical symptoms; however, some of these mutations may change the functional properties or stability of the Hb and lead to a clinical disorder. The thalassemias result from defective globin chain production. They are classified into the α, β, δβ, and δβγ-thalassemias according to the particular globin chains that are defectively synthesized. From a public health point of view, only the α and β-thalassemias are common to be of importance (Calzolari et al., 1999; Weatherall and Clegg, 2001).

The major pathophysiological change of thalassemias is imbalanced globin-chain production. This leads to the destruction of the red blood cell precursors in the bone marrow or peripheral blood result in chronic anemia, splenomegaly, and skeletal deformity due to expansion of the bone marrow (Weatherall, 1998). The homozygous or compound heterozygous states for β-thalassemia have a variable course; however, death occurs mostly in the first few years of life without transfusion. With adequate transfusion and iron chelator administration, the thalassemia patients may have good development and can survive into adulthood. Beta-thalassemias of intermediate severity, such as β-thalassemia/Hb E, have a wide range of clinical spectrum from a condition that is compatible with normal survival and growth into adult life without treatment to a transfusion-dependent thalassemia (TDT). The reasons for this clinical heterogeneity are not fully understood (Olivieri, 1999; Barbara, 2006).

The α-thalassemias are equally heterogeneous. The milder forms (termed α-thalassemia 2 or α^{+}-thalassemias) result from one α globin gene deletion, produce a mild anemia in their homozygous states. While α-thalassemia 1 or α^{0}-thalassemia is associated with an absence of α globin chain synthesis because of the deletion of the two α globin genes on the same chromosome. In homozygous states, it results in the most severe form of thalassemia, namely, Hb Bart's hydrops fetalis. The compound heterozygous states for α thalassemia 2 and α-thalassemia 1 result in Hb H disease which varies in severity; at the more severe end, it may be a TDT.

The thalassemias are extremely heterogeneous at the molecular level; over 200 different mutations of the β globin genes have been found in patients with β-thalassemia, and the α-thalassemias are almost as varied in their molecular pathology. However, global population seems to carry a few common mutations that are unique to a particular area, together with varying numbers of rare ones.

Here we will describe conventional methods for thalassemia diagnosis which first characterized subjects with phenotypic traits associated with thalassemia by using hematological (red blood cell indices) and biochemical tests (Hb analysis) and subsequent DNA analysis for definitive diagnoses. However, this diagnosis approach would not detect individuals with normal or borderline red blood cell indices and/or HbA2 levels which are "silent" forms of thalassemia (Yilmaz et al., 2019). In addition, at least 1,800 mutations causing thalassemia or abnormal hemoglobin variants have been characterized to date, the identification of mutation in samples from subjects suspected of having hemoglobinopathies may require labor-intensive methods (Singh et al., 2020). The application of new technology and high-throughput molecular approaches such as next-generation sequencing (NGS) for screening and accurate diagnosis of hemoglobinopathies is feasible (Li et al., 2020; Ruengdit et al., 2020; Wang et al., 2020; Zhao et al., 2020).

LABORATORY DIAGNOSIS OF THALASSEMIA

Thalassemias and abnormal hemoglobin diagnoses require a combination of laboratory tests including the measurement of red blood cell indices by automatic hematology analyzer, Hb analysis, and quantification of Hb A2 and Hb F. The high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CE) system can distinguish thalassemic diseases and the carriers. It has been widely used to replace the manual technique. These systems give both qualitative and quantitative analyses of Hb components with good precision and reproducibility. They have enabled us to do both prenatal and postnatal diagnoses of thalassemia within the few minutes. Specific thalassemia mutation can be detected by DNA analysis, and various techniques have been used for point mutation detection. Moreover, thalassemia genotyping can be carried out by real-time polymerase chain reaction (PCR) follows by melting curve analysis. Cases that mutation cannot identify by previous molecular analysis technique will be sent for DNA sequencing. And in the last few years, the genome sequencing by NGS has been applied for thalassemia diagnosis as well.

AUTOMATIC HEMOGLOBIN ANALYZERS

Because of less globin production, the thalassemic red blood cells showed microcytic and hypochromic. However, Hb, mean corpuscular volume (MCV), and mean corpuscular Hb (MCH) cannot discriminate between thalassemia trait and iron deficiency or between α- and β-thalassemia (England and Fraser, 1973). Hb analysis is needed to determine α- and β-thalassemia carriers and disease. Automatic HPLC and CE system are sensitive and precise methods for qualitative and quantitative analyses of Hb components in red blood cells (Stephens et al., 2015). The HPLC system is cation exchange and use two dual piston pumps to set gradient sodium phosphate buffers of increasing ionic strength to pass through a column spherical cation exchange resin during a 6.5 min. Hemolysate samples are determined by spectrophotometer that read double wavelength at 415 and 690 nm. The resulting chromatograms are separated in retention time (RT). Similarity, the CE system is based on capillary electrophoresis in free solution from cathode to anode. Hb components are separated

in silica capillaries by their electroosmotic flow and at a high voltage (9,800 V) in electrophoretic mobility in an alkaline buffer. The photometry at an absorbance wavelength 415 nm was used to directly detect Hb fractions. The resulting electrophorograms are divided into 15 zones. Several publications on the automatic hemoglobin analyzers have shown their effectiveness in the investigation of thalassemia and hemoglobinopathies for prenatal and postnatal diagnoses (Tan et al., 1993; Borbely et al., 2013; Khongthai et al., 2019; Li et al., 2019).

HEMOGLOBIN ANALYSIS IN ADULT

Both systems give a good correlation for thalassemia diagnosis in adult. Normal blood samples had Hb concentration = 12 g/dl, MCV = 80 fL, MCH = 27 pg, and HbA2 = 3.5% (**Figures 1A,D**). Thalassemia carriers presented normal Hb concentration level but show low MCV and MCH. Alpha-thalassemia carriers had Hb A2 = 3.5% (**Figures 1B,E**), but β-thalassemia carrier had Hb A2 > 3.5% (**Figures 1C,F**). Under the HPLC system, Hb A2 and Hb E co-elute at the same RT, but in the CE system, Hb A2 and

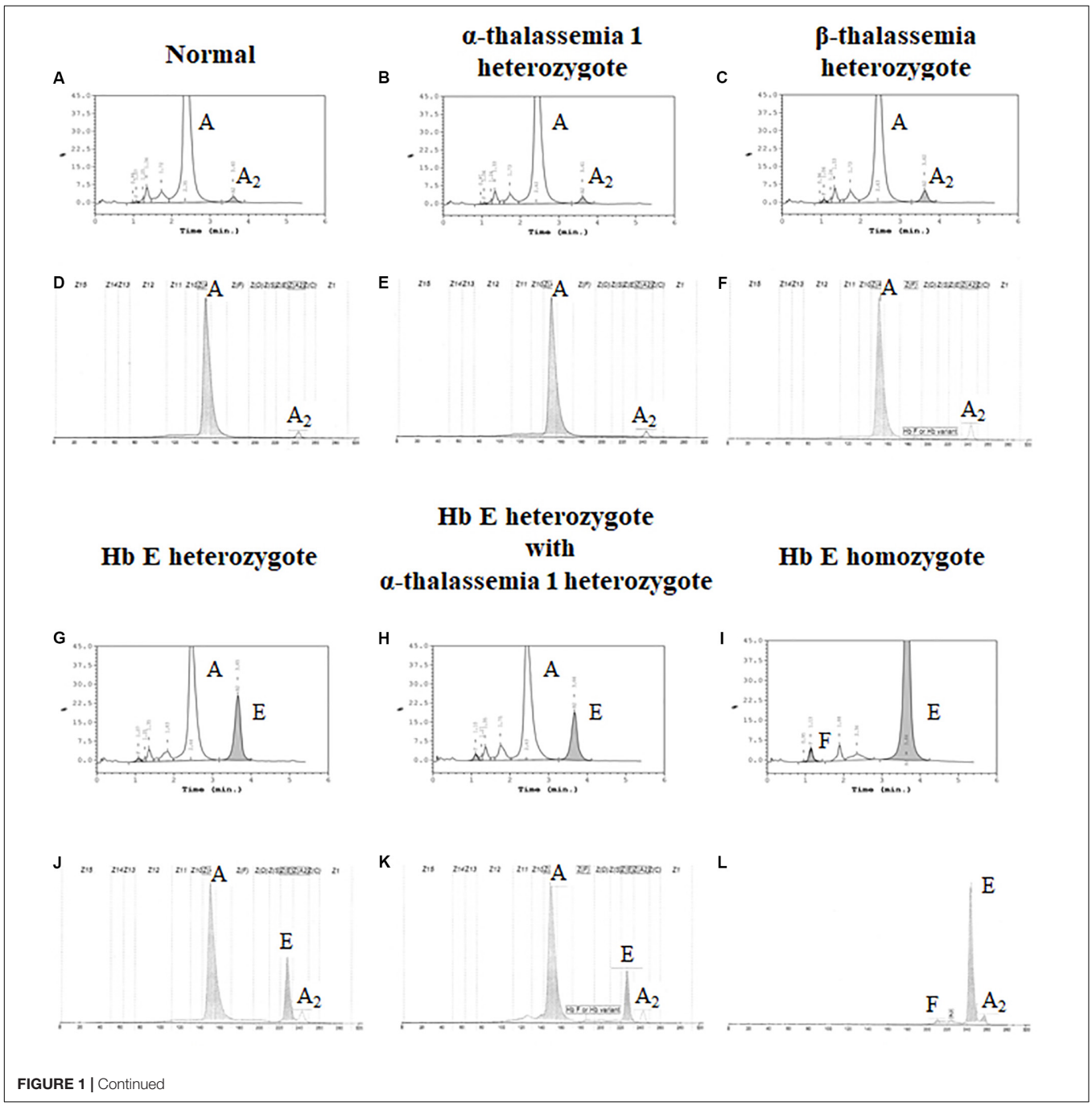

FIGURE 1 | Continued

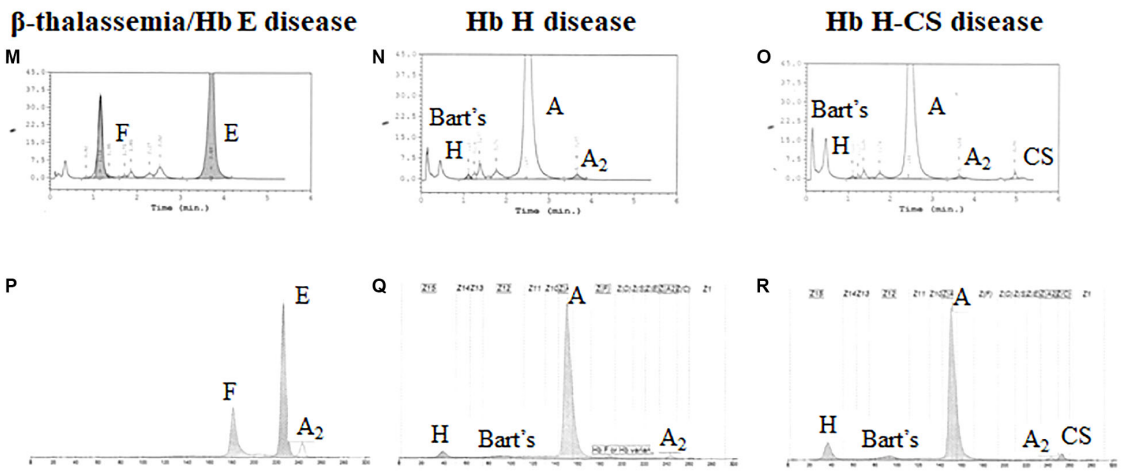

FIGURE 1 | Pattern of hemoglobin analysis in adult blood by the high-performance liquid chromatography (HPLC) system **(A–C,G–I,M–O)** compared with the capillary zone electrophoresis (CE) system **(D–F,J–L,P–R)**.

TABLE 1 | Hemoglobin analysis of adult blood.

Phenotype	Number	Hb Type	Hb A_2 %		Hb E %		Hb F %	
			HPLC	CE	HPLC	CE	HPLC	CE
Normal	45	A_2A	2.6 ± 0.4	2.5 ± 0.4	–	–	0.5 ± 0.7	0.1 ± 0.2
α-thalassemia 1 heterozygote	36	A_2A	2.3 ± 0.2	2.3 ± 0.2	–	–	0.5 ± 0.7	0.3 ± 0.5
β-thalassemia heterozygote	69	A_2A	5.5 ± 1.3	5.4 ± 0.5	–	–	1.5 ± 1.4	0.9 ± 1.4
Hb E heterozygote	85	EA	Not detected	3.5 ± 0.4	27.8 ± 7.5	25.6 ± 1.4	1.2 ± 0.9	0.4 ± 0.8
Hb E heterozygote with α-thalassemia 1 heterozygote	6	EA	Not detected	4.0 ± 0.3	21.9 ± 0.6	16.3 ± 0.8	0.9 ± 0.6	0.5 ± 0.8
Hb E homozygote	56	EE	Not detected	4.1 ± 0.8	90.2 ± 4.9	92.9 ± 3.3	4.3 ± 2.7	2.5 ± 3.1
β-thalassemia/Hb E disease	48	EF	Not detected	4.9 ± 1.6	59.4 ± 12.9	50.3 ± 13.8	31.1 ± 14.5	36.8 ± 13.3
Hb H disease	26	A_2A Bart's H	1.6 ± 1.2	1.0 ± 0.2	–	–	0.6 ± 0.6	0.2 ± 0.3
Hb H-CS disease	9	CSA_2A Bart's H	ND	0.7 ± 0.5	–	–	ND	1.0 ± 0.6
Hb CS homozygote	10	CSA_2A	ND	1.3 ± 0.6	–	–	ND	0.8 ± 0.8

Phenotype	Number	Hb Type	Hb Bart's %		Hb H %		Hb CS %	
			HPLC	CE/Undetected number	HP	CE/undetected number	HPL	CE/undetected number
Normal	45	A_2A						
α-thalassemia 1 heterozygote	36	A_2A						
β-thalassemia heterozygote	69	A_2A						
Hb E heterozygote	85	EA						
Hb E heterozygote with α-thalassemia 1 heterozygote	6	EA						
Hb E homozygote	56	EE						
β-thalassemia/Hb E disease	48	EF						
Hb H disease	26	A_2A Bart's H	Found	1.1 ± 0.7/14	Found	6.7 ± 4.8/0		
Hb H-CS disease	9	CSA_2A Bart's H	Found	4.2 ± 4.1/3	Found	11.3 ± 6.5/3	Found	2.6 ± 1.4/1
Hb CS homozygote	10	CSA_2A	Found/Not found		Found/Not found		Found	3.5 ± 2.5/0

ND, not determined.

Hb E are clearly separated, zone three for Hb A2 and zone four for Hb E. The average amount of Hb E was 27.8 ± 7.5% by HPLC and 25.6 ± 1.4% by CE. In addition, Hb A2 is detected with the average amount of Hb A2 3.5 ± 0.4% under the CE system. In Hb E homozygote, Hb A2 + E was 90.2 ± 4.9% by HPLC and Hb A2 was 4.1 ± 0.8% and Hb E was 92.9 ± 3.3% by CE (**Figures 1I,L**). In β-thalassemia/Hb E disease, Hb A2 + E was 59.4 ± 12.9% by HPLC and Hb A2 was 4.9 ± 1.6% and Hb E was 50.3 ± 13.8%

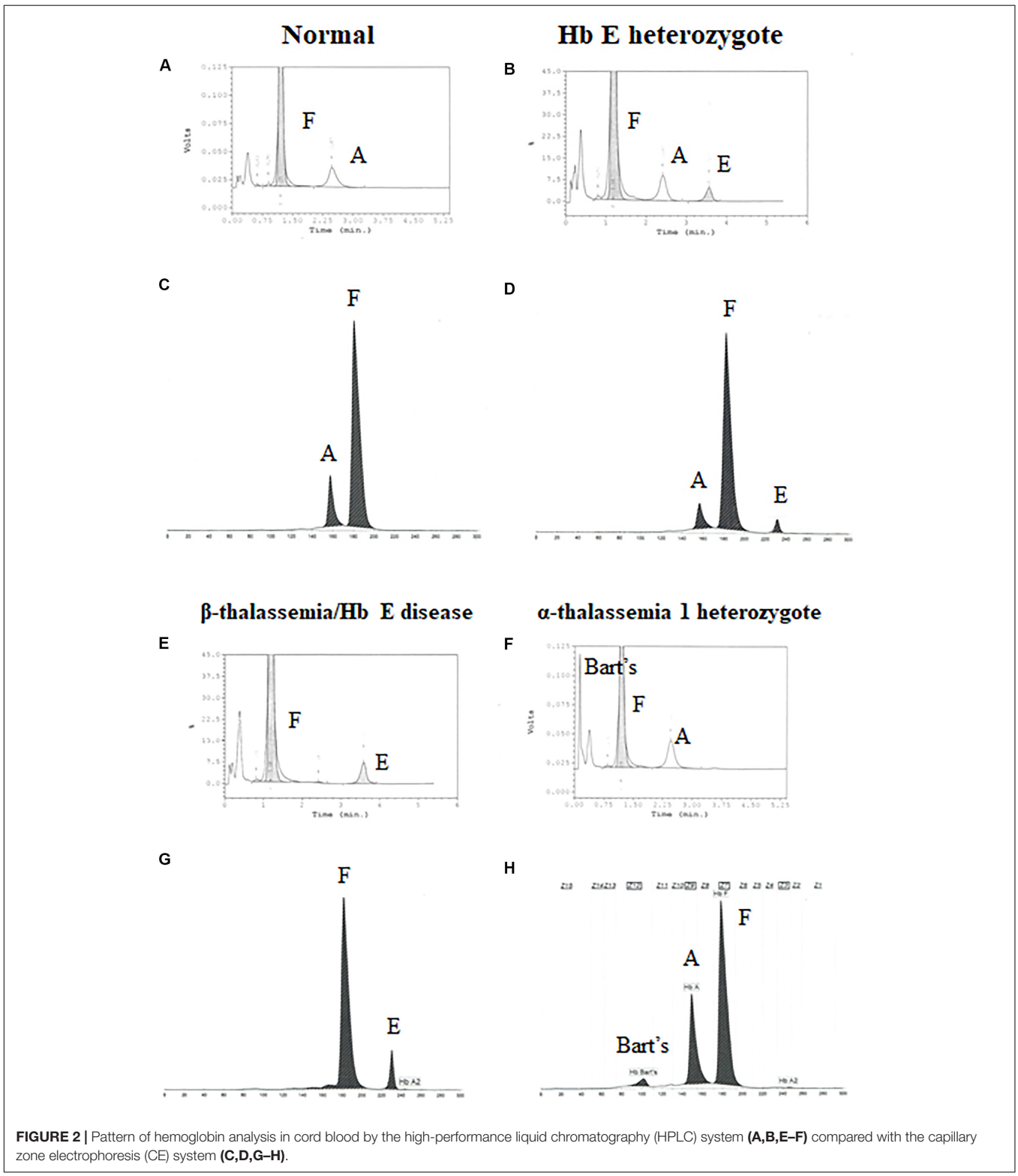

FIGURE 2 | Pattern of hemoglobin analysis in cord blood by the high-performance liquid chromatography (HPLC) system **(A,B,E–F)** compared with the capillary zone electrophoresis (CE) system **(C,D,G–H)**.

by CE (**Figures 1M,P**). In fact, the amount of Hb A2/E is more confusing in the double heterozygote of Hb E and α-thalassemia cases with different numbers of defective α-globin gene. For example, the double heterozygote with Hb E and α-thalassemia 1 presented low Hb E levels, 21.9 ± 0.6% and 16.3 ± 0.8% by HPLC and CE, respectively (**Figures 1G,H,J,K**). The CE system demonstrated Hb E level less than HPLC because of the separated zones of Hb A2 and E. Therefore, criteria for Hb E diagnosis must combine % Hb A2 and E to reduce misdiagnosis (**Table 1**).

In addition, the two systems can also detect Hb Bart's, Hb H, and Hb CS in Hb H and Hb H-CS diseases of which the hemoglobin peaks were shown in **Figures 1N,O,Q,R**. The HPLC system demonstrates the qualitative Hb H and Hb Bart's peaks and quantitative Hb CS (**Figures 1N,O**). However, CE system measures quantitatively Hb H, Hb Bart's, and Hb CS (**Figures 1Q,R** and **Table 1**). Nevertheless, Hb Bart's, Hb H, and Hb CS are unstable and a decreasing level may be found in the blood sample that was kept for a long storage especially at high temperature (Fucharoen et al., 1998b; Winichagoon et al., 2008; Munkongdee et al., 2011; Kingchaiyaphum et al., 2020).

HEMOGLOBIN ANALYSIS IN NEWBORN

Newborns screening for thalassemia can also be diagnosed by HPLC and CE systems. Normal newborn had normal Hb concentration 15.5 ± 1.3 g/dl, MCV 110.0 ± 4.8 fL, and MCH 35.9 ± 1.4 pg and Hb analysis presented FA (**Figures 2A,C** and **Table 2**). Newborns with β-thalassemia heterozygote also has the normal hematological parameters because the β-globin gene is not fully expressed at birth (**Figures 2B,D,E,G** and **Table 2**) (Fucharoen et al., 1998b; Winichagoon et al., 2008; Munkongdee et al., 2011).

In contrast, newborns with α-thalassemia have the abnormal hematological parameters, similarity with adults, because the α-globin gene is expressed in the fetus. Specifically, the MCVs and MCHs of α-thalassemia newborns are significantly lower than those of the normal newborn. In addition, Hb Bart's was obscurely presented in newborn associated with α-thalassemia (**Figures 2F,H** and **Table 2**) (Fucharoen et al., 1998b; Winichagoon et al., 2008; Munkongdee et al., 2011).

INTERACTIONS BETWEEN α-THALASSEMIA AND β-THALASSEMIA

Up to now, more than 1,800 mutations causing hemoglobinopathies have been discovered (Huisman et al., 1997; HbVar, 2019). These mutated α and β globin genes in different combinations lead to over 60 thalassemic diseases (Fucharoen and Winichagoon, 1997; Fucharoen et al., 1998a). The degree of disease severity depends on the complexity of gene interaction, and even identical genotypes patients can have remarkably different levels of severity. This can lead to the difficulty on identification of high-risk pregnancies and provide appropriate genetic counseling for both treatment and prenatal diagnosis.

TABLE 2 | Hemoglobin analysis of cord blood.

Phenotype	Number	Hb Type	Hb (g/dL)	MCV (fL)	MCH (pg)	Hb A %		Hb F %		Hb E %		Hb Bart's %	
						HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE
Normal	339	FA	15.5 ± 1.3	110.0 ± 4.8	35.9 ± 1.4		18.3 ± 6.8		81.6 ± 6.9			0.7 ± 0.4	0.0 ± 0.1
Hb E heterozygote	86	EFA	15.4 ± 1.4	108.6 ± 5.0	35.4 ± 1.6		10.0 ± 3.8		86.1 ± 4.8		3.8 ± 1.3	0.7 ± 0.4	0.0 ± 0.1
Hb E heterozygote with α-thalassemia 1 heterozygote t	6	EFA Bart's	14.4 ± 0.4	87.9 ± 3.6	28.0 ± 1.4		7.6 ± 4.0		83.1 ± 2.7		4.4 ± 4.3	8.6 ± 1.7	4.8 ± 1.0
Hb E homozygote	9	EF	14.5 ± 2.1	103.0 ± 6.7	34.0 ± 2.5		ND	81.4 ± 4.5	ND	8.0 ± 3.5	ND		ND
β-thalassemia/Hb E disease	4	EF	14.8 ± 2.3	105.7 ± 6.3	34.1 ± 2.1		0	93.0	90.2 ± 4.5	2.0	9.2 ± 4.0	1.0	0 ± 0
α-thalassemia 1 heterozygote	17	FA Bart's	13.4 ± 1.0	89.8 ± 3.4	28.0 ± 1.2		23.6 ± 5.3		71.5 ± 5.4			9.2 ± 1.1	4.6 ± 0.5
Hb H disease	2	FA Bart's	7.4	22	30	43.5	ND	52.0	ND	0	ND	24.2	ND
Hb H-CS disease	3	CSFA Bart's	12.0 ± 0.1	97.2 ± 1.7	26.4 ± 0.5	ND	20.0 ± 8.2	ND	46.3 ± 7.0		0	ND	32.1 ± 3.1
Hb CS homozygote	1	CSFA Bart's	12.3	115.0	30.0	13.9	ND	78.5	ND	0	ND	15.0	ND

ND, not determined.

The variability in severity of thalassemias, especially β-thalassemia, involves many gene loci, some of which are directly involved with defects in α, β, or γ globin synthesis, whereas others, related to other genes such as *KLF1*, *BCL11A* (Sripichai and Fucharoen, 2016). A good example is observed in β-thalassemia/Hb E patients who co-inherit α-thalassemia as they will have less unmatched α-globin chains, which result in less symptoms (Kanavakis et al., 1982; Wainscoat et al., 1983; Winichagoon et al., 1985). On the other hand, the co-inheritance of triplicated α-globin genes (ααα) may lead to an increase of globin chain imbalance and severe anemia in β-thalassemia (Galanello et al., 1983).

These findings are important in genetic counseling especially in the high risk couples for β-thalassemia/Hb E who are performing prenatal diagnosis. An example of the family pedigree, including hematological data of the high-risk couples and their offspring, is shown in **Figure 3**. The mother is double heterozygous for Hb E and α-thalassemia 1, while the father is β-thalassemia heterozygote. It is important to characterize the father's specific β-thalassemia mutation. If the mutation is a β^0-thalassemia, there is a 1/4 chance that the future child would be a compound heterozygote for β^0-thalassemia/Hb E. This would result in a moderate or severe clinical symptoms, with iron overload and possible TDT. Beta^0-thalassemia/Hb E child who co-inherit with α-thalassemia 1 would be expected to have less symptoms than β^0-thalassemia/Hb E child who does not carry α-thalassemia and may result in a TDT. Thus, the recommendation for both parents would be DNA analysis for α globin with additional β-globin DNA analysis for the father. Prenatal diagnosis would allow high-risk couples to determine possible adverse outcomes in their pregnancies.

Hb analysis generally do not contribute to the diagnosis of mild forms α-thalassemia, in which one or two (cis or trans) α-globin genes are deleted. The excess β-globin chains

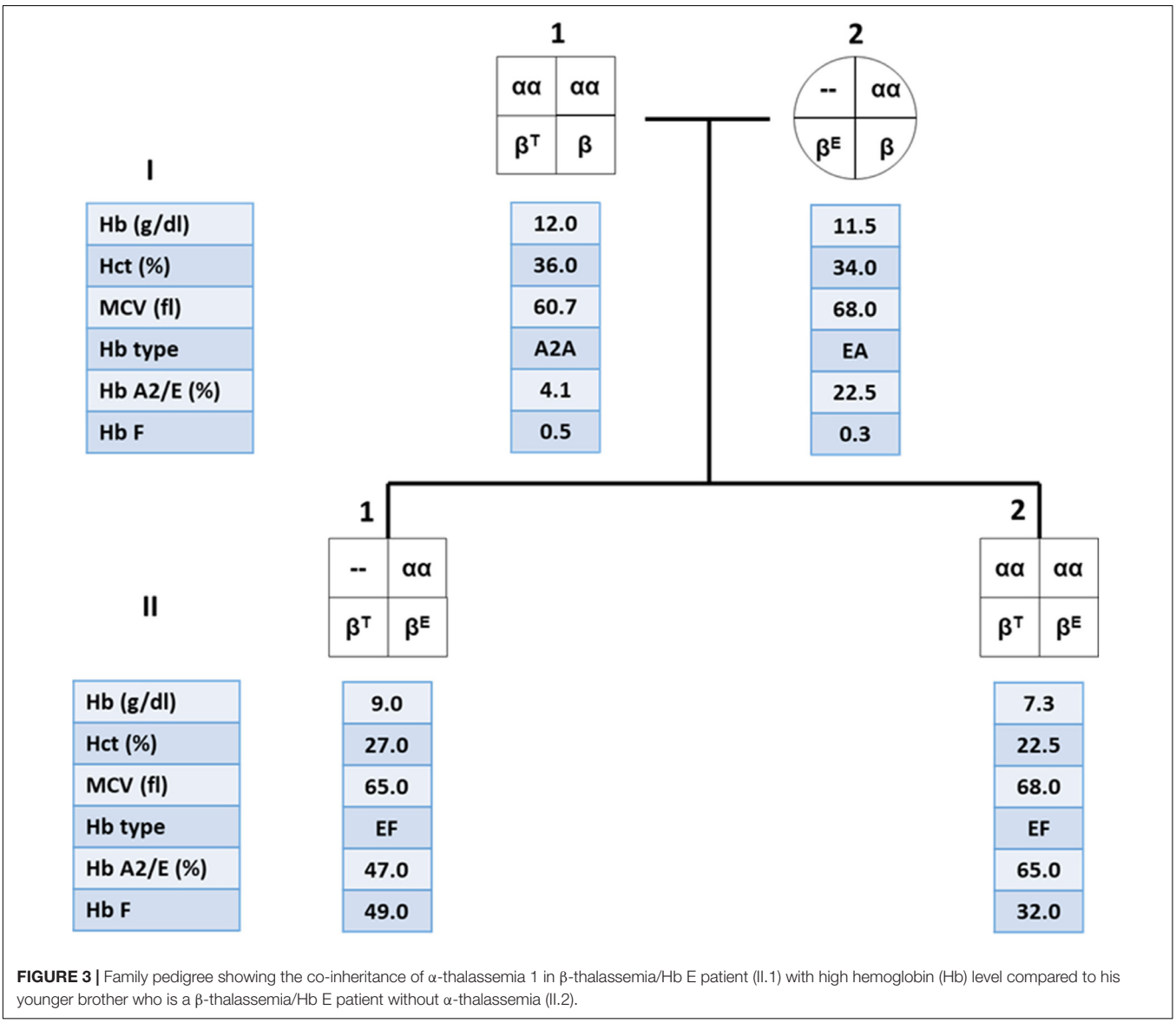

FIGURE 3 | Family pedigree showing the co-inheritance of α-thalassemia 1 in β-thalassemia/Hb E patient (II.1) with high hemoglobin (Hb) level compared to his younger brother who is a β-thalassemia/Hb E patient without α-thalassemia (II.2).

(Hb H molecule) in these α-thalassemia cannot be visualized by Hb analysis. Thus, α-thalassemia is often diagnosed by exclusion, when a subject with microcytic red blood cells, normal Hb analysis and normal iron studies is presumed to have α-thalassemia. Alpha-thalassemia may also be masked in the presence of β-thalassemia which also have microcytic red blood cells. For these subjects, family study and DNA analysis may be the definitive diagnosis, and this is important for genetic counseling.

MOLECULAR ANALYSIS FOR α AND β-THALASSEMIA MUTATIONS

The advent of the PCR has enabled screening for single-base mutations to become simpler (Eisenstein, 1990). Most of thalassemia mutations are point mutation. Point mutations are considered to be single-base substitutions or minor insertions or deletions. **Table 3** showed a summary of common DNA techniques used for point mutation detection. In this paper, we will briefly discuss only common DNA techniques, namely, allele-specific PCR, reverse dot blot (RDB) analysis, real-time PCR with melting curve analysis, and DNA sequencing.

ALLELE-SPECIFIC PCR

This technique employs two primers identical in sequence except for the 3′-terminus base, one of which is complementary to the wild-type and the other for the mutant base; a common primer for the opposite strand must of course be used as well. For primer extension to occur using *Taq* polymerase which has no 3′–5′ exonuclease (proofreading) activity, perfect matching of the primer 3′-terminus with the DNA template must occur. With a normal individual, PCR product will be seen only in the reaction employing the wild-type primer set. A heterozygote will generate a band using both wild-type and mutant primer set, and an individual with homozygous mutation will be negative with the normal and positive with the mutant primer set (**Figure 4**) (Suwannakhon et al., 2019).

REVERSE DOT-BLOT ANALYSIS

The suspected mutation can be identified by hybridization of an allele-specific oligomer (ASO) DNA probe with the PCR product which is immobilized on a membrane filter sheet as a dot. The ASO probe can be radiolabeled with ^{32}P for autoradiography or has attached reporter groups (biotin, digoxigenin, or an enzyme such as horseradish peroxidase) which can subsequently be visualized in a chemiluminescent or colorimetric reaction. Commercial kits have made these non-radioactive

TABLE 3 | Summary of common molecular technique used for point mutation detection.

Known mutation	Unknown mutation
Gel electrophoresis	Mismatched analysis
Allele-specific PCR	Denaturing gradient gel electrophoresis
Dot blot analysis	DNA sequencing
Real-time PCR with melting curve analysis	

All of these techniques developed after gene amplification by polymerase chain reaction (PCR).

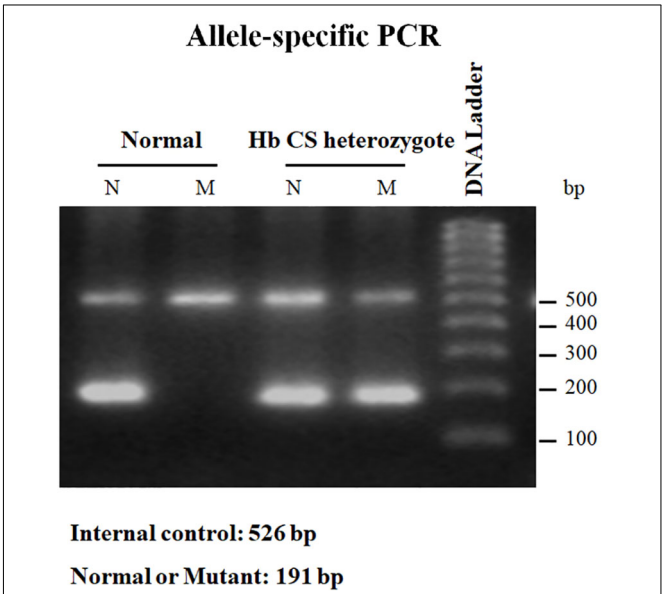

FIGURE 4 | Result of allele-specific PCR showing PCR product of normal individual which can be seen only in the reaction employing the wild-type (N) primer set. While Hb CS heterozygote will generate a band using both wild-type (N) and mutant (M) primer set. Internal control was exhibited at 526 bp.

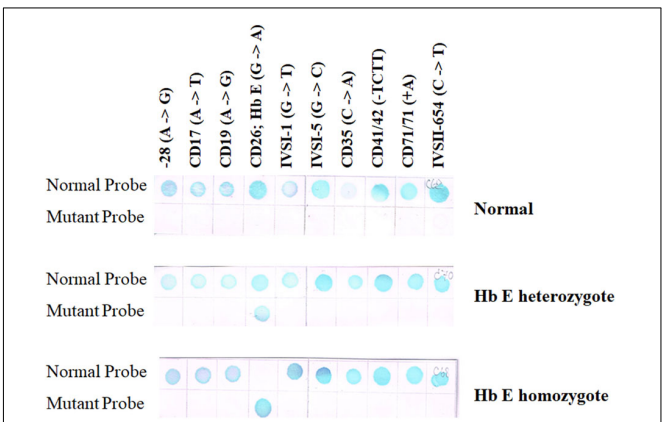

FIGURE 5 | Result of RDB showing a result of normal individual will give positive results with each wild-type sequence but not with any mutant probe (upper panel). Heterozygotes show a reaction with a single mutation dot in addition to the normal dots (middle panel), whereas homozygotes will give a positive dot with that mutant probe but not with its corresponding normal sequence (two mutant dots will be seen if the individual carries two different mutations) together with positive spots for the remaining normal probes (lower panel).

detection methods quite a routine procedure. For each mutation, two hybridization reactions need to be conducted, one with the probe for the mutant sequence and the other with the probe for the normal sequence. The stringency of hybridization has to be optimized for each ASO probe.

A reverse dot-blot analysis has been developed (Maggio et al., 1993; Winichagoon et al., 1995). The ASO probes contain an amino group at the 5′-terminal base which enables them to be covalently attached onto a nylon membrane strip. This is then hybridized with amplified DNA which has been labeled with biotin for colorimetric detection. A normal individual will give positive dots with each wild-type probe but not with any mutant probe (**Figure 5** upper panel). Heterozygotes exhibit a positive with one mutation dot in addition to the normal dots (**Figure 5** middle panel), whereas homozygotes for mutation will give a positive dot with that mutant probe but not with its corresponding normal sequence together with positive spots for the remaining normal probes (**Figure 5** lower panel). A critical requirement for this technique is the optimization of washing temperature for all of the probes. This can be achieved by optimizing the length of each ASO probe.

REAL-TIME PCR WITH MELTING CURVE ANALYSIS

The conventional PCRs give a clear result, but it requires labor-intensive and time-consuming in post-PCR processing steps. The real-time PCR or quantitative PCR (qPCR) is widely used to detect, characterize, and quantify nucleic acids. It is high throughput, automation, and low risk of post-PCR contamination. Currently, the application of real-time PCR with melting curve analysis for thalassemia diagnosis is based on two general approaches, intercalating dye assays and probe-based assays, obtaining a fluorescent signal from the synthesis of product in PCR.

The first approach depends on the fluorescent DNA-intercalating dyes such as SYBR Green I to bind with double-stranded DNA (dsDNA) and undergo a conformational change that leads to an increase in their fluorescence. In the

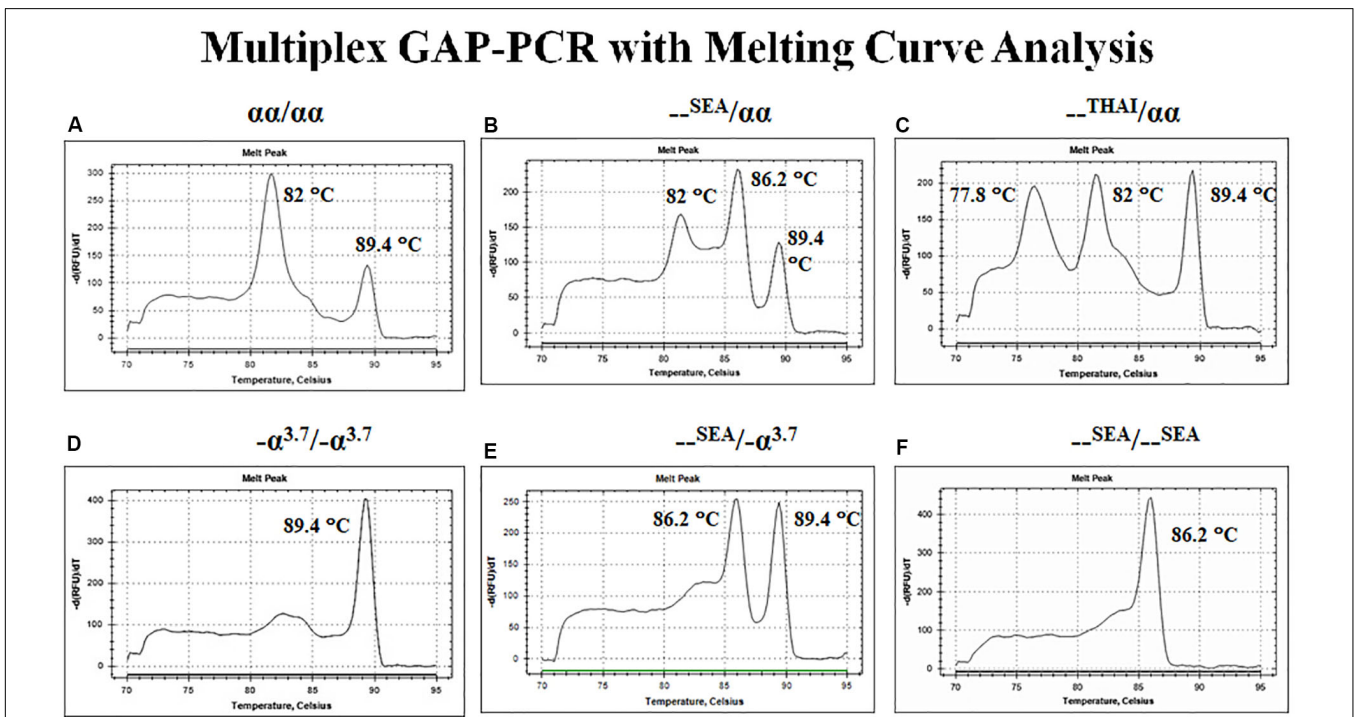

FIGURE 6 | Multiplex GAP–PCR with melting curve analysis for α-thalassemia genotyping. Panel **(A)** shows two peaks of α2-globin and ψζ-globin fragments from normal globin genotype. Panel **(B)** shows three peaks of α2-globin, $-^{SEA}$, and ψζ-globin from α-thalassemia 1 heterozygote ($-^{SEA}$ type). Panel **(C)** shows three peaks of $-^{THAI}$, α2-globin, and ψζ-globin from α-thalassemia 1 heterozygote ($-^{THAI}$ type). Panel **(D)** shows a single peak of $-\alpha^{3.7}$ from α-thalassemia 2 homozygote. Panel **(E)** shows two peaks of $-^{SEA}$ and ψζ-globin from deletional Hb H disease. Panel **(F)** shows a single peak of $-^{SEA}$ from Hb Bart's hydrops fetalis (α-thalassemia 1 homozygote; $-^{SEA}$ type).

TABLE 4 | The genotype results by PCR-NGS among 57,229 cases in Baise, Guangxi, China.

Number of samples	α-Thalassemia	β-Thalassemia	α-Thalassemia with β-thalassemia	Uncomom/novel mutation (not detected by conventional methods)	Hb variants
57,229	10,018	3,141	931	458	479

TABLE 5 | Abnormal hemoglobin detected by NGS analysis in Baise, Guangxi, China.

Hb variants	No. of Samples
Hb Hekinan II	366
Hb J-Wenchang-Wuming	28
Hb Groene Hart	15
Hb Macarena	14
Hb Owari	12
Hb Q-Thailand	11
Hb New York	8
Hb G-Coushatta	6
Hb Saclay	6
Hb Handsw	3
Hb Iraq-Halabja	3
Hb G-Honolulu	2
Hb Greenville-NC	1
Hb J-Broussais	1
Hb J-Lome	1
Hb Parma [A2]	1
Hb Prato	1
Hb Pressath	1
Hb Riccarton – II	1
Hb Wurzburg	1

presence of single-stranded DNA (ssDNA) or the dyes are free in solution, they will not fluoresce. After completion of the amplification reaction, thermal cycler program generated a melt curve by increasing the temperature in small increments and monitoring the fluorescent signal at each step. When 50% of a dsDNA are separate into ssDNA so-called melting temperature (Tm). The difference size or GC content of PCR products demonstrated the difference Tm peak. Therefore, the multiplex GAP-PCR with melting curve analysis was developed for α-thalassemia genotyping. The primers were designed to specifically amplify two deletion fragments, the –SEA and –THAI deletions and two normal fragments, ψζ- and α2-globin gene. The melting curve analysis is able to distinguish α-thalassemia 1 heterozygotes, α-thalassemia 2 homozygotes, Hb H disease, and α-thalassemia 1 homozygote (Hb Bart's hydrops fetalis) as shown in **Figure 6** (Munkongdee et al., 2010).

The second, probe-based assays are now widely used for detection of point mutations. TaqMan assays is fluorescently labeled oligonucleotide probe. The TaqMan assay used the 5′-exonuclease activity of thermostable Taq polymerases. The probe composed a fluorescent reporter at the 5′ end and a quencher at the 3′ end. The fluorescence of the reporter is quenched due to its proximity to the quencher. However, during the annealing/extension step in the PCR reaction, the probe hybridize to the target region. The 5′ to 3′ exonuclease activity of Taq will cleave off the reporter resulting fluorescence signal which is proportional to the amount of PCR product in the sample. This technique can apply for β-thalassemia diagnosis, the multiplex probe-based fluorescence melting curve analysis (FMCA) which is a powerful tool for point mutations detection based on the Tm generated by thermal denaturation of the probe-target hybrid (Huang et al., 2011; Qiuying et al., 2011; Xiong et al., 2011).

DIRECT DNA SEQUENCING

The mutation(s) can be identified by sequencing the PCR product, usually employing the Sanger's dideoxy termination method. This requires the production of a single DNA strand as a template. There are a number of techniques to achieve this. An aliquot of the amplified DNA can be subjected to another round of PCR but in the presence of a single primer strand; or the original PCR product can be denatured and rapidly cooled so that the two strands remain separated; or one of the primer strand is phosphorylated at the 5′-terminus and the PCR product treated with lambda exonuclease which digests 5′-phosphorylated strand in a dsDNA; or biotin can be incorporated into the 5′-terminus of one of the primer strand enabling the PCR product to be adsorbed onto streptavidin-coated magnetic beads, which can then be treated to denature the duplexes and allow removal of the non-biotinylated strands from the beads. Alternatively, the PCR products can be subcloned into a sequencing vector, but this method introduces the possibility of detecting PCR artifacts. It enables the identification of novel or rare mutations present in the population (Sirichotiyakul et al., 2003; Korf and Rehm, 2013).

MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

Multiple ligation-dependent probe amplification (MLPA) is a multiplex PCR method that allows the detection of any deletions or duplications in the screened regions. This technique has been proven to find known and unknown deletions in unsolved cases after performing conventional techniques. The MLPA technique is easy to use and requires only a thermocycler and CE equipment (Schouten et al., 2002; Lei et al., 2019).

MLPA was started with DNA denaturation/hybridization step. DNA was denatured and incubated with a mixture of MLPA probes. MLPA probes consist of two separate oligonucleotides (LPO and RPO). The two probe oligonucleotides were hybridized to adjacent target sequences. Then, the probes can be ligated during the ligation reaction. Only ligated probes will be amplified during the PCR reaction, the number of probe ligation products is a measure for the number of target sequences in the sample. The amplification products were separated using CE.

NEXT-GENERATION SEQUENCING

Advancement of sequencing technology makes an enormous contribution in characterization of the human genome. NGS technologies have gained the capacity to sequence entire human genome in an ultra-high throughput, scalability, and

speed manner at a level that is not possible using Sanger sequencing technology. Most NGS platforms have three general steps: first library preparation using random fragmentation of DNA followed by ligation with custom linkers. Second, library amplification using clonal amplification methods and PCR. Third, sequencing using incorporation of fluorescent-labeled nucleotides by DNA polymerases or ligation processes. NGS has enabled researchers to diagnose and understand complex diseases through whole-genome sequencing, exome sequencing, or targeted gene panels (Yang et al., 2013; Stark et al., 2016). Recently, NGS has been applied for thalassemia screening (He et al., 2017; Shang et al., 2017; Zhang et al., 2019). Target NGS approach was designed to cover entire globin genes coding regions, their key regulatory regions, and modifier genes such as *KLF1*, *BCL11A*, *HBS1L*, and *MYB*. Preliminary data show that NGS may be much more accurate than convention thalassemia diagnosis by complete blood count (CBC), Hb analysis, Hb typing, and selected for genotyping. Preliminary study by PCR-NGS among 57,229 cases was performed in Guangxi, China, and revealed uncommon or novel mutations that could not be detected by conventional methods 458 mutations (**Tables 4**, **5**).

CONCLUSION

Laboratory diagnosis of thalassemia requires a number of tests including red blood cell indices and Hb and DNA analyses. Although low MCV and MCH are a character of thalassemic red blood cells, however, these two red blood cell indices cannot discriminate between thalassemia trait and iron deficiency or between α- and β-thalassemic conditions. Today, Hb analysis may be carried out by either HPLC or CE system. Both qualitative and quantitative analysis for Hb components can obtain from these automatic systems and help to do both prenatal and postnatal diagnoses of thalassemia within a few minutes. DNA analysis have been used for point mutation detection in β-thalassemia and large-deletion detection in α-thalassemia. Limitations of conventional thalassemia diagnosis methods are missed diagnoses due to normal or borderline red blood cell indices and/or Hb A2 levels, various labor-intensive methods may need to identify disease-causing mutation for thalassemia that have more than 1,800 mutations ranging from point mutation to large deletion. Recently, NGS has been introduced to screen for thalassemia. More loci including genetic modifiers which have significant effects on clinical manifestation should be covered in the NGS screening, which is important for precise diagnosis and treatment of thalassemia. However, there are still some limitations of NGS techniques including expensive cost. All service labs were highly recommended to select the technique(s) they are most familiar and most economic one for their daily use.

AUTHOR CONTRIBUTIONS

TM, KP, and PC performed the relevant literature search and wrote the review manuscript. PW and SF reviewed the manuscript for submission and provided additional points for discussion.

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BRIEF RESEARCH REPORT
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An Innovative Multilevel Test for Hemoglobinopathies: TGA/Chemometrics Simultaneously Identifies and Classifies Sickle Cell Disease From Thalassemia

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Introduction: Hemoglobinopathies are the most common genetic disorder wordlwide and because of migrations are become an emerging global health problem. Screening programmes for Sickle cell disease and Thalassemia have been implemented in some countries, but are not a common practice, due to a lack in the accuracy of the methods and to the costs of the analyses.

Objectives: The objective of this study was the application of the thermogravimetry coupled to chemometrics as new screening method to perform an early diagnosis of thalassemia and sickle cell disease.

Methods: Whole blood samples (30 μL) from sickle cell anemia and thalassemia patients were analyzed using the thermobalance TG7 and the resulting curves were compared with those of healthy individuals. A chemometric approach based on Principal Components Analysis (PCA) was exploited to enhance correlation between thermogravimetric profiles and a model of prediction by Partial Least Square Discriminant Analysis (PLS-DA) was developed and validated.

Results: The characteristic profile of the blood sample thermal decomposition and the first derivative of the TG curve showed that patients were clearly distinguished from healthy individuals as a result of different amounts of water and corpuscular fraction of blood. The chemometric approach based on PCA allowed a quick identification of differences between healthy subjects and patients and also between thalassemic and sickle cell anemia subjects. Chemometric tools (PLS-DA) were used to validate a model of prediction to process the thermogravimetric curves and to obtain in 1 h an accurate diagnosis. The TGA/Chemometric test permitted to perform first level test for hemoglobinopathies with the same accuracy of confirmatory analyses obtained by the molecular investigation.

Conclusions: A screening test based on the coupling of thermogravimetry and chemometrics was optimized for the differential diagnosis of hemoglobinopathies. The novel test is able to simultaneously perform a simple and fast diagnosis of sickle cell

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anemia or thalassemia, in a single analysis of few microliters of non-pretreated whole blood at low cost, and with high accuracy. Moreover this method results particularly suitable in pediatric patients as it requires small sample volumes and is able to characterize also transfused patients.

Keywords: hemoglobinopathies, screening, thermogravimetric analysis, chemometrics, thalassemia, sickle cell disease

INTRODUCTION

Hemoglobinopathies are the most common genetic disorder wordlwide, and include the Sickle Cell Disease (SCD) (Schnog et al., 2004; Rees et al., 2010; Kato et al., 2018), caused by structural changes in the globins chains of hemoglobin (Hb), and the thalassemia syndromes (Weatherall and Clegg, 2001a; Thein, 2013) that are disorders of globin chains expression (Schechter, 2008; Steinberg, 2008; Kohne, 2011).

SCD is a disorder of hemoglobin synthesis characterized by the production of an altered form of hemoglobin, hemoglobin S (HbS) for a mutation in the sixth codon of the β globin gene, which results in the substitution of glutamic acid for valine. Sickle hemoglobin, under hypoxic conditions, polymerizes leading to the sickling of the red blood cells. The most severe form is homozygous HbSS (Sickle cell anemia, SCA), but there are other compound heterozygous conditions such as HbS and β-thalassemia, or HbS and other Hb variants. Chronic anemia, hemolysis, and recurrent acute vaso-occlusive crises, characterized by pain and systemic inflammatory response, are the main clinical features (Azar and Wang, 2017; Ware et al., 2017).

Thalassemia syndromes are characterized by the absence or reduced β-globin chain synthesis, are heterogeneous at the molecular level with almost 300 point mutations and deletions classified as severe, mild and silent that can produce clinical and hematological phenotypes of variable severity ranging from the asymptomatic carrier to the severe transfusion-dependent type. Homozygosity or compound heterozygosity for β-thalassemia mutations cause a severe spectrum of anemias called thalassemia intermedia and thalassemia major (Cao and Galanello, 2010).

Hemoglobinopathies are particularly frequent in the countries where the malaria was endemic (Africa and Mediterranean region, Middle East, India, and South and East Asia) as a result of the heterozygote advantage against malaria. In the last decades, due to migrations, the hemoglobinopathies are become an emerging global health problem, and programs of screening for SCD and Thalassemia have been implemented in some countries (WHO report, 2005-2011; Weatherall, 2010; Williams and Weatherall, 2012).

The major limits to the expansion of these screening programs are the high cost and technical complexity of conventional diagnostics methods, specially for the developing countries where hemoglobinopathies are more frequent but poor facility for diagnosis, control and management of the subjects affected by hemoglobin disorders are available (Weatherall and Clegg, 2001b; Cao and Kan, 2012).

Laboratory diagnosis of hemoglobin disorders require a two levels diagnostic protocol (Clarke and Higgins, 2000; Cao et al., 2002; Greene et al., 2015; Sabath, 2017; Aiello et al., 2018). The first level screening tests include: (i) a complete blood count (CBC), since the hematological parameters mean corpuscolar volume (MCV), mean corpuscular hemoglobin (MCH), red blood cell counts (RBC), and red cell distribution width (RDW), are considered indicator in the haemoglobinopathies screening; (ii) the assessment of martial state to exclude the iron deficiency anemia; (iii) the study of hemoglobin fractions HbA_2 and HbF by HPLC or electrophoresis to identify β-thalassemia phenotype, or abnormal peak for the presence of hemoglobin variants.

These methods permit the detection of the most common Hb variants (HbS, HbC, and HbE) that may result in a Hb disorder by interacting with β-thalassemia. Nevertheless, these methods do not detect the β-thalassemia in the newborn period, since HbA_2 does not reach adult levels until about 6 months of age, and cannot be applied in transfused patients. A positive screening test requires to perform the second level tests to confirm the presence of hemoglobinopathy by molecular analysis of the globin genes (Mosca et al., 2009; Giardine et al., 2014; Barret et al., 2017). Therefore, the conventional screening methods for SCD and Thalassemia require cost, equipment and specialized personnel available only in few specialized clinical laboratories (Clarke and Higgins, 2000; Urrechaga et al., 2011).

Thermogravimetric techniques have been largely applied to complex matrix as analytical tool that does not require sample pretreatment nor clean-up (Toth et al., 2010; Fonseca et al., 2012; Materazzi et al., 2015, 2017a; Papadopoulos et al., 2016; Risoluti et al., 2016b, 2017; Bach and Chen, 2017; Catauro et al., 2018) and permits to rapidly obtain both qualitative and quantitative outcomes (Skreiberg et al., 2011; Materazzi et al., 2014a,b; Napoli et al., 2014; Shan-Yang et al., 2015; Marcilla et al., 2017). Recently, a novel approach based on the association of thermogravimetric analysis (TGA) and chemometrics has been proposed in different fields (Pappa et al., 2003; Khanmohammadi et al., 2012; Caramés-Alfaya et al., 2013; Chauhan et al., 2020), including the investigation of biological samples for clinical analysis (Risoluti et al., 2016a, 2018a; Materazzi et al., 2017a). Themogravimetry, in fact, with respect to clinical specimens, presents the advantage of processing samples without requiring any sample manipulation and the resulting curves reflect the characteristic composition of the sample in few microliters of sample. In addition, the coupling with chemometric tools permits to simultaneously consider the TG profile of blood samples and to compare in a click a patient of unknow diagnosis with the developed model of prediction (Risoluti et al., 2018c, 2019).

The objective of this study was the application of the thermogravimetry coupled to chemometrics, the TGA/Chemometric test, as new screening test to perform an early diagnosis of Thalassemia and SCD on few microliters of whole blood. This innovative screening test is cheap, specific, rapid, and applicable also in the early neonatal period, and in transfusion dependent people.

METHODS

Enrollment of Patients

The study included a number of 235 subjects: among these, 120 healthy donors were characterized at the National Health Institute of Rome and 115 patients affected by hemoglobinopathies were followed for diagnosis, management and therapies at the Thalassemia Unit of S. Eugenio Hospital in Rome. The anemic patients included 65 subjects with Sickle Cell Anemia (SCA) diagnosis and 50 subjects affected by Thalassemias.

Blood specimens were collected in ethylene diamine tetracetic acid (EDTA) after informed consent of the patient (provided on request) and according to guidelines established by the Ethical Committee for human subject studies (Helsinki Declaration of 1975, revised in 2008).

Hematological Characterization

The hematological characterization of all the collected samples from healthy subjects and patients was performed by evaluating the parameters RBC counts, Hb values, Hematocrit (Hct), MCV, MCH, and red cell distribution width (RDW) determined with an automated hematology analyzer ADVIA 120 (Siemens, USA). The comparison between the groups was performed by Student *t*-test for unpaired data.

Thermogravimetric Analysis (TGA)

A Perkin Elmer TGA7 Thermobalance (Massachusetts, USA) was used to record the thermogravimetric curves. Non pre-treated whole blood (about 30 μl) was placed into the crucible where temperature was raised from 20 to 800°C, with a 10°C/min heating rate, as the best resolution rate. The atmosphere was air as carrier gas, at a flow rate of 100 ml/min. The Curie-point transitions of standard metals were involved to calibrate the instrumental response, as reported by the equipment recommendations. Reproducibility was checked by collecting three replicates for each sample. Derivative Thermogravimetric data (DTG) were also calculated to compare samples and represent the derivative of the function TG(T) with respect T.

Chemometrics

Multivariate statistical analysis was performed by chemometrics with the aim of investigating correlations among samples and developing a model of prediction of the anemic status. Principal Component Analysis [PCA (Massart et al., 1998a; Ferreiro-González et al., 2018; Risoluti et al., 2018b)] was used as exploratory tool, while the classification model of prediction was pointed out by Partial Least Square Linear Discriminant Analysis [PLS-DA (Savitzky and Golay, 1964; Massart et al., 1998b; Barker and Rayens, 2003; Materazzi et al., 2017b,c)].

A chemometric study was planned with the aim of evaluating the most performing pretreatment to get the lowest root mean square errors (RMSE%) and the highest Specificity (Sp %) and Non Error Rate (NER %) (Murphy, 2012; Otto, 2017). The entire dataset of samples was divided into calibration set (75% of the dataset) and test set (25% of the dataset), while the prediction ability of the model were assessed by processing patients from an external dataset of measurements in order to ensure independency.

Diagnostics and acquisition of the thermogravimetric data were carried out by Pyris software (Thermo Fisher Scientific Inc., Waltham, MA, USA) as ASCII files, which were then imported into Unscrambler package to perform statistical analysis.

RESULTS

Hematological Characterization

The hematological features were determined for all the collected samples from patients with confirmed diagnosis of sickle cell anemia (SCA) and thalassemia (T) and results were compared to those of healthy individuals.

The SCA group included patients with HbS homozygosity (HbSS), and with double heterozygosity (HbS/thalassemia, HbS/HbC, HbS/HbD). The thalassemia group included thalassemia major and intermedia patients, generally transfusion dependent, with different globin mutations β° and β^{+} in both homozygosity and heterozygosity.

Table 1 shows the hematological data expressed as mean ± standard deviation (SD). The group of SCA patients was characterized by a chronic hemolytic anemia with a decrease in the number of RBC and a corresponding decrease in the levels of Hb and Hct. MCV and MCH were significantly decreased, and the RDW values significantly higher, for the presence of hemolysis and reticulocytosis, in sickle cell patients than healthy donors (*p*-values: MCH = 0.01; all other parameters < 0.0001). As expected, the comparison between thalassemic patients and healthy subjects showed significantly differences in all parameters ($p < 0.0001$) with decreased RBC, Hb, Hct, MVC, and MCH values and an higher RDW value.

Despite the investigation of the hematological parameters revealed that patients affected by hemoglobinopathies significantly differ to the population of healthy individuals a statistically difference between SCA and T subjects was observed only for RDW values ($P < 0.05$). Therefore, the hematological parameters alone did not differentiate patients with thalassemia from those with sickle cell anemia. This observation is in agreement with the heterogeneity and wide range of severity of both thalassemia and SCA patients, and in addition a number of HbS/Thal patients are present in SCA group.

Thermogravimetric Analysis

Thermogravimetric analysis was performed on all the collected samples and the TG profile of blood samples was observed under controlled conditions of combustion. The characteristic

TABLE 1 | Hematological characterization of healthy individuals, sickle cell disease (SCA) and thalassemia (T) patients.

	RBC (10^6/mL)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	RDW (%)
CTR	5.3 ± 0.4	14.8 ± 0.8	46.6 ± 2.8	87.4 ± 3.2	27.8 ± 1.4	13.2 ± 0.7
T	4.1 ± 1.1	10.0 ± 1.7	32.0 ± 6.1	79.9 ± 8.0	25.2 ± 3.5	18.3 ± 4.4
SCA	3.8 ± 0.7	9.9 ± 1.3	30.9 ± 3.8	81.8 ± 8.4	26.4 ± 3.3	20.0 ± 4.2
T vs. CTR*	3.3E-09	2.8E-26	1.3E-22	4.5E-07	3.9E-05	1.5E-10
SCA vs. CTR*	4.8E-26	2.1E-44	1.3E-47	7.1E-05	1.0E-02	1.1E-18
SCA vs. T*	1.1E-01	7.7E-01	2.2E-01	2.5E-01	7.5E-02	4.5E-02

**p-value estimated to 95% of confidential level.*

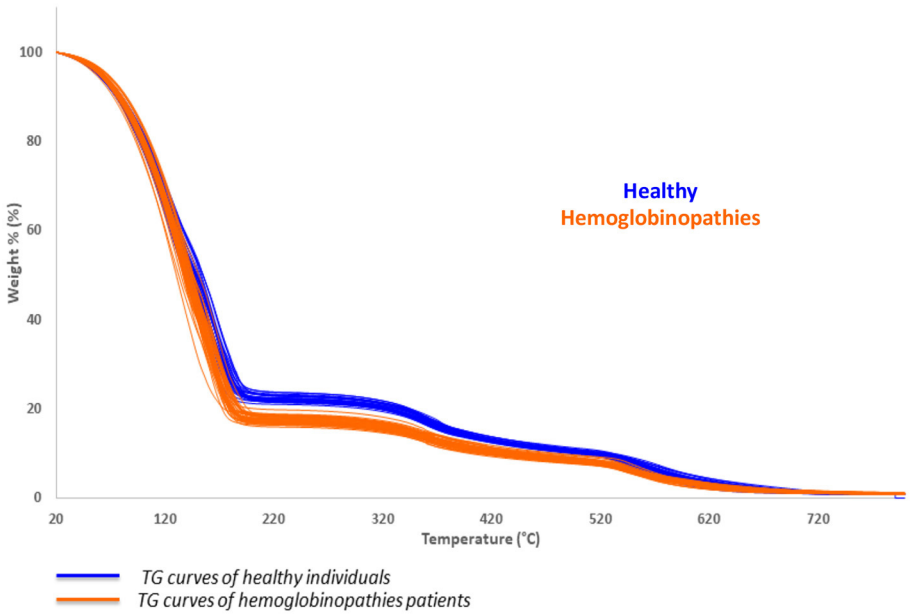

FIGURE 1 | Collected thermogravimetric curves of healthy subjects (blue) and patients affected by hemoglobinopathies (orange).

thermal behavior of all patients affected by hemoglobinopathies (thalassemia and SCA patients) was estimated and compared to the thermal profile of healthy subjects, as reported in **Figure 1**.

A significant shift to lower temperature may be observed in the first thermally induced decomposition step of the patient with hemoglobinopathies (orange), with respect to healthy subjects (blue), as a result of the changing in the mass of the sample. The calculation of the first derivative curve of the TG profile, permitted to highlight all the decomposition steps of the samples and to calculate the percentage weight losses of each process: the first, corresponds to the release of the bulk water while the second to the bound water in the temperature range of about 50–150°C.

The total amount of water calculated for patients affected by hemoglobinopathies (sum of the first and second thermally induced processes) was found to be significantly higher than the percentage of the healthy subjects (*p*-values of 2.7E-22 and 3.7E-31 for thalassemia and SCA patients, respectively). On the contrary, the corpuscular fraction of blood related to the processes occurring at 350 and 530°C, was significantly lower in hemoglobinopathies than the healthy population (*p*-values of 7.6E-13 and 2.2E-11 for thalassemia patients and 1.7E-25 and 1.2E-15 for SCA patients). In addition, the decrease in the corpuscular fraction observed by TGA on hemoglobinopathies subjects (weight losses occurring at 350 and 530°C) is strictly associated to the anemia and hemolysis and is confirmed by the calculated hematological parameters reported in **Table 1**, where RBC, Hct, and Hb were found to be lower than the healthy donors.

The percentages of the bulk water and bound water were respectively higher and lower in SCA and thalassemic patients than the healthy subjects. As a consequence, the bulk/bound water ratio was found to be significant in differentiating SCA from healthy subjects (*p*-value of 4.7 E-03) and thalassemic from healthy individuals (*p*-value of 1.3 E-07). Interestingly, as suggested by the *p*-values reported in **Table 2** where SCA and thalassemic groups are compared, both the two groups showed a similar distribution of the total amount of water (water content) and the corpuscular fraction (2nd and 3rd weight losses) as no statistically differences were observed in the corresponding TG parameters. On the contrary, the distribution of the bulk water

TABLE 2 | Calculated weight losses (%) of each thermally induced decomposition process.

	Water Content (%)	Bulk Water (%)	Bound Water (%)	Bulk/Bound Water ratio	2nd Weight Loss (%)	3rd Weight Loss (%)
CTR	78.1 ± 0.9	46.8 ± 4.0	30.9 ± 4.8	1.6 ± 0.5	11.5 ± 0.7	9.1 ± 0.5
T	82.1 ± 1.7	62.8 ± 8.8	19.3 ± 8.1	4.3 ± 3.0	9.6 ± 1.1	7.8 ± 0.9
SCA	81.6 ± 1.3	55.7 ± 8.6	26.0 ± 8.3	2.8 ± 2.7	9.4 ± 0.9	7.8 ± 0.9
T vs. CTR*	2.7E-22	1.5E-16	2.6E-11	1.3E-07	7.6E-13	2.2E-11
SCA vs. CTR*	3.7E-31	4.2E-09	5.9E-04	4.7E-03	1.7E-25	1.2E-15
SCA vs. T*	8.2E-02	7.3E-05	8.7E-05	7.5E-03	2.5E-01	9.8E-01

**p-value estimated to 95% of confidential level.*

and bound water was found to be significantly different as well as the bulk bound water ratio.

These preliminary results suggested that the themoanalytical investigation of the blood may be more effective in discriminating SCD from thalassemia disease than the hematological features.

Chemometric Analysis

Statistical analysis was performed on the collected thermogravimetric curves, by principal component analysis (PCA), with the aim of highlighting correlations among measurements. The resulting scores plot, obtained after mean centering correction of data, is reported in **Figure 2A**. The mean centering data processing is performed by calculating the average vector of the curves of all the rows and subtracting it point by point from each vector in the dataset. The scores plot reported in **Figure 2A**, clearly shows two separated groups related to the healthy subjects (blue squares) and the patients affected by hemoglobinopathies (orange triangles). The two groups of subjects may be differentiated according to the first principal component that explains the maximum of the variance in the data set (88%). Therefore, the explorative multiparametric analysis of the TG curves performed by PCA (overall explained variance on of 96% using two PCs), permitted to put in evidence differences between healthy and subjects with hemoglobinopathies, as a function of the presence or the absence of the anemia.

A model of prediction was developed by Partial Least Square Discriminant Analysis (PLS-DA) in order to instruct a platform to rapidly identify the anemic status. To this aim, the 75 and 25% of the entire set of samples, were used as calibration and evaluation set, respectively. In addition, with the aim of improving accuracy and sensitivity of the method, different chemometric pre-treatments were investigated. Among these, Mean Centering (MC), Standard Normal Variate (SNV), and a combination of the first derivative of the TG curve and MC or SNV (Murphy, 2012; Otto, 2017).

The figures of merit of the model were calculated to evaluate the prediction ability of the novel test, including the Non Error Rate (NER) expressed as the percentage of samples correctly predicted by the model, the specificity (Sp %) expressed as the percentage of samples predicted as belonging to the correct class and the Root Mean Square Error (RMSE %). As a consequence, the most performing pre-treatment of the TG curves was selected as the one providing the highest values of NER % and Sp % and the lowest values of RMSE % in calibration and validation. The optimized model for the identification of the presence of anemia provided for an accuracy and a specificity of about 100% and the RMSE was 0.2%. for both controls and hemoglobinopathies. The resulting 3D plot of the model is reported in **Figure 2B**.

Development of the Multilevel Test

The second stage of the work focused on the optimization of a comprehensive model of prediction able to simultaneously identify and classify the presence of anemia, discriminating SCD from thalassemia. Preliminarily, a Principal Component Analysis was performed to evaluate the most performing conditions of separation of the samples (**Figure 3A**) and at last a model of prediction by PLS-DA was developed for the final multilevel test (**Figure 3B**).

The model permits to separate samples according to the different class they belong to (Healthy, SCA or thalassemic) and may represent a novel way to perform the screening of hemoglobinopathies with the accuracy (100% of NER for all the classes) and the specificity (100% of Sp for all the classes) of the second level test since all the subjects were correctly differentiated according to PC 1. The analysis of the factor loadings provided the clarification of the variables affecting most the distribution of samples, concluding that the range of temperature 85–190°C associated to the bulk and bound water release is responsible for SCA and thalassemia discrimination. In fact, the prediction ability of the model was estimated by the calculation of the figures of merit reported in **Tables 3–5**, showing that the involvement of the first derivative of the TG curves prior to mean centering pretreatment, permitted to obtain the suitable results in calibration, cross-validation and prediction of external set of samples, providing the lowest error of prediction (RMSE %) of about 0.18, 0.20, and 0.10% for healthy, talassemic and SCA subjects, respectively.

Diagnosis of Difficult Cases of Anemia

The novel test was used to investigate some cases of hemolytic anemias of difficult diagnosis such as in neonatal period and in transfused patients. We have analyzed seven subjects, characterized by hemolytic anemia, negative Coombs tests, and increased LDH value that suggested the presence of an erythrocyte congenital defect as cause of the hemolytic anemia. The TGA/Chemometrics test showed the presence of two cases of SCA and five patients with thalassemia (two of them in the newborn period). The 2nd level tests by molecular analysis of the globin genes confirmed the presence of the globin genes

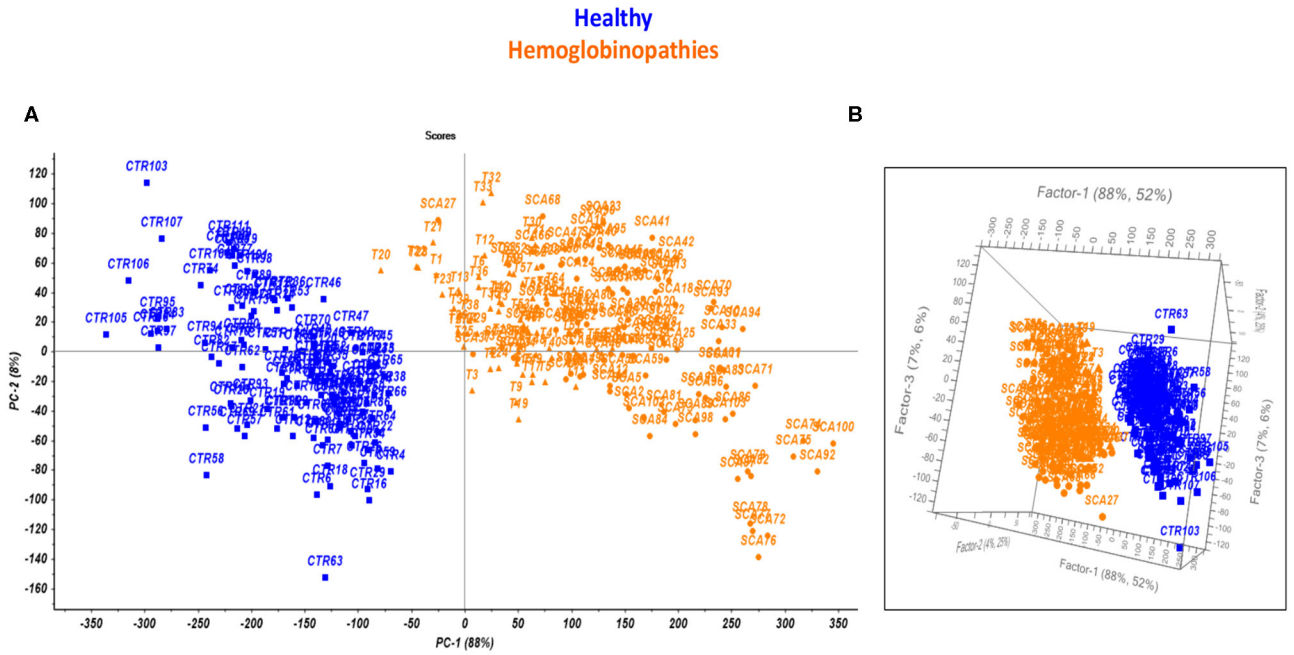

FIGURE 2 | Scores plot performed by PCA **(A)** and PLS-DA model **(B)** on the TG curves for healthy subjects (blue) and patients affected by hemoglobinopathies (orange).

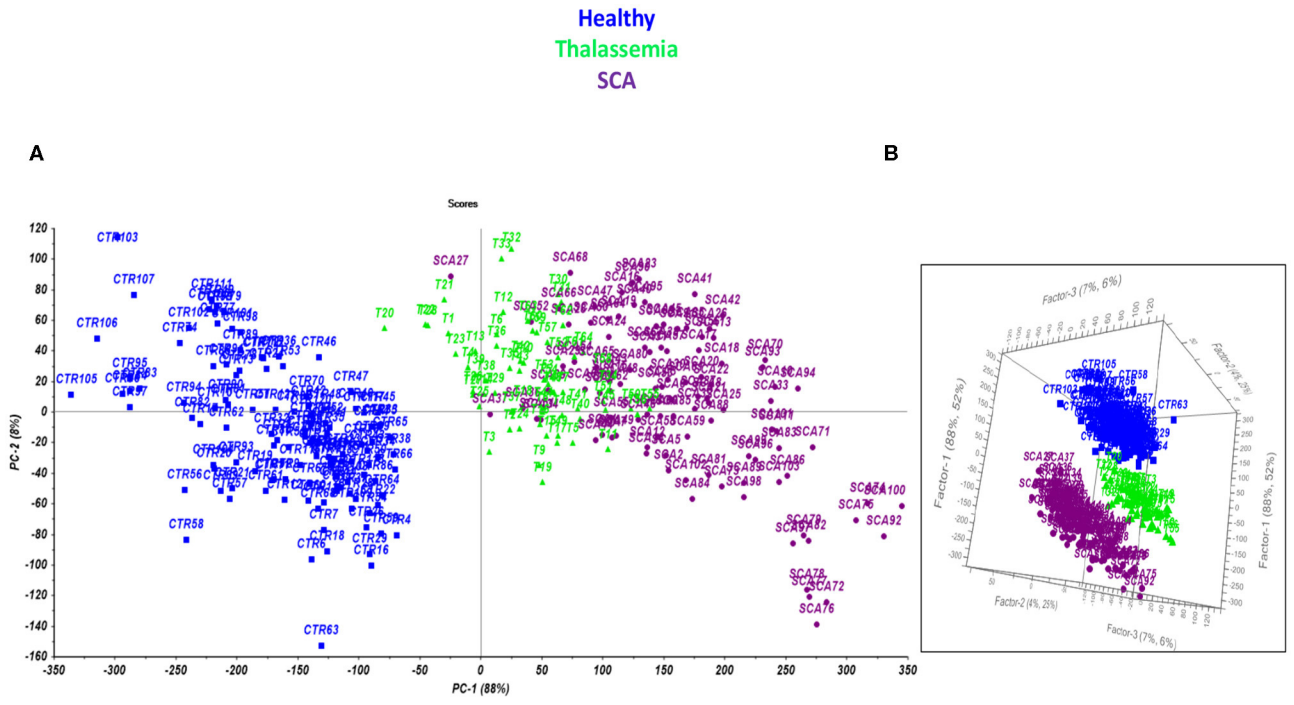

FIGURE 3 | Scores plot performed by PCA **(A)** and PLS-DA model **(B)** on the TG curves for healthy subjects (blue), and patients affected by thalassemia (light green) and SCA (purple).

mutations. All the analyzed patients were found to be correctly predicted by this screening test that permitted a differential diagnosis of two hemoglobinopathies. The application of this new screening method will allow to address patients to the 2nd level confirmatory analysis reducing time and costs for the diagnosis.

TABLE 3 | Calculated Non Error Rate (NER %) of the model in calibration, validation and prediction, obtained using different chemometric pre-treatments.

	Patient	Mean centering	SNV	1st derivative + mean centering	1st derivative + SNV
Calibration	CTR	100.0	100.0	100.0	99.1
	T	100.0	98.4	100.0	95.3
	SCA	100.0	100.0	100.0	100.0
Validation	CTR	100.0	100.0	100.0	93.9
	T	96.9	92.2	95.3	85.9
	SCA	100.0	100.0	100.0	100.0
Prediction	CTR	100.0	100.0	100.0	100.0
	T	100.0	100.0	100.0	100.0
	SCA	100.0	100.0	100.0	100.0

TABLE 4 | Calculated specificity (Sp %) of the model in calibration, validation and prediction, obtained using different chemometric pre-treatments.

	Patient	Mean centering	SNV	1st derivative + mean centering	1st derivative + SNV
Calibration	CTR	98.3	100.0	100.0	98.3
	T	100.0	95.1	98.3	93.3
	SCA	100.0	100.0	100.0	100.0
Validation	CTR	100.0	100.0	100.0	88.6
	T	93.4	85.2	91.8	83.0
	SCA	100.0	100.0	100.0	100.0
Prediction	CTR	100.0	100.0	100.0	100.0
	T	100.0	100.0	100.0	100.0
	SCA	100.0	100.0	100.0	100.0

TABLE 5 | Calculated Root Mean Square Error (RMSE %) of the model in calibration, validation and prediction, obtained using different chemometric pre-treatments.

	Patient	Mean centering	SNV	1st derivative + mean centering	1st derivative + SNV
Calibration	CTR	0.19	0.20	0.18	0.18
	T	0.25	0.23	0.21	0.22
	SCA	0.12	0.10	0.11	0.12
Validation	CTR	0.20	0.20	0.20	0.23
	T	0.25	0.24	0.24	0.28
	SCA	0.13	0.12	0.12	0.12
Prediction	CTR	0.22	0.24	0.18	0.21
	T	0.18	0.18	0.20	0.22
	SCA	0.15	0.16	0.10	0.10

DISCUSSION AND CONCLUSIONS

The disorders of hemoglobin, such as thalassemia and SCD, are today a real global healthy problem. A rapid and correct diagnosis becomes extremely relevant because allows an early preventive care and avoids consequences for affected children mainly in low and middle-income countries. A number of procedures have been proposed for screening hemoglobinopathies based on complete blood counts followed by the analysis of hemoglobin fractions, differential erythrocyte density, differential wicking of Hb S and Hb A through filter paper, a polyclonal antibody-based capture immunoassay, and next-generation sequencing (Korf and Rehm, 2013; McGann et al., 2016; Piety et al., 2016; Barret et al., 2017). Despite these methods contribute significantly in improving the first level test, they lack in 100% accuracy and the cost is prohibitive in low-resource countries.

This study introduces an innovative method to perform the screening of hereditary hemoglobin defects, characterized by severe hemolytic anemia with different severe clinical manifestations. Systematic screening programmes for hemoglobinopaties are not a common practice, due to a lack in the accuracy of the methods and to the costs of the analyses. As a consequence, the diagnosis is usually made when a severe complication occurs, particularly in developing countries. The novelty of this study consists of the accuracy and the simplicity of the test that permits to rapidly perform a differential diagnosis of sickle cell anemia and thalassemia. The study demonstrated that the themoanalytical investigation of the blood was more effective in discriminating SCA from thalassemia disease than the hematological features. The application of a model of prediction by PLS-DA was developed for the final multilevel test and this model permits to separate samples according to the different class they belong to (Healthy, SCA or thalassemic) and may represent a novel way to perform the screening of hemoglobinopathies with the accuracy and the specificity of the second level test since all the subjects were correctly differentiated.

In conclusion the application of TGA/chemometric analysis has proved to be a particularly useful diagnostic tool for the screening of the hemoglobin defects, in a short time and at low cost, also in case of congenital hemolytic anemia of difficult diagnosis. This method results particularly suitable in pediatric patients as it requires small sample volumes and is able to characterize patients subjected to transfusion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Roma 2 of the S. Eugenio Hospital, Rome. Written informed consent for participating in the study and publishing clinical data in an anonymized manner are collected and copies of the informed consent are available on request. In the case of child, written informed consent for participating in the study and publishing clinical data in an anonymized manner was obtained from the participant's legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RR, SMat, and PC conceived the study and wrote the manuscript. FS and LM enrolled the patients and performed

the clinical and laboratory evaluation. GG, SMas, and EC performed analysis. All authors performed the data analysis, contributed to the article, and approved the submitted version.

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REVIEW
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Microfluidics in Sickle Cell Disease Research: State of the Art and a Perspective Beyond the Flow Problem

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Sickle cell disease (SCD) is the monogenic hemoglobinopathy where mutated sickle hemoglobin molecules polymerize to form long fibers under deoxygenated state and deform red blood cells (RBCs) into predominantly sickle form. Sickled RBCs stick to the vascular bed and obstruct blood flow in extreme conditions, leading to acute painful vaso-occlusion crises (VOCs) – the leading cause of mortality in SCD. Being a blood disorder of deformed RBCs, SCD manifests a wide-range of organ-specific clinical complications of life (in addition to chronic pain) such as stroke, acute chest syndrome (ACS) and pulmonary hypertension in the lung, nephropathy, auto-splenectomy, and splenomegaly, hand-foot syndrome, leg ulcer, stress erythropoiesis, osteonecrosis and osteoporosis. The physiological inception for VOC was initially thought to be only a fluid flow problem in microvascular space originated from increased viscosity due to aggregates of sickled RBCs; however, over the last three decades, multiple molecular and cellular mechanisms have been identified that aid the VOC *in vivo*. Activation of adhesion molecules in vascular endothelium and on RBC membranes, activated neutrophils and platelets, increased viscosity of the blood, and fluid physics driving sickled and deformed RBCs to the vascular wall (known as margination of flow) – all of these come together to orchestrate VOC. Microfluidic technology in sickle research was primarily adopted to benefit from mimicking the microvascular network to observe RBC flow under low oxygen conditions as models of VOC. However, over the last decade, microfluidics has evolved as a valuable tool to extract biophysical characteristics of sickle red cells, measure deformability of sickle red cells under simulated oxygen gradient and shear, drug testing, *in vitro* models of intercellular interaction on endothelialized or adhesion molecule-functionalized channels to understand adhesion in sickle microenvironment, characterizing biomechanics and microrheology, biomarker identification, and last but not least, for developing point-of-care diagnostic technologies for low resource setting. Several of these platforms have

already demonstrated true potential to be translated from bench to bedside. Emerging microfluidics-based technologies for studying heterotypic cell–cell interactions, organ-on-chip application and drug dosage screening can be employed to sickle research field due to their wide-ranging advantages.

Keywords: sickle microfluidics, sickle cell disease, exosomes, organ-on-chip, drug screening, cell–cell interactions, endothelialized microfluidics, neutrophil-platelet aggregates

INTRODUCTION

Sickle cell disease (SCD) is a monogenic vascular disorder originating from a single point mutation of beta globin gene of the human hemoglobin, affecting people from African, middle eastern, north Indian and Mediterranean ancestry (Aich et al., 2019). It is estimated that every year about 300,000 children are born with SCD world-wide (Uzunova et al., 2010). At low oxygen concentration, the hemoglobin molecules self-polymerize in long fibers and deform the red blood cells (RBCs) causing more viscous blood flow than normal healthy individual. The very nature of periodic oxygenation-deoxygenation of RBCs leads to recurrent sickling-unsickling of RBCs resulting in persistent presence of sticky RBCs in the blood flow in sickle patients. Vaso-occlusive crises (VOCs) are the major life-threatening acute events of SCD during which the blood flow is obstructed by RBC and other cell aggregates causing severe pain. Other acute complications involve stroke, acute chest syndrome (ACS) and pulmonary hypertension. Persistent inflammation, oxidative stress and activated endothelium facilitate multi-faceted chronic complications involving failure or distress in multiple organs of the body (Ballas et al., 2012; Rees and Gibson, 2012). The disease burden is immense on the quality of life, and chronic pain in adult patients add to the burden from acute complications and end organ damage. While origin of this disease is monogenic, pleotropic effects are present and disease outcomes are clinically variable making the prognosis of the disease unpredictable. Measures to reduce the morbidity and mortality of sickle cell anemia include prophylactic penicillin therapy and substitute in infants and children, and hydroxyurea (HU), the 30-year old FDA approved drug for SCD, in adults (Ataga and Desai, 2018). Very recently, L-glutamine, Crizanlizumab, and Voxelotor have been approved by the US FDA for their efficacy in reducing acute VOC/year in adult population (Ballas, 2020). However, standardization of SCD management plan is difficult as the classification of sickle patients are not straightforward. The only curative treatment of SCD is allogeneic hematopoietic stem cell transplantation, which is expensive and resource-intensive (Aich et al., 2019). Gene therapy approaches under development represent to date potentially definitive cures in SCD, but again price-intensive (Orkin and Bauer, 2019). Therapeutic outcomes are not similar among patients with similar acute or chronic complications and such clinical heterogeneity in the response are yet to be understood from the mechanistic viewpoint. Therefore, elucidating molecular and cellular mechanism of specific complications, identifying novel mediators and pathways and overall addressing patient-specific treatment needs are major areas of improvement in sickle cell research.

In recent decades, the advent of microfluidic platforms has enabled researchers to explore pathophysiological events at the cellular and molecular level in addition of their great utility in diagnostics (Sackmann et al., 2014). Similarly, sickle cell researchers have been involved in utilizing microfluidic technology to understand how VOCs happen during flow of mixture of rigid and non-rigid RBCs, how hypoxia-reoxygenation affects the sickle RBC deformability and their biophysical properties, how microvascular geometry may potentiate occlusion, how non-RBC systemic cells interact with each other and/or vascular endothelium to facilitate endothelial adhesion that aid orchestration of VOC, how blood rheology is distinct for sickle and healthy individuals, how tonic salinity influences sickling of RBCs etc. Additionally, a number of microfluidics-based SCD diagnostic platform have shown promise to be viable to be used in low resource setting with high accuracy and sensitivity (Alapan et al., 2016a; McGann and Hoppe, 2017). The trend of sickle microfluidics research in terms of publication counts can be seen in **Figure 1**, and a complete list with all the devices and applications can be found in **Supplementary Table 1**. can be seen in Being more accurate in recapitulating microvasculature than other *in vitro* platforms, microfluidics pose itself as a versatile platform for aiding both mechanistic and translational studies of SCD. Here, we review the current state of the art of microfluidics studies that are driven toward mechanistic understanding as well as studies that have potential for clinical translation. In this review, we have not included discussion about point-of-care diagnostics, as they have discussed in details elsewhere (Alapan et al., 2016a; McGann and Hoppe, 2017; Ilyas et al., 2020). We classify the devices based on their utility in capturing specific biophysical or physiological events underlying sickle pathobiology and discuss exemplary devices, their characteristics and how their usage are filling in knowledge gaps of molecular and physical aspect of the disease. In the end, we envisage development of novel microfluidics platforms for investigating heterotypic cell–cell interactions in the context of vascular-immune and neuro-immune components of the disease that extends beyond the flow problem. Additionally, we discuss the utility of adopting existing organ-on-chip platforms for studying organ-specific sickle complications, and finally, we highlight the advantage of microfluidics as drug assay and

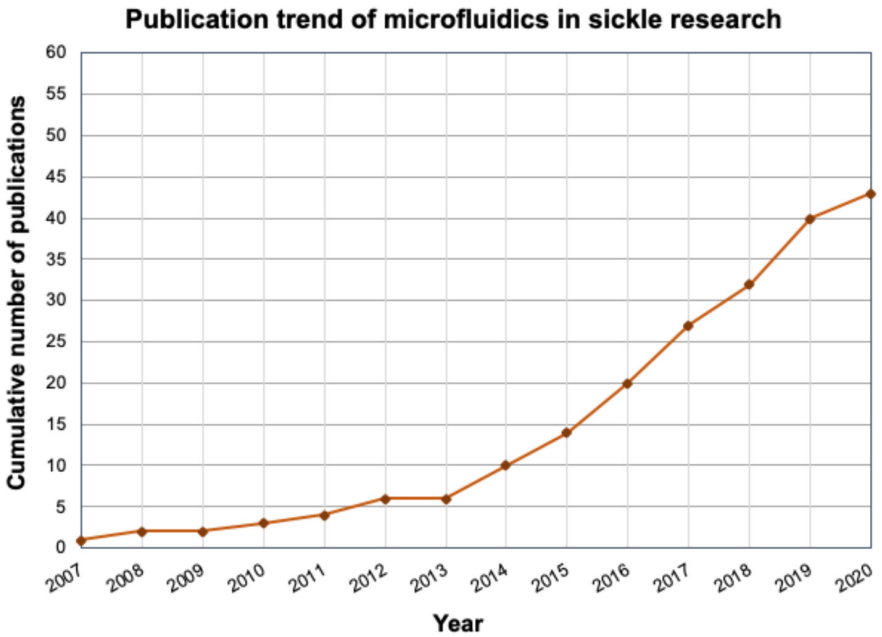

FIGURE 1 | Cumulative trend of publication number of microfluidics-based research in sickle cell field starting at 2007. A rapid progress is observable in sickle microfluidics field. The detailed list is shown in **Supplementary Table 1**.

dosage screening platform for rapid development of clinically effective therapeutics.

CURRENT STATE-OF-THE ART OF MICROFLUIDICS IN SICKLE CELL RESEARCH

Traditionally, acute VOCs were thought to be obstruction only of the capillary blood flow due to aggregation of deformed RBCs originating from the mutated sickle hemoglobin (HbS) polymerization (Manwani and Frenette, 2013). However, in the last two decades, evidences from *in vitro* and *in vivo* studies have demonstrated that the pathophysiology of VOC is complex and the orchestrating events are not limited to merely sickled RBCs aggregating and adhering to endothelium and blocking the blood flow (Kato et al., 2018). A myriad of inflammatory and adhesion activation mechanisms in concert with oxidative stress is inherent to the disease lead to VOC. Sickled RBCs are prone to damage and chronic hemolysis is characteristic feature of SCD. Hemolysis releases hemoglobin and free heme in the intra-vascular space. Extra-cellular hemoglobin consumes nitric oxide, thus, reducing the vascular tone and contributing to the oxidative stress; while free heme adds to the ongoing inflammatory milieu. Deformed RBCs exhibit adhesion molecules on its surface in addition to activation of adhesion molecules (e.g., ICAM-1, VCAM, P-selectin, E-selectin, etc.) on the vascular endothelium. In addition, sickle RBCs and activated endothelium promote sustained pro-inflammatory environment *in vivo*. Non-RBC cells such as platelets, neutrophils and natural killer cells remain activated and contribute to VOC in extreme situation by forming large aggregates with deformed RBCs. Sickle patients exhibit activated neutrophils and platelet-leukocyte aggregates in circulation (Zhang et al., 2016). Murine studies demonstrated that platelet-neutrophil aggregates may be aided by P-selectin (Polanowska-Grabowska et al., 2010), and recent clinical trials targeting P-selectin resulted in positive outcomes in reducing VOC events in sickle patients (Kutlar et al., 2019). Therefore, complex mechanisms underlying VOC extend beyond sickle RBC shape-induced blood flow obstruction and require delineation of molecular and cellular factors/events that involve non-RBC and non-HbS entities.

With the generation of transgenic mouse models of SCD (Berkeley and Townes mice) (Sagi et al., 2018), in the 1990s and early 2000s, the research on VOC relied on observing and quantifying microvascular stasis in dorsal skin venules using intravital microscopy (Kalambur et al., 2004). While *in vivo* studies provide an insight into complex RBC aggregation process or leucocyte rolling that facilitates VOC, the interactions of these cell types with the vascular endothelium depend on multiple factors – which cannot be discerned utilizing these *in vivo* studies. Dissecting roles of RBC deformation, flow shear, distinct cell–cell interactions, adhesion activation, endothelial permeability/dysfunction, immune activation, and converging/diverging vascular bed geometry are essential to fully understand pathophysiology underlying VOC and beyond in SCD in order to develop mechanism-driven targeted therapeutics. Over the last decade, microfluidics have risen to this occasion to enable sickle cell researchers to fabricate and employ simple devices (Horton, 2017) that emulate microvascular dimensions and/or blood cell-endothelial

interactions and facilitate *in vitro* experiments to study RBC sickling and VOC events.

Modeling VOC as RBC Flow Problem in Microvascular Geometry

Higgins et al. (2007) first demonstrated VOC can be recapitulated in microfluidic devices due to deformed RBCs jamming during flow in channels with dimensions both similar to and greater than the size of RBCs (**Figure 2A**). An important feature of the device was that the oxygen concentration could be varied using a gas chamber below the fluidic channel with permeable membrane in between. Occlusion initiation under the influence of low oxygen concentration was found to be a slow process (∼124 s), however, dissolution was a rather rapid event (∼22 s). The larger variability in time to jam larger vessels indicates a stochastic process suggesting that only extreme hypoxic conditions may lead to VOC events. While this study was simple in design and did not capitulate the complexities of cellular interactions and adhesions that contribute to VOC, this study demonstrated – for the first time – the feasibility of utilizing microfluidic devices for quantitative study of VOC (Higgins et al., 2007). Subsequently, several microfluidic devices have been developed and implemented to emulate VOC under physiologic oxygen gradients and identify biophysical markers or predictors of VOC (Wood et al., 2012; Du et al., 2015; Lu et al., 2017). Understanding the kinetics of sickling-unsickling under physiologic conditions and consequential effect on microvascular transit time have been a major drive behind these devices. Utilizing one such device, Wood et al. (2012) demonstrated that a rate of change of conductance of blood flow under constant pressure head but with diminishing oxygen concentration is observable and quantifiable for sickle blood while no such change is observed for blood from healthy or sickle trait individuals (Wood et al., 2012). Additionally, sickle blood treated with 5-hydroxymethyl furfural (5-HMF) exhibited significantly reduced rate of change of conductance compared to untreated sickle blood, demonstrating the utility of measurement of such biophysical markers in microfluidic devices to test small molecules that are potential candidates for sickle cell therapeutics (Wood et al., 2012). Additionally, in a modified version of the same device, the same group incorporated microvascular network that contains channels with dimensions of arterial vessels branching into channels resembling post-venule capillaries (Lu et al., 2017). Study using this device with limited sickle blood samples demonstrated that it is possible to have occlusion events at low oxygen tension in the channels upstream of venule-like capillaries. In addition, these results suggest that occlusion events can occur in the timescale of microvascular transit times. Furthermore, similar device was used to estimate sickle Hb polymer content as a function of RBC oxygen saturation collected from high throughput single cell imaging (**Figure 2B**) (Di Caprio et al., 2019). These observations are crucial for understanding the kinetics of VOC and studying how these events can be interrupted or even slowed down, if not completely stopped from occurring, to facilitate treatment of patients.

With a different approach than channelized flow, Du et al. (2015) investigated sickle RBC flow between micro-pillars with distance among themselves in the size range of RBCs. The motivation was to study the kinetics of sickling and individual RBC deformability under constant pressure and physiologic oxygenation/deoxygenation conditions. Importantly, they defined two parameters to observe sickling kinetics: (a) fraction of RBCs that are sickled at oxygen concentration <5% and (b) delay time (s) required from initiation of deoxygenation to appearance of the first cell taking sickle shape. The RBCs from patients on Hydroxyurea (HU) treatment exhibited significantly longer delay time (>25 s) compared to un-treated patient RBCs, suggesting a beneficial role of HU-treatment in slowing down the sickling process. For combined HU-treated and un-treated group, the sickled RBC fraction under deoxy condition was strongly correlated with the HbS percentage, and capillary obstruction by single RBCs between the micro-pillars exhibited similar correlation (**Figure 2C**) (Du et al., 2015). Such characterization of sickling-unsickling can potentially serve as aide for prognostic assessments and evaluation of therapeutic benefits in sickle cell clinic. Another study using microfluidic device incorporated much widely separated triangular micro-pillars with acute corners in the flow path of deoxygenated RBCs (Loiseau et al., 2015). While mixture of sickle and normal RBCs did not occlude the channels, the acute corners acted as sites of nucleation and deposition of sickle RBC aggregates. This supports the prominent sites of occlusion in sickle mice during hypoxia/reoxygenation being at venular bifurcations (Kalambur et al., 2004). Healthy RBCs did not produce aggregates. However, in presence of cell-free sickle hemoglobin (HbS), both sickle and healthy RBCs formed aggregates and such aggregation were less prominent in presence of only cell free heathy hemoglobin, thus suggesting a role of cell-free sickle HbS in activation of RBC membrane adhesion (Loiseau et al., 2015). As hemolysis-driven extra-cellular HbS is present in high concentration in sickle patients, free HbS may contribute to VOC via promoting RBC aggregation.

The above devices model VOC as an entrapment of deformed and aggregated RBCs due to a combination of shape change of RBCs and vascular bed geometry. They only offer the kinetics of sickling/unsickling as characteristic and predictive measure of VOC. However, such devices do not take into account the distinct or consorted effects of complex interactions among RBC deformation, flow shear and altered microrheology, sickle RBC-endothelial interactions, and inflammation induced endothelial adhesion-dysfunction which play crucial role in the orchestration of both acute crises and chronic inflammatory-vascular complications in SCD.

Devices to Study Sickle RBC Deformability, Blood Rheology, and Biomechanical Mechanisms of RBC Aggregation

Red blood cell deformability is the major biomechanical component that drives the micro and macro-rheological properties of blood (Ballas and Mohandas, 2004). Normal RBCs

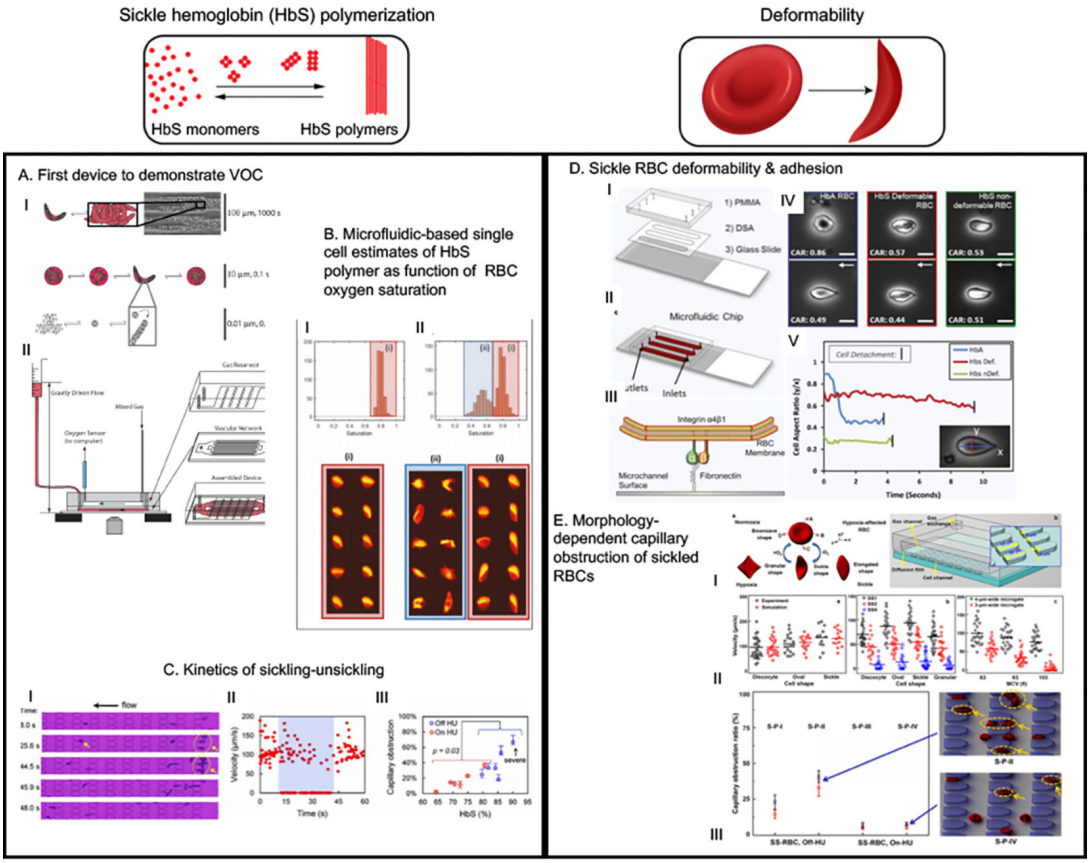

FIGURE 2 | The microfluidic devices in sickle cell research study major molecular and cellular events starting from sickle hemoglobin polymerization and consequent red blood deformability to hemolysis, inflammation, endothelial adhesion and vaso-occlusion (VOC). This figure shows some examples of application of microfluidics in studying HbS polymerization, VOC, and enhanced adhesion of sickled RBCs. **(A)** This was the first device that demonstrated hypoxia induced sickling alone could obstruct microvascular network and demonstrated utility of microfluidics for the study of vaso-occlusion (Horton, 2017). **(B)** The authors utilized high throughput microfluidic single RBC analysis to quantify sickle hemoglobin polymerization in terms of RBC oxygen saturation (Du et al., 2015). **(C)** Transient oxygenation-induced microfluidics-based quantitative single-cell analysis of sickle RBC to understand sickling-unsickling kinetics (Lu et al., 2017). Capillary obstruction percentage correlated with disease severity when hydroxyurea-treated and untreated patient samples were analyzed (III). **(D)** This device incorporated fibronectin-functionalized surface to assay the adhesion characteristics of sickle and normal RBCs (Dominical et al., 2015). They identified that two classes of sickled RBC exist: deformable and non-deformable; and non-deformable sickle RBCs have greater adhesion than deformable sickle RBC. This demonstrated direct correlation of deformation with adhesion (Dominical et al., 2015). **(E)** This study first demonstrated how microfluidics observation of hypoxia-induced morphology variation can help develop simulation-based models to predict experimental biorheological behavior of patient-specific RBCs (Nader et al., 2019). All images presented in this figure have been adapted, modified and/or re-used from original articles as cited. Proper permissions were obtained for the use of the published materials.

are highly deformable in nature owing to their discoid shape and extra surface area compared to a sphere with same cell volume (Mohandas and Chasis, 1993). Therefore, normal RBCs are able to traverse through capillaries that are of much smaller width than discoid-phase RBC width (∼8 μm). Sickle RBCs are stiff and less deformable than normal red cells. Apart from HbS polymerization under deoxygenation, altered cellular membrane properties (e.g., elasticity and viscosity) and increased cytosolic viscosity due to increased HbS concentration contribute to the reduced RBC deformability (Evans et al., 1984; Ballas and Mohandas, 2004). The HbS polymers exhibit bending moduli of about 1,600 more than the RBC membrane can withstand and four times greater than the outside blood pressure, thus making the deformation of sickle RBCs physiological (Di Caprio et al., 2019). Moreover, cyclic sickling-unsickling causes cell membrane damage. Damaged RBC membrane allows for high permeability of cations upon re-oxygenation and consequent over-hydration lead to increased volume-to-surface area ratio which makes the RBCs further rigid (Barabino et al., 2010). From the fluid mechanics point of view, rheology of whole blood (a highly non-Newtonian fluid) in post-capillary venules is largely influenced by RBC aggregation and deformability (Ballas and Mohandas, 2004). Sickle blood viscosity is higher compared to normal blood and distinct characteristics of sickle blood rheology originates due to the contribution from the heterogeneous mixture of RBC subpopulations in the flow even in the oxygenated condition. In an early effort to characterize rheological behavior of sickle RBC suspensions, Kaul et al. (1983)

fractionated sickle RBCs in four sub-populations in terms of their constituent RBC morphology and incremental density: I = least dense reticulocytes, II = discocytes, III = highly dense discocytes, and IV = most dense irreversibly sickled cells (ISCs). Upon deoxygenation, all fractions exhibited similar increase in the levels of viscosity in the bulk viscosity measurements at high shear. However, hemodynamic experiments demonstrate that the peripheral resistance were dramatically increased in the fraction III and IV compared to less dense sub-populations I and II. The significance of this finding lies in the understanding that high and low shear flow may have distinct effects on the RBC aggregation behavior and cumulative resistance achieved in thereafter.

Endothelial adhesion of sickle RBCs is another contributing factor to abnormal blood rheology and blood flow obstruction in SCD. Hoover et al. (1979); Hebbel et al. (1980), and Hebbel (1992) demonstrated *in vitro* that abnormal adhesive interaction of sickle RBCs with the endothelium is present in SCD. Mohandas and Evans (1984) suggested that such adhesion is predominant in the less dense and more deformable RBC population (vs. ISCs), possibly due to greater contact area. This was confirmed by Barabino et al. (1987) by parallel plate flow chamber experiment where the least dense reticulocyte fractions displayed the greatest adhesion to the human endothelial cells. Flowing oxygenated sickle RBCs on a microvascular bed *ex vivo* demonstrated that post-capillary venules are the sites of adhesion of most sickle cells and *in vivo* studies using transgenic sickle mice confirmed similar deposition in post-capillary venules (Kaul et al., 1989b, 1995; Embury et al., 1999). *Ex vivo* observations demonstrated an absence of dense RBC population at sites where there were only adherent cells but no VOC, while at VOC sites a high percentage of dense and ISCs were seen. Based on all these, Kaul et al. (1989a) proposed a 2-step model of VOC. At first, the less dense and deformable sickle cells adhere to the vascular bed making the passage of blood slower. This event promotes hypoxia and consequent jamming of dense and ISCs in the post-capillary venules (which may also have some percentage of deformable adherent cells). However, later it was discovered that leukocyte-mediated adhesion interactions also contribute to the pathogenesis of VOC (Turhan et al., 2002) – discussed later in the review.

Abnormal sickle blood rheology have been studied extensively (Nader et al., 2019). Traditional viscometry, filtration, ektacytometry, micropipette aspiration, atomic force microscopy (AFM), and optical tweezer methods have always been used to measure the rheological properties of blood, RBC deformability, and mechanical and adhesion (with endothelial cells) properties of RBC membranes in isolation from flow (Barabino et al., 2010). However, the earliest example of device mimicking physiological blood flow that quantified the sickle blood rheology was parallel-plate-chamber (Barabino et al., 1987). Recently, sickle researchers have initiated using microfluidics to quantify and characterize both individual cellular biomechanics induced by reduced deformability and collective rheological behavior of sickle RBCs in flow. In an elegant work, Li et al. (2017) characterized individual RBC biomechanics in terms of flow-ability under transient hypoxic conditions in a microfluidic device with capillary channel that contains 15-μm-long, 4-μm-wide, and 5-μm-high micro-pillars as periodic obstacles to the flow (**Figure 2E**). The RBC samples were density-fractionated in similar fashion as Kaul et al. (1983) did previously. The dense and rigid RBCs (III and IV type) were individually blocked in the microgates during transient hypoxia, but less dense and deformable cells found ways to move forward during the flow via changing direction at the face of obstruction. The traversal velocities of density-fractionated cells were interestingly found to be shape-dependent and more surprisingly, the granular-shaped cells showed less velocity than classical sickle-shaped cells. This unexpected observation was also preserved within the density fractions, thus indicating a within-fraction bio-rheology variability to be present in sickle microenvironment. To test the applicability of this device as a patient-specific prognostic monitoring tool, the authors measured capillary obstruction ratio (= total trapped cells/total cells in channel) for samples from patients with and without hydroxyurea treatment. As expected, HU-treated samples showed significantly less capillary obstruction ratio compared to un-treated samples. All these experiments were also simulated with multi-scale RBC model where RBC membrane elasticity and transient hypoxic conditions were factored in a dissipative particle dynamics model. The simulation results were in excellent agreement with the experimental results, thus establishing this framework as a means of characterizing dynamic behavior of patient-specific individual RBCs under transient hypoxic conditions. Several microfluidic devices studying the bio-rheological properties have been conceived in the last few years, as they are tabulated in **Supplementary Table 1**.

Another simple microfluidic chip (Lizarralde Iragorri et al., 2018) was recently developed to study the effect of exerting mechanical stress on single sickle red cells (**Figure 3A**). The device was built with parallel channels that had gradually narrowing walls to squeeze individual cells in flow. The samples were collected at the outlet and then analyzed for quantification of the extra-cellular free hemoglobin as an indicator of RBC lysis under exerted mechanical stress during flow. The results indicated that successive mechanical and increased stress on sickle RBCs can contribute to lysis. A significant observation was that the high-density fraction containing high percentage of ISCs were about 2-fold more susceptible to lysis under such stress compared to low density fraction RBCs from the same sickle blood samples, indicating that less deformable RBCs are more prone to hemolysis under recurrent mechanical stress (Lizarralde Iragorri et al., 2018). Induction of fetal hemoglobin (HbF) can reduce intra-cellular concentration of HbS and thus can reduce sickle RBC sickling (Akinsheye et al., 2011). Analysis of percentage of HbF-positive cells before and after flowing the samples through this microfluidic device indicated the HbF-positive cells were better protected from lysis than HbF-negative cells (Lizarralde Iragorri et al., 2018). More importantly, low-density sickle RBC fractions contained much higher percentage of HbF-positive cells, indicating that higher HbF concentration intra-RBC may restrain RBC density increase, thereby enabling higher deformability and consequently protection from hemolysis. These results demonstrate the

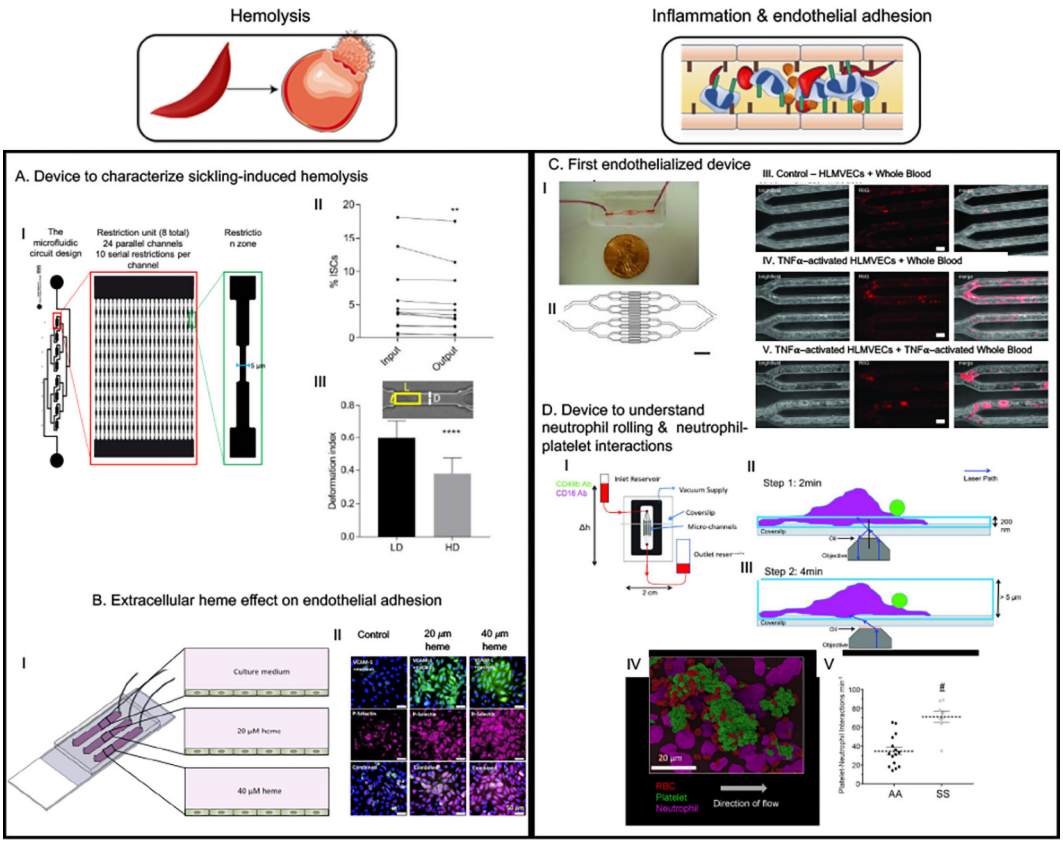

FIGURE 3 | The microfluidic devices in sickle cell research study major molecular and cellular events starting from sickle hemoglobin polymerization and consequent red blood deformability to hemolysis, inflammation, endothelial adhesion and vaso-occlusion (VOC). This figure shows some examples of application of microfluidics in studying hemolysis and inflammation-induced enhanced adhesion of sickled RBCs. **(A)** This device was to demonstrate that less deformable fraction of sickle RBCs are prone to hemolysis when subjected to repeated mechanical stress (Li et al., 2017). **(B)** This device is an endothelialized device where endothelium was activated with free heme simulating hemolysis-driven scenario; higher heme concentration caused more adhesion of RBCs (Mannino et al., 2015). **(C)** "Do-it-yourself" endothelialized device demonstrated that simple off-the-shelf materials can be used to make microfluidic devices to study endothelial adhesion in different geometries relevant to vascular disorders. An important observation was sickle RBCs were more adherent at the bifurcation than in the straight channels and no control RBCs were adhesive at any of the geometry. However, simulating endothelial cells with TNFα did not increase adhesion – indicating a major role of sickled RBCs in adhesion phenomenon (Tsai et al., 2012). **(D)** This device is p-selectin, ICAM-1, and IL-8 -coated to observe neutrophil-rolling and neutrophil-platelet interactions and demonstrated that such interactions are elevated in sickle blood (Embury et al., 2004; Tajima et al., 2009). All images presented in this figure have been adapted, modified and/or reproduced from original articles as cited. All necessary permissions were obtained for the use of the published materials from respective journals.

utility of simple microfluidic devices to infer deformability and biomechanical properties of sickle red cells to correlate with their rheological properties in relation to factors that govern sickling and consequently contribute to vascular pathology.

Microfluidics to Study Adhesion: Endothelialized and Protein-Functionalized Microfluidics for Cell–Cell Interactions

Adhesion of RBCs to the vascular endothelium is an essential pre-cursor to facilitating VOC *in vivo*, however, such interactions are not only mediated by expression of adhesion proteins on activated RBCs and endothelial cells, but also through complex cell–cell interactions among RBCs, leukocytes, platelets and endothelium (Manwani and Frenette, 2013). There have been development of two major approaches in modifying the channel beds of microfluidic devices to delineate the role of interactions among sickle RBCs, endothelium, WBCs, platelets and immune cells in the pathobiology of SCD: (1) using endothelialized channels, and (2) using adhesion molecule functionalized channels. We will here describe the general features, utility, outcomes and promises that both of these types of devices hold.

Endothelialized Devices

Endothelialization refers to forming a 3-dimensional (3D) endothelial monolayer inside a microchannel to investigate endothelial and other cell interactions mimicking physiologically relevant processes (**Figure 3B**) (Mannino et al., 2018). The endothelial monolayer is allowed to form after absorption of one of the extracellular matrix (ECM) proteins such as collagen, fibronectin (FN) or laminin (LN) that increases

the adhesivity of endothelial cells to the microfluidics device material (Myers et al., 2012). These devices help to recapitulate close to physiological interactions between the systemic RBCs, leukocytes and platelets, and in combination with correct microphysiological dimensions can very closely mimic the capillary or arterial microenvironment (Mannino et al., 2018). SCD is characterized by endothelial adhesion and interactions between endothelium, RBC, leukocytes and platelets, and utilizing endothelialized devices offer the best way to characterize such interactions. Lam lab produced the first endothelialized device with lung microvascular endothelial cell-seeded channels resembling post-capillary venule and arterioles and utilized tumor necrosis alpha (TNFα)-induced endothelial adhesion to demonstrate leukocyte adhesion-induced obstruction/decrease of whole blood flow (Tsai et al., 2011). The rate of blood flow further decreased when the leukocytes were primed with TNFα, thus indicating a role of inflammation in vascular stasis. They used this device to demonstrated that whole blood from patients untreated with HU were able to clog up to 60% of microvascular channels even when fully oxygenated whole blood from HU-treated sickle patients only obstructed up to 4–5% of the network – thus providing evidence that HU-treatment reduces whole blood adhesion or viscosity which possibly provides the treatment benefit (Myers et al., 2012; Tsai et al., 2012). Interestingly, a "do-it-yourself" version of the device with modified channel geometry to add bifurcations showed enhanced RBC aggregation at the bifurcations (**Figure 3C**) (Mannino et al., 2015), however, TNFα-induced endothelial activation had minimal contribution and sickle RBCs were sufficient to induce such aggregation – demonstrating utility of this device to quantitatively define mediators of VOCs. Another device with much higher endothelialized surface area of 32 mm^2 (vs. 0.1 mm^2 in other devices) (Mannino et al., 2015) was used to investigate the adhesion characteristics of sickle RBCs when the endothelial monolayer is pre-treated with micromolar concentrations of heme, as hemolysis-driven intravascular heme is known to activate endothelium and to enhance p-selectin-mediated occlusion. Notable findings from this study were twofold: (1) heme induced adhesion and loss of deformability in a concentration-dependent manner, and (2) when patient samples were clustered based on lactate dehydrogenase (LDH) and reticulocytes – the significantly increased adhesion and decreased deformability in response to heme treatment of endothelial monolayer (also in a concentration-dependent manner) were observed for the patients with high LDH and high reticulocyte counts – indicating a possible hemolysis-dependent clinical and biophysical phenotype segregation among these patients (Kucukal et al., 2018a). Thus, endothelialized microdevices offer more relevant microenvironment to study pathophysiological processes compared to microvascular networks without endothelial monolayers – via simulating characteristic interactions among intravascular cellular entities.

Protein-Coated Surface Microfluidics

Functionalized with extracellular (ECM) proteins

Commercially available microfluidic devices utilized fibrinogen and collagen functionalized surfaces for cellular adhesion assays, which are being used in sickle research (Proença-Ferreira et al., 2014; Dominical et al., 2015), and similar devices are being developed in academic settings. Plasma FN and endothelial FN are mediators of adhesion between the endothelium and RBCs/neutrophils through the receptor $\alpha_4\beta_1$. LN is another ECM protein that is also implicated in adhesion process in SCD. Alapan et al. (2014) first demonstrated the utility of FN-treated functionalized microfluidic device to quantitatively describe RBC adhesion and deformability under physiological flow velocities and post-capillary vessel size. Image-based single cell analysis enabled classification of sickle RBCs based on their deformability defined by the aspect ratio change under flow (and also during detachment from adhesive contact) into two classes: deformable and non-deformable. Deformable sickle RBC had significantly lower deformability during flow and at detachment. A field of view analysis of RBCs to find out adherence of RBCs during flow found that the non-deformable sickle RBCs had multiple adhesion sites while deformable sickle RBC had a single adhesion site (**Figure 2D**) (Alapan et al., 2014). Alapan et al. later improved the algorithm to define the deformability with a parameter dynamic deformability index which is basically the computed rate of change of cell aspect ratio under flow till the time when the cell is detached from the adhesion site. A noteworthy finding was that even at high flow above physiological velocities, there were significantly different non-deformable sickle RBCs compared to deformable sickle RBCs adhered to the surface. This is of significance because while a relationship between RBC deformability and adhesion was always thought to originate from the resistance due to deformed aggregates or the activation of adhesion molecules on endothelium, these data for the first time implicate a direct correlation between these two in a more cause-and-effect manner (Alapan et al., 2016c). Finally, the group implemented SCD biochip, an FN or LN-coated RBC adhesion assay device, to assess over one hundred sickle patient sample and demonstrated that the assay had high sensitivity and accuracy in differentiating hemoglobin phenotypes in addition to finding correlation of clinical parameters such as HbS percentage or LDH with number of adhered cells under different flow conditions and different cell subtypes (non-deformable adherent cells) (Alapan et al., 2016b). Modification of SCD biochip has led to user-friendly characterization of hypoxia-induced (Alapan et al., 2016b) or shear-dependent (Kucukal et al., 2018b) sickle RBC adhesion. In one such study, RBCs from sickle male patients with history of priapism exhibited significantly higher adhesion activity compared to RBC from sickle male patients without history of priapism under hypoxia but not with ambient oxygen concentration (Yuan et al., 2019) – implicating the usefulness of the device in revealing clinical phenotypes or biophysical properties not characterized in traditional non-microfluidic adhesion assays.

Recently, another remarkable study using FN-coated microfluidics device presented – for the first time – quantitative characterization of concurrent adhesion mechanics and HbS polymerization kinetics of sickle RBCs with different deformability under controlled hypoxia (Papageorgiou et al.,

2018). The flow shear stress was varied within 0.035–0.085 Pa to allow for sickle RBC adhesion to the FN-functionalized surface of microfluidics device – for the ability to observe the adhesion mechanics in real-time. This microfluidics assay demonstrated that sickle RBCs are more prone to adhesion under hypoxia than normoxia and adhesion propensity is in the following order: highly deformable reticulocytes > deformable discocytes > less deformable discocytes > ISCs. The steps of adhesion dynamics under low shear and hypoxia are as follows:

1. single site adhesion of the sickled cells to FN-coated surface occurs first,
2. the cell flips against single adhesion site to align with the direction of the flow and oscillates with the flow,
3. incremental HbS polymer growth facilitates generation of more adhesion sites via shape change and stops the oscillation of the cell making it permanently adhered.

Further analysis of the discocytes and reticulocyte adhesion phenomena revealed that there is a significant delay between the initial single-site adhesion and final morphological change to sickle shape. The adhesion increases the residence time of the cells under hypoxia which in turn promotes HbS polymerization and consequent shape change-mediated adhesion site increase. Thus, these results demonstrate a mutually synergistic relation between adhesion and HbS polymerization during hypoxia (Papageorgiou et al., 2018). Another important observation is that the most deformable reticulocytes (which are also the most adhesive ones) promote adhesion via HbS polymer-induced protrusions. These outward protrusions to initial membrane/cell boundary causes increase of cell-to-surface contact area thus facilitating enhanced adhesion (vs. mature discocytes and ISCs) possibly due to lower dissociation energy barrier of the lipid bilayer-cytoskeleton of the immature reticulocytes. In an extension of this study, Deng et al. studied the detachment dynamics of the discocytes and ISCs under hypoxia by increasing the shear stress (via changing flow rates of pulsatile flow) up to the point where the single adherent cell detaches from the FN-coated surface (Nader et al., 2019). These critical shear stress required for detachment of single cells represent the adhesion strength and results from the study indicated that the adhesion strength of discocytes were much greater than those of ISCs, thus complementing the prior results of adhesion likeliness being in the same order. Both the studies also incorporated numerical simulations which corroborated the experimental results (Papageorgiou et al., 2018; Deng et al., 2019). Such numerical modeling would be incomprehensible without the leverage of microfluidics to extract certain critical parameters.

Carden et al. (2017) recently utilized both non-endothelialized hypoxia-enabled and endothelialized devices to investigate the effect of infusing intravenous fluids (IVFs) with different tonicities. This approach enabled them to separately determine the effect of IVFs with different tonicity on deformability under normoxic and hypoxic conditions in the former device and also estimate the effect on adhesion using the endothelialized device. This study highlights the relevant advantages of different types of microphysiological devices in the context of decoupling adhesion mechanics and studying deformability. More importantly, they were able to extract an optimum tonicity from these measurements which balances the biophysical changes of RBC and adhesion characteristics, thus indicating translational potential for such approach (Carden et al., 2017).

Adhesion and chemoattractant protein-coated surfaces

Adhesion interaction in SCD is not limited to endothelium and blood cells, rather RBC, platelets and neutrophils also mediate intercellular interactions between themselves to form aggregates in flow (Telen, 2014). Such interactions are mediated primarily by P-selectin that is expressed on endothelial cells and platelets in response to inflammation in SCD (Embury et al., 2004; Polanowska-Grabowska et al., 2010). Neutrophil-platelet-RBC aggregates have been demonstrated to be involved in ACS – a form of acute lung injury in the event of VOC in the lung vasculature (Bennewitz et al., 2017), and Sundd lab utilized a unique microfluidic device functionalized with immobilized p-selectin, intercellular adhesion molecule 1 (ICAM-1) and interleukin 8 (IL-8) (Bennewitz et al., 2014, 2017; Jimenez et al., 2017; Vats et al., 2019) to delineate aspects of neutrophil-platelet interactions in forming such aggregates. ICAM-1 is a major adhesion protein expressed on endothelium responsible for leucocyte-endothelial interaction (Kotteas et al., 2014) and IL-8 is a chemoattractant for the neutrophils (Tajima et al., 2009). Thus, this approach captures the major endothelial interactions with neutrophil and platelet without having the need to using endothelialization approach and provides a better visibility toward specific interactions – as has been demonstrated by the initial study (**Figure 3D**) (Jimenez et al., 2015). Using this approach, the group first demonstrated that P-selectin-mediated neutrophil-platelet interaction is a feature of lung VOC (Bennewitz et al., 2017) and recently, they demonstrated that platelet-derived exosomes that carry IL-1β and caspase-1 can contribute to neutrophil-platelet aggregation, consequently causing lung VOC *in vivo* (Vats et al., 2019). A commercially available E-selectin/ICAM-1-coated microdevice with gradually decreasing width demonstrated that sickle neutrophils were more adherent and occlusive than healthy neutrophils. In the same study sickle RBCs alone were not sufficient to obstruct LN-coated channels and such obstruction was only facilitated by addition of sickle neutrophils to the sickle RBC solution (Dominical et al., 2015). This approach of chemoattractant and adhesive protein functionalized microfluidics, thus, have been successful in validating mediators of VOC *in vitro* and to reveal detailed cell–cell interactions underlying inflammation and its contribution to occlusive adhesion in SCD.

A unique microfluidics-based well-plate platform (White et al., 2015) that utilizes vascular cell adhesion molecule 1 (VCAM-1) for functionalization of the well-plate and pulsatile flow for the assay demonstrated significant adhesion variation in adhesion characteristics of adherent RBCs during pulsatile flow compared to continuous flow. As physiologically blood is pumped in a pulsatile manner, such approaches could enable more appropriate quantification of adhesion characteristics of sickle RBCs. Indeed, this approach proved to be useful in

observing effects of novel drugs to reduce sickle RBC adhesion (White et al., 2016; Lancelot et al., 2017).

CURRENT CHALLENGES AND PROMISING MICROFLUIDICS TO BETTER UNDERSTAND SCD: A PERSPECTIVE FROM ADDRESSING COMPLEX BIOLOGY

While there has been tremendous progress in the field of sickle cell microfluidics during recent years, majority of the studies focus on understanding or evaluating the adhesion mechanics and biomechanics of vaso-occlusion, in addition to vascular cell–cell interactions. Some technologies such as adhesion protein-coated adhesion assay chips are commercially available and there are devices already developed by several engineering groups to tackle such problems. However, it is still a challenge use microfluidic technology to mimic complex biological systems or problems such as SCD. Consequentially, the usage of complex vasculature systems or organ-on-chip systems have not evident in sickle research field. The microenvironment for mimicking *in vivo* conditions requires elaborated engineering solutions. Moreover, protocols for automated quantification, involving a huge amount of data acquisition requires the development of software and data processing. In this sense, these systems are still under development and it is not a plug-and-play technology for biologists. Complex microdevices require experience major experience in developing them – which is probably why there have been only about 44 articles published over more than a decade of sickle cell microfluidics research. Hence, it is understandable that many of microfluidic technologies that have been availed at different disease states, they have not been yet introduced in sickle cell field.

Additionally, many different microdevices with complexity relevant to sickle microenvironment have been used in other disease states, the lack of standardization of outcomes that is relevant to SCD is absent. For example, while concentration gradient microfluidics have been in use for some time, the absence of standardized characteristics of assessing the outcomes of vascular adhesion or other sickle microenvironmental parameters have made such technologies inapplicable so far. Another major hindrance of progress in sickle microfluidics research has been of less involvement of engineers in sickle research field. While in recent years, few engineers have been involved in fundamentally addressing sickle research using microfluidics technology, the long-standing disconnect of the sickle researchers from physician and biologist community with the engineers have yet to be dissolved. With increasing interaction and collaboration between engineers, biologists and clinicians, sickle research field is currently observing a greater progress toward standardizing some adhesion assays. A characteristic feature of SCD is its heterogeneity and clinical variability, which requires any microfluidic technologies to be validated with hundreds of patient samples – which are not easy to obtain. These limitations pose intrinsic obstacles in the rapid development, standardization and ubiquitous implementation of novel microfluidic devices that are possibly already in use in other research fields.

There are critical needs of devices that can recapitulate the sickle microenvironment not only in terms of vascular endothelial beds but also the interaction with non-vascular heterotypic cells such as neuronal or tissue-resident immune cells. Additionally, it is important to capture the complexity of organ systems that are affected by sickle red cells and inflammatory hemolytic microenvironment. Such interactions can only be recapitulated in microfluidics-based organ-on-chip systems – which could be useful to screen drug compounds and their effects on end-organ damages. Such devices have not been so far utilized or incorporated in SCD research field. Additionally, no standardized platforms exist to screen drug compounds for their efficacy. There are multiple research groups are studying small molecule effects on endothelialized system – however, the translatability of such devices into screening different concentrations of drug molecules or synergistic effectiveness of multiple drugs are still absent. Owing to the versatility of microfluidics, current advancement in modular composite fluidic systems, and most importantly, reflecting on the complexity of sickle microenvironment, organ-specific sickle complications and high patient-specific variability in clinical prognosis, we anticipate and emphasize the need for novel and emerging areas of microfluidics-based technology development for: (1) studying exosome-mediated interactions between endothelium and non-systemic cell immune cells, (2) organ-on-chip platform, and (3) parallelized therapeutic testing platforms – all in the context of sickle research.

Need for Novel Microfluidics for Studying Exosome-Mediated Vascular-Immune Interactions in Sickle Microenvironment

Distal homo- and heterotypic cell–cell interactions are often mediated via exosomes (Patil et al., 2019), and recently circulating exosomes have garnered attention as a mediator of sickle pathobiology such as endothelial dysfunction (Khalyfa et al., 2016; Lapping-Carr et al., 2017). Exosomes are extracellular nanovesicles released by numerous cell types into biological fluids and known to be involved regulatory physiological as well as in pathophysiological processes, modifying the functional phenotype of target cells (Patil et al., 2019). While circulating microparticles have been investigated in SCD due to their relevance (depending on source) vascular injuries, reticulocytosis, endothelial activation, endothelial adhesion and platelet activation i.e., the major features of SCD (Hebbel and Key, 2016), only very recently, micro-RNAs from plasma-derived exosomes from a set of children with diverse set of SCD-induced complications have been shown to display a molecular signature reflective of disease severity (Khalyfa et al., 2016). Subsequent analysis of exosomes from sickle children with ACS+ and without ACS- demonstrated that the exosomes from ACS + children could alter endothelial integrity and had induced eNOS expression in these cells, more than exosomes from ACS-children (Lapping-Carr et al., 2017).

The endothelial permeability measurements were done with electrical cell impedance sensors (ECIS). While ECIS is an appropriate tool for such measurements, direct observation of exosome uptake by endothelial cells was absent in this study (Lapping-Carr et al., 2017). Also, in general, how non-systemic and endothelial cells interact in sickle microenvironment is unknown. SCD being a vascular disease with complex vascular-immune and neuro-immune interactions (Aich et al., 2019), it is essential to understand the complex interplay mechanistically for developing targeted therapeutics. For example, mast cell activation contributes to sickle pathobiology (Vincent et al., 2013) and vascular dysfunction (Tran et al., 2019). However, how mast cells and endothelial cells interact in sickle microenvironment is unknown. Mast cell-derived exosomes have been shown to activate endothelial cells to secrete plasminogen activator inhibitor-1 (PAI-1) (Khalid et al., 2005). Elevated levels of PAI-1 have been found in steady state SCD patients (Hagger et al., 1995) which further increases during VOCs (Nsiri et al., 1997). Therefore, complex heterotypic cell–cell interaction networks via exosomes is conceivable in the scope of SCD, investigation of which is warranted. Microfluidics-based approach can help to decipher molecular mechanisms of exosome-mediated intercellular interactions in sickle microenvironment with an added advantage of direct observation of kinetics of the process – due to ease of being able to membrane-separated chambers for co-culture setups. Thus, microfluidic devices that contain engineered cells that produce exosomes in one chamber and another chamber containing cells that uptakes the transferred exosomes through a permeable membrane that separates the two chambers may help to delineate role of exosomes in modulating endothelial dysfunction and/or other heterotypic inter-cellular interactions in the context of SCD. Additionally, such cell–cell interaction device also offers modeling opportunities for studying blood flow-induced endothelial exosome-secretion and its consequential effects on surrounding microenvironment. We envision that this type of devices to study heterotypic cell–cell interactions and their modifications to simulate sickle microenvironment will offer exciting and innovative approaches to elucidate novel molecular and cellular processes and mediators in SCD.

Organ-on-Chip Devices to Study Organ-Specific Complications in SCD

Acute VOC pain crisis is the major feature of SCD, and all currently FDA-approved drugs were approved based on the outcome of reduction of VOC events. However, despite organ-specific complications such as stroke in brain, ACS and pulmonary hypertension in the lung, splenomegaly and auto-splenectomy, kidney-renal complications, leg ulcers etc. contributing to life-threatening and life-disabling aspects of SCD – independent of VOC (**Figure 4**), such complication-specific sickle therapeutics is unavailable due to limited understanding of underlying mechanisms. While current microfluidics devices used in sickle research field can correlate RBC deformation and blood cell-endothelial adhesion well with some clinical parameters to some degree, none captures the organ-specific complex tissue-vasculature interactions and are, therefore, not suitable for studies of organ-specific complications. Integrated microsystems composed of various cell types and extra-cellular matrix and designed with capacity to mimic mechanical, biochemical and functional properties similar to *in vivo* microenvironment are known as organ-on-chip platforms. Organ-on-chip platforms are complex microfluidic systems that feature the physiologically relevant complexity of human biology and can provide more intricate interactions from integration of tissue matrix, vasculature, muscles and other organ-specific cells/tissues in addition to off-chip/on-chip perfusion systems and/or micromachined self-actuating or electro-activated mechanical valves/filtration systems (Zhang B. et al., 2018). Organ-on-chip devices can provide more holistic idea of effects originating from a drug or interventional treatment.

While transgenic sickle mouse models are available that express sickle human hemoglobin (with transgenic control mouse expressing normal human hemoglobin) which recapitulate many features of SCD such as RBC sickling, RBC-leukocyte aggregated-mediated vaso-occlusion, reticulocytosis, hemolysis, inflammation, and acute/chronic pain (Sagi et al., 2018). However, rodent models do not always capture the variety of organ-specific complications. At the same time many drugs that are effective in mouse models, they do not translate into effective therapeutics once tried in humans (Justice and Dhillon, 2016). Additionally, *in vitro* studies with single/two-cell systems do not capture the complexities of human tissue interactions with systemic environment, immune cell responses and other molecular/cellular mediators of complex microenvironment. For example, Rivipansel, a pan-selectin antagonist (selectins are mediators of endothelial adhesion in SCD), demonstrated efficacy in transgenic sickle mouse and endothelial adhesion assay;(Chang et al., 2010) however, the drug failed to demonstrate efficacy in phase III clinical trial in reducing acute VOC crisis events and required opioid usage in sickle children of age 6–11 years (Morris et al., 2013; Telen et al., 2015; Ballas, 2020). While resources are often scarce and disparate for sickle cell research (Lee et al., 2019), such loss of resources in terms of millions of dollars and time of scientists in a path of ineffective drug development adds to the burden of managing this disease. Organ-on-chip platforms may offer faster and less resource-intensive outcome measures of physiologic relevance before the preclinical studies. Since the first lung-on-chip system in 2010 (Huh et al., 2010), major improvements have been made in the field of tissue engineering and microtechnology to build different organ systems such as brain (Bang et al., 2019), lung (Nawroth et al., 2019), kidney (Hagger et al., 1995), spleen (Rigat-Brugarolas et al., 2014; Picot et al., 2015), neural systems (Huval et al., 2015; Pamies et al., 2017; Sharma et al., 2019), and skin (Zhang Q. et al., 2018) among many. The emergence of 3D bioprinting have also been conducive to rapid development of organ-on-chip systems (Miri et al., 2019).

Another aspect of utility of organ-on-chip platforms is addressing the discrepancy and dissimilarities in the form of pathophysiological, genetic and translational landscape for a specific biological event. For example, SCD is a hemolytic

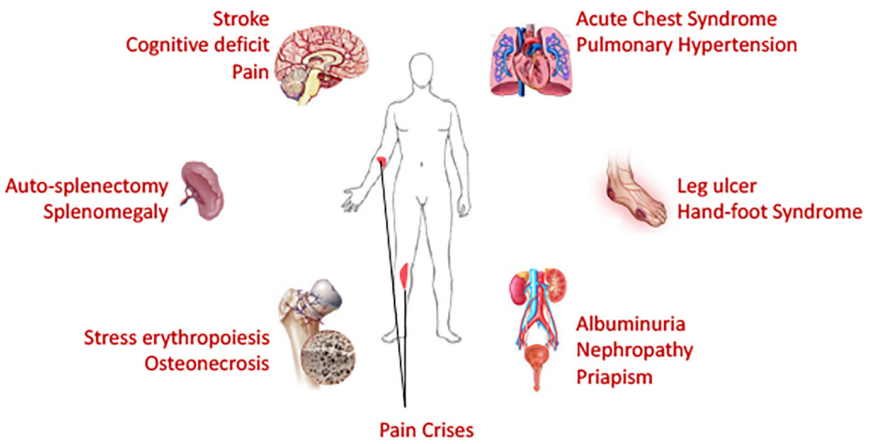

FIGURE 4 | Necessity of organ-on-chip devices: Being a blood disorder with hyperinflammatory and hypercoagulative state, sickle cell disease affects many organs of the body. Chronic and ongoing insults and injuries result in end organ damages. Life threatening acute complications affect brain, lung and spleen; while chronic deleterious effects are observed in many organs. Organ-on-chip devices, if utilized, can provide a holistic view on the macroscopic sickle microenvironment and should facilitate evaluation of efficacy of therapeutic interventions in organ-specific manner. Patient-specific modeling can also be performed via development of organ-on-chip systems from patient-derived stem cells, thus paving the path toward personalized medicine.

anemia and consequent stress erythropoiesis is a major feature of the disease (Zivot et al., 2018). However, erythroid systems respond differently at the molecular and cellular levels in human and mouse (Telen, 2014). While understanding the differences may offer some relief in studying the stress erythropoiesis in transgenic mouse, the inaccessibility of bone marrow coupled with the human/mouse differences makes it difficult to study such processes in real-time. Bone-marrow-on-chip devices using ECM and myeloid cells in concert with vascularized channels (Chou et al., 2020) or human hematopoietic stem cell derived complex 3D microsystems resembling bone-marrow microenvironment (Sieber et al., 2018) may offer insights into mechanisms of the erythropoiesis dysfunction and therapeutic benefits of transfusion or drugs on easing the burden on the hematopoietic niche in SCD. Additionally, such devices will offer benefits to prognostic observation of disease progression at the organ level, which studies are very difficult to perform in humans or mice. Similar on-chip platforms of brain, lung, spleen, skin, peripheral and central nervous system, and kidney with vascularized system are suitable for studies at the microphysiological levels to understand organ-specific comorbidities and disabilities in SCD, such as stroke, ACS, splenomegaly, leg ulcer, pain, renal complications and many more. These devices also enable testing of drug safety, toxicity, efficacy, and relevant biomarker validation in a tissue/organ-specific manner and can provide insights into effects of mono/combination therapeutics (Nawroth et al., 2019). Additionally, organ-on-chip platforms made from patient-specific stem cells can provide insights into the clinical variability and patient-specific therapeutic response, thus, offering a more precision and personalized medicine approach for sickle patients. Finally, body-on-chip devices (Sung et al., 2019) can help with understanding how sickle drugs targeted to reducing HbS polymerization or endothelial adhesion might effect differently at organ-specific level and can provide insights to design combination therapeutics to enhance the treatment outcome (Herland et al., 2020; Novak et al., 2020).

Emerging Concentration-Gradient Microfluidics for Testing Novel Drugs and Dosage Comparison

Novel drug development requires high-throughput screening of the compounds for their efficacy and also once identified, dosage and concentration identification is a major challenge. Dosage evaluations primarily depend on pre-clinical mouse studies or phase I clinical trials – which is time and resource-intensive. The estimated cost to develop a clinically approved drug is 2.5 billion dollars (DiMasi et al., 2016). However, two thirds of the total costs for drug development are spent in the initial research phases (Paul et al., 2010). Also, we have discussed that development of Rivipansel, an anti-adhesion molecule, for a decade as sickle cell drug failed in the phase III clinical trials. Despite renewed interest at the national/international policy level and among pharmaceutical industries for developing sickle drugs, the long history of disparate funding for sickle research (Lee et al., 2019) has limited the identification of drug targets and their subsequent development. Thus, it is imperative to develop novel strategies to speed up and decrease costs for drug screening by eliminating drug candidates as early as possible in the approval process is beneficial, since to fail early is to fail cheaply (Zhang and Radisic, 2017). Microfluidics offers high throughput screening of drug compounds owing to ease of observing cellular behavior, compartmentalization, parallelization and generating concentration gradients. Concentration gradients have been investigated in different microchips applications and since different conditions can be investigated at the same experiment including replicates, resulting in high throughput screening options (Atencia et al., 2009; Cimetta et al., 2010; Wang et al., 2017; Regnault et al., 2018). These microchips are designed to

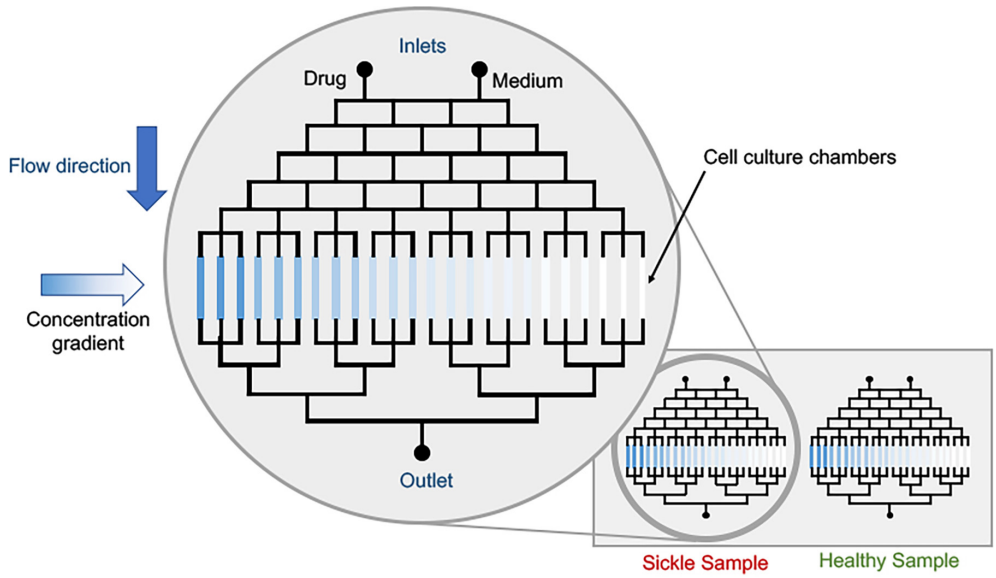

FIGURE 5 | Conceptual schematic of drug dosage screening device. The cell culture platforms can be the current state of sickle microfluidic devices that can measure deformability, adhesion or other characteristic parameters pertaining to sickle pathobiology. Parallelization of healthy and sickle samples is possible via multiplexing.

manipulate fluids in a way where the reagent concentration is known in space and time.

Different concentration gradients can be achieved in different mixing systems: convection and diffusion-based (Oliveira et al., 2016; Regnault et al., 2018; Vit et al., 2019). Convection-based concentration gradients are formed via laminar flow mixing. In this case, the contact between two streams with different concentrations promotes solute mixing and depending on the geometry, a linear concentration gradient can be generated, and diffusion-based concentration gradients have no net flow and mass transport occurs only via diffusion (Regnault et al., 2018). These different strategies to generate concentration gradients, that can vary from linear to exponential ones (Wang et al., 2017) and the design can be selected according to the research purpose. In sickle research context, the use of already developed microfluidic techniques to investigate VOC, RBC adhesion, inflammation and/or blood cell-endothelium interactions coupled with approaches to generate concentration gradients to evaluate dosage values of a single drug; a conceptual device is shown in **Figure 5**. Multiple devices have demonstrated utility in evaluating novel drug effects on HbS polymerization, RBC sickling, RBC membrane damage, microrheology and endothelial adhesion (Du et al., 2015; Lu et al., 2019; Hansen et al., 2020; Man et al., 2020; Noomuna et al., 2020). Moreover, often sickle patients require combination therapeutics. Thus, evaluation of synergistic effect of the combination of two drugs can also be explored, in order to minimize drug administration. Microfluidic tests can be used prior to *in vivo* studies, minimizing animal tests and speeding up the results. Finally, pre-mixing and generating gradients before administering drugs into organ-on-chip devices can provide approaches to evaluate effects on multi-cellular and tissue levels. We propose these approaches be evaluated not only in academic setting, but also for industrial level drug combination evaluation – as we see a surge in the pharmaceutical companies' interest in developing sickle drug (Ataga and Desai, 2018).

CONCLUSION

Sickle cell research field has seen rise in the microfluidics technologies for over a decade now, while organ-on-chip systems in other fields have become viable options to mimic micro- and macro-physiological processes of human biology. A critical understanding of how the future of sickle cell microfluidics research will look like lies at the intersection of three major perspective. Firstly, the decades-old *in vitro* studies performed in cell culture petri dish to investigate basic properties of sickle red cells such as deformability, adhesion and microrheology are now being improved by incorporation of vascular components in microfabricated systems. While we acknowledge that the current state of art of sickle microfluidics have provided us basic devices to study these properties in more physiologically relevant approach that were inconceivable before, we must recognize the need for more complex systems such as heterotypic cell–cell interaction devices and organ-on-chip platforms in enhancing mechanistic understanding of the disease process. Secondly, currently there is limited understanding of the relevant critical components that truly represents suitable or quasi-suitable configuration for specific tissue or vascular architecture and/or combination of cellularized modules in microphysiological and organ-on-chip systems. Therefore, it is imperative that standardization of fabrication processes, validation methods, characterization of relevant molecular and cellular mediators

and physiologically relevant input and output measures needs to be assessed in a more rational manner to establish guidelines that distinguishes good and bad design – with relevance to SCD physiology and by the consensus among the clinicians and researchers in the field. Finally, the future of sickle microfluidics is not solely limited to the development of the device itself, but also requires development of novel imaging, measurement and analytics approaches, in addition to rapidly testing the utility of such devices in a more clinically oriented manner. However, accomplishment of such advancements would require the inter-disciplinary approach from tissue/microfabrication engineers, biologists, hematologists, data acquisition/analytics specialists, imaging scientists, polymer scientists and many other scientific and technological professionals.

The promise of microfluidics in sickle research is multifaceted, however, microfabrication facilities and development of organ-on-chip systems are resource-intensive. Therefore, such endeavors must be facilitated at the national policy-level by ensuring that more funding is available for innovation in countries like Brazil and low/middle-income-countries in Africa where unmet need of disease management for sickle patients is prevalent. Currently the scientific community in the developed countries is racing to find a gene-therapy-based cure for SCD, however, to make such cures affordable for everyone in the long run is quite a challenge. In the meantime, microfluidics can help us to understand novel mechanisms, identify new drug targets, screen novel drug molecules and finally model the drug delivery and safety/efficacy in organ-on-chip platforms. And we should pursue these promising avenues with clear scientific and therapeutic goals to help ease the burden of sickle patients.

AUTHOR CONTRIBUTIONS

AA, LGT, and YL conceived and designed the research, performed literature search, and wrote and edited the manuscript. AA, YL, and DS prepared the figures. DS performed the literature search, wrote and edited manuscript, and prepared **Supplementary Table 1**. SK and DC supervised YL. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2020.558982/full#supplementary-material

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