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ORIGINAL ARTICLE

# Newborn screening by tandem mass spectrometry confirms the high prevalence of sickle cell disease among German newborns

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## Abstract

Sickle cell disease (SCD) is a severe inherited blood disorder associated with significant morbidity and mortality in early childhood. Since simple interventions are available to prevent early fatal courses, SCD is a target condition of several national newborn screening (NBS) programs worldwide, but not in Germany. Traditionally, the diagnosis of SCD is made by high-performance liquid chromatography (HPLC), isoelectric focusing (IEF), or capillary electrophoresis (CE), but globally, most NBS programs in place are based on tandem mass spectrometry (MS/MS). Recently, several publications have shown that MS/MS is an appropriate screening technique to detect hemoglobin patterns suggestive of SCD in newborns, too. We have studied dried blood spot samples of 29,079 German newborns by both CE and MS/MS and observed a 100% congruence of test results. Seven babies had hemoglobin patterns characteristic of SCD (1:4154). Our study confirms that (a) the suitability of MS/MS as an adequate substitute for CE in NBS for SCD and (b) the high prevalence of SCD among German newborns. Our results support the thesis that German newborns should be screened for SCD by MS/MS.

**Keywords** Sickle cell disease · Sickle cell anemia · Newborn screening · Tandem mass spectrometry · Capillary electrophoresis

## Introduction

### Sickle cell disease

SCD is an inherited disorder of hemoglobin. Its biochemical hallmark is the occurrence of the pathological sickling hemoglobin (HbS) at levels (significantly) exceeding 50% of total hemoglobin. HbS is a structural beta globin variant on the basis of the HBB:c.20A > T point mutation. Homozygosity for HBB:c.20A > T results in the total substitution of physiological adult hemoglobin (HbA) by HbS, resulting in SCD-S/S, formerly known as sickle cell anemia. Compound heterozygosity for HbS and another pathogenic beta globin variant leads to the co-occurrence of HbS and the other variant, most commonly associated with a less severe phenotype of SCD in comparison to SCD-S/S. Compound heterozygosity for SCD and a beta thalassemia mutation also results in the phenotype of SCD. The more severe the beta globin expression from the thalassemia allele is reduced, the more severe is the associated sickling disorder. Although many genotypes of SCD have been described over the years, the three most common of which are SCD-S/S, SCD-S/C, and SCD-S/beta$^0$ thalassemia. Globally, they account for more than 90% of all cases of SCD [1–3].

The biochemical diagnosis of SCD is very straightforward and based on the demonstration of the absence of HbA and its substitution by HbS ± a second beta globin variant. Several high-throughput hemoglobin separation methods, including HPLC, CE, and IEF, are appropriate to prove this pathognomonic constellation. The first-tier biochemical test should then be confirmed by another second-tier method to rule out the confusion of HbS with several other hemoglobin variants that have similar biochemical properties. Contemporarily, molecular genetic testing makes the definitive diagnosis [4–6].

SCD patients are prone to life-threatening infections with encapsulated bacteria, in particular pneumococci (so-called overwhelming post-splenectomy infection (OPSI)). Another

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severe early complication is sudden worsening of anemia secondary to a variety of pathophysiological processes including splenic sequestration, aplastic crisis due to infection with erythrotropic viruses, in particular parvovirus B19 and hyperhemolysis [1–3].

Very simple preventive measures (e.g., penicillin prophylaxis, vaccinations, education of parents) are available to avoid these life-threatening complications provided that the diagnosis has been made and that the family and health care professionals are aware of it. This is the rationale for NBS. Various studies have demonstrated the effectiveness of NBS from both the medical and the economic point of view [7–14].

## The German NBS program

In 2016, 792,131 children were born in Germany [15]. The German NBS program currently comprises 15 metabolic and endocrine disorders (Table 1). The most prevalent of which are congenital hypothyroidism (1:3139) and cystic fibrosis (estimated prevalence of 1:3300). All newborns are also screened for auditory defects.

In Germany, NBS is offered to all parents. Although participation is voluntary, the coverage is virtually 100%. NBS is done from heel prick dried blood spots that are sampled between the 36th and 72nd hour of life and analyzed in one of 11 accredited NBS laboratories. In > 80% of cases, results are available within 24 h after sample receipt. Very early preterm newborns are re-screened as soon as they have a corrected gestational age of 32 weeks [16].

## NBS for SCD in Germany

Since 2011, three different studies have investigated the prevalence of SCD among German newborns [17–20]. Our group has found 14 affected newborns in 34,084 births (1:2435) in urban Berlin, Grosse et al. published a prevalence of 1:2385 (7 in 17,018) in the Hamburg Metropolitan area, and Kunz et al. 1:12,613 (3 in 37,838) in the more rural catchment area of the Heidelberg NBS laboratory. In all three studies, different methodological approaches were used. The first-tier method in Berlin was HPLC and confirmation of suspicious results was done by CE. Grosse et al. used HPLC and molecular genetics, respectively, and Kunz et al. took advantage of a TaqMan assay in the first line. Suspicious results were confirmed by PCR and Sanger sequencing. None of these methods is established for any other target disease in the German NBS program.

## Aims

During the last years, several authors have shown that tandem mass spectrometry (MS/MS), which is by far the most frequently used method in German NBS laboratories, is appropriate for high-throughput NBS for SCD as well [21–23]. As a consequence, the aims of this project were to confirm the following:

a) The suitability of MS/MS as an adequate substitute for CE in NBS for SCD and
b) The high prevalence of SCD among German newborns.

**Table 1** Target diseases of the German NBS program (in order of their prevalence in 2015)

| | |
|---|---|
| Hypothyroidism | 1: 3,139 |
| Cystic fibrosis | 1: 3,300 (est.) |
| Phenylketonuria and hyperphenylalaninemia | 1: 4,950 |
| Congenital adrenal hyperplasia | 1: 20,488 |
| Biotinidase deficiency | 1: 61,465 |
| Isovaleric aciduria | 1: 73,758 |
| Galactosemia | 1: 105,368 |
| Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency | 1: 105,368 |
| Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency | 1: 122,929 |
| Glutaric aciduria | 1: 147,515 |
| Maple syrup urine disease | None in 2015 |
| Carnitine palmitoyltransferase I (CPT I) deficiency | None in 2015 |
| Carnitine palmitoyltransferase II (CPT II) deficiency | None in 2015 |
| Carnitine acylcarnitine translocase (CACT) deficiency | None in 2015 |

Please note that the CF program started on 1 September 2016. The given prevalence is thus an estimate

# Methods

## Study design and population

The individual hemoglobin patterns of an unselected cohort of newborns screened at the Berlin NBS laboratory were investigated in a prospective setting. All children born in the catchment area of the Berlin NBS laboratory were considered eligible for the project provided they took part in the routine NBS program. There was no preselection on the basis of an individual's ethnic origin (so-called "universal" NBS). It is noteworthy that, in contrast to the first Berlin pilot study on NBS for SCD [17], the study population did not only contain urban Berlin newborns, but also most children born in the much more rural federal state of Brandenburg. Newborns were excluded from the study if their parents did not provide written consent or if, for any reason, it was not possible to investigate a sample with both methods compared.

For various reasons that are not inherent to the screening methodology (e.g., capacity problems on our research mass spectrometer), it was not possible to cover the entire newborn

cohort born during the study period between 14 November 2015 and 21 September 2016. However, drop-outs occurred completely randomly.

## Target conditions and screening procedure

### Target conditions

The primary target conditions of this study included SCD-S/S, SCD-S/C, SCD-S/$D^{Punjab}$, SCD-S/E, SCD-S/Lepore, SCD-S/$O^{Arab}$, SCD-S/β thalassemia, SCD-S/δβ thalassemia, and SCD-S/HPFH (hereditary persistence of fetal hemoglobin) [4].

### Preparation of samples

Samples for CE and MS/MS were prepared simultaneously. Two 3.2-mm dried blood spots were punched into one well of a 96-well plate and incubated with 83 μl of distilled water at room temperature. After 2 h,

a) 10 μl of the solution was transferred into one well of another 96-well plate for further use in MS/MS and
b) 50 μl of the solution was transferred into one well of an 8-well segment of the CAPILLARYS 2 Neonat FAST™ system (Sebia/PerkinElmer, Rodgau, Germany).

If samples for both, CE and MS/MS, were not processed immediately, they were stored in a refrigerated humidity chamber for a maximum of 72 h.

### MS/MS

Electrospray MS/MS analyses were performed as previously reported. Briefly, the water extract was digested tryptically. For this purpose, 5 μl of acetonitrile (Promochem, Wesel, Germany), 5 μl of 1% formic acid (Fluka, Munich, Germany), and 30 μl of distilled water were added to the hemolysate and incubated for 5 min at room temperature. Then, 15 μl of trypsin reagent (5 mg/ml) in ammonium hydrogen carbonate ($NH_4HCO_3$) solution (1 mol/L) was added (both Sigma-Aldrich, Munich, Germany). The mixture was covered and incubated for 20 min at room temperature and then for 45 min at 37 °C on a microplate shaker (BioSan ThermoShaker PST-60HL-4, Riga, Latvia) at 250 rpm. Afterwards, it was centrifuged at 3000 rpm (Heraeus Cryofuge 5500i, Hanau, Germany) and 20 μl of the supernatant were transferred into a new microtiter plate and diluted with 180 μl of running buffer (mobile phase of the newborn screening kit; Chromsystems, Graefelfing, Germany).

After an additional 10-min incubation at room temperature on a microplate shaker at 250 rpm and 10-min centrifugation at 3000 rpm, the plate was placed into the HTS-PAL autosampler (CTC Analytics, Axel Semrau GmbH & Co. KG, Sprockhoevel, Germany), which was part of QTRAP 4000 mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Prominence HPLC pump system with degasser (Shimadzu Deutschland GmbH, Duisburg, Germany). The Analyst 1.6.2 software (Sciex, Darmstadt, Germany) was used for data acquisition.

Three microliters of the sample was injected into the MS/MS system for flow injection analysis using a flow gradient of 400 μl/min for 0.1 min and 30 μl/min for 0.7 min. Then, the system was flushed with 600 μl/min for 0.2 min to be prepared for the next sample.

Peptide detection was carried out in the "multiple reaction monitoring" (MRM) mode. Table 2 gives an overview about the target peptides and the ion masses.

Raw data were analyzed with the ChemoView 2.0.3 software (Sciex, Darmstadt, Germany) taking advantage of the abundance ratios of the variant peptide and its corresponding wild-type peptide.

**Table 2** MRM target peptides and ion masses

| Tryptic peptide | Target peptide ion (Da) | Target fragment ion (Da) |
|---|---|---|
| HbF γT2 y6 1 | 488.8 | 691.4 |
| HbF γT3 b2 1 | 658.8 | 214.1 |
| HbF γT12 b2 1 | 549.8 | 251.2 |
| HbF γT14 y9 2 | 725.3 | 536.8 |
| HbA βT1 b2 1 | 476.8 | 237.2 |
| HbA βT2 y6 1 | 466.8 | 675.4 |
| HbA βT3 y7 1 | 657.8 | 659.3 |
| HbA αT1 y5 1 | 365.2 | 517.3 |
| HbS βT1 b2 1 | 461.77 | 237.2 |
| HbS βT1 y7 2 | 461.77 | 412.25 |
| HbE βT3 y6 1 | 458.7 | 604.3 |
| HbE βT3 y7 1 | 458.7 | 703.4 |
| HbC βT1 b2 1 | 694.4 | 237.2 |
| HbC βT1 b2 1 | 347.71 | 237.2 |
| HbC βT1 y5 2 | 347.71 | 298.2 |
| HbS_T1 | 461.66 | 472.15 |
| HbA_T1 | 476.67 | 502.15 |
| HbLepore_T2 | 480.16 | 688.2 |
| HbO_T13 | 625.25 | 1001.45 |
| HbA2_T3i | 628.9 | 829.5 |
| HbA_T3 | 657.7 | 887.25 |
| HbD_T13 | 689.22 | 377.08 |
| HbA_T13b3 | 689.72 | 378.05 |
| HbA_T13_2 | 689.9 | 501.3 |
| HbA_T13y9 | 689.85 | 1001.35 |
| HbA2_T14 | 721.4 | 532.9 |

### Capillary electrophoresis

Capillary electrophoresis takes advantages of the discriminative electrophoretic mobility and the electroosmotic flow of charged molecules of different size and form in an alkaline buffer with a specific pH. Capillary electrophoreses of the newborns' hemolysates were carried out on the Sebia CAPILLARYS 2 Neonat FAST™ system. Data acquisition, management, and analysis were conducted with the PHORESIS software. The detailed protocol has been published elsewhere [24].

## Statistics

Assuming that each positive screening result is an independent event and that the mean number of positive screening results is constant over time, it is appropriate to assume a Poisson distribution for statistical calculations. This kind of a discrete probability distribution allows for the calculation of the probability of a given number of independent events in a fixed interval of time provided that the average number of events per interval of time is known. Because the latter prerequisite was not fulfilled, we used hypothetical incidences instead.

## Ethical approval

The Charité University Ethical Review Committee approved the study (reference number EA2/088/11). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

## Care for screening positive newborns

Screening positive newborns were referred to the Department of Pediatric Hematology, Oncology, and Stem Cell Transplantation of the Charité University Children's Hospital for molecular genetic confirmation of the screening result and provision of state-of-the-art medical care including important measures like disease education, penicillin prophylaxis, vaccination, and hydroxycarbamide.

# Results

## Patient recruitment

During the study period, the dried blood spot cards of 29,079 babies born in the German federal states of Berlin and Brandenburg fulfilled the inclusion criteria of the study and were comparatively analyzed by both screening methods. In all cases, evaluable results were obtained. However, very rarely and virtually exclusively concerning the application of capillary electrophoresis, repeated measurements were necessary.

## Comparison of CE and MS/MS

In all 29,079 newborns (100%), identical patterns of hemoglobin were detected by both CE and MS/MS. No incongruent measurement results were obtained. Moreover, all suspected disease states were confirmed by molecular genetic analysis performed as a part of the initial diagnostic workup in the Charité Department of Pediatric Oncology, Hematology and Stem Cell Transplantation. Hence, the specificity of both methods was 100%. False-negative screening results have not come to our attention until to date with a maximum observation time of 33 months since the beginning of the project. However, false-negative results cannot be completely ruled out so far. The new German SCD registry, which started on 15 December 2016 (ClinicalTrials.gov identifier NCT03327428), will help to monitor the quality of SCD NBS in the future.

## Hemoglobin patterns

In seven newborns (7/29,079 = 1/4154 = 2.4/10,000), we found hemoglobin patterns consistent with the diagnosis of SCD (Table 3). In five babies, only the hemoglobins F and S (FS pattern) were present. Most commonly, the underlying genotype is homozygosity for HbS. However, an FS pattern is also observed in patients with SCD-S/$\beta^0$ thalassemia and in some patients with SCD-S/$\beta^+$ thalassemia. SCD-S/$\delta\beta$ thalassemia and compound heterozygosity for HbS and HPFH (SCD-S/HPFH) also have an FS hemoglobin pattern biochemically.

Two newborns showed an FSC pattern, which is pathognomonic for compound heterozygosity for HbS and HbC (SCD-S/C).

**Table 3** Detailed results of the investigations performed in seven screening positive newborns

| No. | Native country | CE | MS/MS | Genotype |
|---|---|---|---|---|
| 1 | Nigeria | FS | FS | SCD-S/S |
| 2 | Ghana | FS | FS | SCD-S/HPFH |
| 3 | Ghana | FS | FS | SCD-S/S |
| 4 | N/D | FSC | FSC | SCD-S/C |
| 5 | Sierra Leone | FSC | FSC | SCD-S/C |
| 6 | Nigeria | FS | FS | SCD-S/S |
| 7 | Democratic Republic of Congo | FS | FS | SCD-S/S |

Beyond, the putative disease states, 211 newborns were identified with hemoglobin patterns in accordance with a heterozygous state for one of several variants. One thirty four showed an FAS pattern, typical of heterozygosity for HbS; 18 showed an FAC pattern that indicates heterozygosity for HbC; 21 children were heterozygous for HbE (FAE pattern); 9 for HbD (FAD pattern); and 29 for various rarer hemoglobin variants (FAX pattern, X = unspecified hemoglobin variant). None of the investigated babies showed an "F-only" pattern which would indicate clinically relevant β thalassemia, e.g., β thalassemia major or severe β thalassemia intermedia.

Molecular genetic analysis revealed that four babies actually had SCD-S/S, one baby had SCD-S/HPFH which is known to cause a very mild/inapparent form of SCD, and two newborns suffered from SCD-S/C.

### Statistics

In the course of the study, seven babies with three different genotypes of SCD were identified. This translates into an arithmetical prevalence of 2.4/10,000. Assuming a Poisson distribution, the probabilities of detecting at least seven affected babies in the study population are only 2.91, 4.91, and 7.62% applying hypothetical prevalences of 1/10.000, 1.125/10.000, and 1.25/10.000. Conversely, the probability that the true prevalence of SCD in the study population is higher than (a) 1/10.000 is 97.09%, (b) 1.125/10.000 is 95.09%, and (c) 1.25/10.000 is 92.38%.

### Costs

In our specific scientific setting, NBS for SCD by MS/MS generated net costs of exactly 3.00 EUR per sample. These costs were composed of the following:

a. Proportionately 0.77 EUR/sample for personnel
b. 1.50 EUR/sample for reagents and running buffer
c. Proportionately 0.42 EUR/sample for hardware purchase (assuming a measuring capacity of 100.000 samples per mass spectrometer and year and a recovery period of 6 years)
d. Proportionately 0.31 EUR/sample for hardware maintenance.

In contrast, the net costs for capillary electrophoresis were 3.58 EUR/sample and composed as follows:

a. Proportionately 0.48 EUR/sample for personnel
b. 3.10 EUR/sample all-inclusive price for hardware leasing, consumables, and maintenance.

## Discussion

In our study, we have found a perfect concordance of screening results obtained by CE and MS/MS. These results contribute to the constantly growing evidence that MS/MS is an appropriate method for NBS for SCD. It can be assumed that all genotypes will be detected by MS/MS that are identifiable with the other well-established methods, i.e., HPLC, CE, and IEF, too. From theoretical considerations, it is suspected that the SCD genotype SCD-S/$\beta^+$ thalassemia has the highest risk of being missed in NBS as it can be confused with SCD heterozygosity if the remaining expression of HbA from the thalassemia allele is high.

By definition, screening is not diagnostic. Screening examinations are supposed to identify individuals at risk for a certain disease who may benefit from early, more sophisticated diagnostic procedures. Like all diagnostic tests, screening tests are expected to provide false-positive and false-negative results. However, in NBS, it is of fundamental importance to choose methods with both a high specificity and a high sensitivity. False-positive test results cause fear and anxiety in families who ought to be happy about having a healthy child. In the worst case, the child-parent relationship will sustain significant damage. The false-positive screening results also waste time and money of the health care system. In contrast, false-negative screening results mean that children who suffer from a significant target disease are missed. This may ultimately result in significant morbidity and mortality. Making the diagnosis later can even be hindered since the negative screening result may suggest that the clinically suspected diagnosis has already been ruled out.

Both CE and MS/MS appear to be excellent NBS methods for SCD with very high sensitivity and specificity. However, there are several arguments favoring MS/MS for the German NBS program over CE and also over HPLC. Most target diseases of the current German NBS are investigated by MS/MS. Thus, MS/MS hardware and expertise are available in every German NBS laboratory, while CE and HPLC are not in use. Moreover, the software algorithm behind MS/MS methodology allows for suppression of heterozygotes [25]. This is interesting as the very strict German genetic testing act prohibits to test minors for heterozygous disease states that are not relevant for the individual during childhood and adolescence like HbS heterozygosity [26]. In Germany, the right for informational self-determination including the right not to know is legally considered a much higher good than the reproductive benefit arising for the family from the identification of an index "patient." This legal position does limit the value of NBS for SCD not only for a certain family but also for the society as a whole, because one major advantage of identifying carriers in a population is to raise awareness for a certain disease.

Unfortunately, NBS for SCD is not possible synchronously with the other endocrine and metabolic target diseases of the German NBS program limiting the recently mentioned benefit of using the same screening technique for both metabolic/

**Table 4** Overview about studies on NBS for SCD in Germany

| | Newborns screened | Affected babies | Reference |
|---|---|---|---|
| Berlin I | 34,084 | 14 | [17, 18] |
| Hamburg | 17,018 | 7 | [20] |
| Heidelberg | 37,838 | 3 | [19] |
| Berlin II | 29,079 | 7 | Present study |
| Total | 118,019 | 31 | |

endocrine disorders and SCD. It is likely that many NBS laboratories will need additional MS/MS machines if NBS for SCD in Germany would be performed by MS/MS. This calls for further research to combine the identification of old and new putative target disorders "in the same shot."

In our specific setting, we screened for SCD by (a) MS/MS at costs of 3.00 EUR per sample and (b) CE at costs of 3.58 EUR per sample. However, these costs are certainly variable and highly dependent on factors like the number of samples screened per laboratory or contracts between a certain laboratory and the medical diagnostic industry. Thus, the cost argument does not irrevocably favor any of both methods.

Including this study, 118,019 newborns have been screened for SCD in four different projects (Table 4). As a result of these studies, 31 babies have been diagnosed with SCD. These data cannot be overlooked. There is a clear demand to introduce NBS for SCD in Germany as soon as possible.

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## Compliance with ethical standards

**Conflict of interest** Novartis has contributed the major funding of the present study. S.L. has received honoraria from Novartis, Nordic Pharma, Celgene, and Bluebird for the scientific presentations and for his participance in Advisory Boards. The other authors declare no conflicts of interest.

**Informed consent** Informed consent was obtained from all patients for being included in the study.

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