Early testicular maturation is sensitive to depletion of spermatogonial pool in sickle cell disease

Cryopreservation of testicular tissues before hematopoietic stem cell transplant (HSCT) is offered by specialized centers worldwide to boys with sickle cell disease (SCD). In order to elucidate whether hydroxyurea (HU) therapy or disease-related factors affect the spermatogonial quantity of SCD patients, we collected clinical data of 29 boys (age range, 2.8-15.1 years) and calculated a Z-score. Our results show that most spermatogonial numbers (n=17) were below the reference values of healthy boys. There was a correlation between the number of spermatogonia and the age at HU initiation (P=0.029, r=0.476). This study suggests that besides factors intrinsic to SCD, an HU exposure in early life may lead to further depletion of spermatogonia reducing the potential for successful fertility preservation.

SCD is the most common corpuscular anemia, with approximately 300,000 affected babies born anually.[1] SCD manifests in hemolytic anemia and episodes of microvascular vaso-occlusion leading to end-organ ischemia-reperfusion injury and organ damage. Treatment to alleviate complications involves HU.[14] In high-resource countries, allogeneic HSCT is proposed as a cure. However, there are significant risks, including acute toxicity and late effects, e.g., infertility caused by previous gonadotoxic conditioning.[3]

As fertility preservation, a growing number of centers worldwide cryopreserve either sperm or testicular tissues of prepubertal boys prior to HSCT.[4,5] Cryopreservation of testicular tissue containing undifferentiated germ cells, termed spermatogonia, remains an experimental approach as protocols to differentiate spermatogonia into sperm are under development.[6] In fertility preservation programs, patients with SCD constitute up to 35% of all patients with non-malignant diseases.[4] Based on limited case series, even before HU therapy, most young men with SCD have semen quality below the World Health Organization standard minimum criteria and spermatogonial numbers in prepubertal boys have been shown to be reduced.[5,7-8] A recent study with a small cohort size concluded that spermatogonial numbers in HU exposed patients (n=17) were not significantly different from those without HU exposure (n=13).[9]

The current study was designed to evaluate if dose, exposure time, age at HU initiation, iron load or disease severity affects spermatogonial quantity in SCD patients. Insights enable clinicians to prospectively counsel patients and parents about the risks for reduced spermatogonial numbers prior to HSCT.

Testicular tissues from 29 boys with SCD (age range, 2.8-15.1 years) participating in the fertility preservation programs Androprotect (German network, n=19) and Nordfertil (Nordic network, n=10) between 2013-2020 were included. Also, testicular tissue from two adult SCD patients (age range, 21.5-48.5 years, University Hospital Münster) were available. All age-appropriate patients and the guardians gave written informed consent for the use of testicular tissues for research. All procedures were in accordance with the Declaration of Helsinki and Ethical approval was obtained from the responsible Ethics Boards.

Hormone levels, testicular volume, and markers of chronic hemolysis were determined, information detailing transfusions, HU therapy and SCD complications was collected, and severity scores were calculated (Table 1; *Online Supplementary Table S1*).[10,11]

Patients underwent open testicular biopsy during

Table 1. Clinical characteristics and Spearman correlations for mean spermatogonial numbers per round tubular cross section and fertility index Z-scores of the 24 patients with testicular samples containing a minimum of 25 evaluated tubules.

Clinical characteristics			S/T Z-score		FI Z-score	
	N	Median (range)	*P*	r	*P*	r
Age	24	7.1 (2.8-15.1)	0.015*	0.492*	0.073	0.373
Hematological parameters						
HbS level, % of total Hb	16	54.2 (9.4-85.3)	0.778.	- 0.077	0.571	- 0.153
HbF level, % of total Hb	13	12.0 (3.0-34.0)	0.553	0.181	0.734	0.105
MCV, fL	15	86.0 (76.0-123.0)	0.512	0.184	0.838	- 0.058
Platelets, G/L	22	294.0 (104.0-1,103.0)	0.497	0.153	0.990	0.003
Neutrophils, G/L	20	4.2 (0.5-7.9)	0.925	0.023	0.853	0.044
Biomarkers of chronic hemolysis						
Hb, mg/L	23	100.0 (74.0-134.0)	0.667	- 0.095	0.645	- 0.101
LDH, U/L	20	158.0 (86.2-262.3)	0.405	0.197	0.046*	0.451*
Biomarker of iron status						
Ferritin, μg/L	19	305.0 (51.0-3,244.0)	0.304	0.249	0.209	0.302
HU therapy†						
Dose, mg/kg	24	22.5 (0.0-45.0)	0.766	- 0.064	0.537	- 0.132
Exposure time, d	24	750.5 (0.0-2,839.0)	0.856.	- 0.039	0.565	- 0.124
Age at HU start, y	24	5.0 (1.0-9.9)	0.029*	0.476*	0.024*	0.490*
Dose multiplied by exposure time, mg x d	24	17,434.0 (0.0-1,01636.0)	0.984	0.004	0.720	- 0.077
Complications						
Pain crisis per year, n	19	0.4 (0.0-4.0)	0.486	0.170	0.158	0.337
Number of ATS, n	24	0.0 (0.0-3.0)	0.703	- 0.082	0.802	0.054
Score (Sebastiani *et al.*)[10]	24	0.4 (0.3-0.6)	0.439	- 0.166	0.748	- 0.069
Score C (Van den Tweel *et al.*)[11]	24	50.0 (5.0-80.0)	0.168	0.291	0.149	0.304

ATS indicates acute thorax syndrome; FI: feritlity index; S/T: spermatogonia per round tubular cross-section; Hb: hemoglobin; HbF: fetal hemoglobin; HbS: sickle hemoglobin; HU: hydroxyurea; y: year; x d: per day; LDH: lactate dehydrogenase; MCV: mean corpuscular volume; *P*: *P*-value and r, Spearman correlation coefficient. *P<0.05. † Washout period of 2 weeks - 4 months was used prior to biopsy for three HU-exposed patients. All 3 patients received transfusions.

which less than 20% of the testicular volume of one testis was sampled. Two-thirds of the tissues were transported to the research center, partly overnight, and were cryopreserved. The remaining third was fixed in formalin in the Nordfertil program. Tissues from the Androprotect program and adult patients were fixed in Bouin's solution. After embedding in paraffin, all tissues were sectioned (3-5 µm), and two independent sections (distance >15 µm) were immunostained with MAGEA4, following published protocols.[12,13] At Karolinska Institutet, a fluorescence microscope (Eclipse E800, Nikon; Japan) was employed for analysis. In Münster, images were captured using the PreciPoint M8 microscope/scanner and subsequently analysed using the ViewPoint light software (1.0.0.9628, PreciPoint, Freising, Germany). Spermatogonia were identified based on their morphology (size, shape), location and MAGEA4 expression (*Online Supplementary Figure S1*). Using a blinded approach, all round tubular cross-sections within the tissue sections were quantified (mean: 94, range, 0-344) and classified as tubules with spermatogonia and tubules only containing somatic Sertoli cells. Mean spermatogonial numbers per round tubular cross section (S/T) were assessed to obtain comparable spermatogonial numbers across samples (*Online Supplementary Table S1*). Moreover, the fertility index (FI) as the percentage of tubular cross-sections containing spermatogonia was determined.

In order to control physiological variation in spermatogonial numbers during development, Z-scores were calculated for S/T and FI using reference means.[14] For statistical analysis, only samples with >25 round tubules were considered, resulting in the inclusion of n=24 prepubertal/pubertal patient samples (*Online Supplementary Table S1*). Spearman correlation coefficient (r) and ROC analysis were performed to determine the relationships between spermatogonial quantity, age and treatment characteristics using IBM SPSS Statistics V26.0 software (IBM Corporation, Armonk, NY; US) and GraphPad Prism Version 8.4.3(471) (GraphPad Software, San Diego, CA, US).

Out of the 29 prepubertal/pubertal samples, the major-

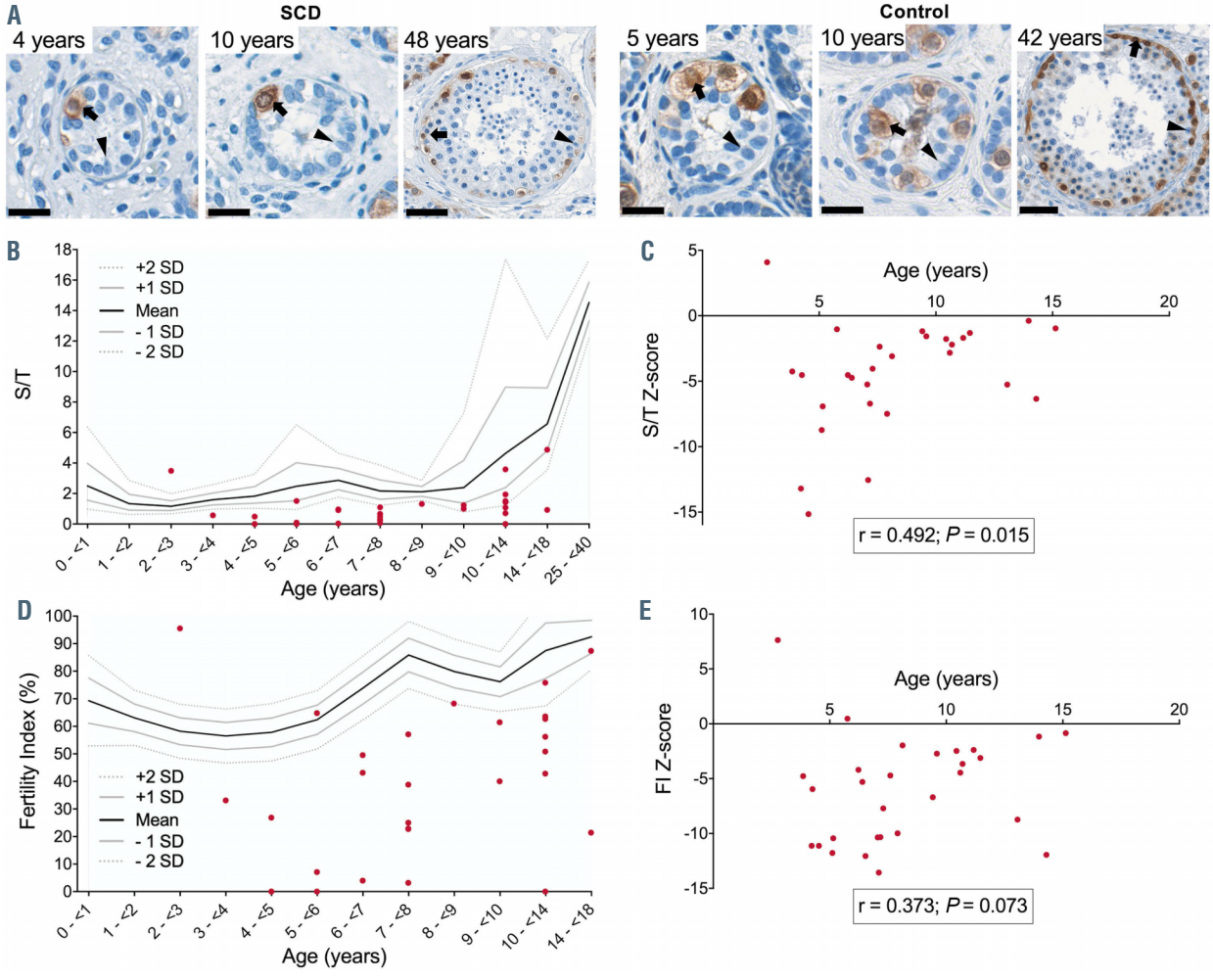

Figure 1. Number of spermatogonia per round tubular cross-section and fertility index in patients with sickle cell disease. (A) Representative images showing MAGEA4-positive spermatogonia (immunohistochemical staining) in patients with sickle cell disease (SCD) and controls (n=3, each) of different ages. Scale bars: 20 µm (4-10 years), 50 µm (42-48 years), arrows indicate spermatogonia and arrowheads Sertoli cells. Scale bars were added to the images using Adobe Photoshop CS2 (Adobe Systems, California, US). Objective: Olympus PlanC N 60x/0.80 (PreciPoint, Freising, Germany). (B) Spermatogonia per round tubular cross-section (S/T) and (C) S/T Z-score as well as the fertilty index (FI) (D) and FI Z-scores (E) by age in 29 patients with SCD. In (B) and (D) data are plotted on lines corresponding Z-scores for the mean reference values (Adapted from Funke *et al.*).[14] A significant correlation exists between younger age and lower S/T Z-scores (*P*=0.015, r=0.492). SD: standard deviation.

ity (n=17) scored below previously published reference values of S/T (Z-score <-3), but only four were devoid of spermatogonia (Figure 1A to C). Both adult patients showed spermatogonial numbers below the reference values (*Online Supplementary Table S1*). Our observations confirm previous reports of severely decreased spermatogonial numbers.[5,7,9] Additionally, most patients (n=21/29) had FI Z-scores below the reference values (Figure 1D and E), illustrating that spermatogonia were only focally present.

In line with Gille *et al.*,[9] no difference was observed between the numbers of spermatogonia in HU-exposed (n=21) and non-exposed groups (n=3) although the small sample size results in limited informative value (*P*=0.9999). Three patients with a wash out period of 2 weeks to 4 months without HU prior to biopsy were included in the HU-exposed group. There was no correlation between spermatogonial numbers and HU dose or exposure time (Table 1; Figure 2A and B).

Importantly, younger age correlated significantly with lower S/T (*P*=0.015, r=0.492) Z-score (Table 1; Figure 1C). Since 2014, an international consensus has recommended HU therapy for all infants with SCD aged 9 months or older to reduce complications.[2] Our observations reflect this recommendation; younger patients had an earlier HU initiation (*P*<0.001, r=0.713), leading to HU exposure at an earlier time of testicular development compared to patients diagnosed before 2014. The young age at the HU initiation further correlated with lower S/T (*P*=0.029, r=0.476) and FI Z-scores (*P*=0.024, r=0.490) (Figure 2C and D). By using ROC analysis, we were able to unveil that an age of 2.4 years at HU initiation showed a good diagnostic value (area under the curve [AUC]: 0.84, 95% confidence interval [CI]: 0.67–1.00) with 75% sensitivity and 82% specificity to identify testicular samples containing very low spermatogonial numbers (S/T Z-score <-7). Therefore, testicular maturation during the first 3 years of life may be especially sensitive to depletion of the spermatogonial pool in SCD patients. Significant correlation however does not prove that early HU initiation determines the low S/T Z-score at very young age.

Older age correlated significantly with higher S/T Z-scores (*P*=0.015, r=0.492) and a cut-off age of 8.8 years identified with 100% sensitivity and 80% specificity (AUC 0.84, 95% CI: 0.64–1.00) S/T Z-scores within the normal range (>-3). Our data confirm that the pubertal increase in spermatogonial numbers does occur in SCD patients in line with a previous report.[8] Also in cases of early HU exposure we cannot exclude that spermatogonia maintain their capacity of expansion at puberty.

Besides the potentially harmful effects of early HU exposure, other factors intrinsic to SCD need to be considered. There is wide variability in the phenotypic severity of SCD. This variation can be explained partly by differences in the total hemoglobin concentration, the mean corpuscular hemoglobin concentration, iron load, coinheritance of α-thalassemia and fetal hemoglobin persistence.[15] In line with this, low hemoglobin values observed in the present study correlated with a high frequency of pain crises (*P*=0.026, r=-0.524). However, we could not show any correlation between spermatogonial numbers and the severity of SCD indicated by the total number of pain crises per year, the number of acute thorax syndromes or SCD severity scores (Table 1; Figure 2E and F). This absence of correlation may be due to the limited

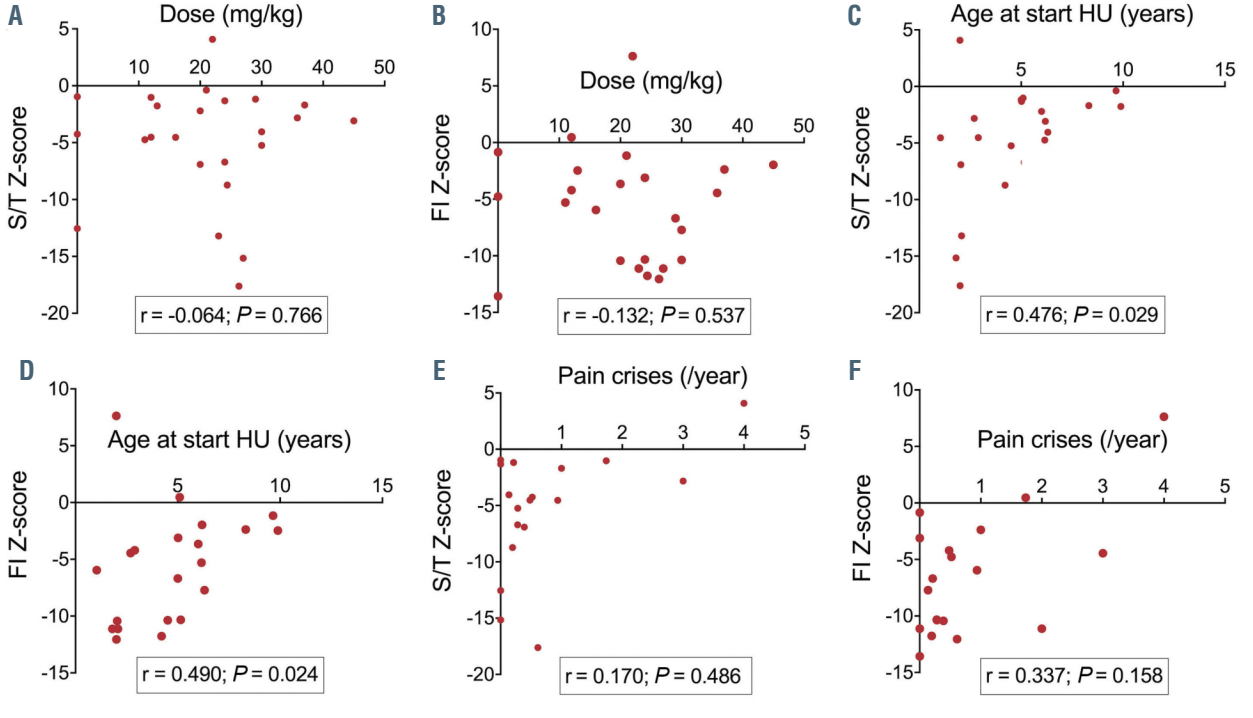

Figure 2. The impact of hydroxyurea dose, age at hydroxyurea therapy initiation and the number of pain crises as disease severity marker on the numbers of spermatogonia per round tubular cross-section and fertility index in patients with sickle cell disease. Graphical display of the numbers of spermatogonia per round tubular cross section (S/T) and fertility index (FI) Z-score by hydroxyurea (HU) dose (A and B), age at HU therapy initiation (C and D) and the numbers of pain crises (E and F). In linear regression analysis, the age at HU therapy initiation reached statistical significance. *P* indicates *P*-value and r Spearman correlation coefficient.

number of patients enrolled. Altogether seven patients were under regular transfusion regimes and six patients received pre-operative transfusions prior to testicular biopsy (Table 1). With limited informative value, the serum ferritin showed no correlation with spermatogonial numbers suggesting that iron load plays no significant role in the testicular toxicity of this patient population.

In summary, most boys with SCD in this study had spermatogonia within their testicular tissue although at reduced numbers. Importantly, an association between reduced spermatogonial numbers and the young age was found while boys older than 8.8 years showed spermatogonial numbers within the normal range. Reduced spermatogonial numbers were further shown to correlate with the age at HU initiation. An age of 2.4 years or less at initiation associated with severely decreased spermatogonial numbers suggesting that spermatogonia and/or testicular somatic cells may be especially sensitive to disturbances such as HU exposure or disease complications. The potential risk of further compromising the already limited fertility by early HU initiation, must be weighed against the overwhelming evidence that HU significantly decreases morbidity and increases the lifespan among SCD patients.[2]

For all boys that are mature enough, it is most important that they are offered sperm cryopreservation. Moreover, testicular function should be studied if HU therapy must be discontinued to increase our knowledge of possible testicular recovery. For prepubertal boys, it is essential that they are given the opportunity to cryopreserve immature testicular tissue prior to HSCT after appropriate counseling regarding potentially decreased spermatogonial numbers.

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References

1. Piel FB, Simon IH, Gupta S, Weatherall DJ, Williams TN. Global burden of sickle cell anaemia in children under five, 2010–2050: modelling based on demographics, excess mortality, and interventions. PLoS Med. 2013;10(7):e1001484.
2. Yawn BP, Buchanan GR, Afenyi-Annan AN, et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. JAMA. 2014;312(10):1033-1048."
3. Walters MC, Hardy K, Edwards S, et al. Pulmonary, gonadal and central nervous system status after bone marrow transplantation for sickle cell disease. Biol Blood Marrow Transplant.

2010;16(2):263-272.
4. Goossens E, Jahnukainen K, Mitchell RT, et al. Fertility preservation in boys: recent developments and new insights †. Hum Reprod Open. 2020;2020(3):hoaa016.
5. Valli-Pulaski H, Peters KA, Gassei K, et al. Testicular tissue cryopreservation: 8 years of experience from a coordinated network of academic centers. Hum Reprod. 2019;34(6):966-977.
6. Neuhaus N, Schlatt S. Stem cell-based options to preserve male fertility. Science. 2019;363(6433):1283-1284.
7. Stukenborg JB, Alves-Lopes JP, Kurek M, et al. Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy. Hum Reprod. 2018;33(9):1677–1683.
8. DeBaun MR. Hydroxyurea therapy contributes to infertility in adult men with sickle cell disease: a review. Expert Rev Hematol. 2014;7(6):767-773.
9. Gille AS, Pondarré C, Dalle JH, et al. Hydroxyurea does not affect the spermatogonial pool in prepubertal patients with sickle cell disease. Blood. 2021;137(6):856-859.
10. Sebastiani P, Nolan VG, Baldwin CT, et al. A network model to predict the risk of death in sickle cell disease. Blood. 2007;110(7):2727-2735.
11. van den Tweel XW, van der Lee JH, Heijboer H, Peters M, Fijnvandraat K. Development and validation of a pediatric severity index for sickle cell patients. Am J Hematol. 2010;85(10):746-751.
12. Alves-Lopes JP, Soder O, Stukenborg JB. Testicular organoid generation by a novel in vitro three-layer gradient system. Biomaterials. 2017;130:76-89.
13. Albert S, Wistuba J, Eildermann K, et al. Comparative marker analysis after isolation and culture of testicular cells from the immature marmoset. Cells Tissues Organs. 2012;196(6):543–554.
14. Funke M, Yang Y, Lahtinen A, et al. Z-scores for comparative analyses of spermatogonial numbers throughout human development. Fertil Steril. 2021;116(3):713-720.
15. Quinn CT. Minireview: clinical severity in sickle cell disease: the challenges of definition and prognostication. Exp Biol Med (Maywood). 2016;241(7):679-688.