Diet and gender influence survival of transgenic Berkley sickle cell mice

The prevalence of sickle cell disease (SCD) is highest in regions in which there is global malnutrition with high infant and early childhood mortality.[1,2] In sub-Saharan Africa, 5-year olds are at the highest risk of death.[1] In tribal communities of India, 20% of children with SCD die by the age of 2 years and 30% die before reaching adulthood.[2] Additionally, individuals with SCD may suffer from disease-related malnutrition as a result of increased demands for absorbed nutrients, caused by multiple pathological factors including higher resting basal metabolic rate.[3,4] Deficiencies of many nutrients, including vitamins, zinc, magnesium, and fatty acids are well-documented in SCD.[3] Nutritional deficiencies may begin during gestation because of poor parental diet, which adds to the existing challenge of examining the effect of diet on survival of individuals with SCD. Transgenic mouse models of SCD including homozygous Berkley (HbSS-BERK) expressing exclusively human sickle hemoglobin replicate the pathophysiology of human SCD and provide an opportunity to examine the effect of dietary interventions across generations.[5] We examined whether a diet enriched in protein, amino acids, fatty acids, and specific critical micronutrients (Table 1) fed to parents and/or offspring would improve the survival of offsprings with SCD.

We used protocols approved by the Institutional Animal Care and Use Committee of the University of Minnesota. HbSS-BERK mice offspring (henceforth called pups) expressing >99% human sickle hemoglobin were used.[5] Because homozygous BERK female mice often do not breed/survive pregnancy,[6] we used heterozygous BERK female (HbAS-BERK) and male HbSS-BERK as breeding pairs (Figure 1). Breeders and pups were randomly assigned to either a sickle diet (SD) or regular diet (RD) (Table 1) to differentiate groups by pup diet, parental diet, and gender (Figure 1). After phenotyping, only the homozygous pups were included in the study. Breeder pairs were divided into two dietary groups, SD and RD, as shown in Figure 1. Compared to the RD, the SD has a higher content of protein and fat calories and contains higher amounts of specific minerals, vitamins, amino acids, and omega-3-fatty acids (Table 1). Pups were weaned 3 weeks after birth and fed the SD for 10 days after weaning and then randomly assigned to either the SD or RD until 150 days of age. A multivariate analysis was performed on the relationship between rates of survival and the following factors of interest: pup gender, pup diet, and parental diet. The outcome variable of interest, survival in days, was examined using Kaplan-Meier plots of estimated survival and Cox regression to obtain hazard ratios (HR) with 95% confidence intervals (95% CI). For Cox regression, the significance of individual factors in multivariate analysis and the significance of overall survival differences were based on Wald and log-rank tests, respectively. Data were analyzed using the 'survival' package in 'R 3.4.0' (R Foundation for Statistical Computing; Vienna, Austria). *P* values <0.05 were considered statistically significant.

We first studied the effect of diet on survival of HbSS-BERK pups. Of 601 pups, 482 were given the SD and 119 were given the RD. At 150 days, 77% of pups on the SD and 51% of pups on the RD were alive (Figure 2A). According to the multivariate analysis, pups fed the SD had significantly prolonged survival compared to pups on the RD (HR: 0.3988; 95% CI: 0.2860-0.5561; $P=6.03\times10^{-8}$).

Dietary supplementation with individual nutrients has been shown to attenuate pathogenic mechanisms and reduce morbidity associated with SCD. A high protein diet attenuated ischemic end-organ damage, increased muscle mass, bone mineral density, and grip strength, and decreased vascular leakage in HbSS-BERK or Townes sickle mice.[7,8] Omega-3-fatty acid supplementation decreased NF-κB activation, adhesion molecule expression, and inflammatory markers and reduced the frequency of vaso-occlusive crises and transfusion requirements in patients with SCD.[9,10] Zinc supplementation improved growth and decreased the incidence of infection.[11] Folic acid is critical for DNA synthesis and does not have body stores. A compensatory increase in erythropoiesis in SCD results in folate deficiency due to over-utilization.[12] In HbSS-BERK mice, arginine supplementation markedly enhanced nitric oxide bioavailability, and decreased both hemolysis and oxidative stress.[13] Arginine supplementation alleviated pain induced by vaso-occlusive crises and decreased narcotic use by >50% in a randomized placebo-controlled clinical trial.[14] In a comparison with placebo administration, L-glutamine supplementation (0.6 g/Kg/day) reduced the average cumulative recurrence of vaso-occlusive crises but significantly increased musculoskeletal, abdominal and chest pain in patients with SCD in a phase III clinical trial.[15] The SD includes these nutrients but in significantly smaller amounts, which may synergize to meet the nutritional requirements in SCD without causing off-target effects. Therefore, supplementation of diet with a combination of multiple nutrients may be more beneficial than the use of individual nutrients at high doses.

We next studied the effects of gender on survival of HbSS-BERK pups. We compared the survival of 338 female pups and 263 male pups independent of their

Table 1. Composition of the sickle diet and the regular diet.

Contents	Sickle Diet	Regular Diet
Protein (% weight)	26.4	18.6
Fat (% weight)	11.1	6.2
Carbohydrates (% weight)	62.5	44.2
Protein (% calories)	27.5	24
Fat (% calories)	26	18
Carbohydrates (% calories)	46.5	58
Arginine (%)	2.52	1
Aspartic acid (%)	2.53	1.4
Glutamic acid (%)	5.16	3.4
Magnesium (%)	0.48	0.2
Sulfur (%)	0.29	0
Zinc (mg/kg)	225	70
Vitamin A (IU/g)	33	15
Vitamin D3 (IU/g)	3.3	1.5
Vitamin E (IU/kg)	200	110
Folic acid (mg/kg)	8	4
Vitamin B12 (μg/kg)	60	80
Choline chloride (mg/kg)	2200	1200

The major components of the individual diets are listed above. The sickle diet was the Sickle Cell Mouse Diet (59M3) (TestDiet, St. Louis, MO, USA). A complete list of its components can be found at: *www.testdiet.com*. The reegular diet was the Teklad Global 18% Protein Rodent Diet (Harlan Laboratories; Madison, WI, USA) A complete list of its components can be found at: *www.envigo.com/resources/datasheets/2018-datasheet-0915.pdf*.

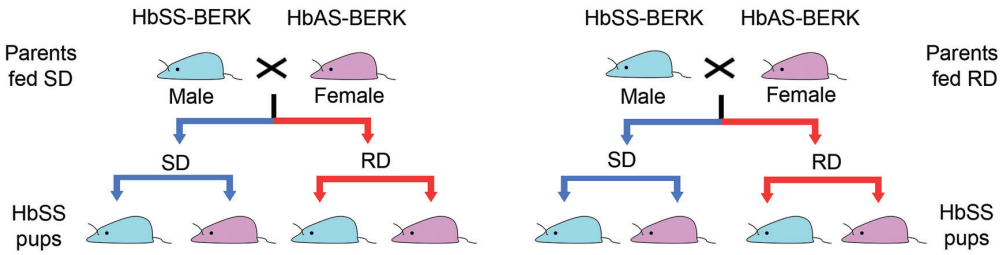

Figure 1. Breeding strategy. Parent pairs comprised male homozygous (HbSS-BERK) and female hemizygous (HbAS-BERK) sickle mice. After phenotyping and genotyping, only homozygous HbSS pups were included in the study. Breeder pairs were divided into two groups according to the diet they were fed: the sickle diet (SD) or the regular diet (RD). Contents of the SD and RD are specified in Table 1. Pups were weaned 3 weeks after birth, fed the SD for 10 days after weaning and then randomly assigned to either the SD or RD until 150 days of age.

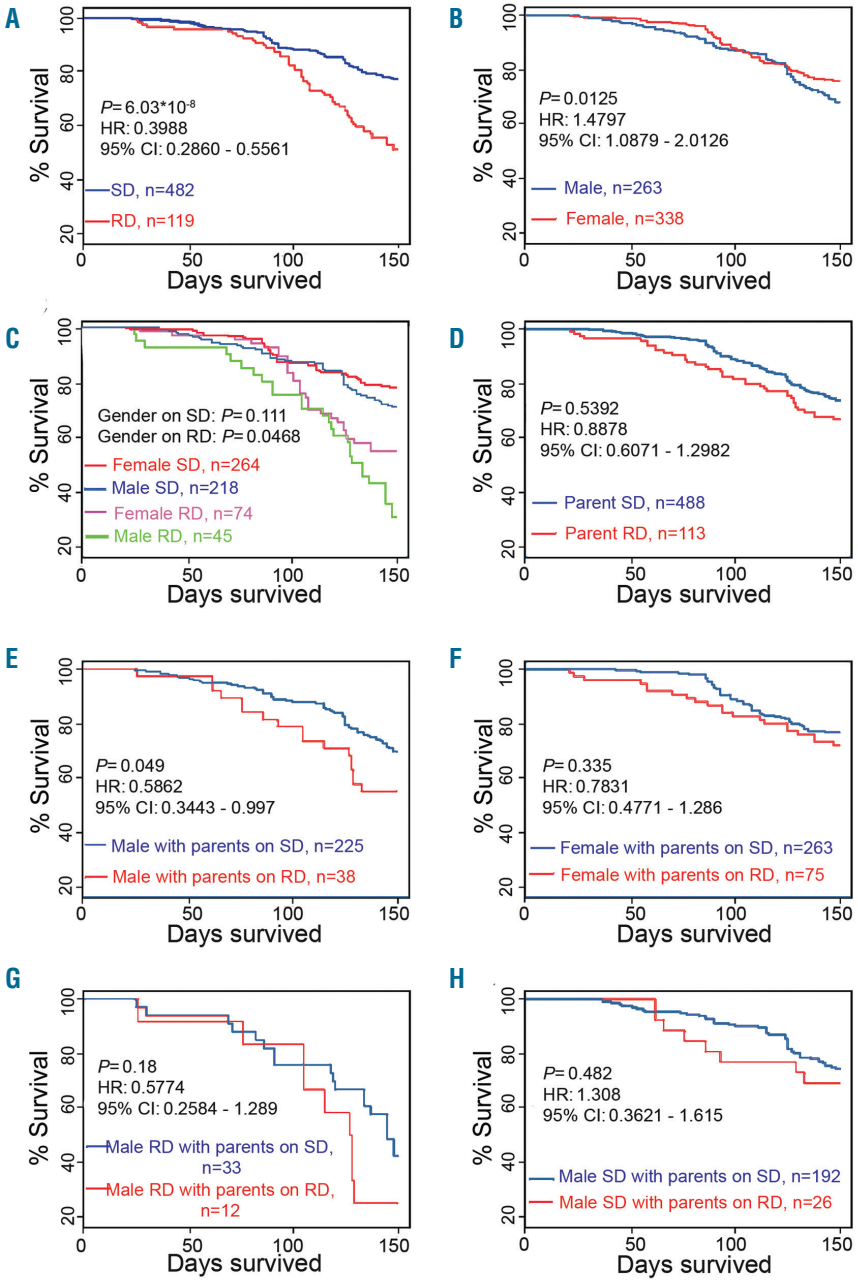

Figure 2. Diet and gender affect the survival of sickle mice. Transgenic homozygous Berkley mice expressing >99% human sickle hemoglobin were used. The protein, fat, mineral, vitamin, amino acid, and omega-3-fatty acid contents were higher in the sickle diet (SD) than in the regular diet (RD). Pups were weaned 3 weeks after birth, fed the SD for 10 days after weaning and then randomly assigned to either the SD or RD until 150 days of age. Multivariate analysis was performed on the relationship between rates of survival and pup gender, pup diet, and parental diet. (A) Survival of pups fed the SD or RD. (B) Survival of male and female pups. (C) Survival of male and female pups fed the RD or SD. (D) Survival of pups born to parents fed the SD or RD. (E) Survival of male pups on the SD or RD. (F) Survival of female pups on the SD or RD. (G) Survival of male pups fed the RD and born to parents fed either the SD or RD. (H) Survival of male pups fed the SD born from parents fed either the SD or RD. HR: hazard ratio; 95% CI: 95% confidence interval.

diets. At 150 days, 76% of females and 68% of males were alive (Figure 2B). In multivariate analysis, females survived significantly longer than males (HR: 1.4797; 95% CI: 1.0879-2.0126; P=0.0125). We then studied the influence of diet on gender-specific survival of HbSS-BERK mice (Figure 2C). Compared to the RD, the SD significantly increased the survival of both males (HR: 0.3411; 95% CI: 0.2167-0.5367; P=3.3x10^{-6}) and females (HR: 0.4424; 95% CI: 0.2821-0.6939; P=3.8x10^{-4}). There was not a statistically significant difference between the survival of females and males on the SD (HR: 1.357; 95% CI: 0.9318-1.976; P=0.111), while there was a significant difference between the survival of males and females on the RD (HR: 1.687; 95% CI: 1.008-2.825; P=0.0468).

Several gender-specific differences may explain the longer survival of females than males. Blood from female donors has lower levels of hemolysis than blood from male donors in the SCD population,[16] which may be due to the effects of gender on hemoglobin F levels,[17] red blood cell membrane stability,[18] and nitric oxide bioavailability.[19] Our observations in sickle mice are complementary to those in a long-term follow up of about 3,800 SCD patients with a median survival of 42 and 48 years in males and females, respectively.[20] The Walk-PHaSST study showed a progressively higher proportion of female SCD patients with increasing age, with a lower degree of hemolysis and vasculopathy, a reason for their prolonged survival.[21] Our observations of improved survival of male mice given an enriched diet suggest the significance of dietary interventions in improving survival of males with SCD.

To determine the effect of parents' diet during gestation on their offsprings' survival, we randomly assigned 601 pups born to SD-fed and RD-fed parents to the SD (488 pups) or the RD (113 pups) (Figure 1). At 150 days, 73% of pups of SD-fed parents and 68% of pups of RD-fed parents were alive (Figure 2D). Pups born to parents fed the SD showed a trend towards increased survival, although parental diet did not have a statistically significant impact on survival of pups of both genders together (HR: 0.8878; 95% CI: 0.6071-1.2982; P=0.5392) (Figure 2D). However, male pups with SD-fed parents survived significantly longer than male pups with RD-fed parents (HR: 0.5862; 95% CI: 0.3443-0.997; P=0.049) (Figure 2E). The survival of female pups with parents fed the SD was similar to that of female pups with parents fed the RD (HR: 0.7831; 95% CI: 0.4771-1.286; P=0.335) (Figure 2F). Controlling for diet, we found that parental diet did not statistically significantly affect the survival of male pups on the SD or RD, but a trend towards improved survival of male pups on the SD born to parents fed the RD was observed (Figure 2G,H).

Maternal diet during gestation and lactation influences long-term physical and mental development of the offspring. Suboptimal maternal diet alters mu opioid receptor and dopamine type 1 receptor binding and affects the inflammatory gene expression profile and behavioral responses to stressors in offspring.[22] Intrauterine inflammation led to gender-specific effects on neuroinflammation and behavior in rodents.[23] Since inflammation plays a critical role in the pathobiology of SCD, it is likely that the effects of the dietary modulations in our study may be mediated via modulation of inflammation and its consequences. Our data provide a strong rationale for analyzing the mechanisms underlying improved survival with dietary interventions.

SCD demonstrates the features of disease-related malnutrition with inflammation according to the guidelines for malnutrition from the European Society of Clinical Nutrition and Metabolism (ESPEN).[24] Disease-related malnutrition coupled with social malnutrition requires attention in resource-limited settings, which may have an additive effect on observed mortality in SCD. Our observations provide the proof of principle that targeting malnutrition with dietary interventions can improve survival. Meta-analyses of multiple prospective studies have shown that food-based dietary manipulations to provide combined increases of nitric oxide, anti-oxidants and vitamins, etc. had significant beneficial effects on reducing the risk of cardiovascular disease and all-cause mortality compared to those associated with the intake of individual supplements.[25,26] Therefore, considering the beneficial effect of several individual supplements, including arginine and glutamine on SCD pathobiology, and our observations that dietary interventions improved the survival of sickle mice, further examination of the mechanisms and effectiveness of individual supplements, compared to an enriched diet, on survival in SCD is required. The survival of male pups was prolonged by giving their parents the SD; this novel observation is potentially clinically significant and warrants further investigation into gender-specific effects of dietary interventions.

Om B. Jahagirdar,[1] Aditya M. Mittal,[1*] Waogwende L. Song-Naba,[1] Ritu Jha,[1] Stacy B. Kiven,[1] Susan T. Thompson,[1] John E Connett[2] and Kalpna Gupta[1]*

**OBJ and AMM contributed equally to this manuscript.*

[1]Vascular Biology Center, Division of Hematology, Oncology & Transplantation, Department of Medicine, University of Minnesota, Minneapolis, MN and [2]School of Public Health, Division of Biostatistics, University of Minnesota, Minneapolis, MN, USA.

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Correspondence: KALPNA GUPTA.
gupta014@umn.edu
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