Hydroxycarbamide exposure and ovarian reserve in women with sickle cell disease in the Multicenter Study of Hydroxycarbamide

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Summary
The application of modern ovarian reserve measures to women with sickle cell disease (SCD) may help answer longstanding questions about whether SCD or hydroxycarbamide (HC; also known as hydroxyurea) affect women’s reproductive lifespan. Anti-Müllerian hormone (AMH), an established marker of ovarian reserve, is used to assess the ovarian follicle pool. We used a standard clinical assay to measure AMH in 285 banked samples from 93 female subjects with haemoglobin SS from the historic Multicenter Study of Hydroxyurea (MSH), which led to the United States Food and Drug Administration approval of HC for adults with SCD. No samples from the randomised portion of the MSH remain, so samples from the decade-long MSH follow-up studies were analysed. Most subjects were exposed to HC (86/93). The median AMH levels were lower in study subjects than in age- and sex-matched reference values. The median AMH levels consistent with diminished ovarian reserve, a risk factor for infertility, occurred in subjects starting at the age of 25–30 years; in healthy women, this occurs after the age of 40 years. In multivariate analysis, taking HC was independently associated with a low AMH (β = 0.001, 95% confidence interval −0.002 to 0.000; P = 0.006). These results suggest that ovarian reserve is prematurely reduced in women with haemoglobin SS and raise the possibility that HC contributes to this finding.

Keywords: fertility, ovarian reserve, sickle cell disease, hydroxyurea, anti-Müllerian hormone.

Sickle cell disease (SCD) is a congenital haemoglobinopathy characterised by anaemia, chronic end-organ damage and early death. Near universal survival through childhood has led to the identification of previously unappreciated and consequential end-organ damage.1–3 Whether the ovaries are injured by SCD or SCD therapies is not established.4 Oxidative and hypoxic-ischaemic injury underlies SCD pathophysiology and causes ovarian injury in other settings.4,5 Sickling in the ovarian vasculature may cause primary ovarian insufficiency, a condition defined in by age <40 years with irregular menses and postmenopausal range follicle-stimulating hormone (FSH) levels, indicating depletion of the egg supply and causing inferSCD treatments may indirectly or directly damage the ovaries and compromise existing oocyte quantity and/or quality. For example, iron overload secondary to chronic transfusions,7,8 hydroxycarbamide (HC; also known as hydroxyurea)9 and non-myeloablative haematopoietic stem cell transplant (HSCT)10,11 may compromise oocyte quantity or quality. Identifying whether SCD or its therapies reduce ovarian reserve matters to patients with SCD and parents of affected children, some of whom decline significant, life-changing treatments because of concern that the therapy will compromise future fertility.12–14 Females are born with a finite number of primordial ovarian follicles, representing their entire egg supply, which are continuously recruited over their lifespan. The recruitment of primordial follicles during ovulation causes a progressive decline in the follicle pool.15 Contemporary measures of this follicle pool, called the ovarian reserve, include direct and indirect measure of the ovaries’ appearance and oocyte...
quantity. Primordial follicles produce anti-Müllerian hormone (AMH), an established biomarker of the oocyte pool that is the earliest and most sensitive marker of ovarian reserve,\(^\text{15}\) and that is used to predict response to ovarian stimulation in women experiencing infertility.\(^\text{16}\) AMH levels of <1-1 ng/ml contribute to the definition of diminished ovarian reserve (DOR), a finding that is normal in women aged >40 years, but is a risk factor for infertility in younger women.\(^\text{17}\) AMH is used to assess iatrogenic damage to ovarian reserve before and after exposure to gonadotoxic therapies.\(^\text{18-21}\) Pretreatment AMH predicts post-therapy levels and helps predict if and when the ovarian follicle pool recovers from chemotherapy.\(^\text{22,23}\)

In men, SCD and its therapies are associated with primary and secondary hypogonadism and compromised sperm quality and quantity,\(^\text{24}\) but little is established about ovarian reserve in women with SCD at baseline. A British study compared women with all genotypes of SCD to age-matched women being evaluated for infertility; AMH levels were lower in women with SCD at a younger age compared to controls with infertility.\(^\text{25}\) Although interpretation of this study is somewhat confounded by the inclusion of diverse SCD genotypes, these findings suggest that women with SCD have accelerated ovarian ageing and a reduced reproductive lifespan.

The effects of HC on ovarian reserve are not yet well defined. HC is considered ‘low risk’ for infertility in women, but ovarian follicles may be sensitive to DNA damage caused by this ‘milder’ agent.\(^\text{26}\) Limited data in mice and humans raise concern about the drug’s effect on oocytes: in a study of wild-type mice treated with HC or placebo, HC-treated mice had lower ovarian weight, oestriadiol levels and ovulation rates. Also, while ex vivo fertilisation rates did not differ between treatment groups, embryos with continuous HC exposure failed to develop to the blastocyst stage.\(^\text{27}\) A single-centre study of ovarian reserve in adolescent girls with haemoglobin SS (HbSS) and HbS\(^\text{2}\) found that teenage subjects treated with HC, but not with supportive care, had DOR (24%, \(n = 8\)), a finding associated with older age and longer duration of HC treatment.\(^\text{9}\) This study raised the possibility that HC may reduce ovarian reserve.

The purpose of this study was to test the hypotheses that (i) women who participated in the Multicenter Study of Hydroxyurea (MSH; ClinicalTrials.gov Identifier: NCT00000586) would have lower AMH levels than age- and sex-matched reference values and (ii) that HC exposure is associated with lower AMH levels. These hypotheses were tested by measuring AMH levels in banked samples from the homogenous population of adult women with HbSS who participated in the follow-up and extension studies of the historic MSH, the randomised controlled trial (RCT) that established HC’s efficacy in adults with SCD, leading to HC’s approval by the United States Food and Drug Administration (FDA).\(^\text{28}\)

Patients and methods

This study was approved by the Johns Hopkins University Institutional Review Board (IRB153296).

Patient cohort and samples

After the MSH RCT concluded, consented subjects were followed in the MSH follow-up and extension studies, a non-randomized cohort study that monitored subjects for late toxicities of HC exposure.\(^\text{29}\) The follow-up and extension studies consisted of 10 annual study visits. Subject age at enrolment in the MSH, but not during follow-up visits, was available. Age at follow-up visits was therefore calculated by adding the following values in years: (i) age at enrolment, (ii) number of years the patient was enrolled in the RCT and (iii) number of years from the RCT’s closure to the time of follow-up visit.

AMH measurement

Through the National Heart, Lung, and Blood Institute (NHLBI) Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC), we obtained clinical data from all MSH participants and banked serum samples from all female participants for whom a serum sample was available.\(^\text{30}\) AMH was measured in serum samples using a Clinical Laboratory Improvement Amendments (CLIA)-certified, ultrasensitive electrochemiluminescent assay (Esoterix Laboratory Services, Calabasas, CA, USA). This assay is used in clinical practice by the reproductive endocrinologists at the Johns Hopkins Fertility Center. The analytic range of the assay is 0-0015–15 ng/ml. The intra- and inter-assay sample precision for AMH determination were 9.3% and 12.9% respectively. AMH levels that were beneath the assay’s limit of detection were run in duplicate when sufficient sample was available for analysis; this did not change results for any sample. Samples with AMH levels beneath the lower limit of detection were assigned a value of zero. DOR is a risk factor for developing primary ovarian insufficiency and associated infertility. The assay’s AMH cut-off for DOR is a level of <1.1 ng/ml.

Statistical analysis

Statistical analysis of the results was conducted in R.\(^\text{31}\) Descriptive analyses are reported using ranges, means and medians as appropriate. Samples were analysed according to subject age at the time of specimen collection. We described the AMH levels in the cohort, comparing median AMH values by age for the cohort to reported age- and sex-dependent reference values for the Esoterix assay. To test the hypothesis that HC treatment in the RCT of the MSH would be associated with lower AMH than placebo treatment at any follow-up visit, we compared
subjects’ age-matched AMH values by original MSH randomisation.

We then analysed outcomes among subjects for whom a study sample was available at the first follow-up visit. The rationale for this approach was that this was the visit at which there was a sample available for 56 of 93 subjects at the first follow-up visit, and no other follow-up visit had more individual subjects available for analysis. A second rationale was that this follow-up visit was closest to the RCT portion of the study. We performed a dichotomous analysis of AMH in this sub-group, comparing subjects with DOR to those without DOR. Univariate and multivariate linear regression analysis was used to estimate the strength of association between HC exposure and AMH levels and between mean corpuscular volume (MCV) and AMH levels in patients while adjusting for possible confounders [age, body mass index (BMI), RCT randomisation]. Increased MCV is often, but not always, associated with HC adherence. Established limitations in the MSH study related to tracking HC use in the follow-up studies led to our use of MCV as a proxy measure of HC adherence and exposure.

Precise HC exposure data in the MSH and follow-up studies are difficult to interpret.29 For this reason, we considered HC exposure in several ways (Fig 1). First, we evaluated AMH levels by age at time of sample in all subjects based on whether they were randomised to HC or placebo in the original MSH RCT. This analysis included 93 subjects and all 285 available samples. Second, we evaluated age-adjusted AMH levels based on whether they were taking HC at the time of the first study visit. This analysis included the 56 subjects each with an existing sample from the first study visit. Third, we compared subjects by age with no HC exposure throughout the study period to those with any exposure who were ≤40 years old at the time of their first available sample. Subjects were defined as having no HC exposure if they were randomised to placebo in the original MSH and never took HC thereafter. This analysis examined the AMH level from the first available sample for each subject.

Results

Descriptive

A total of 93 of 153 original female MSH participants had at least one banked sample available for AMH analysis and there were 285 samples available for analysis of AMH at one or more of nine annual follow-up study visits. Figure 1 provides an overview of the study population, the samples available and how they were used in the analyses performed. According to MSH inclusion criteria, all subjects had HbSS disease and averaged three painful crises per year.28 Among the 93 subjects for whom a serum sample was available for analysis: median (interquartile range, IQR) age at enrolment in the RCT was 30 (37) years and BMI was 21.5 (4.6) kg/m². In all, 53 subjects (57%) were randomised to receive HC during the MSH RCT, and they had a median (IQR) of 25.3 (27) months of HC treatment. During the RCT or in the follow-up and extension studies, most subjects (n = 86) took HC at some point. Seven subjects had no HC exposure up to the time of the first AMH level.

Comparison of female MSH participants with (n = 93) and without (n = 60) an available sample showed no difference in the number of subjects randomised to HC (57% vs. 40%, P = 0.0592), median age at enrolment (30 vs. 31.5 years, P = 0.131) or BMI (21.5 vs. 21.2 kg/m², P = 0.153), indicating no selection bias based on clinically relevant factors. As might be expected, subjects for whom a banked serum sample was available were universally enrolled in the MSH follow-up and extension studies, whereas fewer than half of subjects without a banked sample available had enrolled in the MSH follow-up studies (100% vs. 43%, P < 0.01).

AMH levels are low in adult women with SCD

The median AMH level by subject age at the time of the sample is shown in Fig 2A. At every age, the median AMH levels were lower in women with SCD than reference values for age-matched women. AMH levels consistent with DOR occurred in subjects starting at age 25–30 years. In reference norms, as shown, this occurs after the age of 40 years. Figure 2B shows a trend of higher AMH levels in subjects randomised to placebo compared to HC; this difference was significant at ages 30–35 years (median AMH 1.23 vs. 0.87 ng/ml, P < 0.001) and 40–46 years (median AMH 0.045 vs. 0.05 ng/ml, P = 0.005).

AMH levels at first follow-up visit. Fifty-six subjects (60%) had samples available for the first follow-up visit after the MSH RCT closed. The median (IQR) age of this group was 35.9 (11.5) years. Among these subjects, 33 (59%) were randomised to HC and 23 (41%) to placebo in the RCT; 44 (79%) were taking HC at the time of the visit. In univariate analysis (Fig 3), AMH was significantly lower in women aged ≥35 years, in those taking HC and in those with an MCV of ≥100 fl. Variables associated with DOR were analysed with AMH as a dichotomous variable (Table I). Variables associated with low AMH were older age, taking HC at the time of the sample and MCV.

In a multivariable linear regression analysis controlling for age, BMI and original randomisation, MCV was not associated with low AMH [β = −0.019, 95% confidence interval (CI) −0.045 to 0.008; P = 0.181], but taking HC at the time of the study visit was associated with a lower AMH (β = 0.001, 95% CI −0.002 to 0.000; P = 0.006).

AMH levels are normal in reproductive age women with HbSS and no HC exposure. Very few subjects (n = 7) in this study had no HC exposure documented at any point before a sample was available. Figure 4 compares the median AMH levels in
subjects with any HC exposure to those with no HC exposure and to age- and sex-matched reference values. The small number of subjects makes statistical comparison difficult. The four subjects aged <36 years without HC exposure had AMH comparable to reference values while those with HC exposure had AMH levels that were lower than reference values.

**Discussion**

In adult women with HbSS disease in the MSH, almost all of whom were exposed to HC, expected age-associated decline in AMH occurred, but levels were lower than the median levels in age- and sex-matched reference values. AMH is an established measure of the ovarian oocyte pool. Normal values cover a wide range, and extremely low levels are a risk factor for infertility. In this cohort, the median AMH levels consistent with DOR occurred between 25 and 30 years of age, well before the expected age of ≥40 years. The American College of Obstetrics and Gynecology recently called for further study of AMH in women with risks of premature ovarian ageing or infertility. The preponderance of AMH levels consistent with DOR in women with HbSS and HC exposure need further study.

These findings add to two previous studies of ovarian reserve in SCD. Kopeika et al. also found that AMH was lower in women with SCD and that low AMH occurred at an earlier age in women with SCD compared to controls. However, Kopeika et al. included women with compound heterozygous SCD and few subjects (n = 8) took HC. Also, Kopeika et al. found that most subjects aged 25–30 years had normal AMH, but we identify AMH levels consistent with DOR starting in this age group. This difference might be explained by the exclusive analysis of women with HbSS in our cohort and/or that most subjects in our study had HC exposure. While it is plausible that both severe forms of SCD and HC treatment may accelerate reductions in AMH, we found normal AMH in the few subjects aged 20–35 years without HC exposure. Elchuri et al. studied AMH in adolescent girls with HbSS and HbSβ⁰ and also identified normal AMH in subjects without HC exposure and DOR in some HC-exposed subjects. The presence of normal AMH in two small, HC-naive populations with sickle cell anaemia suggests that the disease alone may not reduce ovarian reserve in young women. Further clarification of the effects of HC on ovarian reserve will be especially important for the emerging generation of young adults who started HC in infancy or early childhood, for girls and women treated in sub-Saharan Africa where both fixed-dose and maximally tolerated dose treatment strategies are under investigation, and in women pursuing fertility preservation interventions before HSCT or gene therapy.
Many patients, caregivers and prescribers are concerned about whether HC affects female fertility. Our results, especially given their harmony with previous publications, may conservatively inform the care of women with sickle cell anaemia. Existing recommendations for initiating infertility evaluations are age dependent: if aged ≥35 years, evaluation is indicated after failure to conceive for ≥6 months of unprotected intercourse, if aged <35 years, evaluation is indicated after ≥12 months of unprotected intercourse. These cut-offs reflect the more rapid age-associated decline in ovarian reserve in healthy women after the age of 35 years.36 DOR is seen in this study population at age 25–30 years, suggesting that standard age-based indications for infertility evaluations may not apply to women with HbSS. Given the
Table I. Comparison of subjects at first follow-up visit by dichotomized anti-Müllerian hormone (AMH) shows that AMH levels consistent with diminished ovarian reserve are associated with age, taking hydroxycarbamide (HC) and higher mean corpuscular volume (MCV).

<table>
<thead>
<tr>
<th>Age at enrolment, years, median (IQR)</th>
<th>AMH ≥1.1 ng/ml (n = 14)</th>
<th>AMH &lt;1.1 ng/ml (n = 42)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at first follow-up visit, years, median (IQR)</td>
<td>24.5 (8.0)</td>
<td>34.5 (9.0)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Months after MSH enrolment to follow-up visit, months, median (IQR)</td>
<td>29.1 (7.5)</td>
<td>39.3 (8.1)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>BMI, kg/m², median (IQR)</td>
<td>20.1 (2.1)</td>
<td>21.3 (7.3)</td>
<td>0.54</td>
</tr>
<tr>
<td>Received HC in RCT, n (%)</td>
<td>7 (50)</td>
<td>26 (62)</td>
<td>0.43</td>
</tr>
<tr>
<td>Months HC exposure during RCT, median (IQR)</td>
<td>11.0 (28.0)</td>
<td>23.5 (33.0)</td>
<td>0.44</td>
</tr>
<tr>
<td>ANC, × 10³/µL, median (IQR)</td>
<td>4.7 (2.2)</td>
<td>5.6 (3.8)</td>
<td>0.75</td>
</tr>
<tr>
<td>MCV, fl, median (IQR)</td>
<td>95.8 (11.9)</td>
<td>105.3 (14.3)</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

ANC, absolute neutrophil count; AMH, anti-Müllerian hormone; MSH, Multicentre Study of Hydroxyurea; BMI, body mass index; IQR, interquartile range; RCT, randomised controlled trial.

†P value was calculated using Wilcoxon rank-sum test for continuous variables and Pearson chi-square test for categorical variables.

*Indicates statistical significance defined as P ≤ 0.05.

Fig 4. Anti-Müllerian hormone (AMH) is normal in young women without hydroxyureabamid (HC) exposure. Bar graphs show an age-stratified comparison of subjects ≤40 years who were never exposed to HC and those exposed to HC. Only one sample per subject is included and the included sample for each subject was their earliest AMH level. AMH levels in subjects aged ≤35 years with no HC exposure is normal for age, while those with any HC exposure are low. The few HC unexposed subjects preclude statistical comparison.

intense interest in biological reproduction captured in studies of the parents of children with SCD and young adults with SCD,12,13,37 counselling about the potential for a reduced reproductive lifespan, at least in women with HbSS treated with HC, may be indicated. Further studies may inform approaches to treatment, fertility preservation, and how HC treatment compares to gonadotoxic HSCT regimens.

The present study has several strengths. Subjects enrolled in the MSH represent a homogenous population of adult women only with HbSS. This is the largest study of AMH levels in girls or women with SCD to date and included mostly HC-exposed subjects. This study addresses a significant challenge in studying ovarian reserve in women with SCD: differentiating the possible effects of HC from those of SCD complications on ovarian reserve. This problem is further confounded by the higher likelihood that adults with more SCD complications at baseline will take HC. In this study population, all subjects met disease severity inclusion criteria to participate in the MSH.38 If disease complications rather than HC treatment accounted for diminishment in ovarian reserve, we would expect lower AMH levels in placebo-treated subjects who had more SCD complications in the MSH.28 This study shows the opposite: placebo-treated subjects had higher AMH levels, thus implicating HC rather than disease complications in the finding of low AMH levels.

This study is limited because the primary outcome of the MSH was unrelated to AMH and the study was not designed to determine whether SCD or HC were associated with AMH levels. HC exposure data are, as noted, difficult to parse in the MSH extension and follow-up studies,29 furthermore nearly the whole population was HC exposed. There are limited definitive conclusions that can be drawn about HC’s effect on ovarian reserve. This study offers no information about the effect of HC on AMH when started in infancy or before puberty, about ovarian reserve in women with compound heterozygous forms of SCD, and lacks historic race- and age-matched controls for comparison. Finally, while AMH is an established marker of ovarian reserve, it does not predict spontaneous pregnancy in women without a diagnosis of infertility.39

The findings from this historic cohort suggest that HbSS and HC therapy are risk factors for early ovarian ageing and a reduced reproductive lifespan. HC remains a fundamental and life changing SCD therapy. Prospective studies are needed to determine whether HC use is associated with poor oocyte quantity or quality during fertility preservation procedures, to definitively determine the effect of HC on ovarian reserve and to establish whether this leads to infertility. Such studies will enhance the informed consent process for HC initiation and may lead to new care standards to shape family planning guidance, specialist referrals to reproductive endocrinology and indications for fertility preservation for girls and women with SCD.
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Conflict of interest
The authors declare no competing financial interests.

Author contributions
Lydia H. Pecker, Sophie Lanzkron and Mindy S. Christianon designed the study, interpreted the data and wrote the paper. Sarah Hussain analysed and interpreted the data and wrote the paper.

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