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

Lydia H. Pecker^{1,2}, Sarah Hussain¹, Mindy Christianson^{2,3}, Sophie Lanzkron¹

¹Division of Hematology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD.

²Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, Baltimore, MD.

³Division of Reproductive Endocrinology, Johns Hopkins University School of Medicine, Baltimore, MD.

Abstract

The application of modern ovarian reserve measures to women with sickle cell disease (SCD) may help answer longstanding questions about whether SCD or hydroxycarbamide affect women's reproductive lifespan. Antimüllerian 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 FDA approval of hydroxycarbamide for adults with SCD. No samples from the randomized portion of the MSH remain, so samples from the decade-long MSH follow-up studies were analyzed. Most subjects were exposed to hydroxycarbamide (86/93). Median AMH levels were lower in study subjects than in age- and sex-matched reference values. Median AMH levels consistent with diminished ovarian reserve, a risk factor for infertility, occurred in subjects starting at age 25–30 years; in healthy women, this occurs after age 40. In multivariate analysis, taking hydroxycarbamide was independently associated with a low AMH (Est Std .001, 95% CI –0.002 – 0.000, $p=.006$). These results suggest that ovarian reserve is prematurely reduced in women with haemoglobin SS and raise the possibility that hydroxycarbamide contributes to this finding.

Keywords

Fertility; Women; Ovarian Reserve; Sickle Cell Disease; Hydroxyurea; Antimüllerian Hormone; Infertility

Corresponding Author: Lydia H. Pecker, MD, Division of Hematology, Johns Hopkins University School of Medicine, 720 Rutland Ave, Ross Research Building, Room 1025, Baltimore, MD 21205, Ph: 410 502-2546, Fax: 410-955-0185, lpecker1@jh.edu.

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Introduction

Sickle cell disease (SCD) is a congenital haemoglobinopathy characterized 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 (Niss *et al*, 2017; Lebensburger *et al*, 2019; Vichinsky *et al*, 2010). Whether the ovaries are injured by SCD or SCD therapies is not established (Chase *et al*, 2009). Oxidative and hypoxic-ischemic injury underlies SCD pathophysiology and causes ovarian injury in other settings (Soleimani *et al*, 2011; Agarwal *et al*, 2005; Chase *et al*, 2009). Sickling in the ovarian vasculature may cause primary ovarian insufficiency, a condition defined in women under 40 years by irregular menses and follicle-stimulating hormone levels in the post-menopausal range, indicating depletion of the egg supply and causing infertility (Chase *et al*, 2009). SCD treatments may indirectly or directly damage the ovaries and compromise existing oocyte quantity and/or quality. For example, iron overload secondary to chronic transfusions (Fung *et al*, 2006; Chang *et al*, 2011), hydroxycarbamide (Elchuri *et al*, 2015) and non-myeloablative haematopoietic stem cell transplant (HSCT) (Pecker *et al*, 2018; Carpinello *et al*, 2018) 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 (Nahata *et al*, 2017; Oyeku *et al*, 2013; Thompson *et al*, 2013).

Females are born with a finite number of primordial ovarian follicles, representing their entire egg supply, that are continuously recruited over their lifespan. The recruitment of primordial follicles during ovulation causes a progressive decline in the follicle pool (Li *et al*, 2014). 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 antimüllerian hormone (AMH), an established biomarker of the oocyte pool that is the earliest and most sensitive marker of ovarian reserve, (Li *et al*, 2014) and that is used to predict response to ovarian stimulation in women experiencing infertility (Visser *et al*, 2012). AMH levels beneath 1.1 ng/mL contribute to the definition of diminished ovarian reserve (DOR), a finding that is normal in women over 40 years, but is a risk factor for infertility in younger women (Pastore *et al*, 2018). AMH is used to assess iatrogenic damage to ovarian reserve before and after exposure to gonadotoxic therapies (Peigné & Decanter, 2014; Perdrix *et al*, 2017; Elchuri *et al*, 2016; Helden & Weiskirchen, 2017). Pre-treatment AMH predicts post-therapy levels and helps predict if and when the ovarian follicle pool recovers from chemotherapy (Su *et al*, 2014; Anderson & Wallace, 2013).

In men, SCD and its therapies are associated with primary and secondary hypogonadism and compromised sperm quality and quantity (Berthaut *et al*, 2017), 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 (Kopeika *et al*, 2019). 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 aging and a reduced reproductive lifespan.

The effects of hydroxycarbamide on ovarian reserve are not yet well defined. Hydroxycarbamide is considered “low risk” for infertility in women, but ovarian follicles are sensitive to DNA damage caused by this “milder” agent (Bedoschi *et al*, 2016). Limited data in mice and humans raises concern about the drug’s effect on oocytes: wild-type mice treated with hydroxycarbamide or placebo found that the hydroxycarbamide treated mice had lower ovarian weight, estradiol levels, and ovulation rates; while ex-vivo fertilization rates did not differ between groups, embryos with continuous Hydroxycarbamide exposure failed to develop to the blastocyst stage (Sampson *et al*, 2010). A single-center study comparing AMH and follicle stimulation hormone in adolescent girls with HbSS and haemoglobin S β^0 (HbS β^0) found that teen subjects treated with hydroxycarbamide, but not with supportive care, had DOR (24%, n=8), a finding associated with older age and longer duration of hydroxycarbamide treatment (Elchuri *et al*, 2015). This study raised the possibility that hydroxycarbamide may reduce ovarian reserve.

The purpose of this study was to test the hypotheses that women who participated in the MSH would have lower AMH levels than age- and sex-matched reference values and (2) that hydroxycarbamide 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 haemoglobin SS (HbSS) who participated in the Follow-up and Extension studies of the historic Multicenter Study of Hydroxyurea (MSH), the randomized controlled trial (RCT) that established hydroxycarbamide’s efficacy in adults with SCD, leading to hydroxycarbamide’s FDA approval (Charache *et al*, 1995a).

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 hydroxycarbamide exposure (Ballas *et al*, 2009). The Follow-up and Extension Studies consisted of 10 annual study visits. Subject age at enrollment 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: a) age at enrollment, b) number of years patient was enrolled in the RCT and c) number of years from the RCT’s closure to the time of follow-up visit.

AMH measurement

Through the NHLBI Biorepository, BioLINCC, we obtained clinical data from all MSH participants and banked serum samples from all female participants for whom a serum sample was available (Giffen *et al*, 2017). The serum samples were measured using a CLIA-certified, ultrasensitive electrochemiluminescent assay (Esoterix Laboratory Services, Calabasas, CA). 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 resulted 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 less than 1.1ng/mL.

Statistical analysis

Statistical analysis of the results was conducted in R(Team, 2019). Descriptive analyses are reported using ranges, means and medians as appropriate. Samples were analyzed 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 hydroxycarbamide treatment in the RCT of the MSH would be associated with lower AMH than placebo treatment at any follow-up visit, we compared age-matched AMH values by original MSH randomization.

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

Precise hydroxycarbamide exposure data in the MSH and follow-up studies is difficult to interpret(Ballas *et al*, 2009). For this reason, we considered hydroxycarbamide exposure in several ways (Figure 1). First, we evaluated AMH levels among all subjects based on whether they were randomized to hydroxycarbamide 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 hydroxycarbamide at the time of the first study visit. This analysis included the 56 subjects with an existing sample from the first study visit. Third, we compared subjects by age with no hydroxycarbamide 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 hydroxycarbamide exposure if they were randomized to placebo in the original MSH and never took hydroxycarbamide thereafter. This analysis examined AMH level from the first available sample for each subject.

Results

Descriptive

Ninety-three 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, samples and those used in the analyses performed. According to MSH inclusion criteria, all subjects had HbSS disease and averaged 3-painful crises per year (Charache *et al*, 1995a). Among the 93-subjects for whom a serum sample was available for analysis: median age at enrollment in the RCT was 30 years (Inter-Quartile Range (IQR) 37) and BMI was 21.5kg/m² (IQR 4.6). Fifty-three (57%) were randomized to receive hydroxycarbamide during the MSH RCT, and they had a median of 25.3 (IQR 27) months of hydroxycarbamide treatment. During the RCT or in the Follow-up and Extension studies, most subjects (n=86) took hydroxycarbamide at some point. Seven subjects had no hydroxycarbamide 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 randomized to hydroxycarbamide (57% vs 40%, p=0.0592), median age at enrollment (30 vs 31.5 years, p=0.131) or BMI (21.5 vs 21.2kg/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 Figure 2A. At every age, AMH levels were lower in women with SCD than reference values for age-matched women. Median AMH levels consistent with DOR occurred in subjects starting at age 25–30 years of age; in reference norms, this occurs after 40 years of age. Figure 2B shows a trend of higher AMH levels in subjects randomized to placebo compared to hydroxycarbamide; this difference was significant at ages 30 – 35 (median AMH 1.23ng/mL vs 0.87ng/mL, p<0.001) and 40 – 46 years old (median AMH 0.045ng/mL vs 0.05ng/mL, p=.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 age of this group was 35.9 (IQR 11.5) years. Among these subjects, 33 (59%) were randomized to hydroxycarbamide and 23 (41%) to placebo in the RCT, and 44 (79%) were taking hydroxycarbamide at the time of the visit. In univariate analysis (Figure 3), AMH was significantly higher in women under age 35 years, not taking hydroxycarbamide and with an MCV less than 100fL. Variables associated with DOR were analyzed with AMH as a dichotomous variable (Table 1). Variables associated with low AMH were older age, taking hydroxycarbamide at the time of the visit, and MCV.

In a multi-variable linear regression analysis controlling for age, BMI and original randomization, MCV was not associated with low AMH (Est Std -0.019 , 95% CI $-0.045 - 0.008$, $p=.181$), but taking hydroxycarbamide at the time of the study visit was associated with a lower AMH (Est Std $.001$, 95% CI $-0.002 - 0.000$, $p=.006$).

AMH levels are normal in reproductive age women with HbSS and no hydroxycarbamide exposure—Very few subjects ($n=7$) in this study had no hydroxycarbamide exposure documented at any point before a sample was available. Figure 4 compares median AMH levels between subjects with any hydroxycarbamide exposure, no hydroxycarbamide exposure and to age- and sex-matched reference values. The small number of subjects makes statistical comparison difficult, however in the four subjects under age 36 years without hydroxycarbamide exposure, AMH was comparable to reference values.

Discussion

In adult women with HbSS disease in the MSH, almost all of whom were exposed to hydroxycarbamide, expected age-associated decline in AMH occurred, but levels were lower than 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, median AMH levels consistent with DOR occurred between 25 and 30 years of age, well before the expected age of 40 more more years. The American College of Obstetrics and Gynecology recently called for further study of AMH in women with risks of premature ovarian aging or infertility (ACOG Committee Opinion No. 773: The Use of Antimüllerian Hormone in Women Not Seeking Fertility Care., 2019). The preponderance of AMH levels consistent with DOR in women with HbSS of reproductive age in this study suggests that women with HbSS and hydroxycarbamide exposure need further study.

Our findings add to two previous studies of ovarian reserve in SCD. Kopeika et al also found that AMH is lower in women with SCD and that low AMH occurred at an earlier age in women with SCD compared to controls (Kopeika *et al*, 2019). However Kopeika et al included women with compound heterozygous SCD, few subjects ($n=8$) took hydroxycarbamide., and found that most subjects ages 25 – 30 years had normal AMH. In contrast, we find AMH levels consistent with DOR starting at 25 – 30 years. 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 hydroxycarbamide exposure. While it is plausible that both severe forms of SCD and hydroxycarbamide treatment may accelerate reductions in AMH, we found normal AMH in the few subjects aged 20 – 35 years without hydroxycarbamide exposure. A previous study of AMH in adolescent girls with HbSS and HbS β^0 similarly found normal AMH in subjects without hydroxycarbamide exposure and DOR in some hydroxycarbamide exposed subjects (Elchuri *et al*, 2015). The presence of normal AMH in two, small hydroxycarbamide naive populations with sickle cell anaemia suggests that SCD itself may not affect ovarian reserve in young women of reproductive age. Further clarification of the effects of hydroxycarbamide on ovarian reserve is especially important for the emerging generation of young adults who started hydroxycarbamide in infancy or

early childhood(Thornburg *et al*, 2012; Hankins *et al*, 2014), for girls and women treated in sub-Saharan Africa where both fixed dose and maximally tolerated dose treatment strategies are under investigation(Akingbola *et al*, 2019), and in women pursuing fertility preservation interventions before HSCT or gene therapy(Pecker *et al*, 2018; Carpinello *et al*, 2018).

Many patients, caregivers and prescribers are concerned about whether hydroxycarbamide affects female fertility. Our results, especially given their harmony with previous publications, may conservatively inform the care of women with HbSS. Existing recommendations for initiating infertility evaluations depends on age: if over age 35-years, work-up is indicated after failure to conceive for 6 or more months of unprotected intercourse, if under age 35-years of age evaluation is indicated after at least 12 of unprotected intercourse. These cut-offs reflect the more rapid age-associated decline in ovarian reserve in healthy women after age 35 years(Practice Committee of the American Society for Reproductive Medicine. Electronic address: ASRM@asrm.org, 2020). Since DOR is seen in our study population at age 25 – 30 years, standard indications for infertility evaluations may not apply to women with HbSS. Given the intense interest in biological reproduction captured in studies of the parents of children with SCD and young adults with SCD(Oyeku *et al*, 2013; Chakrabarti & Bareford, 2007; Nahata *et al*, 2017), counseling about the potential for a reduced reproductive lifespan, at least in women with HbSS treated with hydroxycarbamide, may be indicated. Further studies will inform approaches to treatment, fertility preservation or how hydroxycarbamide treatment compares to gonadotoxic HSCT regimens.

This study has several strengths. Subjects enrolled in the MSH represent a homogenous population of adult women only with HbSS disease. This is the largest study of AMH levels in girls or women with SCD to date and included mostly hydroxycarbamide-exposed subjects. This study addresses a significant challenge in studying ovarian reserve in women with SCD: differentiating the possible effect of hydroxycarbamide from those of SCD complications on ovarian reserve. This is further confounded by the higher likelihood that adults with severe SCD at baseline will take hydroxycarbamide. In our study, the study population met disease severity inclusion criteria to participate in the MSH(Charache *et al*, 1995b). If disease complications rather than hydroxycarbamide treatment accounted for diminishment in ovarian reserve, we would expect lower AMH levels in placebo-treated subjects who had more disease complications in the MSH(Charache *et al*, 1995a). This study shows the opposite: placebo-treated subjects had higher AMH levels thus implicating hydroxycarbamide 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 hydroxycarbamide were associated with AMH levels. Hydroxycarbamide exposure data is, as noted, difficult to parse in the MSH Extension and Follow-Up Studies(Ballas *et al*, 2009), furthermore nearly the whole population was hydroxycarbamide-exposed. There are limited definitive conclusions that can be drawn about hydroxycarbamide's effect on ovarian reserve. This study offers no information about the effect of hydroxycarbamide on AMH when started in infancy or before puberty, about ovarian reserve in women with compound heterozygous forms of SCD, and there are no historic race and age matched controls available 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 (Steiner *et al*, 2017).

The findings from this historic cohort suggest that HbSS and hydroxycarbamide therapy are risk factors for early ovarian aging and a reduced reproductive lifespan. Hydroxycarbamide remains a fundamental and life changing SCD therapy. Prospective studies are needed to determine whether hydroxyurea use is associated with poor oocyte quantity or quality during fertility preservation procedures, to definitively determine the effect of hydroxycarbamide on ovarian reserve and to establish whether this leads to infertility. Such studies will enhance the informed consent process for hydroxycarbamide initiation and may lead to new care standards to shape family planning guidance, specialist referrals to reproductive endocrinology and indications for fertility preservation for affected girls and women.

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Abbreviations

AMH	Antimüllerian Hormone
BMI	Body Mass Index
DOR	Diminished Ovarian Reserve
HbSβ^0	Haemoglobin S β^0
HbSS	Haemoglobin SS
HC	Hydroxycarbamide
HSCT	Haematopoietic Stem Cell Transplant
IQR	Inter-Quartile Range
MSH	Multicenter Study of Hydroxyurea
NHLBI	National Heart, Lung, Blood Institute
POI	Primary ovarian insufficiency
RCT	Randomized Controlled Trial
SCD	Sickle Cell Disease

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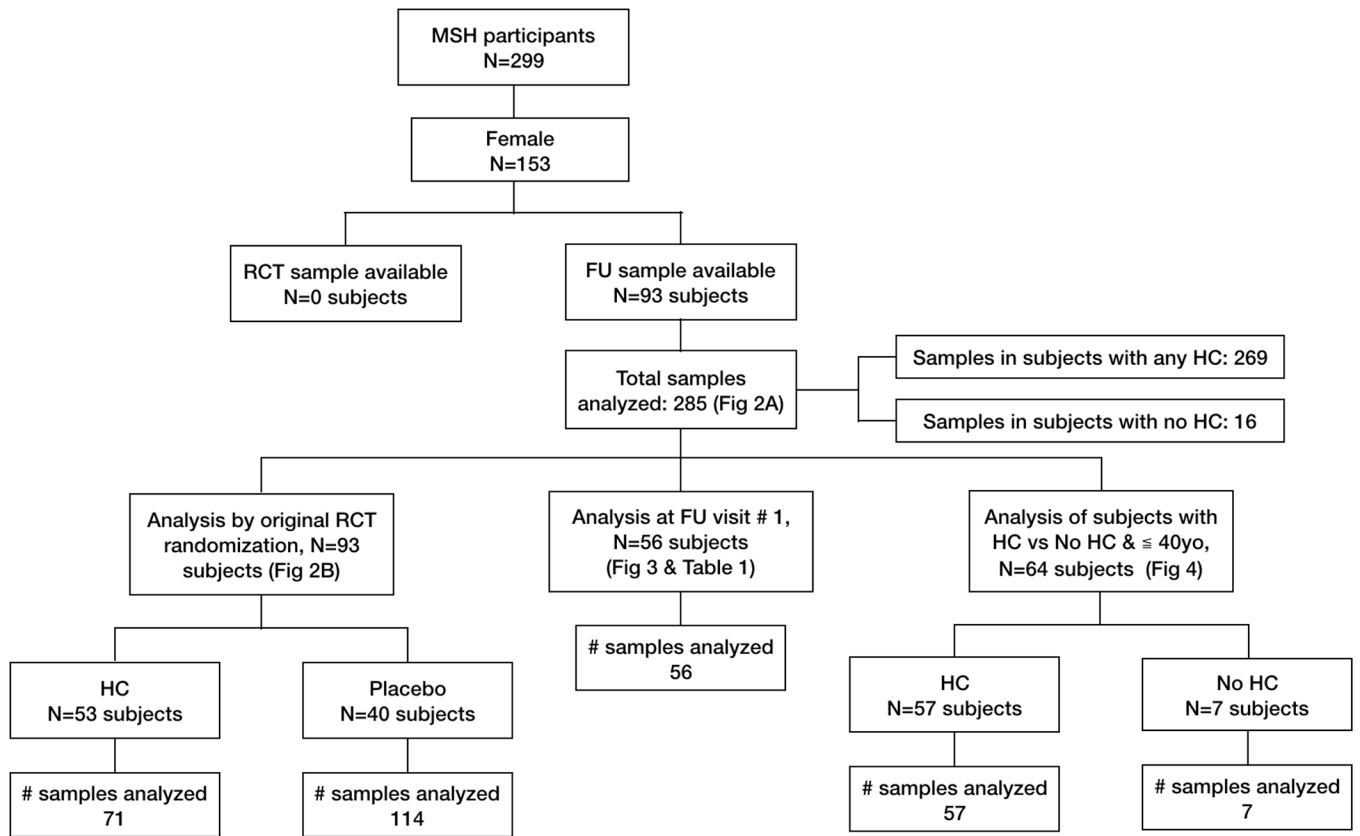
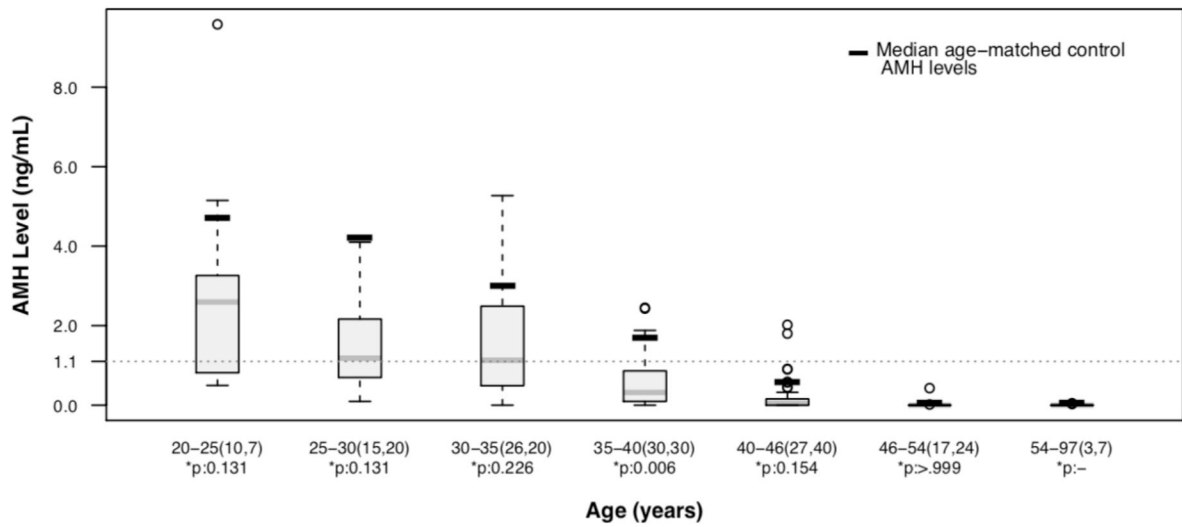


Figure 1. Explanation of study population and sample availability.

This figure outlines the study population, sample availability by hydroxycarbamide exposure, and populations and samples used in study analyses (Figures 2 – 4 and Table 1).

A



B

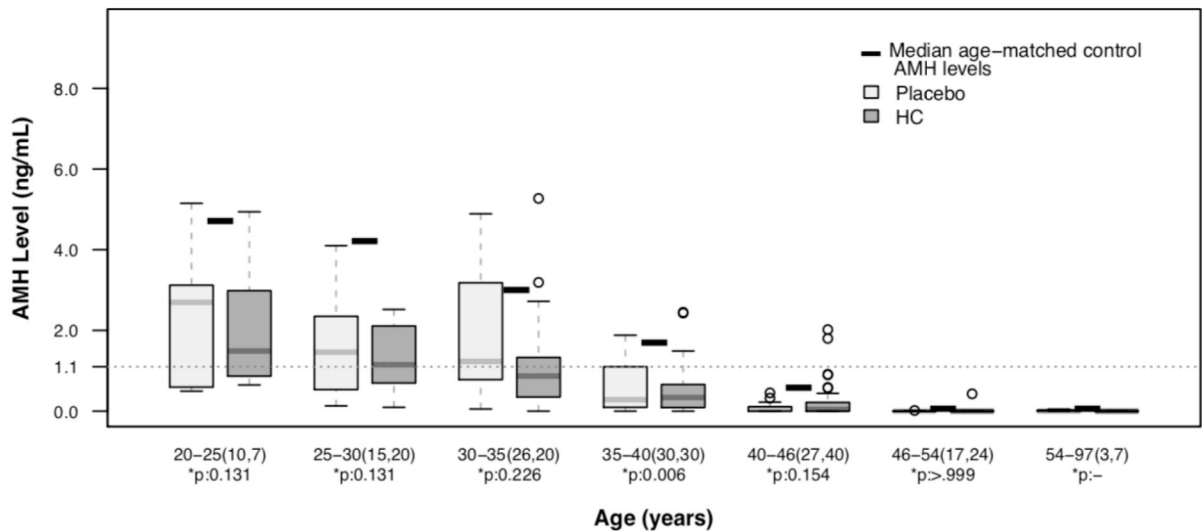


Figure 2. AMH is lower than reference values for age in women with HbSS.

Box plots of 285 AMH levels in 93 subjects by subject age at time of specimen collection. As expected, AMH decreased in an age-dependent manner. Median AMH by age as reported for this assay is indicated by a thick black line. Median AMH for age is lower than median age-dependent values in the general population. The dotted line indicates AMH less than 1.1ng/mL, a level consistent with diminished ovarian reserve, and a risk factor for infertility. **(A)** Most study subjects had an AMH less than 1.1ng/mL by age 30 years. **(B)** Box plots of AMH level by subject age and original randomization in the MSH.

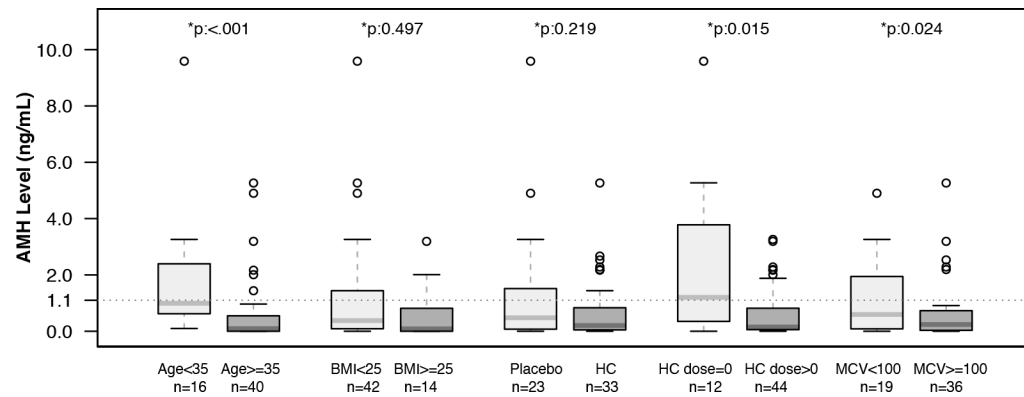


Figure 3. Low AMH is associated with age, taking hydroxycarbamide and MCV.

Box plots of univariate analysis of AMH in the first MSH follow-up visit. Fifty six subjects had a sample available at the first visit. Box plots compare subjects < 35 years of age, with BMI < 25 , by randomization to placebo or hydroxycarbamide in the MSH RCT, hydroxycarbamide use at the time of sample, and MCV < 100 fL. AMH is lower in older subjects, those taking hydroxycarbamide and those with an MCV greater than 100fL.

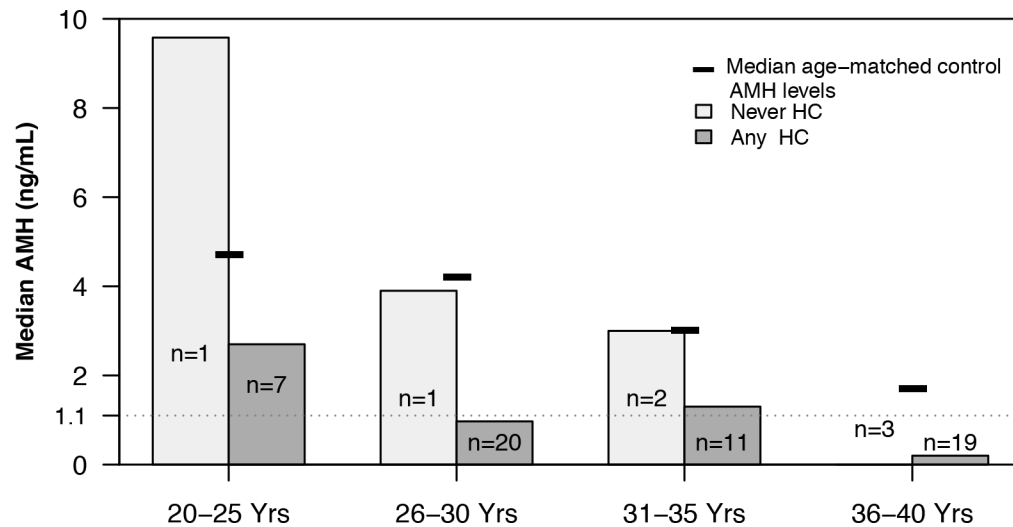


Figure 4. AMH is normal in young women without hydroxycarbamide exposure. Bar graphs show an age-stratified comparison of subjects never exposed to hydroxycarbamide and those exposed to hydroxycarbamide when ≤ 40 years of age. Only one sample per subject is included and the included sample for each subject was their earliest AMH level. AMH levels in subjects 35 years of age or less with no hydroxycarbamide exposure is normal for age while those with any hydroxycarbamide exposure are low. The few hydroxycarbamide unexposed subjects preclude statistical comparison.

Table 1.

Comparison of subjects at first follow-up visit by AMH shows AMH levels consistent with diminished ovarian reserve are associated with age, taking HC and higher MCV.

	<i>AMH</i> <i>1.1</i> <i>(n=14)</i>	<i>AMH < 1.1</i> <i>(n=42)</i>	<i>p-value</i> **
Age at enrollment, years	24.5 (8.0)	34.5 (9.0)	<0.0001
Age at first follow-up visit, years	29.1 (7.5)	39.3 (8.1)	<0.0001*
Months after MSH enrollment to follow-up visit, months median(IQR)	29.5 (8.75)	31(6)	0.35
BMI, kg/m², median (IQR)	20.1 (2.1)	21.3 (7.3)	0.54
Received HC in RCT, n(%)	7 (50%)	26 (62%)	0.43
Taking HC at time of sample, n(%)	8 (57%)	36 (86%)	0.024*
Months HC exposure during RCT, median (IQR)	11.0 (28.0)	23.5(33.0)	0.44
ANC, K/mcL, median (IQR)	4.7 (2.2)	5.6 (3.8)	0.75
MCV, fL, median (IQR)	95.8 (11.9)	105.3 (14.3)	0.03*

** p-value was calculated using Wilcoxon rank-sum test for continuous variables and Pearson Chi-Square test for categorical variables