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ANTI-MÜLLERIAN HORMONE AND SKELETAL AGE IN FEMALE CHILDREN

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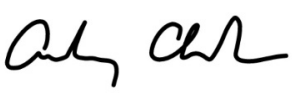

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ANTI-MÜLLERIAN HORMONE AND SKELETAL AGE IN FEMALE CHILDREN

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Anti-müllerian hormone (AMH) plays an important role in sex differentiation of fetuses as well as in fertility in adulthood. During adolescence, AMH levels can be indicative of many disorders of sexual development for both males and females. In adults, AMH levels serve as an indication of the ovarian reserve. In adolescent girls, however, the role of the AMH during human growth and development has not been as well established. As skeletal age has been considered the best singular indication of maturity, and AMH had never previously been described by skeletal age, this study sought to describe patterns of change in AMH in relation to skeletal and chronological age and to assess the influence of skeletal maturation on AMH levels. This secondary data analysis study consisted of a subset of eighty-eight female Fels Longitudinal Study participants ranging in age from 8 to 18 years.

Data analysis was completed in two phases. Phase I was a cross-sectional analysis that utilized linear regression modeling to examine log transformed AMH (AMH_{log}) levels by relative age, a difference between skeletal and chronological age, in a forward selection approach while adjusting for adiposity and cardiometabolic factors. Phase II was a longitudinal data analysis that utilized generalized linear mixed effect modeling to

investigate AMH_{log} levels by relative age, also in a forward selection approach while adjusting for adiposity and cardiometabolic factors.

Findings from Phase I analyses revealed that relative skeletal age was significantly related to AMH_{log} (β= -0.177, SE= 0.053, p= 0.001) while accounting for chronological age. Sex steroid binding globulin was also a significant predictor of AMH_{log} (β= 0.006, SE= 0.002, p= 0.011). Phase II results demonstrated that relative age was significantly related to changes in AMH_{log} (β= -0.073, SE= 0.032, p= 0.023). Also, glucose was significantly associated with the changes in AMH_{log} (β= -0.008, SE= 0.004, p= 0.044) in the adjusted analysis.

In conclusion, AMH_{log} was found to have a similar relationship with skeletal age as it does with chronological age. However, a decrease in AMH_{log} levels was identified in individuals who experienced later maturation among relatively healthy adolescent girls in both cross-sectional and longitudinal analyses.

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BACKGROUND

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Anti-Müllerian Hormone

The function of the anti-müllerian hormone, or AMH, varies depending on the sex and age of an individual. AMH is considered to be essential in the sex differentiation of a fetus as it plays a role in determining which of either the Müllerian duct or Wolffian duct further develops.[1] Both male and female fetuses have bipotential gonads as well as a Müllerian and Wolffian ducts.[2] In developing males *in-utero*, AMH is produced in the Sertoli cells of the testis and initiates the regression of the Müllerian ducts[4] that would otherwise develop into the female reproductive organs.[1] After the production of AMH and regression of the Müllerian duct in the male fetus, the Wolffian duct develops into male reproductive organs. This includes the epididymis, testes, seminal vesicle, and vas deferens.[1,3] Conversely in a female fetus, given the absence of AMH, the Wolffian duct regresses and the Müllerian duct then allows for the development of the female reproductive organs including the fallopian tubes, uterus, and upper vagina.[1,4]

In females, AMH levels are detectable in fetal ovaries at 36 weeks gestation.[5] AMH is produced in the granulosa cells that surrounds the oocyte, or immature egg cell, in the preantral and small antral follicles of the ovary.[1,6] During the preantral and small antral phases of follicular development AMH is secreted,[6] meaning that AMH levels increase as preantral and small antral follicle count increases.[1] Therefore, when the preantral and small antral follicle count gradually declines as a woman's age increases, the decrease in

production and serum level of AMH can be referenced as an indication of a woman's ovarian reserve.[6]

The majority of AMH research has been conducted in regard to fertility and the ovarian reserve in adult women. For adolescent females, AMH research largely focuses on menstrual disorders. Moreover, polycystic ovarian syndrome and AMH levels in adolescences have been widely researched. Common findings report that AMH levels are elevated in adolescents who have polycystic ovaries.[7, 8, 9, 10] Other research areas explore AMH hormone with respect to a variety of other menstrual and chromosomal conditions, such as oligomenorrhea and Turner Syndrome among others. Recently, Smith *et al.* examined the relationship between AMH and pubertal milestones in relatively healthy female children and found AMH to have a significant, non-linear relationship with chronological age.[11] This study is a companion study that proposes to expand the knowledge garnered from the aforementioned research, which examined the relationship between AMH and pubertal sexual milestones, and noted the non-linear relationship with chronological age.[11] In particular, this study aimed to explore AMH levels relative to skeletal maturity, a maturation indicator of the skeleton rather than the maturation of secondary sex characteristics (e.g., breast development), in the same population of relatively healthy female children. Notably, AMH research in adolescents predominantly focuses on those with other pre-existing conditions and literature regarding AMH in healthy adolescent populations is limited.

Skeletal Age

Skeletal age is a measure of skeletal maturation, which further allows for the assessment of the developmental processes of bone.[12] As such, skeletal age is a tool that can

be utilized as an alternative method for evaluating growth and maturity in children. Determining skeletal age is a non-invasive method that typically relies on radiograph images[13] and is reported in years, similar to chronological age. Skeletal age is relatively precise and reliable compared to other methods for evaluating maturation such as sexual maturity.[14] Sexual maturity is assessed using secondary sex characteristics such as pubic hair, breast, or genital development. These methods, although easy, are typically arbitrary and limiting as they can only be assessed after the onset of puberty. For this reason, skeletal age is commonly considered the best singular indicator of maturation.[14]

There are several assessment methods for determining skeletal age including the Fels method for the hand-wrist. The Fels method for the hand-wrist was created by analyzing nearly 1400 radiographic images of Fels participants over the course of several decades. By means of a mathematical process, the Fels method for the hand-wrist combines several indicators of skeletal age to create a single estimate along with a standard error. The creation of the assessment began with identifying potential maturity indicators in the hands and wrist of children. Maturity indicators are centers of ossification[15] and because the skeletal maturation process is similar among all people[13] these centers of ossification can be used to predict chronological age. The potential indictors that were found to be reliable, valid, complete, visually universal, and discriminated within chronological age groups were used in the testing and development of the hand-wrist method.[14, 15] Further testing was completed after the final indicators for the Fels method for hand-wrist skeletal age assessment were identified.[15]

The final Fels method for hand-wrist is comprised of 85 grade maturity indicators including indicators for the radius, ulna, carpals, metacarpals, and phalanges.[14] Furthermore, 13 epiphyseal to diaphyseal ratios of these bones are included in the final method. Chronological age and sex determine how many maturity indicators will be assessed in identifying the skeletal age of an individual. Although some indicators are assessed on a maximum five-grade scale, most of these maturity indicators are either present or absent.[14] The Fels method of skeletal aging is highly replicable with little inter-assessor difference compared to other skeletal age assessment methods.[15]

Anti-Müllerian Hormone during Growth and Development

Patterns of AMH appear to vary based on the stages of growth and development in females. In a healthy female sample, Fong *et al.* found AMH levels to increase from infancy until adolescences, reach a peak at 15.8 years of age, remain plateaued until 25 years of age, and then begin to gradually decline. This suggested childhood follicular dynamics, indicated by AMH levels, may differ from that of adulthood. Furthermore, Fong *et al.* reported notable inter-individual differences in AMH levels across all chronological ages.[16]

Though inter-individual conclusions were not available, Hagen *et al.* identified intra-individual AMH levels as having only minor fluctuations throughout childhood and adolescents in healthy females. For this reason, a random AMH measurement would likely be representative for adolescent females. Results from this study also suggest that AMH levels may serve as an indication of the ovarian reserve in female children as it may serve as a marker of preantral and small antral follicles, similar to the relationship identified in adult females.[17]

Ortega *et al.* identified higher levels of AMH in early postmenarchal girls than levels found in adult women with regular ovulatory cycles similar to findings from Fong *et al.* Results indicate, however, that girls with anovulatory cycles, and therefore higher AMH levels, are responsible for this trend. Inter-individual variability of AMH levels was noted more in girls with anovulatory cycles, however, this may reflect differences in the causes of anovulation. Additionally, findings report that AMH levels serve as an indication of ovarian immaturity. Ortega *et al.* further state that is not yet known if AMH is the cause or consequence of anovulatory cycles. AMH was also found to have a strong correlation with ovarian volume.[18]

In the Smith *et al.* study alluded to previously, the non-linear relationship of AMH over chronological age was found to be potentially affected by abdominal adiposity.[11] Furthermore, the study found that during the pubertal transition, children with higher waist-to-height ratios had elevated predicted AMH levels. Contradictory to what had been identified in adults, it was also noted that declines in AMH levels in children may not reflect a diminished ovarian reserve.[11]

Though the literature is expanding, allowing for the identification of AMH trends by age and a better understanding of the implications of these levels at different developmental stages is needed. Much is still unknown of AMH in regard to growth and development in adolescent females.

Public Health Significance

While AMH levels have been monitored by chronological age in adult women, adolescent boys, and adolescent girls, AMH levels have yet to be described by skeletal age.

As the first to describe this relationship, this study sought to assist in characterizing the role of AMH throughout the childhood of adolescent females. A better understanding of these childhood AMH levels may prove useful in explaining or predicting AMH levels later in adulthood and, therefore, providing more insight on female fertility and the ovarian reserve.

Study Objective

This study aimed to assess the influence of skeletal maturation on AMH levels by examining patterns of change in AMH by skeletal age and chronological age in female children.

METHODS

Study Design

Using data previously collected from the Fels Longitudinal Study, this study was a retrospective secondary data analysis that utilized both cross-sectional and longitudinal analytic methods.

Study Population

The Fels Longitudinal Study originated in 1929 in Yellow Springs, Ohio and continues to this day. It is the world's oldest continuous study of growth, development, and aging.[15] Fels Longitudinal Study participants are followed from enrollment, which is usually birth, until death or infirmity rendering their continued participation impossible.

The initial study subjects of the Fels Longitudinal Study were enrolled while *in-utero* by their pregnant mothers. Some mothers with subsequent pregnancies enrolled the siblings of these initial subjects as well. As the study proceed through the years, the children,

grandchildren, and even great-grandchildren of the earliest subjects were also enrolled in the study.[19]

Study Sample

The subjects for this secondary data analysis study included female children who were included in the Smith *et al*. study, which assessed serial serum AMH levels in healthy adolescent girls and women from the Fels Longitudinal Study. Exclusion criteria for this secondary data analysis included observations in which data were missing for either AMH levels or skeletal age. After exclusions, a data set was available for eighty-eight female Fels Longitudinal Study children who were assessed at least once from 8 years of age to 18 years of age (total observations = 212). Due to the serial nature of the data collection methods, there was not a standard number of observations per participant. Each participant was observed at least once but on no more than 6 different occasions as seen below in Table 1.

Table 1. Repeated Observations from 88 participants

Observation Count	Participant Frequency	Percent Participants	Total Observations
1	36	40.91	36
2	15	17.05	66
3	19	21.59	123
4	6	6.82	147
5	7	7.95	182
6	5	5.68	212
Total	88	100	212

Data Collection

The data for this secondary data analysis were collected from 1990 through 2014. For the general Fels Longitudinal study, data collection falls under the auspices of the National Institutes of Health: R01 HD012252. Stored longitudinal serum samples were assayed for AMH from 2017 to 2018 with the grant support from the Goldhirsh-Yellin Foundation Research Grant, the Dayton Area Graduate Medical Education Community Research Grant, and the National Institutes of Health (R01HD12252). De-identified data, with the necessary variables, was readily available under HSC-SPH-17-0262 (Dr. Miryoung Lee) and a Material and Data Transfer Agreement of the Fels Longitudinal Study (Dr. Stefan Czerwinski, executed on March 27, 2019) between Wright State University and UTHealth.

Anti-Müllerian Hormone Collection Methods

Fasting serum samples were collected in Fels Longitudinal Study visits by means of venipuncture following a minimum eight hours fast. Prior to assaying, serum samples were

stored at -80°C. The Reproductive Endocrine Research Laboratory at the University of Southern California conducted biomarker assays after the frozen, never-thawed samples were shipped from the collection site (Wright State University) in Ohio. The Ultrasensitive AMH ELISA (Ansh Lab, Webster TX) was used to obtain AMH levels. An assay sensitivity of 60 pg/mL was used and the inter-assay coefficient of variation was 9.7% at 1.6 ng/mL and 12.0% at 4.5 ng/mL.[11]

Skeletal Age

Skeletal age was collected at each observation using the Fels method for the hand-wrist as previously described. Relative age was the primary exposure of interest and was created by subtracting a participant's skeletal age from their chronological age at a single point in time. The relative age variable was further categorized into earlier, normal, or later maturation using the mean and standard deviation of the continuous relative age variable. Therefore, relative age was categorized as earlier maturation if values were less than one negative standard deviation from the mean, normal or average maturation if values were greater than or equal to one negative standard deviation from the mean but less than or equal to one standard deviation from the mean, and later maturation if greater than one standard deviation from the mean.

Covariates

The main outcome of interest was AMH levels. As Smith *et al*. identified adiposity to have an effect on the relationship of AMH and chronological age,[11] covariates consisted of adiposity variables, which included body mass index (BMI) percentile, waist circumference, waist to height ratio, and percent body fat total. BMI percentile was further categorized using

the Centers for Disease Control and Prevention's guidelines for defining childhood obesity where underweight was less than the 5th percentile, normal or healthy weight was greater or equal to the 5th percentile but less than the 85th percentile, overweight was greater or equal to the 85th percentile but less than the 95th percentile, and obese was greater or equal to the 95th percentile.[20]

Cardiometabolic characteristics have been known to be associated with growth and sexual maturation.[21] Thus, cardiometabolic covariates were included in the analysis of skeletal maturation and AMH. These variables included Homeostatic model assessment of insulin resistance (HOMA-IR) index, glucose, insulin, systolic blood pressure, systolic blood pressure for age percentile, diastolic blood pressure, diastolic blood pressure for age percentile, triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, sex steroid binding globulin, total testosterone, and free testosterone. Detailed information regarding the measurements of cardiometabolic risk factors can be found in Remsberg *et al.* and Limon *et al.*[21, 22] Lastly, an observation variable was created for participants with greater than one observation to express the range in years from first to last observation.

Data Analysis

The data analysis was conducted in two phases: the first phase was a cross-sectional analysis of observations collected at baseline and the second phase was a longitudinal analysis using all observations. All results were considered significant at a p-value of 0.05 or less. STATA version 16 was utilized for all data analyses.

Phase I: Cross-Sectional Analysis

The cross-sectional analysis began with calculating descriptive statistics for all relevant study variables at baseline. As AMH levels were not normally distributed, the variable was natural log transformed for all analyses. The log transformed AMH levels (AMH_{log}) were plotted against chronological age at baseline to examine patterns of variability. Additionally, mean AMH_{log} was plotted by the categorical variable for relative age (e.g., earlier, normal, later maturation). Linear regressions were used to model the relationship between AMH_{log} and relative age while taking several variables into account using the forward selection approach. The first linear regression model included relative age, age, age^2, and age^3. The second model included all variables from the first model with the addition of an adiposity variable. Lastly, the third and final model included all variables from the first model with the addition of an adiposity variable and cardiometabolic variable.

Phase II: Longitudinal Analysis

Phase II, the longitudinal analysis, began with plotting the AMH_{log} levels against both chronological age and skeletal age across all observations to examine patterns of variability. The generalized linear mixed effect modeling was conducted in a similar method to that of the linear regression modeling for the cross-sectional analysis using a forward selection approach. All generalized liner mixed effect models included a random intercept and an unstructured covariance matrix adjusting for unbalanced serial correlated observations. The first model included relative age, age, age^2, and age^3. The second model included all variables from the first model with the addition of an adiposity variable. Lastly, the third and

final model included all significant variables from the first model with an additional adiposity and cardiometabolic variable.

Human Subjects Considerations

As the study required data that were derived from human subjects, Institutional Review Board (IRB) approval had previously been obtained. All personal identifiers or protected health information were retracted from the data used in this study. Further security of the data was maintained by utilizing a UTHealth firewall.

RESULTS

Phase I: Cross-Sectional Analysis Results

At baseline, AMH levels ranged from 0.46 to 16.20 ng/mL, had a mean of 4.85 ng/mL, and a median of 3.65 ng/mL. Chronological age ranged from 7.71 to 16.91 years with an average of 11.61 years. Skeletal age at baseline ranged from 7.05 to 18.00 years with an average of 12.19 years. The average relative age was -0.57 years. Additionally, 15.91% of participants experienced earlier maturation, 69.32% of participants experienced normal or average maturation, and 14.77% of participants experienced later maturation based on comparisons between their skeletal age and chronological age (i.e., relative age). The majority of participants (64%) were of normal or healthy weight, 3.41% were underweight, 11.36% were overweight, and 12.50% were obese by CDC standards. The average duration between the first and last visit was 4.67 years with a minimum of 1 year and a maximum of 9 years. Additional descriptive characteristics at baseline are shown below in Table 2.

Table 2. Descriptive Characteristics at Baseline

Variable	n	Mean	SD	Min	Max
Anti-Müllerian Hormone (AMH, ng/mL)	88	4.85	3.45	0.46	16.20
Chronological Age (years)	88	11.61	2.75	7.71	16.91
Skeletal Age (years)	88	12.19	3.10	7.05	18.00
Relative Age (years)	88	-0.57	1.19	-3.37	3.01
Weight (kg)	88	44.63	17.56	19.70	109.70
Height (cm)	88	148.77	14.88	118.25	184.00
Body Mass Index Percentile	88	56.31	30.24	0.51	98.97
Waist Circumference (cm)	88	69.98	12.51	51.35	116.95
Waist to Height Ratio	88	0.47	0.06	0.36	0.68
Percent Body Fat Total (%)	76	26.62	7.54	13.27	42.60
Duration* (years)	52	4.67	2.30	1.00	9.00

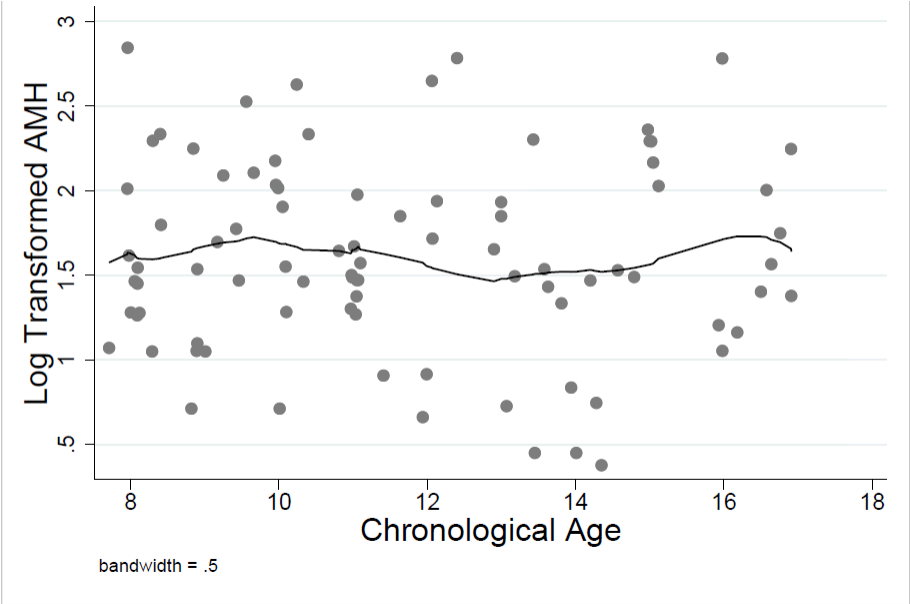
Variable		n	Percent
Relative Age	Earlier Maturation	14	15.91
	Normal Maturation	61	69.32
	Later Maturation	13	14.77
Body Mass Index Percentile	Underweight	3	3.41
	Normal or Healthy Weight	64	72.73
	Overweight	10	11.36
	Obese	11	12.50

*Duration variable was calculated for participants with >1 observation.

A plot of AMH_{log} values by chronological age showed a slight decrease in AMH_{log} at around 13 years of age. Furthermore, the plot revealed three potential inflection points of the

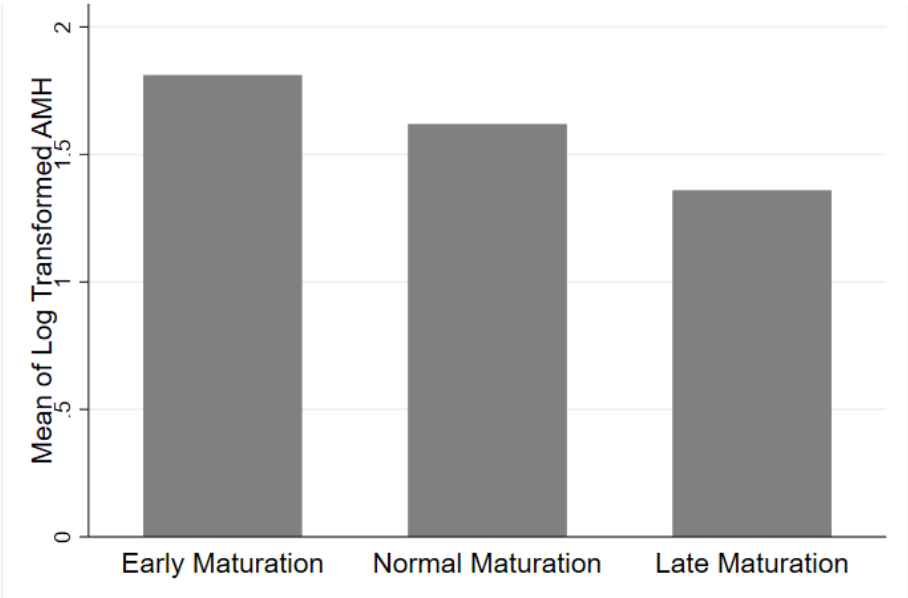
relationship. This was considered when building the linear regression models. The plotted relationship is displayed below in Figure 1.

Figure 1. Log Transformed AMH and Chronological Age at Baseline



Mean AMH_{log} values were graphed by relative age categories of maturation at baseline. Figure 2 below depicts the inverse relationship between mean AMH_{log} values and delayed maturation. Results from a one-way ANOVA, however, revealed no significant differences in mean AMH_{log} values across the three relative age categories ($p = 0.1083$).

Figure 2. Mean Log Transformed AMH by Relative Age Categories at Baseline



Linear Regression Modeling

In the first linear regression model (Model 1), which included relative age, age, age^2, and age^3, relative age had a beta coefficient of -0.124 (standard error [SE] = 0.050) and was significantly associated with AMH_{log} with a p-value of 0.015.

To build onto Model 1 and account for adiposity, four adiposity variables were separately considered for the second linear regression model (Model 2). In Table 3, a beta coefficient, SE, p-value, and R^2 value can be found for each adiposity variable considered for Model 2. None of the adiposity variables that were considered for Model 2 were significant, however, the BMI percentile variable was chosen for Model 2 as it explained the most variability of the response data compared to the other adiposity variables (R^2 = 0.105). BMI percentile was not significantly associated with AMH_{log} (beta = -0.002, p = 0.270) in Model

2. Relative age had a beta coefficient of -0.148 (SE = 0.050) and remained to be significant in Model 2 with a p-value of 0.008.

Table 3. Adiposity Variables Considered for Linear Regression Model 2

Variable	**Beta Coefficient**	**SE**	**p-value**	**R^2**
Body Mass Index Percentile	-0.002	0.002	0.270	0.105
Waist Circumference (cm)	-0.002	0.006	0.742	0.093
Waist to Height Ratio	0.385	1.025	0.708	0.093
Percent Body Fat Total (%)	0.011	0.009	0.225	0.072

To build further onto Model 2 and account for cardiometabolic characteristics, 15 variables were each considered individually for the final linear regression model (Model 3). In Table 4, a beta coefficient, SE, p-value, and R^2 value can be found for each cardiometabolic variable considered for Model 3. As the sex steroid binding globulin (SHBG) variable was significant in the final model (beta = 0.006, p = 0.011) and explained the most variability of the response data compared to the other cardiometabolic variables (R^2 = 0.174), it was incorporated into the final model. All other cardiometabolic variables considered were not significant nor included in the final model. Relative age had a beta coefficient of -0.177 (SE = 0.053) and was significant in the final model with a p-value of 0.001. The final linear regression model explained 17.4% of the variation of AMH_{log} levels. Results for each of the three linear regression models can be found in Table 5.

Table 4. Cardiometabolic Variables Considered for Linear Regression Model 3

Variable	Beta Coefficient	SE	p-value	R^2
HOMA-IR (mg/dL)	0.010	0.045	0.832	0.113
Log Transformed HOMA-IR	0.050	0.136	0.714	0.114
Glucose (mg/dL)	0.005	0.010	0.597	0.108
Insulin (mIU/L)	0.001	0.010	0.926	0.112
Systolic Blood Pressure (mmHg)	-0.014	0.007	0.059	0.146
Systolic Blood Pressure for Age Percentile	-0.003	0.003	0.216	0.124
Diastolic Blood Pressure (mmHg)	0.003	0.007	0.612	0.110
Diastolic Blood Pressure for Age Percentile	0.002	0.003	0.443	0.113
Total Cholesterol (mg/dL)	<0.001	0.002	0.840	0.116
Triglyceride (mg/dL)	<0.001	0.001	0.847	0.116
High Density Lipoprotein Cholesterol (mg/dL)	-0.003	0.007	0.651	0.118
Low Density Lipoprotein Cholesterol (mg/dL)	0.001	0.002	0.602	0.119
Sex Steroid Binding Globulin (nmol/L)	0.006	0.002	**0.011***	0.174
Total Testosterone (ng/dL)	0.005	0.008	0.506	0.123
Free Testosterone (pg/mL)	0.002	0.037	0.958	0.118

*Significant at $\alpha = 0.05$

Table 5. Phase I Linear Regression Analyses for Log Transformed AMH

	Independent Variable	Beta Coefficient	SE	p-value	R^2
Model 1	Relative Age	-0.124	0.050	**0.015***	0.0918
	Age	1.662	1.580	0.296	
	Age^2	-0.149	0.133	0.265	
	Age^3	0.004	0.004	0.242	
Model 2	Relative Age	-0.148	0.054	**0.008***	0.1053
	Age	1.407	1.594	0.380	
	Age^2	-0.127	0.134	0.345	
	Age^3	0.004	0.004	0.318	
	BMI Percentile	-0.002	0.002	0.270	
Final Model	Relative Age	-0.177	0.053	**0.001***	0.1738
	Age	1.849	1.551	0.237	
	Age^2	-0.152	0.130	0.250	
	Age^3	0.004	0.004	0.259	
	BMI Percentile	0.000	0.002	0.946	
	SHBG	0.006	0.002	**0.011***	

*Significant at $\alpha = 0.05$

Phase II: Longitudinal Analysis Results

Plotting AMH_{log} by chronological age across all observations, as seen in Figure 3, resulted in a pattern similar to Figure 1 obtained in the cross-sectional analysis with lower AMH_{log} levels found around 13 years of age. Additionally, there appeared to be three inflection points in the AMH_{log} and chronological age relationship. The plot of AMH_{log} by skeletal age, as seen in Figure 4, also revealed lower AMH_{log} values at around 13 years of age, however, this relationship only displayed two points of inflection.

Figure 3. Longitudinal Plot of Log Transformed AMH by Chronological Age

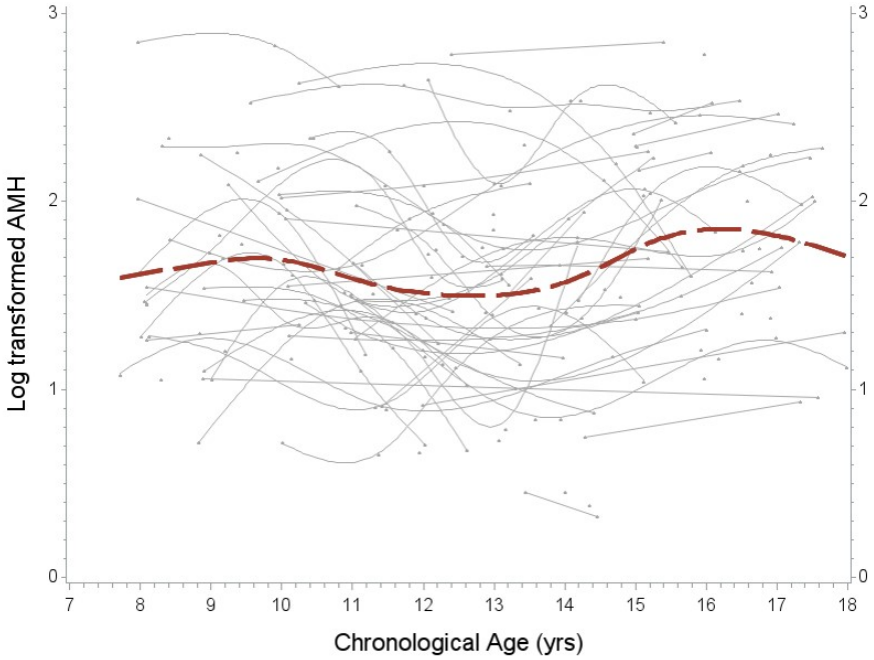
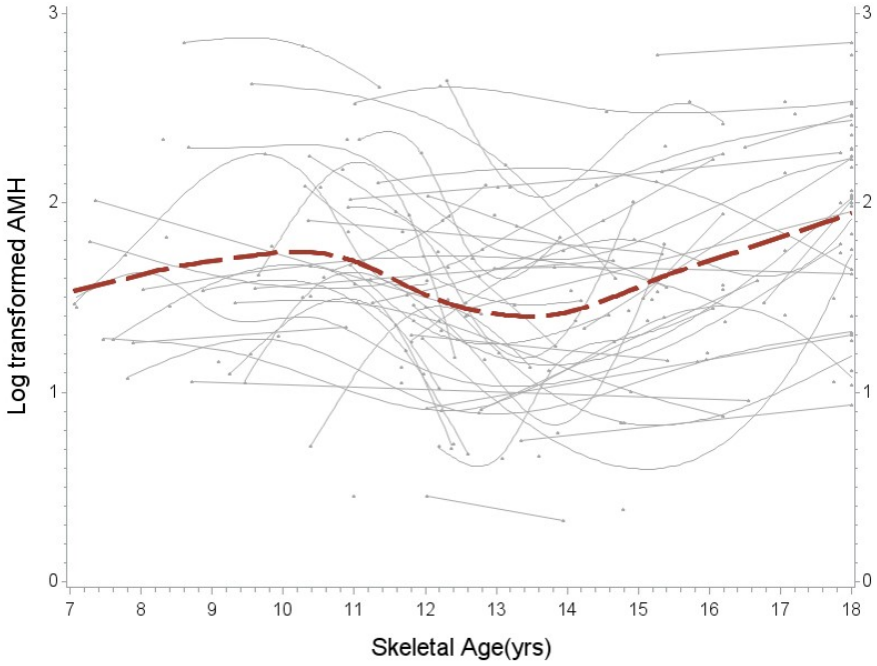


Figure 4. Longitudinal Plot of Log Transformed AMH by Skeletal Age



Generalized Linear Mixed Effect Modeling

In the first generalized linear mixed effect model (Model 1), which included relative age, age, age^2, and age^3, relative age had a beta coefficient of -0.089 (SE = 0.030) and was significant with a p-value of 0.003.

To build onto Model 1 and account for adiposity characteristics, using methods similar to that used in the linear regression modeling, four adiposity variables were separately considered for the second mixed model (Model 2). In Table 6, a beta coefficient, SE, p-value, and Log Likelihood value can be found for each adiposity variable considered for Model 2. Although none of the adiposity variables considered were significant, the percentage body fat variable was added into Model 2 as it had the highest Log Likelihood value compared to the other the adiposity variables. Relative age had a beta coefficient of -0.065 (SE = 0.032) and was also significant in the second model with a p-value of 0.042.

Table 6. Adiposity Variables Considered for Generalized Mixed Effect Linear Model 2

Variable	**Beta Coefficient**	**SE**	**p-value**	**Log Likelihood**
Body Mass Index Percentile	-0.002	0.002	0.186	-104.303
Waist Circumference (cm)	<0.001	0.004	0.925	-105.171
Waist to Height Ratio	0.765	0.667	0.251	-104.518
Percent Body Fat Total (%)	0.008	0.006	0.178	-97.794

To build further onto Model 2, 15 cardiometabolic variables were each considered individually for the final mixed effect model (Model 3). In Table 7, a beta coefficient, SE, p-

value, and Log Likelihood value can be found for each cardiometabolic variable considered for Model 3. Glucose had a beta coefficient of -0.008 (SE = 0.004) and was significant in the model with a p-value of 0.044. Thus, glucose was incorporated into the final model. All other cardiometabolic variables were not significant nor included in the final model.

Table 7. Cardiometabolic Variables Considered for Generalized Mixed Effect Linear Model 3

Variable	**Beta Coefficient**	**SE**	**p-value**	**Log Likelihood**
HOMA-IR (mg/dL)	-0.036	0.019	0.054	-93.982
Log Transformed HOMA-IR	-0.089	0.060	0.137	-94.713
Glucose (mg/dL)	-0.008	0.004	**0.044***	-95.793
Insulin (mIU/L)	-0.007	0.004	0.090	-94.385
Systolic Blood Pressure (mmHg)	-0.005	0.004	0.164	-96.377
Systolic Blood Pressure for Age Percentile	-0.001	0.001	0.343	-96.890
Diastolic Blood Pressure (mmHg)	<-0.001	0.003	0.993	-97.338
Diastolic Blood Pressure for Age Percentile	<0.001	0.001	0.887	-97.328
Total Cholesterol (mg/dL)	-0.001	0.001	0.487	-94.585
Triglyceride (mg/dL)	<0.001	<0.001	0.747	-94.774
High Density Lipoprotein Cholesterol (mg/dL)	0.001	0.003	0.871	-94.813
Low Density Lipoprotein Cholesterol (mg/dL)	-0.001	0.002	0.701	-94.753
Sex Steroid Binding Globulin (nmol/L)	0.001	0.001	0.196	-96.988
Total Testosterone (ng/dL)	0.003	0.002	0.089	-95.304
Free Testosterone (pg/mL)	0.010	0.006	0.092	-95.342

*Significant at $\alpha = 0.05$

Relative age had a beta coefficient of -0.073 (SE = 0.032) and was significant in the final model with a p-value of 0.023. All three linear mixed effect models were significant with a model p-value of less than 0.0001. Results for each of the generalized linear mixed effect models can be found in Table 8.

Table 8. Phase II Generalized Mixed Effect Linear Model Analyses for Log Transformed AMH

	Independent Variable	**p-value**	**Beta Coefficient**	**SE**	**Model p-value**
Model 1	Relative Age	**0.003***	-0.089	0.030	**<0.0001***
	Age	0.702	0.202	0.526	
	Age^2	0.468	-0.031	0.042	
	Age^3	0.292	0.001	0.001	
Model 2	Relative Age	**0.042***	-0.065	0.032	**<0.0001***
	Age	0.925	0.053	0.560	
	Age^2	0.659	-0.020	0.044	
	Age^3	0.441	0.000	0.001	
	Total Percent Body Fat	0.178	0.008	0.006	
Final Model	Relative Age	**0.023***	-0.073	0.032	**<0.0001***
	Age	0.833	0.116	0.551	
	Age^2	0.587	-0.024	0.044	
	Age^3	0.393	0.001	0.001	
	Total Percent Body Fat	0.162	0.008	0.006	
	Glucose	**0.044***	-0.008	0.004	

*Significant at $\alpha = 0.05$

DISCUSSION

Patterns were identified for AMH_{log} by skeletal age and chronological age in female children and study results indicated that the relationship between skeletal age and AMH_{log} showed similar patterns to that of chronological age and AMH_{log}. Additionally, results revealed an inverse relationship between AMH_{log} and delayed skeletal maturation. These results indicate that female children who experienced later maturation also experienced lower AMH_{log} levels.

Results from the final linear regression model of Phase I revealed that, when holding all other variables constant, for each one-year increase in delayed maturation, AMH_{log} decreases by 0.177. This result was similar to what was depicted in preliminary figures of mean AMH_{log} graphed by categorical relative age (Figure 2) in the cross-sectional analysis. Additionally, holding all other variables constant, for each one-unit increase in SHBG, AMH_{log} increases by 0.006. Lastly, 17.38% of the variation of AMH_{log} levels could be explained by this final linear regression model.

In regard to SHBG, we identified a significant, positive relationship with AMH_{log} in the final linear regression model. Literature suggests that AMH and SHBG could be used together in the diagnosis of polycystic ovarian syndrome but that AMH levels are higher in adolescents with polycystic ovaries, whereas SHBG levels are lower.[23] Therefore, the findings from this study are not consistent with literature in this regard. However, given much of existing research and available information about AMH is in regard to adolescent females with pre-existing menstrual or chromosomal conditions, these conflicting results

could potentially be attributable to the relatively healthy study population used compared to those that are typically studied.

The predicted line in the plot of AMH_{log} by chronological age (Figure 3) obtained in the longitudinal analysis of Phase II may be skewed as there are a few data points closer to maximum chronological age of 18 years that seem to cause the predicted line to curve downward as it approaches this maximum value. For this reason, it is possible that the true relationship of AMH_{log} by chronological age would more closely mirror the plot of AMH_{log} by skeletal age which has only two inflection points.

In the final generalized linear mixed effect model, after controlling for all other variables, for every one-year increase in delayed maturation, AMH_{log} decreases by 0.073. Additionally, when holding all other variables constant, for each one-unit increase in glucose, the AMH_{log} decreases by 0.008. Findings from both the cross-sectional and longitudinal analysis similarly reported a decrease in AMH_{log} levels in individuals who experience later maturation.

AMH_{log} plotted against chronological age depicted a peak AMH_{log} level near 16 years of age which was similar to the maximum AMH level value of 15.8 years that Fong *et al*. reported in their longitudinal plot of AMH by chronological age. Fong *et al*. also reported a plateau of AMH level throughout adolescences.[16] However, the results from this study, as mentioned previously, identified two likely inflection points in AMH_{log} levels when plotted by chronological age. Fong *et al*. further noted considerable inter-individual differences in AMH levels. This observation, combined with the small sample size of this study, could provide some explanation as to why the plot for this study varies from findings Fong *et al*.

obtained.[16] Furthermore, in the longitudinal analysis, AMH_{log} had a significant, negative relationship with glucose. Although the literature often reports increased cardiometabolic risks in those with polycystic ovarian syndrome, in a healthy, general population of adolescent females cardiometabolic risk factors were not found to be associated with AMH levels.[24] Therefore, this finding does not closely corroborate with the literature that is currently available.

Limitations

Despite the advantages of using skeletal age as an indication of maturation, skeletal age may only be obtained until 18 years of age as hand ossification is then complete. Once the skeletal age of a participant reaches 18 years of age it cannot exceed that age. Furthermore, although there were 212 observations, the study sample included only 88 participants, which is fairly small sample size that could serve as a limitation. Lastly, as the Fels Longitudinal study consists of largely middle-class, non-Hispanic white participants and for this reason we are unable to generalize the results of this study to a wider, more diverse population.

Strengths

A strength of this study included its longitudinal design. This allowed for the temporal analysis of AMH over several years, in addition to the cross-sectional analysis of AMH at baseline. Also, along with increased precision and accuracy, the Fels method for the hand-wrist is an appropriate skeletal aging method for this study sample of adolescent females in the United States because the skeletal ages from which the Fels method was created are comparable to that obtained from United States national surveys.[15] Additionally,

having access to a data set that offered a multitude of adiposity and cardiometabolic variables allowed for the opportunity to incorporate these variables into the modeling and account for relationships that have been identified in the literature. The most notable strength of this study, however, is its novel nature. As the relationship had not yet been previously described, this study may serve as a foundation for further examination of AMH by skeletal age and skeletal maturation. Lastly, a better understanding of AMH levels in adolescent females has the potential to give insight on fertility and the ovarian reserve as these early childhood AMH levels might prove useful in explaining or predicting AMH levels later in adulthood.

CONCLUSION

Though this study is the first to describe the relationship between AMH and skeletal age, additional research is warranted to corroborate these study findings, and identify the true relationship AMH has with skeletal age and skeletal maturation in adolescent females. Additionally, future studies should analyze the relationship of AMH by skeletal age and chronological age as well as the influence of skeletal maturation on AMH level in other populations of relative healthy adolescent females to identify if these findings persist in a variety of other populations as well.

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