Hormone Phenotypes and the Timing of Pubertal Milestones in a Longitudinal Cohort of Girls

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Age 8 → Age 19
© Menstrupedia

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Hormones During Puberty

Hormone levels change throughout puberty.[1]

1. Gonadotropin-releasing hormone (GnRH) is released at the beginning of puberty.
2. The follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are then released into the bloodstream.
3. LH and FSH stimulate the ovaries to produce estrogen (estradiol, estrone, and estriol) to initiate breast development.
4. The adrenal gland hormones, DHEA-S (dehydroepiandrosterone sulfate) and testosterone, stimulate pubic hair growth. [2]
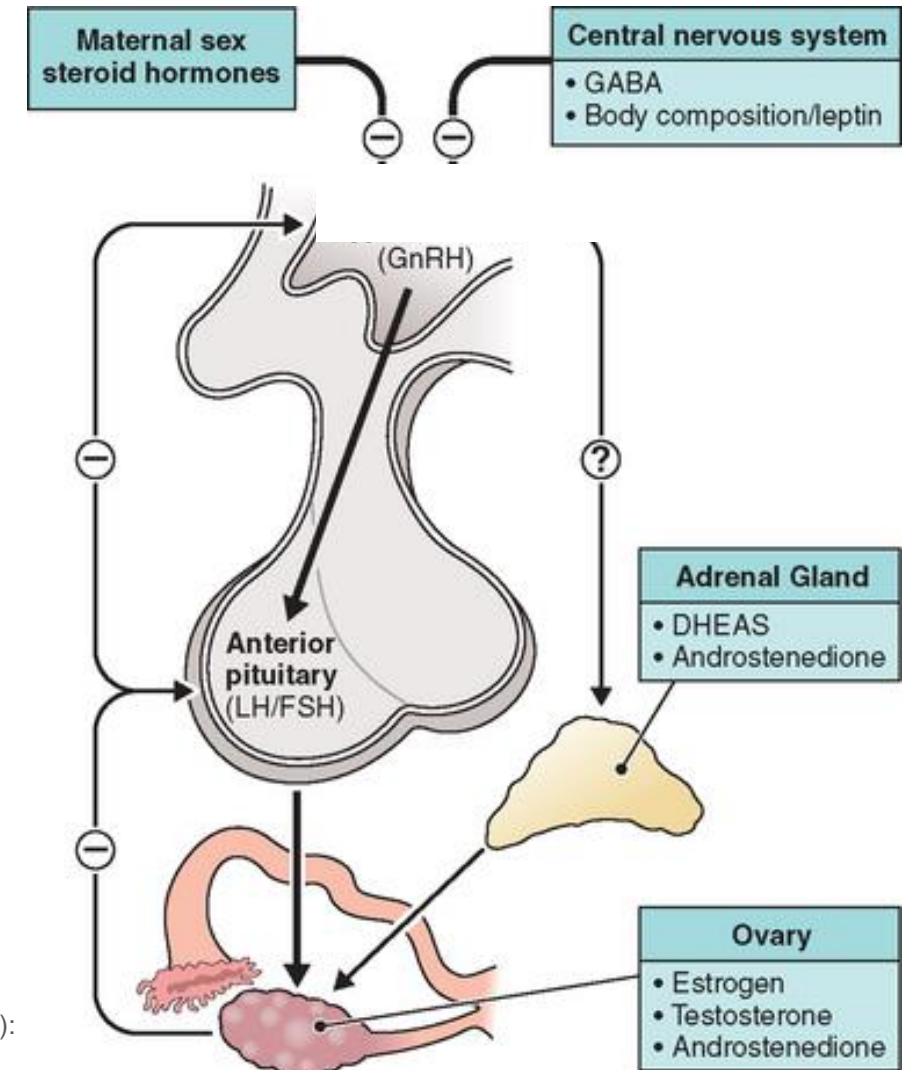
1 Peper JS, Dahl DE. Surging hormones: brain behavior interactions during puberty. Curr Dir Psychol Sci. 2013 April;22(2): 134-139.
2 Braude, P, Hamilton D. Hormone changes during puberty, pregnancy, and menopause. Obstetric and Gyneocologic Dermatology 2008;3:3-12.
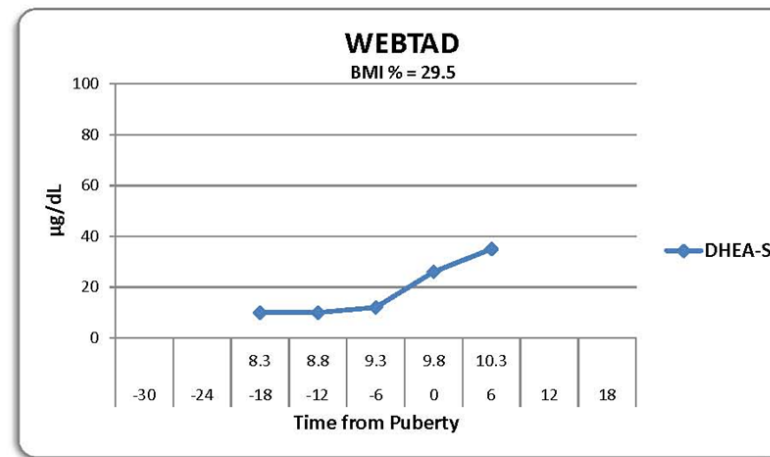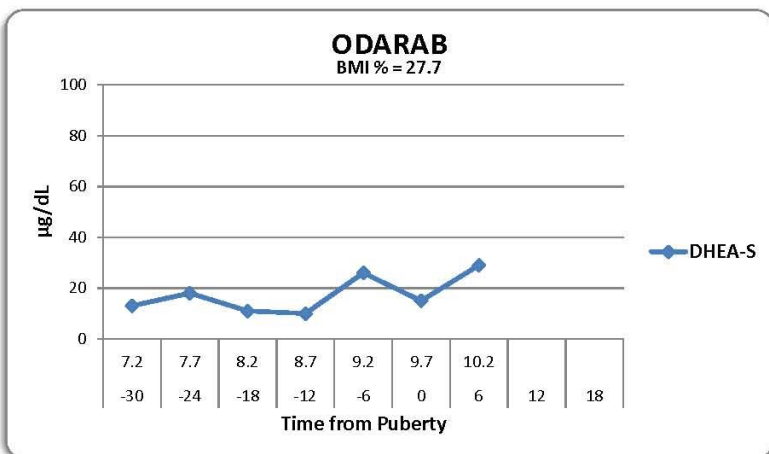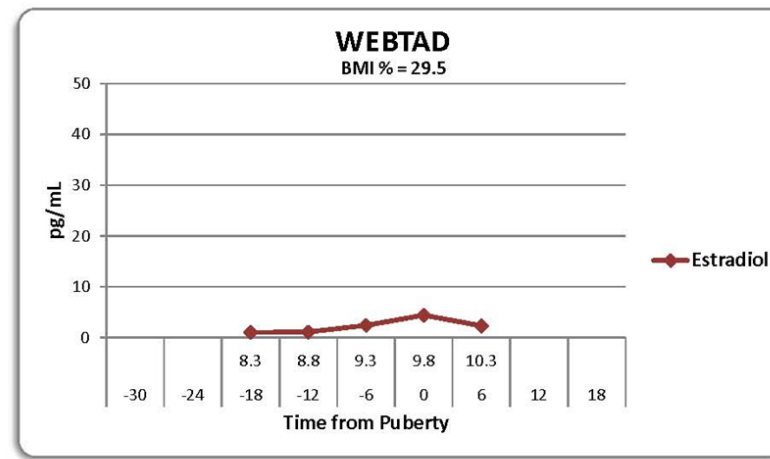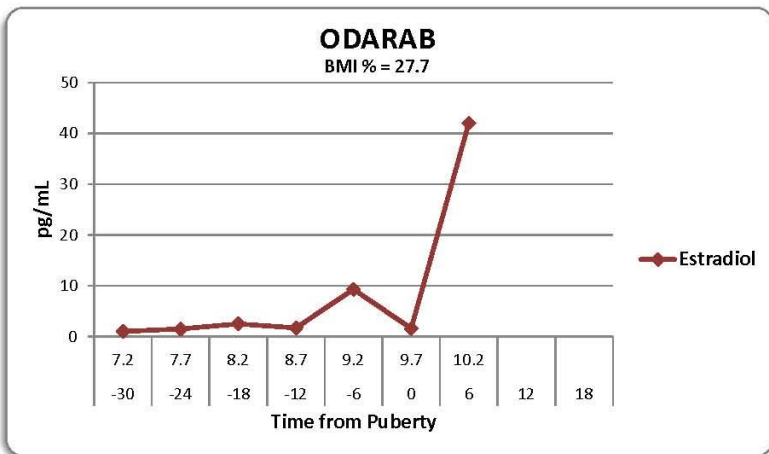
https://abdominalkey.com/normal-puberty-and-pubertal-disorders/

Hormones Attributes

Mean hormone values across time related to thelarche (time=0)*
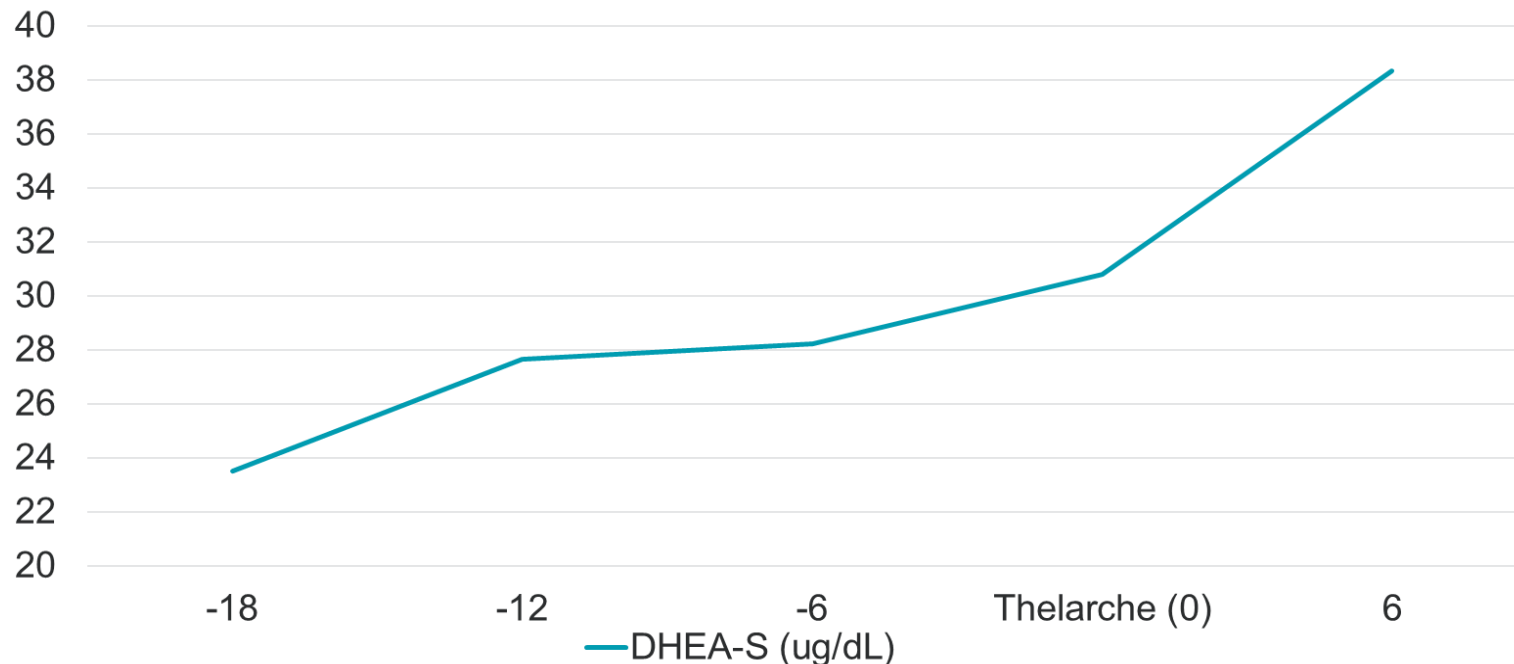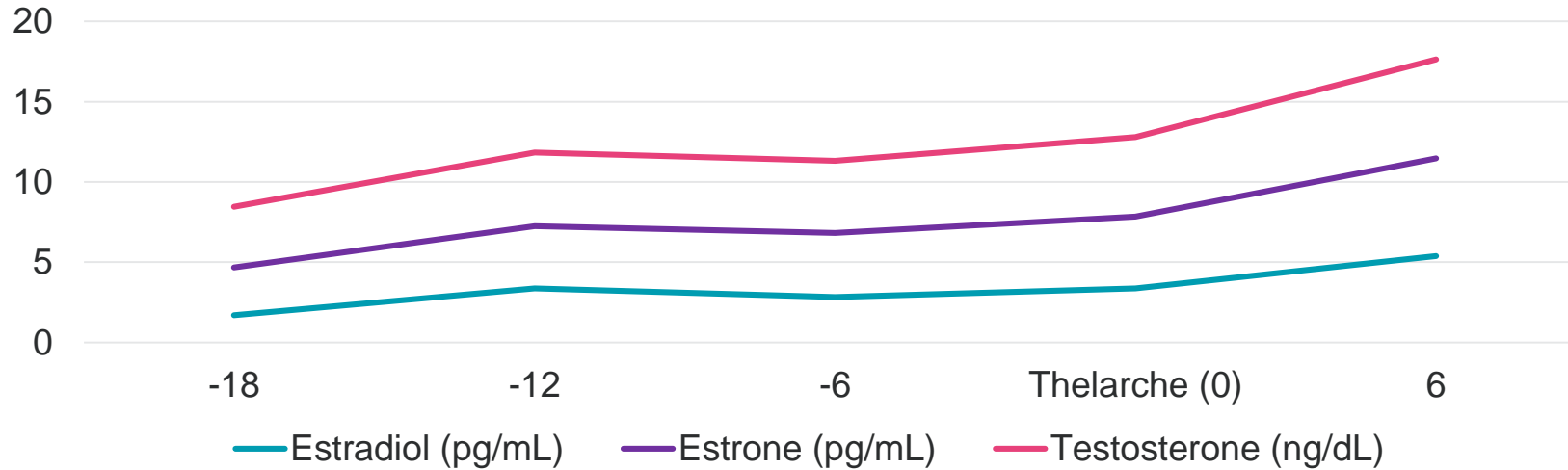
* Note the units of each hormones differ.

Individual Girl's Hormones

Objective

Determine if the hormone levels in girls around the time of thelarche are the same for all girls or if girls have different patterns in increases and decreases in hormone levels.

- Identify peri-pubertal hormone phenotypes (or clusters) in young girls based on hormone levels around thelarche (e.g. estradiol at -6 and 0 or testosterone and estrone at 0).

- Determine if the phenotypes are associated with differences in the ages of pubarche, thelarche and/or menarche.

Study Design – Cincinnati Cohort

BCERP's Puberty Cohort
- Three site, longitudinal, prospective cohort: East Harlem, New York; Greater Cincinnati Area; San Francisco Bay Area
- Recruited girls aged 6-8 from 2004 until 2006 (n=1,239)

Cincinnati Cohort -
- 379 girls were enrolled in the Cincinnati cohort.
- The girls were seen every six months for study visits from 2004-2014.
    - Anthropometric measurements
    - Blood for serum collected
    - Pubarche and thelarche staging
    - Answered questions regarding menarche, exercise, nutrition, etc.
- For this analysis, girls will be excluded if they report taking oral contraceptives or have an underlying hormone condition.
- This analysis included 269 girls with hormone measurements.

Unique Longitudinal Cohort

Most cohorts studying pubertal hormones are cross sectional and based on absolute age or pubertal status rather than timing related to puberty

Cross sectional - looks at hormones of the girls who achieved thelarche vs those who did not or looks at hormones of 12 year olds versus 11 year olds versus 10 year olds regardless of pubertal status.

Longitudinal cohort - ability to determine age of thelarche and link other visits to time before or after thelarche .

	Thelarche Achieved	Menarche Achieved	Age
Girl 1	Yes	No	12
Girl 2	Yes	Yes	10
Girl 3	No	No	10
Girl 4	No	No	9

Visit	1A	1B	2A	2B	3A	3B	4A	4B
Girl 1	-12	-6	Thelarche	+6		Menarche		
Girl 2	-18	-12	-6	Thelarche	+6	Menarche		
Girl 3		-18	-12	-6	Thelarche	+6		Menarche
Girl 4					-18	-12	-6	Thelarche

There were visits beyond 4B included in this analysis.
Girls who entered at -6 were not included.

Pubertal Milestone Measurements

Pubarche

Thelarche
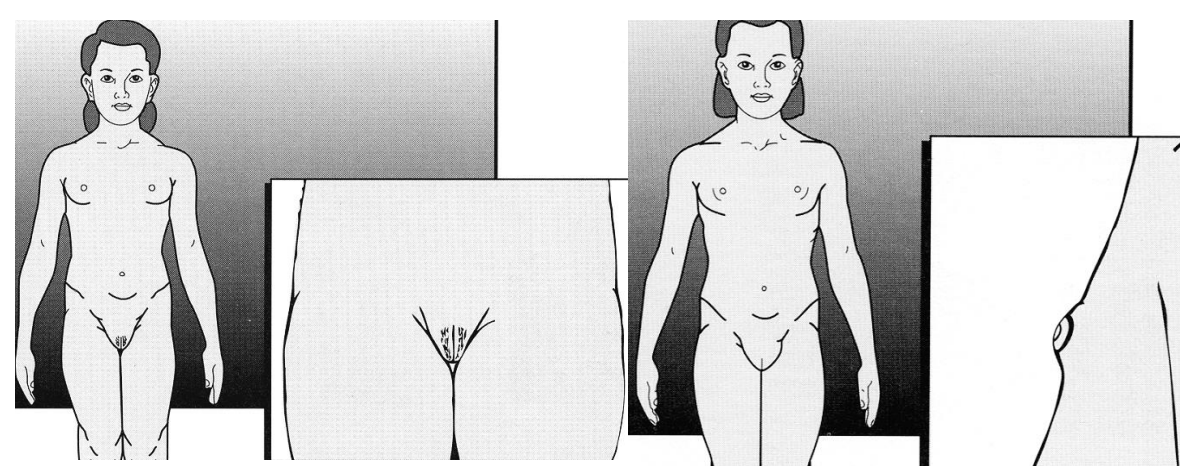
Stage 2: First appearance of hair, which is sparse, straight, or only slightly curled; long, slightly pigmented; downy; and primarily located along the labia.

Stage 2: Breast bud stage, with a small mound formed by the elevation of the breast papilla; areolar diameter enlarges.

Thelarche and Pubarche were achieved when sexual maturation stage 2 or greater was reached.

Age of Pubarche in months–Used accessory light sources to determine the presence or absence of hair in the pubic area.

Age of Thelarche in months –Staff observed and palpated to determine breast stage.

– Staff graded down if all criteria were not met for a stage.
– Girls with inconsistent breast staging were considered the lower stage until consistently the higher stage.
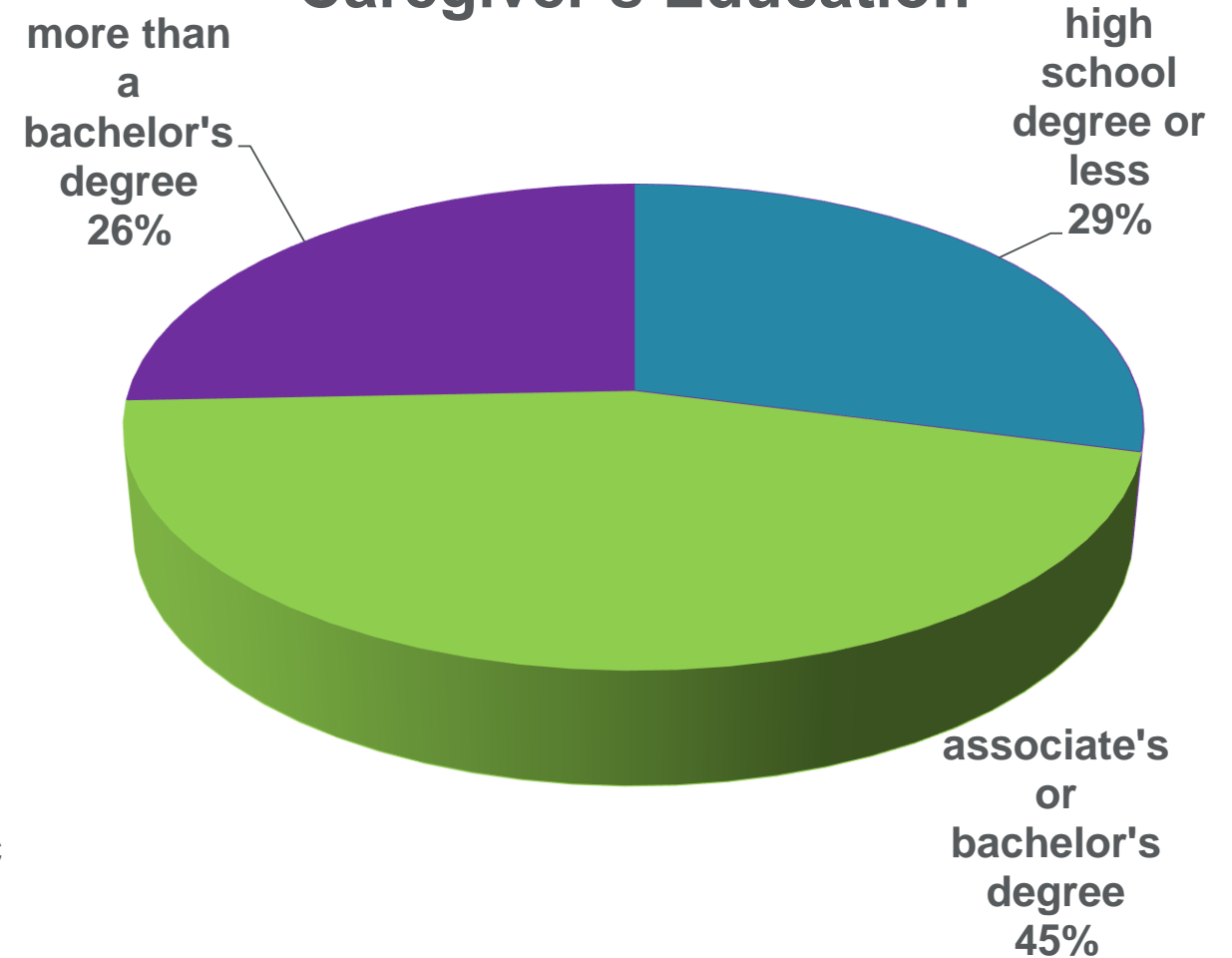
Age of menarche in months - Self-reported from study participant's and/or her caregiver's answers to questions regarding their first menstrual cycle.

Figures attributed to Dr. Frank Biro, 1989

Demographic Information (n=269 girls)
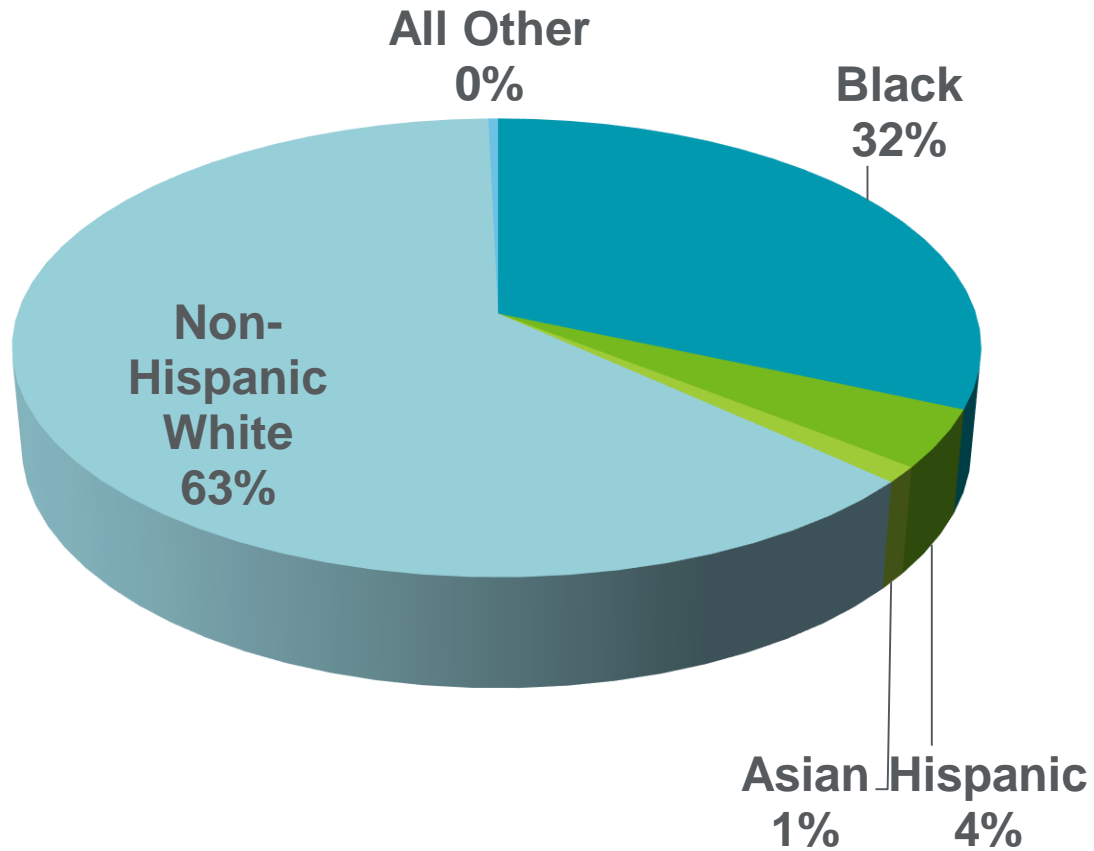
Methodology

Employ an objective and agnostic analysis using Principal Component Analysis followed by Cluster Analysis (PCA-CA) to define hormone phenotypes only looking at the hormones (estradiol, estrone, testosterone and DHEA-S at times -6, thelarche and +6) not any other variables suspected or known to influence puberty such as race or BMI.

1. Pearson Correlations – determine if hormones at the time periods are highly correlated

2. Principal Component Analysis – determine if variable reduction is possible given that hormones at certain time periods are redundant /highly correlated and they measure the same thing

3. Cluster Analysis – classify girls into phenotypes/clusters based on the 12 hormone data points such that girls in one cluster are more similar to each other than girls in another cluster

4. Survival Analysis – determine which phenotypes are associated with different ages of pubertal milestones

Methodology

Principal Components followed by Cluster Analysis

- PCA-CA is a validated statistical approach to identify subgroups or phenotypes. Previously patients were classified into "phenotypes" based on a few well characterized traits or thought to be homogenous.

- PCA-CA phenotypes have been well documented for cardiovascular risk, chronic obstructive pulmonary disease, asthma, and sleep apnea.
  - For each study, different sets of known disease symptoms presented in each of the disease phenotypes.

Cecily S Fassler, Iris Gutmark-Little, Changchun Xie, Courtney M Giannini, Donald W Chandler, Frank M Biro, Susan M Pinney, Sex Hormone Phenotypes in Young Girls and the Age at Pubertal Milestones, *The Journal of Clinical Endocrinology & Metabolism*, Volume 104, Issue 12, December 2019, Pages 6079–6089, https://doi.org/10.1210/jc.2019-00889
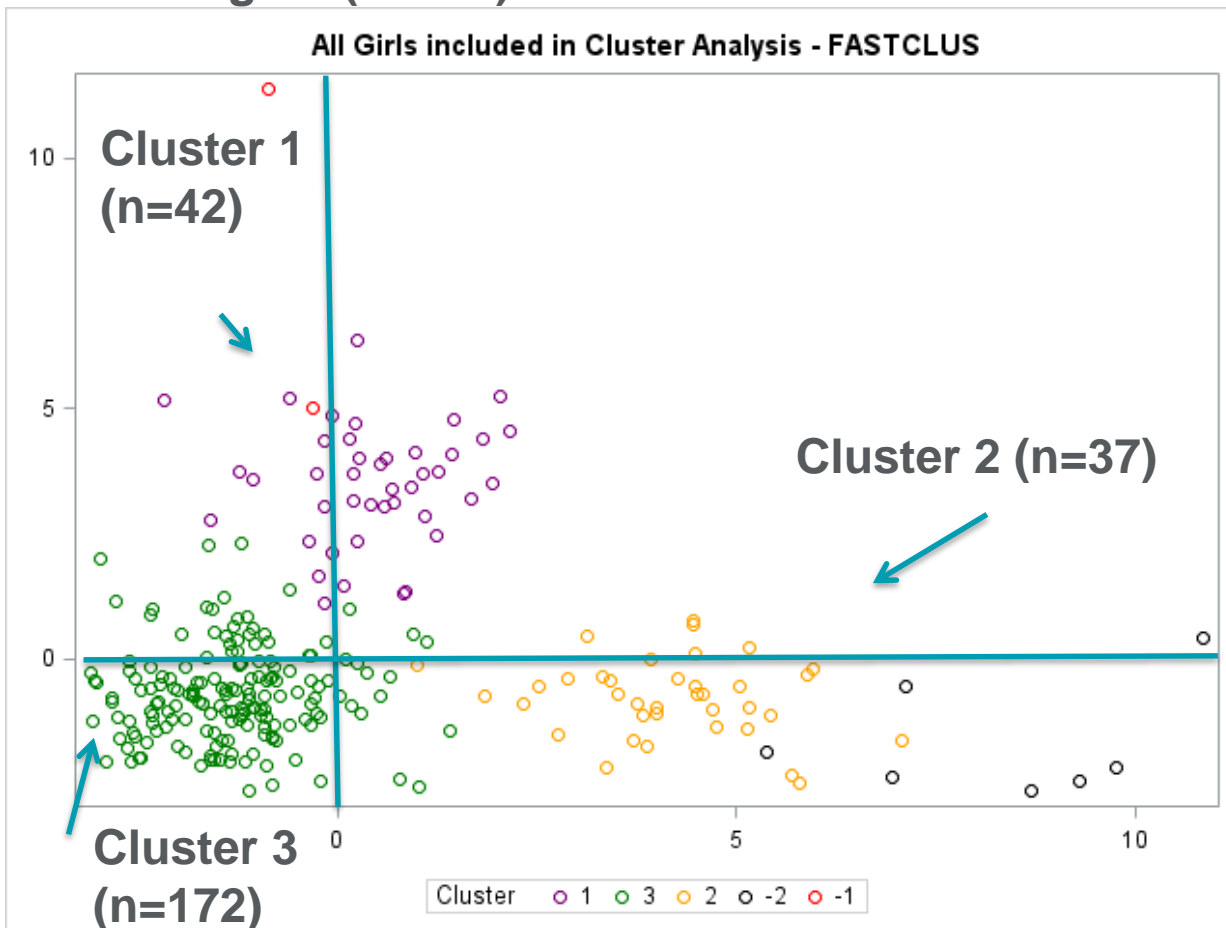
Correlations and Principal Components

- The lack of correlation of E2 at different time periods and the high degree of correlation among the other absolute hormones supports the inclusion of them into PCA-CA.

- PCA did not result in variable reduction of the absolute hormone values.
  - Should use all four absolute hormones measurements at -6, 0 and 6 as objective predictive variables in the cluster analysis.
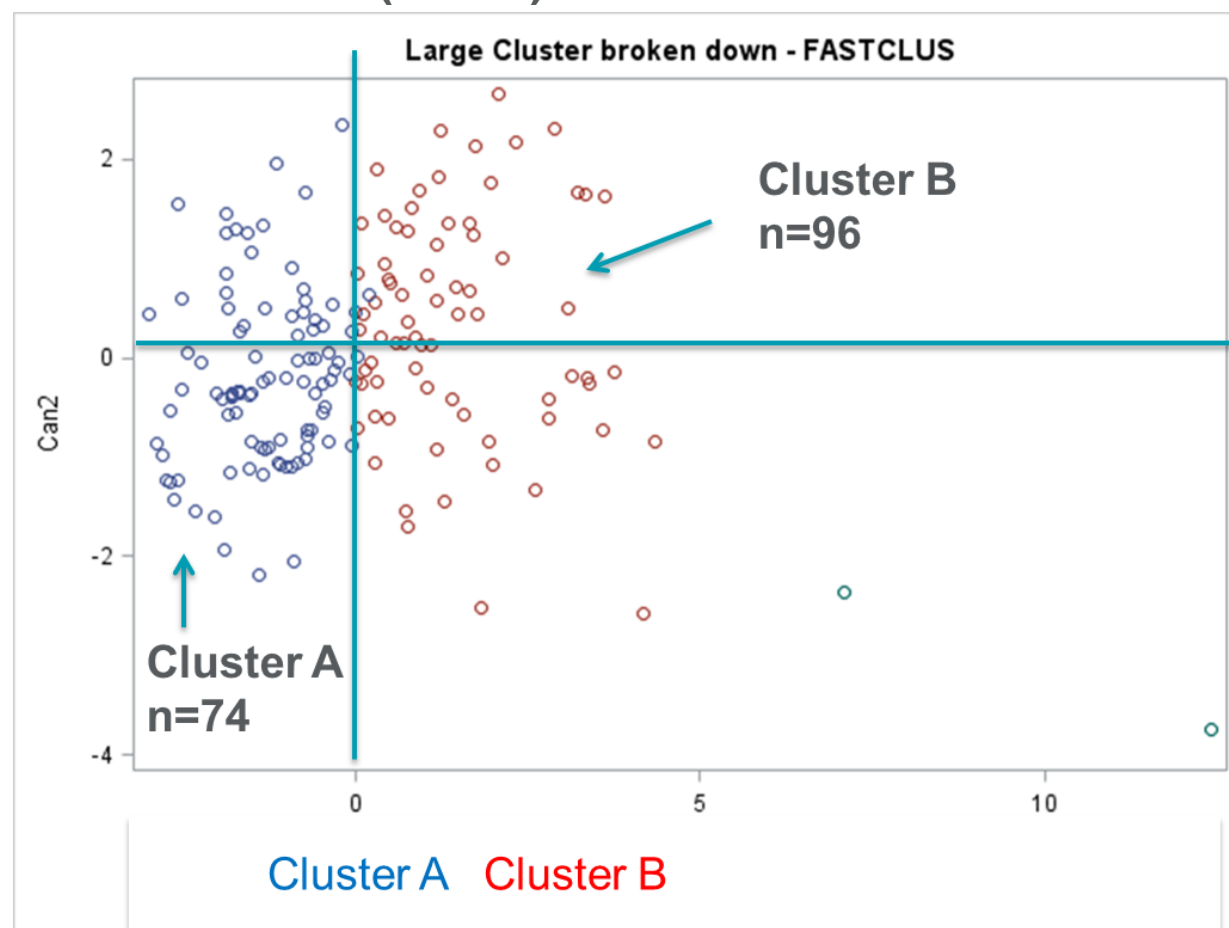
Participant Clustering using CA
based on estradiol, estrone, DHEA-S and testosterone at times =-6,0,6
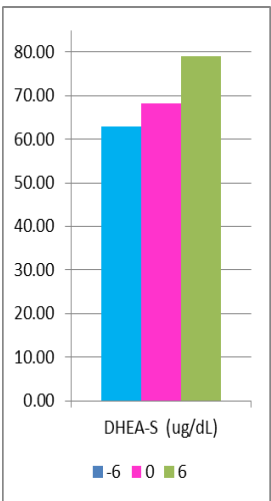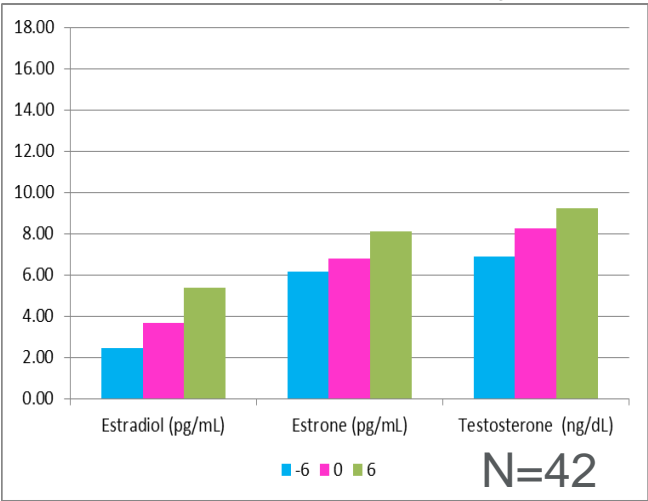
CA on all girls (n=290)

Cluster 1 (n=42)

Cluster 2 (n=37)

Cluster 3 (n=172)

CA on Cluster 3 (n=172)

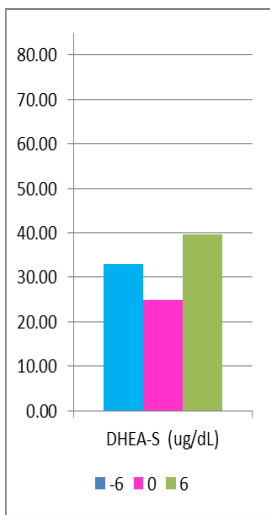
Cluster B n=96
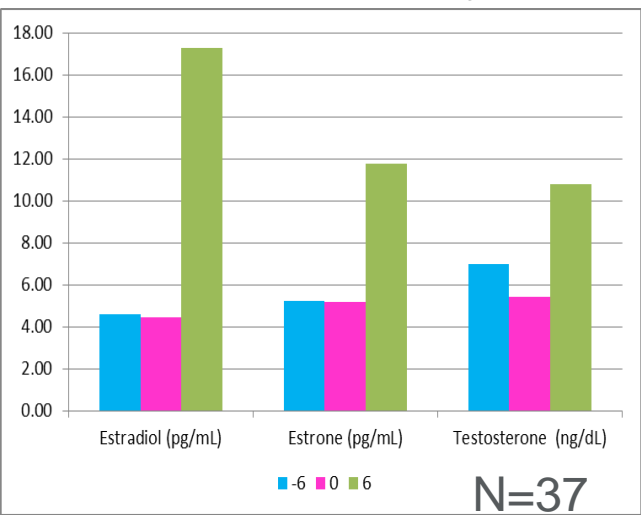
Cluster A n=74

Cluster A   Cluster B

*Assigns each girl to only one cluster and identifies outliers.
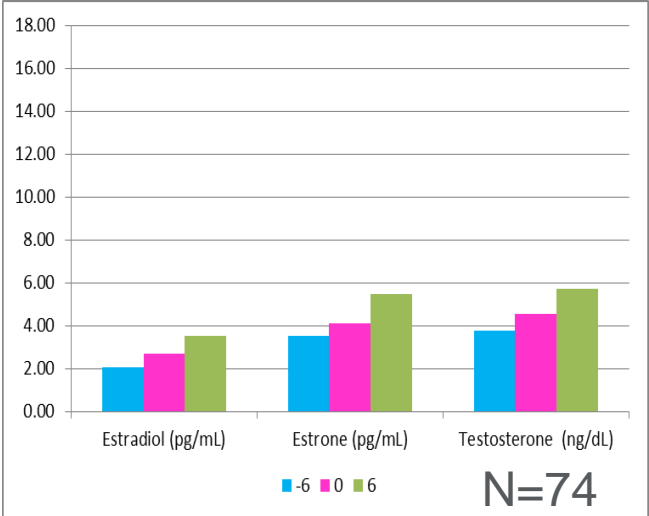
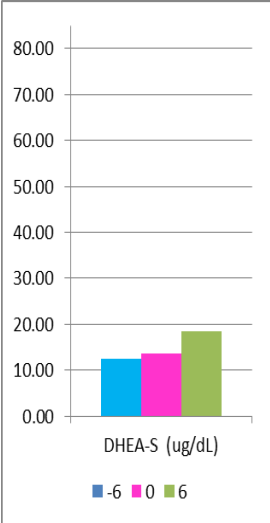# Mean Hormone Values by Phenotype*

Phenotype 1

N=42

Phenotype 2

N=37

Phenotype 3a

N=74

Phenotype 3b

N=96

* Note the units of each hormones differ.

Race and parental education

No statistically significant differences across the phenotypes.

Phenotype 1 **Phenotype 2** **Phenotype 3a** **Phenotype 3b**

Phenotype 1: 35.71% / 64.29%
Phenotype 2: 27.03% / 72.97%
Phenotype 3a: 29.73% / 70.27%
Phenotype 3b: 34.38% / 65.63%

Black
White and all other

Phenotype 1: 24.43% / 40.48% / 38.10%
Phenotype 2: 29.73% / 16.22% / 54.05%
Phenotype 3a: 28.38% / 27.03% / 44.59%
Phenotype 3b: 27.08% / 29.17% / 43.75%
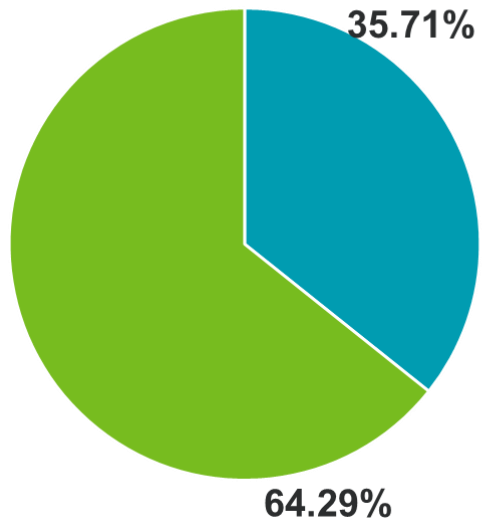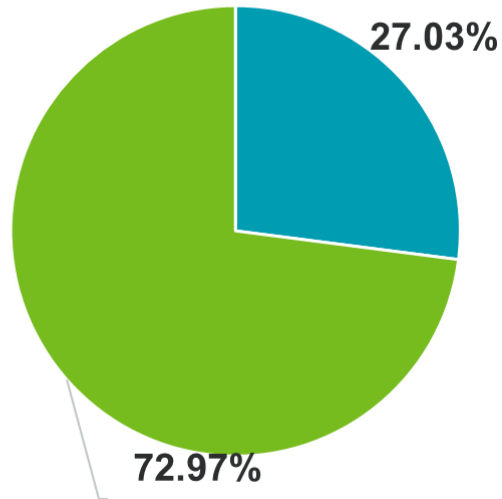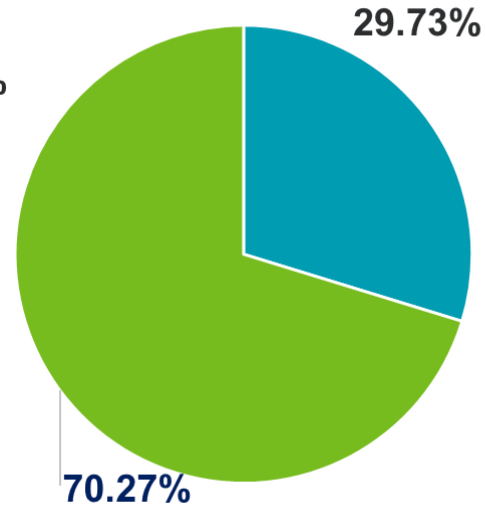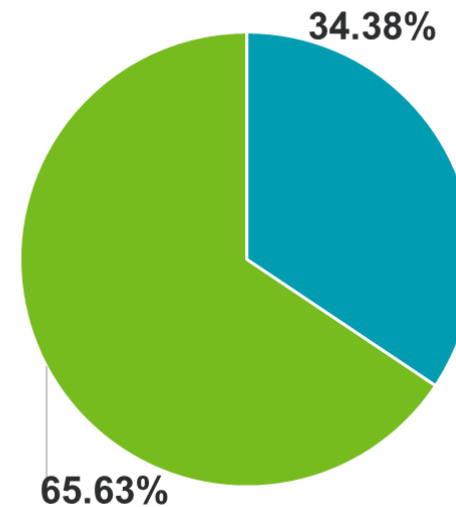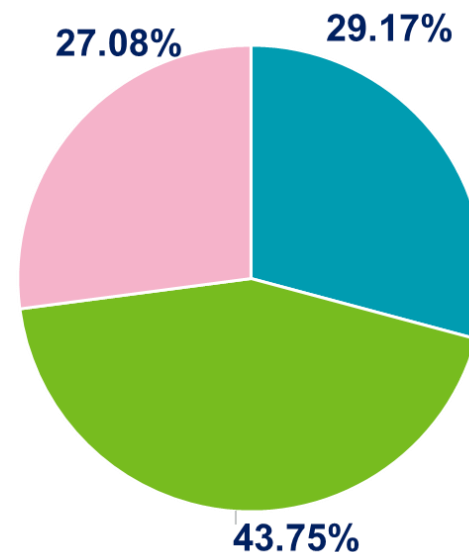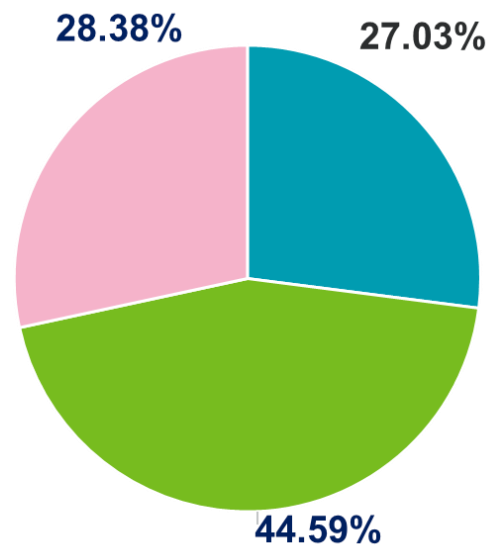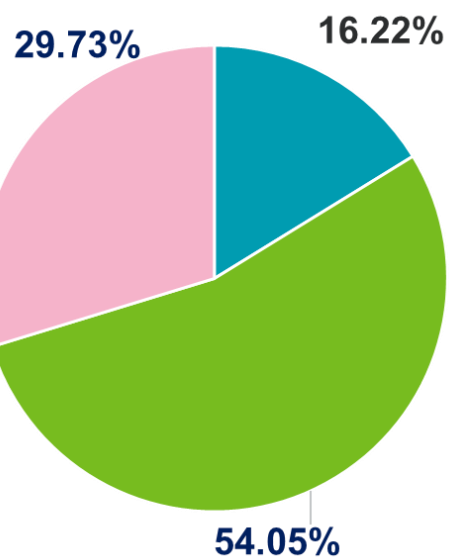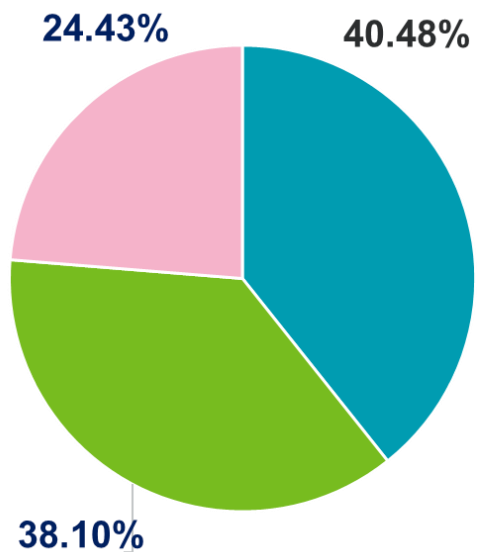
High school degree or less
Associate's or bachelor's degree
More than a bachelor's degree

BMI Percentile

Median BMI Percentile

Phenotype	Value
Phenotype 1	59.08
Phenotype 2	44.6
Phenotype 3a	71.84
Phenotype 3b	70.44
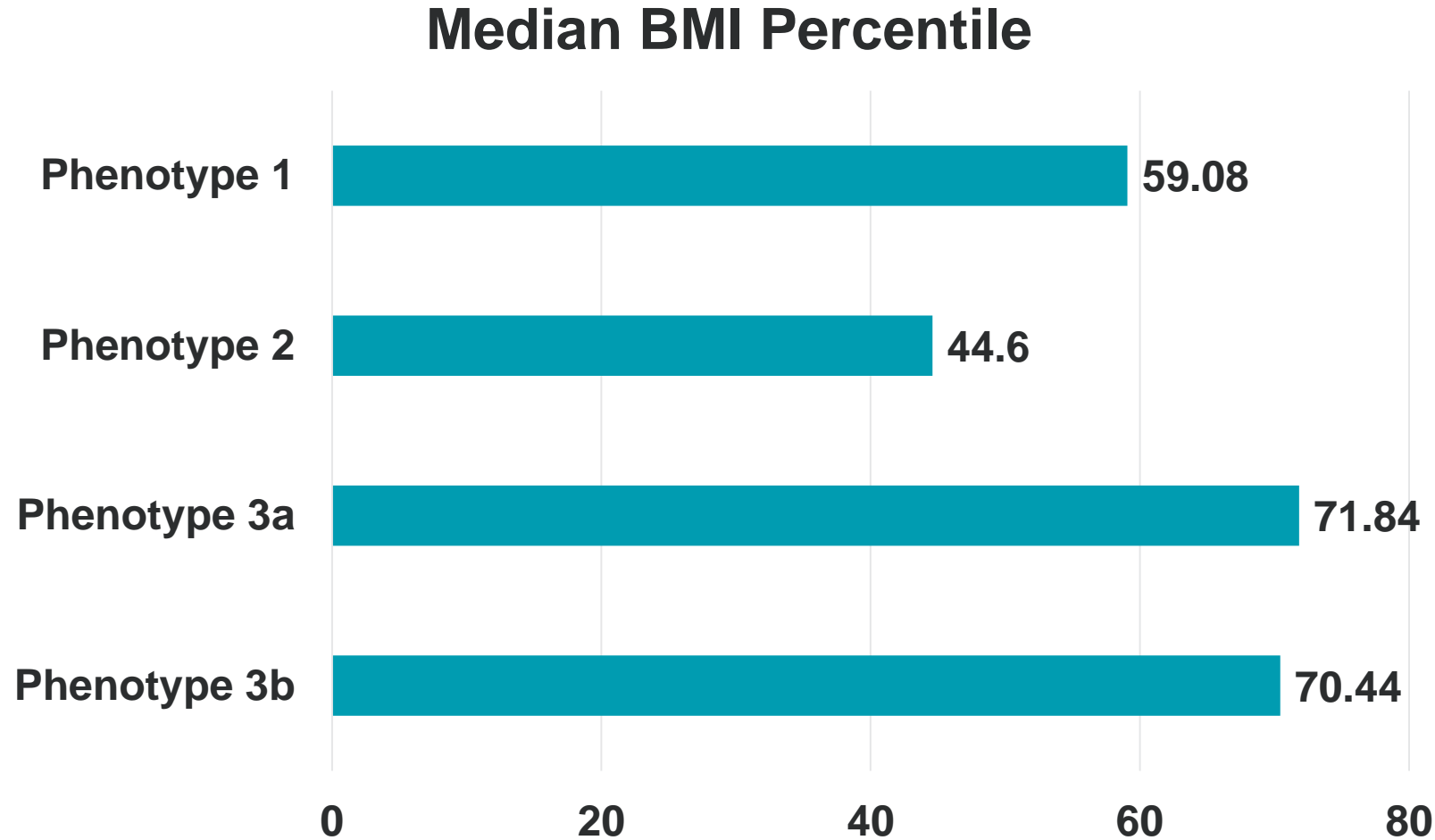
No statistically significant difference in BMI by phenotype but 3a and 3b have the largest BMI%.
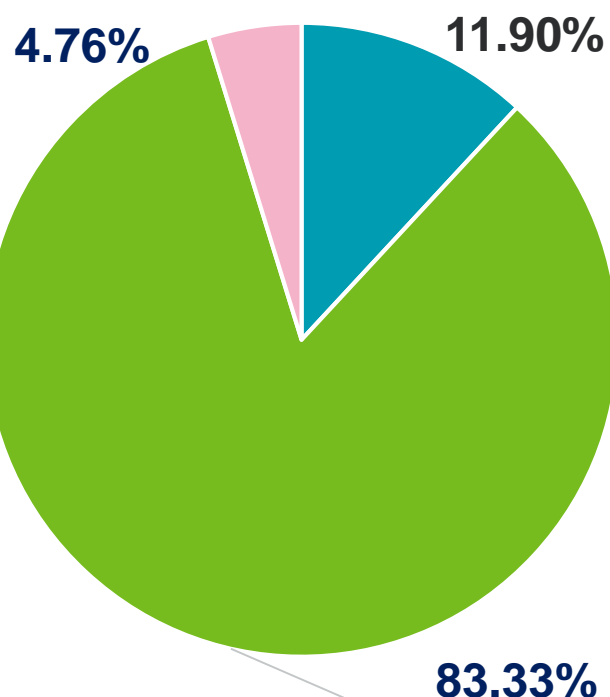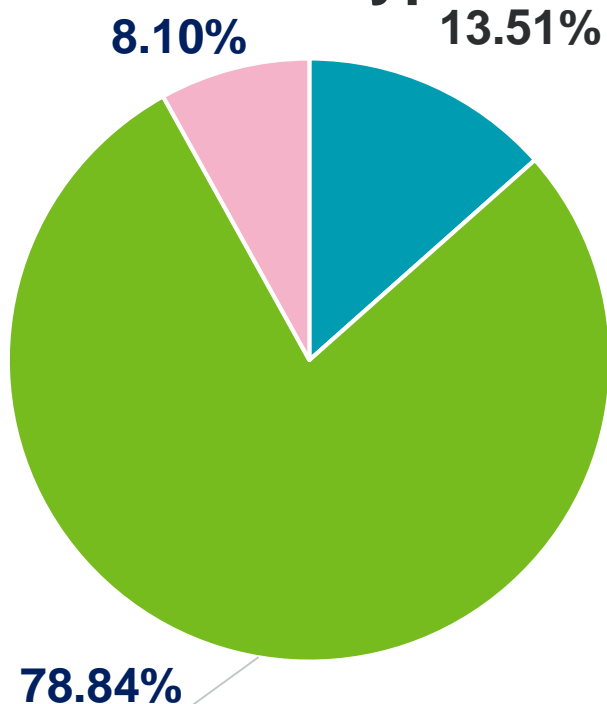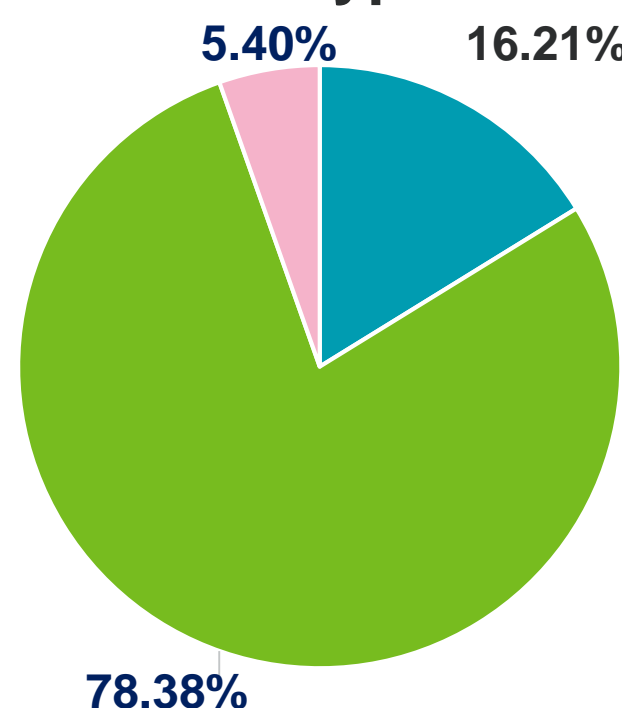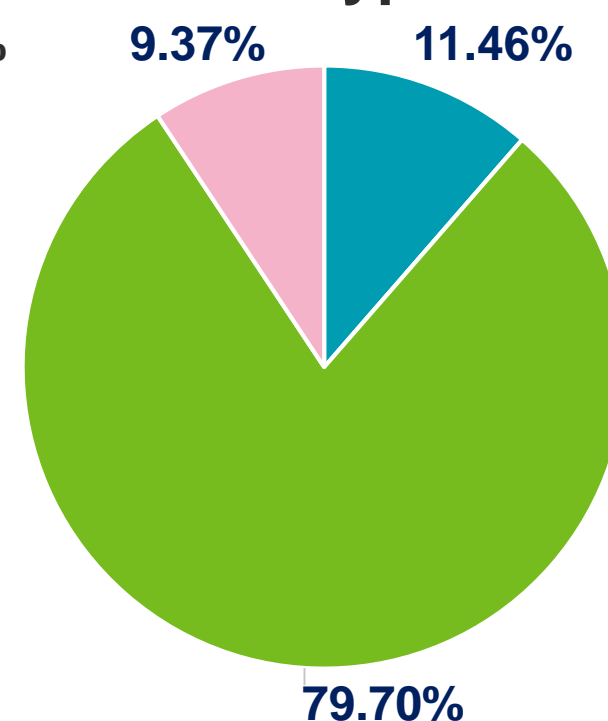
Clustering of phenotypes

- Cluster analysis assigned each girl to a phenotype based on her estradiol, estrone, DHEA-S, and testosterone at each time period (-6,0,6) .

- The hormone levels at the different time periods across the four phenotypes varied greatly indicating hormone levels relative to the timing of thelarche are not the same for all girls.

- No statistical difference in other variables including BMI, race/ethnicity, parental education level and family history of cancer existed among the phenotypes which confirms phenotypes should be based on the hormones at the three time periods.

Associations With Pubertal Milestones from Survival Analysis

Earlier ages of thelarche, pubarche and menarche differed by phenotypes, confirming heterogeneity of hormone phenotypes.

- Phenotype 2 much more likely to experience menarche earlier than all other phenotypes.
- Phenotype 3a and 3b more likely to have an earlier age at thelarche than 1.
- Phenotype 3a is more likely to have an earlier age of pubarche than 3b.

All analyses controlled for race, BMI nearest but before age of the pubertal milestone, and mother's age of menarche. As expected:

- Black girls were twice as likely to reach puberty earlier than other girls.
- Heavier girls were more likely to reach puberty earlier than those with a lower BMI.
- Girls with mother's ages of menarche younger than 12 years old are 50% more likely to reach all three milestones earlier than girls with mother's ages at least 14 years old.

Conclusions

- Classifying hormone heterogeneity prior to puberty is highly informative in unveiling different pathways through puberty.

- The four distinct hormone phenotypes in girls indicate hormones levels relative to the age of thelarche are not the same in all girls and help to explain disparity in the age of onset.

- These findings underscore the need to better understand female sex hormones prior to puberty based on time related to puberty rather than chronological age or pubertal status.

Acknowledgements

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Michigan State University

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Lab Corp

Dr. Walt Chandler

Are the phenotypes predictive of the differing ages of pubertal milestones?

Average age of pubertal events in months by phenotype

Thelarche ⬤
Pubarche ▭
Menarche ◆

High DHEA-S (1)

High estradiol (2)

Not high hormones (3a)

Low hormones (3b)

80 100 110 120 130 140 150

Months

Further Characteristics of the Phenotypes after being agnostically defined by the hormones

- Statistical difference exists between the age of thelarche and the age of pubarche among the four phenotypes.

- Girls in phenotypes 1 and 3b had an average age of menarche statistically later than girls in phenotype 2.

- The tempo of girls in phenotype 2 was statistically shorter than that for girls in phenotype 3b.

- Girls in phenotype 3b were more likely to enter puberty via pubarche rather than thelarche which is different than the other phenotypes.

- No differences between the phenotypes existed for the following characteristics:
  - BMI
  - ethnicity
  - family history of 1st or 2nd degree breast cancer
  - mother's age of menarche
  - caregiver's education

Phenotype 1

- Higher DHEA-S (over 100% higher than the cohort mean at each time period)
- Testosterone values over 50% higher than the cohort mean
- E1 values over 50% higher than the cohort mean
- Large increase in DHEA-S from -6 to 0 and 0 to 6
- Latest age of thelarche
- Latest age of menarche
- Less risk of early thelarche than 3a or 3b
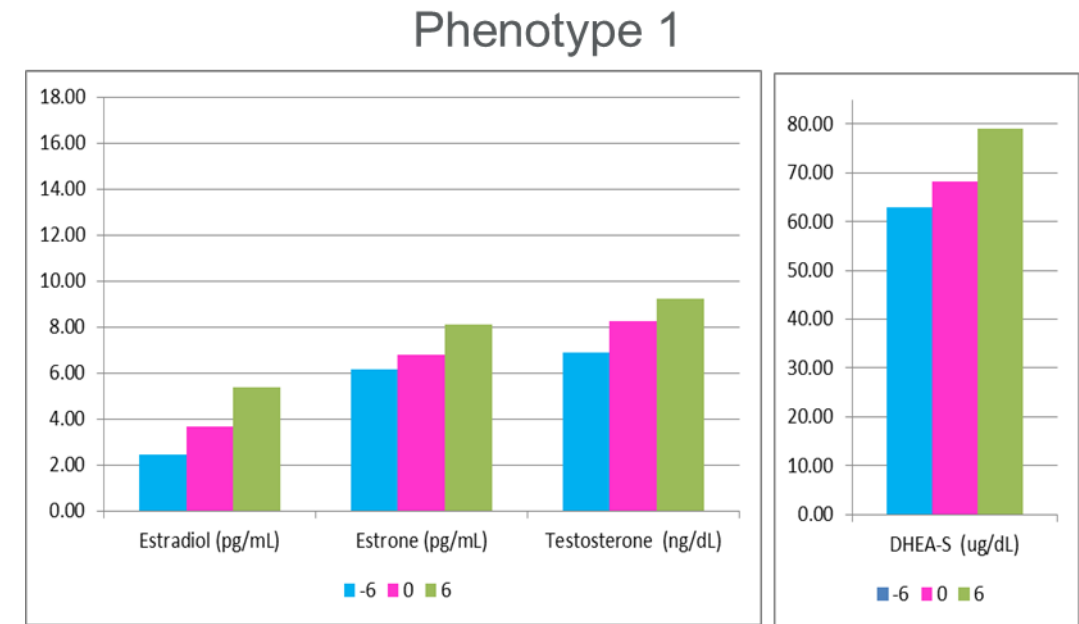
Phenotype 2

- High E2 values across the time period from 30%-200% higher than the mean of the cohort
- E1 90% higher at +6
- Testosterone >50% higher at -6 and +6
- Huge increase in E2 from 0 to 6 (300%)
- Decrease in testosterone, estrone and DHEA-S from -6 to 0, then larger increase in all hormones from 0 to 6
- Earliest to achieve menarche
- Shortest tempo
- 50% greater risk of earlier menarche than other phenotypes
- Less risk of early thelarche than 3b

Phenotype 3a
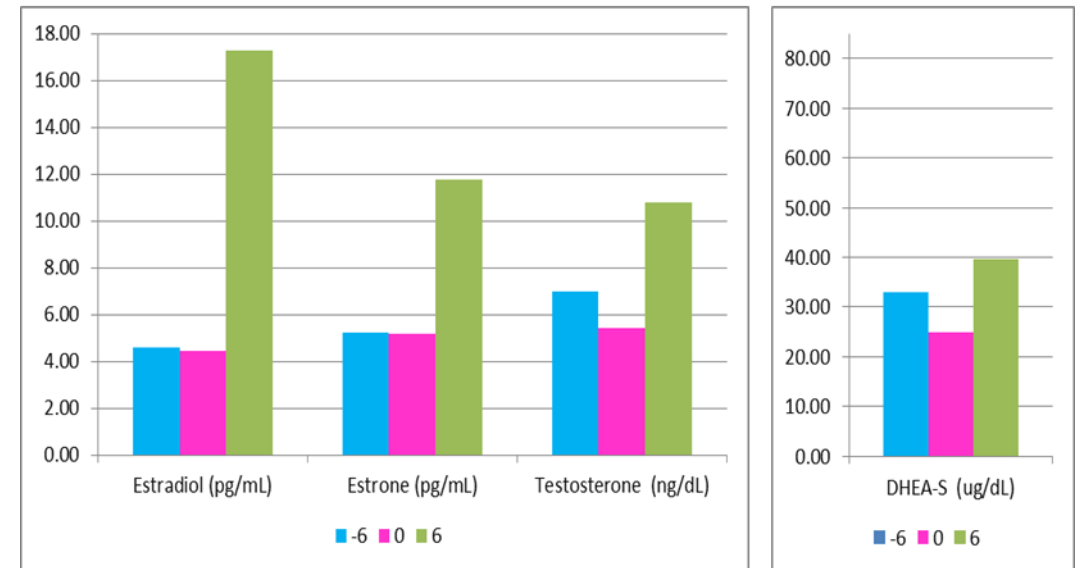
- Hormones lower than the cohort averages by about 10% except
  - 20% lower for DHEA-S at -6
  - >20% lower for all E2 time periods
- Large increase in DHEA-S from -6 to 0
- Earliest to pubarche
- More girls entering pubarche prior to thelarche than other phenotypes
- Greater risk of entering pubarche early

  than 3b

Phenotype 3a
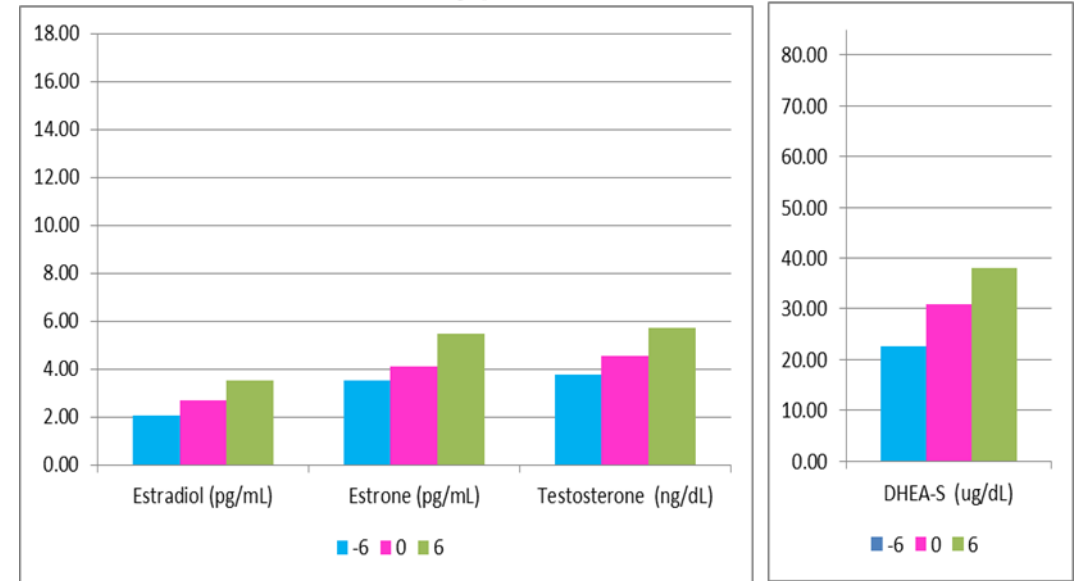
Phenotype 3b

- Over 30% lower levels for all hormones
  - DHEA-S was 50% lower at all 3 time periods
  - E1 was over 40% lower at 6 and
  - E2 at -6 and 6 and E1 at 0 (over 20% lower)
- Minimal changes in the hormones
- Earliest to thelarche
- Latest to pubarche
- Second to last to enter menarche
- Longest tempo
- Fewest girls entering pubarche prior to thelarche

Phenotype 3b
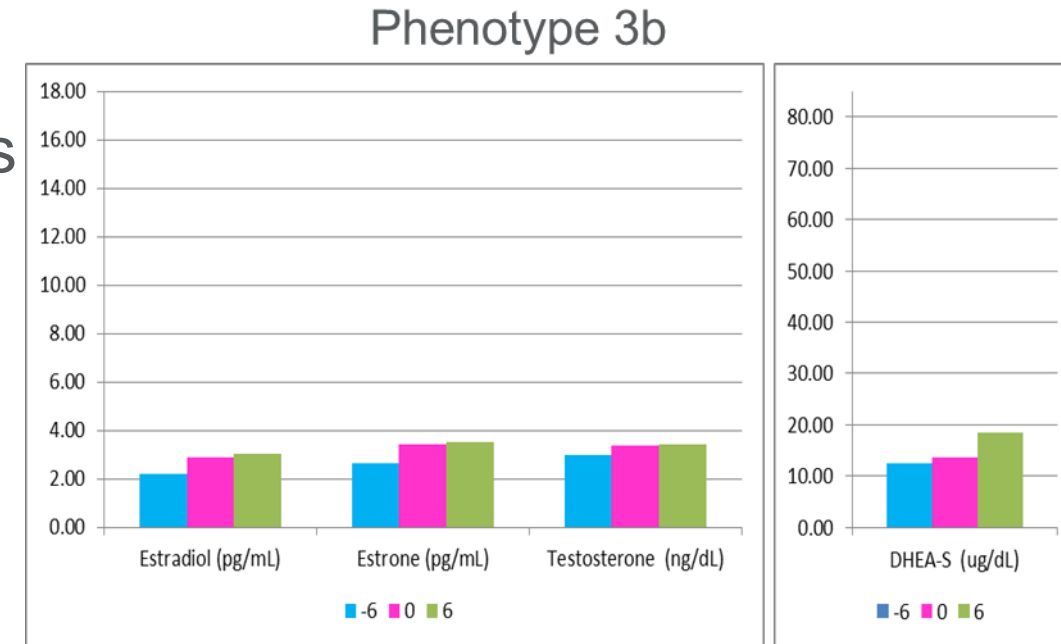
Limitations

Limitations

- Breast tissue is sometimes confused with fat tissue making some question validity of breast maturation staging.
  - Our study staff was trained and certified to assess breast maturation.
  - Cohen's Kappa (0.67) indicated "substantial agreement"

- As with any study there is the potential for volunteer bias.

- One site study leads to lack of generalizability to the United States.
  - The cohort has a similar racial and social economic background to the United States (US).
  - BMI% of the cohort is similar to the NHANEs data making it more generalizable to the entire US.[1]

1 Ogden CL, Carroll MD, Flegal KM. High body mass index for age among US children and adolescents, 2003-2006. *Journal of American Medical Association* 2008;299(20);2401-2405.

Limitations

- Accuracy of recall of age of menarche could be questioned.
  - Questionnaire data on menarche was collected yearly.
  - Recall of menarche is typically high because it is not an arbitrary event.
  - Studies have shown age of menarche recall over 63% accurate after a year or more.[1]

- Lack of age of menarche for 21% of the girls who either dropped out of the study or the study ended prior to them achieving menarche.

1 Bergsten-Brucefors A. A note on the accuracy of recalled of age of menarche. *Jrnl Annals of Human Biology* 1976;3:71-73.

Strengths

Strengths

- The use of HPLC-MS enabled us to evaluate hormones measurements that are typically too low to measure in young girls with earlier methods.

- Esoterix Laboratories (now LabCorp) is qualified by the CDC.[1]
  - Interassay Precision (% of coefficient variation for the low, medium and high control serum samples) for initial 252 girls were all less than the standard expectation of 15%. The interassay precisions follow:
    - Estradiol ≤4.4%, Estrone ≤4.9%, DHEAS ≤8.4%, Testosterone ≤9.9%[1]
  - The average bias estimations from on-going proficiency studies are less than 2%.[1]

1 Biro FM, Pinney SM, Huang B, et al. Hormone Changes in Peripubertal girls. *J Clin Endocrinol Metabl.* 2014 Oct; 99(10): 3829-3835.

Strengths

- Study staff was trained and certified to take standardized anthropometric measurements, with quality assurance procedures.

- A limited number were trained and certified to assess pubertal maturation.
  - Cohen's Kappa = 0.67 for agreement between the examiner and master trainer during 127 dual examinations across the three sites.[1]

1 Biro FM, Galvez MP, Greenspan LC, et al. Pubertal Assessment Method and baseline characteristics in a mixed longitudinal study of girls. *Pediatrics* 2010;126: e583-e590.

Strengths

- Longitudinal analysis
  - First study to quantify hormones in a longitudinal way relative to time to thelarche rather than based on chronological age.
  - Examining hormones based on a chronological age would have diluted the differences in hormone levels.

- Agnostic, objective, and innovative statistical analysis
  - PCA-CA has been used to identify clinical phenotypes in other medical conditions (e.g. COPD, asthma, sleep apnea).
  - Each phenotype included some but not all of the hormones supporting the heterogeneity of the phenotypes.

Future Directions

Future Directions

- Identification of these phenotypes and their relationships to clinical characteristics should be repeated in a larger longitudinal study to replicate these findings and extend them to a more nationally representative population. This would also enable clinical references for the phenotypes to be defined.

- Associate these phenotypes with other outcomes such as migraine headaches and breast cancer.

Windows of Susceptibility for Breast Cancer

"Windows of Susceptibility" are periods when the developing breast tissue is most susceptible to gene-environment interactions and environmental exposures that increase the risk of breast cancer

Bay Area BCERP
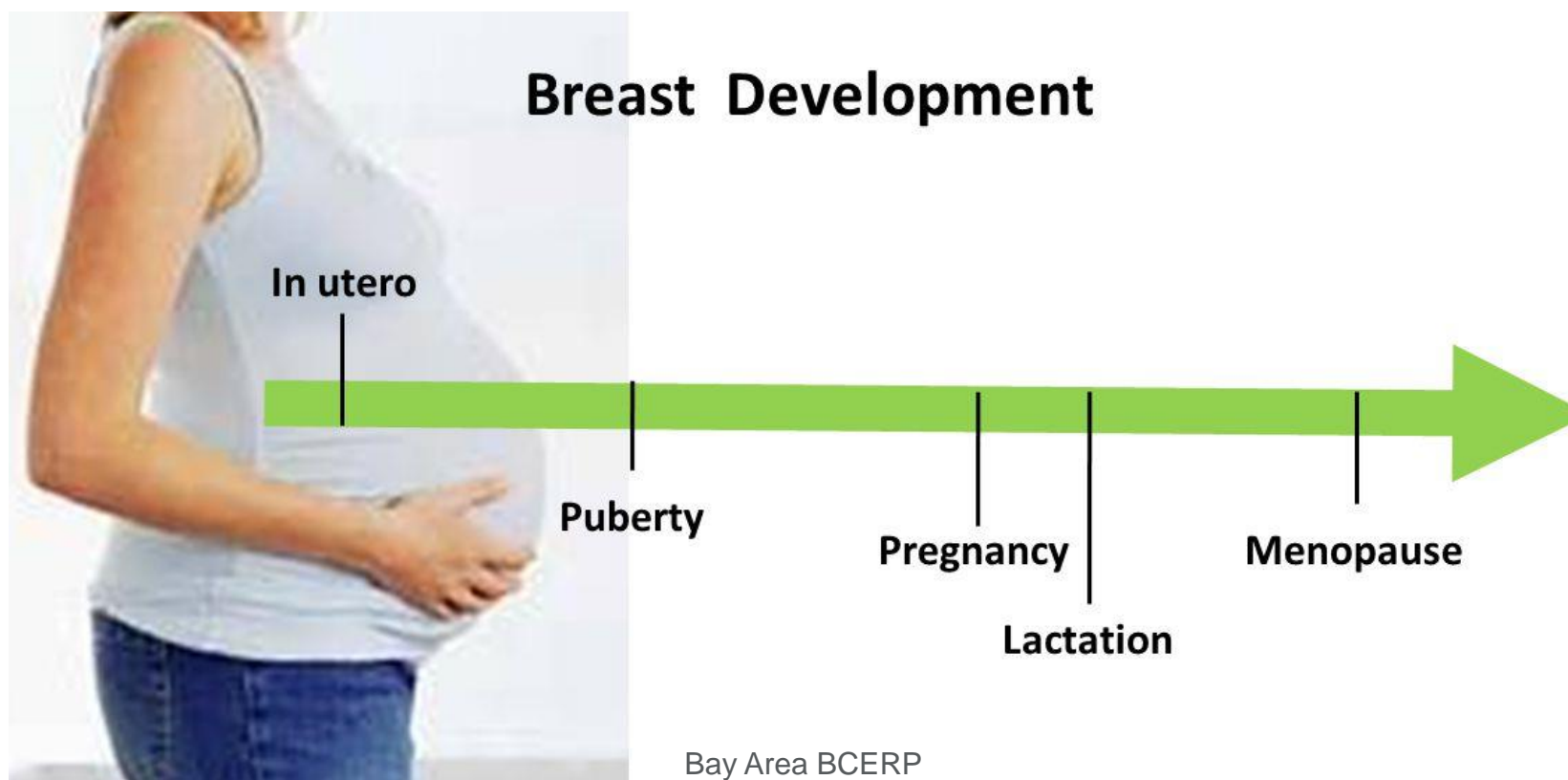
Lifecycle of the Breast
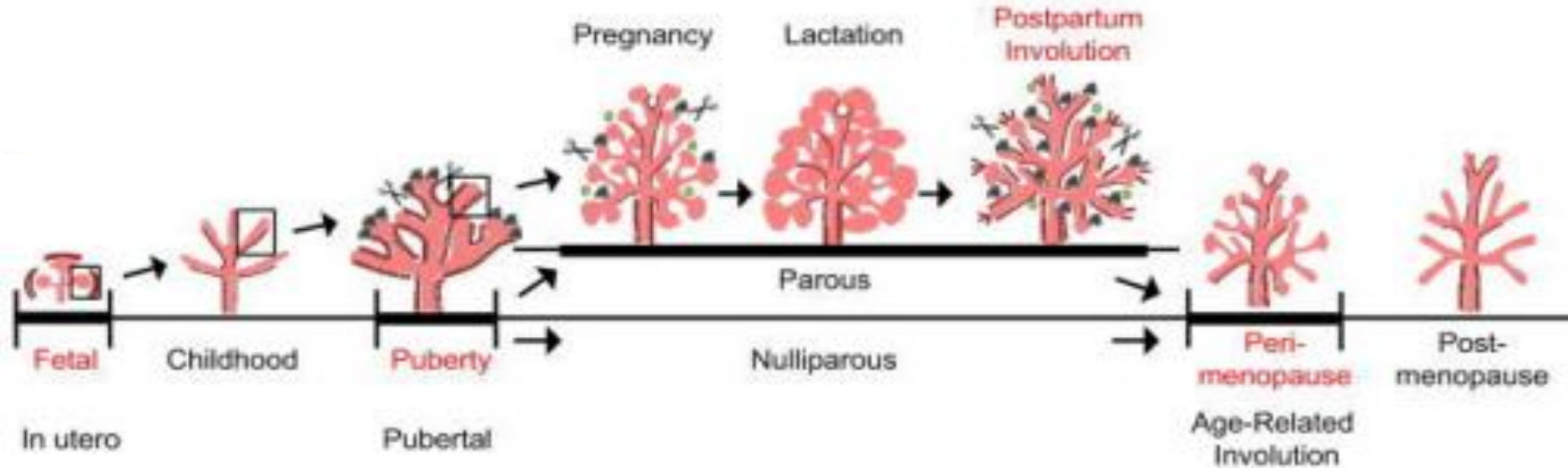
In utero – breast ducts begin to form

Puberty – breast tissue proliferation: lengthening and branching of ducts and development of lobules

Pregnancy – terminal end bud differentiation

Lactation – the milk duct system grows as more lobules form

Menopause – reduction of glandular tissue in the breast

Martinson HA, Lyons TR, Giles ED, Borges VF, Schedin P. Developmental windows of breast cancer risk provide opportunities for targeted chemoprevention. *Experimental cell research*. 2013;319(11):1671-1678

Pubertal Milestones

There are a number of pubertal milestones reached before the end of puberty and full sexual maturation.
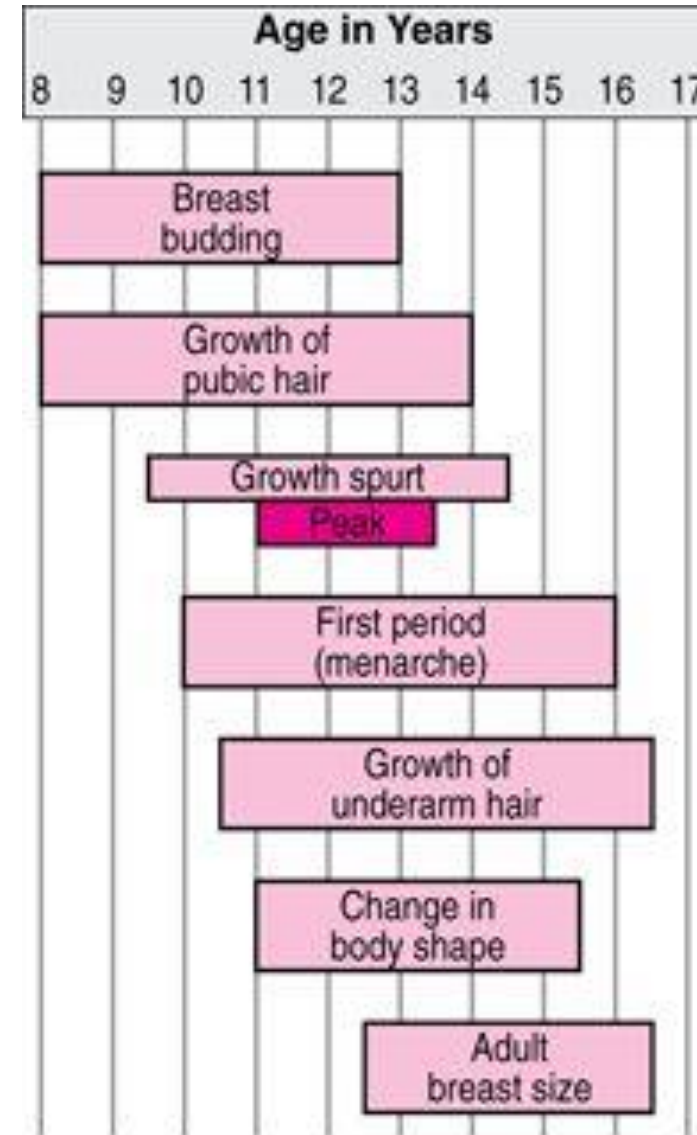
Sex Characteristics

- Primary Sex Characteristics – sex organs responsible for reproduction e.g. ovaries, uterus

- Secondary Sex Characteristics – physical characteristics that are not responsible for reproduction e.g. pubic hair, enlarged breasts, increase height

Objective

- The objective of this research project is to use longitudinal cohort data and quantitative research methods to identify sex hormone phenotypes around the time of thelarche. These analyses will incorporate serum concentrations of up to four hormones (DHEA-S, estradiol, estrone and testosterone) at five different time periods measured in 6 month increments from 18 months prior to 6 months after the age of thelarche.

- A second objective of the research is to determine if the ages of pubertal milestones (thelarche, pubarche, and menarche) are associated with a hormone phenotype.

Study Measurements

BMI% - calculated as weight in kilograms/ height in meters^2

- Derived from the average of two measurements taken at each study visit by trained staff
- Determined using CDC growth charts from 2000
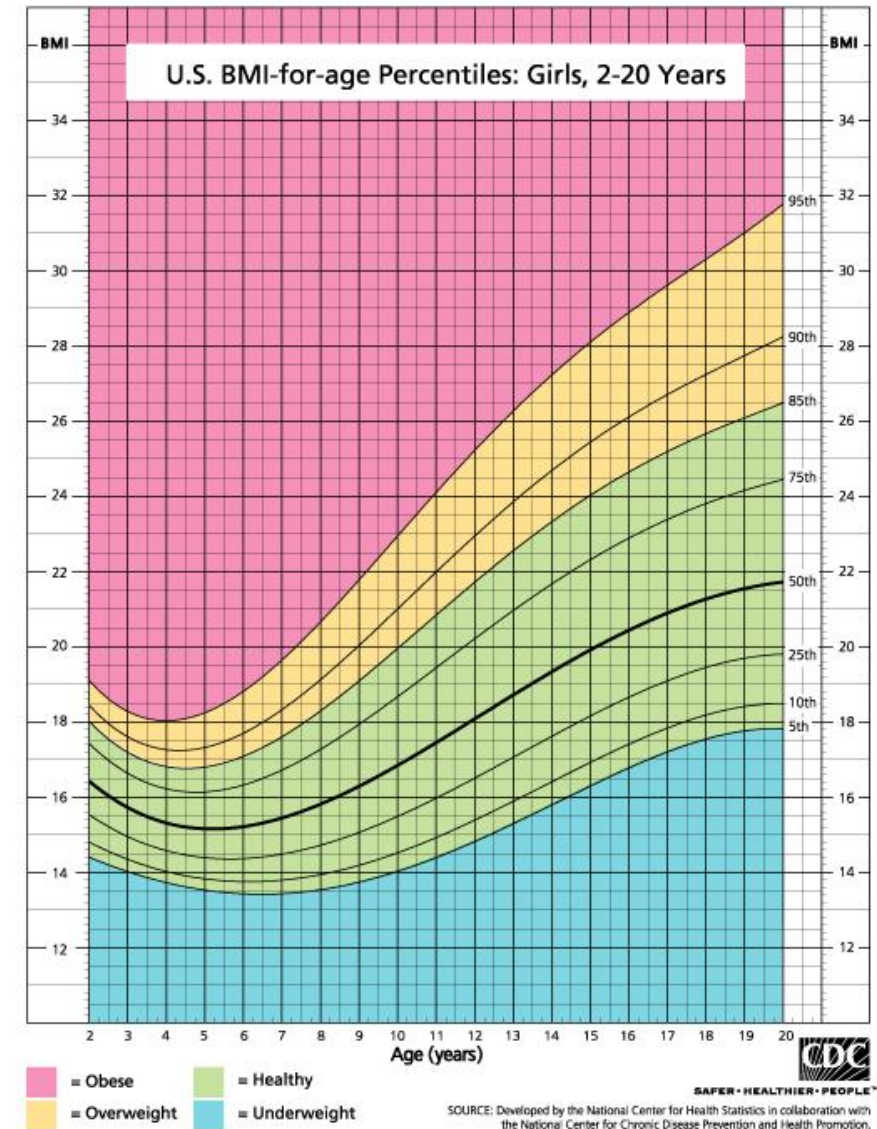
Study Measurements

BMI% vs BMIz score?

- BMIz score is the number of standard deviations away from the mean BMI for an age group. This score can be compared across age groups unlike BMI% and the measure is usually normal in distribution.

- BMI% is the percentage of people who fall below a certain value. However it is likely to not be normal in distribution.

Hormone Attributes

Table 1 - Description of hormones for the study cohort girls across the 5 time periods (-18,-12,-6,0, and 6)

Hormone	N	Median	Mean	Standard Deviation	Minimum	Maximum	LOD	# < LOD	% < LOD
DHEA-S (ug/dL)	920	22.00	30.16	26.57	7.07	211.00	10.00	170	18.48
Estradiol (pg/mL)	856	1.80	3.42	6.26	0.71	114.00	1.00	207	24.18
Estrone (pg/mL)	858	3.60	4.36	3.41	1.77	51.00	2.50	254	29.60
Testosterone (ng/dL)	859	4.10	4.89	3.59	1.77	50.00	3 or 2.5*	242	28.17

All values <LOD imputed with LOD/$\sqrt{2}$

* Two batches were sent off to assay.

Objective

Use principal component and cluster analysis (PCA-CA) to identify, for the first time, distinct hormone phenotypes of girls in a longitudinal cohort.

- Focus on patterns in the data, that could be predictors of risk of early or late pubertal maturation.

- Use an agnostic approach to define the phenotypes by completing the statistical analysis of the sex hormone data only and then looking at other variables to describe the phenotypes.

Correlations

- The lack of correlation of E2 at different time periods and the high degree of correlation among the other absolute hormones supports the inclusion of them into PCA-CA.

- The overall lack of correlation between the differences in the hormone values between the time periods (e.g. the difference in testosterone values between -18 and -12) does not support inclusion of these "change" variables into PCA.

Variable Dimension Reduction
PCA

Results from factor analysis will be used as a guide to determine which variables to be used in cluster analysis to determine a hormone phenotype.

1. PCA of absolute hormones at the time periods -6,0,6 – produced fairly consistent results in the sensitivity analysis (n=260 vs 67).

2. PCA of the differences in hormones between the time period produced one factor including estrone, testosterone and DHEA-S differences between -6 and 0 and the differences between 0 and 6 which is consistent with the lack of correlation

… seems to make sense not to combine the two ways of looking at the hormones (absolute vs differences) and focus on the absolute values for cluster analysis

Participant Clustering
Proc Fastclus – Hormone Values at time =-6,0,6

- Cluster analysis will classify the participants into phenotype groups that exhibit similar clinically relevant hormone values related to thelarche
- Identifies disjoint clusters of observations by distance (nearest centroid)
- Assigns participants to a cluster where the other participants are more similar to them than participants in another cluster. Each participant belongs to only one cluster.
- Uses *k*-means method (least squares)
  - User defines the number of clusters (k)
  - Observations are divided in to the clusters
  - The first k observations (no missing values) are selected as the initial seeds
  - Other observations are then assigned to the closest cluster
  - The cluster center is updated
  - Repeated in an iterative process until all observations are grouped into their closest cluster
- An adjusted distance is computed for missing data (Fastlcus is the only cluster procedure that handles missing data)
- Good for datasets larger than 100
- Very sensitive to outliers and has a method to identify outliers in their own clusters (unlike other cluster procedures)
- Need to standardized the variables first because Fastclus uses an algorithm that emphasizes variables with larger variances.

1-Changing the number of Clusters

CA - n=260; clusters = 6

All Girls included in Cluster Analysis - 6 clusters FASTCLUS
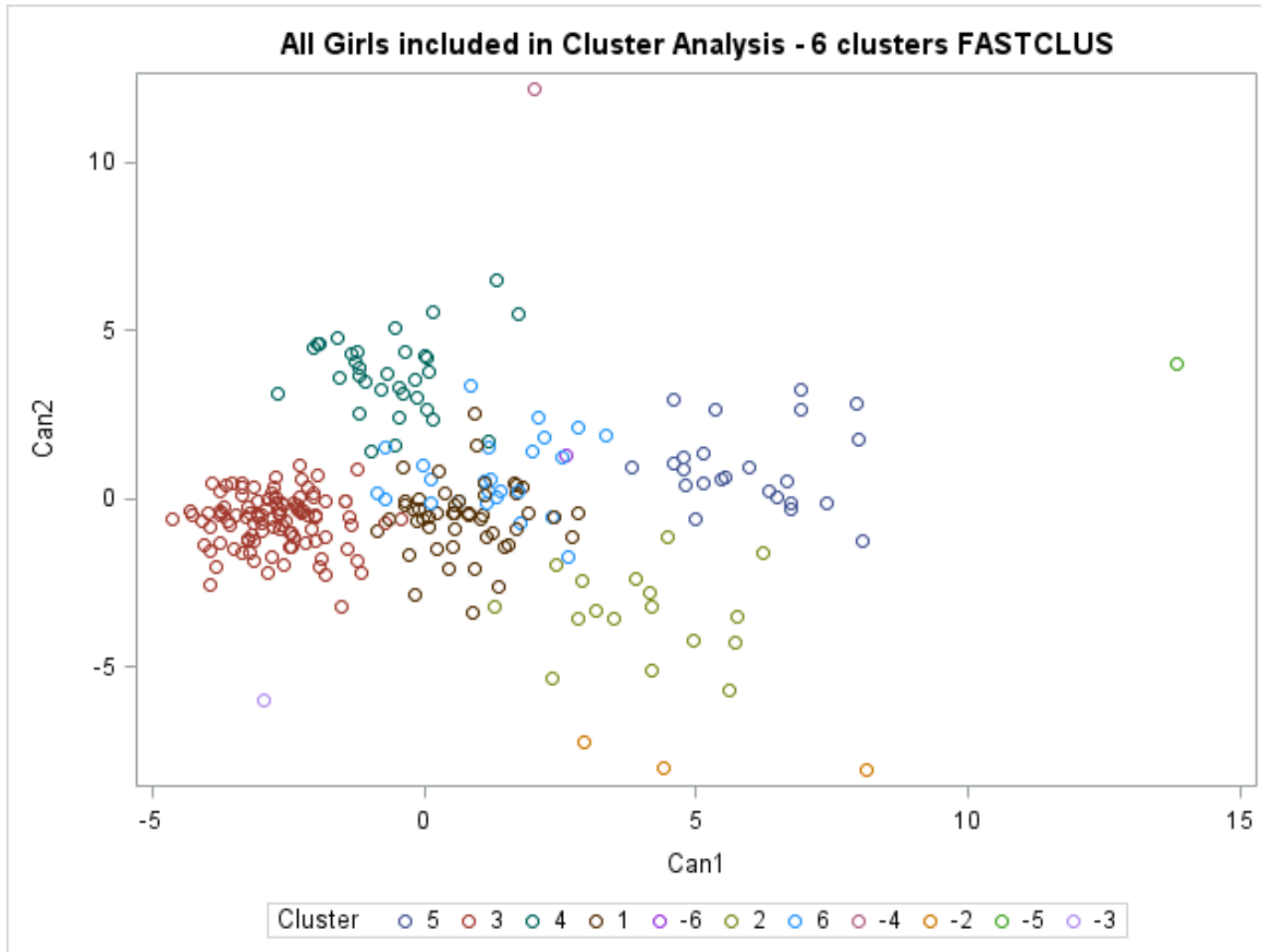
Outliers = 7
Cluster 1 n=49
Cluster 2 n=17
Cluster 3 n=105
Cluster 4 n=33
Cluster 5   n=24
Cluster 6 n=25

- cluster 1 and 6 overlap implying they not distinct

- cluster 2 is really small (perhaps should be outliers?)

- Too many clusters????

1 -Changing the number of Clusters
CA - n=260; 4 clusters and 5 clusters

All Girls included in Cluster Analysis - MAX= 4 CLUSTERS FASTCLUS

Cluster 1 n=151
Cluster 2 n=25
Cluster 3 n=46
Cluster 4 n=29
Outliers n=9

All Girls included in Cluster Analysis - 5 Clusters FASTCLUS

Cluster 1 n=110
Cluster 2 n=24
Cluster 3 n=29
Cluster 4 n=55
Cluster 5 n=34
Outliers n=8
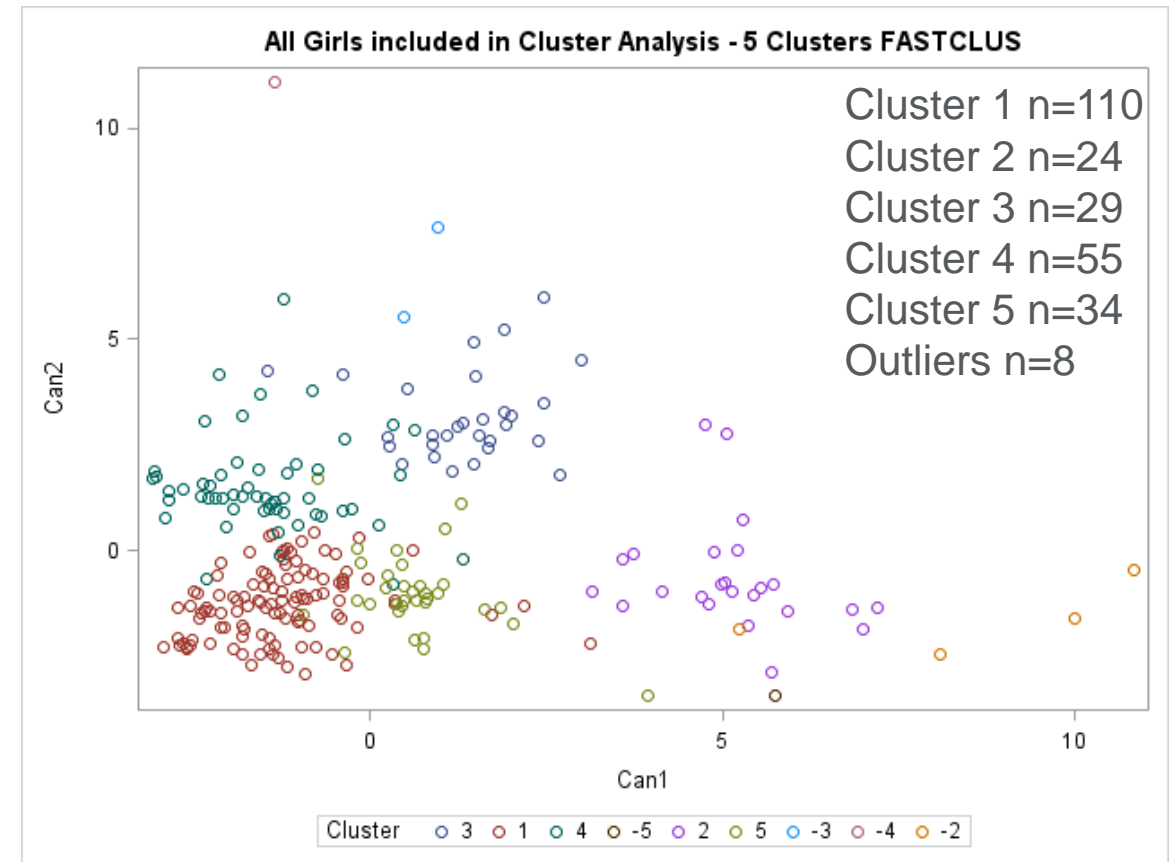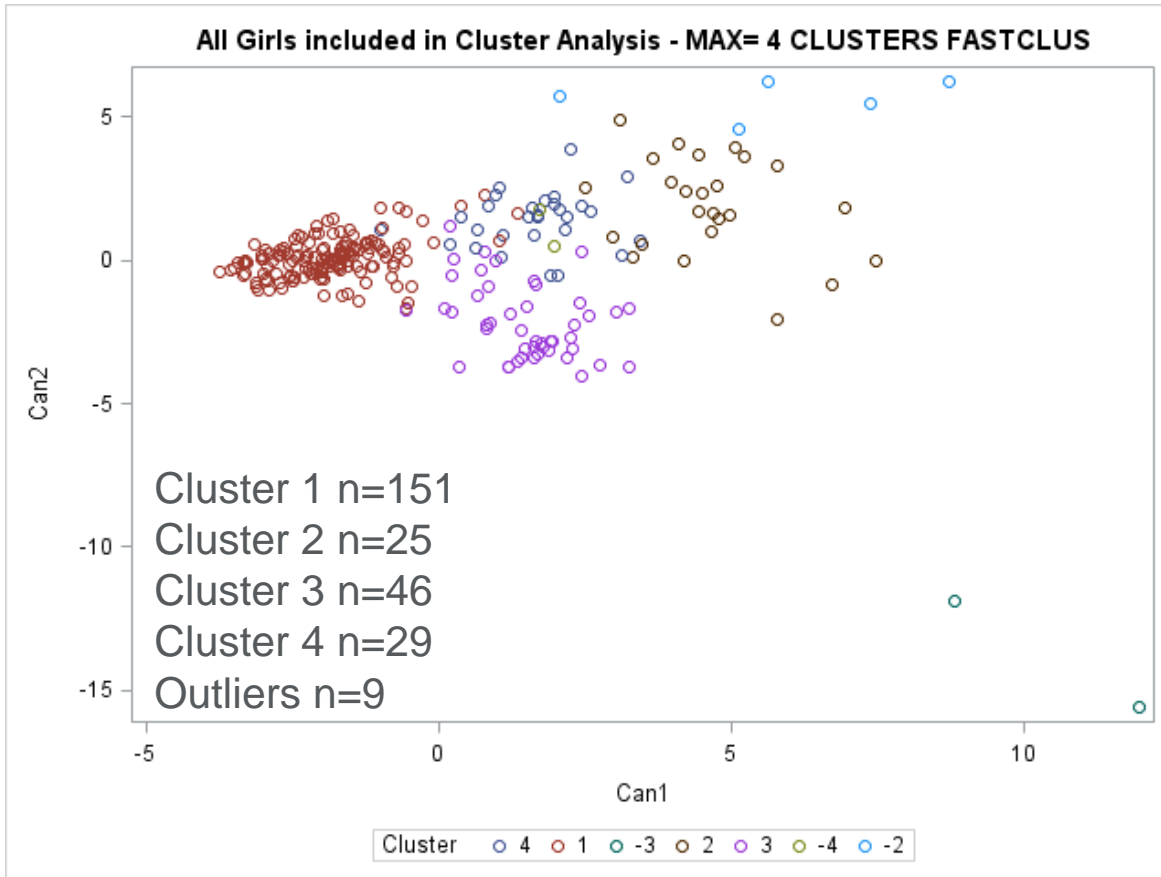
Still seems to be overlap of some of the clusters and there is still one large cluster. What if we look at that large cluster only?

Creating 4-6 clusters of the entire cohort (n=260) did not produce distinct clusters. This reinforces the results from PCA , 3 components that represent 74% of the variance should result in 3 clusters. But are we missing something?

Therefore let's try redoing the PCA-CA on only cluster 3 (N=172)

2 - CA on Cluster 3 (n=172) (mean values)

Hormone Phenotype Objective Predictors

Chracteristics of the 172: girls means reported unless noted.

	Cohort	Cluster 3A Not High	Cluster 3B Low	Significance (P value)*
	N=269	N=74	n=96	
Testosterone -6[‡]	4.48	3.79	2.97	<0.001
Testoserone 0[‡]	4.96	4.55	3.39	<0.001
Testosterone 6[‡]	6.16	5.72	3.44	<0.001
Estrone -6[‡]	4.00	3.54	2.65	<0.001
Estrone 0[‡]	4.47	4.10	3.43	<0.001
Estrone +6[‡]	6.09	5.45	3.53	<0.001
DHEA -6[‡]	28.23	22.51	12.56	<0.001
DHEA 0[‡]	30.79	31.00	13.63	<0.001
DHEA +6[‡]	38.32	38.00	18.55	<0.001
Estradiol -6	2.83	2.07	2.22	<0.001
Estradiol 0[‡]	3.37	2.67	2.88	<0.001
Estradiol +6[‡]	5.38	3.51	3.05	<0.001

* Comparison between clusters using analysis of variance for continuous variables.

Much lower values

Cluster 3a– hormones lower than the cohort averages by about 10% except 20% lower for DHEA-S at -6 and >20% lower for all E2 time periods

Cluster 3b – hormones much lower than the cohort, over 30% lower for all hormones except E2 at -6 and 6 and estrone at 0 (over 20% lower). E2 was over 40% lower at 6 and DHEA-S was 50% lower at all 3 time periods

Phenotypes

Initially....

Hormones were used to define 4 phenotypes of sex hormones in girls around the time of thelarche.

Then....

After phenotypes were formed, additional variables were then examined to further describe the participants in each of the phenotypes.

Phenotype Characteristics - from CA

(mean values)

Sex hormone serum concentrations of the girls according to the four phenotypes identified using principal component based cluster anlaysis
DHEA-S (ug/dL), estrone and estradiol (pg/mL), testosterone (ng/dL)

	Cohort		Phenotype 1 High DHEA-S, T and E1		Phenotype 2 High E2, T and E1		Phenotype 3a No High Hormones		Phenotype 3b All Low Hormones		
	N=269		N=42		N=37		N=74		N=96		
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Testosterone -6[‡]	4.48	2.69	6.89	2.53	7.00	3.22	3.79	1.60	2.97	1.29	<0.0001
Testoserone 0[‡]	4.96	2.76	8.23	2.80	5.40	1.93	4.55	1.55	3.39	1.72	<0.0001
Testosterone 6[‡]	6.16	3.64	9.23	3.22	10.79	4.01	5.72	1.52	3.44	1.26	<0.0001
Estrone -6[‡]	4.00	2.19	6.18	1.16	5.24	2.11	3.54	1.68	2.65	1.27	<0.0001
Estrone 0[‡]	4.47	2.46	6.81	2.37	5.17	2.31	4.10	1.72	3.43	1.98	<0.0001
Estrone +6[‡]	6.09	4.46	8.10	2.39	11.78	2.92	5.45	1.94	3.53	1.72	<0.0001
DHEA-S -6[‡]	28.23	24.99	62.97	25.60	32.93	14.39	22.51	10.93	12.56	6.83	<0.0001
DHEA-S 0[‡]	30.79	26.08	68.13	24.80	24.91	12.42	31.00	10.77	13.63	6.15	<0.0001
DHEA-S +6[‡]	38.32	29.16	79.00	29.29	39.64	19.37	38.00	14.01	18.55	8.13	<0.0001
Estradiol -6	2.83	3.32	2.46	1.75	4.60	2.78	2.07	1.75	2.22	1.96	<0.0001
Estradiol 0[‡]	3.37	4.22	3.68	2.61	4.46	4.71	2.67	2.37	2.88	3.05	<0.0001
Estradiol +6[‡]	5.38	7.16	5.40	4.04	17.25	10.48	3.51	2.60	3.05	3.55	<0.0001

* Baseline values for the cohort given as mean unless noted. P values represent tests for groupwise differences between the phenotypes; values for the phenotypes represent mean values within the phenotypes. Comparison between phenotypes using Kruskal-Wallis for continuous variables and χ^2 for categorical.

‡ Objective predictive variables used for phenotype development.

Much higher values
Higher values
Much Lower values

Phenotype 1 – Higher DHEA-S (over 100% higher than the cohort mean at each time period)
- Testosterone and E1 values over 50% higher than the cohort mean

Phenotype 2 – High E2 values across the time period from 30%-200% higher than the mean of the cohort
- E1 90% higher at +6
- testosterone >50% higher at -6 and +6 vs the cohort mean

Phenotype 3a – hormones lower than the cohort averages by about 10% except
- 20% lower for DHEA-S at -6
- >20% lower for all E2 time periods

Phenotype 3b– Over 30% lower levels for all hormones
- DHEA-S was 50% lower at all 3 time periods
- E1 was over 40% lower at 6 and
- E2 at -6 and 6 and E1 at 0 (over 20% lower)

Characteristics of Participants in each Phenotype (mean values unless noted)

Maturation and clinical characteristics of the girls according to the four phenotypes identified using principal component based cluster anlaysis

DHEA-S (ug/dL), estrone and estradiol (pg/mL), testosterone (ng/dL)

	Cohort N=269		Phenotype 1 High DHEA-S, T and E1 N=42		Phenotype 2 High E2, T and E1 N=37		Phenotype 3a No High Hormones N=74		Phenotype 3b All Low Hormones N=96		
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Age of Thelarche (months)	108.26	13.20	113.24	11.29	112.49	13.16	106.36	12.98	104.05	12.31	<0.0001
Age of Pubarche (months)	118.19	16.24	116.83	19.22	118.69	11.39	113.91	17.16	121.56	15.06	0.0345
Age of Menarche (months)	147.59	13.75	150.70	15.34	142.48	11.84	147.65	14.31	148.63	12.30	0.0606
Tempo (time between	39.01	13.06	36.39	11.50	30.55	10.27	40.50	10.87	45.12	12.58	<0.0001
Pubertal Pathway (%)											0.0017
thelarche before pubarche	69.14%		66.67%		64.86%		64.86%		79.17%		
pubarche before thelarche	17.10%		26.17%		16.22%		28.38%		5.21%		
entered at the same time	7.43%		7.14%		13.51%		4.05%		4.17%		
missing due to censorship	6.32%		0.00%		5.41%		2.70%		11.46%		

* Baseline values for the cohort given as mean unless noted. P values represent tests for groupwise differences between the phenotypes; values for the phenotypes

represent mean values within the phenotypes. Comparison between phenotypes using Kruskal-Wallis for continuous variables and χ^2 for categorical.

- No differences between the phenotypes existed for BMI, ethnicity, family history of breast cancer, mother's age of menarche or caregiver's education.
- Pairwise comparisons proved the age of menarche to be different between Phenotypes 1 and 2.

Phenotype 1 –latest age of thelarche (5 months) but earlier pubarche by 2 months, latest to menarche, more girls entering pubarche prior to thelarche.

Phenotype 2 - later age of thelarche , earliest to achieve menarche, shortest tempo,

Phenotype 3a –Early to thelarche and earliest to pubarche, more girls entering pubarche prior to thelarche.

Phenotype 3b -Earliest to Thelarche, latest to pubarche, longest tempo

Changes in hormone levels from one time window to the next (mean values unless noted)

Maturation and clinical characteristics of the girls according to the four phenotypes identified using principal component based cluster anlaysis
DHEA-S (ug/dL), estrone and estradiol (pg/mL), testosterone (ng/dL)

| | Cohort | | Phenotype 1 High DHEA-S, T and E1 | | Phenotype 2 High E2, T and E1 | | Phenotype 3a No High Hormones | | Phenotype 3b All Low Hormones | | |
| | N=269 | | N=42 | | N=37 | | N=74 | | N=96 | | |
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Δ Testosterone from -6 to 0	1.26	4.68	1.81	4.75	-4.95	6.46	1.64	3.45	1.34	3.42	0.0045
Δ Testosterone from 0 to 6	2.38	5.50	3.54	4.31	12.76	10.09	1.97	2.94	0.20	3.60	<0.0001
Δ Estrone from -6 to 0	1.32	3.74	1.24	4.71	-0.73	4.62	1.65	2.87	1.72	3.35	0.1052
Δ Estrone from 0 to 6	1.88	4.87	2.84	3.76	11.80	5.10	1.98	3.55	0.08	4.53	<0.0001
Δ DHEA-S from -6 to 0	9.68	24.85	17.15	21.26	-12.71	31.87	14.52	22.22	3.27	11.11	0.0001
Δ DHEA-S from 0 to 6	15.14	26.71	27.27	41.84	28.38	39.36	15.37	21.16	8.90	14.42	0.1014
Δ E2 from -6 to 0	1.21	8.31	2.13	3.72	1.66	10.04	1.33	5.53	2.26	5.80	0.4542
Δ E2 from 0 to 6	2.19	11.79	1.94	7.54	25.74	22.10	1.46	5.97	1.13	6.63	0.0006

* Baseline values for the cohort given as mean unless noted. P values represent tests for groupwise differences between the phenotypes; values for the phenotypes represent mean values within the phenotypes. Comparison between phenotypes using Kruskal-Wallis for continuous variables and χ^2 for categorical.

Δ = change in hormone value

Phenotype 1 –large increase in DHEA-S from -6 to 0 and 0 to 6

Phenotype 2 – huge increase in E2 from 0 to 6, decrease in testosterone, estrone and DHEA-S from -6 to 0, larger increase all hormones from 0 to 6.

Phenotype 3a –larger increase in DHEA-S from -6 to 0 than the cohort

Phenotype 3b –very few changes in the hormones over the time periods

Increase between time periods
Decrease in values between -6 to 0 then an larger increase from 0 to 6

Survival Analysis

Survival analysis (Cox proportional-hazard models) were conducted in SAS using PHReg and controlling for covariates.

$$h(t,x)=h_0(t)\exp(\beta x)$$

such that $h_0(t)$ is the underlying hazard function which is independent of x, x is the covariate and t stands for time. A unit increase in x multiplies the hazard by $\exp(\beta)$ or e^{β} for all values of t, β is assumed the same for all individuals.

Assumption of proportional hazards – additive changes in a variable cause corresponding multiplicative changes in the hazard function e.g. ratio of hazards for two individuals over time is constant

Girls who have not yet reached the pubertal milestone during the study or were lost to follow up prior to achieving it will be right censored.

Survival Analysis

Hazard Ratio ($\exp(\beta)$) is the the measure of effect or risk of suffering the event/outcome.

- A ratio <1 indicates a reduced risk of reaching the pubertal outcome (eg delayed menarche) e.g HR=.88 means a 22% decreased risk for reaching menarche for every unit increase in mother's age of menarche.

- A ratio >1 indicates an increased risk of reaching menarche (eg early menarche) e.g. HR=1.44 means a 44% risk of reaching menarche every for unit of increase in DHEAS at -6 months.

- A ratio =1 indicates no relationship between the predictor and the age of pubertal outcome outcome.

Survival Analysis

Cox- Proportional model advantages

- does not require a certain probability function[43,44]

- may include multiple covariates

- may include continuous and/or categorical covariates

- allows for the inclusion of girls who are right censored

- allows for interactions between covariates

- time varying covariates e.g. BMI% that changes over time vs only including the BMI% at the -12 month study visit

Survival Analysis

Cox-Proportional model assumption

Proportional hazards – additive changes in a variable cause corresponding multiplicative changes in the hazard function e.g. ratio of hazards for two individuals over time is constant

- Plot of the log-negative-log of the Kaplan Meier estimates of the survival function against the log of time should be parallel if the hazard is constant over time for a categorical predictor. [44,45]
- If a variable fails the assumption, will include a multiplicative with time.

Survival Analysis

Non-parsimonious Model

Besides the phenotypes, the models will include the following even if they are insignificant because they are known risk markers for early pubertal timing

- Race
- BMIz - nearest but before age of the pubertal milestone
- Caregiver's education
- Mother's age of menarche

- Multiplicative interaction with time included if a variable failed the proportional hazard assumption

Risk Estimates of Pubertal Milestones

Proportional hazard ratio analysis of risk factors for age at pubertal milestones

Variable	Age of Thelarche (months)			Age of Pubarche (months)			Age of Menarche (months)		
	Hazard Ratio	Wald's 95% CI	p-vlaue	Hazard Ratio	Wald's 95% CI	p-vlaue	Hazard Ratio	Wald's 95% CI	p-vlaue
Phenotypes			<0.0001			0.012			0.1254
DHEA-S (1) vs high estradiol (2)	0.93	0.59 1.48	0.7600	0.91	0.57 1.46	0.7013	0.55	0.32 0.93	0.0264
DHEA-S (1) vs no high hormones (3a)	0.63	0.43 0.93	0.0196	0.77	0.52 1.15	0.2042	0.88	0.57 1.35	0.555
DHEA-S (1) vs all low hormones (3b)	0.45	0.31 0.66	<0.0001	1.32	0.91 1.93	0.1493	0.87	0.57 1.33	0.514
high estradiol (2) vs no high hormones (3a)	0.68	0.45 1.03	0.0656	0.85	0.56 1.29	0.4433	1.61	1.02 2.54	0.0427
high estradiol (2) vs all low hormones (3b)	0.48	0.32 0.73	0.0006	1.45	0.96 2.18	0.0752	1.59	1.01 2.52	0.0466
no high hormones (3a) vs all low hormones	0.71	0.52 0.97	0.0337	1.71	1.24 2.35	0.0012	0.99	0.70 1.41	0.9647
Race (all other vs black)	0.67	0.51 0.88	0.0256	0.01	0.00 0.06	<0.0001	0.01	0.00 0.30	0.0092
BMIZ closest to outcome	7.18	2.65 19.51	0.0001	1.16	1.05 1.30	0.0056	1.57	1.36 1.81	<0.0001
Mother's age of menarche (years)			0.055			0.0837			0.1327
Under 12 vs ages 12-14	1.34	0.96 1.87	0.0905	1.15	0.81 1.62	0.432	1.20	0.83 1.74	0.3418
Under 12 vs at least 14	1.68	1.10 2.57	0.0168	1.58	1.04 2.42	0.0336	1.61	1.01 2.59	0.0468
ages 12-14 vs at least 14	1.26	0.89 1.77	0.1931	1.38	0.98 1.95	0.0664	1.35	0.91 1.99	0.1334
Caregiver's education level			0.9763			0.6314			0.3051
High school or less vs at least an associate's or bachelor's degree	1.00	0.67 1.49	0.9967	0.88	0.58 1.33	0.5491	0.73	0.45 1.18	0.1946
High school or less vs master's degree or more	0.97	0.68 1.38	0.8618	0.84	0.59 1.20	0.3376	0.97	0.65 1.44	0.8747
At least an associate's or bachelor's degree vs master's degree or more	0.97	0.70 1.35	0.8568	0.95	0.68 1.33	0.7829	1.33	0.90 1.96	0.1478
race*age of milestone in months				0.96	0.94 0.98	0.0004	0.97	0.945 0.994	0.0166
bmiz closest to outcome* age of milestone in months	0.99	0.98 1.00	0.0036						

- 1, 2 and 3a were less likely to enter early pubarche than 3b and 1 was also less likely than 3a.
- 3a was much more likely to enter thelarche earlier than 3b.
- 2 was 50% more likely to enter menarche earlier vs all 3 other phenotypes.
- White girls were half as likely to enter puberty early than black girls.
- Being heavier increased the likelihood of entering puberty early.
- Girls whose mother's who entered puberty prior to 12 years old had a much higher chance of entering early puberty than those whose mothers entered after 14 years old.

Characteristics of Participants in each Phenotype (mean values unless noted)

Maturation and clinical characteristics of the girls according to the four phenotypes identified using principal component based cluster anlaysis
DHEA-S (ug/dL), estrone and estradiol (pg/mL), testosterone (ng/dL)

	Cohort		Phenotype 1 High DHEA-S, T and E1		Phenotype 2 High E2, T and E1		Phenotype 3a No High Hormones		Phenotype 3b All Low Hormones		
	N=269		N=42		N=37		N=74		N=96		
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Age of Thelarche (months)	108.26	13.20	113.24	11.29	112.49	13.16	106.36	12.98	104.05	12.31	<0.0001
Age of Pubarche (months)	118.19	16.24	116.83	19.22	118.69	11.39	113.91	17.16	121.56	15.06	0.0345
Age of Menarche (months)	147.59	13.75	150.70	15.34	142.48	11.84	147.65	14.31	148.63	12.30	0.0606
Tempo (time between thelarche and menarche in months)	39.01	13.06	36.39	11.50	30.55	10.27	40.50	10.87	45.12	12.58	<0.0001
BMIZ	0.33	1.02	0.29	0.96	0.14	0.97	0.42	1.10	0.40	1.03	0.5245
BMI Percentile	58.97	29.56	57.67	27.62	52.47	28.56	61.46	30.70	61.60	30.29	0.3667
Mother's Age of Menarche (%)											0.4507
less than 12 years old	20.07%		14.29%		27.03%		25.68%		14.58%		
at least 12 years but less than 14 years old	59.85%		66.67%		51.35%		55.41%		64.58%		
at least 14 years or older	20.07%		19.05%		21.62%		18.92%		20.83%		
Ethnicity (%)											0.7755
Black	31.60%		35.71%		27.03%		29.73%		34.38%		
Hispanic, White, Asian, All Other	68.40%		64.29%		72.97%		70.27%		65.63%		
First or Second Degree Maternal Family Member Breast Cancer Diagnosis (%)											0.8573
diagnosis of breast cancer	12.64%		11.90%		13.51%		16.21%		11.46%		
no diagnosis of breast cancer	80.30%		83.33%		78.84%		78.38%		79.70%		
missing	7.06%		4.76%		8.10%		5.40%		9.37%		
Caregiver's education (%)											0.4303
high school degree or less	29.00%		40.48%		16.22%		27.03%		29.17%		
associate's or bachelor's degree	45.35%		38.10%		54.05%		44.59%		43.75%		
more than a bachelor's degree	25.62%		21.43%		29.73%		28.38%		27.08%		
Pubertal Pathway (%)											0.0017
thelarche before pubarche	69.14%		66.67%		64.86%		64.86%		79.17%		
pubarche before thelarche	17.10%		26.17%		16.22%		28.38%		5.21%		
entered at the same time	7.43%		7.14%		13.51%		4.05%		4.17%		
missing due to censorship	6.32%		0.00%		5.41%		2.70%		11.46%		

* Baseline values for the cohort given as mean unless noted. P values represent tests for groupwise differences between the phenotypes; values for the phenotypes represent mean values within the phenotypes. Comparison between phenotypes using Kruskal-Wallis for continuous variables and χ^2 for categorical.

Δ = change in hormone value

Phenotype 1 – (High DHEA-S) latest age of thelarche (5 months) but earlier pubarche by 2 months, latest to menarche, more black

Phenotype 2 – (High E2) later age of thelarche (4 months), very lean girls, less black vs all other

Phenotype 3a – (Not High) Early to thelarche and earliest to pubarche, more obese girls.

Phenotype 3b – (Low) Earliest to Thelarche, latest to pubarche (longest time in between the two), more obese girls, more black

Assumptions of Tests

- Pearson correlation coefficient measures linear correlation between two continuous variables,
    - ranges between -1 and 1
    - based on covariance strength, normal and linear and not too many outliers
    - Can use Spearman's Rank if not linear or normal
- Chi Square for categorical variables. Look at the proc freq race*education (use Fischer's exact if expected value is <5 in proc freq of race*education) – independent categories, mutually exclusive, non-parametric (so no distribution assumption), groups are of equal sizes, not large amounts of categories (e.g.20), not paired e.g. mother and child,
- T Test assumptions – equal variance, random independent samples, normal if a small sample size

Study Measurements

Effect Modifier or Confounder?

- Modifier – magnitude of the effect on the outcome differs depending on a third variable; true relationship
  - e.g. a treatment may work for men but not women so sex is an effect modifier, UV induced cancer rate is higher for those with a rare hereditary defect
  - report individual stratified findings (they will be significantly different from each other)

- Confounder – variable has an association with both the exposure and the outcome but does not lie in the causal pathway; false relationship
  - e.g. birth order and mother's age with outcome of down syndrome (if you stratify you see it is not birth order but age that effects the likelihood of down syndrome)
  - Stratify (these findings will be similar but different by at least 10% from the total finding) and use Mantel-Haenszel pooled odds ratio or risk ratio
  - Multivariable modeling

$$\widehat{RR}_{cmh} = \frac{\sum \frac{a_i(c_i+d_i)}{n_i}}{\sum \frac{c_i(a_i+b_i)}{n_i}} \qquad \widehat{OR}_{cmh} = \frac{\sum \frac{a_i d_i}{n_i}}{\sum \frac{b_i c_i}{n_i}}$$

Background – Hormones not included

- SHBG – declines during puberty
- Insulin-like Growth Factor -1 (IGF-1) – does not increase until later stages of thelarche and closer to menarche
- DHEA – 98% of circulating DHEA is in the form of DHEAS, DHEA and DHEAS levels run parallel and DHEAS is more stable to measure.
- Androstenedione – is either secreted or converted into testosterone, has few effects of its own.
- Estrogen – group of similar female hormones: estradiol, estrone, estiol (minor effect, best detected after a 24 urine collection)

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An oral dissertation defense in partial fulfillment of the requirements for the
degree of Doctor of Philosophy in the Division of Epidemiology, Department
of Environmental Health, University of Cincinnati College of Medicine

DISSERTATION COMMITTEE:
Susan Pinney, PhD (Chair, Advisor)
Frank Biro, MD
Iris Gutmark-Little, MD
Changchun Xie, PhD

Agenda

- Background
- Methods
- Objective, Hypothesis and Aims
- Aim 1
- Aim 2
- Aim 3
- Limitations
- Strengths
- Future Directions

Background

Puberty

- The beginning of puberty in girls is considered the beginning of breast development, thelarche.
- There are several pubertal milestones besides thelarche:
  - Pubarche – first appearance of pubic hair
  - Menarche – first menstrual bleeding, occurs about two years after thelarche
- Puberty can last 1-7 years.
- Puberty starts on average between the ages of 8 and 13.
- Puberty alters a girl's body into one with full sexual capabilities.
- Girls experience changes in their physique, hormones levels, and brain development.
- Wide disparity in age of onset and tempo in girls as well as the pathway of puberty.

Age of Menarche

- Girls are experiencing menarche at an earlier age than in previous generations.[1]
  - Girls born prior to 1920 had an average age of menarche of 13.3 years.[1]
  - Girls born between 1980 and 1984 reported an average age of menarche of 12.4 years.[1]
  - Girls born between 1998 and 1999 reported a median age of menarche of 12.25 years.[2]
- Risk factors linked to this earlier age of menarche include higher BMI (body mass index),[1] race/ethnicity (African American),[3] and endocrine disrupters.[4]

1 Euling SY, Herman-Giddens ME, Lee PA, et al. Examination of US puberty-timing data from 1940 to 1994 for secular trends: Panel findings. Pediatrics 2008;121(Suppl 3): S172-91
2 Biro FM, Pajak A, Wolff MS, Pinney SM, Windham GC, Galvez MP, Greenspan LC, Kushi LH, Teitelbaum SL. Age of menarche in a longitudinal US Cohort. J Pediatr Adolesc Gynecol. 2018; 31; 339-345.
3 Cabrera SM, Bright GM, Frane JW, et al. Age of thelarche and menarche in contemporary US females: a cross sectional analysis. J Pediatr Endocrinol Metab. 2014 January;27(0): 47-51.
4 Ouyang F, Perry MJ, Venners SA, Chen C, Wang B, et al. Serum DDT, age of menarche, and abnormal menstrual cycle length. Occupational and Environmental Medicine. 2005 Dec; 878.

Health Risks due to Early Menarche

- Early menarche has been linked with depression in the later teenage years,[1] earlier sexual activity,[2] eating disorders[1] and substance abuse.[1]

- Poor health outcomes later in life include increased risk of heart disease, higher BMI, increased fasting insulin and other risks of heart disease.[3]

- Early menarche is the most widely known and established risk factor for breast cancer.[3]

  – 9% decrease in risk for every year of delayed of menarche in premenopausal women.[4]

  – 4% decrease in risk for every year of delayed menarche in post menopausal women.[4]

  – The cost of treating breast cancer was greater than $16.5 billion in 2010. Costofcancer.org

1 Mendle J, Turkheimer E, Emery R. Detrimental psychological outcomes associated with early pubertal timing in adolescent girls. Developmental Review 2007;151-171.
2 Kaltiala-Heino R, Kosunen E, Ripela M. Pubertal timing, sexual behaviour and self-reported depression in middle adolescence. Journal of Adolescence 2003; 26: 531-545.

3 Widen E, Silventoinen K, Sovio U, et al. Pubertal Timing and Growth Influence Cardiometabolic Factors in Adult Males and Females. *Diabetes Care* 2012 Apr; 35(4): 850-856.
4 Clavel-Chapelon F. Differential effects of reproductive factors on the risk of pre- and postmenopausal breast cancer: results from a large cohort of French women. Br J Cancer 2002;86(5): 723-727.

Hypothesis and Specific Aims

Previous Look at Individual Girl's Hormones
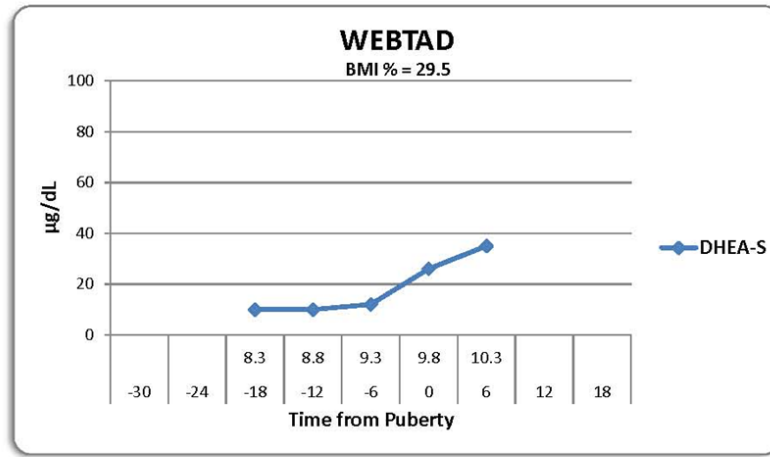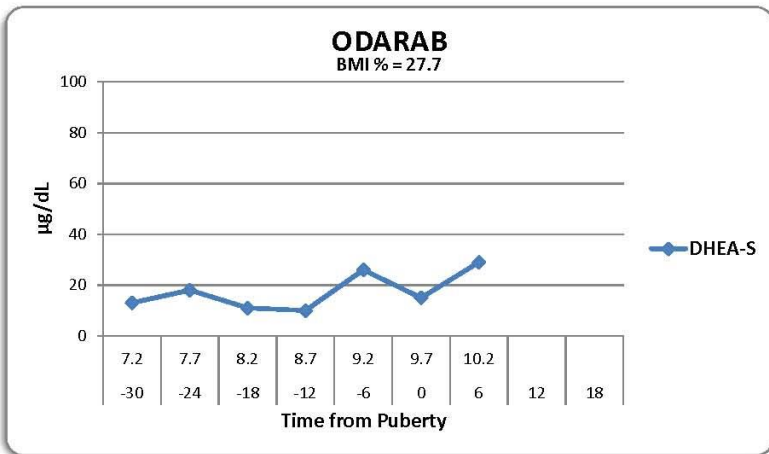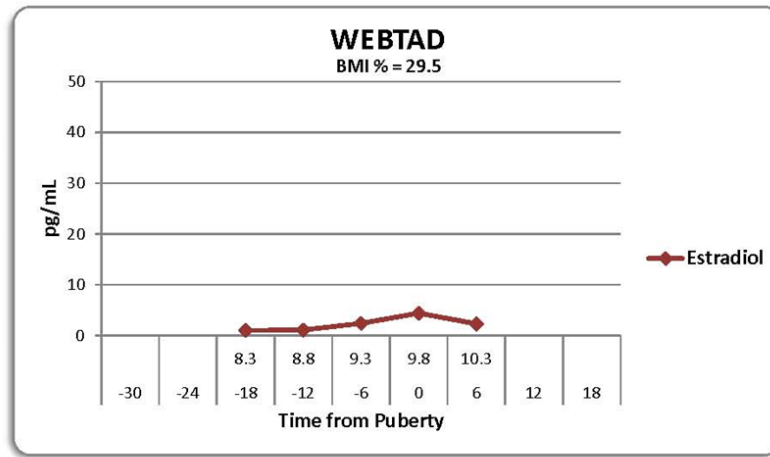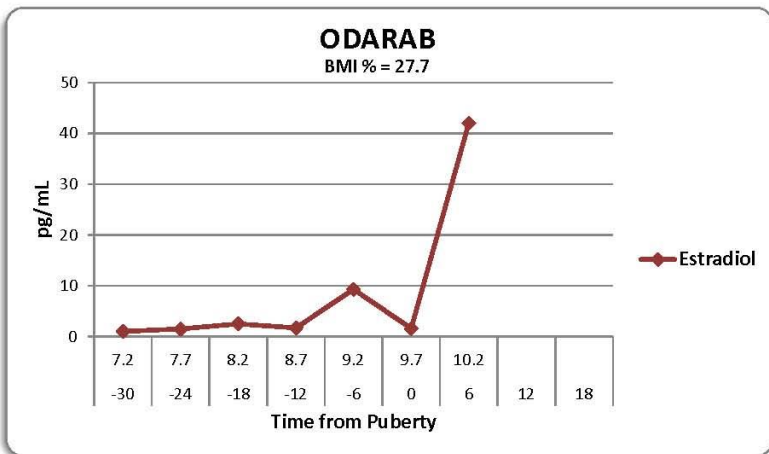
Hypothesis

In young girls, relative levels or changes in DHEA-S, estrone, estradiol, and testosterone around the time of thelarche, when considered together as an individual hormone phenotype, are directly related to the age at pubertal milestones (thelarche, pubarche and menarche).

Specific Aims

- **Specific Aim 1** – Describe the serum DHEA-S, estradiol, estrone and testosterone levels in girls measured in 6 month increments from 18 months prior to 6 months after the age of thelarche.

- **Specific Aim 2** –Classify phenotypes of the sex hormones using the hormone levels across the different time windows relative to thelarche.

- **Specific Aim 3** – Use multivariable survival analysis to determine which among the sex hormone profile phenotypes (consisting of DHEA-S, estradiol, estrone and testosterone) are predictive of ages of thelarche, pubarche, and menarche to allow researchers to further understand why some girls experience pubertal milestones at an earlier age than others.

Methods

Time Windows Relative to Thelarche

Five time windows relative to thelarche visit were defined.

Time Period	-18 months	-12 months	-6 months	Thelarche (0)	6 months
Defined as..	From -21 to < -15	-15 ≤ to < -9	-9 ≤ to < -3	-3 ≤ to < 3	3 ≤ to < 9

-21 months   -15 months   -9 months   -3 months   3 months   9 months

Thelarche, time=0
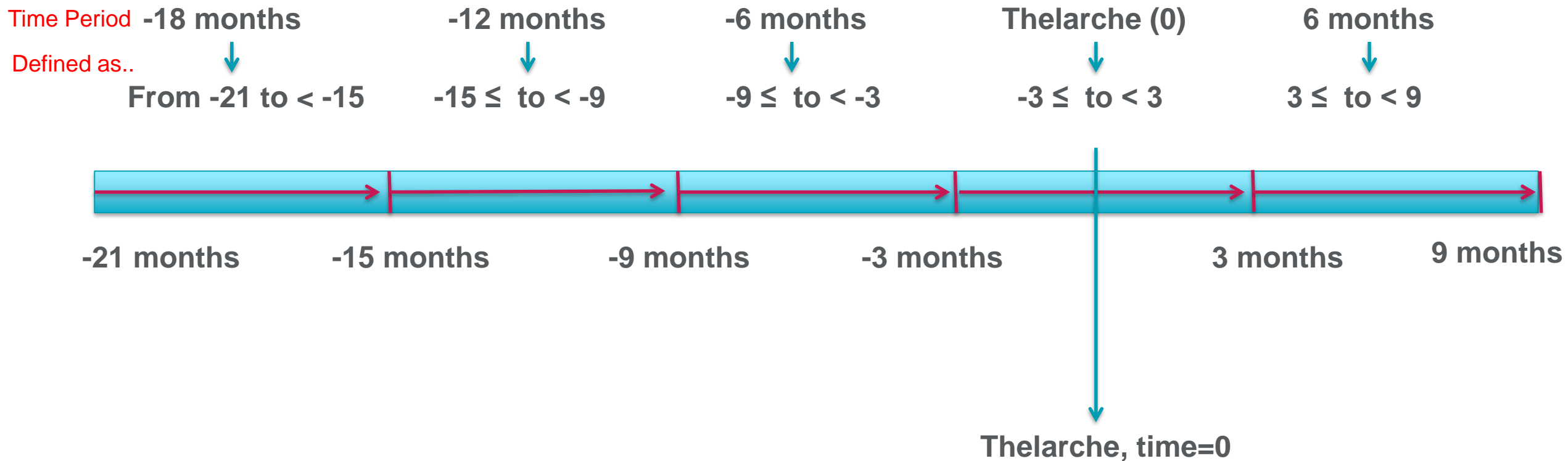
Included in this analysis (n=269 girls)

Eligibility of Girls

Cincinnati Cohort (n=379)

	n	Eligible Girls	Observations	Total Hormones
Hormone 2015 Dataset		253	975	3480
Hormone 2016 Dataset		205	425	1360
Deletion of Observations		303	1307	4840
Remove duplicates for a girl at the same visit		303	1307	4836
Keep only Hormones in -18 to +6 time period	-4	299	1046	3902
Keep girls with at least two hormone measurements during -18 to +6	-30	269	1009	3764
Keep only 1 visit per bucket*		269	935	3493

Sex Hormone Measurements

Serum hormone levels (continuous)

- Samples were frozen at -80℃.

- Analysis performed by Esoterix Labs.

- Estradiol, Estrone and Testosterone were measured by High Performance Liquid Chromatography with tandem mass spectrometry (HPLC-MS) which is very sensitive compared to other methods.

- Radioimmuno assay (RIA) was used to measure DHEAS for one batch and HPLC-MS for a second batch.

- Missing data was due to missing a study visit, refusing to have blood drawn or insufficient amount of blood serum to measure.

- Serum concentrations of these hormones are known to change during puberty.

Sex Hormone Measurements

Serum hormone levels (continuous)

Lower Limit of Quantification (LLOQ) is the value at which the coefficient of variation (standard deviation / mean) <20% (<25% for DHEAS).

- Measure of precision at low analyte levels
- If the measurement was <LLOQ (but not canceled or insufficient), the LLOQ/$\sqrt{2}$ was used.

LLOQ	1st Batch n=252	2nd Batch n=51
DHEA-S μg/dL	10	10
Estradiol pg/mL	1	1
Estrone pg/mL	2.5	2.5
Testosterone ng/dL	3	2.5

Aim 1

Aim 1

Describe the serum DHEA-S, estradiol (E2), estrone (E1) and testosterone (T) levels in girls measured in 6 month increments from 18 months prior to 6 months after the age of thelarche.

Maturation Information

Average Age of Thelarche – 108.26 months (9.02 years)
Average Age of Pubarche – 118.19 months (9.85 years)
Average Age of Menarche – 147.52 months (12.29 years)
Average tempo – 39 months (3.25 years)

Pubertal Pathway
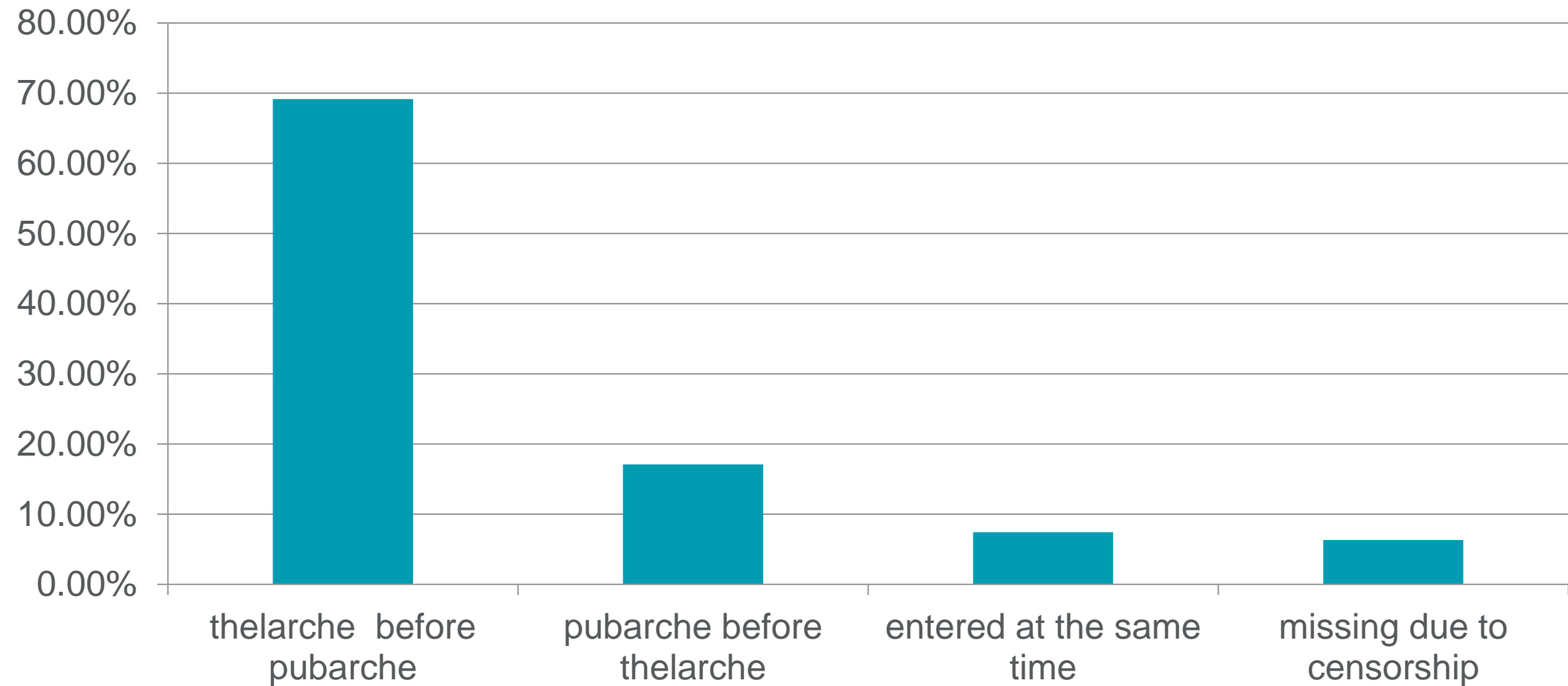
Aim 2

Aim 2

Classify phenotypes of the sex hormones using hormone levels across the different time points relative to thelarche.

Two or more hormone data points used together as a phenotype in this analysis may refer to:

- two or more measurements of a certain hormone in an individual girl at different time points (such as the change in E2 from time=-18 to -12 months relative to thelarche)
- measurements of at least two hormones taken at the same or different time points in time (E2 and DHEA-S at time=-6) relative to thelarche.

Correlations
Absolute Hormones

- All significant correlations are positive
- Lack of correlation of E2 with itself or other hormones, suggesting different longitudinal phenotypes.
- DHEA-S is highly correlated with itself across all time periods.
- Estrone is highly correlated with itself across all time periods.
- Testosterone is highly correlated with itself across all time periods.
- In general, DHEA-S, estrone and testosterone are highly correlated with each other across the time periods.

- No correlation between DHEA-S and E2
- General lack of correlation between E2 and testosterone
- Correlation between E2 and estrone at corresponding time periods (eg. -18 with -18)

Correlations
Differences in Hormone Values Between Time Periods

Correlations of Difference in Hormones Between Time Periods

Standarized differences of the hormones between two time periods

Positivley Correlated - R>0.25
Negatively Correlated - R>0.25

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

- In general, there is a lack of either positive or negative correlations, implying girls with big changes in a hormone between time periods did not experience a big change in another hormone, suggesting different longitudinal phenotypes.
- For all hormones the change between -18 to -12 is highly inversely correlated with next time period change of -12 to -6.
- For each time period estrone is negatively correlated with the change in the next time period.
- No correlation between DHEA-s and estrone or E2, suggesting different longitudinal phenotypes.

Variable Dimension Reduction
Principal Component Analysis (PCA) - Proc Factor

- Variable reduction method

- Shared variance between several variables is explained in fewer unobserved variables (factors)

- Look for the number of factors that explain approximately 70% or more of the variance

- Variables are loaded on the factors by eigenvalues (>0.35). A high absolute value of an eigenvalue indicates a higher correlations with the factor.

Variable Dimension Reduction
PCA

- PCA does not handle missing data

- Looked at only time periods -6,0 and 6 due to the large amount of missing data at time periods -18 and -12

- 9 girls were lost as all of their hormone measurements were outside of times -6, 0 and +6.

- Need to use Proc MI to handle the missing data as some girls might no have had blood drawn at every visit or the assays were too low to quantify.

- Ran Proc Mi for 30 imputations to ensure quality of the estimated values

- Factor analysis for each of the 30 imputed dataset

- Averaged the factor loadings (eigenvalues) across the 30 factor analysis results

Variable Dimension Reduction
PCA

Sensitivity Analysis - Factor Analysis on a smaller more complete data set to compare the results

- – N=260 – all the girl included in the hormone analysis with at least some data at -6,0 and 6 (30 imputed data sets then averaged the 30 factor analysis results)
- – N=67 - all the girls with values for all four hormones at three time periods (-6, thelarche,+6 therefore no missing data)

Variable Dimension Reduction
PCA -Hormone Values at time=-6,0,6 – Only 3 of 12 factors shown

Factor loading from principal component analysis of hormones (n=260)

Factor loadings greater that 35 are flagged by an '*'.

Average of loadings from 30 PCAs of 30 Imputations

	Factor 1	Factor 2	Factor 3
Testosterone -6	70*	34	6
Testosterone 0	73*	26	31
Testosterone +6	55*	63*	7
DHEA-S -6	87*	9	4
DHEA-S 0	88*	2	3
DHEA-S +6	87*	16	8
Estrone -6	61*	46*	36
Estrone 0	60*	35	60*
Estrone +6	33	80*	20
Estradiol -6	0	40*	67*
Estradiol 0	11	32	85*
Estradiol +6	5	82*	26

*Hormones log transformed

	Factor 1	Factor 2	Factor 3
Variance Explained by Factor	0.501302	0.1541	0.086107
Cumulative Variance	0.501302	0.655402	0.741509

Factor loading from principal component analysis of hormones (n=67)

Sensitivity Analysis
Factor loadings greater that 35 are flagged by an '*'.

n=67 (Girls who have all hormone values at times =-6,0,6

	Factor 1	Factor 2	Factor 3
Testosterone -6	81*	5	8
Testosterone 0	80*	14	10
Testosterone +6	78*	-4	35*
DHEA-S -6	86*	8	4
DHEA-S 0	87*	12	7
DHEA-S +6	91*	4	14
Estrone -6	79*	35	12
Estrone 0	59*	54*	26
Estrone +6	60*	22	63*
Estradiol -6	20	81*	-4
Estradiol 0	-9	82*	26
Estradiol +6	8	15	94*

*Hormones log transformed

All factors had eigenvalues >1

	Factor 1	Factor 2	Factor 3
Variance Explained by Factor	0.5314	0.1413	0.0944
Cumulative Variance	0.5314	0.6727	0.7671

PCA to Cluster (CA)

- PCA did not result in variable reduction of the absolute hormone values.
  - Should use all four absolute hormones measurements at -6,0 and 6 as objective predictive variables in the cluster analysis as all the variables loaded onto the first three components that explained >74% of the variance.

- Use the subset of girls (n=260) with hormone data between -6 and 6 as the imputed data resulted in factors very similar to the reduced data set in the sensitivity analysis.
  - Factor 1 – same except estrone at 6
  - Factor 3 of N=260 the same as factor 2 of n=67

- Three factors imply 3 possible clusters

Participant Clustering
Proc Fastclus – Hormone Values at time =-6,0,6

- Assigns participants to a cluster where the other participants are more similar to them than participants in another cluster.

- Each participant belongs to only one cluster.

- Very sensitive to outliers and has a method to identify outliers in their own clusters (unlike other cluster procedures)
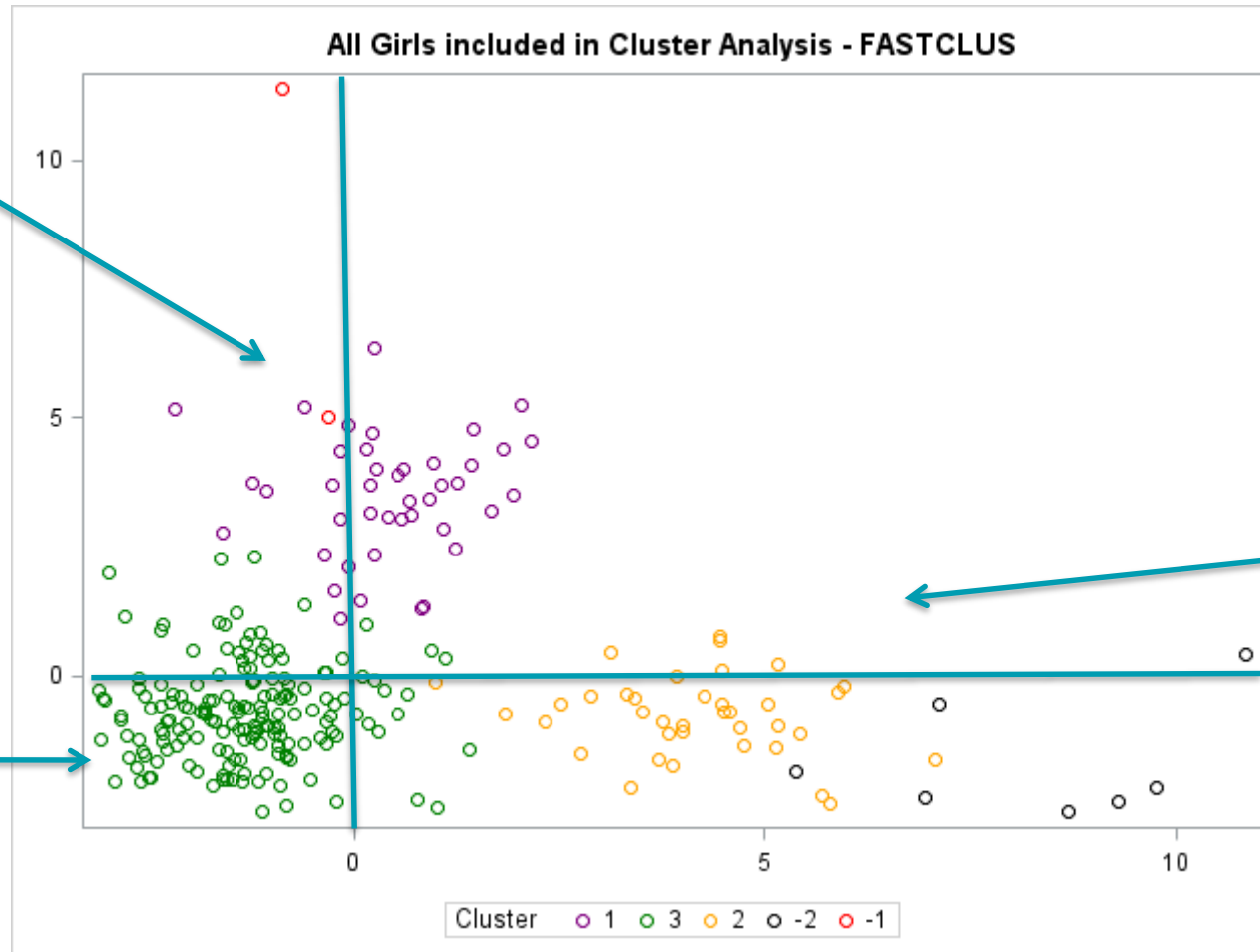
Participant Clustering
CA - Hormone Values at time =-6,0,6

N=260 (results for n=67 produced similar cluster plot)

Cluster 1 (n=42)

Cluster 3 (n=172)

Cluster 2 (n=37)

3 distinct clusters were produced

Canonical variable 1 discriminates between cluster 3 and cluster 2

Canonical variable 2 discriminates between cluster 1 and cluster 2 and 3

Clusters -1 (n=2) and -2 (n=7) represent outliers that do not fit into any cluster

Mean hormone values for each cluster

Hormone phenotype objective predictors
Sex hormone serum concentrations of girls according to the three phenotypes identified using principal component anlaysis-based cluster anlaysis
DHEA-S (ug/dL), estrone and estradiol (pg/mL), testosterone (ng/dL)

	Cohort	Cluster 1 High DHEA, T and E1	Cluster 2 High E2, T and E1	Cluster 3 No High Hormone Values	Significance (P value)*
	N=269	N=42	n=37	n=172	
Testosterone -6 ‡	4.48	6.89	7.00	3.33	<0.001
Testosterone 0 ‡	4.96	8.23	5.40	3.88	<0.001
Testosterone 6 ‡	6.16	9.23	10.79	4.48	<0.001
Estrone -6 ‡	4.00	6.18	5.24	3.06	<0.001
Estrone 0 ‡	4.47	6.81	5.17	3.68	<0.001
Estrone +6 ‡	6.09	8.10	11.78	4.39	<0.001
DHEA -6 ‡	28.23	62.97	32.93	17.33	<0.001
DHEA 0 ‡	30.79	68.13	24.91	21.78	<0.001
DHEA +6 ‡	38.32	79.00	39.64	27.63	<0.001
Estradiol -6	2.83	2.46	4.60	2.12	<0.001
Estradiol 0 ‡	3.37	3.68	4.46	2.79	<0.001
Estradiol +6 ‡	5.38	5.40	17.25	3.26	<0.001

* Baseline values for the cohort given as mean unless noted. P values represent tests for groupwise differences between the phenotypes; Comparison between phenotypes using Kruskal-Wallis for continuous variables and χ^2 for categorical.

Considerably higher values than cohort and other phenotypes

Cluster 1
- Higher DHEA (over 100% higher than the cohort mean at each time period)
- T and E1 values (over 50% higher than the cohort mean)

Cluster 2
- High E2 values across each time period from 30% to 200% higher than the mean of the cohort
- E1 90% higher at +6
- T >50% higher at -6 and +6 vs the cohort mean)

Cluster 3
- lower hormone values (approximately >20% lower) across the time period vs the mean of the entire cohort.

3 clusters or not….

- Two clusters are very well defined by hormone values.

- One cluster (cluster 3) is a large group that is appears tightly clustered but not well defined except the hormone values are lower than the other clusters and the cohort as a whole.   Could it possibly be broken into 2 or more clusters?

PCA on Cluster 3 (n=172) vs the cohort

PCA on the cluster still shows all variables loading and suggests using all 12 as predictive variables in CA

Factor loading from principal component anlaysis restricted to girls in cluster 3 (n=172)

Factor loadings greater that 35 are flagged by an '*'.

Average of loadings from 30 PCAs of 30 Imputations

	Factor 1		Factor 2		Factor 3	
Testosterone -6	59	*	28		5	
Testosterone 0	63	*	37	*	22	
Testosterone +6	48	*	64	*	-3	
DHEA-S -6	81	*	-1		5	
DHEA-S 0	83	*	7		5	
DHEA-S +6	78	*	27		5	
Estrone -6	44	*	40	*	43	*
Estrone 0	47	*	32		57	*
Estrone +6	21		80	*	21	
Estradiol -6	-9		4		65	*
Estradiol 0	5		19		70	*
Estradiol +6	-9		72	*	28	

*Hormones log transformed

All factors had eigenvalues >1

Factor loading from principal component anlaysis of hormones (n=260)

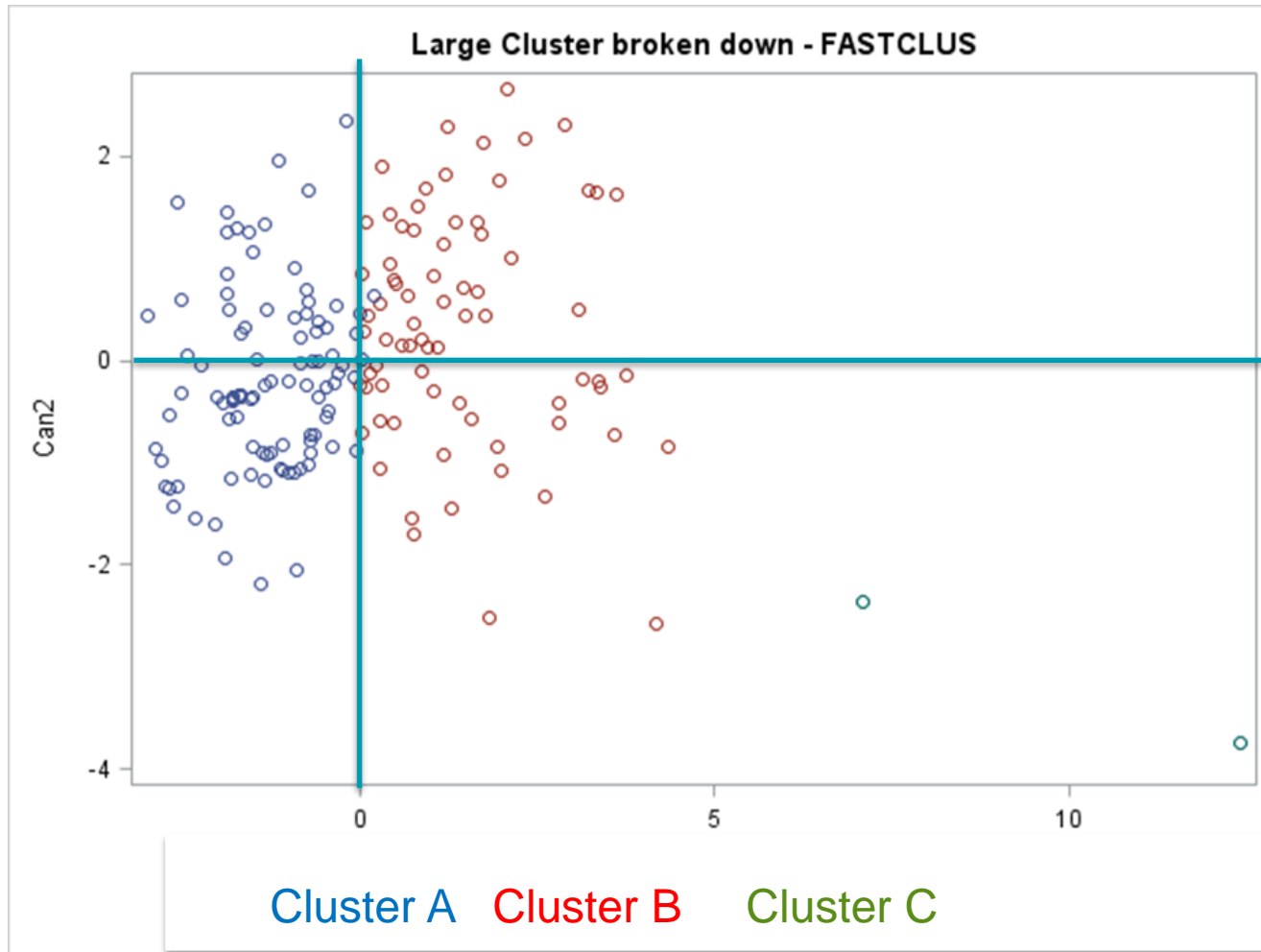**Factor loadings greater that 35 are flagged by an '*'.**

Average of loadings from 30 PCAs of 30 Imputations

	Factor 1		Factor 2		Factor 3	
Testosterone -6	70	*	34		6	
Testoserone 0	73	*	26		31	
Testosterone +6	55	*	63	*	7	
DHEA-S -6	87	*	9		4	
DHEA-S 0	88	*	2		3	
DHEA-S +6	87	*	16		8	
Estrone -6	61	*	46	*	36	
Estrone 0	60	*	35		60	*
Estrone +6	33		80	*	20	
Estradiol -6	0		40	*	67	*
Estradiol 0	11		32		85	*
Estradiol +6	5		82	*	26	

*Hormones log transformed

All factors had

CA on Cluster 3 (n=172)

Cluster A n=74
Cluster B n=96
Cluster C n=2

Clusters A and B seem to be a large cluster cut in half.

Canonical Variable 1 discriminates between Cluster A and B

Cluster C is just too small and are the outliers.

Canonical Variable 2 does not discriminate between clusters

Clustering to Phenotype

- Phenotype 1 = Cluster 1, n=42 (from CA n=260)
- Phenotype 2 = Cluster 2, n=37 (from CA n=260)
- Phenotype 3a = Cluster 3A, n=74 (from CA on n=172)
- Phenotype 3b = Cluster 3B, n=96 (from CA on n=172)

- Outliers are not included in any clusters or phenotypes.

Further Characteristics of the Phenotypes after being agnostically defined by the hormones

- Statistical difference exists between the age of thelarche and the age of pubarche among the four phenotypes.
- Girls in phenotypes 1 and 3b had an average age of menarche statistically later than girls in phenotype 2.
- The tempo of girls in phenotype 2 was statistically shorter than that for girls in phenotype 3b.
- Girls in phenotype 3b were more likely to enter puberty via pubarche rather than thelarche which is different than the other phenotypes.
- No differences between the phenotypes existed for the following characteristics:
  - BMI
  - ethnicity
  - family history of 1st or 2nd degree breast cancer
  - mother's age of menarche
  - caregiver's education

Conclusions

- Classifying hormone heterogeneity prior to puberty is highly informative in unveiling different pathways through puberty.

- These analyses are the first to apply PCA-CA methods to longitudinal sex hormone data of girls going through puberty.

- PCA-CA did not result in variable reduction of the absolute hormone values but did identify four meaningful phenotypes of hormones levels in young girls in relationship to the timing of thelarche.

- The four distinct hormone phenotypes in girls indicate hormones levels relative to the age of thelarche are not the same in all girls and help to explain disparity in the age of onset.

- These findings underscore the need to better understand female sex hormones prior to puberty based on time related to puberty rather than chronological age or pubertal status.

Aim 3

Aim 3

Use multivariable survival analysis to determine which among the sex hormone profile phenotypes (consisting of DHEA-S, estradiol, estrone and testosterone) are predictive of age of thelarche, pubarche, and menarche to allow researchers to further understand why some girls experience pubertal milestones at an earlier age than others.

Survival Analysis

Cox proportional-hazard models (probability or likelihood of the event of interest) were conducted in SAS using PHReg and controlling for covariates.

– do not require a certain probability function

– may include multiple covariates

– may include continuous and/or categorical covariates

– allows for the inclusion of girls who are right censored

– allows for interactions between covariates

Assumption of proportional hazards – additive changes in a variable cause corresponding multiplicative changes in the hazard function e.g. ratio of hazards for two individuals over time is constant

Girls who have not yet reached the pubertal milestone during the study or were lost to follow up prior to achieving it will be right censored.

Survival Analysis (unadjusted)

Thelarche
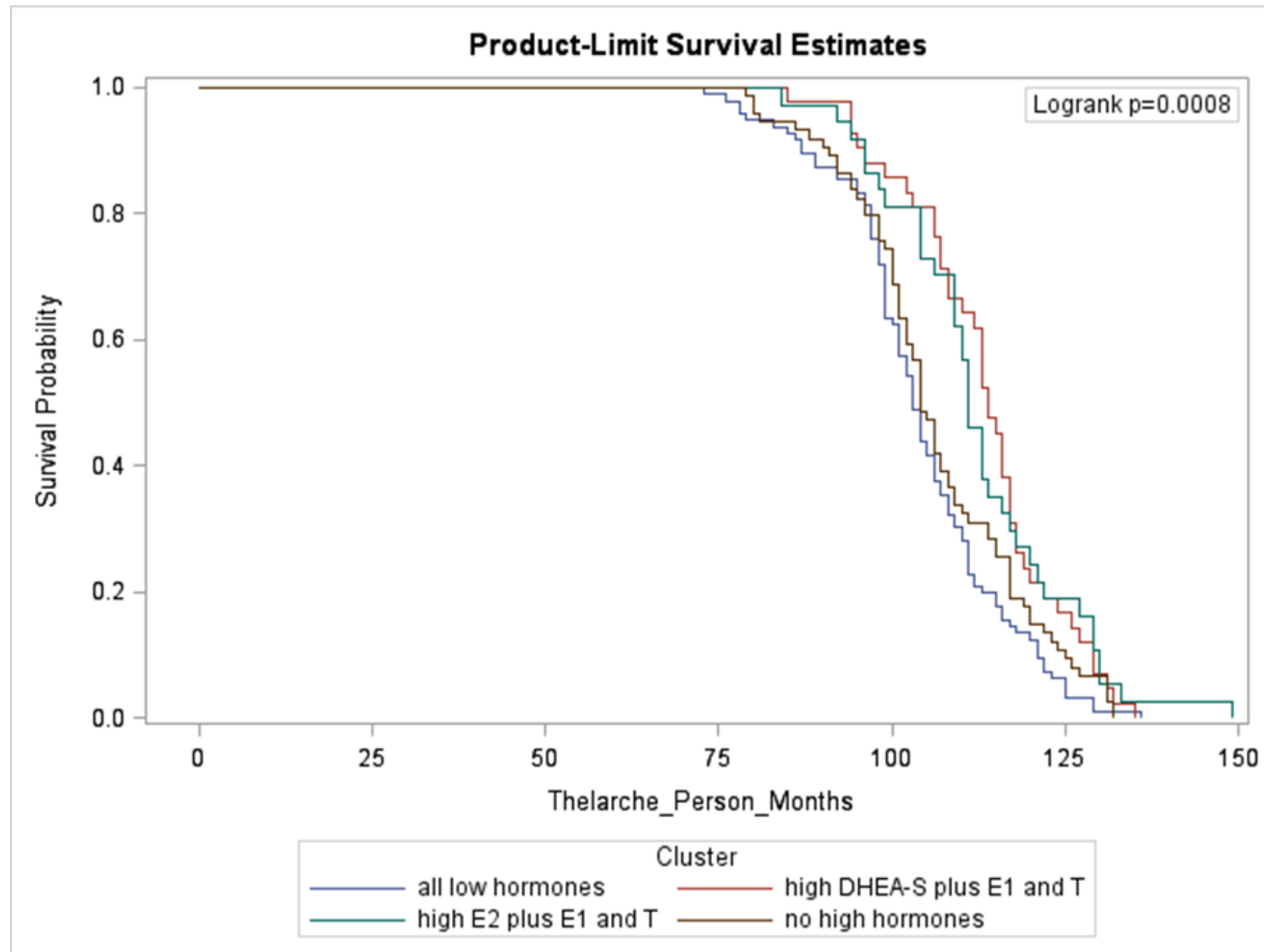
Survival Analysis (unadjusted)

Pubarche
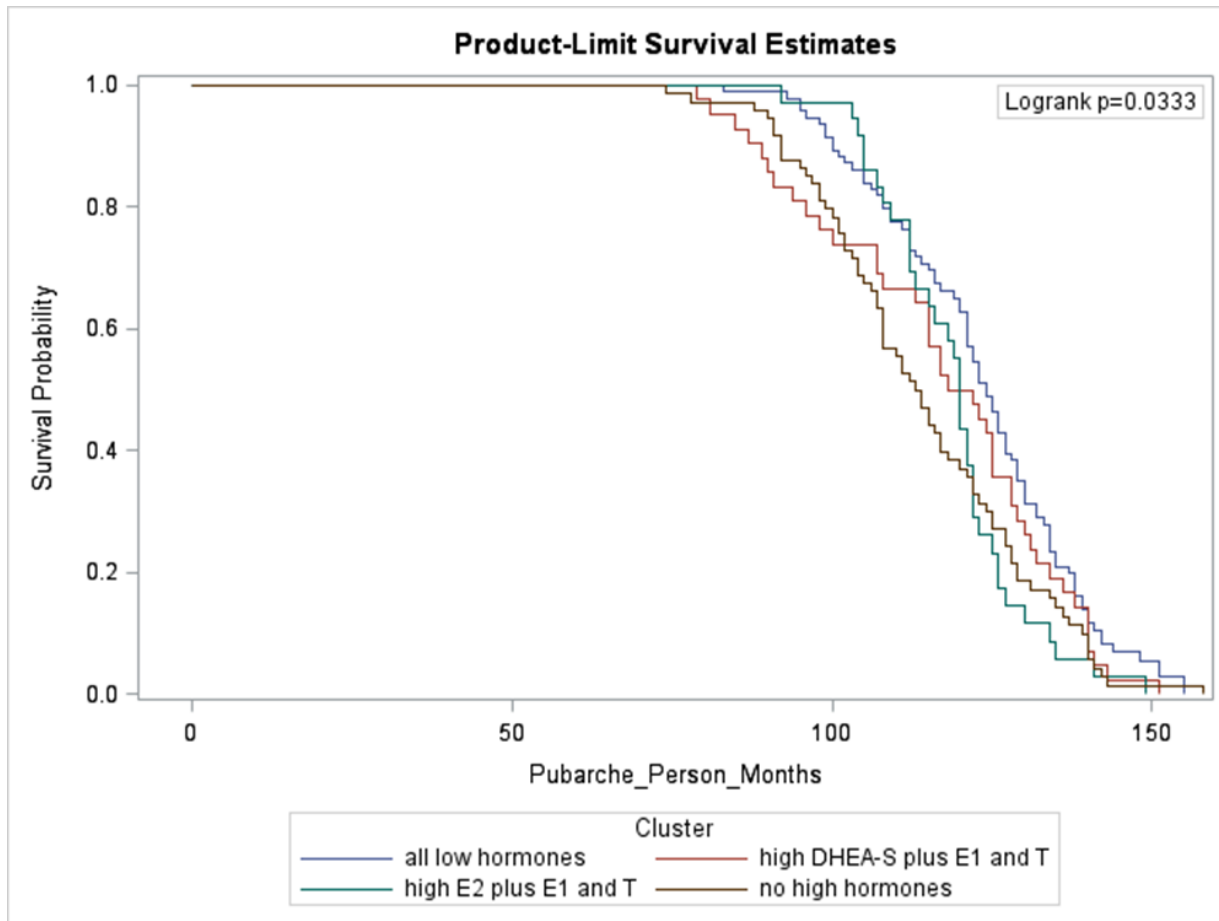
Menarche
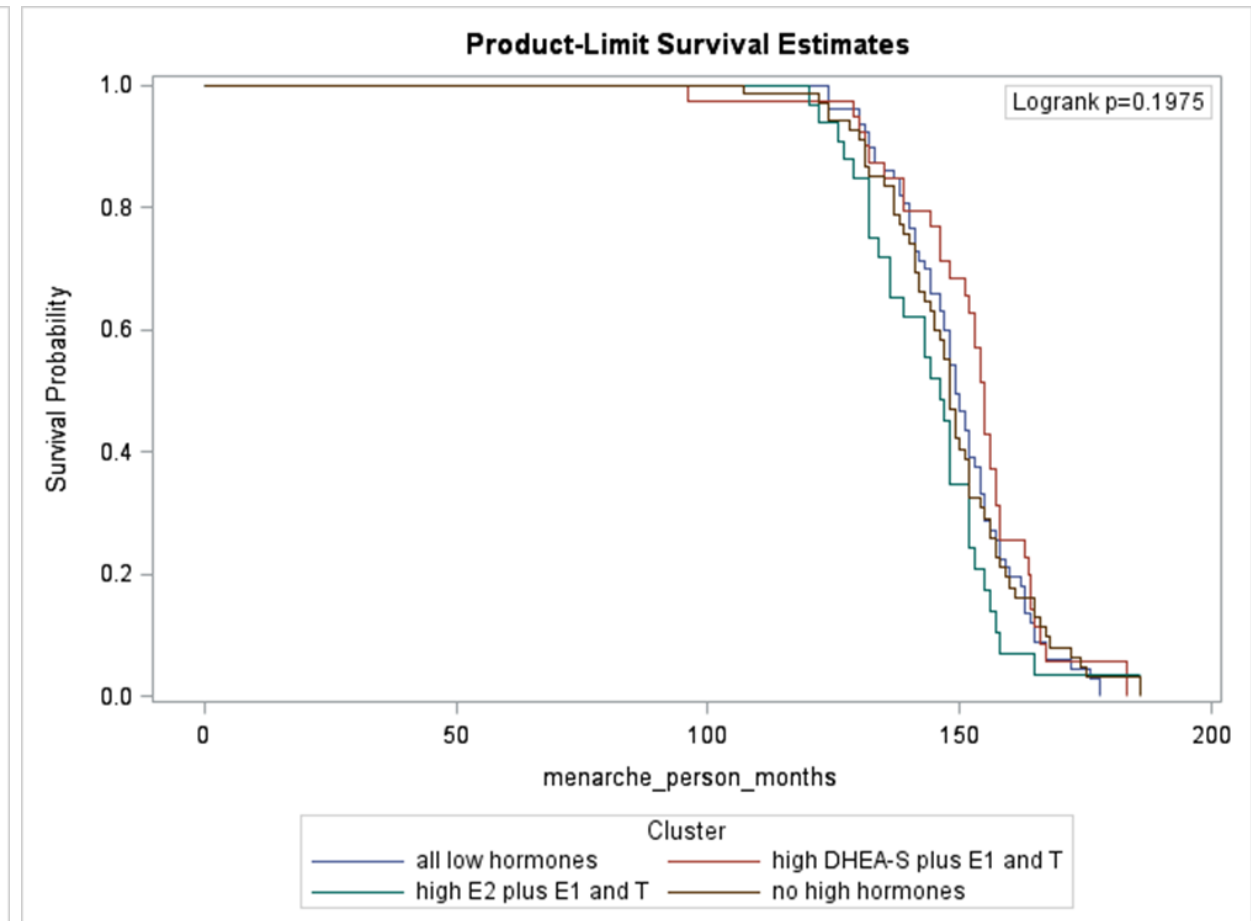
Risk Estimates by Phenotypes

Thelarche   Pubarche   Menarche

Clark O; Djulbegovic B. Forest plots in excel software(Data sheet). 2001.Available at www.evidencias.com.
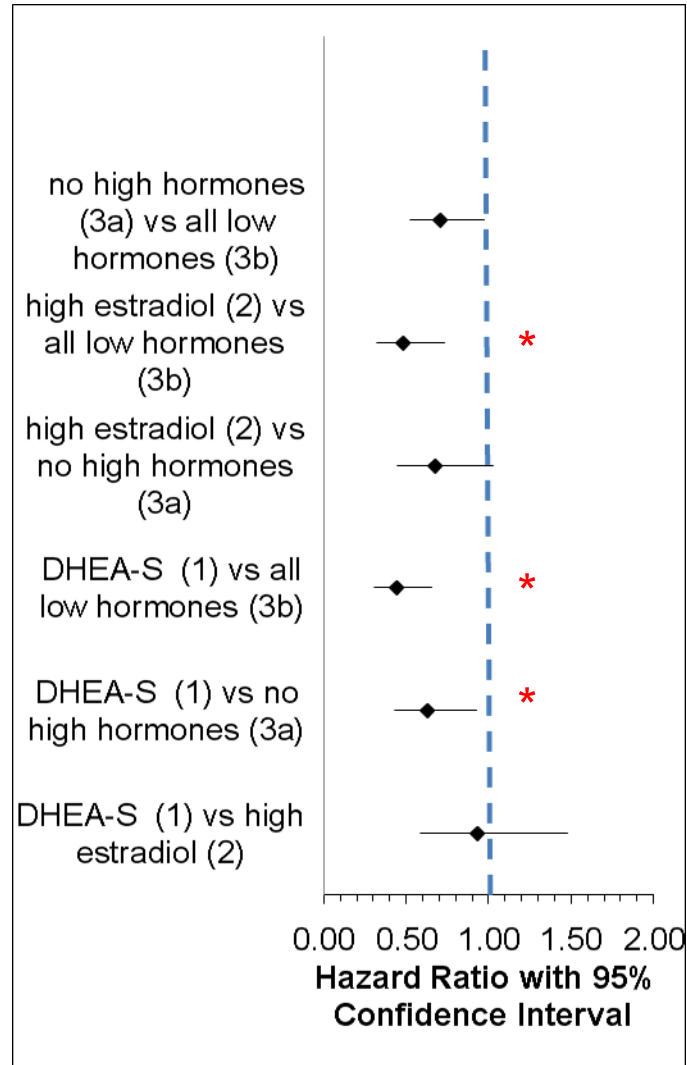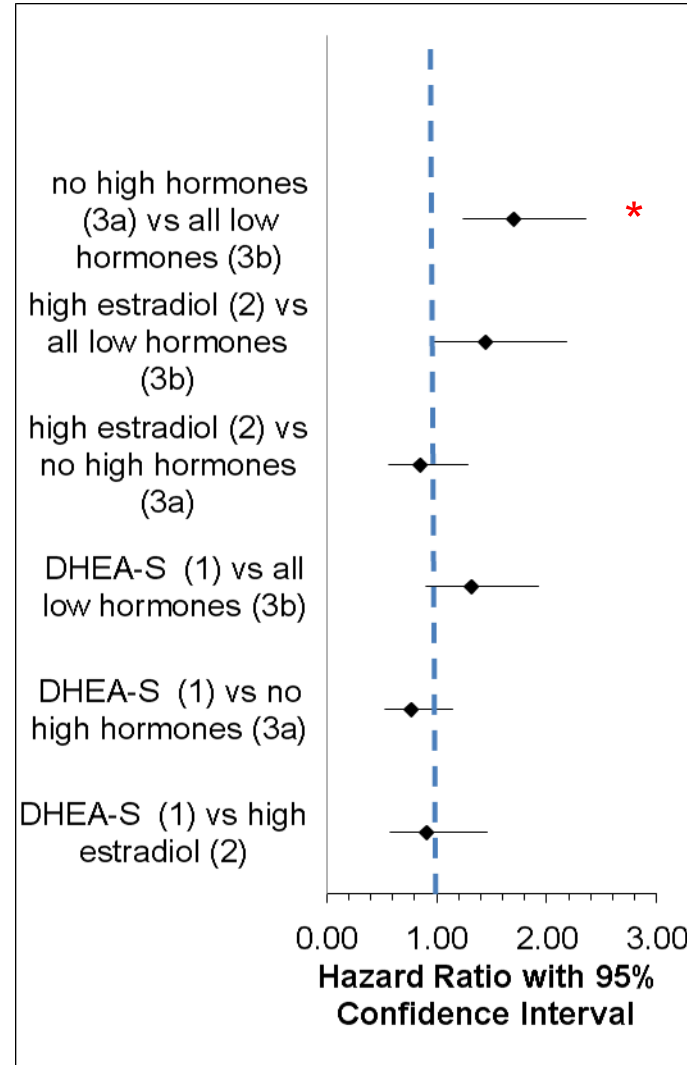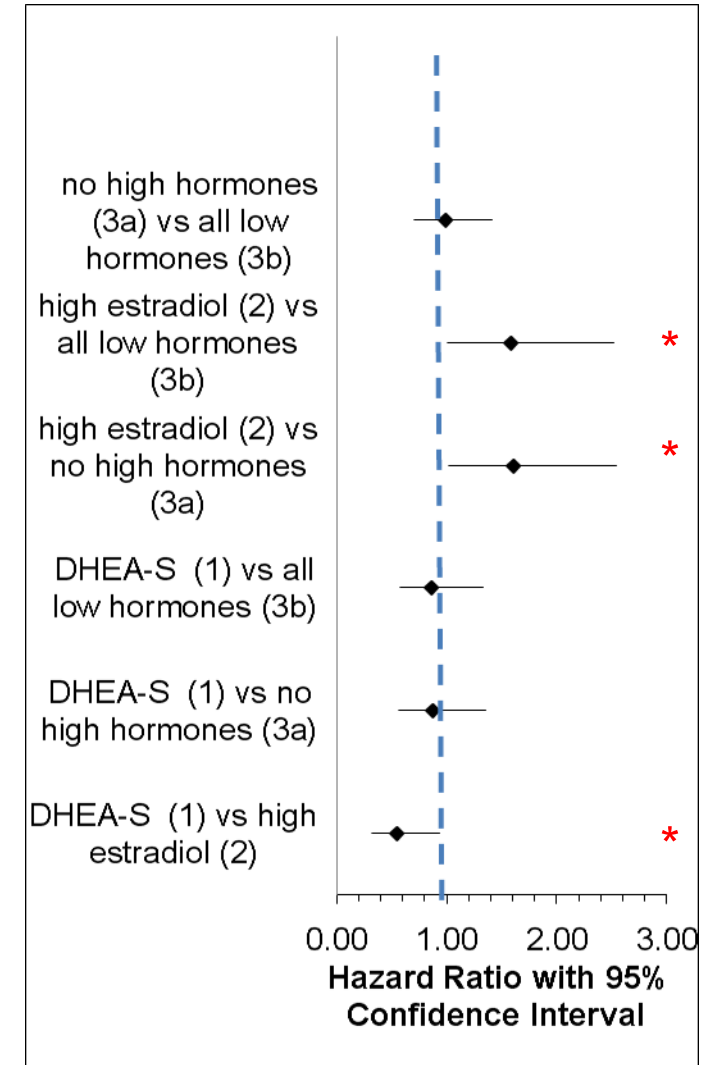
Conclusions

- Risk of earlier age of thelarche, pubarche and menarche differed by phenotypes, confirming heterogeneity of hormone phenotypes.

- Girls with mother's ages of menarche younger than 12 years old had a 50% increased risk of reaching all three milestones earlier than girls with mother's ages at least 14. This possibly indicates a genetic influence.

Summary

Participant Clustering
CA - Hormone Values at time =-6,0,6

CA on all girls (n=290)

CA on Cluster 3 (n=172)

Cluster 1 (n=42)

Cluster 2 (n=3...

Cluster 3 (n=172)

Cluster B n=96

Cluster A n=74

Cluster A   Cluster B

CA on Cluster 3 (n=172)

Large Cluster broken down - FASTCLUS
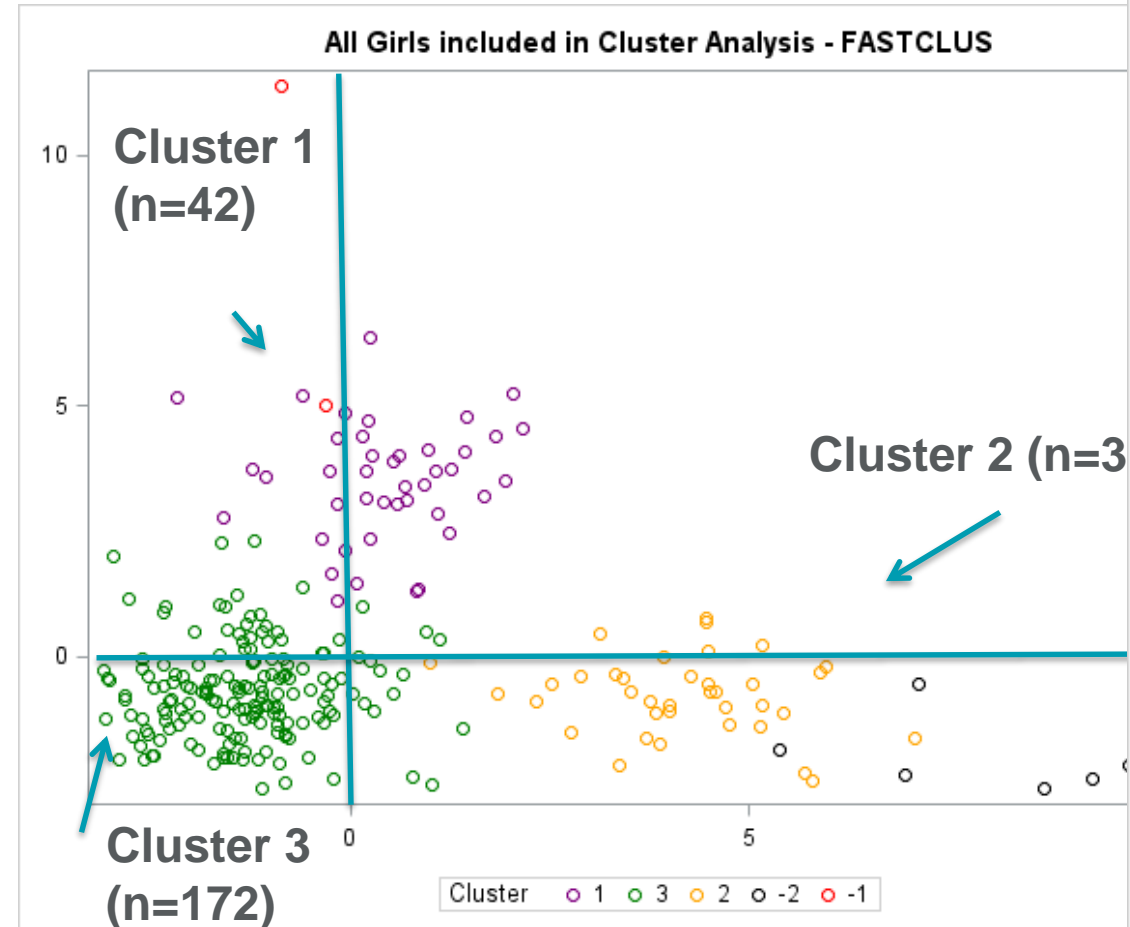
Cluster B
n=96
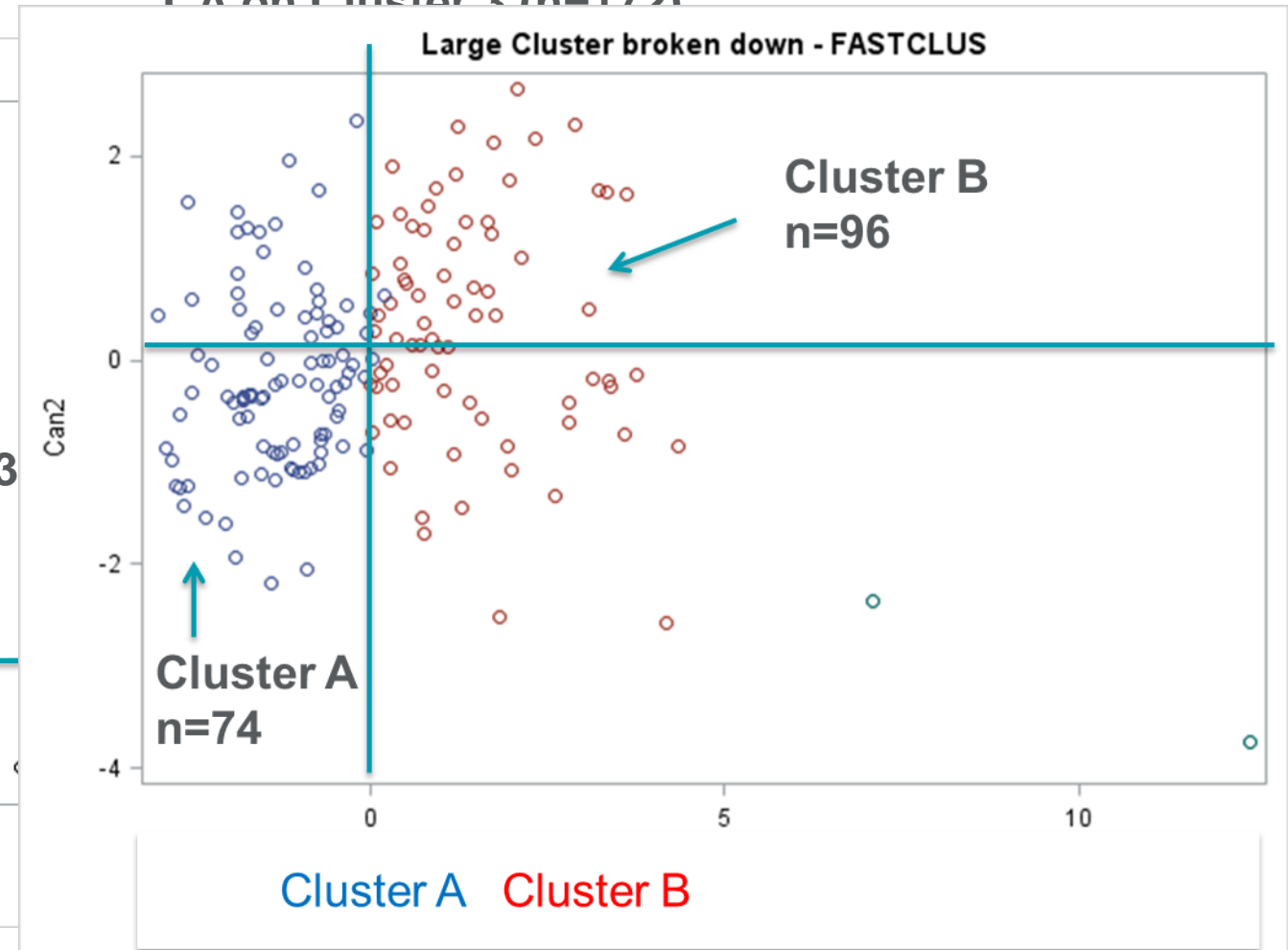
Cluster A
n=74

Cluster A   Cluster B
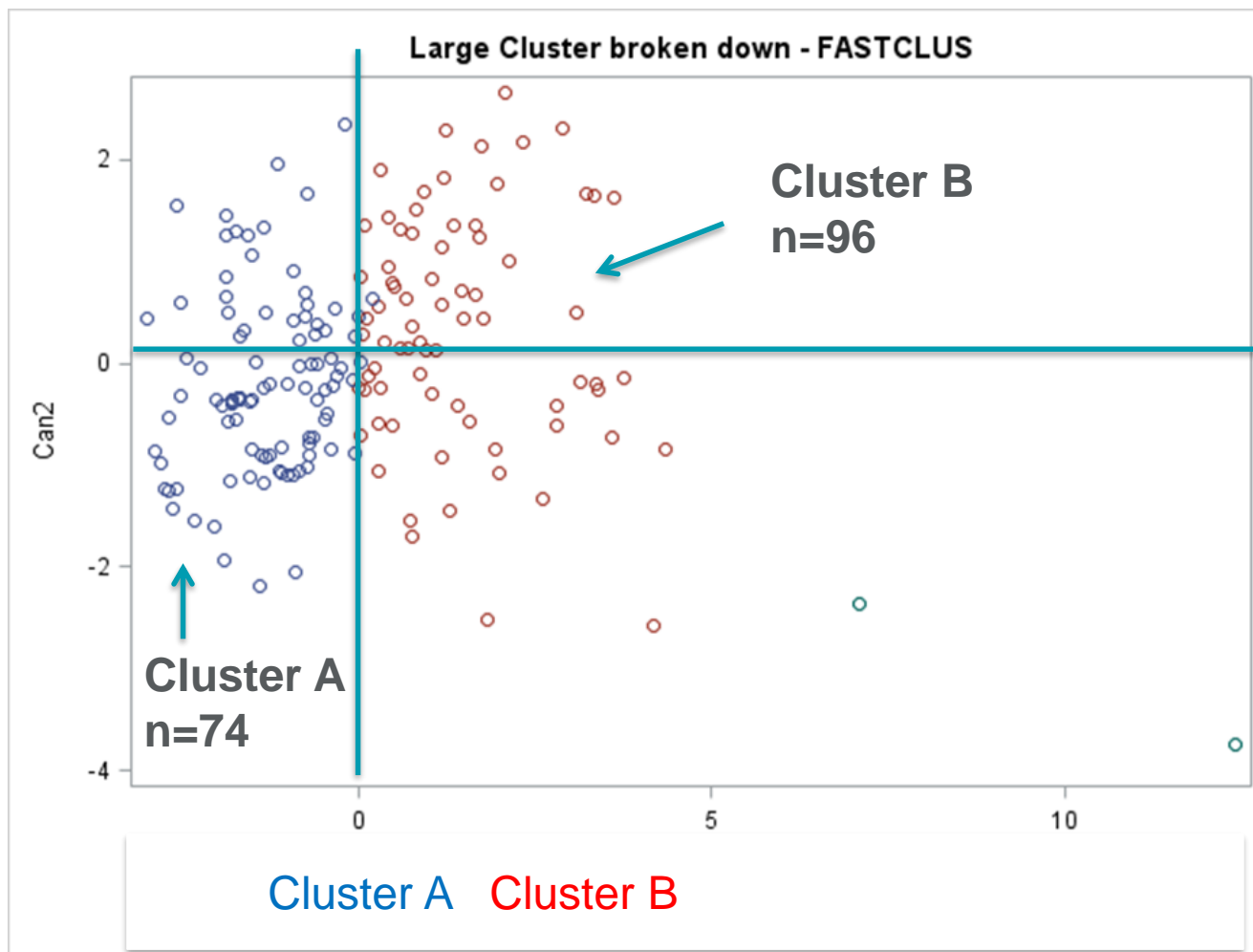
Race and parent's education by Phenotype

Phenotype 1 | **Phenotype 2** | **Phenotype 3a** | **Phenotype 3b**

Phenotype 1: 35.71% / 64.29%
Phenotype 2: 27.03% / 72.97%
Phenotype 3a: 29.73% / 70.27%
Phenotype 3b: 34.38% / 65.63%
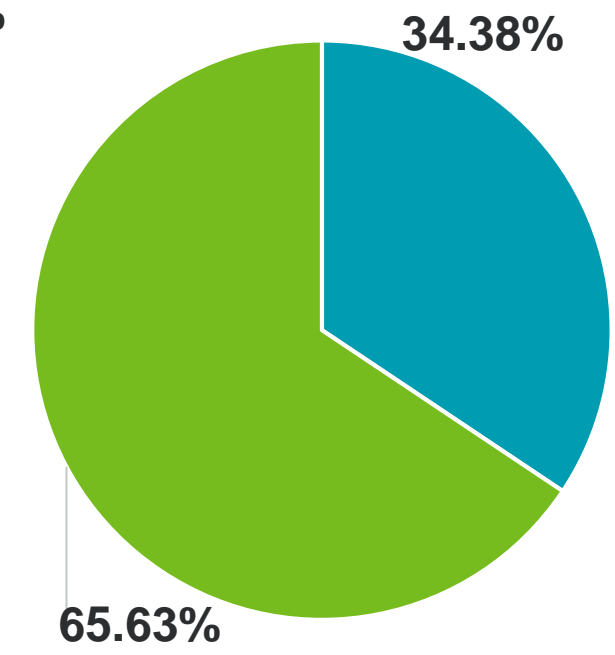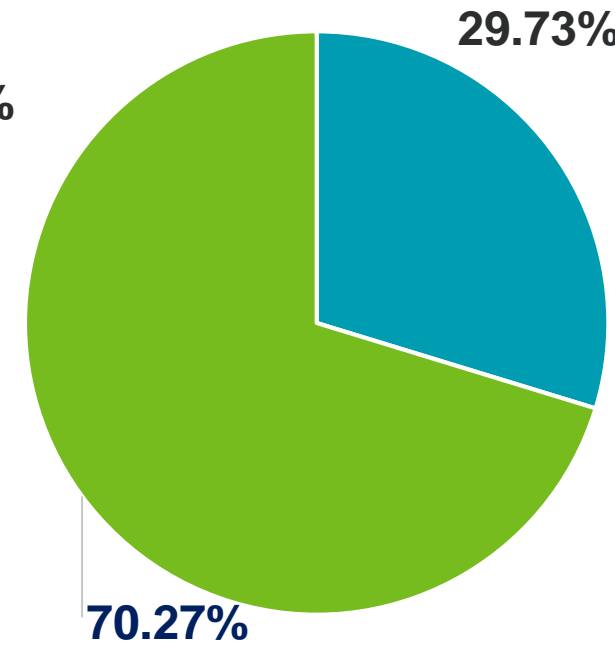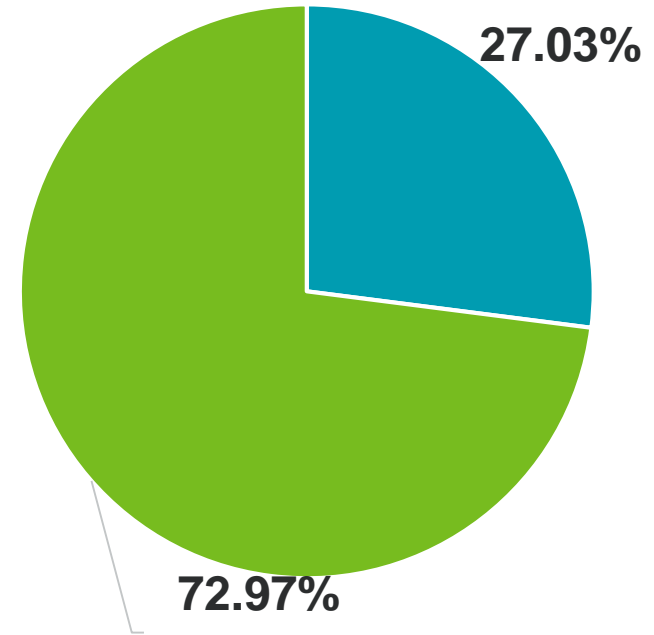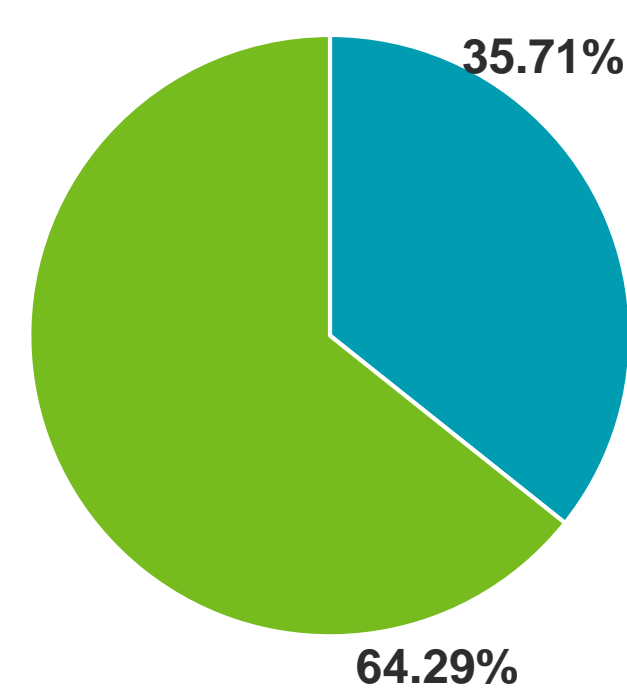
Black
White and all other

No statistically significant difference in race by phenotype.
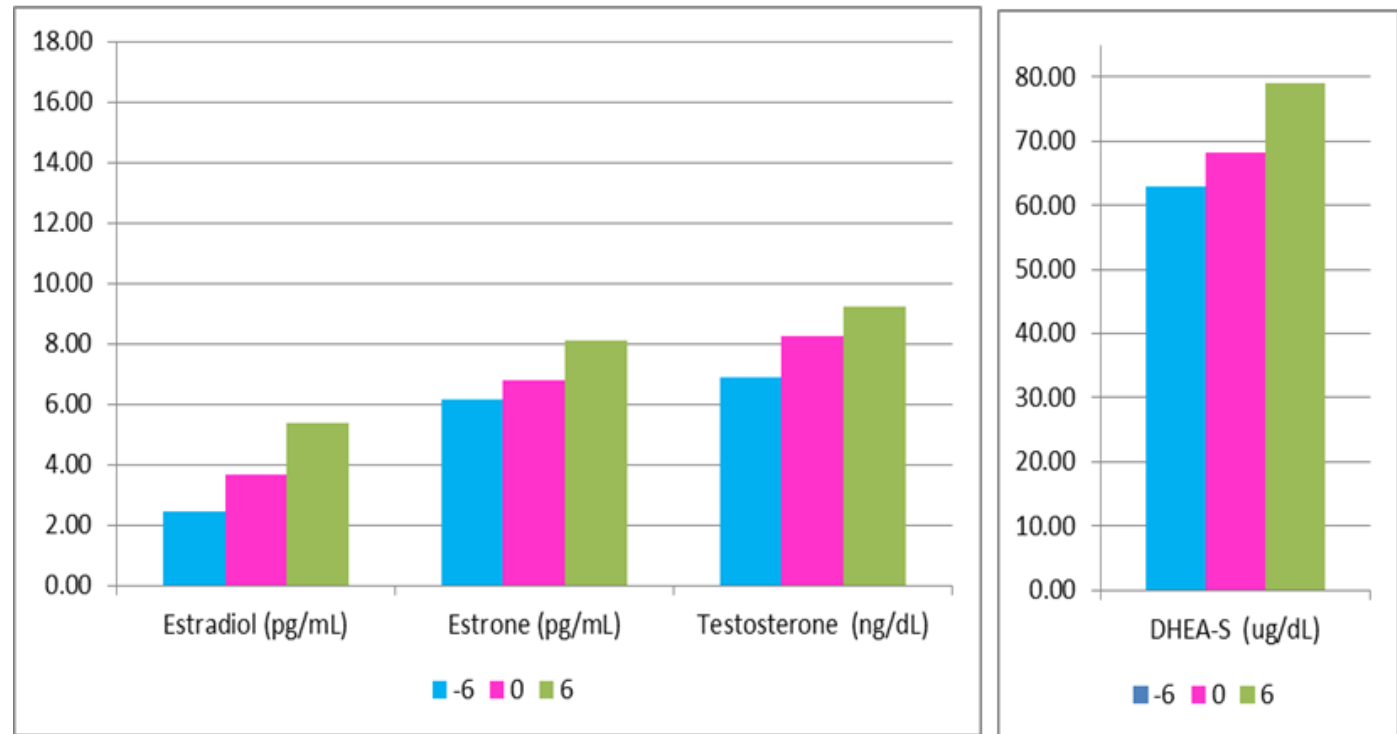
Parent's Education by Phenotype

Phenotype 1

24.43% 40.48%

38.10%

Phenotype 2

29.73% 16.22%

54.05%

Phenotype 3a

28.38% 27.03%

44.59%

Phenotype 3b

27.08% 29.17%

43.75%
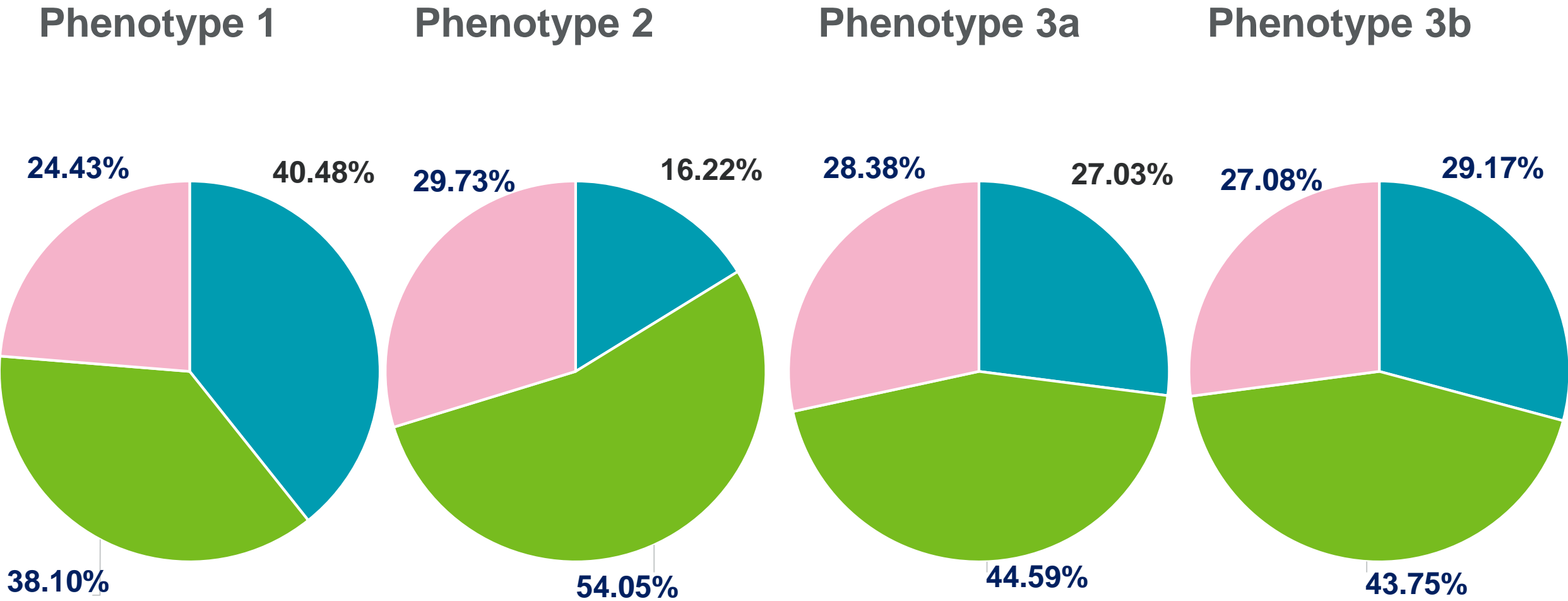
High school degree or less

No statistically significant difference in education by phenotype.

Phenotype 1

- Oldest age at thelarche
- Oldest age at menarche
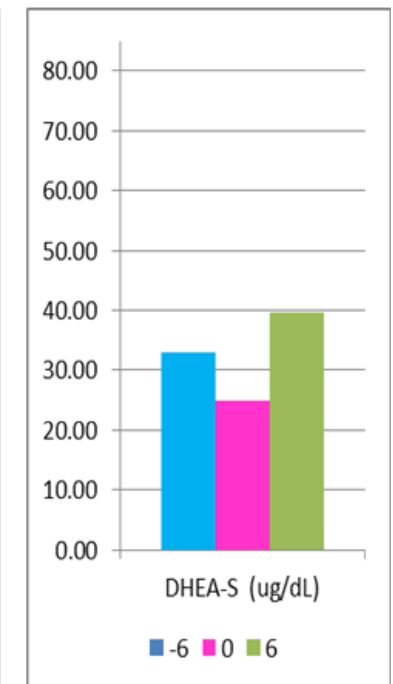- Less likely to enter thelarche early compared to 3a or 3b
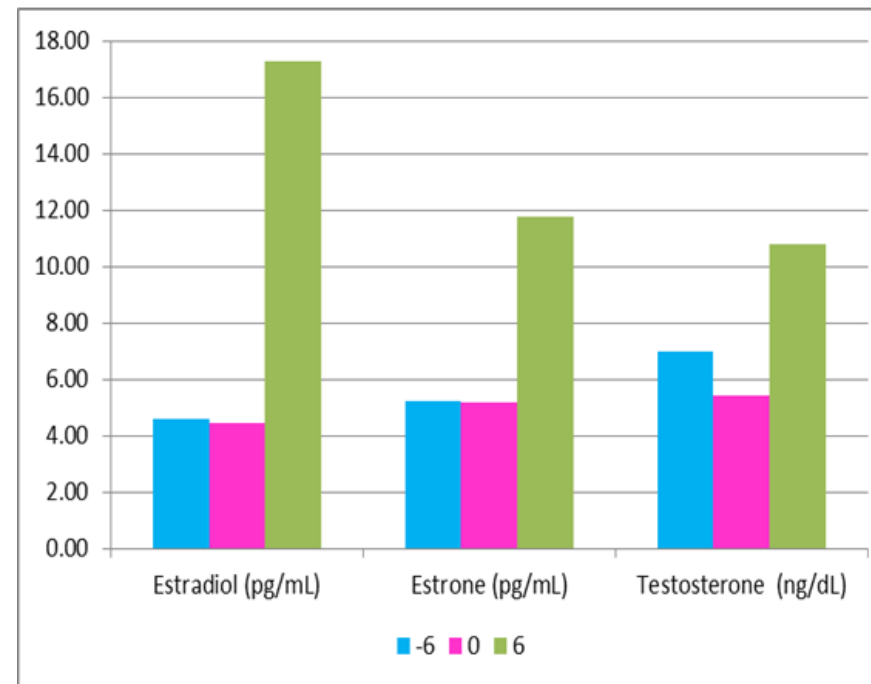

Phenotype 1

Phenotype 2

- Shortest tempo
- Youngest age at menarche
- 50% greater risk of earlier menarche than other phenotypes
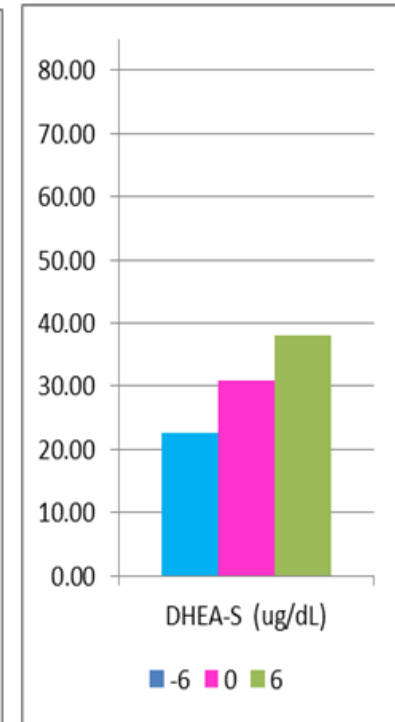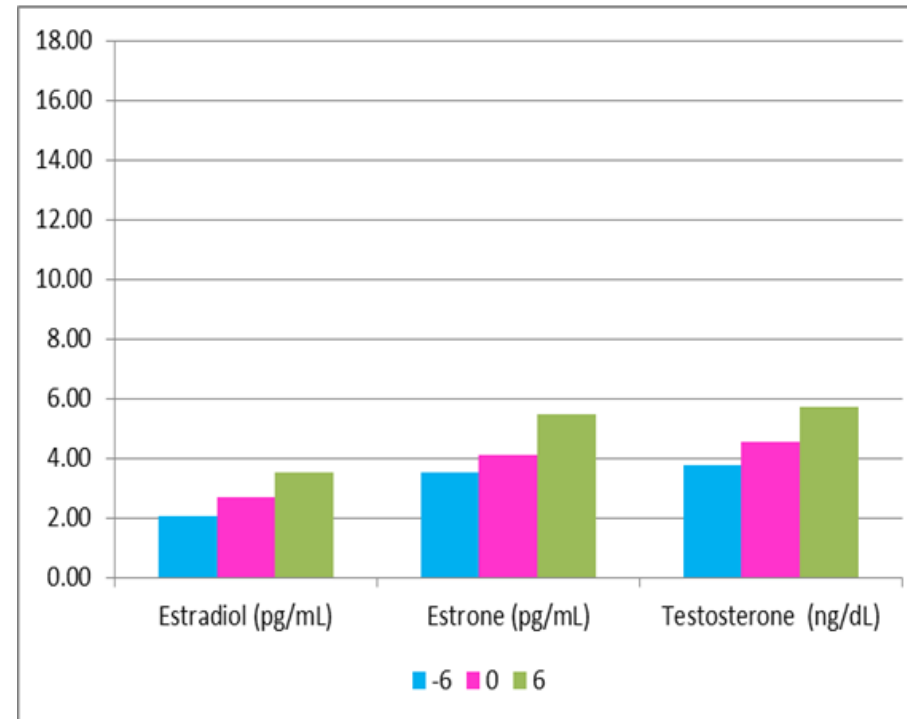- More likely to enter thelarche later than 3b

Phenotype 2

Phenotype 3a

- Youngest age at pubarche

Phenotype 3b

- Youngest age at thelarche
- Second to oldest age at menarche
- Longest tempo
- Oldest age at pubarche

Hip Pocket??