Published in final edited form as:

J Pediatr Adolesc Gynecol. 2017 October ; 30(5): 535–539. doi:10.1016/j.jpag.2017.02.003.

Amphiregulin as a Novel Serum Marker of Puberty in Girls

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Abstract

OBJECTIVES—Amphiregulin is a member of the epidermal growth factor family. In breast tissue, amphiregulin is a mediator of estrogen and progesterone signaling. The objectives were to examine the relationship of amphiregulin levels during peripuberty with estrogen levels.

METHODS—The participants in this analysis were a subset from a longitudinal study of pubertal maturation, the Breast Cancer and the Environment Research Program. They were recruited between ages six and seven. Blood specimens were selected for hormone analysis between 24 months prior to and six months after breast development. Serum amphiregulin levels were analyzed by ELISA.

RESULTS—Amphiregulin levels were measured in 188 girls; 8.5% had a maternal history of breast cancer. 30.9% of samples were below the limit of detection. Amphiregulin levels were greatest at 18 months prior to onset of breast development ($p < 0.006$), and the rise in estrone levels between -24 and -18 months was correlated with the increase in amphiregulin levels in the same time period ($p = 0.0002$). After adjustment for time relative to breast development, amphiregulin levels were associated with maternal breast cancer ($p = 0.024$). Tracking of amphiregulin levels was highly significant ($p < 0.0001$) within a given individual.

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Financial Disclosure: The authors have no financial relationships relevant to this article to disclose. Conflict of Interest: The authors have no conflicts of interest to disclose.

CONCLUSIONS—Amphiregulin levels peaked at 18 months prior to onset of breast development, were temporally related to the rise in serum estrone, and were significantly associated with maternal history of breast cancer. Elevated amphiregulin levels at puberty may be a predictor of increased breast cancer risk.

Introduction

Amphiregulin (AREG) is a member of the epidermal growth factor family of proteins and binds exclusively to the epidermal growth factor receptor (EGFR).¹ AREG expression is impacted by cytokines, hormones, and growth factors² and acts in multiple tissues and functions to promote cellular differentiation and proliferation, especially where branching takes place during growth and development.³ In breast tissue, AREG is a mediator of estrogen and progesterone signaling.^{4–6} It contributes to mammary epithelium proliferation, ductal elongation, and terminal end bud formation during puberty.⁷

The breast bud, breast stage 2,⁸ is the most commonly used metric to determine the onset of pubertal maturation in girls.⁹ This is a time of rapid proliferation in the breast, with ductal outgrowth and formation of terminal end buds.¹⁰ When considering puberty as a window of susceptibility for breast cancer risk,¹¹ the role of AREG during puberty potentially becomes more important through its action on cellular proliferation. Additionally, AREG has been associated with a ten-fold up-regulation in preneoplastic hyperplasia of the breast,¹² and additional associations between AREG and breast cancer have been reported.^{1,12–15} Since AREG expression is found to increase in puberty, as well as types of breast cancer and their precursors,^{16,17} it is important to consider potential relationships between AREG, puberty, and breast cancer risk.

The purpose of this paper is (1) to examine changes in AREG levels in peripubertal girls relative to breast development and (2) to explore if maternal history of breast cancer is associated with AREG levels.

Methods

The participants in the study were the Cincinnati subset of a larger cohort of girls enrolled in a longitudinal epidemiologic study of pubertal maturation, the Breast Cancer and the Environment Research Program (BCERP). The projects of the BCERP have been conducted through three sites: Mount Sinai School of Medicine, Kaiser Permanente of Northern California, and Cincinnati Children's Hospital Medical Center/University of Cincinnati. Study aims and design have been described in detail elsewhere.¹⁸ The participants at the Cincinnati site were recruited through the Breast Cancer Registry of Greater Cincinnati, as well as a convenience sample recruited through public and parochial schools in the greater Cincinnati metropolitan area. Participants were recruited between six and seven years of age, and the enrollment process at the Cincinnati site lasted two years. Girls were seen every six months between 2004 and 2010, with a visit window of four weeks, and annually until 2014. This study was approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center. Informed written consent was obtained from parents or guardians and assent from participants.

A limited number of female staff were trained and certified in assessment of pubertal maturation with breast palpation, incorporating the criteria established by Marshall and Tanner for breast maturation.⁸ In the first years of the study, 127 dual maturation assessments had been performed, with 87% agreement with a master trainer.¹⁸ Early morning fasting blood specimens were drawn at every visit, and serum was frozen at -80°C . After the participant had attained puberty (defined as breast stage 2), specimens were identified for hormone analysis. We used specimens collected from the first visit when pubertal development was recognized, as well as 6, 12, 18, and 24 months before and six months after breast development. Participants were eligible for this sub-study if they had a specimen available at pubertal onset, as well as one or more specimens between six to 24 months before onset of puberty. Up to four specimens were analyzed for each participant.

Maternal history of breast cancer was gathered from a questionnaire administered to the parent/guardian in years one, five, and nine of the study, with specific questions asking if the biologic mother had been diagnosed with breast cancer.

Serum AREG levels were analyzed by AmphiregulinDuoSet ELISA System (R & D Systems, Minneapolis, MN). The capture antibody, 100 μL of 2.0 $\mu\text{g/mL}$ mouse anti-human AREG, was plated in each well and blocked for one hour with 1% bovine serum albumin in phosphate buffered saline (PBS). 100 μL of samples, standards, and negative control were plated in duplicate and incubated for two hours at room temperature. Each well was aspirated and then washed three times with 0.05% Tween-20 in PBS. The detection antibody, 100 μL of 100 ng/mL biotinylated goat anti-human AREG in PBS, was added to each well and incubated for 2 hours. 100 μL of a 1/200 dilution of Streptavidin-HRP was added per well and incubated for 20 minutes, and then 100 μL of substrate 3,3', 5,5'-tetramethylbenzidine was added to each well for 20 minutes, followed by the addition of 50 μL of 2N H_2SO_4 . Absorbance of standards and samples were measured at 450 nm with background subtraction. The limit of detection (LOD) for AREG was 15.6 pg/mL. Inter-assay variability was 4.7–9.9%, and intra-assay variability was 3.3–5.5%.¹⁹ Estrone data were obtained from our earlier studies¹⁹ using high performance liquid chromatography with tandem mass spectrometry.

Statistical analysis

The initial statistical analysis modeled the odds of a girl having an AREG level less than the LOD at each time point. We also conducted analyses to determine if the values differed by time relative to onset of puberty (defined as onset of breast maturation) and by maternal history of breast cancer. We applied a mixed-effects mixed-distribution model, which is an advanced statistical modeling technique specifically designed for analyzing repeated data with clumping at less than LOD.²⁰ This approach does not require imputation of data points less than the LOD, but rather models them as a separate component. For the AREG levels observed above LOD, this approach models their linear relationship with maternal history, age at onset of puberty and relative timing of pubertal onset. The model accounts for repeated data collection by implementing individual random effects for both components and the potential correlations between the random effects for two components. The AREG values were calculated from least square means for the detectable levels and plotted against

time relative to onset of breast maturation. To evaluate individuals' tracking relative to AREG levels, a Markov transition model was used, with adjustment for timing relative to onset of breast development.²¹

A "lasagna plot"²¹ was constructed to depict longitudinal trajectories of amphiregulin, using logarithmic categories of amphiregulin levels.

Role of the Funding Source—None of the funding sources had a role in the study design; in the collection, analysis, or interpretation of data; in the writing of, or in the decision to submit the paper for publication.

Results

Amphiregulin levels were obtained for 188 girls; 39 had two samples, 58 had three samples, and 91 had four samples analyzed. AREG levels varied significantly by time relative to onset of puberty ($p = 0.0007$) (Table, Figures 1 and 2). In a post hoc analysis to evaluate the relationship of time period to AREG levels, AREG levels were significantly greater at 18 months (mean level 68 pg/mL) prior to clinically observable breast maturation, breast stage 2, when contrasted to other time periods, $p < 0.0056$ (Figure 1). AREG levels were not related to BMI z-score ($r = -0.020$) or to race ($p = 0.57$). There were no correlations between AREG levels at -18 months with serum estradiol obtained at -18 months ($r = -0.060$) or at onset of breast development ($r = -0.009$) or with estrone levels at either time (-18 months, $r = 0.102$ and onset of puberty, $r = -0.050$) (p values 0.41 to 0.93). However, there was a concomitant increase in estrone and AREG levels between -24 and -18 months before breast tissue detection ($r = 0.450$, $p = 0.024$); no other six-month change in estrone or in estradiol was associated with any other six-month change in AREG between -24 and zero months.

There were 24 participants (13%) who had AREG levels below the LOD at all time points. There was no association between those with levels of AREG below the LOD and age of pubertal onset ($p = 0.81$). In our cohort, 30.9% (173/559) of all specimens were below the LOD; this is significantly lower than results from a recent study in adults,²³ which reported 66.7% of specimens (89/129) were below the LOD, $p < 0.001$.

There were 16 participants (8.5%) who had a mother diagnosed with breast cancer; mean age of diagnosis of breast cancer was 38.5 years, whereas mean age of all mothers at time of the survey was 45.4 years. Within this peripubertal period, AREG levels were greater in those with a maternal history of breast cancer ($p = 0.024$), especially at 6 months prior to breast development (Figure 2).

Results of the Markov transition revealed that, after adjustment for time relative to puberty onset, AREG levels tracked (that is, relative position of levels compared to other participants) for a given participant (beta coefficient 0.652, $p < 0.0001$).

Discussion

This is the first study to examine AREG levels in girls around the time of pubertal maturation; it identified the rise in AREG levels 18 months prior to onset of detectable breast development. Thus, it may complement other newly proposed serum markers of pubertal/breast development, such as insulin-like peptide 3, a marker of theca cell functioning,²⁴ and anti-Mullerian hormone, a marker of ovarian reserve.²⁵ During puberty, there is rapid expansion of rudimentary breast tissue through proliferation and differentiation of epithelial and stromal components. These changes occur, in part, through EGFR and one of its ligands, AREG. In animal models, AREG is the only EGFR ligand that is up-regulated at puberty.²⁶ This raises the possibility that the detected rise in AREG may act to promote breast development. The rise in AREG in this study occurred at the same time as the rise in estrone. Since AREG is regulated by estrogen,⁴⁻⁶ this raises the possibility that estrone may have a role in early regulation of AREG.

We selected AREG as a growth factor of interest based on studies that reported the role of AREG in normal mammary gland development in animal models.^{4,7,27} Animal studies have noted that estrogen is necessary for breast duct elongation and branching, whereas progesterone is necessary for further ductal branching and formation of terminal end buds. Amphiregulin is a mediator of estrogen as well as progesterone effects on breast tissue. A limited number of manuscripts have reported on AREG levels in normal human subjects. Lemos-Gonzalez reported on AREG levels in 45 adult blood donors, as well as two groups with cancer; they noted a mean AREG value of 19.6 pg/ml in donors (gender and age unspecified).²⁸ Peterson reported results from two studies and noted there was no significant association between AREG detection comparing women with and without breast cancer, utilizing two different cut points for AREG.²³ The majority of women without breast cancer in their two studies have levels of AREG below the LOD (54/85 and 86/129).^{23,29} It may be difficult to compare concentrations of AREG between the three human studies above, given that the reported LOD was slightly different between the three studies, 15.6 (our study) vs 20 (Peterson's studies) pg/ml.^{23,29} Of note, our higher rate of detection might be secondary to our slightly lower LOD, or the higher rates could reflect greater extracellular release of AREG and consequent elevated serum levels during rapid proliferation of the pubertal breast. Peterson also performed serial serum measurements in seven women and reported that levels did not vary across the menstrual cycle.²⁹ Thus, it is of interest that we did not note an association with estrogen values, nor did Peterson²⁹ detect a difference across the menstrual cycle. Although Peterson²⁹ did not analyze tracking in that group, their data suggest high consistency in levels within a given person, and our analyses noted strong tracking within an individual. The strong evidence of tracking in these peripubertal girls, coupled with higher AREG levels in girls associated with maternal breast cancer, may point to an underlying genetic mechanism which may confer increased risk of breast cancer. Given the young age at diagnosis of breast cancer in their mothers, these families could represent a heritable genetic variant, a pattern noted in younger women with breast cancer.³⁰ Although the previous statement is speculative, a recent paper reported that amphiregulin is a critical mediator of estrogen response in estrogen receptor-positive breast cancer.³¹

A previous study reported that expansion of breast epithelial progenitor cells was regulated by AREG in a mouse model.³² Several authors have commented on AREG actions during puberty, a generally accepted window of susceptibility to factors that increase breast cancer risk.^{1,4,6,13,16,17} Amphiregulin expression is higher in certain types of breast ductal carcinomas in situ, as well as invasive breast carcinomas,^{16,17} and might serve as a potential target for breast cancer therapy.³³ Of note, AREG gene expression is down-regulated by BRCA1, a tumor suppressor gene, and has been proposed as one mechanism explaining how loss of BRCA1 function leads to breast cancer.³⁴ Two single-nucleotide polymorphisms within the *AREG* (amphiregulin) gene were identified³⁵ as a significant locus for breast density in a recent meta-analysis of genome-wide association studies (GWAS) examining mammographic density and breast cancer. In a review of The Cancer Genome Atlas (TCGA) database^{36,37} (<http://www.cbioportal.org/>), 4% (34/825) of all breast cancer subtypes revealed alterations in *AREG*, including amplification, as well as RNA up- or down-regulation, including 9% of Her2, 7% of luminal A/B, and 0% of basal-like subtypes. The lack of *AREG* alterations in basal-like tumors may indicate a role for *AREG* dysregulation in the etiology of Her2 and Luminal A/B, but not basal-like breast cancer.

There are several potential limitations to this study. The main limitation of this study was the sample size in those with a maternal history of breast cancer (N = 16); many mothers of the study participants are younger than the usual age range of breast cancer onset, which could lead to misclassification of breast cancer heritable risk that would bias downward the effect estimate for family history of breast cancer. The younger age of the mothers of the girls also meant that those with breast cancer would have a young age at diagnosis, although the mean age at diagnosis was much younger than the mean age of all mothers. Additionally, a larger sample number and the addition of histologic data from mothers with breast cancer might allow association with specific breast cancer subtypes. A second limitation was that our measurement of AREG was analyzed in serum specimens. Amphiregulin is membrane bound in its inactive form and is activated by ADAM17, a disintegrin and metalloproteinase.³⁸ Serum AREG assays may not fully capture the expression of activated AREG in breast tissue. Given the sampling frequency of six months, estrone may rise as early as six months prior to or at the same time as the rise in AREG. This relationship may represent the regulation of AREG directly by estrone or indirectly through either a common process or two concurrent events. Additionally, we measured AREG in specimens around the time of pubertal breast maturation and may have missed a second induction of AREG levels with ovulation and associated production of progesterone. Lastly, our reported LOD was slightly more sensitive than the adult studies (15.6 vs 20 pg/ml), which could increase the likelihood of detection. Future directions could include identification of specific types of breast cancer diagnoses in mothers, examination of the relationship of AREG to early markers of breast cancer, and obtaining additional measurement of AREG at the time of menarche or after ovulation.

This study reports the association of AREG levels with onset of breast development in a longitudinal cohort of girls. Amphiregulin could serve as an early biomarker of pubertal onset, given its temporal proximity to breast development, as well as the onset of the pubertal growth spurt, both early manifestations of estrogen's effects. Additionally, we found an association between AREG levels and maternal history of breast cancer.

Amphiregulin may serve as a mitogen that promotes normal breast proliferation during puberty and abnormal proliferation in breast cancer. Understanding the biologic factors associated with AREG tracking and the relationship of AREG to onset of breast development, as well as to several neoplastic processes in breast tissue, may help to identify the underlying mechanistic basis of AREG as a potential early biomarker of breast cancer risk.

Acknowledgments

The authors would like to thank the research staff, study helpers, participants and their families. They would also like to thank the public and parochial schools in the greater Cincinnati metropolitan area, as well as the Breast Cancer Registry of Greater Cincinnati, for their permission to let us recruit study participants at the Cincinnati site.

Funding Sources: All phases of the study were supported by U01ES019434 (NIEHS); U01ES12770 and U01ES019453 (NCI and NIEHS); P30ES006096 (NIEHS); UL1RR026314 (USPHS); and R21ES017315 (NIEHS).

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Figure 1. Median amphiregulin levels relative to breast maturation

Amphiregulin levels peak at 18 months prior to palpable breast tissue. The rise in serum amphiregulin is coincident to the rise in serum estrone levels (arrow).

Figure 2. Serum amphiregulin levels by months relative to breast development and by maternal history of breast cancer

This is a lasagna plot, which allows the reader to observe longitudinal trajectories (Swihart 2010). Grey tones are used to categorize logarithmic values of amphiregulin, by time relative to breast maturation, and maternal history of breast cancer. Those with a maternal history of breast cancer have greater levels of amphiregulin, especially immediately prior to the appearance of palpable breast tissue.

Table 1

Median and least squares mean levels of amphiregulin, and number under the limit of detection (LOD), by time relative to breast maturation

Months relative to breast maturation	N	N < LOD (%)	Median	Least squares mean
-24	83	39 (47.0)	11.3	42.5
-18	88	25 (28.4)	39.6	68.3
-12	125	33 (26.4)	27.6	48.1
-6	104	31 (29.8)	25.9	40.8
onset	159	45 (28.3)	31.7	42.5