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Normal range of serum Amphiregulin in healthy adult human females

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ABSTRACT

Objectives: Prior to large studies in breast cancer patients, we have sought to establish the normal range of a potential serum biomarker, Amphiregulin, in healthy women and to determine whether sampling during the menstrual cycle influences the detected Amphiregulin levels.
Design and methods: Serum Amphiregulin levels were quantified using a commercially available ELISA in 85 normal female donors.
Results: The range of circulating Amphiregulin was 0–4467 pg/mL. The majority of women had no detectable circulating Amphiregulin ($n = 54$), and only five women had levels exceeding 500 pg/mL. Serum Amphiregulin levels did not vary significantly during the menstrual cycle ($n = 7$ women).
Conclusions: Detection of circulating Amphiregulin in a significant minority of healthy women suggests that it may not have the specificity necessary for a population screening tool; however its potential utility for monitoring response to treatment or disease progression should be examined in breast cancer cases.

1. Introduction

Amphiregulin (AREG) is a ligand for the Epidermal Growth Factor Receptor (EGFR). It was initially identified as a secreted factor in the ERα-positive MCF7 breast cancer cell line [1] and was shown to be estrogen-responsive in these cells [2]. In the mouse, Amphiregulin expression is required for normal mammary gland development [3], and is a direct transcriptional target of ER-alpha [4] and a key effector of estrogen [5,6] for this developmental process. In addition, a surge of Amphiregulin production in ovarian follicular fluid, driven by luteinizing hormone, is believed to be an important precursor of ovulation [7]. Modest cyclic changes in Amphiregulin have also been reported in the endometrium [8].

In breast cancer, Amphiregulin is highly expressed in estrogen receptor positive tumors, where it is cleaved at the cell surface by TACE/ADAM17 to release a soluble EGFR ligand which drives tumor cell proliferation [9]. In addition to breast tumors, AREG is highly expressed in several other cancers, including ovarian [10], endometrial [11], colorectal, head and neck squamous cell carcinoma (HNSCC) and non-small cell lung carcinoma (NSCLC), and has been reported to play a role in proliferation, survival, angiogenesis, invasion and metastasis [12].

Because tumors frequently have a leaky vasculature and because Amphiregulin is efficiently shed into the extracellular spaces of the tumor, it may prove to be a potentially useful serum biomarker for the presence or recurrence of various types of cancer, or for monitoring response to therapy. In order to be useful as a cancer biomarker, the reference range for Amphiregulin in the cancer-free population must first be determined. In this study, we evaluated serum samples from 85 women to determine the normal range of circulating Amphiregulin. In addition, to exclude potential cyclic effects due to hormonal fluctuations, we performed serial analysis of circulating Amphiregulin levels in 7 women over a complete menstrual cycle. We find that Amphiregulin levels are below the threshold of detection in approximately two thirds of the women and also that serum Amphiregulin levels do not vary substantially under the influence of cyclic hormonal changes during the menstrual cycle.

2. Methods

2.1. Subjects

Serum samples were acquired in batches from anonymous donors recruited by two commercial repositories (Innovative Research, Novi, MI and Promeddx, Norton, MA). All studies were carried out with the approval of the Institutional Review Board of the Albert Einstein College of Medicine. Donors were non-pregnant women with no current or prior cancer diagnosis, and no history of diabetes, hepatitis B or C, or HIV.

2.2. ELISA analysis

The human Amphiregulin DuoSet ELISA Development System (R & D Systems, Minneapolis, MN) was used to analyze Amphiregulin levels in serum samples according to the manufacturer's instructions. Briefly,

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100 μL of 2.0 μg/mL mouse anti-human Amphiregulin antibody was used as the capture antibody for each well. The coated plates were blocked with 300 μL of 1% BSA (Reagent Diluent Concentrate 2, R & D Systems) for 1 hour. Two-fold serial dilutions of recombinant human Amphiregulin (1000 pg/mL–15.125 pg/mL) in 1% BSA were used to create a seven point standard curve. One hundred microliters of samples, standards and negative control were added per well, in duplicate, and incubated for 2 hours at room temperature. Each well was aspirated and washed three times with 0.05% Tween-20 in PBS (pH 7.2–7.4) after each incubation step until the developing step. One hundred microliters of 100 ng/mL biotinylated goat anti-human Amphiregulin was used as the detection antibody for each well and incubated for 2 hours. One hundred microliters of a 1/200 dilution of Streptavidin-HRP (R&D systems) per well was added and incubated for 20 minutes. Finally, to develop the assay, 100 μL of substrate solution TMB (3,3′,5,5′ -tetramethylbenzidine) was added to each well for 20 minutes followed by the addition of 50 μL of Stop Solution (2 N HCL). Absorbance of standards and samples were measured at 450 nm using a FLUOstar OPTIMA plate reader (BMG Labtech). The average absorbance of the 1% BSA negative control wells was subtracted from the readings of all standards and samples. Then, the $\log_{10}$ of each standard concentration was plotted against the absorbance, and a four parameter logistic (4-PL) curve fit was created using GraphPad Prism software. The concentration of each unknown sample was then interpolated from the standard curve. If samples were diluted, the concentration interpolated from the standard curve was multiplied by the dilution factor.

2.3. Statistical analysis

All data were analyzed using GraphPad Prism (version 5.03).

3. Results

3.1. Stability of Amphiregulin in serum samples

We have previously described the use of a commercially available ELISA assay for the quantification of human Amphiregulin in cell culture supernatants [9]. In this study, we adapted the assay for use with human serum samples. One major barrier to use of a protein analyte as a serum biomarker is the sensitivity of the marker to degradation or precipitation during freezing and thawing. We selected a serum sample from our cohort and subjected it to a series of five freeze–thaw cycles. ELISA analysis of aliquots withdrawn after each thaw indicated that the measured level of Amphiregulin did not change significantly (Fig. 1).

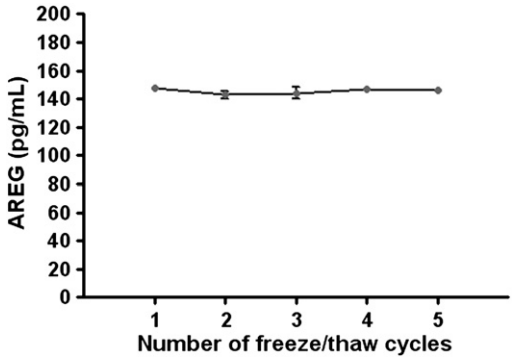

Fig. 1. Stability of serum Amphiregulin following repeated freeze–thaw cycles. A sample of human serum was subjected to freezing on dry ice, followed by thawing to room temperature and extraction of an aliquot for analysis. Amphiregulin levels in aliquots after five such cycles were determined by ELISA.

3.2. Circulating Amphiregulin in healthy subjects

To determine the range of circulating Amphiregulin concentrations in the serum of healthy human females, we procured frozen serum samples from two commercial repositories. The associated demographic parameters available with these samples included age and, in some cases, race. Donors had no history of cancer or diabetes and samples tested negative for HIV and hepatitis B and C. Based on analysis with dilutions of recombinant Amphiregulin we determined that the sensitivity threshold of this ELISA assay was 20 pg/mL. Of the 85 women tested, 54 (64%) had levels below this threshold of detection. Sixteen women had circulating Amphiregulin levels between 21 and 100 pg/mL, and 15 women had levels in excess of 100 pg/mL (Fig. 2A). A breakdown of the data by age and race of the women is shown in Fig. 2B. Using age 50 as an approximate cut-off for menopause, there was no significant difference in the distribution of serum Amphiregulin levels between pre- and post-menopausal women.

3.3. Influence of menstrual cycle on circulating Amphiregulin levels

Because Amphiregulin is known to be regulated by both estrogen [2] and luteinizing hormone [7], one potential concern about its suitability as a biomarker is that levels of circulating Amphiregulin might vary substantially under the influence of the hormonal changes during the menstrual cycle. In that case, blood draws might need to be carefully timed in premenopausal women in future biomarker trials in cancer patients. To address this question, we evaluated the levels of circulating Amphiregulin in seven women over the course of a complete menstrual cycle. One woman was selected as she had no detectable circulating Amphiregulin (Fig. 3, gray line). In this case, there was no elevation of circulating Amphiregulin above the threshold of detection on any day of the cycle. In the other six cases, the levels of circulating Amphiregulin were stable over the length of each menstrual cycle.

4. Discussion

In this study, we determined the range of circulating Amphiregulin in the serum of healthy women and found that this was not subject to substantial intra-individual variation depending on the stage of the menstrual cycle. The range of circulating Amphiregulin was 0–4467 pg/mL; however the majority of women had no detectable circulating Amphiregulin ($n = 54$), and only five women had levels exceeding 500 pg/mL. In addition, Amphiregulin quantification was stable to repeated freeze–thaw cycles.

Lemos-Gonzalez et al. described the concentrations of serum Amphiregulin in a cohort of 22 NSCLC patients, 41 HNSCC patients and 45 normal donors [13]. They reported a range of 0 to 85.8 pg/mL in the normal donors, 0 to 74.8 pg/mL in NSCLC and 0 to 45 pg/mL in the HNSCC patients, values that are considerably below the upper limits of Amphiregulin detected in the normal females in our study. It is noteworthy that only 5 of the 65 cancer patients in their study was female and no gender breakdown for the normal donors was provided, suggesting the high levels of Amphiregulin observed in our cohort may be more common in females. Two other studies have examined serum Amphiregulin in NSCLC patients without any cancer-free control group. In these studies, which included a higher proportion of female patients, ranges of Amphiregulin between 0–375 pg/mL [14] and 0–2034 pg/mL [15] were reported.

Tumor-expressed Amphiregulin has emerged as a potential prognostic or predictive marker in non-small cell lung cancer and colorectal cancer, both of which are frequently driven by activation of the EGFR signaling pathway. In NSCLC, high levels of Amphiregulin were associated with responsiveness to the small molecule EGFR inhibitor, gefitinib [16], while in colorectal cancer, high levels of

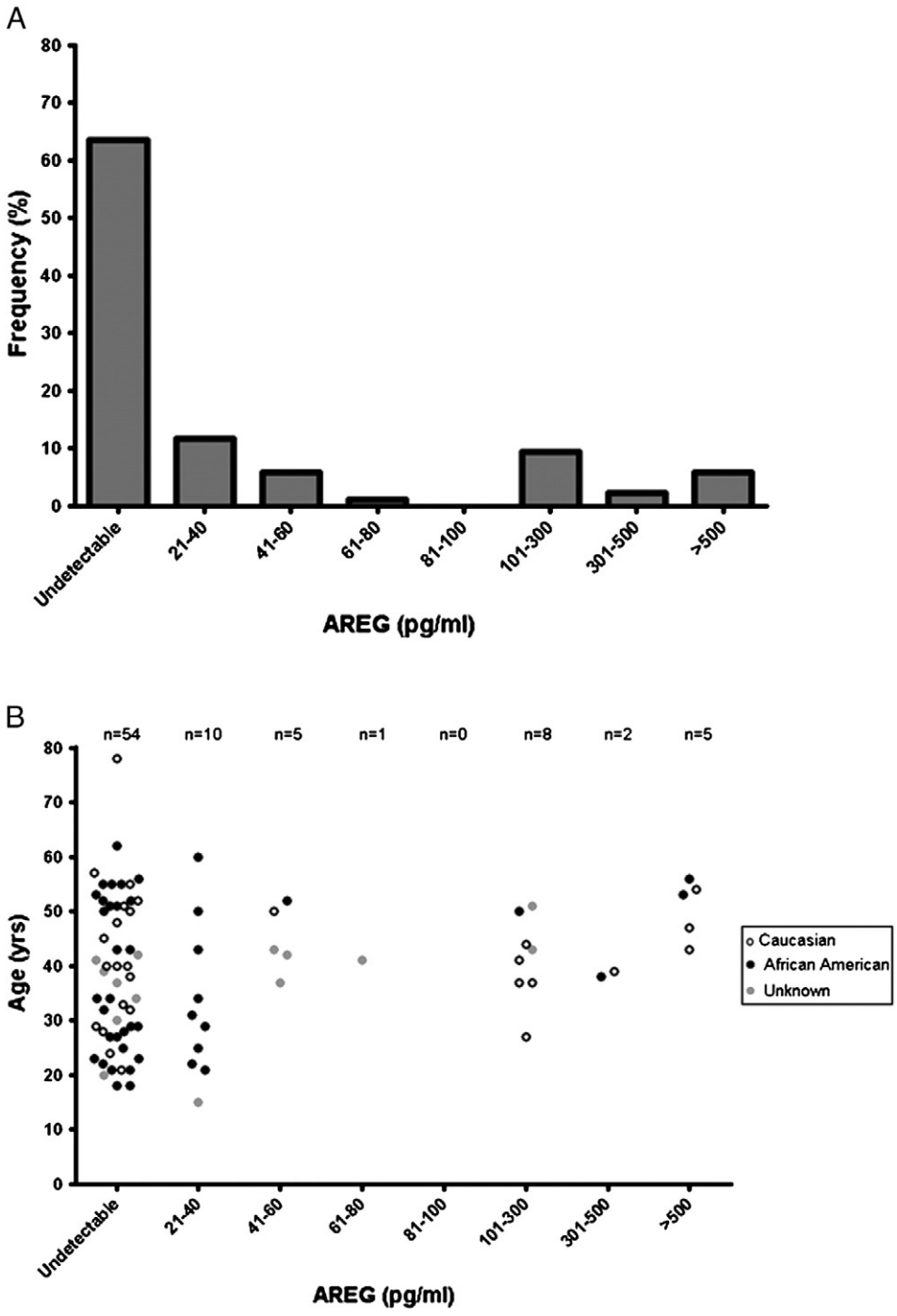

Fig. 2. Serum Amphiregulin levels in healthy human females. A. Histogram showing the distribution of serum Amphiregulin levels among the study population. B. Analysis of serum Amphiregulin levels by age and race. The threshold of detection of the assay was 20 pg/mL.

tumor Amphiregulin were predictive of response to the EGFR blocking antibody, cetuximab [17–19]. Two small studies which examined the utility of serum Amphiregulin as a predictor of gefitinib response in NSCLC have yielded conflicting results, with one study suggesting that high levels of serum Amphiregulin were associated with response to gefitinib [15], and the other finding that high levels were associated with gefitinib insensitivity [14].

In those cases where high Amphiregulin levels were detected in the serum in the women in our cohort, the tissue or cell of origin remains unclear. Early work by Plowman et al. demonstrated that AREG mRNA is highly expressed in human placenta and ovary [20], while analysis of the raw microarray data from a larger set of tissues [21] suggest that Amphiregulin mRNA may also be highly expressed in lung. The extent to which the Amphiregulin produced locally within these tissues may contribute to total serum Amphiregulin is not known. Various leukocytes, including eosinophils [22], basophils [23] and mast cells [24] can be stimulated to produce Amphiregulin, raising the possibility that in some cases the high levels of Amphiregulin detected in the serum samples may result from an immune response. Although the donors in our study were believed to be drawn from the healthy population, we cannot formally exclude the possibility that one or more donors may have had cancer at the time of donation.

Assays such as PSA for prostate cancer and CA125 for ovarian cancer [25] have had a pronounced effect on the management of these diseases, but similarly useful markers in breast cancer are lacking. These data pave the way for a more detailed study of the potential

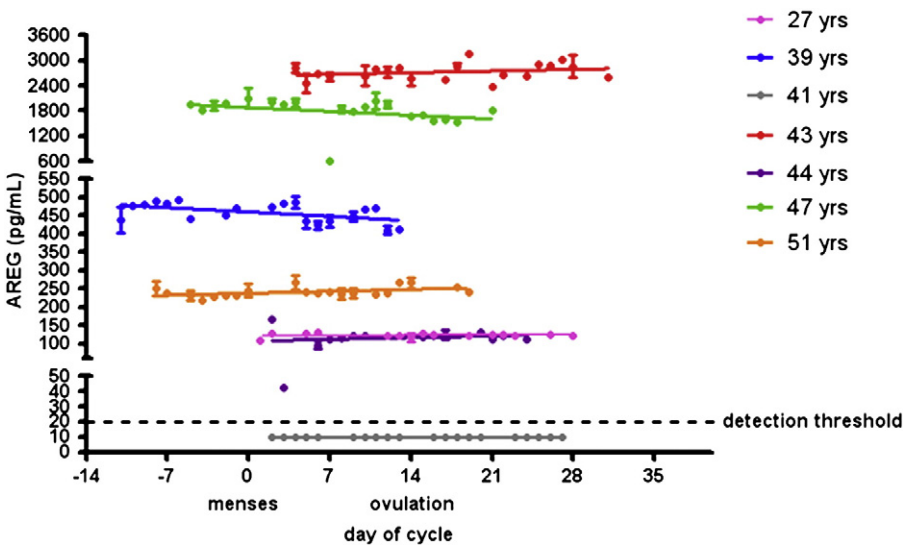

Fig. 3. Analysis of serum Amphiregulin levels during seven complete menstrual cycles. Amphiregulin levels from subjects of the indicated ages are shown. Date of onset of menses was known for all women and the ovulation date was estimated to be 14 days later.

utility of serum Amphiregulin as a biomarker in breast cancer patients. The high levels of Amphiregulin we observed in some women in our healthy cohort suggest that serum Amphiregulin may not have the necessary sensitivity and specificity for use as a population screening tool; however it may be found to have utility in selected subsets of breast cancer patients or in women at high risk of developing breast cancer.

Acknowledgments

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