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Amphiregulin as monitoring biomarker for life-threatening acute graft-versus-host disease: secondary analysis of two prospective clinical trials

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Running Head: AREG as GVHD Monitoring Biomarker

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- Najla El Jurdi, Armin Rashidi, Connor Demorest, and Angela Panoskaltsis-Mortari have nothing to disclose.
- Brian C. Betts holds a patent (WO2015120436A2) related to CD4+ T cell pSTAT3 as a marker and therapeutic target of acute GVHD. Holds a provisional patent (WO2017058950A1) related to the use of JAK inhibitors for rejection and GVHD prevention. Neither BCB nor his institution have received payment related to claims described in the JAK/STAT3 patents. Holds a patent for CD83 CAR T use in immunology and oncology. BCB, UMN, and Moffitt Cancer Center have received licensing revenue related to this IP.
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- Michael Pratta and John Galvin are employees and shareholders in Incyte Corporation.
Patients with life-threatening acute graft-versus-host disease (aGVHD) often have severe symptoms related to organ/tissue damage, although the symptom severity does not universally reflect the risk of aGVHD-related mortality.(1, 2) Biomarkers can serve as complementary non-invasive measurements of aGVHD risk.(3, 4) Using blood samples from established repositories, including samples from the Chronic GVHD Consortium and the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) 0302 and 0802, we have previously demonstrated that circulating amphiregulin (AREG) can risk-stratify patients at the onset of acute GVHD.(5, 6) AREG is a protein that belongs to the EGF family. It is a signaling molecule that plays a key role in cell growth, differentiation, and survival. Amphiregulin is produced by a variety of cells, including epithelial cells, fibroblasts, and immune cells, and it binds to the epidermal growth factor receptor (EGFR) on the surface of target cells.(7) In the present study, we assess AREG as a monitoring biomarker when measured during two prospective studies, a University of Minnesota (UMN) trial testing urinary-derived human chorionic gonadotropin/epidermal growth factor (uhCG/EGF) in supportive care for Minnesota high risk aGVHD in the first line setting or patients with aGVHD receiving second line therapy (NCT02525029), and in patients with steroid-refractory aGVHD receiving ruxolitinib in the REACH1 study (NCT02953678).

Longitudinal plasma samples were cryopreserved at study baseline and on study visit days 7, 14, 28, and 56 from patients enrolled in both the uhCG/EGF study (N=51) and REACH1 (N=60). All study participants signed informed consent documents approved by respective Institutional Review Boards indicating consent for collection of blood samples and data in accordance with the Declaration of Helsinki. In samples collected during uhCG/EGF treatment, AREG was measured using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), and ST2 and REG3a were measured using a multiplex Luminex-based array (R&D Systems) at the Cytokine Reference Laboratory at UMN. Plasma samples from REACH1 were analyzed for concentrations of AREG, ST2, and REG3a using the microfluidic ProteinSimple Ella platform (Bio-Techne, San Jose, CA) at Incyte Laboratories. Correlation of AREG levels between the AREG ELISA and microfluidic immunoassay were determined using the Pearson correlation coefficient with log-transformed values using a subset of samples (N=47), tested at Incyte Laboratories. The correlation of AREG values between the platforms was very high (r=0.89, p<0.001, Supplemental Figure 1). Comparisons of biomarker concentrations between response groups were performed using nonparametric one-way analysis of variance (Kruskal-Wallis test). Patients who died before day 28 of the study were considered non-responders. Analyses of change of biomarkers from baseline to subsequent study days were performed using nonparametric matched pairs analysis from the baseline value with Bonferroni correction for multiple testing. Statistical significance for longitudinal analysis was thus declared at \( p=0.0125 \). Biomarker cutoffs relevant for survival at study baseline were identified using recursive partitioning, dichotomizing groups into values that show the maximum difference in survival, with a difference of \( p=0.05 \) by the log-rank test determined to be statistically significant. This was performed within each trial for dichotomization within the individual study populations, as well as with the combined cohort to identify a value of AREG that would be informative across both platforms. Ann Arbor scores were calculated according to the formula published by Levine et al.(4)
The baseline characteristics of the clinical trial participants are shown in Table 1. The majority of patients enrolled on both studies had grade III/IV aGVHD (79% for uhCG/EGF and 63.3% for REACH1) and an Ann Arbor 3 biomarker score (65% for uhCG/EGF and 85% for REACH1) at the start of the study, predicting a high risk of mortality in these patients with aGVHD. In patients treated with uhCG/EGF who had a complete response (CR) at Day 28 of therapy, AREG decreased 3-fold from baseline to Day 56 (mean, 98 vs 32 pg/mL, p=0.006, Figure 1A). AREG did not significantly change over time in patients with a partial response (PR) or no response (NR) to uhCG/EGF. Baseline AREG >212 pg/mL was associated with a rapidly fatal course, with median survival of 62 days, p<0.006, Figure 1B). Across the entire range of baseline AREG values in the UMN study (6.3 – 821.4 pg/mL), the risk ratio for death was 10.9, 95% CI 1.9 – 49.7, p=0.009. The biomarker patterns were similar for REACH1. In those achieving a CR, AREG decreased 2.8-fold from baseline to Day 56 (mean, 174.7 vs 63.6 pg/mL, p=0.007, Figure 1C). AREG also decreased 2.0-fold over time in patients treated with ruxolitinib who had a very good partial response (VGPR) or PR to treatment (mean baseline 288.2 vs 146.1 pg/mL at Day 56, p=0.017) but no change over time in those with progressive disease (PD). Patients on REACH1 with a baseline AREG > 336 pg/ml had a rapidly fatal course, with a median survival of 74 days, p=0.005 (Figure 1D). Across the entire range of baseline AREG values in REACH1 (34.6 – 1,654 pg/mL), the risk ratio for death was 7.7, 95% CI 1.7 – 29.5, p=0.01. In multivariate analyses, only response at day 28 and baseline AREG with the cutoff determined by recursive partitioning were independent predictors of survival in both cohorts (Figures 2A and 2B). Patients treated with uhCG/EGF with a high baseline AREG had a 4.17-fold increased risk of death, and patients treated with ruxolitinib and a high baseline AREG had a 2.72-fold increased risk of death. When combining the cohorts to find a cutoff of AREG that is useful across the two platforms, an AREG level of 330 pg/ml or greater identified patients at high risk of early mortality (Supplemental Figures 1B-C).

Using samples collected during two prospective clinical trials on two different measurement platforms, we conclude that AREG is a useful monitoring biomarker for patients with life-threatening aGVHD. AREG levels were higher in REACH1 than the UMN uhCG/EGF study (baseline median 170 pg/ml vs 53.6 pg/ml, p<0.001), which could reflect differences in assay, severity of illness, or both. We suspect differences are due to severity of aGVHD, especially considering the very high proportion of patients with Ann Arbor 3 biomarkers in REACH1. Of note, significant biomarker changes did not occur within the first week, and ST2 and REG3a levels did not show statistically significant changes during the course of the study (Supplemental Figure 2), making the value of early biomarker in the first 1-2 weeks of therapy uncertain. Our analysis shows that between AREG, ST2, and REG3a, AREG levels most closely track with clinical response. Measurement of AREG may therefore be the most useful biomarker to assess for potential GVHD flare in the context of clinical events where response is difficult to assess, such as when medication side effects, GI infections, or dietary changes make clinical staging difficult to interpret. A proposed framework for using AREG to supplement clinical staging is shown in Figure 2C.

AREG has been implicated in a number of physiological processes, including tissue repair, wound healing, pregnancy, and cancer.(8) AREG is increased in circulation during aGVHD(5, 6),
Although its tissue expression is more complex. Increased AREG protein expression in cutaneous aGVHD is associated with a high mortality risk, although skin AREG expression does not correlate with serum AREG.(9) In contrast to the skin, gastrointestinal AREG protein expression is high during normal conditions but decreases during aGVHD or inflammatory bowel disease. GI expression of AREG also does not correlate with serum AREG.(10) AREG mRNA expression is significantly higher in the rectosigmoid mucosa of patients with lower gastrointestinal tract aGVHD compared to healthy controls, suggesting it may still reflect a stress or damage response to inflammation even though protein expression decreases.(11)

While we had previously hypothesized that AREG in circulation may be from damaged tissues, recent mouse and human evidence suggests it may also be produced by circulating immune cells during aGVHD. Ito et al recently showed that alloreactive CD4 T cells upregulate AREG expression during murine GVHD. AREG-deficient donor T cells caused less mucosal damage, spared intestinal stem cells, and reduced mortality compared to wild-type donor T cells.(12) We have also recently observed that high peripheral blood mononuclear cell expression of AREG mRNA is associated with low likelihood of response to aGVHD therapy, although the specific cell subset that was associated with this observation could not be determined with our bulk cell analysis.(13) T cells may indeed be a contributor to circulating AREG based upon work showing marked upregulation of AREG after T cell receptor stimulation.(14) In our phase II study of uhCG/EGF, we found a positive correlation between circulating AREG and cell-bound AREG on CD4+ and CD8+ central memory T cells, CD4+ effector memory T cells, double positive T cells, and plasmablasts.(15) Further work to determine the peripheral blood cellular source of AREG is needed.

In summary, circulating AREG serves as a blood biomarker that most closely tracks with longitudinal clinical response when measured in two prospective clinical trials of life-threatening aGVHD. AREG can be tested reliably on different platforms, making it feasible to implement in hospital clinical laboratories. Measuring AREG levels could offer a supplementary tool for assessing mortality risk when aGVHD first appears, even within clinically high-risk subsets. It may also help distinguish between potential aGVHD flare-ups and other clinical conditions that might confuse the diagnosis (please refer to our case report for a pertinent, real-world clinical example).(16) However, further research is required to confirm these findings. In the future, circulating AREG should be studied in other T-cell inflammatory contexts to determine its specificity for a GVHD activity versus other conditions.
REFERENCES


TABLES

Table 1. Patient Baseline Characteristics

<table>
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<tr>
<th>Characteristic</th>
<th>uhCG/EGF (N=52)</th>
<th>Ruxolitinib (N=60)</th>
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<td>Male, n (%)</td>
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<td>III</td>
<td>29 (52)</td>
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<td>IV</td>
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<td>1 (2)</td>
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<tr>
<td>Ann Arbor 2 biomarkers, n %</td>
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<tr>
<td>Ann Arbor 3 biomarkers, n %</td>
<td>34 (65)</td>
<td>51 (85)</td>
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</table>

FIGURE LEGENDS

Figure 1. AREG can risk stratify clinically high-risk patients at study baseline, and AREG decreases over time in patients who respond to GVHD therapy. Longitudinal plasma AREG is shown by treatment response in panels A and C. The optimal AREG cutoff for survival in each study is shown in panels B and D. Abbreviations: CR = complete response, NR = no response, PD = progressive disease, PR = partial response, VGPR = very good partial response.

Figure 2. Only AREG at study baseline and day 28 response are independent predictors of survival in multivariate analyses. The results of the multivariate analysis for the UMN uhCG/EGF study are shown in panel A, and results for REACH1 are shown in panel B. A proposed framework for using AREG as a GVHD biomarker in the first and second line acute GVHD setting is shown in panel C. Abbreviations: CR = complete response, LGI = lower GI, NR = no response, PD = progressive disease, PR = partial response, RR= risk ratio, VGPR = very good partial response.
### UMN (uhCG/EGF)

- **Age (Unit RR)**
- **Age (Range RR)**
- **LGI Stage (No LGI vs LGI GVHD)**

**Day 28 Response (NR vs CR/PR)**
- AREG >212 pg/mL: RR, 4.94, P=0.02
- ST2 >292 ng/mL: RR, 2.50, P=0.13
- REG3a >13.5 ng/mL: RR, 4.40, P=0.1

### REACH1 (ruxolitinib)

- **Age (Unit RR)**
- **Age (Range RR)**
- **LGI Stage (No LGI vs LGI GVHD)**

**Day 28 Response (PD vs CR/VGPR/PR)**
- AREG >336 pg/mL: RR, 9.14, P<0.001
- ST2 >188 ng/mL: RR, 0.39, P=0.17
- REG3a >3.6 ng/mL: RR, 0.36, P=0.21

### AREG

- **<33 pg/mL**
  - Standard risk
  - <330 pg/mL
  - Low 6-month mortality
- **≥33 pg/mL**
  - High risk
  - ≥330 pg/mL
  - High 6-month mortality