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

Patients with life-threatening acute graft-*versus*-host disease (GvHD) often have severe symptoms related to organ/tissue damage, although the severity of the symptoms does not universally reflect the risk of acute GvHD-related mortality.[1,2] Biomarkers can serve as complementary, non-invasive measurements of acute GvHD 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 previously demonstrated that circulating amphiregulin (AREG) levels can be used to risk-stratify patients at the onset of acute GvHD.[5,6] AREG is a protein that belongs to the epidermal growth factor (EGF) family. It is a signaling molecule that plays a key role in cell growth, differentiation, and survival. AREG is produced by a variety of cells, including epithelial cells, fibroblasts, and immune cells, and it binds to the EGF 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 patients with Minnesota high-risk acute GvHD in the first-line setting or patients with acute GvHD receiving second-line therapy (NCT02525029), and in patients with steroid-refractory acute GvHD receiving ruxolitinib in the REACH1 study (NCT02953678).

Plasma samples were collected and cryopreserved longitudinally 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 the 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 an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA), and suppression of tumorigenicity 2 (ST2) and regenerating islet-derived 3-alpha (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, USA) at Incyte Laboratories. The correlation of AREG levels measured by the AREG ELISA and microfluidic immunoassay was determined using the Pearson correlation coefficient with log-transformed values from a subset of samples (n=47), tested at Incyte Laboratories The correlation of AREG values between the platforms was very strong (r=0.89, P<0.001) (*Online Supplementary Figure S1*). Biomarker concentrations were compared between response groups 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 changes of biomarker levels 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 the longitudinal analyses was thus declared at P=0.0125. Biomarker cutoff values relevant for survival at study baseline were identified using recursive partitioning, dichotomizing groups according to 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. The recursive partitioning was performed within each trial for dichotomization within the

Table 1. Patients' baseline characteristics.

Characteristic	uhCG/EGF, N=52	Ruxolitinib, N=60
Age in years, median (range)	55 (2-72)	52 (18-73)
Male, N (%)	39 (75)	31 (52)
MAGIC acute GvHD grade, N (%) II III IV	 11 (21) 29 (52) 12 (27)	 22 (36.7) 24 (40.0) 14 (23.3)
Ann Arbor 1 biomarkers, N (%)	7 (14)	1 (2)
Ann Arbor 2 biomarkers, N (%)	11 (21)	8 (13)
Ann Arbor 3 biomarkers, N (%)	34 (65)	51 (85)

uhCG/EGF: urinary-derived human chorionic gonadotropin/epidermal growth factor; MAGIC: Mount Sinai Acute GVHD International Consortium; GvHD: graft-*versus*-host disease.

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 participants in the clinical trials are shown in Table 1. The majority of patients enrolled on both studies had grade III/IV acute GvHD (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 acute GvHD. In patients treated with uhCG/EGF who had a complete response 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 levels did not change significantly over time in patients with a partial response or no response to uhCG/EGF. A baseline AREG >212 pg/mL was associated with a rapidly fatal course, with a 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% confidence interval [95% CI]: 1.9-49.7; *P*=0.009). The biomarker patterns were similar in REACH1. In those patients achieving a complete response, AREG levels decreased 2.8-fold from baseline to day 56 (mean, 174.7 *vs.* 63.6 pg/mL; *P*=0.007) (Figure 1C). AREG levels also decreased 2.0-fold over time in patients treated with ruxolitinib who had a very good partial response or partial response to treatment (mean baseline AREG concentration was 288.2 *vs.* 146.1 pg/mL at day 56; *P*=0.017) but there was no change over time in patients with progressive disease. 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 (Figure 2A, B). Patients treated with uhCG/EGF with high baseline AREG levels had a 4.17-fold increased risk of death, and patients treated with ruxolitinib and a high

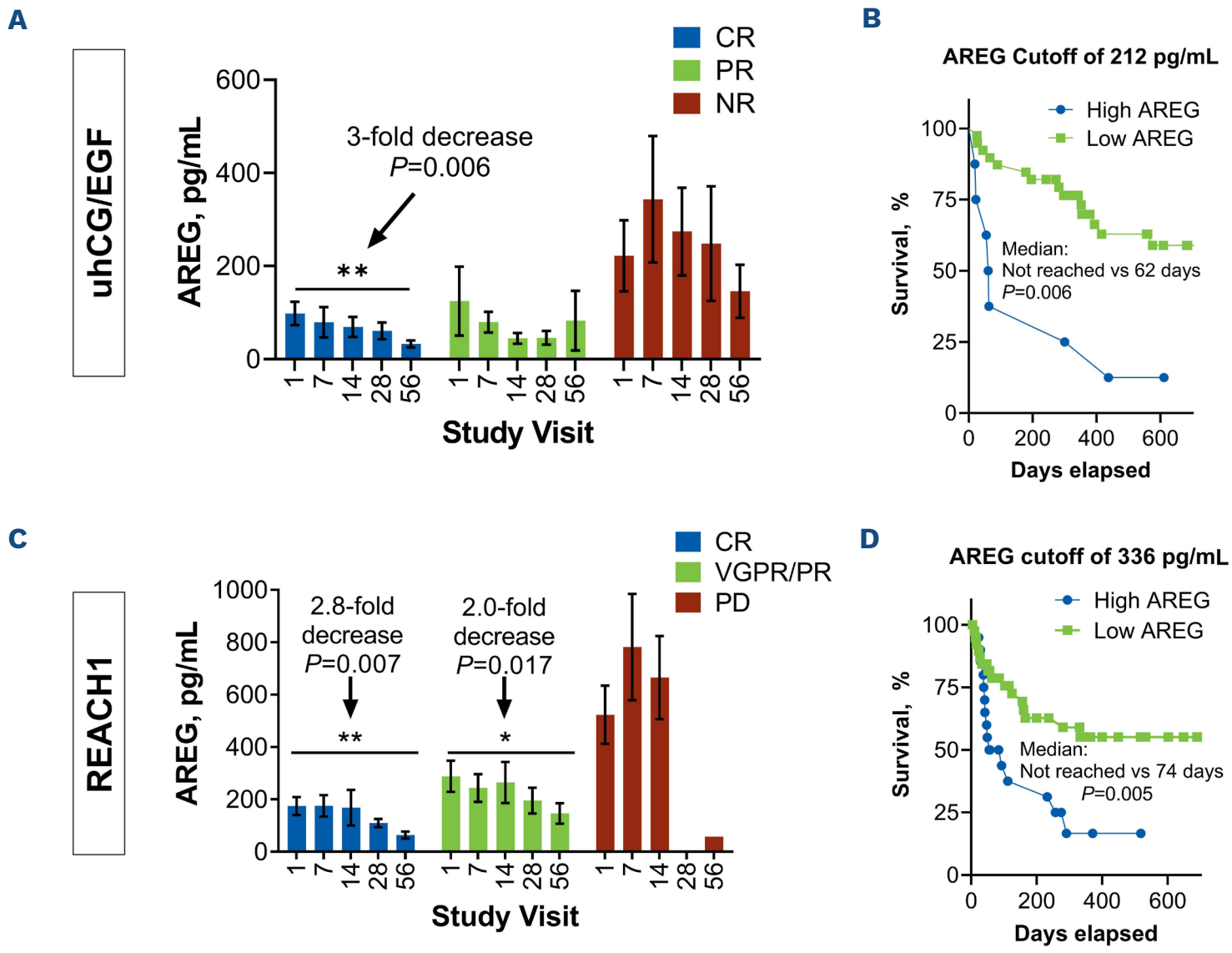

Figure 1. Amphiregulin levels can stratify clinically high-risk patients at study baseline and decrease over time in patients who respond to graft-*versus*-host disease therapy. (A, C) Longitudinally measured plasma amphiregulin (AREG) levels are shown by treatment response for patients from the uhCG/EGF study (A) and REACH1 study (C). (B, D) The optimal AREG cutoff for survival in each study is shown for patients from the University of Minnesota urinary-derived human chorionic gonadotropin/epidermal growth factor study (B) and REACH1 study (D). uhCG/EGF: urinary-derived human chorionic gonadotropin/epidermal growth factor; CR: complete response; PR: partial response; NR: no response; VGPR: very good partial response; PD: progressive disease.

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 (*Online Supplementary Figure S1B, 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 acute GvHD. AREG levels were higher in REACH1 than in the UMN uhCG/EGF study (baseline median, 170 pg/mL *vs.* 53.6 pg/mL, respectively; $P<0.001$), which could reflect differences in assays, severity of illness, or both. We suspect differences are due to severity of acute GvHD, 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 (*Online Supplementary Figure S2*), making the value of early biomarkers in the first 1-2 weeks of therapy uncertain. Our analysis shows that between AREG, ST2, and REG3a, AREG levels track clinical response most closely. AREG concentrations may therefore be the most useful biomarker to assess for potential GvHD flares in the context of clinical events in which response is

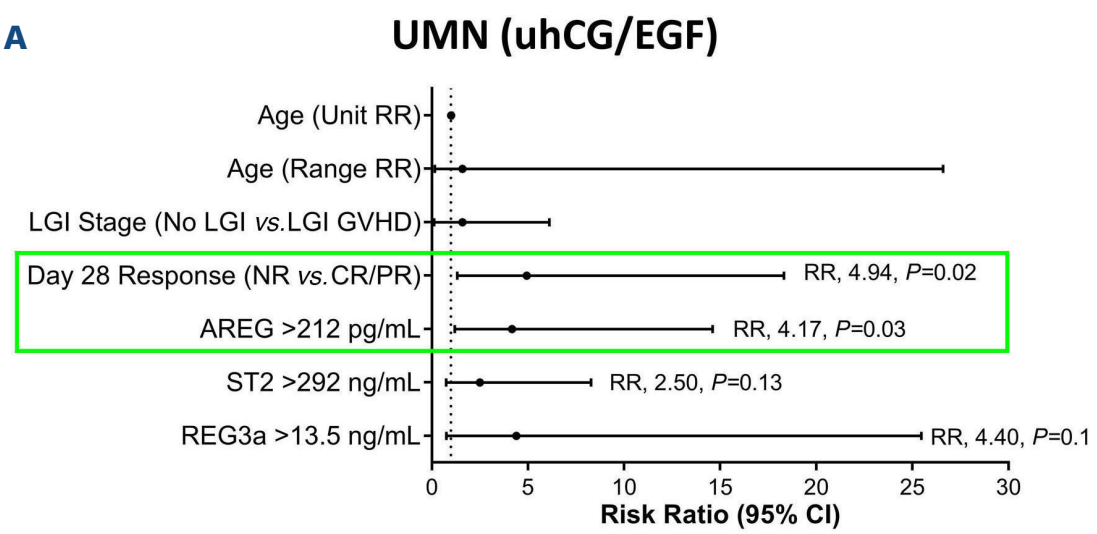

Figure 2. Only amphiregulin levels at study baseline and the day 28 response assessment are independent predictors of survival in multivariate analyses. (A, B) The results of the multivariate analysis for the University of Minnesota urinary-derived human chorionic gonadotropin/epidermal growth factor study (A) and for the REACH1 study (B) are shown. (C) A proposed framework for using plasma amphiregulin (AREG) levels as a graft-*versus*-host disease biomarker in the first- and second-line acute graft-*versus*-host disease setting. UMN: University of Minnesota; uhCG/EGF: urinary-derived human chorionic gonadotropin/epidermal growth factor; RR: risk ratio; LGI: lower gastrointestinal tract; GVHD: graft-*versus*-host disease; NR: no response; CR: complete response; PR: partial response; ST2: suppression of tumorigenicity 2; Reg3a: regenerating islet-derived 3-alpha; 95% CI: 95% confidence interval; PD: progressive disease; VGPR: very good partial response.

difficult to assess, such as when medication side effects, gastrointestinal infections, or dietary changes make clinical staging difficult to interpret. A proposed framework for using AREG measurements 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 the circulation during acute GvHD,[5,6] although its tissue expression is more complex. Increased AREG protein expression in cutaneous acute GvHD is associated with a high mortality risk, although skin AREG expression does not correlate with serum AREG values.[9] In contrast to the skin, gastrointestinal AREG protein expression is high during normal conditions but decreases during acute GvHD or inflammatory bowel disease. Gastrointestinal expression of AREG also does not correlate with serum AREG concentration.[10] *AREG* mRNA expression is significantly higher in the rectosigmoid mucosa of patients with lower gastrointestinal tract acute GvHD compared to that in 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 the circulation may come from damaged tissues, recent evidence from mice and humans suggests that it may also be produced by circulating immune cells during acute GvHD. 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 a low likelihood of response to acute GvHD 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, as measured in two prospective clinical trials of life-threatening acute GvHD. AREG can be tested reliably on different platforms, making it feasible to assay in hospital clinical laboratories. Measuring AREG levels could offer a supplementary tool for assessing mortality risk when acute GvHD first appears, even within clinically high-risk subsets. It may also help to distinguish between potential acute GvHD 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 acute GvHD activity *versus* other conditions.

Authors

Shernan G. Holtan,[1] Najla El Jurdi,[1] Armin Rashidi,[1,2] Brian C. Betts,[1] Connor Demorest,[3] John P. Galvin,[4] Margaret L. MacMillan,[5] Daniel J. Weisdorf,[1] Angela Panoskaltsis-Mortari[5] and Michael A. Pratta[4]

[1]University of Minnesota, Adult Blood and Marrow Transplant & Cell Therapy Program, Minneapolis, MN; [2]Fred Hutchinson Cancer Center, Seattle, WA; [3]Biostatistics Core, Masonic Cancer Center, University of Minnesota, Minneapolis, MN; [4]Incyte Corporation, Wilmington, DE and [5]University of Minnesota, Pediatric Blood and Marrow Transplant & Cell Therapy Program, Minneapolis, MN, USA

Correspondence:
S. HOLTAN - sgholtan@umn.edu

https://doi.org/10.3324/haematol.2023.283215

Received: April 17, 2023.
Accepted: September 5, 2023.
Early view: September 14, 2023.

Disclosures

SGH has received research support from Incyte Corporation and Vitrac Therapeutics and serves as a clinical trial adjudicator for CSL Behring. BCB holds a patent (WO2015120436A2) related to $CD4^+$ T-cell pSTAT3 as a marker and therapeutic target of acute GvHD and holds a provisional patent (WO2017058950A1) related to the use of JAK inhibitors for rejection and GvHD prevention. Neither BCB nor his institution has received payment related to claims described in the JAK/STAT3 patents. He holds a patent for CD83 CAR T-cell use in immunology and oncology. BCB, UMN, and Moffitt Cancer Center have received licensing revenue related to this IP. MLM has received support from FATE and Incyte Corporation and served as a consultant to Equilium. DW has received research support from FATE and Incyte Corporation. MP and JG are employees of and shareholders in Incyte Corporation. NEJ, AR, CD, and AP-M have no conflicts of interest to disclose.

Contributions

SGH designed the study, provided samples, performed analyses, and wrote the manuscript, NEJ, AR, BCB, JPG, MLM, DJW, and APM supervised sample analysis and edited the manuscript, CD performed analyses, MAP provided samples, performed analyses, and edited the manuscript.

Acknowledgments

The authors are grateful to Mr. Michael Ehrhardt in the University of Minnesota Cytokine Reference Laboratory for his laboratory analysis.

Funding

Funding for laboratory analysis of UMN blood samples was provided by grants (Regenerative Medicine Minnesota and UMN BRAINS award to SGH). Funding for laboratory analysis of blood samples from REACH1 was provided by Incyte Corporation. Biostatistical analysis was supported by National Institutes of Health grant P30 CA77598 utilizing the Biostatistics Core of the Masonic Cancer Center, University of Minnesota and the Center for Advancing Translational Sciences of the National Institutes of Health award number UL1TR002494.

Data-sharing statement

Reasonable requests for data may be sent to the author for correspondence (SGH).

References

1. Zeiser R, Blazar BR. Acute graft-versus-host disease - biologic process, prevention, and therapy. N Engl J Med. 2017;377(22):2167-2179.
2. Holtan SG, Yu J, Choe HK, et al. Disease progression, treatments, hospitalization, and clinical outcomes in acute GVHD: a multicenter chart review. Bone Marrow Transplant. 2022;57(10):1581-1585.
3. Srinagesh HK, Levine JE, Ferrara JLM. Biomarkers in acute graft-versus-host disease: new insights. Ther Adv Hematol. 2019;10:2040620719891358.
4. Levine JE, Braun TM, Harris AC, et al. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. Lancet Haematol. 2015;2(1):e21-29.
5. Holtan SG, Khera N, Levine JE, et al. Late acute graft-versus-host disease: a prospective analysis of clinical outcomes and circulating angiogenic factors. Blood. 2016;128(19):2350-2358.
6. Holtan SG, DeFor TE, Panoskaltsis-Mortari A, et al. Amphiregulin modifies the Minnesota Acute Graft-versus-Host Disease Risk Score: results from BMT CTN 0302/0802. Blood Adv. 2018;2(15):1882-1888.
7. Singh SS, Chauhan SB, Kumar A, et al. Amphiregulin in cellular physiology, health, and disease: potential use as a biomarker and therapeutic target. J Cell Physiol. 2022;237(2):1143-1156.
8. Zaiss DMW, Gause WC, Osborne LC, Artis D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. Immunity. 2015;42(2):216-226.
9. Schultz B, Miller DD, DeFor T, et al. High cutaneous amphiregulin expression predicts fatal acute graft-versus-host disease. J Cutan Pathol. 2022;49(6):532-535.
10. Amin K, Yaqoob U, Schultz B, et al. Amphiregulin in intestinal acute graft-versus-host disease: a possible diagnostic and prognostic aid. Mod Pathol. 2019;32(4):560-567.
11. Holtan SG, Shabaneh A, Betts BC, et al. Stress responses, M2 macrophages, and a distinct microbial signature in fatal intestinal acute graft-versus-host disease. JCI Insight. 2019;5(17):e129762.
12. Ito T, Takashima S, Calafiore M, et al. Donor-derived amphiregulin drives CD4+ T cell expansion and promotes tissue pathology after experimental allogeneic BMT. Blood. 2022;140 (Suppl 1):1152–1153.
13. Holtan SG, Hoeschen AL, Cao Q, et al. Facilitating resolution of life-threatening acute GVHD with human chorionic gonadotropin and epidermal growth factor. Blood Adv. 2020;4(7):1284-1295.
14. Qi Y, Operario DJ, Georas SN, Mosmann TR. The acute environment, rather than T cell subset pre-commitment, regulates expression of the human T cell cytokine amphiregulin. PLoS One. 2012;7(6):e39072.
15. Holtan SG, Hoeschen A, Cao Q, et al. Phase II, open-label clinical trial of urinary-derived human chorionic gonadotropin/ epidermal growth factor for life-threatening acute graft-versus-host disease. Transplant Cell Ther. 2023;29(8):509.e1-509.e8.
16. Newell LF, Holtan SG. Acute GVHD: think before you treat. Hematology Am Soc Hematol Educ Program. 2021;2021(1):642-647.