Progression of Disease Within 24 Months in Follicular Lymphoma Is Associated With Reduced Intratumoral Immune Infiltration

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PURPOSE Understanding the immunobiology of the 15% to 30% of patients with follicular lymphoma (FL) who experience progression of disease within 24 months (POD24) remains a priority. Solid tumors with low levels of intratumoral immune infiltration have inferior outcomes. It is unknown whether a similar relationship exists between POD24 in FL.

PATIENTS AND METHODS Digital gene expression using a custom code set—five immune effector, six immune checkpoint, one macrophage molecules—was applied to a discovery cohort of patients with early- and advanced-stage FL (n = 132). T-cell receptor repertoire analysis, flow cytometry, multispectral immunofluorescence, and next-generation sequencing were performed. The immune infiltration profile was validated in two independent cohorts of patients with advanced-stage FL requiring systemic treatment (n = 138, rituximab plus cyclophosphamide, vincristine, prednisone; n = 45, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone), with the latter selected to permit comparison of patients experiencing a POD24 event with those having no progression at 5 years or more.

RESULTS Immune molecules showed distinct clustering, characterized by either high or low expression regardless of categorization as an immune effector, immune checkpoint, or macrophage molecule. Low-programmed death-ligand 2 (PD-L2) was the most sensitive/specific marker to segregate patients with adverse outcomes; therefore, PD-L2 expression was chosen to distinguish immune infiltration (ie, high PD-L2) FL biopsies from immune infiltration (ie, low PD-L2) tumors. Immune infiltration tissues were highly infiltrated with macrophages and expanded populations of T-cell clones. Of note, the immune infiltration subset of patients with FL was enriched for POD24 events (odds ratio [OR], 4.32; c-statistic, 0.81; P = .001), validated in the independent cohorts (rituximab plus cyclophosphamide, vincristine, prednisone; OR, 2.95; c-statistic, 0.75; P = .011; and rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone), with the latter selected to permit comparison of patients experiencing a POD24 event with those having no progression at 5 years or more.

CONCLUSION Assessment of immune-infiltration by PD-L2 expression is a promising tool with which to help identify patients who are at risk for POD24.

INTRODUCTION Follicular lymphoma (FL) is the most common indolent non-Hodgkin lymphoma. FL is typified by a prolonged, relapsing-remitting course, and median overall survival may extend beyond 18 years. The FL International Prognostic Index (FLIPI) is useful for comparing outcomes in clinical trials, but fails to specifically predict the 15% to 30% of patients who experience an early relapse, which is associated with shorter survival. An accepted measure of high-risk patients who experience an aggressive course is defined by progression of disease within 24 months of diagnosis (POD24). POD24 has predicted overall survival of 26% to 50% by 5 years. A pooled analysis of more than 5,000 patients from 13 randomized trials confirmed POD24 as an early clinical end point of poor survival in FL. A pretherapy clinicogenetic risk model was specifically designed to sensitively predict POD24, termed the POD24 prognostic index (POD24-PI), using a modified definition of POD24 for which the risk-defining event was POD24 after first-line treatment initiation. POD24-PI remains untested outside of the originally published populations, and there are unanswered questions regarding the underlying tumor microenvironment (TME) in those who are at high risk of a POD24 event. Better understanding...
of the immunobiologic factors that constitute high-risk FL at
diagnosis has been identified as a top priority.9

The Immune Survival Score (ISS) demonstrated differ-
tential expression of specific gene expression signatures
that represent intratumoral immune cells in patients with
good or poor outcomes.10 A notable feature of that study
was that it included patients with early- and advanced-
stage FL, some of whom were initially treated with ob-
servation alone. The study population therefore reflected
the clinical heterogeneity of FL and, hence, was more
likely to represent the breadth of the intratumoral immune
response. Although influential, this 2004 study was before
the rituximab era, did not test for POD24, and did not take
into consideration potentially actionable immune mole-
cules, such as those involved in the programmed cell
death 1 (PD-1) axis.

In some solid tumors, low levels of immune infiltration—
for example, as measured by T-cell and myeloid cell
infiltration—convey inferior outcomes to conventional
therapy compared with high immune infiltration (im-
mune infiltration11,12). Furthermore, clinical responses
to PD-1 axis blockade occur most often in patients with
an immune infiltration13 intratumoral immunophenotype.14
This suggests that PD-1 axis blockade reverses the local
immunosuppression that has developed as an adaption to
counteract antitumor immunity within the TME.15 The bi-
ologic importance of the TME in FL pathogenesis is well
established;16-19, however, characterization of FL into im-
mune infiltration versus immune infiltration10 phenotypes
and the relationship between immune infiltration and POD24
has not been previously investigated.

PATIENTS AND METHODS
Patient Populations

Patient characteristics are described in the Data Supple-
ment. The discovery cohort (n = 132 patients) was iden-
tified from a prospectively maintained clinical lymphoma
database (Data Supplement) containing patients at the
Princess Alexandra Hospital (PAH) and included patients
with early- and advanced-stage disease. The latter received
treatment or observation as per published criteria.20,21
PAH is a metropolitan hospital with a catchment of ap-
proximately one million. All 198 consecutive patients with
available tissue and a new diagnosis of FL between January
2001 and December 2015 were assessed for eligi-
bility. Eligible patients were age 18 years or older with newly
diagnosed histologic grade I to IIa FL with available formalin-
fixed paraffin-embedded tissue from a pretreatment di-
agnostic biopsy. Median follow-up was 6.67 years.

The British Columbia Cancer Agency (BCCA) cohort con-
stisted of 138 patients with FL from a population-based
registry diagnosed between 2004 and 2009 with symp-
tomatic advanced-stage grade FL I to IIa that required
treatment with rituximab plus cyclophosphamide, vincristine,
and prednisolone immunochemotherapy.22 The second
validation cohort consisted of 45 patients with symptomatic
advanced-stage grade FL I to IIa treated with rituximab
plus cyclophosphamide, vincristine, doxorubicin, and
prednisolone from the German Low Grade Lymphoma
Study Group 2000 (GLSG2000) trial.23 These patients were
recruited between 2000 and 2010 and were specifically
selected, including the availability of tissue, to permit
a comparison of the 29 patients who experienced a POD24
event with 16 who experienced no progression within
5 years.23 Validation cohorts were included in the original
POD24-PI description.8 This study was approved by the
relevant institutional regulatory boards in concordance with
the Declaration of Helsinki.

Sequencing, Gene Expression, and Multispectral
Immunofluorescence Imaging

A targeted sequencing panel of 11 genes was used to
identify FL-relevant mutations.22,24,25 Gene expression used
a NanoString custom code set of 12 clinically pertinent
immune effector (CD137, CD4, CD7, CD8A, TNFα), immune
checkpoint (PD-1, PD-L1, PD-L2, TIM3, LAG3, FOXP3), and
macrophage (CD68) molecules (Data Supplement).26,27
We performed high-throughput T-cell receptor (TCR) rep-
ertoire sequencing as published (Adaptive Biotechnologies,
Seattle, WA).28 Multispectral immunofluorescence (MIF)
used the Opal Multiplex Assay (PerkinElmer, Waltham, MA)
on a tissue microarray.29 The Data Supplement provides
additional details.

Statistics

In the discovery cohort, POD24 is defined as primary-
refractory disease (less than partial response), progres-
sion, transformation, or relapse within 24 months after
diagnosis. Deaths not a result of FL were excluded. In the
validation cohorts, time was from the initiation of therapy.
There were 19 individuals from the original GLSG2000
cohort who were not evaluable for POD24 that were ex-
cluded from the analysis (six deceased and 13 lost to follow-
up). Categorical data were compared with Fisher’s exact or
χ² test, when appropriate, and continuous data using two-
tailed paired t tests. POD24-PI was calculated as originally
described.8 We performed hierarchical clustering with
Euclidean distances and visualized using R statistical
software (https://www.r-project.org/). Additional details on
statistical methods are provided in the Data Supplement.

RESULTS

Immune Infiltration Shows Distinct Clustering in
FL Samples

A heat map was made using nonhierarchical clustering of
immune effector, immune checkpoint, and macrophage
gene expression in the discovery cohort (Fig 1). This
showed distinct clustering of FL samples, which was
characterized by either high or low molecule expression,
irrespective of their designation as an immune effector,
immune checkpoint, or macrophage marker. We then assessed individual genes from the targeted immune panel to dichotomize patients into high- and low-risk subsets for adverse outcomes—defined as progression, relapse, transformation, or death from any cause—over the 6.5-year period of follow-up for each immune gene in the discovery cohort. Use of this continuous variable enabled the identification of a binary cutoff. We applied a regressive partitioning model (Data Supplement).27 The optimum cutoff point was calculated for each marker individually. Four immune markers—PD-L2, TNFα, CD4, and CD68—had significant hazard ratios ($P < .05$; Table 1) using unadjusted $P$ values. Of note, all four biomarkers, regardless of their designation as immune effectors (TNFα, CD4), checkpoints (PD-L2), or macrophage markers (CD68), were associated with a poor outcome in the group with low expression. After adjustment for multiple genes, two markers—the immune checkpoint PD-L2 and the immune effector TNFα—had significant corrected $P$ values. The most significant adverse immune marker, also showing the best specificity and sensitivity, was PD-L2 (cutoff normalized count, 139.0; median count, 237; 25th to 75th percentile, 128 to 348).

**Tissues With High Levels of Immune Infiltration Have a Distinct Immunobiology**

We chose PD-L2 gene expression to compare the immunobiology of tissues that were dichotomized into immune infiltration$^{44}$ and immune infiltration$^{15}$ nodes. PD-L2 is an immune checkpoint molecule that shows broad dynamic range and that we expected to be present within malignant and nonmalignant cells.29 To confirm this, we quantified the relative distribution of PD-L2 molecules between B cells and non–B cells in seven fresh deaggregated FL diagnostic nodes. CD20+ and non-CD20+ cells were sorted using fluorescence-activated cell sorting and PD-L2 was quantified using quantitative polymerase chain reaction. This showed that PD-L2 gene expression was distributed in both cell populations, but that the proportion of PD-L2 was higher in non-CD20+ cells relative to CD20+ cells within the node ($P = .015$; Fig 2A). Using the PD-L2 cutoff as defined by the partitioning model previously, we observed

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**FIG 1.** Unsupervised hierarchical clustering identifies immune infiltration$^{44}$ and immune infiltration$^{15}$ follicular lymphoma (FL) tissues. Immune effector, immune checkpoint, and macrophage gene expression in the FL tissue discovery cohort (n = 132) was digitally quantified by NanoString. Green denotes low and red indicates high gene expression. Genes were categorized as follows: immune effector (CD137, CD4, CD7, CD8A, TNFα); immune checkpoint (PD-1, PD-L1, PD-L2, TIM3, LAG3, FOXP3); and macrophage (CD68) molecules. POD, progression of disease; POD24, progression of disease within 24 months.
significantly lower expression of immune effector, immune checkpoint, and macrophage molecules in the immune infiltration\(^{10}\) phenotype compared with immune infiltration\(^{11}\) (Fig 2B). In contrast, housekeeper genes did not show this, which indicates that clustering was not reflecting tissue RNA quality or quantity. To compare the expression of a range of immune molecules across tissues, we stratified FL tissues by the top and bottom quartiles of PD-L2 gene expression. This highlighted that, in these tissues, immune molecule expression correlated with each other, with clear grouping of tissues into those expressions that were either immune infiltration\(^{10}\) or immune infiltration\(^{10}\) (Fig 2C). To establish whether this was also true of T-cell protein expression, we analyzed flow cytometry that was performed on the diagnostic biopsy. This demonstrated a higher proportion of CD3\(^+\) T cells (mean, 41% v 30%; \(P = .016\)) in immune infiltration\(^{11}\) versus immune infiltration\(^{10}\) tumors (Fig 2D).

**TME Contains Abundant Clonally Expanded T-Cell Populations and PD-L1—Expressing Macrophages in Immune Infiltration\(^{11}\) FL Tissues**

We performed TCR repertoire sequencing on FL tissues (Fig 3A). This demonstrated an increase in productive clonality—a measure of repertoire unevenness as a result of clonal expansions—among the immune infiltration\(^{11}\) tissue subset compared with the immune infiltration\(^{10}\) subset (0.0065 v 0.0032; \(P = .037\)). Next, to examine protein expression in situ, we performed MIF using representative markers of immune effectors, immune checkpoints, and macrophages on a separate cohort of 21 FL tissue microarray tissues (Ochsner Health System, New Orleans, LA). Samples were stratified by PD-L2 NanoString gene expression, and samples with the highest quartile (n = 6; 6 of 6 immune infiltration\(^{11}\)) and lowest quartile (n = 5; 5 of 5 immune infiltration\(^{10}\)) of PD-L2 were stained for expression of CD8 and PD-L1. Quantitative analysis used a computer-learning algorithm (Data Supplement). These analyses (Figs 3B and 3C) demonstrated differences between immune infiltration\(^{11}\) and immune infiltration\(^{10}\) tumors in the density of CD8 and PD-L1 (both \(P < .02\)). CD68 was used to localize macrophages. A high proportion of PD-L1 staining (percent total fluorescence units) was on CD68+ macrophages (median, 31.5%; range, 15.7% to 55.7%) and this was higher in immune infiltration\(^{11}\) versus immune infiltration\(^{10}\) tumors (Fig 3D; 41.7% v 22.8%; \(P = .032\)).

**Immune Infiltration and POD24**

POD24 occurred in 24.4% of patients in the PAH cohort and was a powerful predictor of 5-year mortality (55.3% v 90.8%; hazard ratio, 0.24; \(P < .001\)). POD24 rarely occurred in those with localized disease compared with those with advanced-stage disease (8.1% v 28.7%; \(P = .009\)). Using PD-L2 level as defined by the partitioning model previously as a marker of immune infiltration, levels were compared between POD24+/−ve groupings. This showed that immune infiltration\(^{10}\) FL tissues were enriched in POD24 events (Fig 4A). Findings were consistent in the subset of 79 patients with symptomatic advanced-stage FL (Fig 4B and Data Supplement). To validate these findings, we compared results with two independent populations—the BCCA and GLSG2000 cohorts—using regression partitioning (Fig 4A, 4B, and Data Supplement). For patients in the discovery cohort, 45.7% with low PD-L2 had POD24 versus 16.3% with high PD-L2 (odds ratio [OR], 4.32; 95% CI, 1.81 to 9.67; c-statistic, 0.81; \(P = .001\)).
the BCCA validation cohort, POD24 was observed in 46.7% of low PD-L2 versus 24.0% of high PD-L2 (OR, 2.95; 95% CI, 1.23 to 6.97; c-statistic, 0.75; \( P = .011 \)), and for the GLSG2000 cohort, values for PD-L2 low and high were 54.2% versus 14.3% (OR, 7.09; 95% CI, 1.77 to 26.27; c-statistic, 0.88; \( P = .011 \)), respectively. The GLSG2000 validation cohort was specifically restricted to high-risk patients—that is, a POD24 event had occurred—and low-risk patients—no POD event within 5 years. Taken together, the results are consistent with low PD-L2 identifying a subset of patients with FL who are enriched for POD24.

PD-L2 expression was not significantly different between early- versus advanced-stage disease (\( P = .56 \)). POD24 events were rare in early-stage FL (two of 27) and there was no difference in POD24 events between high and low PD-L2 subsets (\( P = .34 \); OR, 5.25; 95% CI, 0.22 to 103.3). We next tested the proportion of POD24 events occurring in the PAH discovery cohort stratified by FLIPI and ISS (Data Supplement). This showed that patients with high-risk FLIPI (score, 3 to 5) were enriched in POD24 events (33.9% with high-risk FLIPI v 15.8% with low-risk FLIPI; OR, 3.1; 95% CI, 1.5 to 6.19; \( P = .0033 \)), whereas no significant enrichment was observed by ISS (OR, 2.3; 95% CI, 0.93 to 5.37; \( P = .068 \)). The overlap between low PD-L2 and both high-risk FLIPI and high-risk ISS was relatively modest (Data Supplement). We next constructed
integrated prognostic models (Data Supplement). This demonstrated that combining immune infiltration with FLIPI or ISS led to a modest increase in specificity—for example, compared with immune infiltration alone, the specificity of a combined FLIPI–PD-L2 score to correctly identify POD24 increased from 83.7% to 90%.

Mutational Profile Is Similar Between Immune Infiltration$^{\text{Hi}}$ and Immune Infiltration$^{\text{Lo}}$ Tissues

To investigate potential associations between immune infiltration$^{\text{Hi}}$ and immune infiltration$^{\text{Lo}}$ phenotypes across a range of relevant genetic aberrations, we compared the proportions of mutations in BCL2, KMT2D, EZH2, ARID1A, MEF2B, TNFRSF14, EP300, TP53, FOXO1, CREBBP, and CARD11 in immune infiltration$^{\text{Hi}}$ and immune infiltration$^{\text{Lo}}$ nodes. Mutations were detected in equal proportions (Fig 5), which was consistent with the mutational profile not influencing the presence of immune infiltration$^{\text{Hi}}$ or immune infiltration$^{\text{Lo}}$ FL phenotypes.

DISCUSSION

The host intratumoral immune response is critical to the pathogenesis and outcome of FL.\textsuperscript{10,16-19,30} We have found that expression of immune checkpoint and immune effector molecules showed distinct clustering of FL samples, characterized by either high or low immune infiltration, regardless of their categorization as an immune effector,
immune checkpoint, or macrophage molecule. Low PD-L2 was the most sensitive/specific immune molecule to segregate patients into those with or without a combined end point of progression, relapse, transformation, or death. Therefore, high PD-L2 expression was chosen to distinguish immune infiltrationHI from low PD-L2 expressing immune infiltrationLO FL tumors. Using a variety of techniques, immune infiltrationHI hot and immune infiltrationLO cold phenotypes were demonstrated to have a distinct underlying immunobiology, particularly with regard to infiltration by expanded populations of clonal T cells and PD-L1–expressing macrophages. The outcome of FL remains highly heterogeneous, and there is increasing emphasis on early predictors of outcomes, such as POD24. There is limited understanding of the relationship between the intratumoral immune microenvironment and POD24 in FL. Using a discovery/validation approach, we demonstrated that low PD-L2 expression, as a marker of low immune infiltration, is enriched in early events—that is, related to POD24—and is indicative of a more aggressive immunobiology. Of interest, the immune infiltrationLO subset of patients with FL was markedly more enriched for POD24 compared with patients with high-risk FLIPI. There was only modest overlap between immune infiltrationLO FL and high-risk FLIPI groupings, indicating that PD-L2 expression captures a different subgroup of patients.

We have previously shown that the TCR repertoire is related to prognosis in diffuse large B-cell lymphoma.28 Of interest, in the current study, T cells within immune infiltrationLO cases are enriched in progression of disease within 24 months (POD24) events. The proportion of POD24 events occurring in the (A) Princess Alexandra Hospital (PAH) discovery cohort (B) patients in the PAH discovery cohort with active treatment, advanced stage (C) British Columbia Cancer Agency (BCCA) validation cohort and (D) German Low Grade Lymphoma Study Group 2000 (GLSG2000) validation cohort were stratified by the degree of immune infiltration. OR, odds ratio.

FIG 4. Distribution of gene mutations in immune infiltrationHI and immune infiltrationLO follicular lymphoma lymph nodes. Relative frequency of FL relevant mutations stratified by immune infiltrationHI and immune infiltrationLO gene expression. No significant differences were seen for any mutation.

FIG 5. Low immune infiltration cases are enriched in progression of disease within 24 months (POD24) events. The proportion of POD24 events occurring in the (A) Princess Alexandra Hospital (PAH) discovery cohort (B) patients in the PAH discovery cohort with active treatment, advanced stage (C) British Columbia Cancer Agency (BCCA) validation cohort and (D) German Low Grade Lymphoma Study Group 2000 (GLSG2000) validation cohort were stratified by the degree of immune infiltration. OR, odds ratio.
Intratumoral Immune Infiltration and POD24 in FL

tumors exhibited decreased TCR clonality. One explanation is that this may reflect reduced T-cell clonal expansion in response to reduced neoantigen production or an impaired ability to present antigen; however, this remains to be formally tested. Additional studies are required to compare the TCR repertoire with tumor mutational burden—for example, by whole-genome sequencing—and to identify the antigen specificity of individual expanded T-cell clones to better assess the mechanistic implications of the T-cell expansions in FL.

In the discovery cohort (early- and advanced-stage disease), the POD24 risk defining event was calculated as time since diagnosis, as per the original definition. Of importance, findings in the discovery cohort were validated in advanced-stage patients who were treated in a uniform manner (rituximab plus cyclophosphamide, vincristine, and prednisolone for BCCA; and rituximab plus cyclophosphamide, vincristine, doxorubicin, and prednisolone for GLSG2000) in whom the POD24 risk defining event was calculated as time since initiation of therapy. Taken together, immune infiltration seems to apply to the original and modified definitions of POD24.

Our data indicate that patients with biopsies showing reduced immune infiltration are more likely to experience POD24 events. Although specificity was high, this was at the expense of sensitivity, which indicates that immune infiltration fails to capture a subset of patients who will develop POD24. This makes it a valuable predictor in certain clinical situations—for example, when testing poorly tolerated intensive regimens—but with the proviso that some patients who might potentially benefit from treatment intensification will be missed. Additional improvements, including the integration of additional biomarkers, are needed to capture these patients. Furthermore, POD24 only reflects events that occur over a specific time period. Clinicogenetic scores that reflect a different aspect of tumor biology can predict the risk of progression after induction immunotherapy, which (as well as POD24) is a relevant end point regarding treatment efficacy and overall survival. Of note, the frequency of mutations in genes that are known to be commonly mutated in FL was no different between immune infiltration and immune infiltration tissues, indicating that the degree of immune infiltration is capturing aspects of FL biology that are distinct from its mutational profile. Huet et al identified a 23-gene signature characteristic of B-cell centroblasts that was prognostic independently of FLIPI which underlines that, in addition to the TME, tumor B-cell biology contributes to the clinical aggressiveness of FL. Frontline treatment options for FL are expanding, hence additional refinements in the dichotomization of immune infiltration, along with combination with other clinicogenetic and gene expression tissue markers, as well as cell-free circulating DNA and functional imaging, should be prioritized as part of collaborative efforts to develop integrated pretherapy models that sensitively and accurately predict both POD24 and risk of relapse/progression in FL so as to maximize clinical utility.

Until now, studies that characterize the degree of immune infiltration have been largely restricted to solid cancers. Those with immune infiltration tumors have better outcomes with conventional therapy and checkpoint blockade. Of interest, similar to our findings in FL, low PD-L2 expression is superior to PD-L1 as a measure of immune infiltration that has been demonstrated to be adversely associated with outcomes in metastatic melanoma treated with conventional—that is, non–checkpoint blockade—therapy. Furthermore, studies in melanoma and other solid cancer subtypes have demonstrated that PD-L2 is a marker of adaptive immune resistance that outperforms PD-L1 as a measure of antitumor immunity and interferon gamma signaling. We have recently demonstrated that PD-L1 and PD-L2 are expressed on macrophages in Hodgkin lymphoma and diffuse large B-cell lymphoma, where they are functionally active in impairing antilymphoma effector immunity. In the current study, using a combination of gene expression, flow cytometry, and MIF, we show that in FL—a setting in which gene amplification of PD-1 ligands rarely occurs—both PD-L1 and PD-L2 are predominantly expressed in nonmalignant cells. Although checkpoint ligands seem to be an informative strategy by which to stratify immune infiltration, the findings of this study should be seen as a reflection of the importance of the degree of immune infiltration to the immunobiology of FL, rather than as a definitive statement of the importance of a particular immune biomarker or a particular technique—over another to characterize immune infiltration in FL tissues. Choice of PD-L2 gene expression was determined on the basis of disease outcome rather than immune parameters. Additional studies are required to explore the immunobiology of FL in more depth, and it is necessary also to validate approaches that are more accessible than PD-L2 gene expression that might be applicable to the diagnostic laboratory, such as immunohistochemistry.

The majority of patients with FL do not experience a response to checkpoint blockade. It remains to be tested whether segregating FL by immune infiltration helps predict immunotherapy responsiveness, and whether conversion of immune infiltration into immune infiltration tumors sensitizes FL to immunotherapies as proposed in solid tumors. Understanding the differential mechanisms of resistance that are operative in immune infiltration and immune infiltration FL will be critical.

In summary, we demonstrate that FL can be characterized into immune infiltration or immune infiltration immunophenotypes. Assessment of immune infiltration seems to be a promising tool with which to help identify patients who are at risk for POD24.
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