Identification of a Cytokine Biomarker for Prognostic Modeling of Breast Cancer-Related Lymphedema

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Running Title: IFN-α2A as a breast cancer-related lymphedema biomarker

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Authors' Disclosures of Potential Conflicts of Interest

The authors have no competing interests to disclose.

Abstract

Lymphedema is a chronic complication of breast cancer treatment, and early intervention is crucial to reduce morbidity. This study evaluated the role of blood-based cytokine biomarkers in the prognostication of breast cancer-related lymphedema (BCRL) to improve risk prediction. A secondary analysis of inflammatory biomarkers for BCRL was performed using a previously published cohort of 147 breast cancer patients who had undergone serum cytokine profiling during their treatment at the Princess Margaret Cancer Centre from 2010-2014. Prognostic cytokine variables for lymphedema were selected by regression analysis and independence from known clinical risk factors. Regression-based modeling was employed to integrate prognostic variables for the prediction of lymphedema occurrence. We identified the immunostimulatory cytokine IFN-α2A as a potential biomarker for lymphedema development (OR 3.10, 95% CI 1.05-9.51, p=0.042), independent from known clinical risk factors. Furthermore, Kaplan-Meier analysis demonstrated 3-year lymphedema-free survival of 95% (90-100%) vs. 85% (77-94%) for below vs. above median concentrations of IFN-α2A (p=0.026). In combination with an established clinical risk regression-based model, patients identified as high risk based on clinical factors alone were able to be correctly reclassified as low risk by IFN-α2A in 31% (8/26) of cases. Our combined logistic regression model using both IFN-α2A and clinical risk score achieved an AUC of 0.895 (95% CI: 0.796 - 0.971) and Brier Score of 0.101 (95% CI: 0.061 - 0.149), representing a favourable improvement compared to the logistic regression model using clinical risk factors alone. IFN-α2A in combination with established clinical risk factors may be useful for improving BCRL prognostication.

Significance

The cytokine IFN-α2A was identified as a potentially complementary biomarker to improve the stratification of high- and low-risk patients for breast cancer-related lymphedema. This will help

enable earlier intervention to reduce long-term morbidity for those at high risk for lymphedema while minimizing burdensome interventions for those at low risk.

Introduction

Lymphedema is a debilitating, incurable complication of breast cancer treatment, characterized by progressive swelling of the arm, shoulder, neck, trunk, or breast. It typically occurs after radiation therapy or axillary surgical interventions,[1] during which nearby lymph nodes are damaged or removed. This leads to impairment of lymphatic drainage[2] and an accumulation of extracellular fluid in surrounding tissues,[3] resulting in an enlargement in limb circumference, chronic inflammation,[4] tissue fibrosis, and abnormal adipocyte proliferation.[5]

This lymphatic dysfunction and related pathological changes pose a substantial burden on many patients; indeed, the median incidence of breast cancer-related lymphedema (BCRL) within 3 years of treatment is estimated to be 20%, with 10% to 64% of such individuals reporting upper body symptoms.[6] In particular, patients of older age, those who identify as Black or Hispanic, and those who received neoadjuvant chemotherapy have been shown to be at greater risk of BCRL.[7] Patients with BCRL often suffer from a spectrum of physical, medical, and psychosocial consequences that greatly affect their quality of life and day-to-day functioning. Potential issues include recurrent soft tissue infections, discomfort, disability, and psychological distress related to body image and social relationships. Although there are conservative physical strategies and emerging surgical procedures that can manage BCRL, there is currently no cure. Maintenance treatment for BCRL — which is often burdensome and time-consuming — must be continued on a lifelong basis.[8]

With the onset and severity of BCRL varying widely amongst patients,[9] there is an increasing interest in identifying biomarkers to predict the risk of developing this condition. Inflammatory cytokines in particular show considerable promise as prognostic biomarkers, given that they are

involved in numerous molecular mechanisms associated with the pathophysiology of BCRL. Current evidence shows that the early phase of acute inflammation caused by radiotherapy or surgery results in the activation and infiltration of immune cells including CD8+ T cells, T-helper cells, dendritic cells, macrophages, and neutrophils. This occurs alongside a local accumulation of interstitial fluid; eventually, the inability of the lymphatic system to accommodate this fluid overload incites a cascade of chronic inflammatory events.[10] Such events include the impaired transit and excessive accumulation of activated inflammatory/immune cells as well as the release of cytokines within the edematous environment,[11] leading to chronic changes that irreversibly transform surrounding tissues through fibrosis and excess adipose deposition.[12] These late effects caused by an inflammatory cascade mean that lymphedema can be diagnosed months or even years after treatment.

Several recent studies have investigated the presence and potential roles of specific cytokines in lymphedematous tissues from murine models or human samples. In a study using a specific bioassay to conduct transcriptional profiling of human skin, numerous cytokine genes including *IL4*, *IL6*, *IL10*, and *IL13* were found to be upregulated in lymphedema samples.[13] In another study of patients who underwent breast cancer surgery, common variants in the cytokine genes *IL4*, *IL10*, and *NFKB2* were significantly associated with BCRL.[14] Furthermore, in a mouse-tail model of lymphedema, the inhibition of Th2 cytokines IL-4 and IL-13 prevented the T-cell inflammatory response, which led to decreased fibrosis and improved lymphatic function.[15] In addition to IL-4 and IL-13, the cytokine TGF-β1 has also been shown to be a key promoter of fibrotic remodelling and collagen deposition in secondary lymphedema.[16–18] Other cytokines such as G-CSF, GM-CSF, IFN-α, IL-10, IL-12p40, IL-15, IL-17A, IL-1β, IL-2, IL-3, IL-6, and MIP-1β have been found to be elevated prior to cancer treatment in patients who later developed BCRL at 12 months post-radiation therapy compared to those who did not develop BCRL.[19, 20] Together, these findings

strongly suggest that differences in cytokine expression play an important role in the development of secondary lymphedema.

Previously, we developed a multivariable linear regression model that used age, body mass index (BMI), mammographic breast density, number of pathological lymph nodes, and axillary lymph node dissection (ALND) to predict the development of BCRL.[21] In this current study, we further investigated the association of 17 baseline serum cytokines with the occurrence of BCRL in a previously reported cohort of mainly early-stage breast cancer patients prospectively evaluated for cytokine levels and the development of fatigue during radiation therapy.[22] We sought to identify the most important cytokine biomarkers amongst these 17 that can predict lymphedema occurrence independently of clinical factors, and in doing so, we aimed to develop more accurate models for personalized prognostication of BCRL. To our knowledge, this is the first study to integrate a cytokine biomarker with a lymphedema risk prediction model. These models have the potential to be used by healthcare professionals to more accurately predict BCRL risk for patients prior to radiation therapy, and enable clinicians to implement early interventions in recognition of lymphedema risk. Thus, our work will help enable better prognostication of BCRL for early treatment and prevention when necessary, and contribute to a growing body of knowledge surrounding biological markers and immune-targeted therapies for secondary lymphedema.

Materials and Methods

Research Ethics

This secondary analysis was approved by the Research Ethics Board of the University Health Network. There was no patient or public involvement in the design, conduct, reporting, interpretation, or dissemination of this study. The IFN-α2 dataset from the University of Texas MD Anderson Cancer Centre was registered with ClinicalTrials.gov (NCT number: 02949726).

Study Population

We performed a secondary analysis of a previously reported prospective cohort study on inflammatory biomarkers in breast cancer patients undergoing adjuvant radiation therapy in a tertiary care setting;[22] a subset of this cohort has also been analyzed for non-cytokine biomarkers of lymphedema.[23] A total of 152 breast cancer patients were recruited to this longitudinal cohort study between February 2010 to July 2014 at the Princess Margaret Cancer Centre, University Health Network in Toronto, Ontario, Canada.[22] All study participants underwent primary tumour resection and radiation therapy (with or without chemotherapy), were aged 18 or older, and had either ductal carcinoma in situ, invasive ductal carcinoma, or lobular carcinoma confirmed by histology.[22] Patients with hematologic conditions or previous bone marrow transplant were excluded,[22] to avoid confounding from underlying immune dysregulation and chronic inflammation which may impact the results of cytokine profiling. The sample size was predetermined based on the prior prospectively recruited cohort. Findings were subsequently compared to an independent dataset.

Demographic data and clinical characteristics for all participants were collected at enrolment from electronic health records, illustrating a population representative of mainly early-stage breast cancer patients within a Canadian academic hospital setting. Data included age, body mass index (BMI), tumour characteristics (such as tumour and nodal staging), as well as cancer treatment characteristics. For this study, we performed a secondary review of lymphedema-associated risk factors including mammographic breast density assessed with the Breast Imaging-Reporting and Data System (BI-RADS) as well as lymphedema outcomes. The range of patient follow-up time was between 0.2 to 11.8 years from the date of cancer surgery. Data collection was completed and independently reviewed by WS, MSH, and JK.

Lymphedema Measurement

Lymphedema was clinically diagnosed by a medical provider and quantified as an increase in arm volume from baseline in the affected arm compared to the contralateral unaffected arm. Circumferential arm measurements were conducted with a standardized protocol, with measurements conducted at 7 standard points. Volume was calculated based on these measurements and recorded as both absolute (millilitres) and percent (%) increase. Measurements were routinely recorded by trained physiotherapists and occupational therapists using standard dictation templates and collected for this analysis.

Serum Cytokine Quantification

Serum samples were collected via phlebotomy from 147 of the 152 patients. Phlebotomies were conducted within the same 4-hour period for each patient to reduce the effect of diurnal variations.[24] Baseline (pre-radiotherapy) cytokine levels were selected for analysis in this study.

As previously reported, the levels of 17 cytokines were measured to evaluate their association with fatigue,[22] including CRP, interferon-gamma (IFN-γ), interferon-alpha 2a (IFN-α2A), interleukin-10 (IL-10), interleukin-17A (IL-17A), IL-1b, interleukin-1 receptor antagonist (IL-1RA), interleukin-4 (IL-4), interleukin-6 (IL-6), interferon c-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinases-2 (MMP-2), matrix metalloproteinases-9 (MMP-9), SDF-1α, transforming growth factor beta 1 (TGF-β1), tumor necrosis factor receptor II (TNF-RII), and TNF-α (Table S1). Cytokine levels were measured using custom multiplexed electrochemiluminescence immunoassays (Meso Scale Discovery, Gaithersburg, MD) and recorded as concentrations (pg/mL). The protocol was described by Shi et al.[22] As previously described in the literature, many of these cytokines have also been shown to impact lymphatic function, and therefore we selected them as possible predictors for lymphedema risk modelling in this study.

Biostatistical Analysis & Model Development

Five participants were excluded from our dataset of 152 patients given that they lacked baseline serum cytokine measurements, yielding a final sample size of 147 patients. Amongst these 147 patients, 24 were lacking BMI data and 38 were lacking mammographic breast density data. These missing data were substituted by mean imputation (i.e. mean BMI of 26.5 kg/m^2 and mean BI-RADS score of 2.5 were imputed for missing values).

Data analyses were performed using SAS, version 9.4 by SAS Institute Inc (RRID: SCR_008567) and R, version 4.4.1 by The R Foundation for Statistical Computing. Descriptive statistics were performed on this dataset. Frequency (percentage) is provided for categorical variables while mean (SD), median (Q1, Q3) and range (min, max) are presented for continuous variables.

Non-parametric Wilcoxon Rank-Sum testing was applied to determine the associations between candidate cytokines and established clinical risk factors, defined as per our prior study.[21] Cytokines without significant association with the clinical risk assessment ($p>0.05$) were selected for univariate logistic regression. A Kaplan-Meier curve of 3-year lymphedema-free survival with a log-rank test p-value was generated using a median cytokine concentration threshold. Net reclassification improvement was used to assess the performance of our integrated two-step clinical risk model.

Multivariable modeling was performed using logistic regression, yielding binary outcomes for lymphedema presence (i.e., lymphedema present or absent). Variables assessed included the 5-factor clinical risk model score[21] and IFN-α2A. The five factors in the clinical risk score were age, BMI, mammographic breast density, number of pathological lymph nodes, and ALND, as shown in the equation below from our previous study:[21]

$$\text{Lymphedema volume} = -329 + [4 \times \text{Age}] + [10 \times \text{BMI}] - [37 \times \text{Mammographic Breast Density}] + [13 \times \text{No. of Pathological Lymph Nodes}] + [99 \times \text{ALND Treatment Use}]$$

We calculated the 5-factor clinical risk scores for each study participant, which were dichotomized as a high risk (score > 200) or low risk (score ≤ 200) based on the prior study.[21,23] IFN-α2A was dichotomized by median threshold. We then utilized one iteration of 5-fold cross-validation to train and evaluate performance for prediction of lymphedema occurrence. The multivariable logistic regression model was developed from the scikit-learn library (RRID: SCR_002577) in Python. Training and performance evaluation using 5-fold cross-validation were completed. Performance metrics (e.g. area under the receiver operating characteristic curve (AUROC), sensitivity, specificity, Brier score) were calculated and used to plot calibration and ROC curves with the Matplotlib library (RRID: SCR_008624). Non-parametric 95% confidence intervals for all metrics were determined with bootstrapping, involving 1000 iterations of resampling with replacement.

IFN-α2 Comparison Dataset

We performed a secondary analysis of an independent breast cancer patient cohort from the University of Texas MD Anderson Cancer Centre. This cohort has been previously described including its eligibility criteria.[20] A total of 62 study subjects with pre-radiotherapy measurements of plasma IFN-α2 were eligible for this analysis. The plasma IFN-α2 levels at this timepoint have not been previously published. Plasma IFN-α2 was quantified using the Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel 96-well plate assay (catalog #: HCYTA-60K, Millipore Sigma, St. Louis, MI, USA) as per manufacturer's protocol. Subjects were categorized by their lymphedema status at 18 months after cancer surgery. A two-sided Mann Whitney test was used to evaluate the difference between subjects who did or did not develop lymphedema; $P<0.05$ was selected as the significance level.

Data Availability

The computer code is available on GitHub.

Anonymized data may be shared with other researchers on request by contacting the corresponding author. Requesters will be required to sign a data sharing agreement.

Results

Patient Characteristics

The clinical characteristics of these 147 patients have already been published,[22] including BMI, mammographic breast density, and presence of lymphedema for a sub-group of the study population (Table 1).[23] The mean age of the study population was 56.1 years, with the mean BMI being 26.5 kg/m^2. The distribution of mammographic breast densities included 51% (56) with lower-density and 49% (54) with higher-density breasts. The majority (70.1%) of patients (104 of 147) were diagnosed with Tis/T1 breast cancer. The mean number of pathological nodes was 0.9, with 5.4 nodes removed on average. The majority (89.1%) of patients (131 of 147) had lumpectomy as their primary surgery, and 23.1% of patients (34 of 147) underwent axillary lymph node dissection (ALND). Hypofractionated radiation (42.4 Gy/16 fractions) was delivered to 68.7% of patients (101 of 147), while all other patients received conventional fractionated radiation (50Gy/25 fractions). Nearly half (48.2%) of the patients (71 of 147) received a radiotherapy boost. Over a third (35.4%) of patients (52 of 147) received neoadjuvant or adjuvant chemotherapy prior to radiation therapy, and 63.9% of patients (94 of 147) received adjuvant hormone therapy. Lymphedema was observed in 10.2% of patients (15 of 147). The median follow-up time was 4.3 years from the date of cancer surgery.

Cytokine Prioritization

Using our established clinical model,[21] the study population was stratified into clinically low risk (n = 121) and clinically high-risk (n= 26) groups. Wilcoxon rank-sum testing was conducted between these two groups for each of the 17 candidate cytokines, to identify those cytokines that are independent from known clinical factors (Table 2). Cytokines demonstrating significant

association with the known clinical risk groups (CRP, IL-1β, IL-1RA, IL-4, IL-6, IP-10, MCP-1, TNF-RII, and TNF-α) were identified as redundant with the clinical data and therefore eliminated from further analysis. Cytokines without significant association with the known clinical risk groups (IFN-γ, IFN-α2A, IL-10, IL-17A, MMP-2, MMP-9, SDF-1α, and TGF-β1) were classified as independent and considered for further evaluation.

Identification of Top Candidate Cytokines for Lymphedema Occurrence

The eight cytokines identified as independent from known clinical risk factors based on Wilcoxon rank-sum testing were further analyzed using univariate logistic regression for lymphedema occurrence (Table 3). Odds ratios (ORs) were calculated for each cytokine, and those with $p<0.05$ (IFN-α2A, IL-17A, and IFN-γ) were deemed to be significantly associated with lymphedema occurrence. Amongst these three cytokines, IFN-α2A had the highest OR and was therefore selected as our top candidate for analysis of 3-year lymphedema-free survival (LFS) and model development.

IFN-α2A as a Predictor for Lymphedema Occurrence

We generated a Kaplan-Meier curve to assess LFS as a function of median value of IFN-α2A levels (Fig. 1). At 3 years post-cancer surgery, 95.2% of the low IFN-α2A group were lymphedema-free, while only 85.0% of the high IFN-α2A group were lymphedema-free ($p=0.026$).

In an effort to validate the association of IFN-α2A with lymphedema, we assessed an independent cohort of 62 breast cancer patients who were treated with lumpectomy or mastectomy in addition to ALND and radiation therapy at the University of Texas MD Anderson Cancer Centre. In this cohort, 50 patients were clinically diagnosed with lymphedema, at 18 months follow-up. Similarly, plasma IFN-α2 was measured prior to radiotherapy and there was a trend towards increased IFN-α2 levels in patients who developed lymphedema, although this trend was not shown to be significant (Fig. 2).

We then added IFN-α2A to our established model, thus creating an integrated two-step clinical risk model with IFN-α2A risk confirmation. The first step involves a clinical risk assessment using the five previously identified clinical risk factors of age, BMI, mammographic breast density, number of pathological lymph nodes, and ALND, and the second step involves assessment of IFN-α2A level. We assessed the performance of this new two-step model using net reclassification improvement (Table 4). For patients assessed to be at high risk for lymphedema based on clinical factors, IFN-α2A helped correctly reclassify 8 out of 26 patients (31%) who are actually at low risk for developing lymphedema but may continue to incorrectly classify 2/26 patients (8%) as having high risk of lymphedema. For patients assessed to be low risk for developing lymphedema based on clinical factors, IFN-α2A correctly reclassified 2/121 (2%) of patients as having high risk for developing lymphedema but incorrectly reclassified 55/121 (45%) as being high risk.

Determining the Additive Predictive Value of IFN-α2A

To specifically quantify the contribution of IFN-α2A to lymphedema prediction relative to the established clinical risk score, three distinct logistic regression models with combinations of these predictors were developed. All models were trained on the full cohort (N=147) using continuous inputs of calculated 5-factor clinical risk score and IFN-α2A levels. Model A predicted lymphedema using the clinical risk score. Model B predicted lymphedema using the IFN-α2A Level. Model C predicted lymphedema using both clinical risk group and IFN-α2A level. Both continuous predictors were standardized (z-score transformed) to facilitate model convergence and comparison of coefficients. A balanced class-weight parameter was used to account for the low prevalence of lymphedema (15/147, 10.2%), thereby permitting the calculation of informative performance metrics for the minority class. Non-parametric 95% confidence intervals (CIs) were generated using 1,000 bootstrap resamples. The performance metrics for the three models are presented in Table 5.

As a univariable predictor (Model B), IFN-α2A demonstrated poor discriminatory capacity, with an AUC of 0.672 (95% CI: 0.528 - 0.806). The continuous clinical risk score (Model A) served as a very strong baseline, achieving an AUC of 0.875 (95% CI: 0.758 - 0.963).

The combined model using both IFN-α2A and clinical risk score (Model C) achieved the highest performance across all key metrics. Its AUC of 0.895 (95% CI: 0.796 - 0.971) and Brier Score of 0.101 (95% CI: 0.061 - 0.149) both represent a favourable improvement over Model A.

In threshold-dependent metrics, Model C demonstrates a higher accuracy (0.871) than Model A (0.844) and improved specificity (0.871 vs. 0.841) while maintaining the same high sensitivity (0.867).

While the clinical risk score is the dominant predictor variable, this analysis indicates that IFN-α2A may be useful to provide additive predictive utility, improving discrimination (AUC), calibration (Brier Score), and overall accuracy in a combined model.

Discussion

Differential cytokine expression is an important feature in the development of secondary lymphedema. In this study, we evaluated the association of 17 baseline serum cytokines with the occurrence of BCRL in a cohort of mainly early-stage breast cancer patients, which is the most commonly presenting breast cancer patient population in developed countries, in order to identify key cytokine biomarkers for lymphedema prediction. We observed that IFN-α2A had the greatest association with lymphedema occurrence (OR 3.10 (1.05, 9.51), p = 0.042), independent from previously established clinical risk factors. This association was evaluated in an independent cohort of breast cancer patients from another institution, and a trend was observed towards elevated baseline IFN-α2 levels in patients who went on to develop lymphedema, although the trend in this second cohort was not found to be statistically significant. By incorporating IFN-α2A

into our established BCRL model, we were able to create an integrated two-step clinical risk model with IFN-α2A risk confirmation, correctly reclassifying patients as low risk who were previously classified as high risk by clinical factors alone. Together, these results highlight the potential value of using IFN-α2A to improve BCRL prognostication.

Type I interferons include IFN-α, IFN-ω, and IFN-β, of which IFN-α2A belongs to the IFN-α subtype. Although the causal role of IFN-α2A in BCRL has not been previously investigated, past research from existing literature has shown that IFN-α inhibits lymphangiogenesis *in vitro* by suppressing lymphatic endothelial cell (LEC) growth, proliferation, and migration in a dose-dependent manner, in addition to promoting LEC apoptosis.[25] The inhibition of lymphangiogenesis has been found to induce lymphedema in mice,[26] suggesting a possible mechanism by which IFN-α contributes to the development of BCRL.

IFN-α is also known to stimulate the production of T-helper 1 (Th1) cells, which secrete the pro-inflammatory cytokines IFN-γ, IL-1β, IL-2, and TNF-α.[27] These other cytokines induced by IFN-α may also impact the progression of lymphedema through various mechanisms. The increased presence of IFN-γ, IL-2, and TNF-α contributes to the cycle of chronic inflammation observed in lymphedema.[12] In addition to its role in inflammation, IFN-γ also inhibits lymphangiogenesis in a similar manner as IFN-α, by acting on LECs.[25] IL-1β and TNF-α have been shown to dampen lymphatic pumping.[2] Linking these findings in the clinical context, IFN-α2A, IL-1β, IL-2, and TNF-α have been found to be elevated in lymphedema patients.[20, 28]

In light of the relevance of IFN-α in existing literature on lymphedema and its significance in our statistical analysis, we selected IFN-α2A as a key cytokine biomarker to incorporate into our BCRL models. By using IFN-α2A as a measure for secondary risk confirmation in our two-step model, patients who were previously identified as having high risk based on clinical factors alone were correctly reclassified as low risk by IFN-α2A in 31% (8/26) of cases. This improvement brings

forward a practical role for IFN-α2A in clarifying the risk status of those labelled as high risk based solely on clinical risk factors; patients in this group could feasibly undergo baseline bloodwork to detect IFN-α2A levels prior to their cancer treatment to avoid false positives based on clinical risk prediction alone.

However, it is important to note that IFN-α2A may incorrectly reclassify 55/121 (45%) of low-risk patients as being high-risk. Therefore, it should not be used as a marker for risk clarification in low-risk patients. The poorer performance of IFN-α2A on this low-to-high risk reclassification is likely a reflection of the outcomes of our dataset, as the majority of patients in our study population did not develop lymphedema. This is one of the limitations of our study, and it is possible that the performance and thresholding for IFN-α2A risk determination may be optimized using additional data.

A primary goal of this study was to determine if pre-treatment IFN-α2A levels, as a continuous variable, could improve lymphedema risk prediction when added to an established clinical risk score. To isolate this effect, we compared the performance of three logistic regression models: the clinical score alone (Model A), the IFN-α2A level alone (Model B), and a combined model (Model C).

The results, presented in Table 5, provide several key insights. First, as a univariable predictor, the continuous IFN-α2A level demonstrated poor discriminatory capacity (AUC 0.672 [95% CI: 0.528 - 0.806]), confirming it has little to no diagnostic value on its own. In contrast, the continuous clinical risk score served as strong baseline predictor, achieving a high AUC of 0.875 (95% CI: 0.758 - 0.963).

The central finding is that the combined model using IFN-α2A in addition to the clinical risk score yielded the best performance. The point estimates for key metrics improved relative to clinical risk score alone: Accuracy (0.871 vs. 0.844), Specificity (0.871 vs. 0.841), PPV (0.448 vs. 0.382), AUC

(0.895 vs. 0.875), and Brier Score (0.101 vs. 0.106). This consistent improvement suggests that IFN-α2A, even as a weak standalone predictor, provides complementary information that enhances the primary clinical model.

It is critical, however, to interpret these findings in the context of their statistical precision. The 95% confidence intervals for the primary metrics of the univariable and combined models show considerable overlap. This overlap indicates that while the *point estimates* indicate performance improvement, our study is statistically underpowered to declare a definitive, significant difference between the models. This imprecision is a direct mathematical consequence of the low prevalence of lymphedema (N=15) in our cohort, which results in wide confidence intervals.

This analysis has two other important limitations. First, all models were trained and evaluated on the same 147-patient cohort. Without an independent validation set, the reported performance metrics are likely optimistic and susceptible to overfitting. The true value of this analysis is not in the absolute performance of the combined model, but in the *relative improvement* observed over the univariable clinical risk score model.

Despite these limitations, this analysis provides quantitative evidence that IFN-α2A holds utility as a complementary, rather than a primary, biomarker. It supports the two-step clinical model proposed earlier, where the biomarker is not used for initial screening but rather to refine the risk assessment for patients already stratified by clinical factors. Future studies with larger, independent cohorts are essential to validate these findings and more precisely quantify the real-world clinical utility of IFN-α2A in lymphedema risk prediction.

This study presents further unique strengths and limitations. To our knowledge, it is the first study to integrate a cytokine biomarker with clinical risk factors in prognostic modelling for lymphedema. The models we developed were informed by high-quality clinical and serum data, with lymphedema outcomes, which were uniformly assessed by trained health professionals using a

standardized dictation template. Moreover, we had a large overall sample size of 147 participants comprising a broad population of mainly early-stage breast cancer patients, which is a notable strength given that the only other comparable study in the literature had a sample size of only 40 patients.[20] The 17-plex cytokine panel allowed for the assessment and comparison of multiple cytokines potentially involved in lymphedema. In particular, IFN-α2 was also identified by Vang et al.[20] as significantly elevated at the pre-surgery timepoint in breast cancer patients who went on to develop lymphedema at 12 months post-radiotherapy, which supports our results. To compare to the pre-radiotherapy findings in our study, our secondary analysis of pre-radiotherapy IFN-α2 levels in Vang et al.'s cohort also showed a trend towards elevated IFN-α2 in patients who later developed lymphedema by 18 months post-treatment. Although this association did not reach statistical significance (p=0.157), the trend nevertheless helps to corroborate our findings. Further validation of our models will be done in the future, which will allow us to further examine the performance of these models and assess their generalizability.

It is also important to acknowledge the limitations of this study, of which outcome sample size was the primary one. Consistent with the literature, only 10.2% (15/147) of our mostly early-stage breast cancer patients went on to develop lymphedema, which may limit the generalizability and predictive ability of our models. Another weakness to consider is that our lymphedema outcomes were recorded using tape measurements of arm circumferences, whereas the study by Vang et al.[20] used more advanced methods by measuring lymphedema with perometry and quantifying dermal backflow with near-infrared fluorescent lymphatic imaging. However, it is noted that tape measurements may be more accessible and translatable to clinical centres globally. Finally, there are additional cytokines that may be involved in lymphedema that were not included in the 17-plex panel, which may warrant future exploration.

In summary, our study identified IFN-α2A as a potentially complementary cytokine biomarker for lymphedema, and demonstrated the value of using this cytokine in predictive risk models for

BCRL, in conjunction with five established clinical risk factors. Notably, IFN-α2A correctly reclassified 31% (8/26) of cases as low risk which were previously classified as high risk by clinical factors alone. Identifying low risk patients in high clinical risk groups is of utmost clinical importance, given that recent clinical guidelines recommend escalation of preventive and prophylactic compression sleeve management and/or surgical management in high clinical risk groups. However, these upfront strategies can be costly and burdensome if employed on all patients identified as high risk by clinical factors alone. Our literature review additionally suggests a potential role of IFN-α2A in mediating lymphedema, upon which additional research would be necessary to further elucidate its mechanisms. The clinical variables used in our model are already routinely collected as part of breast cancer care, and baseline bloodwork for cytokine detection would be a feasible screening method to offer to clinically high-risk patients. Those confirmed to be at high risk can work with their healthcare providers to better tailor their cancer treatments in recognition of lymphedema as a likely side effect, and to enable earlier preventive management including compressive therapy, physiotherapy, and prophylactic surgical procedures such as lymphaticovenous anastomosis, while those identified as low risk by IFN-α2A can be spared these burdensome and costly interventions.

Acknowledgements

The authors thank Fasila Johri, MB BCh, BAO, BScN, from the Department of Radiation Oncology at the University of Toronto (Canada) for her support with data collection. Ms. Hossack reports grants from Princess Margaret Cancer Centre, Princess Margaret Radiation Medicine Program, Canadian Institutes of Health Research, Cancer Care Ontario, Canadian Cancer Society, GlaxoSmithKline, Elekta, Novartis and grants from Province of Ontario and University of Toronto during the conduct of the study. Dr. Shaitelman reports grants from Artidis, grants from Exact Sciences, and grants from NIH outside the submitted work. Dr. Aldrich reports grants from NIH/NCI (CA201487) during the conduct of the study. Dr. Kwan reports grants from University of Toronto Hold'em for Life Early Career Professorship. Dr. Kwan's research supported by 2024 AACR-AstraZeneca Career Development Award for Physician-Scientists, In Honor of José Baselga, Grant Number 24-20-12-KWAN. All other authors have no disclosures.

Authors' Contributions

Alison J. Wu: Writing – original draft, Writing – review & editing, Visualization. **Neil Lin:** Formal analysis, Methodology, Software, Visualization. **Jie Su:** Formal analysis, Visualization. **Cherie Lin:** Writing – review & editing. **Madison-Shira Hossack:** Investigation. **Wei Shi:** Investigation, Writing – review & editing. **Farnoosh Abbas-Aghababazadeh:** Supervision, Writing – review & editing. **Wei Xu:** Supervision. **Benjamin Haibe-Kains:** Supervision. **Simona F. Shaitelman:** Supervision. **Melissa B. Aldrich:** Supervision. **Fei-Fei Liu:** Supervision, Writing – review & editing. **Jennifer Y. Y. Kwan:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing.

References

1. Vicini F, Shah C, Arthur D: The Increasing Role of Lymphedema Screening, Diagnosis and Management as Part of Evidence-Based Guidelines for Breast Cancer Care. Breast J 22:358–359, 2016

2. Aldrich MB, Sevick-Muraca EM: Cytokines are systemic effectors of lymphatic function in acute inflammation. Cytokine 64:362–369, 2013

3. Paskett ED, Dean JA, Oliveri JM, et al: Cancer-related lymphedema risk factors, diagnosis, treatment, and impact: a review. J Clin Oncol Off J Am Soc Clin Oncol 30:3726–3733, 2012

4. Cheung L, Han J, Beilhack A, et al: An experimental model for the study of lymphedema and its response to therapeutic lymphangiogenesis. BioDrugs Clin Immunother Biopharm Gene Ther 20:363–370, 2006

5. Zampell JC, Aschen S, Weitman ES, et al: Regulation of adipogenesis by lymphatic fluid stasis: part I. Adipogenesis, fibrosis, and inflammation. Plast Reconstr Surg 129:825–834, 2012

6. Hayes SC, Johansson K, Stout NL, et al: Upper-body morbidity after breast cancer. Cancer 118:2237–2249, 2012

7. Montagna G, Zhang J, Sevilimedu V, et al: Risk Factors and Racial and Ethnic Disparities in Patients With Breast Cancer–Related Lymphedema. JAMA Oncol 8:1195–1200, 2022

8. Rockson SG, Keeley V, Kilbreath S, et al: Cancer-associated secondary lymphoedema. Nat Rev Dis Primer 5:22, 2019

9. Smoot B, Cooper BA, Conley Y, et al: Differences in limb volume trajectories after breast cancer treatment. J Cancer Surviv Res Pract 10:772–782, 2016

10. Brown S, Dayan JH, Kataru RP, et al: The Vicious Circle of Stasis, Inflammation, and Fibrosis in Lymphedema. Plast Reconstr Surg 151:330e–341e, 2023

11. Tabibiazar R, Cheung L, Han J, et al: Inflammatory manifestations of experimental lymphatic insufficiency. PLoS Med 3:e254, 2006

12. Bowman C, Rockson SG: The Role of Inflammation in Lymphedema: A Narrative Review of Pathogenesis and Opportunities for Therapeutic Intervention. Int J Mol Sci 25:3907, 2024

13. Lin S, Kim J, Lee M-J, et al: Prospective Transcriptomic Pathway Analysis of Human Lymphatic Vascular Insufficiency: Identification and Validation of a Circulating Biomarker Panel. PLOS ONE 7:e52021, 2012

14. Leung G, Baggott C, West C, et al: Cytokine Candidate Genes Predict the Development of Secondary Lymphedema Following Breast Cancer Surgery. Lymphat Res Biol 12:10–22, 2014

15. Avraham T, Zampell JC, Yan A, et al: Th2 differentiation is necessary for soft tissue fibrosis and lymphatic dysfunction resulting from lymphedema. FASEB J Off Publ Fed Am Soc Exp Biol 27:1114–1126, 2013

16. Di S, Ziyou Y, Liu N-F: Pathological Changes of Lymphedematous Skin: Increased Mast Cells, Related Proteases, and Activated Transforming Growth Factor-β1. Lymphat Res Biol 14:162–171, 2016

17. Baik JE, Park HJ, Kataru RP, et al: TGF-β1 mediates pathologic changes of secondary lymphedema by promoting fibrosis and inflammation. Clin Transl Med 12:e758, 2022

18. Sano M, Hirakawa S, Suzuki M, et al: Potential role of transforming growth factor-beta 1/Smad signaling in secondary lymphedema after cancer surgery. Cancer Sci 111:2620–2634, 2020

19. Vang A, Chan W, Shaitelman S, et al: Plasma Cytokine Levels as Prognostic Biomarkers and Reparative Microsurgery Outcome Measures for Breast Cancer-related Lymphedema. J Vasc Surg Venous Lymphat Disord 11:447–448, 2023

20. Vang AR, Shaitelman SF, Rasmussen JC, et al: Plasma Cytokines/Chemokines as Predictive Biomarkers for Lymphedema in Breast Cancer Patients. Cancers 15:676, 2023

21. Kwan JYY, Famiyeh P, Su J, et al: Development and Validation of a Risk Model for Breast Cancer–Related Lymphedema. JAMA Netw Open 3:e2024373, 2020

22. Shi W, Misra S, Li M, et al: Inflammatory Biomarkers, Hematopoietic Stem Cells, and Symptoms in Breast Cancer Patients Undergoing Adjuvant Radiation Therapy. JNCI Cancer Spectr 4:pkaa037, 2020

23. Lin C, Su J, Wu AJ, et al: External Validation of a 5-Factor Risk Model for Breast Cancer–Related Lymphedema. JAMA Netw Open 8:e2455383, 2025

24. Altara R, Manca M, Hermans KC, et al: Diurnal rhythms of serum and plasma cytokine profiles in healthy elderly individuals assessed using membrane based multiplexed immunoassay. J Transl Med 13:129, 2015

25. Shao X, Liu C: Influence of IFN- α and IFN- γ on Lymphangiogenesis. J Interferon Cytokine Res 26:568–574, 2006

26. Mäkinen T, Jussila L, Veikkola T, et al: Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nat Med 7:199–205, 2001

27. Lee HL, Jang JW, Lee SW, et al: Inflammatory cytokines and change of Th1/Th2 balance as prognostic indicators for hepatocellular carcinoma in patients treated with transarterial chemoembolization. Sci Rep 9:3260, 2019

28. de Schenquer DGD, Schenquer N: Serum Immunoglobulins, IL-1β, IL-2, and IFN-γ Gamma Level in Patients with Lymphedema Treated with Ortho-β-Hydroxy-Ethyl Rutosides (HR). Arch Med Res 32:129–135, 2001

Tables

Table 1. Clinical characteristics of the study population.

Characteristic	n=147
Age	
Mean (SD)	56.1 (10.9)
Median (Q1, Q3)	54.7 (48.3, 62.9)
Range (min, max)	(32.3, 83.7)
BMI	
Mean (SD)	26.5 (4.7)
Median (Q1, Q3)	26.2 (22.9, 29.0)
Range (min, max)	(17.7, 41.9)
Missing	24
Mammographic Breast Density (BI-RADS)	
Almost Entirely Fatty (A or 1: <25%)	8 (7)
Scattered Areas of Fibroglandular Density (B or 2: <50%)	48 (44)
Heterogeneously Dense (C or 3: >50%)	45 (41)
Extremely Dense (D or 4: >75%)	9 (8)
Missing	37
T Category	
Tis	31 (21)
T1	73 (50)
T2	36 (24)
T3	7 (5)
N stage	
Nx	19 (13)
N0	91 (62)
N1	31 (21)
N2	4 (3)
N3	2 (1)
Number of Pathologic Nodes	
Mean (SD)	0.9 (2.5)
Median (Q1, Q3)	0 (0, 1)
Range (min, max)	(0, 19)
Number of Nodes Removed	

Mean (SD)	5.4 (7.1)
Median (Q1, Q3)	2 (1, 7)
Range (min, max)	(0, 35)
Primary Surgery	
Lumpectomy	131 (89)
Mastectomy	16 (11)
Axillary Surgery	
None	31 (21)
Sentinel Lymph Node Biopsy (SLNB)	82 (56)
Axillary Lymph Node Dissection (ALND)	34 (23)
Chemotherapy	
None	95 (65)
Neoadjuvant	12 (8)
Adjuvant	40 (27)
Hormone Therapy	
No	53 (36)
Yes	94 (64)
Radiotherapy Volume and Fractionation	
Local-Hypofractionation	101 (69)
Local-Conventional fractionation	11 (7)
Locoregional-Conventional fractionation	35 (24)
Radiotherapy Boost	
No Boost	76 (52)
Boost	71 (48)
Lymphedema Presence	
No	132 (90)
Yes	15 (10)
Follow-Up Time (Years)	
Median (Q1, Q3)	4.3 (2.6, 7.4)
Range (min, max)	(0.2, 11.8)

Table 2. Identification of redundant cytokines. Cytokine levels are reported in pg/mL. Wilcoxon rank-sum test was performed between candidate cytokines at baseline and established clinical risk assessment. Bolded p-values are statistically significant (p<0.05).

	Clinically Low Risk (n=121)	**Clinically High Risk (n=26)**	**p-value**
CRP			**<0.001**
Mean (SD)	5544675.9 (14857482.2)	11808917.7 (16157727.7)	
Median (Q1, Q3)	2004950.0 (936682.7, 4048530.2)	5656149.6 (2931687.5, 11712601.8)	
Range (min, max)	(75201.2, 108604645.1)	(464254.2, 72167262.6)	
IFN-γ			0.63
Mean (SD)	14.5 (20.3)	14.3 (17.5)	
Median (Q1, Q3)	9.5 (6.2, 15.3)	8.8 (5.4, 14.7)	
Range (min, max)	(1.6, 176.7)	(3.6, 85.9)	

IFN-α2A			0.21
Mean (SD)	1.2 (0.5)	1.4 (0.5)	
Median (Q1, Q3)	1.2 (0.9, 1.5)	1.4 (0.9, 1.9)	
Range (min, max)	(0.3, 2.7)	(0.3, 2.2)	
IL-10			0.20
Mean (SD)	0.3 (0.4)	0.5 (0.7)	
Median (Q1, Q3)	0.3 (0.2, 0.4)	0.3 (0.2, 0.5)	
Range (min, max)	(0.0, 4.6)	(0.1, 4.0)	
IL-17A			0.12
Mean (SD)	5.3 (3.1)	5.9 (2.4)	
Median (Q1, Q3)	4.6 (3.4, 6.4)	5.8 (4.7, 7.2)	
Range (min, max)	(0.5, 20.6)	(1.7, 11.0)	
Missing	1	0	
IL-1β			**0.029**
Mean (SD)	0.1 (0.3)	0.1 (0.1)	
Median (Q1, Q3)	0.1 (0.1, 0.1)	0.1 (0.1, 0.2)	
Range (min, max)	(0.0, 2.3)	(0.0, 0.6)	
Missing	8	0	
IL-1RA			**0.003**
Mean (SD)	293.6 (142.4)	492.8 (350.8)	
Median (Q1, Q3)	254.8 (210.7, 352.0)	384.7 (239.7, 667.1)	
Range (min, max)	(107.3, 891.8)	(131.4, 1689.2)	
IL-4			**0.029**
Mean (SD)	0.1 (0.1)	0.1 (0.0)	
Median (Q1, Q3)	0.1 (0.1, 0.1)	0.1 (0.1, 0.1)	
Range (min, max)	(0.0, 0.5)	(0.0, 0.2)	
IL-6			**<0.001**
Mean (SD)	1.2 (0.9)	2.2 (1.5)	
Median (Q1, Q3)	1.0 (0.8, 1.3)	1.8 (1.2, 2.2)	
Range (min, max)	(0.4, 6.2)	(0.7, 7.0)	
IP-10			**0.044**
Mean (SD)	631.1 (548.2)	938.3 (946.1)	
Median (Q1, Q3)	487.9 (364.1, 682.2)	571.8 (474.8, 919.1)	
Range (min, max)	(157.2, 4383.4)	(274.5, 3847.7)	
MCP-1			**0.022**
Mean (SD)	344.4 (130.4)	433.9 (190.7)	
Median (Q1, Q3)	314.0 (260.5, 409.6)	421.0 (294.9, 556.0)	
Range (min, max)	(82.2, 831.0)	(184.1, 929.2)	
MMP-2			0.53
Mean (SD)	126834.3 (32862.6)	121152.8 (29129.7)	

Median (Q1, Q3)	122858.8 (102564.0, 140855.3)	114577.9 (107030.2, 135099.4)	
Range (min, max)	(67232.8, 213678.4)	(67003.3, 213764.1)	
MMP-9			0.72
Mean (SD)	203369.2 (133213.7)	259364.0 (417722.9)	
Median (Q1, Q3)	164523.7 (112435.8, 267914.5)	184501.0 (91113.3, 218304.8)	
Range (min, max)	(38911.7, 727209.1)	(47832, 2210355)	
SDF-1α			0.81
Mean (SD)	211.5 (270.6)	257.6 (339.5)	
Median (Q1, Q3)	170.2 (118.3, 224.8)	172.8 (119.3, 249.9)	
Range (min, max)	(38.0, 2839.3)	(47.6, 1829.3)	
Missing	9	0	
TGF-β1			0.12
Mean (SD)	17663.6 (6899.3)	19458.3 (5634.4)	
Median (Q1, Q3)	16919.1 (13000.9, 21342.8)	19306.2 (15326.8, 23757.7)	
Range (min, max)	(5121.8, 49386.5)	(9819.6, 32431.5)	
TNF-RII			**0.005**
Mean (SD)	6171.5 (2872.3)	8573.5 (4617.7)	
Median (Q1, Q3)	5617.9 (4382.1, 7472.3)	7551.6 (5280.7, 10247.9)	
Range (min, max)	(1982.7, 22633.7)	(3294.6, 23783.9)	
TNF-α			**0.019**
Mean (SD)	3.3 (1.2)	4.1 (1.6)	
Median (Q1, Q3)	3.3 (2.6, 3.8)	4.3 (2.7, 4.5)	
Range (min, max)	(1.1, 9.3)	(1.6, 8.5)	

Table 3. Univariate logistic regression of candidate cytokines for lymphedema occurrence. Bolded p-values are statistically significant (p<0.05).

Cytokine	OR (95% CI)	p-value	N
IFN-α2A	3.10 (1.05, 9.51)	**0.042**	147
IL-17A	1.17 (1.01, 1.36)	**0.027**	146
IL-10	1.14 (0.29, 2.41)	0.77	147
IFN-γ	1.03 (1.01, 1.05)	**0.021**	147
MMP-2	1.00 (1.00, 1.00)	0.86	147
MMP-9	1.00 (1.00, 1.00)	0.51	147
SDF-1α	1.00 (0.99, 1.00)	0.75	138
TGF-β1	1.00 (1.00, 1.00)	0.89	147

Table 4. Net reclassification improvement by IFN-α2a. Green indicates correct reclassification by IFN-α2A. Red indicates incorrect reclassification by IFN-α2A. LE: Lymphedema.

		Clinical Risk Assessment		
		High Risk	Low Risk	Total
IFN-α2A	High Level	LE: 10 No LE: 6	LE: **2** No LE: **55**	73
	Low Level	LE: **2** No LE: **8**	LE: 1 No LE: 63	74
	Total	26	121	147

Table 5. Performance comparison of logistic regression models to determine the additive value of IFN-α2A. Model A (Clinical Risk only), Model B (IFN-α2A only), and Model C (Combined). Metrics are reported as Point Estimate (95% CI).

Model Performance Measures	**Model A:** **Clinical Risk Only**	**Model B:** **IFN-α2A Only**	**Model C:** **Combined**
Sensitivity	0.867 (0.600 - 1.000)	0.800 (0.533 - 1.000)	0.867 (0.600 - 1.000)
Specificity	0.841 (0.773 - 0.902)	0.553 (0.462 - 0.636)	0.871 (0.803 - 0.924)
Accuracy	0.844 (0.782 - 0.898)	0.578 (0.503 - 0.646)	0.871 (0.816 - 0.918)
PPV	0.382 (0.250 - 0.524)	0.171 (0.111 - 0.247)	0.448 (0.296 - 0.615)
NPV	0.982 (0.949 - 1.000)	0.959 (0.890 - 1.000)	0.983 (0.950 - 1.000)
AUC ROC	**0.875** (0.758 - 0.963)	0.672 (0.528 - 0.806)	**0.895** (0.796 - 0.971)
Brier Score	**0.106** (0.066 - 0.155)	0.219 (0.172 - 0.270)	**0.101** (0.061 - 0.149)

Figure Legends

Fig. 1 Kaplan-Meier curve of lymphedema-free survival (LFS) by IFN-α2A. A median threshold was used to define the high and low groups by IFN-α2A level. Log-rank p-value is 0.026.

Fig. 2 IFN-α2 levels in breast cancer patients. Pre-radiotherapy IFN-α2 was measured in 62 breast cancer patients. Patients were grouped by their lymphedema (LE) status at 18 months after cancer treatment. Data is plotted as median with interquartile range. Two-tailed p-value is 0.157 (Mann Whitney test).

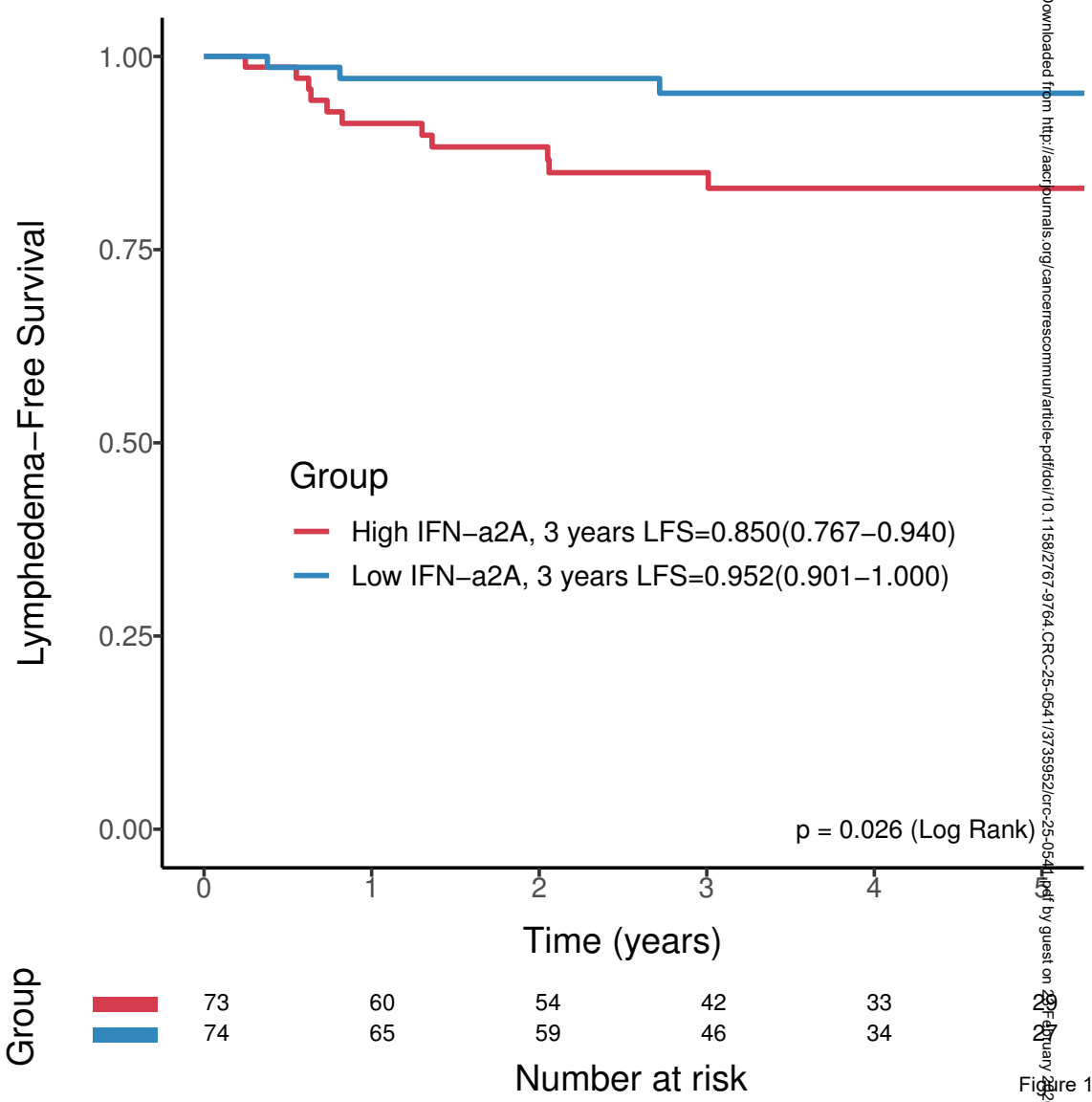

Figure 1

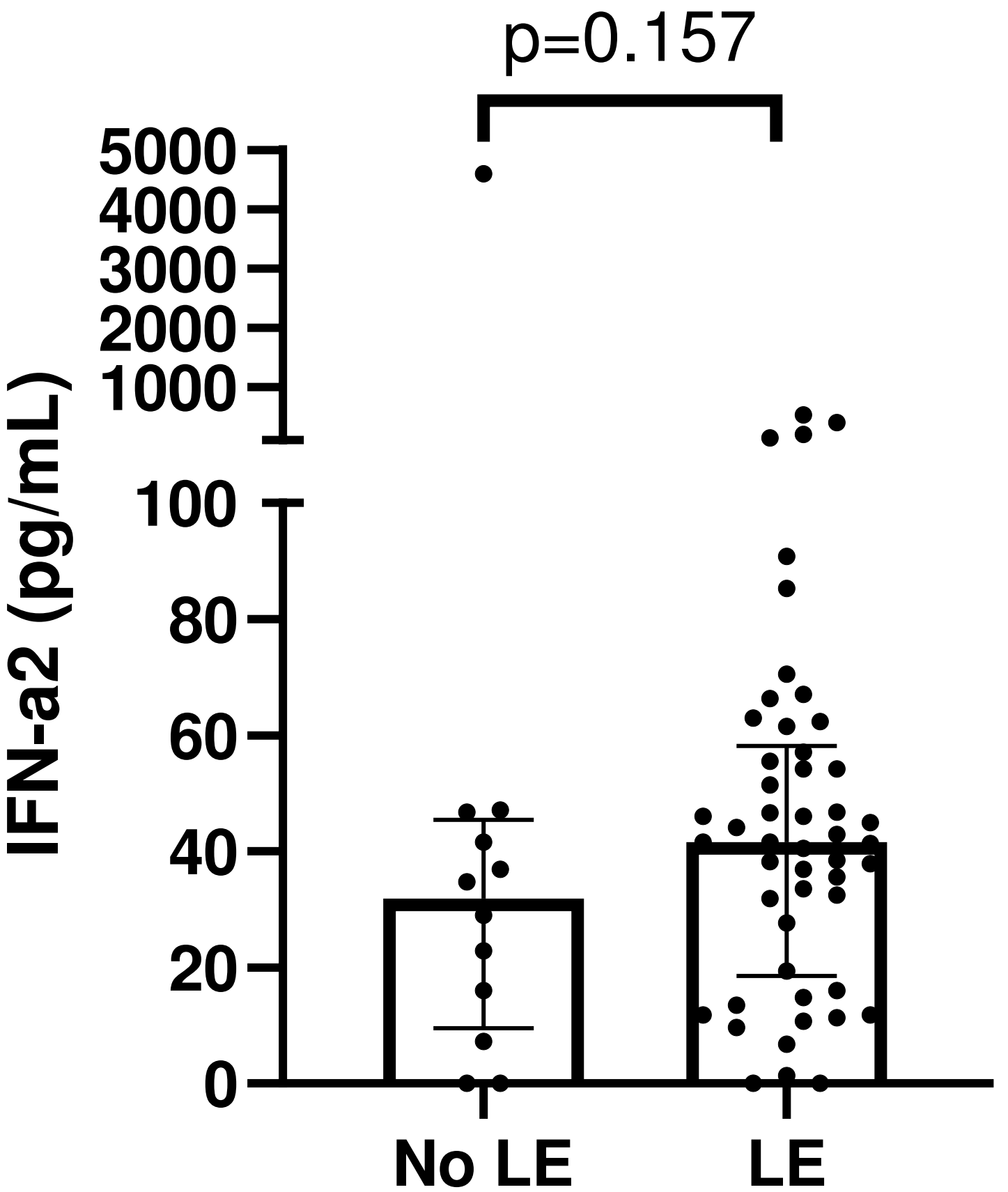

Figure 2