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Gastroenteropancreatic Neuroendocrine Tumors:
Potential for New Therapeutic Targets

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Gastroenteropancreatic Neuroendocrine Tumors: Potential for New Therapeutic Targets

By

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ABSTRACT

Gastroenteropancreatic Neuroendocrine Tumors:
Potential for New Therapeutic Targets

By Alexandra G. Lopez-Aguiar

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are highly vascular tumors. The role of pro-angiogenic factors (STAT3, VEGF, and HIF-1α) in the growth of these tumors, and their association with known prognostic markers (CD31 and Ki-67), adverse clinicopathologic factors, and disease recurrence after resection remains unclear. The purpose of this study was 1) to utilize neuroendocrine tissue samples from Emory through pathologic re-review of STAT3, VEGF, HIF-1α, CD31, and Ki-67 expression to assess the associations between these biomarkers and GEP-NET recurrence; and 2) to use Ki-67 to further stratify low grade pancreatic neuroendocrine tumors (PanNETs), a subset of GEP-NETs, to more accurately predict recurrence of disease.

All patients with non-metastatic primary GEP-NETs who underwent curative-intent resection from 2000-2013 were included. Immunohistochemistry was performed using tissue microarrays made in triplicate by a pathologist blinded to all other clinicopathologic variables. STAT3, VEGF, and HIF-1α were categorized into high vs. low expression; CD31 was dichotomized at the median value, and Ki-67 was grouped by the World Health Organization's classification system. The primary outcome was 3-year recurrence-free survival (RFS).

Of 144 GEP-NETs resected, STAT3 expression was high in 12 (8%), VEGF was high in 19 (13%), HIF-1α was high in 2 (1%), CD31 was above the median in 71 (50%), Ki-67 was $\geq$3% in 14 (10%). Lower 3-year RFS was associated with high STAT3 expression (55% vs. 84%; p=0.003), CD31 above the median (75% vs. 86%; p=0.043), and Ki-67$\geq$3% (51% vs. 84%; p<0.001). High STAT3 expressing tumors were also more likely to have a Ki-67$\geq$3% (42% vs. 7%; p<0.001). Even when controlling for high STAT3 and CD31 expression, Ki-67$\geq$3% had a 4-fold increase in risk of recurrence (HR 4.1; p=0.006). Moreover, when further stratifying Ki-67 index among low grade PanNETs, a Ki-67 of 1-2.99% was associated with a decreased RFS compared to a Ki-67<1% (70% vs. 97%; p=0.005). This finding persisted on multivariable analysis (HR 8.6; p=0.045), controlling for tumor size, margin positivity, lymph node involvement, and advanced T-stage.

In conclusion, while multiple biomarkers are associated with worse RFS in GEP-NETs, Ki-67, in particular, may be used to further stratify and predict aggressive behavior for these tumors.

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INTRODUCTION

Neuroendocrine tumors (NETs) are a heterogeneous group of rare neoplasms that arise from cells that comprise both the endocrine and nervous systems of the human body. In normal cellular conditions, complex neuroendocrine cells function by receiving signals from the nervous system and responding through the production and release of hormones to control various bodily functions. In tumorigenic conditions, neuroendocrine cells grow disproportionately, often releasing hormones outside of the usual feedback loops and invading nearby tissue. While many NETs are indolent and slow-growing, some can behave quite aggressively, with reported 5-year survival rates as low as 14% for certain tumor sites.(1) Although NETs can develop in the majority of organs, they are most commonly seen in the small intestine and pancreas.(2) Indeed, several distinct NET organ sites are frequently grouped together for analysis and treatment. One such group of NETs is the cluster of tumors collectively referred to as the gastroenteropancreatic neuroendocrine tumors (GEP-NETs).

GEP-NETs are a sub-group of neuroendocrine tumors consisting of lesions located in the stomach, small intestine, and pancreas. Although rare, the incidence of GEP-NETs is steadily increasing, with approximately 8,000 patients per year diagnosed in the U.S.,(3) many of whom have their tumors discovered incidentally on cross sectional imaging for other diagnoses.(4) The World Health Organization (WHO) classifies GEP-NETs into two main categories: well-differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs), each with variable degrees of tumor aggressiveness. As a result, the heterogeneity in clinical presentation and outcomes of GEP-NETs creates unique challenges for their

management, particularly in deciding on extent of surgical resection or surgical resection versus surveillance.(5, 6)

As with many tumors, GEP-NETs have been shown to grow through a variety of interconnected angiogenic and proliferative pathways. One such pro-angiogenic pathway is the JAK-STAT pathway, which functions by up-regulating cell migration and growth among tumor endothelial cells.(7) Similarly, activation of the proliferative ERK pathway is also found in many GEP-NETs, leading to the inhibition of apoptosis and thus increasing tumor progression.(8) Although these pathways are extremely complex and a complete understanding of their intricate interactions is yet to be determined, certain biomarker proteins have been isolated with particularly important roles. Three of these proteins, STAT3, VEGF, and HIF-1α, may serve as potential therapeutic targets for GEP-NET management; and two of these proteins, CD31 expression and Ki-67 index, may serve as potential prognostic markers.

When considering the targetable angiogenic and proliferative pathway proteins, STAT3, or Signal Transducer and Activator of Transcription 3, functions as a transcription factor activated by the JAK pathway that up-regulates the expression of genes critical to cell survival, proliferation, and angiogenesis, including the VEGF and HIF-1α genes.(9, 10) VEGF, or vascular endothelial growth factor, is a similarly potent protein that aids in tumorigenesis through the formation of blood vessels to provide adequate blood supply to tumor tissue.(11, 12) HIF-1α, or hypoxia-inducible factor 1a, behaves as a subunit of a heterodimeric transcription factor that regulates cellular function during hypoxic conditions.(13) When activated, it transcribes genes involved in angiogenesis, glucose transport and metabolism, inflammation, and apoptosis. Although

all three of these proteins have been shown to be expressed in low quantities in most normal tissue, they are conversely overexpressed in many tumor cells. Indeed, their targeted blockade has also exhibited promise both *in vitro* and *in vivo* with regard to tumor growth inhibition.(14-17) Thus, they may serve a future therapeutic role in the management of GEP-NETs.

The prognostic proteins, CD31 and Ki-67, have also been shown to be useful in understanding and predicting tumor behavior. CD31, or cluster of differentiation 31, is correlated with worse survival and tumor recurrence in several cancers.(18, 19) It is used to evaluate the degree of angiogenesis in tissues, with elevated expression identified in rapidly growing and highly vascular lesions.(20, 21) Ki-67 is a nuclear protein and marker of cellular proliferation, functioning as a diagnostic tool and prognostic indicator by predicting decreased survival and tumor recurrence for multiple cancers.(22) According to the WHO, which grades NETs based on cellular differentiation, mitotic count, and Ki-67 index,(5, 23) Ki-67 index stratifies NETs into: (i) low-grade tumors (Ki-67 <3%), (ii) intermediate-grade tumors (Ki-67 3-20%), and (iii) high-grade (Ki-67 >20%) carcinomas.(24, 25) Ki-67 index, in particular, has been shown to be highly correlated with clinical outcome, perhaps more so than other known histopathologic features.(26-29) Together, CD31 and Ki-67 may serve not only to predict poor clinical outcomes in patients with GEP-NETs, but also to help select high-risk patients for surgical resection and future adjuvant trials.

Due to the rarity of this disease, data on GEP-NETs and the biomarkers involved in their propagation have been sparse. Moreover, although many resected NETS are considered to be well-differentiated (low-to-intermediate grade) tumors treated primarily

with surgical resection,(5, 30, 31) the clinical behavior of these well-differentiated tumors is heterogeneous, with recurrences reported in 10-54% of patients and median overall survival (OS) ranging from 51 to 79 months.(4, 30, 32, 33) The purpose of this study was thus to use a large collection of GEP-NET tissue samples to examine the expression of angiogenic and proliferative biomarkers and their association with recurrence-free survival, as well as to further differentiate outcomes even amongst low-grade NETs. The approach for addressing this research question was through two aims: 1) to evaluate the expression and prognostic value of STAT3, VEGF, and HIF-1α, the expression and prognostic value of CD31 and Ki-67, and to assess the association between the two among GEP-NETs; and 2) to further stratify low-grade pancreatic neuroendocrine tumors (a common location of NET growth and the largest disease site among the available tissue samples) by Ki-67 proliferative index to better describe the heterogeneity of this group with regard to recurrence of disease after surgical resection.

METHODS

All patients with primary, non-metastatic gastrointestinal neuroendocrine tumors who underwent surgical resection at Emory University from January 1, 2000 to December 31, 2013 were identified. Baseline demographic, preoperative, intraoperative, postoperative, and pathologic data were collected retrospectively via medical record review of all patients. Pre-operative comorbidities were defined using the Charlson Comorbidity Scoring System, and staging was assigned as per the American Joint Committee on Cancer 7th edition guidelines.(34) Data regarding neoadjuvant and adjuvant therapy, disease recurrence, and survival were additionally collected. Recurrence of disease was specifically determined according to review of patient medical records, radiographic reports on surveillance imaging, and/or biopsy results. Institutional review board approval was obtained prior to data collection, and survival information was verified with the Social Security Death Index when appropriate.

Only patients who had surgery with curative-intent and with available tissue samples for pathologic re-review were included in the analysis. Tissue microarrays (TMAs) were created in triplicate from the archived formalin-fixed, paraffin-embedded archived tissue blocks. Standard immunohistochemistry was performed. The slides were stained for the following biomarkers: VEGF (clone VG-1, Abcam Biotechnology Company, Cambridge MA), HIF-1α (clone H1alpha67, Novus Biologicals, Littleton, CO), STAT3 (clone F-2, Santa Cruz Biotechnology, Dallas, TX), CD31 (clone RM0032-1D12, Abcam Biotechnology Company, Cambridge MA), and Ki-67 (clone MIB-1, DAKO Agilent Pathology Solutions, Santa Clara, CA) using antigen retrieval and the Leica Bond Autostainer (Leica Biosystems, Wetzlar, Germany) and counterstained with

hematoxylin. The TMA slides were scanned at ×40 magnification on the Leica Aperio AT2 bright field instrument (Leica Biosystems, Wetzlar, Germany) for computerized quantitation image analysis. An experienced and dedicated gastrointestinal pathologist at Emory University, who was blinded to all other clinicopathologic variables for each tissue sample, supervised the analysis.

All statistical analyses were performed using SPSS version 23.0 (Armonk New York Software, IBM Inc.). Statistical significance was predefined as $p<0.05$, and all 30-day mortalities were removed from survival analysis to more reliably assess oncologic-specific survival.

Aim 1

Study Population

All patients with primary, non-metastatic gastroenteropancreatic neuroendocrine tumors (GEP-NETs) who underwent surgical resection were identified. Pathologic re-review was undertaken for the following biomarkers: VEGF, HIF-1α, STAT3, CD31, and Ki-67. Patients with distant metastases or other malignancies, multifocal disease, and R2 resections were excluded from analysis.

Biomarker Expression

VEGF, HIF-1α, and STAT3 biomarker expression were determined as the percentage of tumor cells with immunoreactive nuclei or cytoplasm. Depending on the tumor cellularity, the number of total cells counted ranged from 600-1800. Intensity labeling was scored based on the percentage of cells that stained with a particular

biomarker, where 0 = none (less than 1% of cells staining); 1 = weak (1-10% of cells staining); 2 = moderate (11-50% of cells staining); and 3 = strong (51-100% of cells staining). An overall score for each tissue sample was calculated as [(1 + the sum of the intensity of each triplicate)/3]. The score categories were then grouped into low and high expression: low expression corresponded to $\leq$ 10% of cells staining and a "none" or "weak" intensity grade; high expression, conversely, corresponded to >10% of cells staining and a "moderate" or "strong" intensity grade.

CD31 expression was determined by microvessel density measurement as defined by the number of endothelial cells expressing the CD31 ligand per square micrometer of intra-tumoral capillaries or small venules. Expression was grouped according to the median recorded measurement, as seen in prior studies.(35)

Ki-67 expression was determined as the percentage of tumor cells with immunoreactive nuclei. An overall score for each sample was calculated as the [(sum of the expression percentage of each triplicate)/3]. Scores were grouped according to the established WHO classification system, where low grade corresponds with <3% Ki-67 staining, intermediate grade with 3-20% staining, and high grade with >20% staining.

Statistical Analyses

Descriptive and comparative analyses were performed on the entire cohort. Recurrence-free survival (RFS) was calculated from the date of operation to the date of recurrence diagnosis. Chi-squared analyses and Fisher's exact tests were used to compare categorical variables, and Student's t-test was used for continuous variables, where indicated. Univariable and multivariable Cox regression analyses were performed to

assess the association of individual clinicopathologic factors with RFS. Kaplan-Meier survival plots for RFS were created, and survival experience between groups was compared using log-rank tests.

Aim 2

Study Population

All patients with primary, non-metastatic PanNETs who underwent surgical resection were identified. Pathologic re-review was undertaken. For the purpose of this study, only patients with well-differentiated tumors and a Ki-67 <3% (low-grade) were included in the analysis. Patients with distant metastases or other malignancies, multifocal disease, and R2 resections were excluded from analysis.

Ki-67 Expression

Ki-67 expression was determined as the percentage of tumor cells with immunoreactive nuclei. Depending on the tumor cellularity, the number of total cells counted ranged from 600-1800. An overall score for each sample was calculated as the [(sum of the expression percentage of each triplicate)/3].

Statistical Analyses

Descriptive and comparative analyses were performed on the entire cohort. Recurrence-free survival (RFS) was calculated from the date of operation to the date of recurrence diagnosis. Chi-squared analyses and Fisher's exact tests were used to compare categorical variables, and Student's t-test was used for continuous variables, where

indicated. Univariable and multivariable Cox regression analyses were performed to assess the association of individual clinicopathologic factors with RFS. Kaplan-Meier survival plots for RFS were created, and survival experience between groups was compared using log-rank tests.

RESULTS

Aim 1

Patient Variables

Of 265 patients with surgically resected gastrointestinal neuroendocrine tumors available for pathologic re-review, 144 patients had curative-intent, non-metastatic primary GEP-NETs, of which 8 were gastric, 13 were duodenal, 7 were located in the ampulla, 82 in the pancreas, 27 in the small bowel, and 7 in the appendix. Baseline demographics and clinicopathologic features of this study cohort are summarized in Table 1.1. The mean age was 55 years, 41% (n=59) were male, and 69% (n=96) were Caucasian. Average tumor size was 2.8 cm, 49% (n=48) of patients had lymph node positive disease, 15% (n=21) underwent R1 resection, and 97% (n=97) were well-differentiated on pathologic analysis. Of all disease recurrences, 64% occurred at distant sites. Only 7 patients received systemic therapy, 3 of which were treated neoadjuvantly.

Pathologic Re-review of Biomarker Data

Among the 144 patients with GEP-NETs, 13% (n=19) had high VEGF expression, 1% (n=2) had high HIF-1α expression, and 8% (n=12) had high STAT3 expression. Fifty percent (n=71) had CD31 expression above the median, and 10% (n=14) of the cohort had a Ki-67 index $\geq$3% (Table 1.1). When comparing patients with CD31 expression above versus below the median, there was no difference in biomarker expression between groups (Table 1.2).When comparing patients with a Ki-67 index of $\geq$3% versus <3%, there was no difference in VEGF or HIF-1α expression. However, 36%

of GEP-NETs with a Ki-67 ≥3% had high STAT3 expression compared to only 5% of GEP-NETs with a Ki-67 <3% (p=0.001) (Table 1.3).

Although high STAT3 expression was correlated with a Ki-67 ≥3% (p=0.001), there was no difference among high versus low STAT3 expression groups with regard to VEGF and HIF-1α expression. Likewise, STAT3 expression was also not found to be associated with other clinicopathologic factors such as lymph node positivity, final resection status, tumor differentiation, tumor grade, necrosis, lymphovascular invasion, or perineural invasion (Table 1.4).

Recurrence-Free Survival Analysis:

Median follow-up was 37 months (IQR 7.4-60.1). On Kaplan-Meier analysis, there was no association between VEGF expression and 3-year recurrence-free survival (RFS) (76% vs. 82%; p=0.098; Figure 1.1a) or HIF-1α expression and 3-year RFS (50% vs. 82%; p=0.476; Figure 1.1b). High STAT3 expression, however, was associated with a worse 3-year RFS compared to low STAT3 expression (55% vs. 84%; p=0.003; Figure 1.1c). Similarly, CD31 expression above the median (75% vs. 86%; p=0.043; Figure 1.1d) and Ki-67 ≥3% (51% vs. 84%; p<0.001; Figure 1.1e) were also correlated with decreased RFS compared to CD31 expression below the median and Ki-67 <3%, respectively.

Predictors for Recurrence-Free Survival

On univariable Cox regression analysis, high STAT3 expression (HR 3.6, 95% CI 1.5-9.1; p=0.006), CD31 expression above the median (HR 2.3, 95% CI 1.0-5.1,

p=0.049), and Ki-67 index ≥3% (HR 5.2, 95% CI 2.1-13.2; p<0.001) were all associated with decreased RFS. However, in a multivariable model consisting of STAT3, CD31, and Ki-67, only Ki-67 index ≥3% remained associated with an increased risk of recurrence (HR 4.1, 95% CI 1.5-11.0, p=0.006) (Table 1.5). Indeed, even multivariable analysis evaluating STAT3 and Ki-67 index alone also only found Ki-67 ≥3% to be correlated with worse RFS (HR 3.9, 95% CI 1.4-10.8, p=0.008). Of note, other factors traditionally associated with cancer recurrence, such as lymph node and margin positivity, were excluded from the multivariable model due to missing data and the small number of recurrence events available for analysis (n=26).

Aim 2

Patient Variables

Of the 144 patients with gastroenteropancreatic neuroendocrine tumors and tissue available for pathologic re-review, 82 (57%) had well-differentiated, non-metastatic pancreatic tumors that underwent curative-intent resection. Ten patients with a Ki-67 index of ≥ 3% were excluded, leaving 72 (50%) patients with primary resected PanNETs with a Ki-67 index of <3% available for analysis. Baseline demographics and clinicopathologic features of this study cohort are summarized in Table 2.1. Mean age was 55 years, 46% (n=33) were male, and 74% (n=53) were white. Mean tumor size was 3.2 cm, 26% (n=19) of PanNETs were considered functional tumors, 51% (n=37) were located in the body/tail of the pancreas, and 71% (n=51) were resected via an open procedure. Sixty-four (89%) underwent R0 resections, while only 8 (11%) underwent R1 resections. There were no R2 resections within our study cohort, and 90% of all

recurrences were at distant sites. Only 5 of 72 patients received systemic therapy, 3 of which were treated neoadjuvantly.

Pathologic Re-review and Ki-67 Data

Among the 72 well-differentiated PanNETs with a Ki-67 index of <3%, Ki-67 was further stratified into 3 initial groups after pathologic re-review: Group A: <1% (n=43, 60%), Group B: 1-1.99% (n=23, 32%), and Group C: 2-2.99% (n=6, 8%) (Table 2.2). Representative IHC KI-67 slides for this grouping are shown in Figure 2.1. These groups were well-matched in baseline demographic and operative variables, including age, race, comorbidities, location of PanNET, tumor size, and operative blood loss ($p>0.05$). However, the groups displayed key pathologic differences. Groups B and C were characterized by advanced T-stage (44 and 67%, respectively) compared to Group A (12%) ($p=0.003$), as well as increased incidence of lymphovascular invasion (LVI) (83% in Group C vs. 23% in Group A) ($p=0.007$) (Table 2.2).

Recurrence-Free Survival Analysis:

Median follow-up was 39 months (IQR 7.1-60.3). On Kaplan-Meir analysis, 3-year recurrence-free survival (RFS) for Group A was 97%, while RFS for Group B and C was 71% and 67%, respectively ($p=0.018$) (Figure 2.2a).

Ki-67 Index as a Guide for Re-stratification

Given the similarity in pathologic characteristics and RFS between Groups B and C, these groups were combined together to form two final subsets of patients with well-

differentiated, low-grade PanNETs for subsequent survival analysis: Group A, Ki-67 <1% (n=43, 60%) and Group B+C, Ki-67 1-2.99% (n=29, 40%). Analysis revealed that Group A had a 3-year RFS of 97%, while Groups B+C had a decreased 3-year RFS of 70% (p=0.005) (Figure 2.2b).

Predictors for Recurrence-Free Survival

On univariable Cox regression analysis, Ki-67 index of 1-2.99% (HR 7.1, 95% CI, 1.5-33.8; p=0.014), tumor size, final resection status, perineural invasion (PNI), lymph node positivity, and advanced T-stage were each associated with worse recurrence-free survival (Table 2.3). On multivariable analysis, R1 resection and Ki-67 (HR 8.6, 95% CI 1.0-70.7, p=0.045) remained independently significant, even when taking into account these other adverse clinicopathologic factors (Table 2.3).

DISCUSSION

GEP-NETs are highly vascular tumors that are progressively increasing in incidence. Multiple pro-angiogenic and proliferative biomarkers have been implicated in the growth of these tumors, although their roles have not been clearly identified. When considering the first aim of this study, we showed that elevated STAT3, CD31, and Ki-67 expression are all associated with worse RFS, and that STAT3 and Ki-67 expression are directly correlated with each other. We also found that even after controlling for high STAT3 and CD31 expression, Ki-67 $\geq$3% was independently associated with an increased risk for disease recurrence. Together, these discoveries highlight the complexity and importance of these pro-angiogenic biomarkers for GEP-NET growth, as well as their need for further study.

Although the oncogene, STAT3, regulates the expression of many genes necessary for tumor survival and proliferation, its analysis to date has been limited to *in vitro* and *in vivo* studies. Activation of STAT3 has been observed in multiple types of tumors, including leukemia, breast, head and neck, and prostate cancer;(36-38) and numerous studies have shown that inhibition of STAT3 decreases tumor growth by inducing apoptosis in both cell lines and xenograft models.(14, 15, 39) However, examination of the role of STAT3 specifically in neuroendocrine tumors remains in its early stages. In a study by Nikolakopoulou *et al.*, STAT3 was implicated in the feedback loop of neuroendocrine cells, affecting key functions of these cells, as well as their resistance to damage.(40) Conversely, a study by Hofsli *et al.*, which performed transcript profiling by cDNA microarray analysis in an effort to identify new neuroendocrine-specific genes, found that neuroendocrine tumor cells were down-

regulated in their expression of STAT3 when compared to non-neuroendocrine tumor cells.(41) While the rationale for this finding was attributed to the slow-growing and less invasive nature of neuroendocrine tumors compared to many other epithelial cancers, STAT3 was still recognized as a potential therapeutic target given its constitutive activation in tumor versus normal cells.(41) The current study supports the findings of Hofsli in that there was an overall small number of high STAT3 expressers in our cohort of patients (n=12; 8%). However, despite these small numbers, high STAT3 expression was found to be associated with worse RFS both on Kaplan-Meier (55% vs. 84% 3-yr RFS; p=0.003) and Cox univariable analysis (HR 3.6; p=0.006). This corroborates the findings of a retrospective study by Zhang and colleagues, which demonstrated that increased STAT3 expression among both early and late stage gastric cancer patients was associated with worse overall survival ($p<0.001$ and p=0.026, respectively).(42) Thus, STAT3 may represent a promising therapeutic target for GEP-NETs.

When evaluating VEGF, another biomarker involved in the proliferation of most tumor cells, and one that is crucial to angiogenesis, the findings of this study were inconclusive. While multiple studies, such as that by Moghaddam *et al.*, support the relationship between VEGF and tumor growth and metastasis,(10-12) our study did not find a statistically significant association between VEGF expression and the measured outcome of RFS (76% vs. 82%; p=0.098). Likewise, while VEGF has been widely acknowledged to be a downstream target of STAT3,(9, 10, 43) we did not find an association between STAT3 and VEGF expression among our GEP-NET tissue samples. However, 25% of high STAT3 expressers had high VEGF expression compared to only 12% of low STAT3 expressers. Although this was not a statistically significant finding

(p=0.197), the trend toward association between these two biomarkers may have been limited by low power.

The last targetable biomarker, HIF-1α, also had inconclusive findings. HIF-1α is a known heterodimeric transcription factor that regulates angiogenesis and glucose metabolism during the hypoxic conditions typically seen in fast-growing tumor cells.(13) While studies have shown that HIF-1α is both a transcription factor for the activation of VEGF and a downstream target of STAT3,(13, 43) only 2 patients in this cohort were found to have high HIF-1α expression. Thus, neither the association between HIF-1α and RFS, nor the association between HIF-1α and other pro-angiogenic biomarkers could be adequately assessed.

While STAT3, VEGF, and HIF-1α represent the targetable proteins examined in this study, CD31 expression and Ki-67 index alternatively represent the proliferative and prognostic markers examined. CD31 and Ki-67 cannot be directly inhibited, yet the extent of their expression may be used to help guide management strategies. CD31, which serves as an adhesion molecule and marker of microvascular density, increases with the rise in angiogenesis that occurs during tumor growth. CD31 has been shown to be associated with worse survival and tumor recurrence in several cancers. For example, a study by Zhao *et al.* reported that among surgically resected non-small cell lung cancer patients, high CD31 expression was associated with worse disease-free survival compared to low CD31 expression.(18) Our study had similar findings to Zhao, as patients whose tumors had CD31 expression above the median had a 2-fold increase in risk for recurrence versus those with CD31 below the median (p=0.049). Moreover, on Kaplan-Meier analysis, above-median CD31 expressers were associated with a 75% 3-

year RFS compared to 86% for below-median CD31 expressers (p=0.043). However, when applied to a multivariable model, the association did not maintain its significance. CD31 was also not found to be linked to the expression of any other examined biomarkers, despite evidence to the contrary in prior studies. Indeed, Lee *et al.*, as mentioned above, suggested an association between STAT3 and CD31, while Marinaccio *et al.* proposed a correlation between CD31 and Ki-67.(20) Thus, the role of CD31 and its relation to other pro-angiogenic biomarkers among GEP-NETs remains unclear.

Ki-67 index, the final biomarker examined in the first aim of this study, is a particularly useful prognostic indicator, as multiple studies, including Ko *et al.*'s study of gastric cancer and Ladstein *et al.*'s study of melanoma, have demonstrated that elevated expression of Ki-67 is associated with worse survival.(44) Indeed, in accordance with these results, the current study demonstrated that among the 14 GEP-NET patients with a Ki-67 $\geq$3%, 3-year RFS was only 51% compared to 84% for those with a Ki-67 <3% (p<0.001). Furthermore, when considering the association between Ki-67 expression and other biomarkers, a Ki-67 $\geq$3% was found to be correlated with high STAT3 expression (p=0.001). This, too, corroborates results from the literature, as a study by Lee *et al.* on lung cancer xenografts revealed that by inhibiting STAT3 with the drug brassinin, both Ki-67 and CD31expression were down-regulated in tumor tissue.(45) Even after adjustment for STAT3 and CD31 in a multivariable model, only Ki-67 index remained associated with decreased RFS (HR 4.1; p=0.006). These results suggest that Ki-67 index is the predominant factor for predicting aggressive behavioral phenotypes among GEP-NETs.

In the second aim of this study, we examined the role of Ki-67 index among PanNETs, a large subset of GEP-NETs. Our findings showed that Ki-67 may be used to further risk stratify low-grade PanNETs, as a Ki-67 index of 1-2.99% was associated with worse recurrence-free survival compared to a Ki-67 index <1%. Despite evidence that well-differentiated, low-grade PanNETs are uniformly noted to have improved recurrence and overall survival compared to high-grade PanNETs, i.e. pancreatic NECs, there remains significant heterogeneity in outcomes amongst these well-differentiated tumors.(30, 32) The absence of a reliable risk stratification scheme to discriminate amongst well-differentiated, low-grade PanNETs has hindered the ability to adequately predict disease recurrence after surgical resection.(32, 46) Furthermore, with up to 80% of resected PanNETs being grouped as well-differentiated (grade 1 or grade 2), this limits the ability to guide individualized post-operative management and surveillance.(31, 32, 47, 48) Ki-67 index has been shown to be a particularly sensitive histopathologic marker for a more aggressive clinical course.(27, 49) However, the current method of using broad Ki-67 categories to define PanNETs may obscure the true prognostic value of this variable, particularly within the low-grade cohort.(28) Indeed, our findings suggest that, even among well-differentiated, low-grade PanNETs, a Ki-67 index of 1-2.99% is independently associated with an 8-fold increase in risk of disease recurrence when compared to tumors with a Ki-67 index of <1%, even after accounting for other adverse clinicopathologic variables.

Previous studies have attempted to determine the optimal Ki-67 index cut-off point to predict outcomes for low-to-intermediate grade PanNETs.(50) For example, Lowe and colleagues demonstrated that a Ki-67 index of >10%, rather than >3%, better

predicts lymph node metastases and poor overall survival (OS).(27) Another study by Hamilton *et al*. found that a Ki-67 index of >9% predicts a higher likelihood of disease recurrence and worse OS.(51) Lastly, a Ki-67 index cut-off of 7.5% was the recommendation of Goodell *et al*.(52) However, these studies include both low and intermediate grade PanNETs in their analyses, rather than focusing solely on the well-differentiated, low grade subset. Since our findings suggest that there is heterogeneity in disease recurrence when stratifying by Ki-67 index even among such low-grade PanNETs, it may be inappropriate to increase the Ki-67 cutoff to classify more patients into the "low-grade" group. Partly due to the rarity of PanNETs, there are currently limited studies that focus on this Ki-67 <3% subset.

In our study, PanNET patients with a Ki-67 <1% had improved RFS compared to patients with a Ki-67 index of 1-2.99%, with a 97% RFS at 3 years versus 70%, respectively. While no studies to our knowledge focus on the effect of Ki-67 index on RFS in well-differentiated, low-grade PanNETs, a study by Boyar Cetinkaya *et al*. did show a significant difference in 5-year OS for patients with PanNETs with a Ki-67 <2% (75.2%) versus a Ki-67 of 3-20% (55.8%) (p=0.04).(4) This difference continued on analysis of 10-yr OS in this study, with a Ki-67 <2% associated with a 68.9% survival compared to a 46.5% survival for a Ki-67 of 3-20% (p=0.03).(4) Another study by Miller *et al*. also looked at OS in patients whose NETs were graded based on the WHO/European Neuroendocrine Tumor Society (ENETS) stratification scheme.(48) In this study, those classified as having low-grade tumors had an OS of 87% compared to 83% and 50% for the patients with intermediate-grade and high-grade tumors, respectively.(48) However, this study did not focus solely on PanNETs, but rather on all

gastrointestinal NETs. Finally, a study by Panzuto *et al*. also identified Ki-67 index as a risk factor for disease progression in PanNETs.(53) In this study, intermediate (Ki-67 3-20%) and high-grade (Ki-67 >20%) PanNETs were associated with a 3.43 ($p<0.001$) and 1.52 ($p=0.074$) fold increased risk for progression compared to low-grade (Ki-67 ≤2%) PanNETs, respectively.(53)

This study is the first to our knowledge to evaluate the expression and prognostic value of STAT3, VEGF, HIF-1α, CD31, and Ki-67 biomarkers in the context of GEP-NETs, and to evaluate the use of Ki-67 index to further risk stratify and predict outcomes in well-differentiated, low-grade PanNETs. Although further studies are necessary, our results suggest that STAT3 shows particular promise as a potential future therapeutic target for GEP-NETs. Likewise, Ki-67 stands out as the most reliable prognostic indicator, and thus may be used to further stratify current classification systems for assigning NET grade and designating treatment strategies.

These findings may also be used to guide future management of low-grade PanNETs given the unclear current National Comprehensive Cancer Network (NCCN) guidelines regarding surgical resection versus observation for small well-differentiated, low-grade PanNETs.(34) Our results could perhaps be extrapolated to support surgical resection of ≤2 cm PanNETs if the measured Ki-67 index is >1% on pre-operative biopsy, as tumors with a Ki-67 index >1% may display more aggressive behavior. Indeed, this study demonstrated higher recurrence rates after resection for such tumors compared to those <1%. Moreover, post-operative surveillance could be increased to include imaging at shorter intervals rather than 1+ year intervals, as per current standard of care.(34) Ultimately, this proposed stratification scheme could be included into future

adjuvant trials for the development of targeted post-operative therapy, as the armamentarium for PanNETs continues to improve.

There are several limitations of this study. First, its retrospective design, using data from a single institution with a relatively small number of patients with tissue available for analysis, restricts the ability to generalize the results. While the available tissue represents one of the larger cohorts of GEP-NETs in current existence, there were only 265 tissue samples available for analysis prior to all exclusion criteria. This limited any desired subset analyses and may have skewed some of the observed biomarker relationships. Moreover, inter-biomarker interactions and interactions between biomarkers and other adverse clinicopathologic factors could not fully be explored due to inadequate power.

Another limitation is that the tissue samples used for analysis were obtained from surgically resected specimens, which inherently selects the lower grade, earlier stage histopathologies conducive to surgery. This could, in part, explain the relatively few tissue samples found to have high biomarker expression. Moreover, pathologic re-review was only performed for a priori selected biomarkers: STAT3, VEGF, HIF-1α, CD31, and Ki-67, which left data on many other important biomarkers in the intricate GEP-NET angiogenic and proliferative pathways unexplored. Likewise, additional histopathologic factors such as lymphovascular and perineural invasion were not re-reviewed by pathologists, and were thus dependent on the original surgical pathology reports, from which they were frequently missing. This, too, limited our subset analyses.

Lastly, the creation of TMAs from random areas in each tumor sample may have underestimated biomarker expression, as hot spot analysis is more commonly used in the

clinical setting. The applicability of our findings is further limited by the numerous and unstandardized methods that currently exist for measuring biomarkers like Ki-67, as well as their reportedly variable accuracy and reliability.(52, 54) As a result, further studies are necessary to replicate our results and continue to elucidate these important biomarkers roles and interactions with GEP-NETs.

CONCLUSION

This study comprises one of the largest cohorts of surgical patients with gastroenteropancreatic neuroendocrine tumors in the literature to evaluate the interactions between key angiogenic and proliferative biomarkers and their roles as prognostic markers for disease recurrence. It is also the first study to our knowledge to assess the use of Ki-67 index, in particular, to further risk stratify and predict outcomes in well-differentiated, low-grade pancreatic neuroendocrine tumors.

By pathologically re-reviewing surgical tissue samples using tissue microarray blocks stained for VEGF, HIF-1α, STAT3, CD31, and Ki-67 biomarker expression was measured among patients with non-metastatic gastroenteropancreatic tumors. STAT3, CD31, and Ki-67 were all found to be associated with worse recurrence-free survival among resected tumors. However, when evaluating inter-biomarker relationships, only STAT3 and Ki-67 expression were directly correlated with each other. Even after accounting for other angiogenic and proliferative biomarkers, Ki-67 index remained the only protein associated with increased risk of disease recurrence. These findings may influence future treatment recommendations and grading systems for GEP-NETs.

When further evaluating Ki-67 index within low-grade pancreatic neuroendocrine tumors, a Ki-67 of 1-2.99% was associated with a worse recurrence-free survival compared to those with a Ki-67 of <1%, even after controlling for other adverse factors. Such findings suggest that low grade pancreatic neuroendocrine tumors may be further risk stratified by Ki-67 proliferative index. This may have implications for the incorporation of Ki-67 into future grading systems, treatment recommendations, and surveillance protocols as the management for PanNETs continues to evolve.

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TABLES

Table 1.1. Baseline Demographics, Clinicopathologic Variables, and Biomarker Data of Patients with Primary Gastroenteropancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection from 2000-2013 (n=144).

Baseline/Operative Variables	Patients with Primary GEP-NETs (n=144)
Age (yrs.), mean ± SD	55 ± 14
Male, n (%)	59 (41)
BMI (kg/m^2), mean ± SD	29 ± 7
Comorbidities, n (%)*	
0	60 (42)
1	46 (32)
≥2	37 (26)
Race, n (%)	
White	96 (69)
Black	40 (29)
Other	4 (3)
Tumor Size (cm), mean ± SD	2.8 ± 2.6
Location of Tumor, n (%)	
Ampulla	7(5)
Appendix	7 (5)
Duodenum	13 (9)
Pancreas	82 (57)
Small Bowel	27 (19)
Stomach	8 (6)
EBL (mL), mean ± SD	256 ± 343
Pathologic Data	
Lymph Node Status, n (%)	
Negative	52 (35)
Positive	48 (33)
Missing	45 (31)
Final Resection Status, n (%)	
R0	123 (85)
R1	21 (15)
Tumor Differentiation, n (%)	
Well	97 (67)
Moderate	1 (1)
Poor	2 (1)
Missing	44 (31)
Tumor Grade, n (%)	
G1	37 (26)
G2	19 (13)
G3	2 (1)
Missing	86 (60)
Ki-67 Index Category, n (%)	
<3%	28 (20)

3-20%	22 (15)
>20%	2 (1)
Missing	92 (64)
Mitotic Rate Category, n (%)	
<2	50 (35)
2-20	12 (8)
>20	0 (0)
Missing	82 (57)
Necrosis, n (%)	
Negative	38 (26)
Positive	7 (5)
Missing	99 (69)
Lymphovascular Invasion, n (%)	
Negative	47 (33)
Positive	54 (37)
Missing	43 (30)
Perineural Invasion, n (%)	
Negative	52 (36)
Positive	29 (20)
Missing	63 (44)
Biomarkers	
VEGF, n (%)	
Low	125 (87)
High	19 (13)
HIF-1α, n (%)	
Low	142 (99)
High	2 (1)
STAT3, n (%)	
Low	132 (92)
High	12 (8)
CD31, n (%)ǂ	
Below Median	71 (50)
Above Median	71 (50)
Ki-67 Index, n (%)	
<3%	130 (90)
≥3%	14 (10)

Abbreviations: SD, standard deviation; BMI, body mass index; EBL, estimated blood loss
*Comorbidities are defined as any concurrent medical condition, including but not limited to, heart disease, chronic pulmonary disease, diabetes, renal disease, and liver disease as per the Charlson Comorbidity Scoring System.
ǂ CD31 staining was missing from two samples, leaving only 142 patients with data available for analysis.

Table 1.2. Distribution of Biomarkers among Patients with Low-Grade Non-metastatic Primary Gastroenteropancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection from 2000-2013, Stratified by CD31 Expression.

Biomarkers	CD31 Below Median n=50	CD31 Above Median n=50	p-value*
VEGF, n (%)			0.805
Low	62 (87)	61 (86)	
High	9 (13)	10 (14)	
HIF-1α, n (%)			1.000
Low	70 (99)	70 (99)	
High	1 (1)	1 (1)	
STAT3, n (%)			0.763
Low	66 (93)	64 (90)	
High	5 (7)	7 (10)	

* Statistical significance is indicated by a $p<0.05$.

Table 1.3. Distribution of Biomarkers among Patients with Low-Grade Non-metastatic Primary Gastroenteropancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection from 2000-2013, Stratified by Ki-67 Index.

Biomarkers	Ki-67 <3% n=130	Ki-67 ≥3% n=14	p-value*
VEGF, n (%)			0.399
Low	114 (88)	11 (79)	
High	16 (12)	3 (21)	
HIF-1α, n (%)			1.000
Low	128 (99)	14 (100)	
High	2 (1)	0 (0)	
STAT3, n (%)			0.001
Low	123 (95)	9 (64)	
High	7 (5)	5 (36)	

* Statistical significance is indicated by a $p<0.05$.

Table 1.4. Distribution of Biomarkers and Clinicopathologic Variables among Patients with Low-Grade Non-metastatic Primary Gastroenteropancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection from 2000-2013, Stratified by STAT3 Expression.

Biomarkers	STAT3 Low n=132	STAT3 High n=12	p-value*
VEGF, n (%)			0.197
Low	116 (88)	9 (75)	
High	16 (12)	3 (25)	
HIF-1α, n (%)			1.000
Low	130 (99)	12 (100)	
High	2 (1)	0 (0)	
Ki-67 Index, n (%)			0.001
<3%	123 (93)	7 (58)	
≥3%	9 (7)	5 (42)	
Pathology Data			
Lymph Node Status, n (%)			0.736
Negative	47 (52)	4 (44)	
Positive	43 (48)	5 (56)	
Final Resection Status†, n (%)			0.384
R0	114 (86)	9 (75)	
R1	18 (14)	3 (25)	
Tumor Differentiation, n (%)			0.194
Well	87 (98)	10 (91)	
Moderate	1 (1)	0 (0)	
Poor	1 (1)	1 (9)	
Tumor Grade, n (%)			0.166
G1	34 (65)	3 (50)	
G2	17 (33)	2 (33)	
G3	1 (2)	1 (17)	
Necrosis, n (%)			0.296
Negative	33 (87)	5 (71)	
Positive	5 (13)	2 (29)	
Perineural Invasion, n (%)			0.155
Negative	48 (68)	4 (40)	
Positive	23 (32)	6 (60)	
Lymphovascular Invasion, n (%)			0.058
Negative	45 (50)	2 (18)	
Positive	45 (50)	9 (82)	

* Statistical significance is indicated by a $p<0.05$.

†R0 resection refers to negative margins on pathologic review of the specimen, while R1 resection refers to positive margins on pathologic review of the specimen;

Table 1.5. Association of Biomarker Expression with Recurrence of Disease in Patients with Non-metastatic Primary Gastroenteropancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection from 2000-2013.

Variables	Univariable Analysis RFS HR (95% CI)	p-value	Multivariable Analysis* RFS HR (95% CI)	p-value
VEGF				
Low	Ref		--	--
High	2.1 (0.9-5.3)	0.106		
HIF-1α				
Low	Ref		--	--
High	2.0 (0.2-15.1)	0.486		
STAT3				
Low	Ref		Ref	
High	3.6 (1.5-9.1)	0.006	2.1 (0.8-5.8)	0.140
CD31				
Below Median	Ref		Ref	
Above Median	2.3 (1.0-5.1)	0.049	2.1 (0.9-4.8)	0.068
Ki-67 Index				
<3%	Ref		Ref	
≥3%	5.2 (2.1-13.2)	<0.001	4.1 (1.5-11.0)	0.006

Abbreviations: RFS, recurrence-free survival; HR, hazard ratio;
*Variables were included on multivariable analysis based on statistical significance on univariable analysis.

Table 2.1. Baseline Demographics and Clinicopathologic Variables of Patients with Primary Pancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection at a Single Institution from 2000-2013 (n=72).

Baseline Variables	Patients with Primary PanNETs (n=72)
Age (yrs.), mean ± SD	55 ± 13
Male, n (%)	33 (46)
BMI (kg/m^2), mean ± SD	29 ± 8
Comorbidities, n (%)*	
0	39 (54)
1	24 (33)
≥2	9 (13)
Race, n (%)	
White	53 (74)
Black	14 (19)
Other	4 (7)
ASA class, n (%)	
1	3 (4)
2	33 (46)
3	30 (42)
4	2 (3)
Functional Tumor, n (%)	19 (26)
Insulinoma	17 (24)
Glucagonoma	1 (1)
Gastrinoma	1 (1)
CgA (ng/L), mean ± SD	125 ± 183
Operative/ Pathologic Data	
Tumor Size (cm), mean ± SD	3.2 ± 3.0
Location of Tumor in Pancreas, n (%)	
Head/uncinate	28 (39)
Neck	7 (10)
Body/Tail	37 (51)
Surgical Technique, n (%)	
Open	51 (71)
Laparoscopic	9 (13)
Other	12 (17)
Type of Resection, n (%)	
Enucleation	10 (14)
Classic pancreatoduodenectomy	6 (8)
Pylorus preserving pancreatoduodenectomy	14 (19)
Central pancreatectomy	6 (8)
Distal pancreatectomy	36 (50)
EBL (mL), mean ± SD	216 ± 162
Ki-67 Index, n (%)	
< 1%	43 (60)
1 – 1.99%	23 (32)

2 – 2.99%	6 (8)

Abbreviations: SD, standard deviation; BMI, body mass index; ASA, American Society of Anesthesiologists; CgA, chromogranin A; EBL, estimated blood loss
*Comorbidities are defined as any concurrent medical condition, including but not limited to, heart disease, chronic pulmonary disease, diabetes, renal disease, and liver disease as per the Charlson Comorbidity Scoring System.

Table 2.2. Distribution of Covariates among Patients with Low-Grade Non-metastatic Primary Pancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection from 2000-2013 Stratified by Ki-67 Index (n=72).

Covariates	Group A Ki-67 <1% (n=43)	Group B Ki-67 1-1.99% (n=23)	Group C Ki-67 2-2.99% (n=6)	p-value*
Age (yrs.), mean ± SD	57 ± 14	52 ± 12	50 ± 10	0.261
Male, n (%)	18 (42)	12 (52)	3 (50)	0.709
Race, n (%)				0.522
White	30 (71)	19 (83)	4 (67)	
Black	9 (21)	4 (17)	1 (17)	
Other	3 (71)	0 (0)	1 (17)	
BMI (kg/m^2), mean ± SD	30 ± 8	27 ± 6	28 ± 8	0.498
Comorbidities, n (%)				0.591
0	22 (51)	13 (57)	4 (67)	
1	14 (33)	9 (39)	1 (17)	
≥2	7 (16)	1 (4)	1 (17)	
Tumor size (cm), mean ± SD	2.8 ± 2.6	3.7 ± 3.7	4.4 ± 1.9	0.327
Location of PanNET, n (%)				0.418
Head/uncinate	15 (35)	11 (48)	2 (33)	
Neck	3 (7)	4 (17)	0 (0)	
Body/tail	25 (58)	8 (35)	4 (67)	
Surgical Technique, n (%)				0.065
Open	28 (65)	19 (83)	4 (67)	
Laparoscopic	6 (14)	2 (9)	1 (17)	
Other	9 (21)	2 (9)	1 (17)	
Type of Resection, n (%)				0.629
Enucleation	5 (12)	5 (22)	0 (0)	
Classic PD	3 (7)	2 (9)	1 (17)	
PPPD	8 (19)	5 (22)	1 (17)	
Central pancreatectomy	2 (5)	3 (13)	1 (17)	
Distal pancreatectomy	25 (58)	8 (35)	3 (50)	
EBL (mL), mean ± SD	188 ± 124	257 ± 213	300 ± 265	0.417
AJCC T-Stage, n (%)				0.003
T1	18 (43)	10 (44)	0 (0)	
T2	19 (45)	2 (9)	2 (33)	
T3	5 (12)	10 (44)	4 (67)	
Mitotic Rate, n (%)				0.480
<2	17 (85)	10 (77)	5 (100)	
2-20	3 (15)	3 (23)	0 (0)	
Final Resection Status, n (%)†				0.099
R0	41 (95)	18 (78)	5 (83)	
R1	2 (5)	5 (22)	1 (17)	
Lymphovascular Invasion, n (%)				0.007
Negative	27 (77)	9 (50)	1 (17)	
Positive	8 (23)	9 (50)	5 (83)	
Perineural Invasion, n (%)				0.391
Negative	26 (77)	12 (71)	5 (100)	
Positive	8 (24)	5 (29)	0 (0)	
Lymph Node Positive, n (%)	6 (21)	6 (32)	1 (25)	0.696

Abbreviations: SD, standard deviation; BMI, body mass index; PanNET, pancreatic neuroendocrine tumor; PD, pancreatoduodenectomy; PPPD, pylorus preserving

pancreatoduodenectomy; EBL, estimated blood loss; AJCC, American Joint Committee on Cancer;
†R0 resection refers to negative margins on pathologic review of the specimen, while R1 resection refers to positive margins on pathologic review of the specimen;
* Statistical significance is indicated by a $p<0.05$.

Table 2.3. Association of Clinicopathologic Factors with Recurrence of Disease in Patients with Non-metastatic Primary Pancreatic Neuroendocrine Tumors who underwent Curative-Intent Resection from 2000-2013.

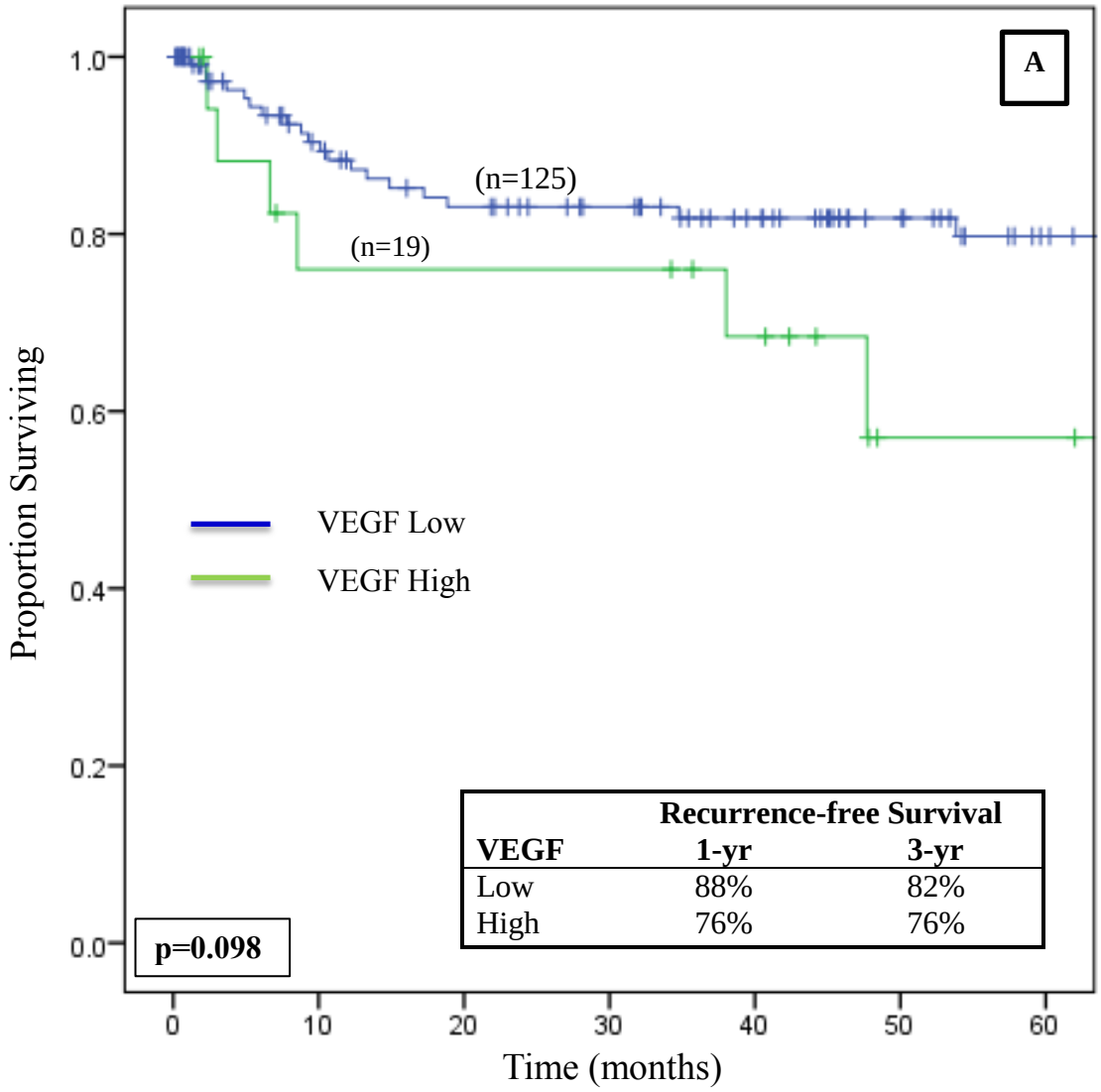
Variables	Univariable Analysis RFS HR (95% CI)	p-value	Multivariable Analysis* RFS HR (95% CI)	p-value
Age	1.0 (0.9-1.0)	0.078	--	--
Gender			--	--
Male	Ref			
Female	1.8 (0.5-6.6)	0.343		
Race			--	--
White	Ref			
Black	1.8 (0.5-6.9)	0.407		
Other	0.0 (0.0-0.0)	0.986		
BMI (kg/m^2)	0.9 (0.8-1.1)	0.309	--	--
Comorbidities			--	--
0	Ref			
1	1.2 (0.3-4.2)	0.817		
≥2	0.0 (0.0-0.0)	0.980		
Functional Tumor	0.05 (0.0-2.4E+28)	0.931		
Type of Resection			--	--
Enucleation	Ref			
PD	1.3 (0.1-14.2)	0.842		
PPPD	0.9 (0.1-8.9)	0.940		
Central pancreatectomy	0.0 (0.0-0.0)	0.985		
Distal pancreatectomy	0.4 (0.0-3.8)	0.442		
EBL (mL)	1.0 (1.0-1.0)	0.064	--	--
Ki-67 Index				
<1%	Ref		Ref	
1-2.99%	7.1 (1.5-33.8)	0.014	8.6 (1.0-70.7)	0.045
Tumor Size (cm)	1.1 (1.0-1.3)	0.032	1.0 (0.9-1.2)	0.695
Final Resection Status[†]				
R0	Ref		Ref	
R1	5.1 (1.3-20.2)	0.019	9.3 (1.3-66.8)	0.026
Mitotic Rate (per 10 HPF)			--	--
<2	Ref			
2-20	1.9 (0.4-10.5)	0.451		
Lymphovascular Invasion			--	--
Negative	Ref			
Positive	133.3 (0.2-89108)	0.140		
Perineural Invasion			--	--
Negative	Ref			
Positive	6.3 (1.1-34.8)	0.035		
Lymph Node Positive	5.9 (1.6-21.5)	0.007	6.1 (1.0-38.0)	0.054
Advanced T Stage				
T1/T2	Ref		Ref	
T3	25.1 (3.2-199.9)	0.002	3.9 (0.4-39.3)	0.249

Abbreviations: RFS, recurrence-free survival; HR, hazard ratio; BMI, body mass index; PD, pancreatoduodenectomy; PPPD, pylorus-preserving pancreatoduodenectomy; EBL, estimated blood loss; HPF, high power fields;
†R0 resection refers to negative margins on pathologic review of the specimen, while R1 resection refers to positive margins on pathologic review of the specimen;
*Variables were included on multivariable analysis based on statistical significance on univariable analysis. Perineural invasion was not included in the final multivariable model because of missing data.

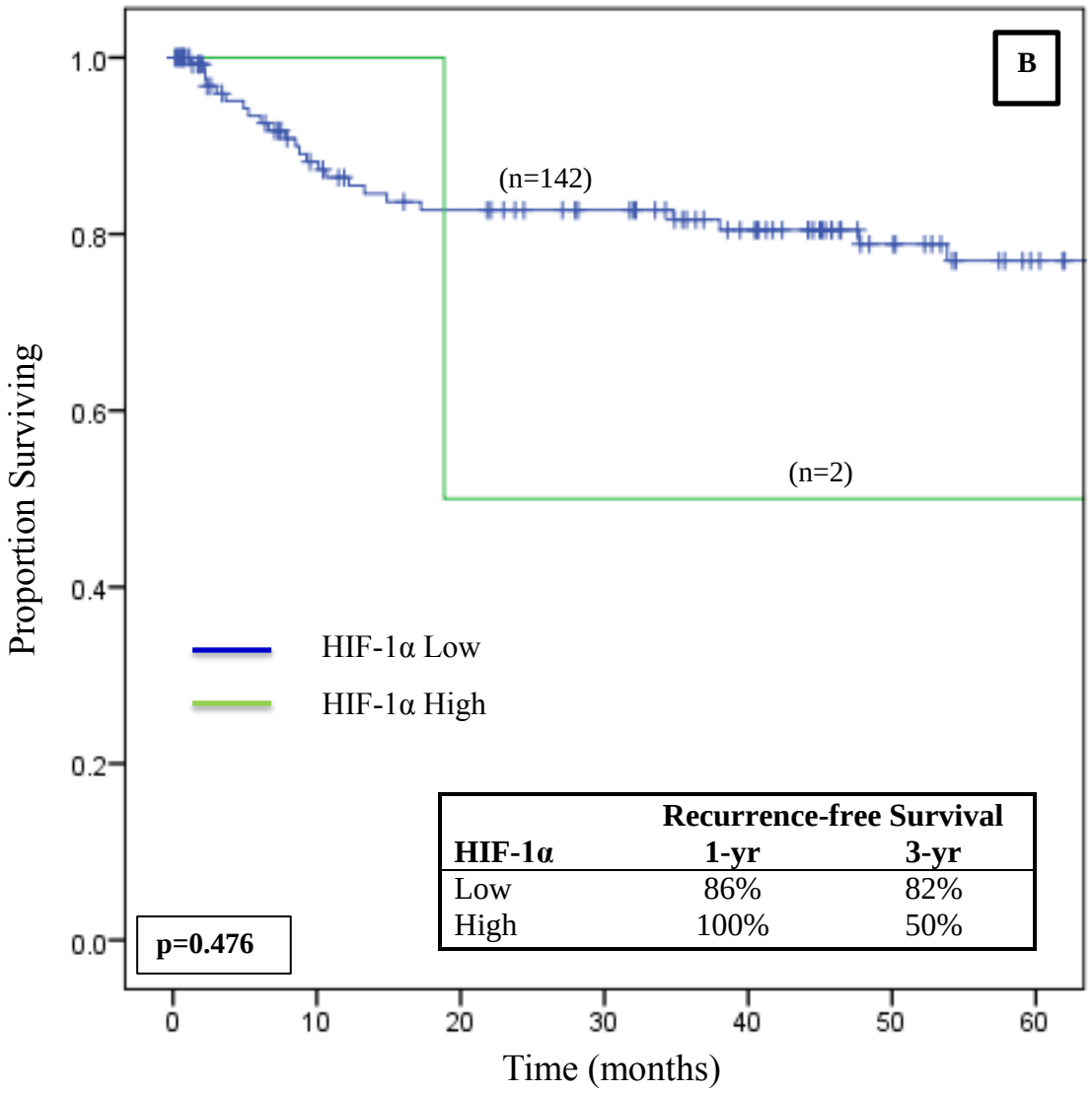
FIGURES

<u>Figure 1.1a-e</u>. Kaplan- Meier Survival Curve for Recurrence-Free Survival among Biomarker Groups.

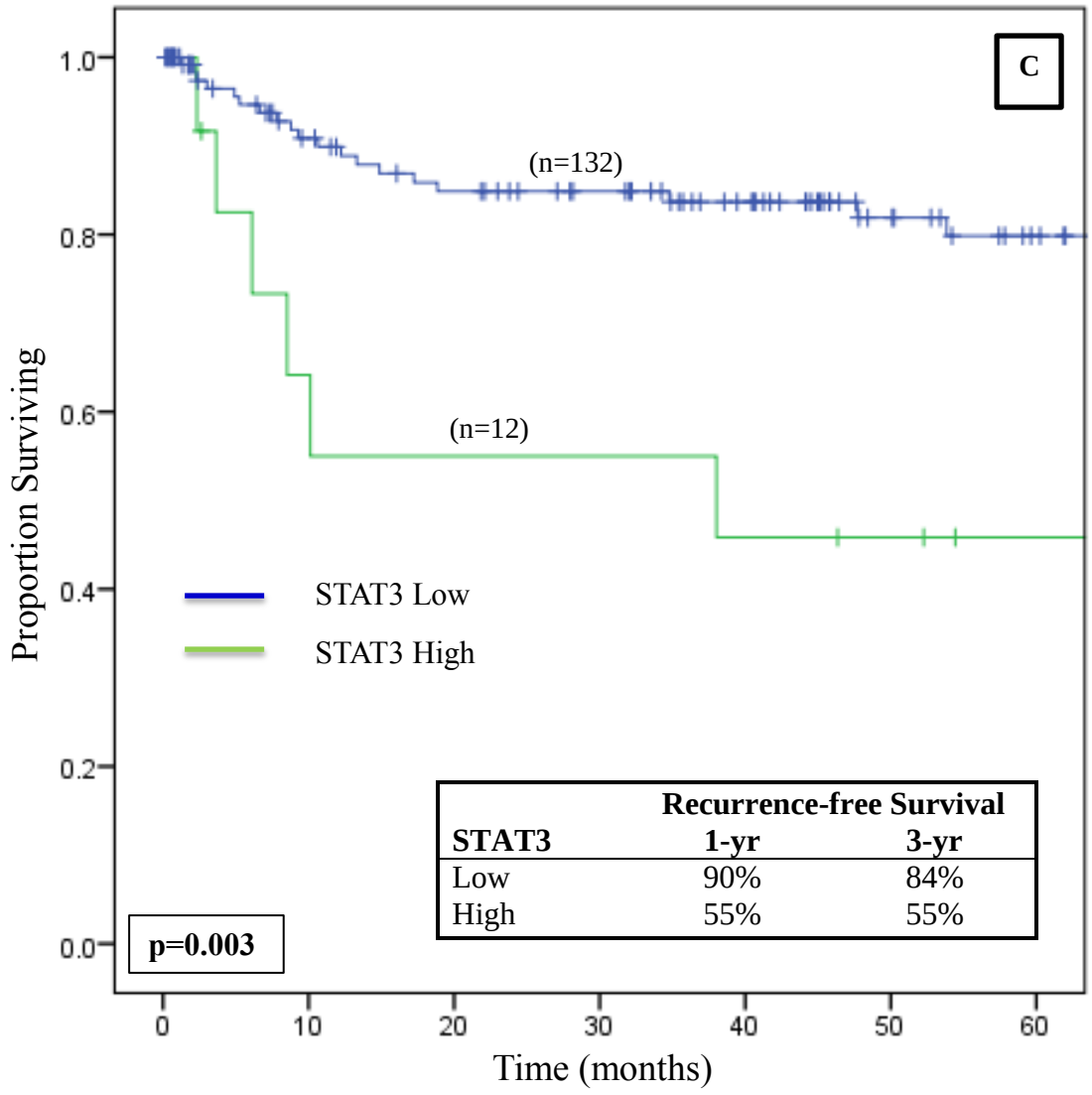
1.1a. There is no statistically significant difference in recurrence-free survival (RFS) among GEP-NET patients with high VEGF expression (n=19) versus those with low VEGF expression. Log-rank p-value = 0.098;
1.1b. There is no association between HIF-1α expression and RFS among patients with GEP-NETs. Log-rank p-value = 0.476.
1.1c. High STAT3 expression is associated with a 55% 3-year RFS compared to 84% for low STAT3 expression among patients with GEP-NETs. Log-rank p-value = 0.003.
1.1d. CD31 expression above the median is associated with a 75% 3-year RFS compared to 86% for CD31 expression below the median among patients with GEP-NETs. Log-rank p-value = 0.043.
1.1e. Ki-67 index ≥3% is associated with a significantly worse RFS compared to a Ki-67 <3% among patients with GEP-NETs. Log-rank p-value < 0.001.



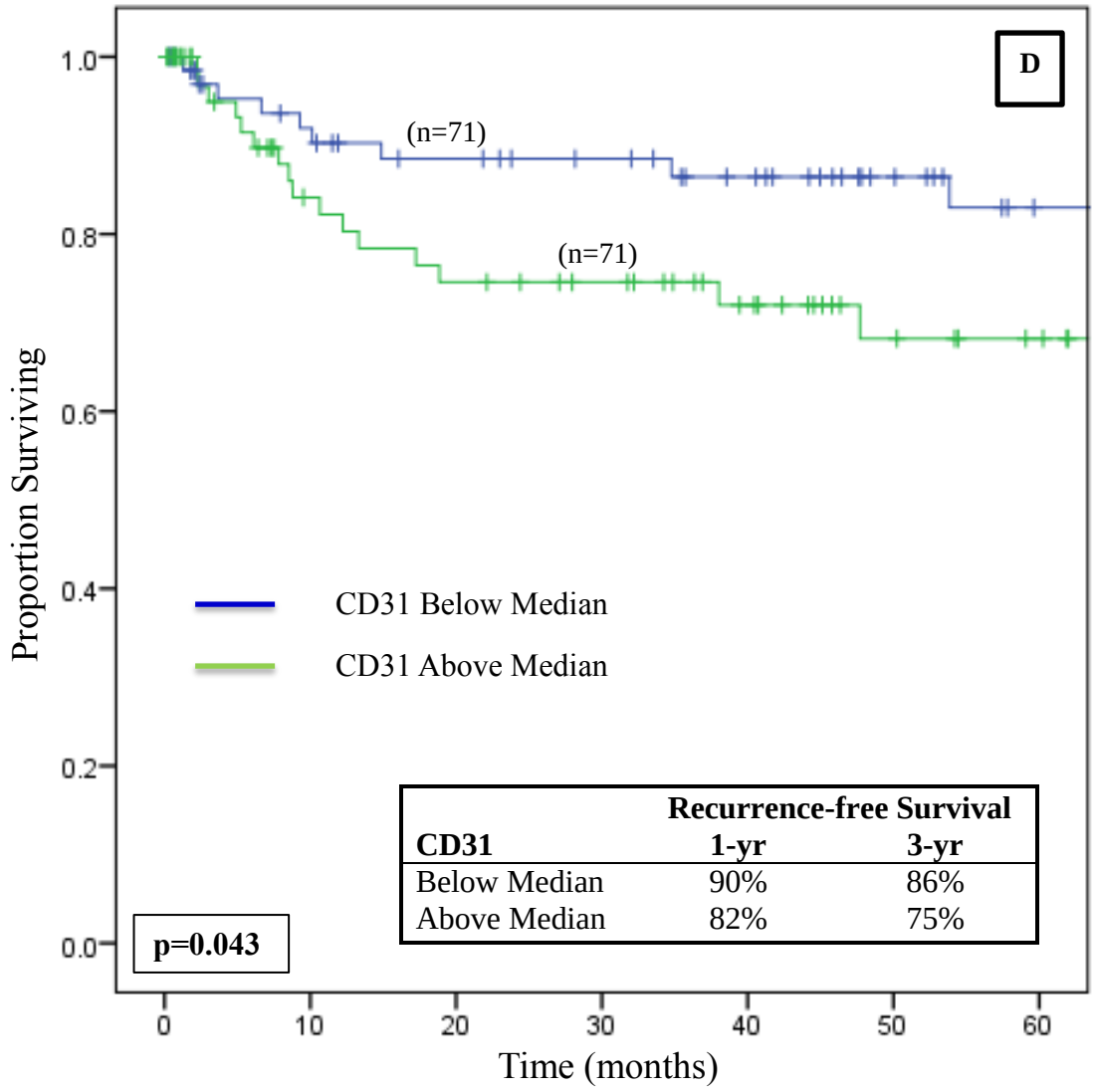
	Recurrence-free Survival	
VEGF	**1-yr**	**3-yr**
Low	88%	82%
High	76%	76%



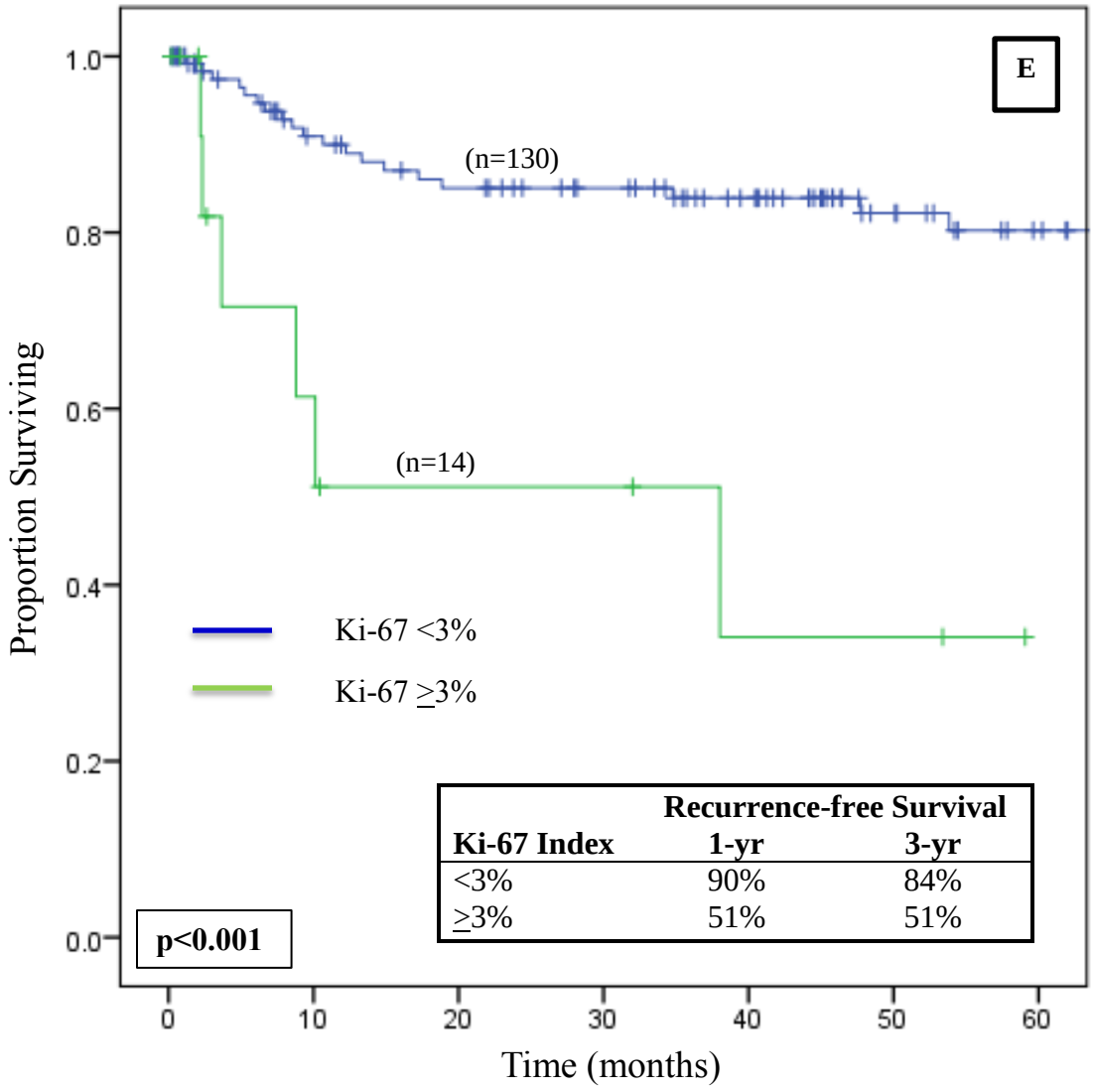
HIF-1α	Recurrence-free Survival 1-yr	3-yr
Low	86%	82%
High	100%	50%



	Recurrence-free Survival	
STAT3	**1-yr**	**3-yr**
Low	90%	84%
High	55%	55%



	Recurrence-free Survival	
CD31	**1-yr**	**3-yr**
Below Median	90%	86%
Above Median	82%	75%



Ki-67 Index	Recurrence-free Survival 1-yr	3-yr
<3%	90%	84%
≥3%	51%	51%

<u>Figure 2.1</u>. Re-stratification of Ki-67 Based on Histologic Analysis by a Pathologist blinded to All Other Clinicopathologic Variables using Tissue Microarray Blocks made in Triplicate.

Patients with low-grade, Ki-67<3% were further stratified into 3 initial groups based on immunohistochemical expression: Group A (Ki-67<1%), Group B (Ki-67 1-1.99%), and Group C (Ki-67 2-2.99%)

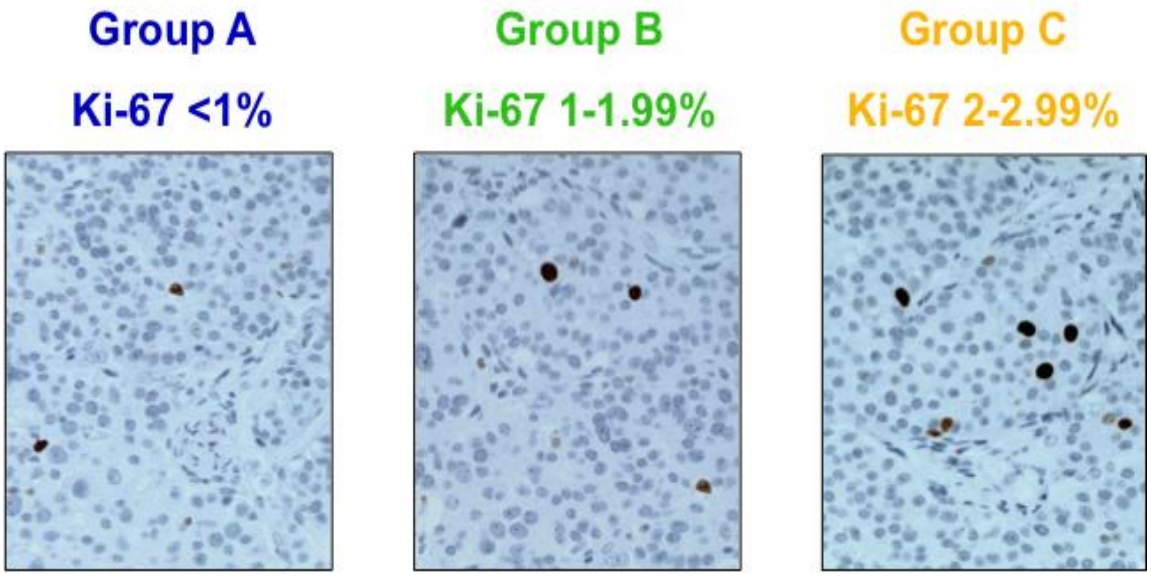
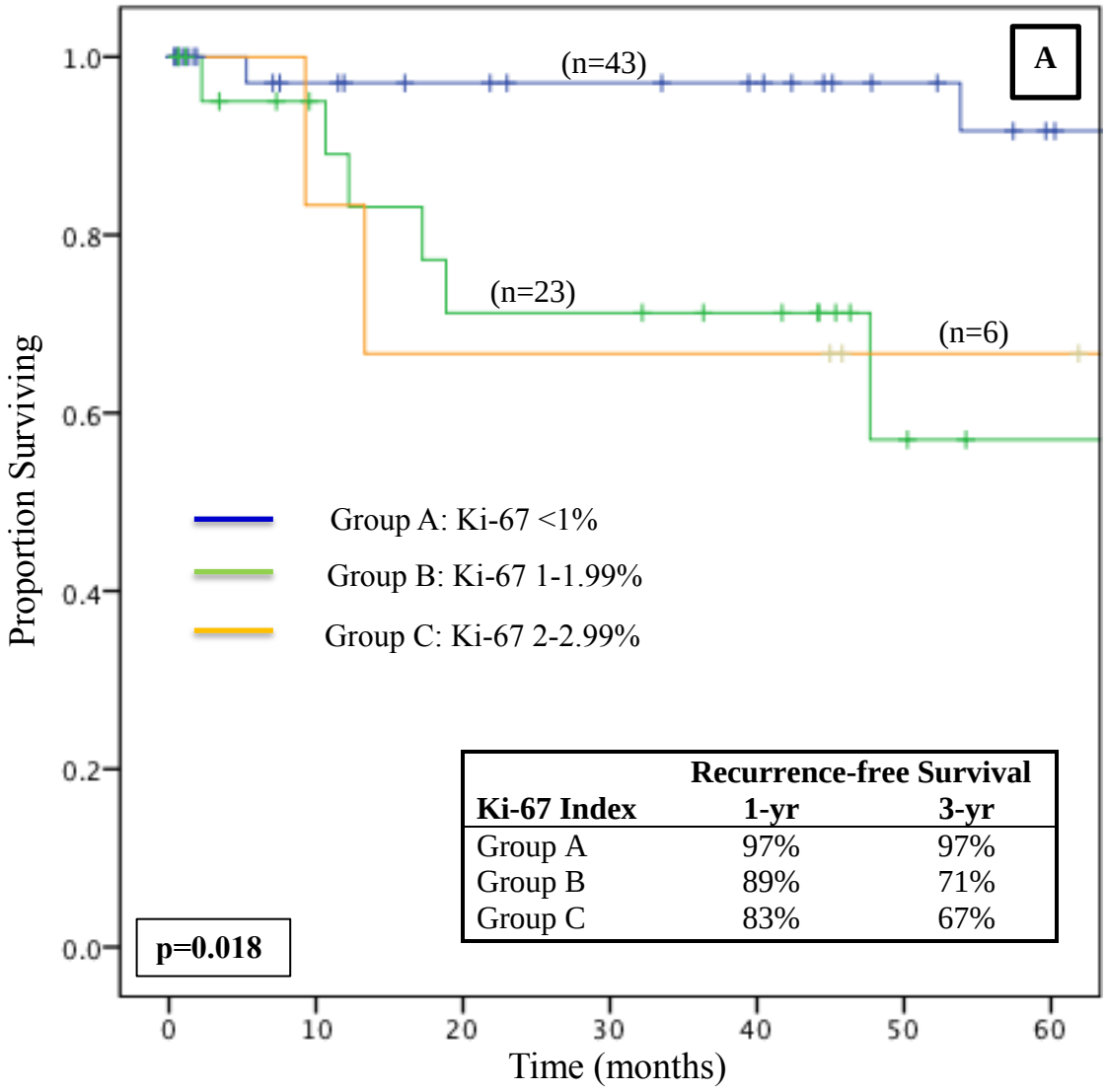


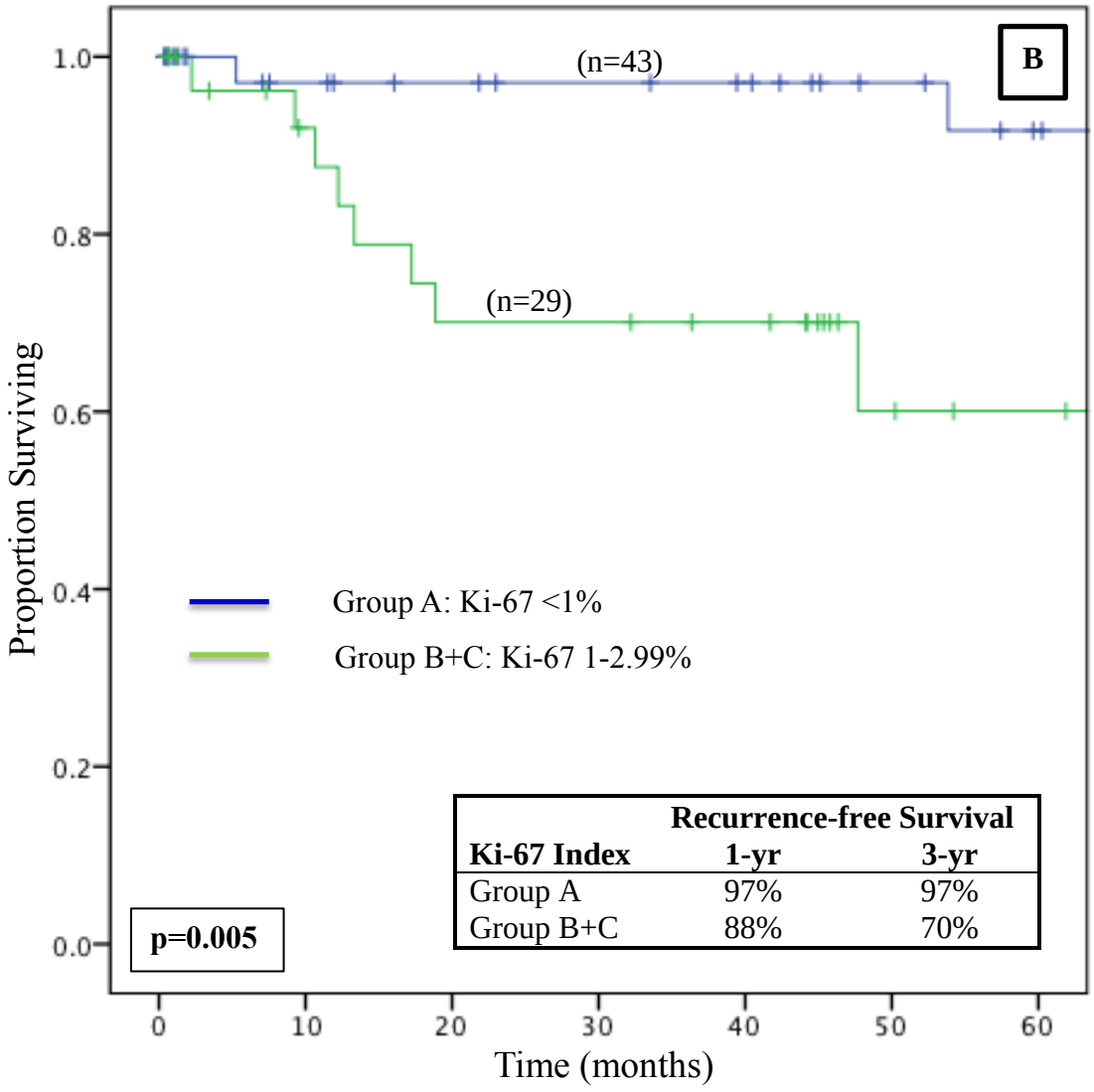
Figure 2.2a-b. Kaplan- Meier Survival Curve for Recurrence-Free Survival in Re-Stratified Ki-67 Index Groups.

2.2a. There is no statistically significant difference between Group B (Ki-67 1-1.99% (n=23)) and Group C (Ki-67 2-2.99% (n=6)), but both are associated with worse RFS compared to Group A (Ki-67 <1% (n=43)). Log-rank p-value = 0.018;

2.2b. The combined Groups B and C (Ki-67 1-2.99% (n=29)) show a 27% decrease in 3-year RFS compared with Group A (Ki-67 <1% (n=43)). Log-rank p-value = 0.005.



	Recurrence-free Survival	
Ki-67 Index	**1-yr**	**3-yr**
Group A	97%	97%
Group B	89%	71%
Group C	83%	67%


B
(n=43)
(n=29)
Proportion Surviving
1.0
0.8
0.6
0.4
0.2
0.0
Group A: Ki-67 <1%
Group B+C: Ki-67 1-2.99%
Recurrence-free Survival
Ki-67 Index 1-yr 3-yr
Group A 97% 97%
Group B+C 88% 70%
p=0.005
0 10 20 30 40 50 60
Time (months)