Gastric Lavage Malignant Cells (yGL) and Hypohemoglobinemia (yAnemia) as New Systems of Tumor Regression Grading and Prognostic Prediction for Gastric Cancer After Neoadjuvant Treatment

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Abstract. *Background/Aim:* Although reckoned necessary for survival benefit, neoadjuvant chemotherapy (NAC) of gastric cancer (GC) patients has so far provided questionable results. Consequently, searching for new and clearer systems of response to NAC, post-NAC re-evaluation and prognostic prediction appears essential. The purpose of this study was to examine endogastric cytopathology and hemoglobin level count as new features, potentially useful for GC patients after NAC. *Patients and Methods:* Between April 2012 and October 2018, 21 of 116 patients with resectable GC received NAC and were investigated for the presence of free-floating malignant cells in their gastric lavage (yGLI) and the development of hypohemoglobinemia (yAnemia). *Results:* yGLI and yAnemia were found in 11 and 12 patients, respectively. yGLI correlated with the traditional parameters of tumor regression ($p=0.0424$). Both yGLI and yAnemia were found to be independent predictive factors of overall and progression-free survival ($p\leq 0.0364$). *Conclusion:* In the light of our results, the yGLI and yAnemia appear two promising, simple and interesting clinicopathological features which should always be examined for better clarifying GC patients' response to NAC.

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Key Words: Neoadjuvant chemotherapy, gastric cancer, gastric lavage, neoadjuvant treatment, neoadjuvant radiotherapy, oncology, cytopathology.

Despite continuous decrease and effort in screening and faster diagnosis, according to the latest estimates on incidence and mortality provided by the International Agency for Research on Cancer (IARC), gastric carcinoma (GC) (including cardia and non-cardia tumors) maintains a predominant position worldwide; in fact, in both sexes combined, it is responsible for over 1 million new cases in 2018 and an estimated 783,000 deaths, making it the fifth most frequently diagnosed cancer (after lung, female breast, prostate and colorectal cancer) and the third leading cause of neoplastic mortality (after lung and colorectal cancer) (1). Differing from other gastrointestinal malignancies where multistep carcinogenesis is well-recognized (as for colorectal cancer), to date sporadic GC still has a less known and understood pathobiology (2-6).

Surgery still represents the mainstay of therapy but, except for early-stage disease (including early GC; EGC), it cannot assure an effective long-term recovery and must be conducted in concert with peri-operative treatment (7-11). Neoadjuvant chemotherapy (NAC), however, despite initial enthusiasm, has sooner showed conflicting results and limitations against this type of cancer (including gastric and junctional lesions) especially in terms of survival (12-16).

To clarify these doubts, ascertain its role and, consequently, optimize the management of GC patients, recently evaluation of NAC effectiveness has been addressed with several new systems and markers, both theoretical and practical (15-18). With this purpose, in our study GC patients submitted to NAC were investigated regarding the presence of free malignant cells in their gastric lavage and the development of hypohemoglobinemia, hereinafter referred as yGL and yAnemia, respectively. Our results suggest that investigation of these two parameters could be useful for evaluating response to NAC in GC patients.

Materials and Methods

Patients. Between April 2012 and October 2018, the yGL samples obtained from 116 GC patients with staging and prognostic intent were prospectively analyzed. Enrolment was performed according to the ethical standards written in the Declaration of Helsinki. Informed consent was obtained from all participants during their stay at the Division of General and Emergency Surgery of St. Andrea Hospital, Faculty of Medicine and Psychology, Sapienza University, Rome, Italy. The procedure, cytomolecular analysis and pathoprognostic significance of GL and yGL in GC patients have already been investigated as previously described (19-23). In particular, yGL was collected from all patients prior to surgery and tumor manipulation. After completion of neoadjuvant treatment (NAT) including NAC alone, neoadjuvant radiotherapy (NAR) alone, or both, but before surgical act, routine blood tests were ordered for each patient; anemia (yAnemia) was diagnosed with hemoglobin (Hgb) levels below 13.5 g/dl for men and 12 g/dl for women, as universally acknowledged (24). Traditional methods for evaluating the therapeutic response to NAT included clinicolaboratory tests (endoscopy and serum markers such as CEA, Ca 19.9 and Ca 72.4), radiology (contrast-enhanced computed and positron emission tomography) and histology through the 4-tiered tumor regression grading system (TRG) as developed by Becker and colleagues (25). Post-NAT pathology of surgical specimens was described following the 7th and 8th edition of AJCC TNM Staging System (26,27). Patients were followed-up from hospital discharge until December 2018.

Statistical analysis. Statistical analysis was performed using MedCalc Statistical Software version 18.11 (MedCalc Software bvba, Ostend, Belgium). Categorical variables were compared using the Chi-square test, whereas continuous data were compared through the Student's *t*-test. Four types of survival were investigated: median overall survival rate (OS), defined as the time from GL collection to death from any cause, progression-free survival (PFS) as the time elapsed between GL retrieval and metastatic tumor progression -but not local or regional progression- or death from any cause, disease-free survival (DFS) or recurrence-free survival (RFS), as the span of life between GL and any recurrence (local or regional), distant metastasis or death due to any cause and, eventually, time to tumor progression (TTP) which differs from PFS by including only cancer-related death (28, 29). All the survival curves were performed in accordance with the Kaplan-Meier method and the log-rank test was used to evaluate statistical significance. Comparison among yGL, yAnemia and the traditional clinico-radio-histologic methods evaluating NAC response (which, hereafter, will be abbreviated as yCRH) were performed in terms of OS using a logistic regression tool. Univariate and multivariate analyses were adopted (with one-way or two-way ANOVA test and Cox proportional hazards model, respectively) to assess significant association and independency, respectively, among predictive factors. A *p*-value ≤ 0.05 was considered statistically significant.

Results

Traditional peri-operative clinicopathological parameters. Only 21 of 116 GC patients received NAT before curative surgery and have been accordingly included in this study; the other 95 were excluded. Their major clinicopathological

characteristics are summarized in Table I. Male-to-female ratio was 2.5:1, average age 64.3 years. Fifteen tumors were proximal (at gastric cardia, fundus, and corpus), only 6 distal (at antrum and pylorus). According to Siewert classification, 6 cancers were type 1, 3 type 2, and 4 type 3. Eighteen patients were given NAC alone, other 3 combined NAC-NAR. Fluorouracil (5FU), including its oral form capecitabine (XEL), was the chemotherapy agent most frequently adopted (100% of patients), followed by platinum (PL) (19 cases); PL-5FU association was given in 17 patients. Epirubicin-cisplatin-5FU was the regimen most frequently used (7 patients), followed by epirubicin-oxaliplatin-XEL (4 participants) and FOLFOX (leucovorin, oxaliplatin, and 5FU). As for CRH response to NAT, only 6 patients ameliorated, while 4 aggravated, and 11 showed stable disease. At Chi-square test, proximal tumor site associated well with NAC ($p=0.0495$), 5-FU ($p=0.0495$ as well), and PL-based regimen ($p=0.0002$). Fourteen (66.7%) cancers resulted locally advanced (stage yIII and yIV) and required open total gastrectomy in 71.4% of cases taking a mean operative time of 245.3 min and an average number of 24.5 loco-regional lymph nodes (Table I). Rates of early postoperative morbidity and peri-operative mortality were 28.6% and 19%, respectively, necessitating re-hospitalization for one patient and emerging re-surgery in 3 cases. Eleven patients went through adjuvant treatment. Interestingly, among the 4 EGCs, 3 were yGL1 and 2 of these have died within the end of this study (30).

The yGL parameter. At cytopathological analysis, free malignant cells were found in 11 GL samples (yGL1 cases), whereas no cell was seen in other 10 (yGL0 samples) (Table I). yGL1 was significantly associated with poorer OS ($p=0.0431$) at Chi-square test (Table II). In particular, at a median follow-up of 14.5 months (range=1-66), 10 of 15 GC patients who died were yGL1. Moreover, associations of yGL1 with AT (including both AC and AR) $p=0.0489$, AC alone ($p=0.0489$), and combined AC-AR ($p=0.0207$) were also statistically significant. This finding is probably explained by the fact that most aggressive cancers, which were yGL1, necessitated AT as further treatment (Table II). More specifically, in comparison with yCRH and yAnemia, yGL achieved significant results in terms of OS at logistic regression analysis ($p=0.0424$ with an overall model fit of $p=0.0391$). In detail, the 3 of the 6 GC patients showing yCRH amelioration were yGL1 and 2 of these have died at the aforementioned median OS; on the other hand, also 2 of the 3 yGL0 with yCRH amelioration have died. Concerning the 15 patients with stable or worsening yCRH, 8 were yGL1 and 7 yGL0; out of these patients, 8 and 4, respectively, died. The Kaplan-Meier model showed that yGL1 patients had shorter OS compared to yGL0 counterpart (13 vs. 33 months, respectively) and this finding achieved a customary level of

Table I. Main clinicopathological features of the 21 gastric cancer (GC) patients treated with neoadjuvant treatment followed by curative surgery and adjuvant therapy.

Gender	Male: 15 Female: 6	Lauren classification	Intestinal type: 9 Non-intestinal type: 12
Race	Caucasian: 20 Latino American: 1	2010 WHO classification	Tubular type: 10 Non-tubular type: 11
Age (mean)	64.3 years (range=41-83)	Signet ring cells	Presence: 2 Absence: 19
Severe comorbidities	Presence: 8 Absence: 13	LVI	LVI0: 7 LVI1: 14
Tumor site	Proximal*: 15 Distal*: 6	PnI	PnI0: 15 PnI1: 6
Siewert type	Type 1 and 2: 9 Type 3 and non-Siewert cancers: 12	LNR	0: 4 >0: 17
NAT	NAC alone: 18 NAC-NAR combined: 3	Number of sampled lymph nodes (mean)	24.5 (range=7-62)
NAC regimen	ECF: 7 EOX: 4 FOLFOX: 2 PL and 5-FU/XEL-based: 17 PL-based: 19 5-FU/XEL-based: 21	Gastrectomy type	Distal: 6 Total: 15
Clinico-radio-histologic response (CRH) to NAT	Amelioration: 6 Stability or worsening: 15	Operative time (mean)	245.3 min (range=155-405)
ASA class	1-2: 7 3-6: 14	PLS (mean)	14.8 days (range=7-52)
yGL	GL0: 10 GL1: 11	30-day postoperative complications	Occurrence: 6 Absence: 15
yAnemia	Development: 12 Absence: 9	90-day postoperative complications	Occurrence: 1 Absence: 20
ypT	T1a: 1 T1b: 3 EGC (T1a + T1b): 4 T1-T2: 5 T3-T4: 16 N0: 4 N1-3: 17 M0: 17 M1: 4	Re-surgery	Accomplishment: 3 Unnecessity: 18
ypN		Re-hospitalization	Necessity: 1 Unnecessity: 20
ypM		Peri-operative mortality	Occurrence: 4 AC alone: 5 AC-AR combined: 6
yStage	1: 5 2-4: 16		
G	G1: 3 G2-4: 18		

*Distal site include antro-pyloric cancers; proximal site include cardio-fundic and gastric body carcinomas; NAT, neoadjuvant treatment; NAC, neoadjuvant chemotherapy; NAR, neoadjuvant radiotherapy; ECF, epirubicin, cisplatin and 5-FU; EOX, epirubicin, oxaliplatin and XEL; FOLFOX, leucovorin, 5-FU and oxaliplatin; PL, platinum; 5-FU, 5-fluorouracil; XEL, xeloda; ASA, American Society of Anesthesiologists Classification; LVI, lymphovascular invasion; PnI, peri-neural invasion; LNR, metastatic lymph node ratio; PLS, postoperative length of stay; AT, adjuvant treatment; AC, adjuvant chemotherapy; AR, adjuvant radiotherapy.

statistical significance ($p=0.0223$) (Figure 1). At univariate analysis, there was a strong relationship of yGL1 with OS ($p=0.040$ at one-way ANOVA) and yAnemia ($p=0.0045$ at two-way ANOVA) (Table III). Furthermore, multivariate analysis demonstrated yGL to be an independent predictive factor of OS ($p=0.0331$ with an overall fit of $p=0.0014$, Table IV), PFS, and DFS (for each endpoint $p=0.0289$ with an overall model fit of $p=0.0009$, Table V).

The yAnemia parameter. Following NAT but before surgery, 12 patients developed yAnemia, whereas Hgb levels were

normal in 9 cases (Table I). No significant association of yAnemia with the traditional parameters for NAT response evaluation (indexed in Table I) was shown by chi-square test. Moreover, contrarily to the yGL, no association was shown between yAnemia and yCRH, in terms of OS, at logistic regression analysis ($p=0.2279$ with an overall model fit of $p=0.0391$). On the other hand, as mentioned above, univariate analysis revealed that yGL1 was a significant predictive factor for yAnemia ($p=0.0045$ at two-way ANOVA) (Table III); in particular, 5 (45.45%) of the 11 yGL1 patients presented yAnemia as well. Moreover, Kaplan-Meier analysis showed

Table II. Correlation of the presence of free-floating tumor cells in gastric lavage fluid (yGLI) with the traditional clinicopathological features of gastric cancer.

OS	$p=0.0431$
AT	$p=0.0489$
AC alone	$p=0.0489$
AC-RT	$p=0.0207$
All the other parameters listed in Table I	$p>0.05$

OS, Overall survival; AT, adjuvant treatment (both AC and AR); AC, adjuvant chemotherapy; AC-RT, adjuvant chemo-radiotherapy. p -Values shown in bold are statistically significant ($p\leq 0.05$).

Table III. Univariate analysis of free-floating tumor cells of gastric lavage fluid (yGLI) as a predictive factor for overall survival (OS) and post-neoadjuvant treatment anemia (yAnemia).

OS	$p=0.040$ (one-way ANOVA)
yAnemia	$p=0.0045$ (two-way ANOVA)
All the other parameters listed in Table I	$p>0.05$ (one- and two-way ANOVA)

p -Values shown in bold are statistically significant ($p\leq 0.05$).

significant differences of OS, PFS, DFS, and TTP between yAnemia and yHgb normal count group (Figure 2). In fact, yAnemia patients had shorter OS than those with normal Hgb levels (8.7 vs. 28 months, $p=0.0379$), as well as poorer PFS-DFS (8.7 vs. 28 months, $p=0.0230$) and TTP (11.6 vs. 31.3 months, $p=0.0370$). Furthermore, at multivariate analysis, yAnemia resulted as an independent predictive factor of OS ($p=0.0364$ with an overall fit of $p=0.0014$, Table IV), PFS and DFS (for each endpoint $p=0.0239$ with an overall model fit of $p=0.0009$, Table V).

Discussion

NAC of GC represents an important but complex topic. Given the promising but not satisfactory results of adjuvant therapy, in the last decade NAC has been adopted worldwide for its theoretical potential to shrink tumor volume, increase the rate of tumor-free surgical margins, and to destroy microscopic disease that is already present at distant sites (12). Such a successful spread followed the publication of 2 famous randomized controlled trials (RCTs) on GC NAC, the MAGIC and FNCLCC-FFCD trials (9, 10). Upon initial appraisal, both studies showed a survival advantage of patients treated with NAC plus curative surgery, compared to surgery alone. However, a closer examination of patients' enrolment and assignation demonstrated a heterogeneity of primary site location (75% GCs, 25% between lower esophagus and junctional cancers), a greater proportion on

Figure 1. The Kaplan-Meier method demonstrated different overall survival (OS) between patients with (yGLI) and without (yGL0) free-floating tumor cells in their gastric lavage fluid.

initial cancers in the NAC-surgery leg compared to surgery alone, and a low percentage (42%) of adequate lymph node dissection (D2 lymphadenectomy) in surgery alone group, in MAGIC study. In FNCLCC-FFCD study, only 25% of patients had a tumor located in the stomach (64% junctional and 11% lower esophagus cancers) and results from D2 dissection performed in both arms were not provided (9, 10). Finally, the survival benefit resulted stronger for lower esophagus and gastroesophageal (MAGIC trial) or gastroesophageal cancers only (FNCLCC-FFCD trial) rather than the proper GCs (12). In the light of the reported data, it appears evident that adoption of NAC for GC has been introduced without a clear and evidence-based medicine-related demonstration (12). Furthermore, the disadvantage of NAC for GC may be over-diagnosis; in the MAGIC trial, in fact, the target patients had clinical stage II disease, but all patients had clinical T2 tumors (14). Moreover, the present prospective study on a small number of patients also included both junctional and proper gastric lesions. As a consequence, further RCTs with a more precise GC patient selection are required to assess the efficacy of NAC in this type of malignancy. Besides, there is also the need for new systems to determine the real efficacy of NAC. The current yCRH parameters (such as serum tumor antigens, histologic TRG, RECIST criteria of conventional image studies, PERCIST criteria from functional imaging, repeat staging laparoscopy) undoubtedly help compare initial with post-treatment patients' disease (15, 25). However, since these parameters focus on a specific field of investigation, they do not offer a global appraisal of patient condition. In this manner, when they provide contradictory results, evaluation of the treated

Figure 2. Kaplan-Meier curves illustrating different overall survival (OS) (A), progression-free survival (PFS) / disease-free survival (DFS) (B) and time to tumor progression (TTP) (C) for gastric cancer (GC) patients who showed deficiency (yAnemia) or physiological levels of hemoglobin (yHgb normal count) after neoadjuvant treatment.

patients becomes more complicated and creates confusion even for prognostic prediction (31-36). With this regard, several reliable tissue and serum markers have already been identified, or are still under investigation, as predictors of the response of GC patients to NAC (13, 16-18, 37-40). In our study we suggest 2 further pertinent tests for post-NAC reassessment and prognostic prediction of patients with resectable GC: the yGL and yAnemia clinicopathological features. As previously demonstrated, GL represents an excellent source of GC cytologic and molecular biomarkers (such as free malignant cells, microRNAs and long non-coding RNAs) because these are directly released by the

tumor without being eliminated by the liver (41). Moreover, yGL cytomicular analysis has provided interesting results in terms of classification, staging, screening and prognosis of this cancer (42-44). As shown in our study, the yGL status also appears to be an important and useful parameter to be applied to the clinicopathologic re-appraisal of GC patients following NAC. In fact, at univariate analysis, yGL1 statistically correlated with OS and yAnemia (Table III) and, moreover, at multivariate analysis it resulted an independent predictive factor of OS (Table IV), PFS and DFS (Table V). Interestingly, logistic regression analysis showed that yGL could also be adopted as an auxiliary system of TRG and post-NAC re-

Table IV. Multivariate analysis of predictive factors for overall survival (OS).

Independent variables	b	SE	Wald	p-Value	Exp(b)	95% CI of EXp(b)
yGL	3.9899	1.8724	4.5410	0.0331	54.0514	1.3773-2121.2673
yAnemia	3.5728	1.7077	4.3775	0.0364	35.6175	1.2534-1012.1625
Response to NAT	0.7324	2.0480	0.1279	0.7206	2.0801	0.0376-115.1763
PL-5FU-based NAC	-3.2553	3.1549	1.0647	0.3022	0.0386	0.0001-18.6959
AT	-2.4976	4.2078	0.3523	0.5528	0.0823	0.0000-314.1089
Re-hospitalization	4.9843	3.4080	2.1390	0.1436	146.1027	0.1835-116309.7658
Re-surgery	4.7664	5.0731	0.8828	0.3474	117.5005	0.0056-2445258.7179
Operation time	0.0314	0.0227	1.9185	0.1660	1.0319	0.9871-1.0787
PLS	-0.0097	0.0588	0.0274	0.8685	0.9903	0.8824-1.1114
yR	7.3884	3.0008	6.0620	0.0138	1617.0924	4.5121-579546.0347
yM	-1.8734	1.8575	1.0171	0.3132	0.1536	0.0040-5.8554
yStage	0.2678	2.3556	0.0129	0.9095	1.3070	0.0129-132.2497

Overall model fit: $p=0.0014$

b, Regression coefficient beta; SE, standard error; Wald, b/SE²; Exp(b), equals e to the power of each beta value; CI, confidence interval; yGL, presence of free malignant cells in gastric lavage fluid after NAT (or NAC alone); NAT, neoadjuvant treatment; PL-5FU, platinum-fluorouracil; NAC, neoadjuvant chemotherapy; PLS, postoperative length of stay; yR, status of surgical margins after NAT (or NAC alone); yM, distant metastases after NAT (or NAC alone). *p*-Values written in bold are statistically significant ($p \leq 0.05$).

Table V. Multivariate analysis of predictive factors for progression-free survival (PFS) and disease-free survival (DFS).

Independent variables	B	SE	Wald	p-Value	Exp(b)	95% CI of EXp(b)
yGL	4.1291	1.8903	4.7716	0.0289	62.1235	1.5284-2525.1439
yAnemia	3.7679	1.6679	5.1033	0.0239	43.2911	1.6467-1138.0858
Response to NAT	0.6542	2.0473	0.1021	0.7493	1.9237	0.0348-106.3753
PL-F based NAC	-3.0438	3.1266	0.9477	0.3303	0.0477	0.0001-21.8523
AT	-2.2976	4.1623	0.3047	0.5809	0.1005	0.0000-350.9034
Re-hospitalization	5.0883	3.3875	2.2563	0.1331	162.1141	0.2120-123967.9200
Re-surgery	4.4407	4.9935	0.7908	0.3738	84.8354	0.0048-1510647.4292
Operation time	0.0323	0.0226	2.0354	0.1537	1.0328	0.9880-1.0796
PLS	-0.0068	0.0585	0.0136	0.9070	0.9932	0.8856-1.1138
yR	7.4337	2.9825	6.2123	0.0127	1692.1010	4.8942-585025.8296
yM	-1.9559	1.8792	1.0833	0.2980	0.1414	0.0036-5.6260
yStage	0.2856	2.3750	0.0145	0.9043	1.3306	0.0127-139.8511

Overall model fit: $p=0.0009$

b, The regression coefficient beta; SE, standard error; Wald, b/SE²; Exp(b), equals e to the power of each beta value; CI, confidence interval. *p*-Values written in bold are statistically significant (≤ 0.05).

evaluation. Concerning hypohemoglobinemia, most previous studies dealt with pre-NAC, pre- or post-surgery anemia: such a feature often correlated with histologic TRG and was demonstrated as an important predictor of postoperative survival for GC patients (45-47). Conversely, post-NAC Hgt levels as pathoprognostic factor (yAnemia) have been rarely investigated (48, 49). In our study, yAnemia patients had significantly shorter OS, PFS-DFS, and TTP, compared to those with normal Hgb count (Figure 1). Furthermore, at multivariate analysis, this category was an independent predictive factor of OS (Table IV), and PFS-DFS (Table V).

Conclusion

The yGL and yAnemia, two non-conventional markers, can help optimize re-evaluation and prognostic prediction of patients with resectable GC submitted to NAC. In fact, yGL1 as well as yAnemia were associated with a poorer survival. More specifically, both markers were shown as independent predictive factors of poor OS, PFS and DFS (Table V). Despite its limitations, our study is the first to provide significant evidence about the application of yGL and yAnemia parameters in the prognosis of GC patients after NAC. Future

studies are warranted to evaluate yGL and yAnemia in larger cohorts of GC patients, investigating both pre-NAT/pre-operative and post-NAT/post-operative disease and preferably distinguishing junctional from proper gastric lesions.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

All the Authors agreed with the content of the article. Dr. Virgilio conceived of the presented research. Dr. Virgilio, Dr. Fegiz and Dr. Mercantini performed the clinical part of the research. Dr. Giarnieri, Mrs. Montagnini and Prof. Giovagnoli performed the cytopathological analysis. Dr. Proietti and Dr. D'Urso contributed to the interpretation of the results. Dr. Virgilio wrote the manuscript and performed the statistical analyses. Dr. Balducci and Dr. Cavallini helped supervise the entire project.

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Received December 25, 2018

Revised January 17, 2019

Accepted January 22, 2019