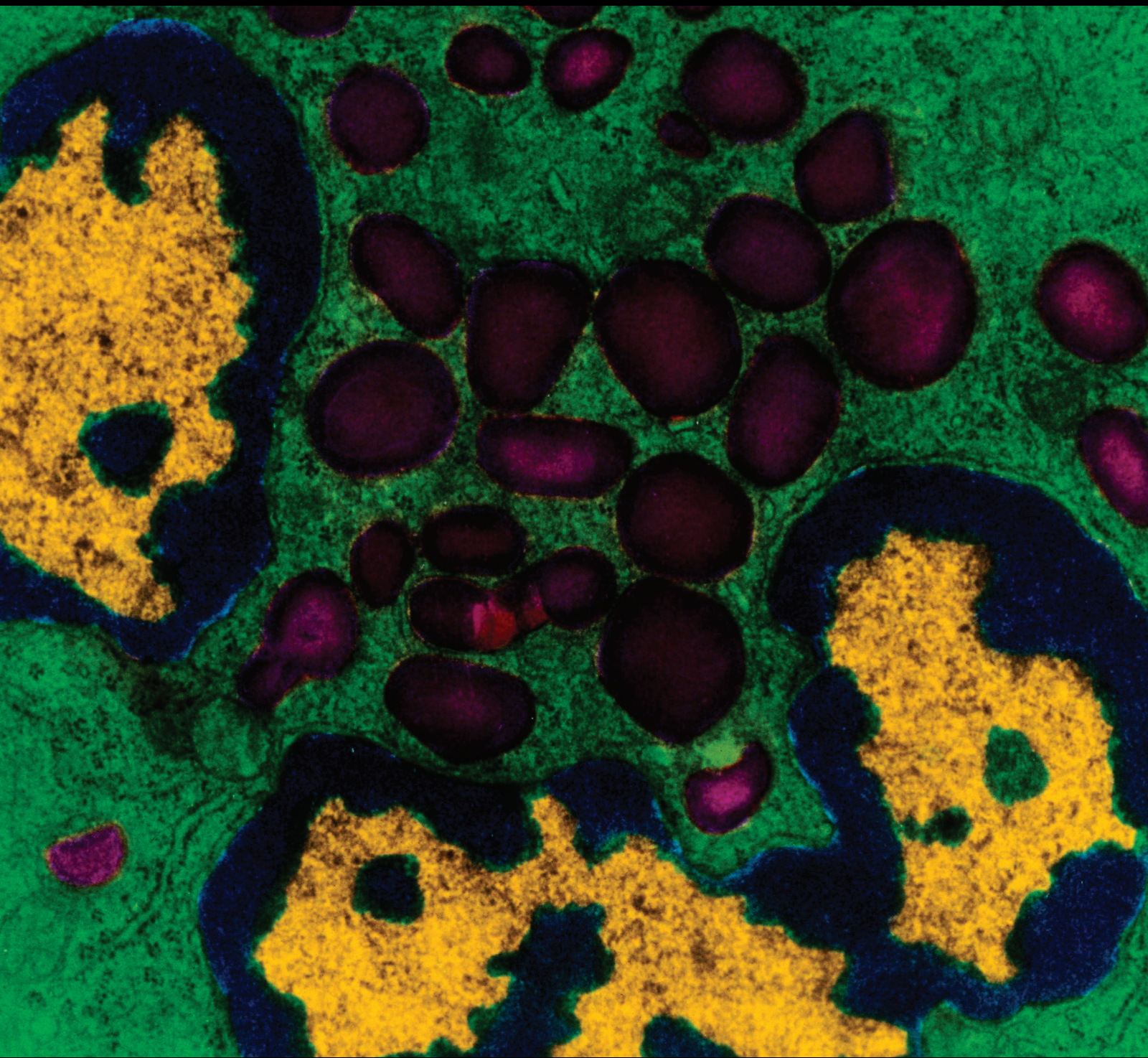
Mediators of Inflammation

Inflammatory Biomarkers in Cancer

Guest Editors: Tomoki Nakamura, Czar L. Gaston, Krishna Reddy, Shintaro Iwata, and Jun Nishio



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Editorial

Inflammatory Biomarkers in Cancer

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In this special issue, we investigate the relationship between inflammation and cancer. Inflammation plays a critical role in the progression of cancers. Several biomarkers have been reported as useful marker for clinical cancer behavior. In this issue, authors reported possible biomarker for the development and prognosis of several cancers including esophageal cancer, breast cancer, colorectal cancer, ovarian cancer, and myelofibrosis.

P.-C. Chen and J.-F. Feng looked at 323 patients with resectable esophageal squamous cell carcinoma (ESCC) and proposed a prognostic staging system for cancer specific survival based on markers of inflammation. Patients with high levels of C-reactive protein, neutrophil to lymphocyte ratio, and platelet count to lymphocyte ratio (stage I3) had significantly poorer survival compared to patients with one or more of these inflammatory parameters within normal limits. The predictive value of their system was maintained in different TNM stages of ESCC and would be a useful tool for clinicians.

M. Zajkowska et al. compared levels of VEGF, M-CSF, and CA 15-3 of 120 patients with breast carcinoma with a control group composed of 60 patients with benign breast tumors and 60 healthy volunteers. They found that expression of VEGF had the highest sensitivity and specificity in differentiating stage 1 breast cancer from the control group, while a combination of VEGF and CA 15-3 had the highest sensitivity and specificity in detecting stage III and stage IV breast cancer. Their results contribute to the growing evidence for the utility of these biomarkers as diagnostic tools in breast cancer.

M. Rutka et al. evaluate the diagnostic accuracy of five different fecal markers for detection of precancerous and cancerous lesions of the colorectum in a prospective, colonoscopy study and report sensitivity and specificity of each of these markers. They established that sensitivity of M_2 Pyruvate Kinase, iFBOT, and Hb/Hp complex to be high in colorectal carcinoma but decreased in adenomas ≥ 1 cm in size. They recommend combined use of M_2PK, iFBOT, and FC for detecting larger adenomas. The authors suggest these noninvasive fecal screening tests for low risk patients or as a part of a two-step screening process with colonoscopy being the gold standard.

Z. Liu et al. addressed whether the expression of Notch3, a type of Notch receptor which activates the PI3K/Akt/mTOR signaling pathway, and ribosomal S6 kinase (S6K), a downstream effector of the PI3K/Akt/mTOR pathway, correlated with the clinical features and prognosis in ovarian epithelial cancer. Their results showed that Notch3 and pS6K expression associated with clinical stage and pathological grading, resulting the association with poorer survival. They concluded that Notch3 and pS6K are potential biomarkers and therapeutic targets in ovarian epithelial cancer.

D. Sollazzo et al. investigated whether the concentration of circulating calreticulin in plasma calreticulin in healthy subjects and in patients with myelofibrosis (MF) differs. The authors demonstrated that the concentration of calreticulin is higher in patients with MF compared to healthy subjects. In contrast, circulating calreticulin levels and mutation status or JAK2V617F burdens were not correlated. The authors

conclude that high circulating calreticulin levels in MF reflects chronic systemic inflammation.

We hope that researchers enjoy the reading of this special issue related to inflammation and biomarkers in cancer. Undoubtedly, the presence of an association between systemic inflammation and a poor prognosis has been established.

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Research Article

Circulating Calreticulin Is Increased in Myelofibrosis: Correlation with Interleukin-6 Plasma Levels, Bone Marrow Fibrosis, and Splenomegaly

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Myelofibrosis (MF) is a clonal neoplasia of the hemopoietic stem/progenitor cells associated with genetic mutations in the Janus kinase 2 (*JAK2*), myeloproliferative leukemia virus oncogene (*MPL*), and calreticulin (*CALR*) genes. MF is also characterized by a state of chronic inflammation. Calreticulin (CRT), as a multifunctional protein, is involved in a spectrum of cellular processes including inflammation, autoimmunity, and cancer initiation/progression. Based on this background, we hypothesised that in MF circulating CRT might reflect the inflammatory process. In the present study we show that circulating CRT is increased in MF patients compared to healthy controls. Also, in MF, CRT levels highly correlate with bone marrow fibrosis, splenomegaly, and Interleukin-6 (IL-6) plasma levels. In turn, higher IL-6 levels also correlated with disease severity in terms of increased spleen size, bone marrow fibrosis, number of circulating $CD34^+$ cells, and lower hemoglobin values. These results demonstrate that the circulating CRT takes part in the inflammatory network of MF and correlates with aggressiveness of the disease.

1. Introduction

Myelofibrosis (MF) is a Philadelphia-negative myeloproliferative neoplasm (Ph-neg MPN) that may arise de novo (Primary Myelofibrosis, PMF) or after Essential Thrombocythemia (ET; PET-MF) and Polycythemia Vera (PV; PPV-MF). MF is a rare blood cancer with an incidence of about 0.58 new cases per 100.000 people per year but with higher prevalence because of a chronic and disabling course leading always to death due to progression and disease-related or treatment-related complications. It is a clonal disorder of the hemopoietic stem/progenitor cells which is clinically characterized by worsening constitutional symptoms, progressive splenomegaly, bone marrow (BM) fibrosis, and cytopenias as well as by an increased risk to develop thrombotic complications and acute leukemia [1–3].

Driver mutations in Janus kinase 2 (*JAK2*), calreticulin (*CALR*), and myeloproliferative leukemia virus oncogene (*MPL*) have been reported. However, less than 10% of patients have nonmutated *JAK2*, *MPL*, and *CALR* genes ("triple-negative"). Regardless of molecular status, all patients have dysregulation in the JAK/STAT signaling [2–6].

Together with molecular abnormalities, MF is characterized by abnormal expression of several proinflammatory and immunoregulating cytokines secreted by activated leukocytes and platelets/megakaryocytes. This inflammatory microenvironment has emerged as a key player in MF pathogenesis [7–12].

Physiologically, CRT was first described as an endoplasmic reticulum protein responsible for Ca^{2+} homeostasis and glycoprotein folding; currently, CRT is recognized as a multifunctional chaperone detected in other cellular compartments, as well as extracellularly, where it is involved in cell proliferation, phagocytosis, apoptosis, adhesion, and innate and adaptive immune processes including cancer cell elimination by immunogenic cell death and fibrosis [13]. CRT overexpression is linked to various pathological conditions including chronic inflammatory diseases, autoimmunity,

fibrosis-related disorders, and malignant evolution [14–18]. In MF, the mutated CRT protein was found to constitutively activate the MPL receptor signaling [19, 20].

Given the CRT involvement in inflammation, fibrosis, and cancer, we hypothesised that in MF circulating CRT might reflect the inflammatory process. Here, we characterized the circulating CRT levels of MF patients. Moreover, we investigated the correlation between CRT levels and various clinical and laboratory parameters.

2. Materials and Methods

2.1. Study Population. Peripheral blood (PB) was obtained from 30 patients with MF in chronic phase and from 10 healthy age-matched volunteers. The diagnosis of MF was made according to WHO 2008 criteria [21]. Patients and controls provided written informed consent for the study. This study was approved by the medical Ethical Committee of the University Hospital of Bologna and was conducted in accordance with the Declaration of Helsinki.

2.2. Assay of Circulating Proteins. Here we analyzed the plasma levels of CRT in patients/controls. EDTA-anticoagulated PB was centrifuged for 15 minutes at 1000 ×g within 30 minutes of collection. The plasma was then collected and stored at −80°C until quantification. CRT was evaluated by a commercially available ELISA assay (Cusabio Biotech Co., Wuhan, China), according to the manufacturer's instructions. Briefly, a standard curve of 100 μL aliquots of known concentrations of recombinant CRT was run and triplicate 100 μL samples were added to the wells. CRT binding was detected using a biotin/avidin system. The plates were then assessed by ELISA on a plate reader at 450 nm. The Ciraplex™ immunoassay kit/Human 9-Plex Array (Aushon BioSystems, Billerica, MA, USA) was used for the measurement of circulating IL-6 and TNF-α.

2.3. Molecular Pattern. Molecular analyses were assessed at diagnosis or before treatment's start on DNA obtained from granulocytes. Driver mutations were analyzed as previously described [22]. Specifically, $JAK2^{V617F}$ mutation was evaluated with ipsogen *JAK2* MutaQuant Kit. The percentage of mutant $JAK2^{V617F}$ allele was expressed as the ratio of $JAK2^{V617F}$ copies to total copy number (CN) of *JAK2* (CN of $JAK2^{V617F}$ + CN of *JAK2* wild type). *CALR* exon 9 sequencing was performed by Next Generation Sequencing (NGS) approach with GS Junior (Roche-454 platform); analysis was carried out with AVA Software (GRCh38 as referenced). Rare *CALR* mutations identified by NGS were confirmed by Sanger sequencing. *MPL* mutations were investigated by ipsogen *MPL W515K/L* MutaScreen Kit and by Sanger sequencing (for *MPLS505N* and other secondary exon 10 mutations).

2.4. Cytogenetic Analysis. Chromosome banding analysis was performed on BM cells by standard banding techniques according to the International System for Human Cytogenetic Nomenclature [23]. At least 20 metaphases were required. Unfavorable karyotype was defined according to the Dynamic International Prognostic Score System (DIPSS) plus [24] and included complex karyotype or single or two abnormalities including +8, −7/7q-, i(17q), −5%5q-, 12p-, inv(3), or 11q23 rearrangement.

2.5. Statistical Analysis. Statistical analyses (Wilcoxon test and Spearman correlation analysis) were performed using GraphPad (GraphPad Software Inc., La Jolla, CA). All p values were considered statistically significant when $p \leq 0.05$ (two-tailed).

3. Results

A total of 30 MF patients were investigated: $JAK2^{V617F}$- (16 cases), *CALR*- (10 cases), and *MPL*- (3 cases) mutated. One patient was triple-negative. *CALR*-mutated patients were type 1 (8 cases) and type 2 (2 cases). Patients characteristics are shown in Table 1. Fifteen patients were at diagnosis. Thirteen patients received previous therapies for MF (hydroxyurea (10 cases) and ruxolitinib (3 cases)); however, at the time of the study, they were untreated for at least 2 months.

As shown in Figure 1(a), we found significantly higher CRT plasma levels in MF patients as compared with healthy subjects (median, 5.2 ng/mL, and range, 1.4–25, versus median, 1.8 ng/mL, and range, 1.2–3.7; $p = 0.0028$). Comparing CRT plasma levels of $JAK2^{V617F}$ and *CALR*-mutated patients, no significant differences were observed (Figure 1(b)). Even though few patients were studied, CRT plasma levels of MPL-mutated and triple-negative patients were superimposable to the other MF patients (Figure 1(b)). CRT plasma levels of patients at diagnosis were not significantly different from those of the other patients. No correlation was observed between circulating CRT levels and hemoglobin levels, white blood cells/platelets count, and circulating $CD34^+$ cells number.

Along with CRT plasma levels, circulating TNF-α (median: 2.62 pg/mL; range: 0.05–9.37) and IL-6 (median: 33.3 pg/mL; range: 8.7–258.9) were also increased in MF patients as compared to healthy subjects (median, 0.26 pg/mL, and range, 0–0.84, and median, 6.37 pg/mL, and range, 4.5–32.8, resp.; $p = 0.008$) (Figures 1(c) and 1(d)). TNF-α and IL-6 plasma levels were not affected by mutational status and allele burden (data not shown). Interestingly, in MF, irrespective of patients being at diagnosis or not, there was a positive correlation between the plasma levels of CRT and BM fibrosis ($p = 0.038$; $r = 0.39$), splenomegaly ($p = 0.0089$; $r = 0.47$), and circulating IL-6 ($p = 0.028$; $r = 0.42$) (Figures 2(a), 2(b), and 2(c)). This correlation was also irrespective of mutational status (comparing $JAK2^{V617F}$-mutated and *CALR*-mutated patients). In turn, IL-6 plasma levels correlated with BM fibrosis ($p = 0.0056$; $r = 0.49$), splenomegaly ($p = 0.018$; $r = 0.46$), and the number of circulating $CD34^+$ cells ($p = 0.029$; $r = 0.48$) and correlated negatively with hemoglobin values ($p = 0.047$; $r = -0.39$; Figures 3(a), 3(b), 3(c), and 3(d)).

4. Discussion

There has been a lack of understanding regarding the role of soluble CRT in MF. The first result of the study is

Table 1: Patients characteristics according to mutational status. Compared to CALR-mutated patients, patients with $JAK2^{V617F}$ mutation were older ($p = 0.01$) and had higher hemoglobin levels ($p = 0.04$).

Characteristics	$JAK2^{V617F}$-mutated patients (number = 16)	CALR-mutated patients (number = 10)	MPL-mutated patients (number = 3)	"Triple-negative" patients (number = 1)
Median age, years (range)	73 (67–84)	65.5 (44–82)	76 (72–76)	67
Male sex, number (%)	9 (56)	6 (60)	1 (33.3)	0 (0)
Median allele burden, % (range)	89 (0.4–99)	56.5 (52–98)	NA	NA
Median WBC, $\times 10^9$/L (range)	10.6 (2.5–157.6)	7.15 (2.3–48.3)	6.2 (4.9–25)	6.7
Median hemoglobin, g/dL (range)	11.1 (8.6–15.1)	9.3 (7.7–14)	9.7 (7.2–9.9)	8.5
Median platelet count, $\times 10^9$/L (range)	280 (41–507)	195.5 (86–419)	196 (46–303)	632
High/intermediate 2 IPSS category, number (%)	9 (56)	6 (60)	3 (100)	1 (100)
Unfavorable karyotype, number (%)	8 (50)	2 (20)	2 (66.6)	1 (100)
Diagnosis, number (%)				
PMF	8 (50)	6 (60)	3 (100)	1 (100)
PET	3 (19)	2 (20)	—	—
PPV	5 (31)	2 (20)	—	—
BM fibrosis grade ≥ 2, number (%)	12 (75)	10 (100)	2 (66.6)	0 (0)
Patients with splenomegaly, number (%)	14 (87.5)	8 (80)	3 (100)	0 (0)

WBC: white blood cells; IPSS: International Prognostic Scoring System; PMF: primary myelofibrosis; NA: not available. Splenomegaly was evaluated by palpation as cm below costal margin. Only patients with a spleen palpable ≥ 5 cm below costal margin by palpation were considered as carrying splenomegaly.

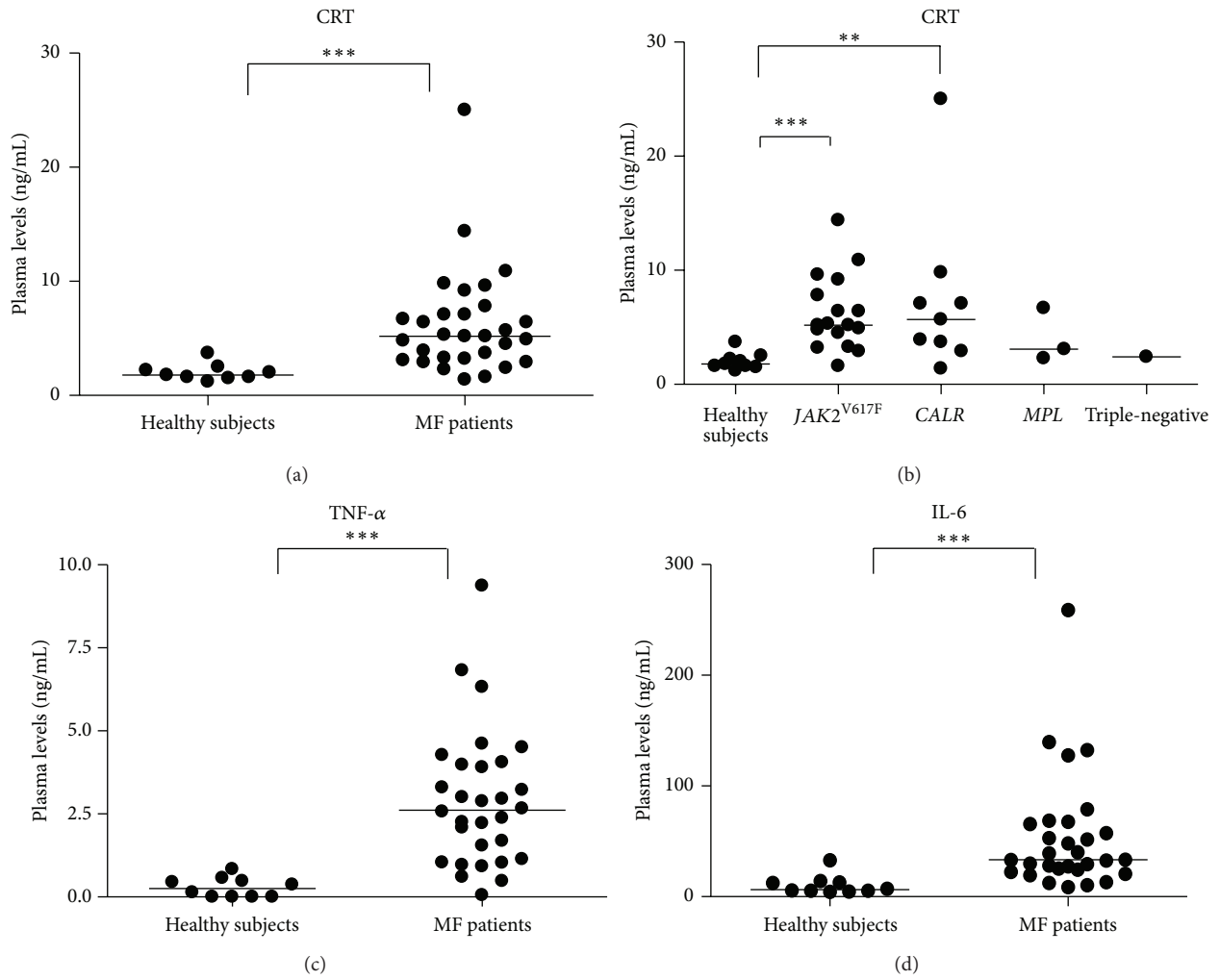

Figure 1: *Analysis of the circulating levels of CRT, IL-6, and TNF-α proteins*. The CRT plasma levels of total MF (a) or MF subdivided into *JAK2*^{V617F}-mutated ($n = 16$), *CALR*-mutated ($n = 10$), *MPL*-mutated ($n = 3$), and triple-negative-mutated ($n = 1$) groups (b) were measured by ELISA. Compared with age-matched controls (HD; $n = 10$), CRT plasma levels were significantly increased in MF patients ($p = 0.0028$). Of note, there was no significant difference between the mutated groups. Irrespective of mutational status, TNF-α (c) and IL-6 (d) blood plasma levels were also increased in MF ($p = 0.008$). For all graphs, one symbol represents one individual, and the height of the bar represents the median value. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

that in MF CRT plasma levels are increased compared to healthy controls. CRT has been found to have a preferential expression in megakaryocyte/platelets either from normal subjects or from patients with Ph-neg MPN (and regardless of mutation status) [25]. Therefore, these cells, which show abnormal number/function in MF, are likely to be the major contributors to the augmented amount of circulating CRT. Previous studies support the hypothesis that extracellular and soluble CRT is mainly released from dead, dying, or inflamed/stressed cells [13–16]. Consequently, the high CRT levels detected in MF may primarily be due to the chronic inflammatory state that characterizes both the marrow and peripheral niches and reflect impairment in tissue homeostasis.

The second result is that CRT plasma levels are equally increased in JAK2^{V617F}-mutated and CALR-mutated MF patients. In this study, we used an antibody that is directed against the N terminus of CRT and is expected to label both mutated and unmutated proteins. Therefore, the circulating protein that was detected in *CALR*-positive patients is likely to be the sum of mutated (hemopoietic restricted) and unmutated molecules. This datum may suggest that the acquisition of mutations in the *CALR* gene, although causing the hyperactivation of the MPL receptor [19, 20], does not induce an increased circulating CRT amount.

Herein, we therefore demonstrated that CRT protein levels were found to directly correlate with the clinical aggressiveness of the disease in terms of larger spleen size and more severe marrow fibrosis. In addition, we found a direct correlation between circulating plasma levels of CRT and IL-6, one of the most potent proinflammatory cytokines which is upregulated in MF [26]. In turn, higher IL-6 levels also correlated with disease severity in terms of increased spleen size, marrow fibrosis, number of circulating CD34^{+} cells,

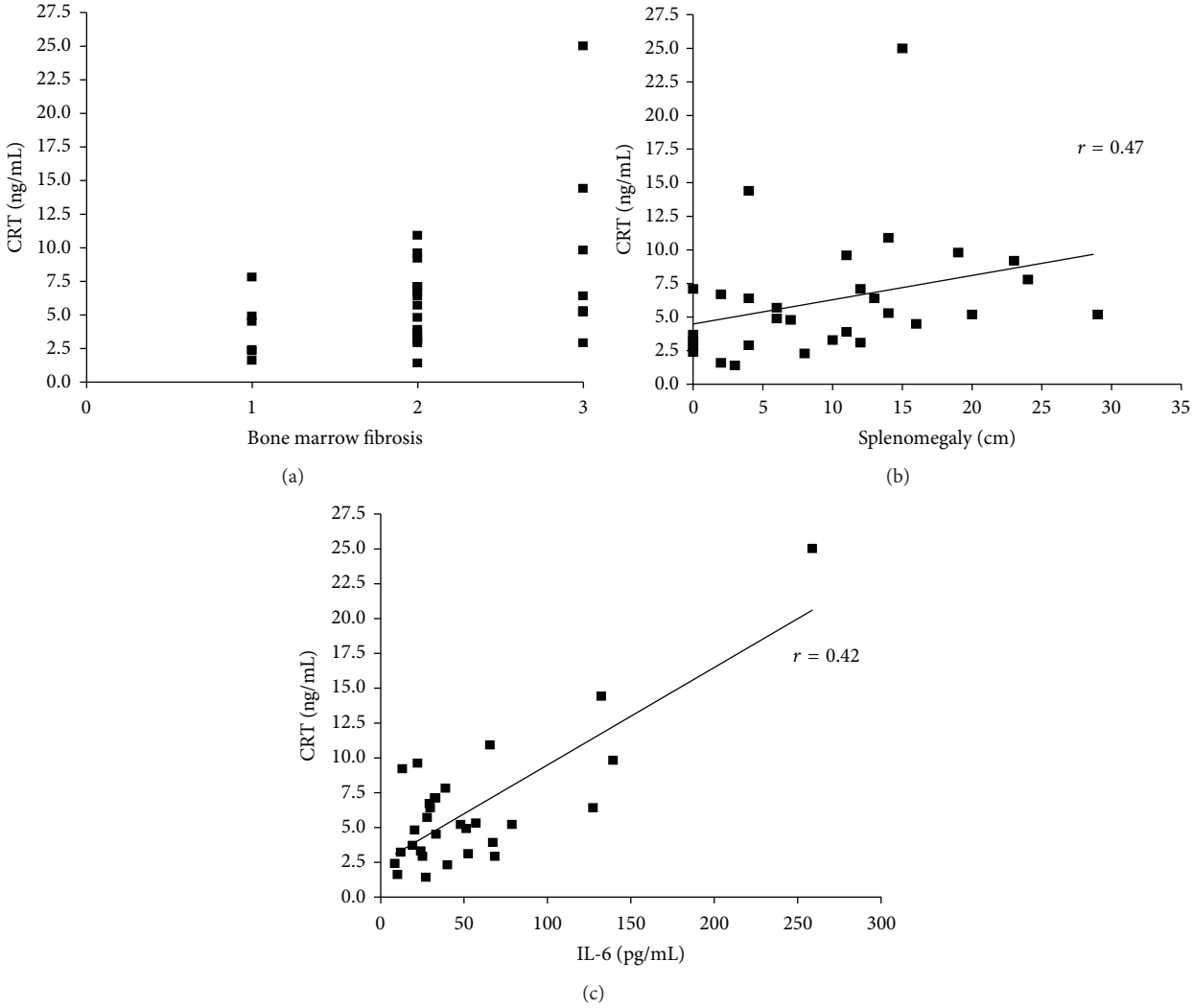

Figure 2: *Correlation between CRT plasma levels and BM fibrosis, splenomegaly, and circulating IL-6*. Circulating CRT positively correlates with fibrosis, splenomegaly, and soluble IL-6 in MF. Scatterplots demonstrating correlation between the plasma levels of CRT and BM fibrosis ($p = 0.038$; $r = 0.39$), splenomegaly ($p = 0.0089$; $r = 0.47$), and circulating IL-6 ($p = 0.028$; $r = 0.42$) in MF patients (a, b, and c, resp.) are shown. x-axis of (a) shows BM fibrosis scale.

and lower hemoglobin values. Even though these correlations were weak (r always below 0.5), all together point out the involvement of CRT in the inflammatory network and in disease aggressiveness. The correspondence between CRT and IL-6 plasma levels may be at least partially justified by the recent discovery that soluble CRT induces active mRNA transcription through MAPK and NF-kB activation in macrophages, thereby augmenting their IL-6 and TNF-α production [27]. In addition, recently, conditioned media from cells expressing type I mutant *CALR* have been shown to exaggerate cytokine production from normal monocytes [28]. It is therefore likely that in MF the increased circulating CRT may contribute to the disease-related inflammation/fibrosis through positively enhancing IL-6 production. By contrast, despite the fact that TNF-α is a negative regulator of CRT expression [29], no correlation was observed between circulating CRT and TNF-α plasma levels in our MF patients, suggesting the presence of a TNF-α-independent mechanism of regulation.

Taken together, our data highlight the role of this protein in the inflammatory network of MF. A mutual interaction among CRT and other inflammatory cytokines including IL-6 may indeed contribute to the generation/maintenance of inflammation/fibrosis of MF.

Potential limitation of the present study is the small sample size of patients. Nonetheless, our data create the rational basis for future studies investigating the role of circulating CRT in the inflammatory network of MF and other Ph-negative MPNs in larger cohorts of patients. Notably, due to correlation with fibrosis and splenomegaly, circulating CRT measurement may be useful in clinical practice.

5. Conclusion

We conclude that the elevated plasma CRT levels in MF patients parallel the degree of disease activity and inflammatory state. This may identify patients with more severe disease who might benefit from tailored therapy.

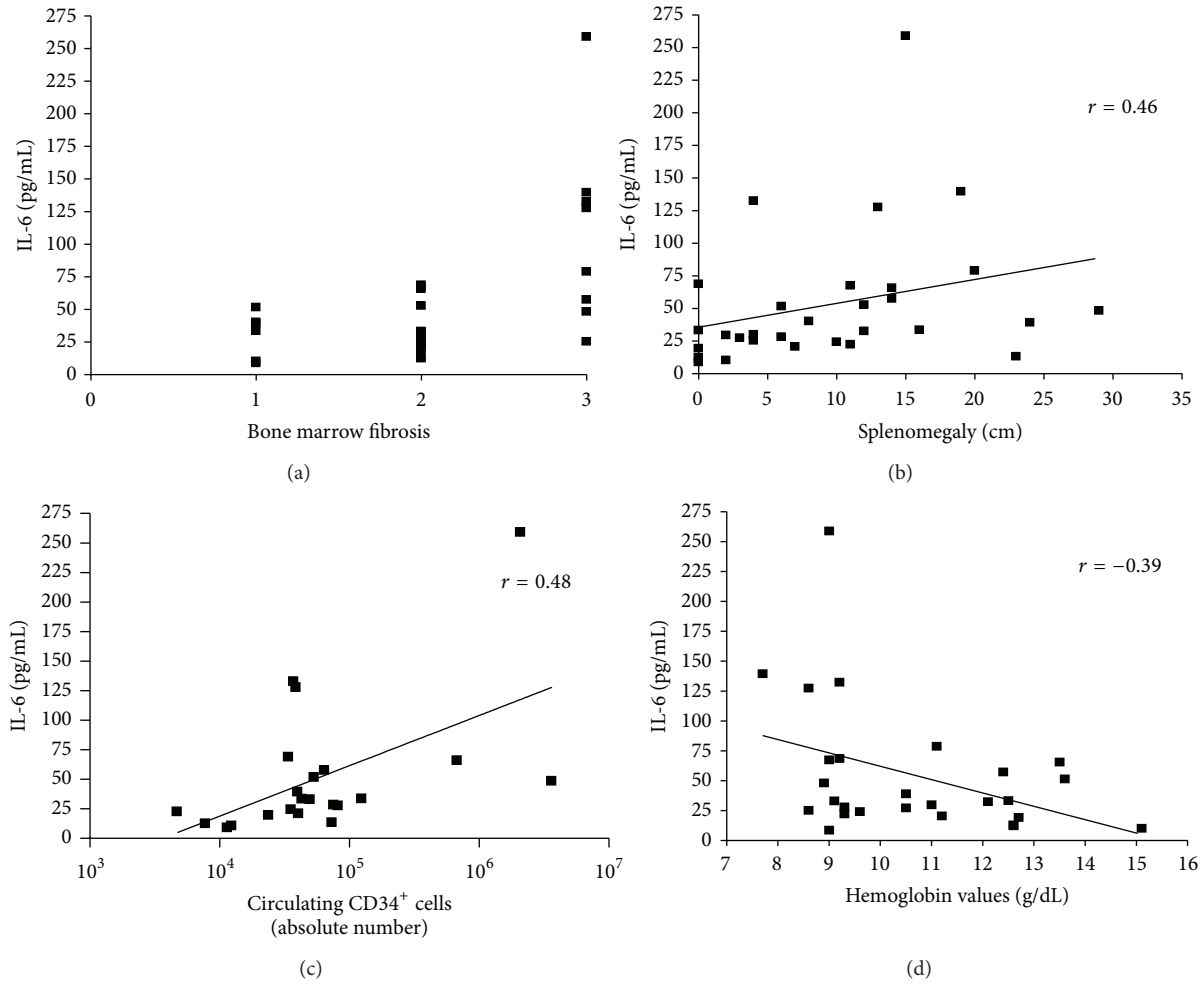

Figure 3: *Correlation between IL-6 plasma levels and BM fibrosis, splenomegaly, number of circulating CD34$^+$ cells, and hemoglobin values.* Irrespective of mutational status, IL-6 plasma levels correlated with BM fibrosis ($p = 0.0056$; $r = 0.49$), splenomegaly ($p = 0.018$; $r = 0.46$), and the absolute number of circulating CD34$^+$ cells ($p = 0.029$; $r = 0.48$) and negatively correlated with hemoglobin values ($p = 0.047$; $r = -0.39$); (a, b, c, and d, resp.). x-axis of (a) shows BM fibrosis scale.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Daria Sollazzo and Lucia Catani designed the study; Nicola Polverelli, Nicola Vianelli, and Francesca Palandri recruited the patients and coordinated their samples collection; Daria Sollazzo, Dorian Forte, Margherita Perricone, and Marco Romano performed the laboratory work and analyzed data; Nicola Vianelli and Michele Cavo supervised the study; Daria Sollazzo, Lucia Catani, and Francesca Palandri wrote the manuscript. Daria Sollazzo, Dorian Forte, Francesca Palandri, and Lucia Catani contributed equally to this work.

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Research Article

Diagnostic Power of Vascular Endothelial Growth Factor and Macrophage Colony-Stimulating Factor in Breast Cancer Patients Based on ROC Analysis

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Breast cancer (BC) is the most common malignancy in women. Vascular endothelial growth factor (VEGF) has been described as an important regulator of angiogenesis which plays a vital role in the progression of tumor. Macrophage colony-stimulating factor (M-CSF) is a cytokine whose functions include regulation of hematopoietic lineages cells growth, proliferation, and differentiation. We investigated the diagnostic significance of these parameters in comparison to CA15-3 in BC patients and in relation to the control group (benign breast tumor and healthy women). Plasma levels of the tested parameters were determined by ELISA and CA15-3 was determined by CMIA. VEGF was shown to be comparable to CA15-3 values of sensitivity in BC group and, what is more important, higher values in early stages of BC. VEGF was also the only parameter which has statistically significant AUC in all stages of cancer. M-CSF has been shown to be comparable to CA15-3 and VEGF, specificity, and AUC values only in stages III and IV of BC. These results indicate the usefulness and high diagnostic power of VEGF in the detection of BC. Also, it occurred to be the best candidate for cancer diagnostics in stages I and II of BC and in the differentiation between BC and benign cases.

1. Introduction

Breast cancer (BC) is an important health problem worldwide. Each year the incidence rate of this disease increases significantly. In 2015, only in the United States about 231,840 women were diagnosed with BC and 40,290 of them died [1]. This disease may appear at any age, yet a particularly high risk is related to females after 50 years of age, what is correlated with menopausal hormonal changes [2].

The crucial factor influencing a successful treatment and survival rate of BC patients is early diagnosis. Biochemical detection of this tumor is nowadays based on markers such as CA 15-3, CEA, and CA 27.29 [3]. In view of their insufficient specificity and sensitivity at the initial type of BC, scientists around the world perform intensive research to find better biomarkers whose levels would correlate with the presence and stage of the studied disease. We assumed that these factors may be cytokines: vascular endothelial growth factor (VEGF) and macrophage colony-stimulating factor (M-CSF).

VEGF has been described as an important regulator of angiogenesis, a crucial process of tumor invasion and progression [4]. Significantly increased levels of VEGF have been found in the serum or plasma of patients suffering from breast and gynecological tumors, for example, ovarian or cervical, as well as other kinds of cancers [5]. The *in vitro* and *in vivo* studies performed so far presented that the overexpression of this cytokine leads to cancer growth and metastasis, while the inhibition of VEGF resulted in the suppression of tumor development [6].

In contrast, M-CSF is a cytokine whose functions include regulation of hematopoietic lineages cells growth, proliferation, and differentiation [7]. M-CSF is produced pathologically by cancer cells. The overexpression of this cytokine has been detected in a variety of tumors, female reproductive tract cancers and breast, renal, colorectal, pancreatic, prostate, and head and neck tumors, and has been correlated with poor prognosis [8, 9]. What is interesting, circulating level of M-CSF has been found to be useful as a method of estimating patients' survival rates.

As VEGF and M-CSF play a significant role in carcinogenesis, the aim of the present study was to investigate the diagnostic power of the selected cytokines and a comparative marker CA 15-3 in breast tumor detection.

In this paper, the use of healthy volunteers and women with benign breast lesions together as a one control group better reflects the current population of women. The data obtained in this work may prove the usefulness of the analyzed parameters (separately and together) in the detection of BC, as a new diagnostic panel.

2. Materials and Methods

2.1. Patients. Table 1 shows the tested groups. The study included 120 breast cancer (BC) women diagnosed by the oncology group. The breast cancer patients were treated in the Department of Oncology, Medical University of Bialystok, Bialystok, Poland. Tumor classification and staging were done in accordance with the International Union Against Cancer Tumor-Node-Metastasis (UICC-TNM) classification. The breast cancer histopathology was established in all cases by tissue biopsy of mammary tumor or after surgery from tumor cancer tissues (all patients with *ductal adenocarcinoma*). The pretreatment staging procedures included physical and blood examinations, mammography, mammary ultrasound scanning, breast core biopsies, and chest X-rays.

In addition, radioisotopic bone scans, examination of bone marrow aspirates, and CT scans of brain and chest were performed when necessary. None of the patients had received chemotherapy or radiotherapy before blood sample collection.

The control group included 120 patients (60 patients with benign breast tumor, *adenoma, intraductal papilloma, fibroadenoma, mastopatia*, and 60 healthy untreated women) who underwent mammary gland examination performed by a gynecologist prior to blood sample collection. In addition, mammary ultrasound scanning was performed in all cases. The benign breast tumor histopathology was established in all cases by tissue biopsy of mammary tumor or after surgery.

The study was approved by the local Ethics Committee in Medical University of Bialystok (R-I-002/239/2014). All the patients gave their informed consent for the examination.

2.2. Biochemical Analyses. Venous blood samples were collected from each patient into a heparin sodium tube, centrifuged at 1000 rpm for 15 min to obtain plasma samples and stored at –85°C until assayed. The tested parameters were measured with the enzyme-linked immunosorbent assay (ELISA) (VEGF and M-SCF, Quantikine Human Immunoassay, R&D Systems Inc., Minneapolis, MN, USA) and chemiluminescent microparticle immunoassay (CMIA) (CA 15-3, Abbott, Chicago, IL, USA). According to the manufacturer's protocols, duplicate samples were assessed for each standard, control, and sample.

Table 1: Characteristics of breast cancer patients and control groups: benign breast tumor and healthy women.

	Study group	Number of patients
Tested group	*Breast cancer patients*	120
	Ductal adenocarcinoma	120
	Median age (range)	54 (34–72)
	Tumor stage	
	I	29
	II	30
	III	31
	IV	30
	Menopausal status	
	(i) Premenopausal	51
	(ii) Postmenopausal	69
Control group	*Benign breast tumor patients*	60
	Adenoma	21
	Intraductal papilloma	18
	Fibroadenoma	11
	Mastopatia	10
	Median age (range)	44 (26–71)
	Menopausal status	
	(i) Premenopausal	29
	(ii) Postmenopausal	31
	Healthy women	60
	Median age (range)	48 (23–73)
	Menopausal status	
	(i) Premenopausal	26
	(ii) Postmenopausal	34

The intra-assay coefficient of variation (CV%) of CA 15-3 is reported to be 2.2% at a mean concentration of 27.0 U/mL (SD = 0.6). VEGF is reported to be 4.5% at a mean concentration of 235 pg/mL (SD = 10.6). M-CSF is reported to be 3.4% at a mean concentration of 227 pg/mL (SD = 7.7).

The interassay coefficient of variation (CV%) of CA 15-3 is reported to be 2.6% at a mean concentration of 27.0 U/mL (SD = 0.7). VEGF is reported to be 7.0% at a mean concentration of 250 pg/mL (SD = 17.4). M-CSF is reported to be 3.1% at a mean concentration of 232 pg/mL (SD = 7.3).

2.3. Statistical Analysis. In this analysis we have used healthy volunteers and women with benign breast lesions together as a one control group. This is in accordance with the latest published papers especially for ROC analysis [10–13]. Statistical analysis was performed by using STATISTICA 12.0. We have defined the receiver-operating characteristics

Table 2: Diagnostic criteria of tested parameters and in combined analysis with CA 15-3 in breast cancer patients.

Tested parameters	Diagnostic criteria (%)	Breast cancer				
		Total group	Stage I	Stage II	Stage III	Stage IV
VEGF	SE	76.25	75	75	85	70
	SP	85	85	85	85	85
M-CSF	SE	60	25	35	85	95
	SP	90	90	90	90	90
CA 15-3	SE	83.75	65	75	95	100
	SP	75	75	75	75	75
VEGF + CA 15-3	SE	96.25	90	95	100	100
	SP	65	65	65	65	65
M-CSF + CA 15-3	SE	91.25	80	85	100	100
	SP	67.5	67.5	67.5	67.5	67.5
VEGF + M-CSF + CA 15-3	SE	96.25	90	95	100	100
	SP	57.5	57.5	57.5	57.5	57.5

(ROC) curve for all the tested parameters and CA 15-3. The construction of the ROC curves was performed using GraphROC program for Windows and the areas under ROC curve (AUCs) were calculated to evaluate the diagnostic accuracy and to compare AUCs for all tested parameters separately and in combination with a commonly used tumor marker (CA 15-3). Statistically significant differences were defined as comparisons resulting in $p < 0.05$.

The *cut-off* values were calculated by Youden's index (as a criterion for selecting the optimum *cut-off* point) and for each of the tested parameters they were as follows: VEGF, 70.25 pg/mL; M-CSF, 394.38 pg/mL; and CA 15-3, 18.30 U/mL.

3. Results

Table 2 shows the sensitivity (SE) and specificity (SP) of the investigated parameters and CA 15-3. We indicated that the SE of the tested parameters in the total cancer group was the highest for CA 15-3 (83.75%) and slightly higher than that for VEGF (76.25%) and M-CSF (60%). Among all parameters, the highest SE in stage I of cancer was observed for VEGF (75%), in stage II of BC it was observed for VEGF and CA 15-3 (75%, equal for both parameters), and in stages III and IV of BC it was observed for CA 15-3 (95% and 100%, resp.).

The diagnostic SP of the tested parameters was the highest for M-CSF and VEGF (90% and 85%, resp.) and was higher than that for CA 15-3 (75%).

The combined analysis for VEGF or M-SCF with CA 15-3 in the total group of BC resulted in a high increase in SE in both cases (96.25% and 91.25%, resp.). A similar range in the total BC group was obtained for the combination of VEGF, M-SCF, and CA 15-3 (96.25%). In all combinations, SP dropped slightly in comparison to the analysis of single parameters.

The relationship between the diagnostic SE and SP is illustrated by the ROC curve. The area under the ROC curve (AUC) indicates the clinical usefulness of a tumor marker and its diagnostic power. It also quantifies the overall ability of the test to differentiate between the individuals with the disease and those without it. All data related to AUCs in different stages of BC (I–IV) are included in Table 3.

We noticed that the VEGF area under the ROC curve (0.729) in the total group of breast cancer was higher than the area of CA 15-3 (0.698) and M-CSF (0.645), statistically significantly larger in comparison to AUC = 0.5, borderline of the diagnostic usefulness of the test ($p < 0.001$ in all cases). The combined analysis of VEGF or M-SCF with CA 15-3 in the total group of BC resulted in a slight increase in AUCs in both cases (0.753 and 0.699, resp.), but a maximum range in the total BC group was obtained for the combination of VEGF, M-SCF, and CA 15-3 (0.754) ($p < 0.001$ in all cases) (Figure 1).

In stage I of BC the highest AUC of all the tested parameters was found in VEGF (0.691) and it was the only parameter which was statistically significantly larger in comparison to AUC = 0.5 ($p < 0.002$) (Figure 2).

In stage II of BC the highest AUC of all tested parameters was also observed in VEGF (0.716; $p < 0.001$). The combined analysis of VEGF with CA 15-3 (0.629; $p = 0.043$) and combination of all tested parameters showed a slight decrease in AUC (0.629; $p = 0.042$) (Figure 3).

In stage III of BC the highest AUC of all the tested parameters was observed in CA 15-3 (0.819; $p < 0.001$) and it was slightly higher than VEGF (0.818; $p < 0.001$) and M-CSF (0.811; $p < 0.001$). The combined analysis of VEGF or M-CSF with CA 15-3 showed an increase in AUC values (0.878 and 0.850, resp.) ($p < 0.001$ in both cases), but the maximum range in stage III of BC was obtained for the combination of VEGF, M-SCF, and CA 15-3 (0.879; $p < 0.001$) (Figure 4).

In stage IV of BC the highest AUC of all the tested parameters was found in CA 15-3 (0.893; $p < 0.001$) and it was higher than M-CSF (0.834; $p < 0.001$) and VEGF (0.690; $p = 0.008$). The combined analysis of VEGF with CA 15-3 or all tested parameters showed an increase in AUC values (0.908; $p < 0.001$ in both cases), but the maximum range in stage IV of BC was obtained for the combination of M-SCF and CA 15-3 (0.921; $p < 0.001$) (Figure 5).

Table 3: Diagnostic criteria of ROC curve for tested parameters and CA 15-3.

Tested parameters	AUC	SE	95% C.I. (AUC)	p (AUC = 0.5)
ROC criteria in breast cancer (total group)				
VEGF	0.729	0.0400	0.650–0.807	<**0.001**
M-CSF	0.645	0.0436	0.559–0.730	**0.009**
CA 15-3	0.698	0.0410	0.618–0.779	<**0.001**
VEGF + CA 15-3	0.753	0.0377	0.679–0.826	<**0.001**
M-CSF + CA 15-3	0.699	0.0409	0.618–0.779	<**0.001**
VEGF + M-CSF + CA 15-3	0.754	0.0377	0.679–0.827	<**0.001**
ROC criteria in breast cancer (stage I)				
VEGF	0.691	0.0616	0.570–0.811	**0.002**
M-CSF	0.396	0.0655	0.267–0.524	1.889
CA 15-3	0.494	0.0647	0.367–0.621	1.073
VEGF + CA 15-3	0.595	0.0680	0.462–0.729	0.161
M-CSF + CA 15-3	0.455	0.0611	0.336–0.575	1.535
VEGF + M-CSF + CA 15-3	0.596	0.0679	0.463–0.729	0.1561
ROC criteria in breast cancer (stage II)				
VEGF	0.716	0.0524	0.613–0.818	<**0.001**
M-CSF	0.539	0.0639	0.414–0.664	0.544
CA 15-3	0.586	0.0665	0.456–0.716	0.196
VEGF + CA 15-3	0.629	0.0637	0.504–0.754	**0.043**
M-CSF + CA 15-3	0.568	0.0632	0.444–0.691	0.285
VEGF + M-CSF + CA 15-3	0.629	0.0637	0.505–0.754	**0.042**
ROC criteria in breast cancer (stage III)				
VEGF	0.818	0.0483	0.724–0.913	<**0.001**
M-CSF	0.811	0.0484	0.716–0.906	<**0.001**
CA 15-3	0.819	0.0490	0.723–0.915	<**0.001**
VEGF + CA 15-3	0.878	0.0376	0.804–0.952	<**0.001**
M-CSF + CA 15-3	0.850	0.0436	0.765–0.936	<**0.001**
VEGF + M-CSF + CA 15-3	0.879	0.0375	0.805–0.952	<**0.001**
ROC criteria in breast cancer (stage IV)				
VEGF	0.690	0.0717	0.549–0.831	**0.008**
M-CSF	0.834	0.0461	0.744–0.925	<**0.001**
CA 15-3	0.893	0.0450	0.805–0.982	<**0.001**
VEGF + CA 15-3	0.908	0.0390	0.832–0.985	<**0.001**
M-CSF + CA 15-3	0.921	0.0368	0.848–0.993	<**0.001**
VEGF + M-CSF + CA 15-3	0.908	0.0387	0.832–0.984	<**0.001**

p, statistically significantly larger AUCs compared to AUC = 0.5.

4. Discussion

Angiogenesis is a vital blood vessel formation process in tumor progression and nutrition. VEGF is considered to be an important factor in promoting angiogenesis and cell proliferation in many pathological conditions. High levels of VEGF have been found in different kinds of tumors, for example, gastric [14] or colorectal cancer [15], and also in gynecological malignancies such as ovarian [16] or cervical cancer [17]. High plasma levels of VEGF have been also found in breast cancer [5].

Tumor growth is influenced by a variety of external and internal factors. Our immune system (producing growth factors and cytokines) is one of the most important mediators involved in tumor development. M-CSF belongs to the group of hematopoietic growth factors (HGFs) which are overexpressed in many tumors. The main function of M-CSF is regulation and differentiation of hematopoietic progenitor cell growth. Its high levels have been found in gastric [18] and pancreatic cancer [19]. It has also been found in many types of gynecological malignancies, for example, ovarian [20, 21], cervical [22], or endometrial cancer [23], and it has also been found in breast cancer [8].

Sensitivity (SE) measures the proportion of positives that are correctly identified. In this study, the SE for CA 15-3 was the highest in the total group of breast cancer patients. However, in stages I and II of cancer it was the highest for VEGF which is much more important because such a

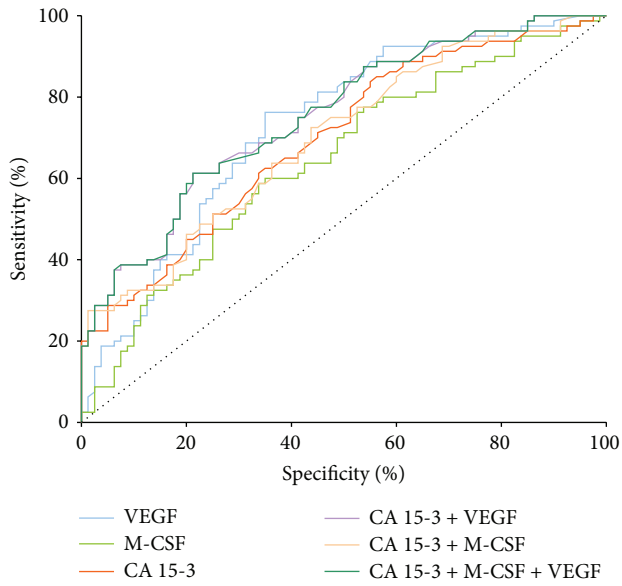

Figure 1: Diagnostic criteria of ROC curve for tested parameters and in combination with CA 15-3 in total BC group.

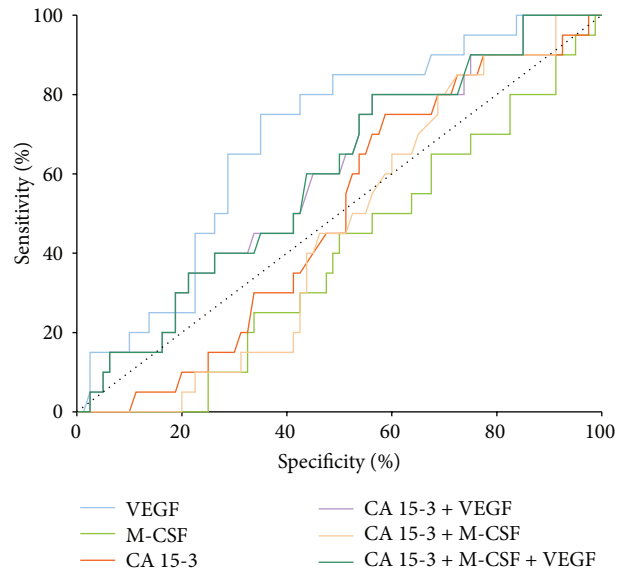

Figure 2: Diagnostic criteria of ROC curve for tested parameters and in combination with CA 15-3 in stage I of BC.

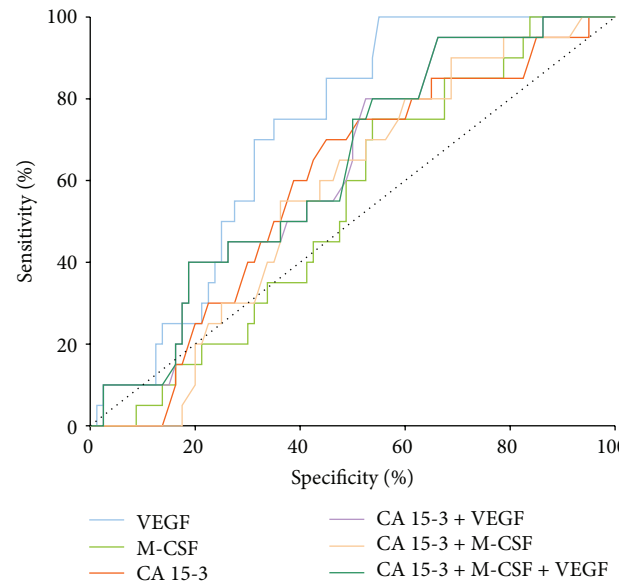

Figure 3: Diagnostic criteria of ROC curve for tested parameters and in combination with CA 15-3 in stage II of BC.

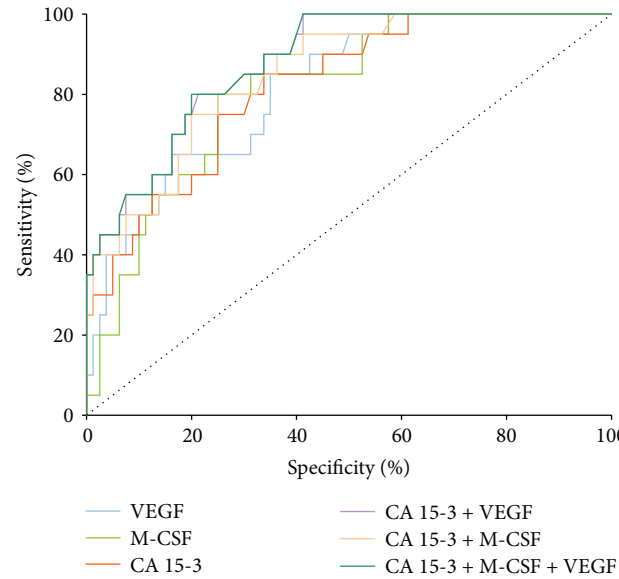

Figure 4: Diagnostic criteria of ROC curve for tested parameters and in combination with CA 15-3 in stage III of BC.

high sensitivity (75%) allows us to confirm the occurrence of breast cancer in the earliest stages, while contributing to an increase in cancer detection, the course of which is often asymptomatic. Earlier diagnosis is associated with a greater chance of survival as well as quality and length of life of patients with BC. Similar data were observed in our previous studies [5, 8], where CA 15-3 had also the highest values in the total group, but what is more important is the fact that VEGF had a higher value in stage I of BC. However, in opposition to this paper, statistical analysis of those previous publications was conducted on groups of "breast cancer patients versus healthy women" only.

Other researchers, such as Motawa El Husseini et al. [24], have also indicated very high SE (83.93%) and SP (96.67%) for VEGF in BC diagnostics, but they conducted their study on 51 BC patients and only 30 healthy volunteers as a control group.

We have also observed similar data in other types of cancer, for example, in ovarian cancer [16]. Other researchers, for instance, Kozłowski et al. [25] in esophageal cancer (SE, 83%; SP, 70%) or Cao et al. [26] in lung cancer (SE, 81.8%; SP, 84.2%), have obtained similar results for VEGF.

The AUC represents the overall accuracy of a test, with a value approaching 1.0 indicating perfect SE and SP. According

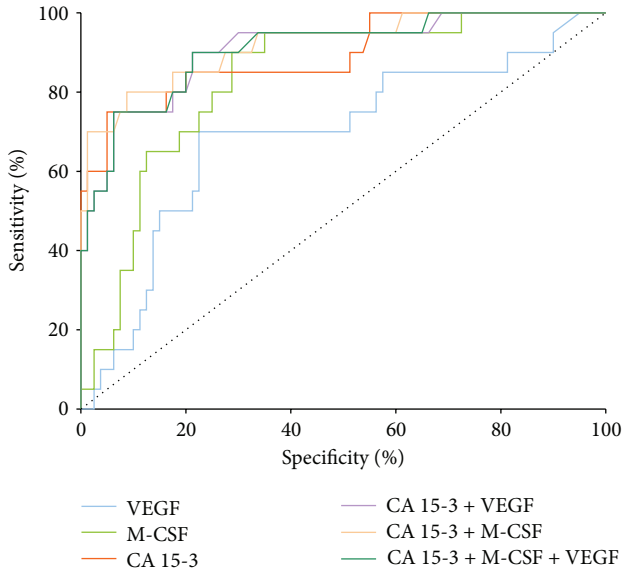

Figure 5: Diagnostic criteria of ROC curve for tested parameters and in combination with CA 15-3 in stage IV of BC.

to this study, the ROC area of VEGF was the largest of all the tested parameters (even higher than CA 15-3 which is nowadays commonly used in the diagnosis of BC) and is the only parameter for which AUC was statistically significantly larger in comparison to AUC = 0.5 in all stages of BC (I–IV), not only in the total group. This is very important as it indicates higher usefulness of VEGF compared to CA 15-3 in the differentiation between BC and benign breast tumor.

Our results showed that the diagnostic power (AUC) of the tested parameters, especially VEGF, in the total group of BC patients was slightly lower than the one obtained by Zhang et al. [27] (0.788). The discrepancy between our research and that study may be related to a different number of patients involved in those studies. Other researchers such as Motawa El Husseini et al. [24] have obtained a higher AUC value for VEGF (0.938), but the control group in their study comprised only healthy women.

The diagnostic power of VEGF in the course of other tumors, for example, studies conducted in lung cancer by Cao et al. [26], revealed a slightly higher AUC value (0.855) than our outcome, which may be associated with different types of cancer. Other researchers, for example, Kozłowski et al. [25], have obtained slightly higher results for VEGF (0.865) in esophageal cancer. This may result from the fact that they conducted their study on 30 healthy volunteers in control group (without benign cancer patients). High importance of VEGF in those types of tumors points out that this cytokine seems to be a good biomarker for a variety of cancers, as shown by other researchers. In stage I of BC the highest AUC of all tested parameters was observed for VEGF. In our previous study in BC [28], which comprised BC patients and only healthy women as a control group, the highest AUC value was found for CA 15-3 (0.7068) and it was the only parameter for which AUC was statistically significantly larger in comparison to AUC = 0.5 ($p = 0.002$). Present statistical analysis with new, combined control group revealed even better results for tested cytokine (VEGF is a better marker than CA 15-3), which is additionally in opposition to the previous results obtained for M-CSF. In our other study [21] conducted in ovarian cancer, which compared M-CSF to HE4 and CA 125, the AUC value in stage I was 0.7676 ($p < 0.001$) and was significantly higher than that in this study.

In stage II of BC, only VEGF and the combined analysis of VEGF and CA 15-3 had a statistically significantly larger AUC in comparison to AUC = 0.5. In our previous study in BC [28] all the tested parameters had significant values (which might be related with the composition of the control group, only healthy subjects). In the study on ovarian cancer [21] the value of AUC for M-CSF was higher (similarly to stage I) than that in this study, but the control group in this study also comprised only healthy women.

In stages III and IV of BC, all the tested parameters had statistically significantly larger AUC in comparison to AUC = 0.5. In our previous study in BC [28] all the tested parameters also showed significant values similarly to the study conducted in ovarian cancer [21].

The combined analysis of VEGF or M-CSF with CA 15-3 resulted in an increase in SE and AUC values, which may be useful in the future diagnosis of this cancer. This study is also similar to our previous paper, indicating diagnostic usefulness of this biomarkers panel in cancer diagnostics. Better parameters were obtained in the combination of VEGF than M-CSF and CA 15-3. The combination of all three parameters did not affect the significant increase in SE, SP, or AUC, which may lead to the assumption that the combination of VEGF and CA 15-3 may be the best diagnostic panel in the diagnosis of BC.

5. Conclusions

In conclusion, our present results indicate the usefulness and a high diagnostic power of VEGF in the detection of breast cancer. Among the tested parameters, VEGF occurred to be the best candidate for cancer diagnostics (better than commonly used tumor marker, CA 15-3) especially in stages I and II of BC as well as in the differentiation between BC and benign breast tumor. M-CSF has shown low SE in stages I and II and was comparable to CA 15-3 and VEGF, SE, and AUC values in stages III and IV of BC. VEGF, especially in the combination with CA 15-3, showed the highest usefulness and diagnostic power in the detection of breast cancer and may indicate a new panel of biomarkers used in early diagnosis of BC.

Ethical Approval

This work was conducted in accordance with the Declaration of Helsinki (1964).

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Overexpression of Notch3 and pS6 Is Associated with Poor Prognosis in Human Ovarian Epithelial Cancer

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Notch3 and pS6 play important roles in tumor angiogenesis. To assess the expression of Notch3 and pS6 in Chinese ovarian epithelial cancer patients, a ten-year follow-up study was performed in ovarian epithelial cancer tissues from 120 specimens of human ovarian epithelial cancer, 30 specimens from benign ovarian tumors, and 30 samples from healthy ovaries by immunohistochemistry. The results indicate that the expression of Notch3 and pS6 was higher in ovarian epithelial cancer than in normal ovary tissues and in benign ovarian tumor tissues ($p < 0.01$). In tumor tissues, Notch3 expression and pS6 expression were negatively associated with age ($p > 0.05$) but positively associated with clinical stage, pathological grading, histologic type, lymph node metastasis, and ascites ($p < 0.05$ or $p < 0.01$). A follow-up survey of 64 patients with ovarian epithelial cancer showed that patients with high Notch3 and pS6 expression had a shorter survival time ($p < 0.01$), in which the clinical stage ($p < 0.05$) and Notch3 expression ($p < 0.01$) played important roles. In conclusion, Notch3 and pS6 are significantly related to ovarian epithelial cancer development and prognosis, and their combination represents a potential biomarker and therapeutic target in ovarian tumor angiogenesis.

1. Introduction

Ovarian cancer represents one of the most aggressive neoplastic diseases in women, and 75% patients are diagnosed at advanced stage due to the lack of biomarkers for early diagnosis [1]. In 2012, ovarian cancer occurred in 239,000 women and caused 152,000 deaths worldwide and was more common in North America and Europe than in Africa and Asia [2]. Until now, the molecular etiology of this cancer has remained mostly unknown and therefore it is of great importance to explore the association of key proteins with poor prognosis in human ovarian epithelial cancer (the major histological type of ovarian cancer).

Notch signaling is a highly conserved cell-cell communication system present in multicellular organisms and has been characterized for its well-established role in a variety of physiological and pathological processes, including cancer development [3]. Notch3, a type of Notch receptor (Notch1, Notch2, Notch3, and Notch4), plays an important role in promoting ovarian tumorigenesis, cancer progression, and chemotherapy resistance via activating the PI3K/Akt/mTOR signaling pathway [4]. Ribosomal S6 kinase (S6K), a downstream effector of the PI3K/Akt pathway, is frequently activated in human ovarian cancer [5] and is significantly more prevalent in malignant tumors than in benign lesions. pS6 kinase is also involved in other aspects of cancer progression in addition to its well-established role in regulating proliferation and cell survival [5–7].

Although the roles of Notch3 and S6K in cancer development have been studied, no study has been carried out to combine the expression of Notch3 and S6K in relation to the prognosis of human ovarian epithelial cancer. It is known that

Notch3 and S6K may complement their common functions in cancer development, but their roles in specific tumors are unique and context-dependent [8–11]. In the present study, we first investigated the expression of Notch3 and S6K in human ovarian epithelial cancer, to verify their expression related to clinicopathological features and prognosis in human ovarian epithelial cancer and to further evaluate their potential value as biological markers of aggressiveness in ovarian cancer, with the goal of improving the management of ovarian cancer patients.

2. Materials and Methods

2.1. Ethics Statement. Patient samples were obtained with written informed consent in accordance with ethics committee requirements at the participating institutes and the Declaration of Helsinki. Permission to carry out the study was obtained from the Institutional Review Board (IRB) of the Second Affiliated Hospital of Nanchang University.

2.2. Tissue Samples. Tissue samples were collected from 120 patients with ovarian epithelial cell carcinoma who underwent surgical resection at the Second Affiliated Hospital of Nanchang University between 1998 and 2008 (age range 36–68 years, median 49 years). All patients were histopathologically diagnosed based on clinical protocols, and none of them received presurgery chemotherapy or immunotherapy. Of the 120 patients, 41 patients (at stage I + II) underwent a hysterectomy + bilateral oophorectomy + omentum resection + appendectomy + pelvic lymph node dissection; 79 patients with advanced ovarian cancer (III + IV) underwent cytoreductive surgery, pelvic lymph node dissection, or pelvic lymph node biopsy; 37 patients had lymphatic metastasis and 70 patients had evident ascites. The histological results revealed that 77 patients had serous carcinoma and 43 patients had mucinous carcinoma; 17 tumors showed a high degree differentiation, 40 showed moderate differentiation, and 63 showed poor differentiation based on pathological grading.

In this study, 30 patients with benign ovarian cystadenoma (14 serous cystadenoma and 16 mucinous cystadenoma) were selected to perform a tumor stripping operation or unilateral salpingo-oophorectomy (age range 23–46 years, median 35 years). Another 30 patients (with either uterine fibroids, adenomyosis, or other nonovarian diseases) underwent hysterectomy + bilateral or unilateral oophorectomy and were selected as the control group (age range 46–69 years, median 58 years).

In the ovarian epithelial cell carcinoma group, 44 patients received combination chemotherapy of cisplatin + adriamycin + cyclophosphamide and 64 patients received carboplatin + paclitaxel. Twelve patients did not receive any postsurgery chemotherapy (see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/5953498).

2.3. Immunohistochemistry. Each tissue was fixed in formalin and embedded in paraffin and then sectioned and mounted on glass slides. After dewaxing in xylene and dehydration in graded alcohol, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Then, the sections were subjected to antigen retrieval in a microwave oven at 700 W for 20 min in 10 mol/L citrate buffer solution (pH 6.0). After that, 10% goat serum albumin was applied for 20 min. Overnight incubation was carried out at 4°C with the following primary antibodies: rabbit polyclonal Notch3 (1 : 50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p70S6k (1 : 50 dilution; Cell Signaling Technology, Beverly, MA, USA). Then, sections were incubated with the appropriate secondary antibodies at room temperature for 60 min and washed in phosphate-buffered saline (PBS). Diaminobenzidine (DAB) was used as the chromogen, and the sections were counterstained with hematoxylin. Samples incubated with PBS instead of primary antibodies were used as negative controls [12].

2.4. Evaluation of Immunostaining. All stained sections were evaluated and scored independently by two pathologists with no prior knowledge of the clinicopathological outcomes of the patients. The mean percentage of positive cells was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Final histological (h) scores were obtained for each case by multiplying the percentage and the intensity score. Protein expression levels were further analyzed by classifying h values as negative (−): 0-1, positive (+): 2–4, or strongly positive (++): 5–7 [12].

2.5. Statistical Analysis. SPSS 19.0 software was used for the statistical analysis. The significance of the relationships between Notch3 and pS6 expression and clinicopathological parameters was evaluated using the Wilcoxon and Kruskal-Wallis tests and Spearman's rank correlation. Survival rates were calculated using the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis was used to identify independent prognostic factors for survival rates using the Cox proportional hazards regression model. p values < 0.05 were considered statistically significant [11–13].

3. Results

3.1. Expression of Notch3 and pS6 in Different Ovarian Tissues. The immunohistochemistry results show that Notch3 was mainly expressed in the cytoplasm and/or nucleus of ovarian epithelial cancer cells, while pS6 was mainly expressed in the cytoplasm (data not shown). In Figure 1 and Table 1, Notch3 protein was detected in normal ovarian tissue, ovarian cystadenoma, and ovarian epithelial cancer at different level. The positive expression rates of Notch3 in normal ovarian tissue, ovarian cystadenoma, and ovarian epithelial cancer were 16.7% (5/30), 70.0% (21/30), and 91.7% (110/120), respectively. Notch3 expression in ovarian epithelial cancer was significantly higher than in normal ovarian tissue ($p < 0.01$) and ovarian cystadenoma ($p < 0.01$), and Notch3 expression in ovarian cystadenoma was much higher than in normal ovarian tissue ($p < 0.01$).

Similar to Notch3 expression, the expression of pS6 in ovarian epithelial cancer (108/120, 90%) was significantly higher than in normal ovarian tissue (5/30, 16.7%) ($p < 0.01$) and ovarian cystadenoma (23/30, 76.7%) ($p < 0.01$),

Table 1: The protein expression of Notch3 and pS6 in normal ovarian tissue, ovarian cystadenoma, and ovarian epithelial cancer.

Characteristics	Cases, n	Notch3 expression, n			p value	pS6 expression, n			p value
		−	+	++		−	+	++	
Normal ovarian tissue	30	25	5	0		25	5	0	
Ovarian cystadenoma	30	9	15	6	0.000	7	17	6	0.000
Ovarian epithelial cancer	120	10	30	80		12	49	59	

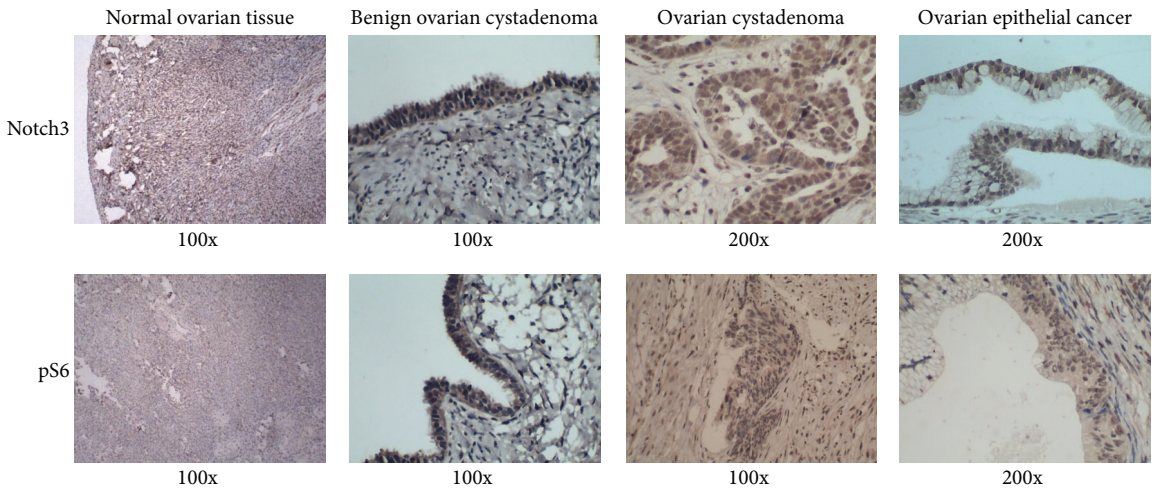

Figure 1: Evaluation of the protein expression of Notch3 and pS6 in normal ovarian tissue, ovarian cystadenoma, and ovarian epithelial cancer using immunohistochemistry.

and a significant increase in pS6 was observed in ovarian cystadenoma compared to normal ovarian tissue ($p < 0.01$).

3.2. Correlation between the Clinicopathological Features and Expression of Notch3 and pS6. The relationship between ovarian epithelial cancer clinical stage and signaling molecule expression (Notch3 and pS6) was analyzed in Table 2. We found that Notch3 expression and pS6 expression were negatively associated with age ($p > 0.05$) but were positively associated with clinical stage, pathological grading, histological type, lymph node metastasis, and ascites. As shown in Table 2, Notch3 expression and pS6 expression were higher in stage III-IV than in stage I-II ($p < 0.01$, $p < 0.01$); similarly, Notch3 expression and pS6 expression were stronger with higher pathological grading compared to low pathological grading ($p < 0.01$, $p < 0.01$). The expression of Notch3 and pS6 was higher in serous cystadenocarcinoma, lymph node metastasis, and ascites than in mucinous cystadenocarcinoma ($p < 0.01$, $p < 0.05$) and in the absence of lymph node metastasis ($p < 0.01$, $p < 0.01$) and ascites ($p < 0.01$, $p < 0.01$).

The correlation analysis of Notch3 expression and pS6 indicated a positive correlation between these two proteins in ovarian epithelial cancer ($r_s = 0.668$, $p < 0.01$) (Tables 3 and 4).

3.3. Survival Analysis of Notch3 and pS6 Expression. A follow-up survey was performed on 64 patients with ovarian epithelial cancer who had received chemotherapy (carboplatin and paclitaxel) after surgery. Of these 64 patients, 46 patients died and 18 patients were censored or truncated. The shortest and longest survival times for these patients were 1 month and 102 months (with an average of 35.16 months), and the accumulated 1- to 5-year survival rates of the patients were 0.55, 0.36, 0.36, 0.28, and 0.21, respectively (Figure S1).

Based on Notch3 and pS6 protein expression, the 64 patients were divided into three groups: low Notch3 and pS6 expression (−−, $n = 18$), moderate Notch3 and pS6 expression (+− or −+, $n = 4$), and high Notch3 and pS6 expression (++, $n = 42$). As shown in Table 4, the overall survival of patients with low Notch3 and pS6 expression was longer (81.9 months), while the groups with high and moderate Notch3 and pS6 expression had a shorter survival time of 12.3 months and 16.8 months, respectively ($\chi^2 = 41.479$, $p < 0.01$).

3.4. Multiple Factor Cox Regression Analysis of the Survival Rate. Table 5 shows the results of multiple factor Cox regression analysis of the survival rates of the 64 patients. When the analysis was performed, the survival time and dead/alive ratio were used as dependent variables, while age (<50 years = 1, ≥50 years = 2), clinical stage (stage I-II = 1, stage III-IV = 2), pathological grading (G1 = 1, G2 = 2, G3 = 3), histologic type (serous cystadenocarcinoma = 1, mucinous cystadenocarcinoma = 2), lymph node metastasis (yes = 1, no = 2), ascites (yes = 1, no = 2), Notch3 expression (negative staining = 0, positive staining = 1, strongly positive staining = 2), and pS6 expression (negative staining = 0,

Table 2: Correlation between the protein expression of Notch3 and pS6 proteins and clinicopathological parameters in patients with ovarian epithelial cancer.

Characteristics	Cases, n	Notch3 expression, n			p value	pS6 expression, n			p value
		−	+	++		−	+	++	
Age, yrs									
<50	79	7	19	53	0.947	6	37	36	0.558
≥50	41	3	11	27		6	12	23	
Clinical stages									
I~II	41	8	17	16	0.000	7	22	12	0.001
III~IV	79	2	13	64		5	27	47	
Pathological grading									
G1	17	7	7	3	0.000	5	7	5	0.001
G2	40	3	17	20		6	22	12	
G3	63	0	6	57		1	20	42	
Histologic type									
Serous cystadenocarcinoma	77	1	19	57	0.006	3	31	43	0.011
Mucinous cystadenocarcinoma	43	9	11	23		9	18	16	
Lymph node metastasis									
Yes	37	0	5	32	0.001	0	5	32	0.000
No	83	10	25	48		12	44	27	
Ascites									
Yes	70	2	9	59	0.000	2	21	47	0.000
No	50	8	21	21		10	28	12	

Table 3: Association between the expression of Notch3 and pS6.

Notch3	pS6				r_s	p
	−	+	++	Total		
−	7	3	0	10		
+	5	22	3	30	0.668	0.000
++	0	24	56	80		
Total	12	49	59	120		

positive staining = 1, and strongly positive staining = 2) were the independent variables; the level of the variate was 0.05.

Among all the clinicopathological features, clinical stage III-IV was found to be a significant indicator of poor overall survival (hazard ratio (HR), 5.398; 95% confidence interval (CI) 1.154–25.259; $p = 0.032$) compared with stage I-II. Moreover, the Notch3 expression was also significantly associated with poor overall survival in these patients (HR, 8.362; 95% CI 2.154–32.461; $p = 0.002$) (Table 5).

As ascites is a key finding in cancer, we analyzed the relationship between the coexpression of Notch3 and pS6 expression and the presence of ascites. Higher expression of Notch3 and pS6 was associated with a higher positive rate of ascites (Table 6); the positive rates of ascites in patients with high, moderate, and low expression of Notch3 and pS6 were 82.1%, 51.9%, and 27.0%, respectively. The χ^2 test indicated that the expression level of Notch3 and pS6 has a significant positive correlation with ascites in these groups ($\chi^2 = 28.448$, $p < 0.01$).

4. Discussion

The Notch signaling cascade is critical for cell proliferation, differentiation, development, and homeostasis [13], and deregulated Notch signaling is found in various diseases (e.g., T-cell leukemia, breast cancer, prostate cancer, colorectal cancer and lung cancer, and central nervous system malignancies) [14]. However, the mechanism of its regulation in ovarian cancer is unclear.

In our study, Notch3 expression in ovarian epithelial cancer was significantly higher than in benign cystadenoma and normal ovarian tissues ($p < 0.01$, Table 1) and was associated with clinical stage, pathological grading, histologic type, lymph node metastasis, and ascites ($p < 0.01$ or $p < 0.05$), suggesting that the Notch signaling pathway is in an activated state and probably plays an important role in the development of ovarian epithelial cancer [1, 8, 12, 13, 15, 16].

It is known that cancer occurrence is a comprehensive consequence of disorders in multiple signaling transduction pathways [9]. It has been shown that the PI3K/AKT signaling pathway is the key downstream mediator of Notch signaling; when Notch ligands activate the Notch signaling pathway, mTOR activates the downstream effectors S6k and eukaryotic translation initiator 4E binding protein 1 (4EBP1). Activated S6K phosphorylates the ribosomal protein pS6 and enhances the synthesis of the translation regulator p4EBP1 to regulate protein synthesis [5, 17, 18]. Therefore, Notch3 expression and pS6 expression play important roles in PI3K/AKT/mTOR signaling and ovarian epithelial cancer development. Our data also indicate a strong positive correlation between Notch3 expression and pS6 expression ($r_s = 0.668$, $p < 0.01$; Table 3).

Table 4: The survival distribution of patients with different Notch3 and pS6 expression.

Notch3 and pS6	Average survival (month)	Overall survival		
		95% CI	χ^2	p
−−	81.916 ± 6.541	69.095–94.737		
−+ or +−	16.750 ± 2.136	12.563–20.937	41.479	<0.01
++	12.338 ± 1.947	8.521–16.155		
Total	35.162 ± 4.985	25.391–44.932		

Table 5: Multiple COX regression analysis of patients with ovarian epithelial cancer.

Variable	B	SE	Wald	p	HR	95% CI
Clinical stages	1.686	0.787	4.586	0.032	5.398	1.154–25.259
Notch3	2.124	0.692	9.418	0.002	8.362	2.154–32.461

Table 6: Relationship between the coexpression of Notch3 and pS6 expression and ascites.

Notch3 and pS6	No ascites		Ascites		Total	χ^2	p
	n	%	n	%			
−−	27	73.0	10	27.0	37		
−+ or +−	13	48.1	14	51.9	27	28.448	0.000
++	10	17.9	46	82.1	56		
Total	50	41.7	70	58.3	120		

In our follow-up survey of 64 patients with ovarian epithelial cancer (Table 4), the patients with high Notch3 and pS6 expression only survived for an average of 12.3 months, while patients with moderate and low Notch3 and pS6 expression survived for 16.8 months and 81.9 months, respectively ($\chi^2 = 41.479$, $p < 0.01$). The clinical stage ($p < 0.05$) and Notch3 expression ($p < 0.01$) were more important than other clinicopathological features (Table 5). In addition, the occurrence of ascites in patients with a high level of Notch3 and pS6 expression was significantly higher than in the other groups, suggesting that a high level of Notch3 and pS6 expression may be associated with peritoneal implantation and spreading (Table 6).

In summary, although some studies have indicated that Notch3 or pS6 alone could be used as indicator of cancer development and prognosis [5, 11, 19], our results indicate that Notch3 and pS6 together have a strong relationship with the clinicopathological features of ovarian epithelial cancer and overall patient survival. However, Notch3 is not the only protein upstream of PI3K/AKT/mTOR signaling, and pS6 is not the only effector of PI3K/AKT/mTOR signaling [20, 21]. Moreover, the association analysis of Notch3 and pS6 (Table 3) indicated that five pS6 negative patients expressed moderate levels of Notch3 (4.2%), and three Notch3 negative patients expressed moderate levels of pS6 (2.5%). Therefore, the combined assessment of Notch3 and pS6 expression is a better choice of prognostic biomarker for overall survival in ovarian epithelial cancer than Notch3 or pS6 alone.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper and regarding the funding that they have received.

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Research Article

Diagnostic Accuracy of Five Different Fecal Markers for the Detection of Precancerous and Cancerous Lesions of the Colorectum

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Background. Colorectal cancer (CRC) is the second deadliest malignancy worldwide. This study aimed to compare the diagnostic accuracy of different fecal markers in the detection of colorectal adenomas and cancer. *Methods.* Stool samples of patients referred to colonoscopy were collected for the analysis of tumor M_2 pyruvate kinase (M_2PK), human hemoglobin (Hb), hemoglobin/haptoglobin (Hb/Hp) complex, fecal calprotectin (FC), and matrix metalloproteinase-9 (MMP-9). *Results.* Sensitivity and specificity of M_2PK for adenomas sized > 1 cm were 60% and 67.5% and for CRC were 94.7% and 67.5%. Sensitivity and specificity of iFOBT for adenomas sized ≥ 1 cm were 80% and 72.5% and for CRC were 94.7% and 72.5%. Sensitivity and specificity of Hb/Hp complex for adenomas sized ≥ 1 cm were 80% and 52.9% and for CRC were 100% and 52.9%. Sensitivity of FC and MMP-9 for CRC was 77.8% and 72.2%. Combined use of M_2PK, iFOBT, and FC resulted in a sensitivity and specificity of 95% and 47.5% for the detection of adenomas sized ≥ 1 cm. *Discussion.* In CRC, sensitivity of M_2PK, iFOBT, and Hb/Hp complex proved to be high. Combined use of M_2PK, iFOBT, and FC may be valuable in the detection of large adenomas.

1. Introduction

Colorectal cancer (CRC) incidence and mortality rates vary markedly worldwide. Globally, CRC is a third most common cancer, being a significant leading cause of cancer death in both genders [1]. Furthermore, the incidence of CRC is increasing in Central European countries [1]. The Hungarian mortality rates for CRC proved to be the highest among men in Europe in 2012 [2].

The vast majority of CRC cases are sporadic colon cancers characterized by a multistep carcinogenic process [3]. Advanced adenomas greater than 10 mm in diameter with high-grade dysplasia or with more than 20% villous component are considered to be the clinically relevant precursors of CRC. However, the long premalignant phase of sporadic CRCs provides a good opportunity for successful screening and intervention.

Colonoscopy is considered the gold standard of CRC screening tools. However, mainly due to the invasive nature of colonoscopy, the acceptance of this type of screening method among patients is low. The most commonly used noninvasive screening method for CRC is the guaiac fecal occult blood test (gFOBT) based on the detection of hemoglobin peroxidase activity in the stool. However, the sensitivity and the specificity of this test are not good enough to safely rule out the presence of CRC or adenomas which is why there is a great need for a better noninvasive marker for these conditions. In the case of proximal malignant lesions, hemoglobin/haptoglobin (Hb/Hp) detection can be superior to Hb detection alone since Hb/Hp complex remains stable over the entire course of the large bowel in comparison to Hb degraded on the way [4–6]. M_2 pyruvate kinase (PK) is a biochemical form of PK which is a key enzyme in cancer cell metabolism [7]. M_2PK is expressed in normal

proliferating cells, embryonic cells, adult stem cells, and cancer cells [8]. Elevated levels of M_2PK have been detected in colonic adenocarcinoma [9]. Calprotectin is a calcium-binding and zinc-binding protein complex that is abundant in the cytosol of inflammatory cells [10, 11]. Fecal calprotectin (FC), a biomarker of intestinal inflammation, has been in clinical use for years in inflammatory bowel disease [11–13]. FC has been shown to be elevated in CRC and has been suggested to be for screening high risk groups for CRC [14].

Matrix metalloproteinase (MMP) is a large family of calcium-dependent zinc-containing endopeptidases responsible for tissue remodelling and degradation of the extracellular matrix components, including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan, in multiple disease settings including malignant processes. MMP-9 subtypes are believed to play a crucial role in the progression and metastasis formation of many tumors, including CRC [15].

Since the majority of the abovementioned tests are not officially recommended in the CRC screening guidelines and some of them have not been tested previously, the aim of this study was to compare the diagnostic accuracy of different fecal markers in the detection of precancerous and cancerous lesions of the colorectum and to find the most accurate for CRC screening.

2. Methods

2.1. Patient Population and Study Protocol. Patients from the 1st Department of Medicine, University of Szeged, who were referred for colonoscopy were invited to participate in the study. Data on symptoms, smoking habits, family history, and current medication were collected. Every patient was informed about the study details and asked to sign written consent. The patients were instructed for sample collection and handling. All patients were asked to collect stool samples one day before administration of bowel preparation. Plastic containers were provided for feces collection. After bringing the samples at the lab of the clinic, they were frozen at −20°C until further analysis. Patients did not have to keep a special diet and were told to take their usual medications. Selection of the patient groups with adenomas sized <1 cm and ≥1 cm and CRC was based on the endoscopic and histological finding. The stool testing for M_2PK, iFOBT, FC, and MMP-9 was carried out by a single trained person who was blinded to the results of the colonoscopy.

The study was approved by the Regional and Institutional Human Medical Biological Research Ethics Committee of the University of Szeged.

2.2. Measurement of Fecal M_2PK and iFOBT. A combined rapid immunochromatographic lateral flow test was used for simultaneous detection of enzyme biomarker M_2PK and human hemoglobin (combined M_2PK and HB, 2 in 1 Quick Test, ScheBo® Biotech). For these measurements, stool samples were thawed and a special stick capturing 4 mg of stool was loaded. These tests are based on visual inspection of colors at test and control lines. The result is exclusively qualitative (detection limit of M_2PK was 4 U/mL; detection limit of Hb was 15 ng/mL).

2.3. Measurement of Fecal Hb and Hb/Hp Complex. Hb/Hp complex was determined from stool samples with a visual immunochromatographic quick test: ColonView Hb and Hb/Hp fecal occult blood test (Biohit HealthCare; detection limit of Hb was 15 ng/mL; detection limit of Hb/Hp was 4 ng/mL).

2.4. Measurement of FC and Fecal MMP-9. For FC measurements, fecal specimens were thawed at 4°C. FC level was quantified by using enzyme-linked immunosorbent assay (Quantum Blue, BÜHLMANN Laboratories Ltd., Schönenbuch) according to the manufacturer's instructions. For MMP-9 measurements, 1 g of fecal samples was diluted, mixed, homogenised in 4 mL of ice-cold Tris-buffer (0.15 M NaCl + 20 mM Tris-HCl, pH 8.3), and then centrifuged. MMP-9 was also measured by quantitative enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK) [16].

2.5. Colonoscopy and Histological Examination. Diagnosis was based on the endoscopic and histopathological findings. Colonoscopies were performed by three experienced endoscopists (TM, ZSZ, and FN) who were blinded to fecal tests results. Carcinomas were classified according to the Dukes staging system and location. Adenomatous polyps were classified according to histopathological characteristics, size (large polyps: ≥1 cm; small polyps: <1 cm), and location. All colonoscopy biopsies were examined by an expert pathologist (LT). The diagnoses were reported using the standard WHO classification of colorectal neoplasia. In addition to their size, all polypoid lesions were classified as hyperplastic polyps or adenomas, being further classified according to their histological pattern as tubular, tubulovillous, villous, or serrate adenomas.

2.6. Statistical Analysis. CRCs and adenomas were analysed separately. The diagnostic value of fecal markers for detecting adenomas and CRCs was assessed by calculating the sensitivity and the specificity of the test. Correlations between FC and MMP-9 and endoscopic findings were determined by ANOVA method. The cut-off levels, specificity, and sensitivity between CRC and control groups were calculated using the receiver operating characteristic (ROC) analysis. All statistical analyses were carried out using STATA 9 (StataCorp, TX, 2005). P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Patient Population. Ninety-five consecutive in- and outpatients admitted for total colonoscopy between September 2014 and April 2015 were prospectively enrolled in the study. Indications for colonoscopies were abdominal complaints, bloody stool, family history of CRC, and prior colorectal adenoma. Patients with active gastrointestinal bleeding, menstruation, and past history of total colectomy were excluded from the study. Study groups were defined on the basis of the result of colonoscopy and histological evaluation.

Mean age was 67 years (range: 21–92) in study population. 57 female and 38 male patients were in these three groups,

Table 1: Demographic characteristics of the study population.

Demographic data	All patients (95)	Control group (40)	Adenoma group (36)	Cancer group (19)
Female/male	38/57	19/21	14/22	5/14
Age (years)	67 (21–92)	67 (21–87)	68 (51–81)	65 (44–92)
Current smokers	13 (13.7%)	4 (10%)	5 (13.9)	4 (21.1)
Comorbidities				
Hypertension	54 (56.8%)	23 (57.5%)	22 (61.1%)	9 (47.4%)
Diabetes mellitus	21 (22.1%)	7 (17.5%)	8 (22.2%)	6 (31.6%)
Hyperlipidaemia/hypercholesterinemia	22 (23.2%)	9 (22.5%)	11 (30.6%)	2 (10.5%)
Cardiovascular disease	25 (26.3%)	11 (27.5%)	10 (27.7%)	4 (21.1%)
Cerebrovascular disease	13 (13.7%)	6 (15%)	4 (11.2%)	3 (15.8%)
Hyper/hypothyroidism	13 (13.7%)	5 (12.5%)	7 (19.4%)	1 (5.3%)
Pulmonary disease	6 (6.3%)	4 (10%)	2 (5.6%)	0
Gout	11 (11.6%)	5 (12.5%)	5 (13.9%)	3 (15.8%)
Autoimmune disease	4 (4.2%)	0	3 (8.3%)	1 (5.3%)
Malignant disease (simultaneously)	3 (3.2%)	1 (2.5%)	2 (5.6%)	0
Hepatitis (B, C)	2 (2.1%)	1 (2.5%)	1 (2.8%)	0
Diverticulum	24 (25.3%)	11 (27.5%)	10 (27.8%)	3 (15.8%)
Haemorrhoids	20 (21.1%)	11 (27.5%)	7 (19.4%)	2 (10.5%)

respectively. Demographic characteristics of the study population are summarized in Table 1. Family history of CRC was reported by 26 patients. Considering therapy, 26 patients received aspirin or clopidogrel and 4 received acenocoumarol or heparin at the time of the investigation.

3.2. Colonoscopic and Histological Findings. Forty of the 95 patients included in the study represented the control group without any premalignant or malignant findings on endoscopy. Nine of the control patients presented with initial diverticulosis without any sign of inflammation. Colonoscopic findings in the remaining patients of the control group were totally normal.

Thirty-six patients were diagnosed with adenomas (adenoma group). In the adenoma group, 16 patients presented with adenomas sized <1 cm and 20 with adenomas sized ≥1 cm. Adenomas sized <1 cm were equally located at the proximal and the distal part of the colon. The location of adenomas sized ≥1 cm in the majority (65%) of the patients was the proximal part of the colon. In twenty-three adenomatous cases, a histologic sample was obtained. In the remaining thirteen cases, the samples were less than 1 cm and did not suggest the presence of malignancy. Based on the histological assessment of the samples ($n = 23$), in 78.3% of the cases (in 18 patients), the adenomas were with low-grade dysplasia; in 13% (in 3 patients), adenomas were with high-grade dysplasia; and in 8.7% (in 2 patients) there were hyperplastic polyps. In 56.5% of the patients the adenomas were of the tubular type, in 4.3% they were of the villous type, and in 30.4% they belong to the tubulovillous type.

Cancer was found in 19 cases, and, according to their histological evaluation, the tumors were identified as adenocarcinomas. In 89% of the patients, the cancer was located in the distal colon (in 10 patients in the rectum and in 7 patients in the sigmoid colon). In the remaining 2 cases, the tumor was located in the distal part of the transverse colon. 28.8% of these patients had a family history of CRC. The numbers of patients having different stages of cancer according to Dukes classification are shown in Table 2.

Table 2: The numbers of patients having different stages of cancer according to Dukes classification.

Dukes stage	Patients
Carcinoma in situ	1
Dukes A	3
Dukes B	9
Dukes C	1
Dukes D	5

3.3. Diagnostic Accuracy of Fecal Markers in Adenomas and CRCs. M_2PK was positive in 32.5% of the patients with normal colonoscopy, in 43.7% with adenomas sized <1 cm, in 60% with adenomas sized ≥1 cm, and in 94.7% with CRCs. M_2PK sensitivity for adenomas sized >1 cm was 60%, and specificity was 67.5%. Sensitivity and specificity for CRC were 94.7% and 67.5%. Sensitivity and specificity for iFOBT for adenomas sized ≥1 cm were 80% and 72.5% and for CRC were 94.7% and 72.5%. The Hb/Hp (Hb and Hb/Hp ColonView Biohit test) complex was positive in 47.1% of the patients with normal colonoscopy, in 50% with hyperplastic polyps, in 54% with adenomas sized <1 cm, in 80% with adenomas sized ≥1 cm, and in 100% with CRC. Sensitivity and specificity of Hb/Hp complex for adenomas sized ≥1 cm were 80% and 52.9% and for CRC were 100% and 52.9%.

FC and MMP-9 differed significantly between the control and CRC group ($p = 0.022$; $p < 0.001$); however, no difference was found in FC and MMP-9 concentrations between the control and the adenoma groups. FC was significantly lower in adenomas sized <1 cm compared to CRCs but did not differ when compared to adenomas sized ≥1 cm with CRCs

Table 3: Sensitivities, specificities, and positive and negative predictive values of the fecal markers.

Parameters	M_2-PK_{ScheBo}	HB_{SchBo}	HB/HP_{biohit}	Calprotectin	MMP-9
Sensitivity					
Adenoma sized ≥1 cm	60	80	80.0		
CRC	94.7	94.7	100.0	77.8	72.2
Adenoma sized ≥1 cm + CRC	76.9	87.2	90.9		
Specificity					
Adenoma sized ≥1 cm	67.5	72.5	52.9		
CRC	67.5	72.5	52.9	70.0	95.0
Adenoma sized ≥1 cm + CRC	67.5	72.5	52.9		
PPV (%)					
Adenoma sized ≥1 cm	80	59.2	42.9		
CRC	85.7	62	52.9	53.8	86.6
Adenoma sized ≥1 cm + CRC	69.7	75.5	65.2		
NPV (%)					
Adenoma sized ≥1 cm	77.1	96.6	85.7		
CRC	96.4	96.6	100.0	87.5	88.3
Adenoma sized ≥1 cm + CRC	75	85.3	85.7		

($p = 0.022$, $p = 0.089$). MMP-9 proved to be significantly lower compared to either adenomas sized <1 cm with CRCs or adenomas sized ≥1 cm with CRCs ($p \leq 0.001$ and $p \leq 0.001$).

Sensitivity of FC for CRC was 77.8%, while specificity for CRC was 70%. The cut-off value of FC for the detection of CRC was 128.5 μg/g (AUC = 0.77, $p = 0.001$). Sensitivity of MMP-9 for CRC was 72.2%, while specificity was 95%. The cut-off value of MMP-9 for the detection of CRC was 1.12 ng/g (AUC = 0.77, $p < 0.001$).

Using combinations of fecal markers, the highest sensitivity for detection of adenomas sized ≥1 cm was revealed when combining M_2PK, iFOBT, and FC (with the cut-off of 128.5 μg/g) resulting in a sensitivity and specificity of 95% and 47.5% for the detection of adenomas sized ≥1 cm.

Sensitivities, specificities, and positive and negative predictive values of the fecal markers are summarized in Table 3.

We did not find any relationship between platelet aggregation inhibitor therapy and positive results of the different hemoglobin tests (logistic regression: Hb_{ScheBo} $p = 0.4$; Hb/Hp_{Biohit} $p = 0.609$).

4. Discussion

CRC is a major health problem worldwide. Despite being a good candidate for screening due to its detectable premalignant lesions, mortality rates of CRC are still significant in Hungary [17]. Early detection by an accurate, noninvasive, cost-effective, simple-to-use screening technique is central to decrease the incidence and mortality of this disease. Patient discomfort, invasiveness, embarrassment, high cost, and considerable expertise and equipment required for the procedure may all limit the appeal of this screening technique and the increasing number of examinations puts a huge burden on the gastroenterologists. Thus, there is still an unmet need for suitable noninvasive biomarkers to screen for CRC.

In this prospective colonoscopy-controlled study, we assessed the sensitivity, specificity, and positive and negative predictive values of different noninvasive fecal markers for the detection of adenomas and CRC. For adenomas sized ≥1 cm, iFOBT showed the highest sensitivity and M_2PK the highest specificity. For CRC, M_2PK and Hb/Hp complex showed the highest sensitivity and fecal MMP-9 the highest specificity. FC and fecal MMP-9 concentrations did not differ between the control and the adenoma group, although they proved to be beneficial mainly in the detection of adenomas sized ≥1 cm and CRC. In CRCs, the sensitivities of FC and MMP-9 were 78% and 72%, with specificities of 70% and 95%. The combination of M_2PK, iFOBT, and FC increased their sensitivity for the detection of adenomas sized ≥1 cm up to 95%.

The study has some limitations. First, we collected stool samples before performing colonoscopy; thus, we were blinded to the findings and the number of high-grade adenomas finally proved to be low. We do not know whether there would be associations between adenomas and fecal markers if the number of adenomas with high-grade dysplasia would be higher. Second, M_2PK and Hb tests and the Hb/Hp complex were all qualitative tests based on a chromatographic method interpreted visually which may limit their assessment in case of borderline results. Therefore, it may be difficult to compare the results with those of FC and MMP-9. However, these tests are simple, do not require specific laboratory equipment, and therefore are less expensive than the quantitative methods.

The guaiac-based FOBT (gFOBT) is the oldest and most commonly used noninvasive test for detecting CRC [18, 19]. Although the test is relatively inexpensive and easy to perform, false-positive and false-negative results compose its main limitation resulting in limited sensitivity for detecting cancer and advanced adenomas [20]. The Hb/Hp complex shows higher stability against degradation than Hb itself. Sieg

et al. revealed that Hb/Hp complex has a comparable sensitivity to fecal Hb for CRCs (87% for both) and higher sensitivity for adenomas (76% versus 54%) [4]. However, these tests are based on the bleeding property of the adenomas. Since early-stage cancers or advanced adenomas are unlikely to bleed continuously, 100% of clinical sensitivity cannot be achieved with the use of these tests. That is why the identification of novel fecal-based biomarkers is important.

M_2PK is expressed by proliferating cells, in particular the tumor cells being direct target of several oncoproteins. Among the first studies assessing the sensitivity of M_2PK for the detection of CRC, Shastri et al. revealed that fecal M_2PK assay had sensitivity and specificity of 81.1 and 71.1% for diagnosing CRC at a cut-off value of 4 U/mL whereas FOBT showed a sensitivity of 36.5% and specificity of 92.2% for CRC. They concluded that M_2PK is a poor screening biomarker, due to its low specificity [21]. However, a meta-analysis including 17 studies performed between 2006 and 2010 found the mean fecal M_2PK sensitivity and specificity to be 80.3% and 95.2% for CRC and a sensitivity of 44% for adenomas >1 cm [22].

According to our results, M_2PK, Hb, and Hb/Hp tests show better sensitivity in the detection of CRC than advanced adenomas. The study by Kim et al. revealed that the sensitivity of iM_2PK, an immunochromatographic qualitative method for fecal M_2PK for CRC, was 92.8% and for adenomatous lesions the sensitivity was 69.4% [23]. Compared with M_2PK ELISA, iM_2PK exhibited significantly enhanced sensitivity for CRC (97.5% versus 80%, $p = 0.03$).

FC is valuable in differentiating functional and organic bowel diseases. FC was shown to be more sensitive (79%) but less specific (72%) for CRC and adenomatous polyps as a combined group than gFOBT [24]. MMP-9 is an important member of the gelatinases involved in the development of several human malignancies [25]. Yang et al. found that MMP-9 expression in colon cancer tissues was significantly higher than that in corresponding distal normal mucosa tissue [15]. However, the sensitivity of MMP-9 detected in feces has not been examined previously. Our results revealed a moderate sensitivity of 72% and a good specificity of 95% for fecal MMP-9 in CRC. However, neither FC nor fecal MMP-9 provided valuable information on the detection of adenomas.

In this study, we compared the sensitivity and specificity of several fecal markers for the detection of colorectal cancers. The strengths of this study are the design that allowed directly calculating sensitivity and specificity of the different fecal markers, since every patient underwent colonoscopy after stool sample collection. This was the first time when five biomarkers were simultaneously studied. Fecal M_2PK has the advantage that it detects both bleeding and nonbleeding tumors and adenoma. Conversely, fecal M_2PK does not have false-positive results due to various noncancerous sources of bleeding. Furthermore, FC, MMP-9, and fecal M_2PK are also sensitive to intestinal inflammation (inflammatory bowel disease, diverticulitis) increasing the proportion of false-positive cases. In this study, we performed examinations for patients with GI symptom(s) not as a part of screening process because by this method we could disclose false-positive results and could determine specificity data as well.

In our cohort, the highest sensitivity and specificity were achieved by the use of combined M_2PK and iFOBT test in the detection of CRC. FC seems to be a useful adjuvant to the investigation of patients at high risk for colorectal neoplasia, while fecal MMP-9 may be a promising factor for detection of CRC. Although, in CRC, sensitivity of M_2PK, iFOBT, and Hb/Hp complex proved to be high, in adenomas sized ≥1 cm, sensitivity decreased significantly. Therefore, none of these markers are unique for detection of precancerous lesions of the colorectum. However, our result revealed that combined use of M_2PK, iFOBT, and FC may be valuable in the detection of large adenomas.

We recommend these noninvasive fecal tests in low-risk patients and in patients who do not have comorbidities. Results of FOBT may be false positive if the source of bleeding is not an adenoma or a malignant disease (diverticulitis, hemorrhoids, and anticoagulant therapy). However, inflammatory diseases of the colon (diverticulitis, different infections, and inflammatory bowel diseases) and extraintestinal cancer (cancer in the hepatobiliary tract, pancreas) or inflammation (hepatitis) may affect the results of the inflammatory marker test; thus, in these cases, we recommend colonoscopy as a one-step investigation. High-risk patients (who had at least one relative with early CRC or adenoma or had at least two relatives with CRC or adenoma) with symptoms or patients who have early (under the age of 60) CRC or adenoma among their relatives should also undergo colonoscopy. However, it is not questionable whether continued efforts are needed to discover effective tests to identify patients with nonhereditary risk factors and to develop invasive and cost-effective screening modalities.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Klaudia Farkas and Tamás Molnár contributed equally to this study.

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Research Article

A Novel Inflammation-Based Stage (I Stage) in Patients with Resectable Esophageal Squamous Cell Carcinoma

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Background. Inflammation plays a key role in cancer. In the current study, we proposed a novel inflammation-based stage, named I stage, for patients with resectable esophageal squamous cell carcinoma (ESCC). *Methods*. Three hundred and twenty-three patients with resectable ESCC were enrolled in the current study. The I stage was calculated as follows: patients with high levels of C-reactive protein (CRP) (>10 mg/L), neutrophil-to-lymphocyte ratio (NLR) (>3.5), and platelet-count-to-lymphocyte ratio (PLR) (>150) were defined as I3. Patients with two, one, or no abnormal value were defined as I2, I1, or I0, respectively. The prognostic factors were evaluated by univariate and multivariate analyses. *Results.* There were 112 patients for I0, 97 patients for I1, 66 patients for I2, and 48 patients for I3, respectively. The 5-year cancer-specific survival (CSS) in patients with I0, I1, I2, and I3 was 50.0%, 30.9%, 18.2%, and 8.3%, respectively (I0 versus I1, $P = 0.002$; I1 versus I2, $P = 0.012$; I2 versus I3, $P = 0.020$). Multivariate analyses revealed that I stage was an independent prognostic factor in patients with resectable ESCC ($P < 0.001$). *Conclusion*. The inflammation-based stage (I stage) is a novel and useful predictive factor for CSS in patients with resectable ESCC.

1. Introduction

The cancer incidence and mortality have been increasing worldwide. Esophageal cancer (EC) is one of the most common cancers and remains the 4th leading cause of cancer death [1]. There are two major histologic types of EC: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is the most common pathological type in China [2, 3]. However, the prognosis for patients with ESCC is still poor [3]. Therefore, assessing the prognostic factors in ESCC patients will become more and more important.

Recent reports revealed that inflammation plays an important role in cancer [4, 5]. Therefore, a series of inflammation-based biomarkers, such as C-reactive protein (CRP), neutrophil-to-lymphocyte ratio (NLR), and platelet-count-to-lymphocyte ratio (PLR), have been analysed in various cancers [6–11]. However, the prognostic values of these biomarkers in patients with ESCC remain uncertain [12–17]. In addition, most of these studies only evaluated one or two biomarkers without considering others. In the current study, therefore, we proposed a novel inflammation-based stage, named I stage (combination of CRP, NLR, and PLR), for predicting the prognosis for patients with resectable ESCC.

2. Patients and Methods

A retrospective analysis was conducted for patients with ESCC in our hospital from January 2005 to December 2008. Patients with ESCC were confirmed by histopathology. All patients underwent surgery with curative esophagectomy and standard lymphadenectomy. Patients who had received preoperative therapy were excluded. Patients with any form of acute infection or chronic inflammatory disease were also excluded. At last, 323 patients were enrolled in our study. In the current study, a cancer-specific survival (CSS) analysis was ascertained. The last follow-up was on 30 June 2013. This study was approved by the Ethical Committees of Zhejiang Cancer Hospital (Hangzhou, China). All patients were staged according to the 7th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging [18].

Table 1: Clinicopathological characteristics for patients with ESCC.

	Cases (n, %)
Age (years, mean ± SD)	59.1 ± 7.9
Gender	
Female	42 (13.0)
Male	281 (87.0)
Tumor length (cm, mean ± SD)	4.3 ± 1.9
Tumor location	
Upper	17 (5.3)
Middle	151 (46.7)
Lower	155 (48.0)
Differentiation	
Good	44 (13.6)
Moderate	216 (66.9)
Poor	63 (19.5)
T grade	
T1	55 (17.0)
T2	55 (17.0)
T3	179 (55.4)
T4	34 (10.6)
N stage	
N0	174 (53.9)
N1	87 (26.9)
N2	37 (11.5)
N3	25 (7.7)
TNM stage	
I	81 (25.1)
II	104 (32.2)
III	138 (42.7)
I stage	
I0	112 (34.7)
I1	97 (30.0)
I2	66 (20.4)
I3	48 (14.9)
CRP (mg/L, mean ± SD)	9.7 ± 13.5
NLR (mean ± SD)	3.3 ± 2.8
PLR (mean ± SD)	160.9 ± 70.6

Routine laboratory results (including CRP, neutrophil, lymphocyte, and platelet count) were extracted in retrospective medical records. The definitions of NLR and PLR were described as follows: NLR is neutrophil-to-lymphocyte ratio and PLR is platelet-count-to-lymphocyte ratio. The cut-off values for CRP, NLR, and PLR were 10 mg/L, 3.5, and 150 according to the previous studies [12, 13, 16, 17]. Therefore, the I stage was calculated as follows: patients with high levels of CRP (>10 mg/L), NLR (>3.5), and PLR (>150) were defined as I3. Patients with two, one, or no abnormal value were defined as I2, I1, or I0, respectively.

2.1. Statistical Analysis. The 5-year CSS was analysed by the Kaplan-Meier method. Univariate and multivariate Cox analyses were performed to analyse the prognostic factors.

Table 2: The relationship between I stage and clinicopathological characteristics.

	I stage 0 (n = 112)	I stage 1 (n = 97)	I stage 2 (n = 66)	I stage 3 (n = 48)	P value
Age (years)					0.817
≤60	66	58	37	25	
>60	46	39	29	23	
Gender					0.375
Female	18	14	5	5	
Male	94	83	61	43	
Tumor length (cm)					<0.001
≤3	45	31	9	4	
>3	67	66	57	44	
Tumor location					0.488
Upper	8	4	1	4	
Middle	51	49	28	23	
Lower	53	44	37	21	
Vessel involvement					0.385
Negative	99	79	54	38	
Positive	13	18	12	10	
Perineural invasion					0.043
Negative	98	70	52	40	
Positive	14	27	14	8	
Differentiation					0.310
Good	17	10	12	5	
Moderate	80	65	41	30	
Poor	15	22	13	13	
T stage					<0.001
T1	33	18	3	1	
T2	23	14	11	7	
T3	50	58	42	29	
T4	6	7	10	11	
N stage					<0.001
N0	71	55	32	16	
N1	30	30	12	15	
N2	6	9	12	10	
N3	5	3	10	7	
TNM stage					<0.001
I	46	21	9	5	
II	31	41	22	10	
III	35	35	35	33	

Pearson correlation analyses were performed to analyse the correlation. Receiver operating characteristic (ROC) curves were plotted to determine the accuracy of CRP, NLR, and PLR. A $P < 0.05$ was considered to be statistically significant. Statistical analyses were conducted with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

Clinicopathologic characters were shown in Table 1. The mean CRP, NLR, and PLR were 9.7 ± 13.5 (mg/L), 3.3 ± 2.8, and 160.9 ± 70.6, respectively. The histograms of CRP,

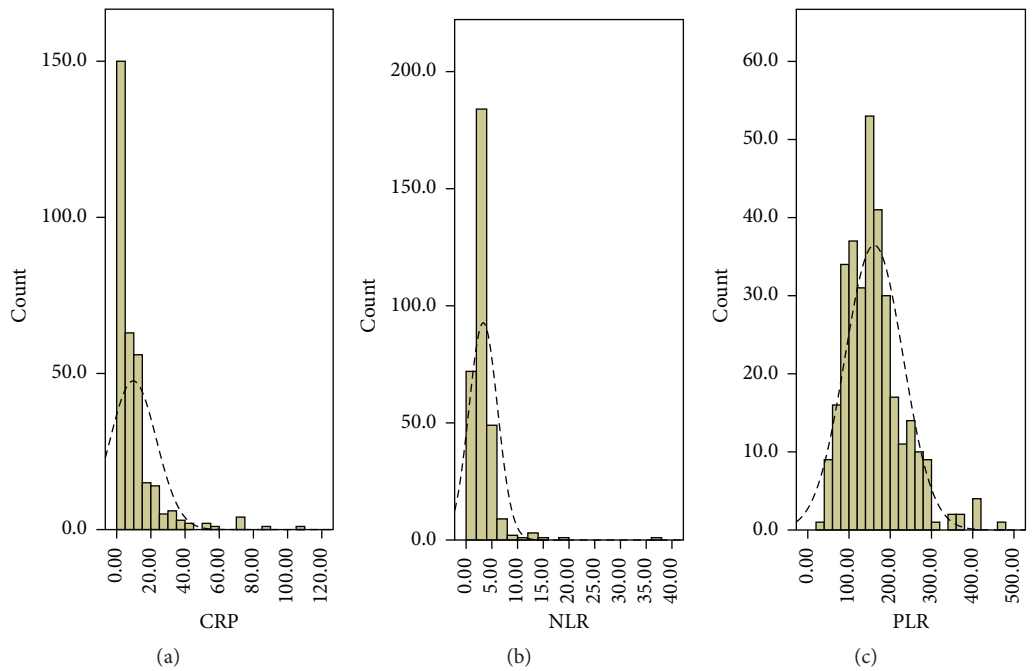

Figure 1: The histograms of the CRP (a), NLR (b), and PLR (c).

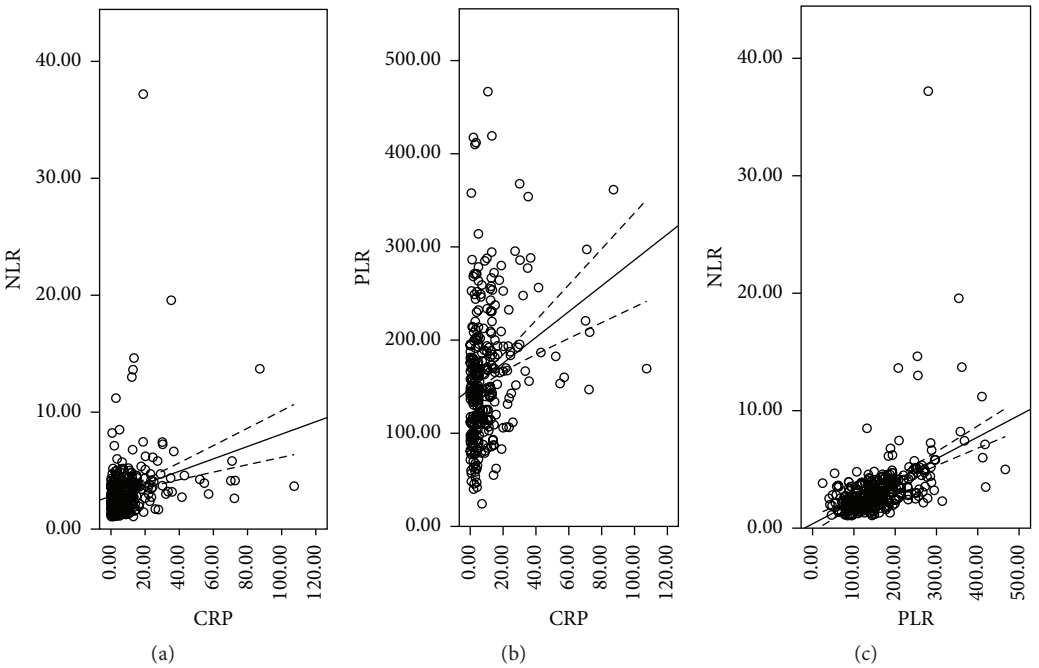

Figure 2: Pearson correlation analysis. Positive correlations in CRP and NLR ($r = 0.258$, $P < 0.001$; (a)), CRP and PLR ($r = 0.265$, $P < 0.001$; (b)), and NLR and PLR ($r = 0.470$, $P < 0.001$; (c)).

NLR, and PLR were shown in Figure 1. There were significant positive correlations in CRP and NLR ($r = 0.258$, $P < 0.001$), CRP and PLR ($r = 0.265$, $P < 0.001$), and NLR and PLR ($r = 0.470$, $P < 0.001$) (Figure 2). ROC curves for CSS prediction were shown in Figure 3. The area under the curve (AUC) was 0.713 (95% CI: 0.653–0.772, $P < 0.001$) for CRP, 0.650 (95% CI: 0.589–0.711, $P < 0.001$) for NLR, and 0.685 (95% CI: 0.626–0.744, $P < 0.001$) for PLR.

Of the 323 patients, 112 (34.7%) were allocated an I stage 0, 97 (30.0%) were allocated an I stage 1, 66 (20.4%) were allocated an I stage 2, and 48 (14.9%) were allocated an I stage 3, respectively. The relationships between the I stage

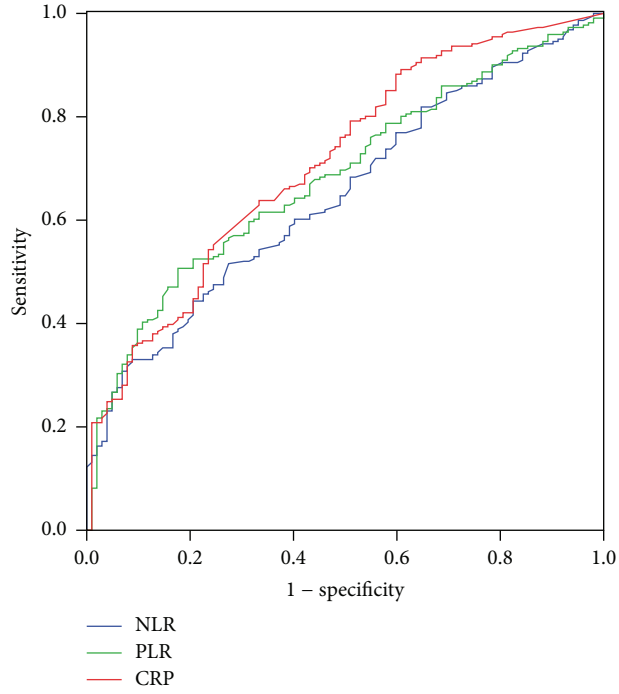

Figure 3: ROC curves for CSS prediction. The area under the curve (AUC) was 0.713 (95% CI: 0.653–0.772, $P < 0.001$) for CRP, 0.650 (95% CI: 0.589–0.711, $P < 0.001$) for NLR, and 0.685 (95% CI: 0.626–0.744, $P < 0.001$) for PLR.

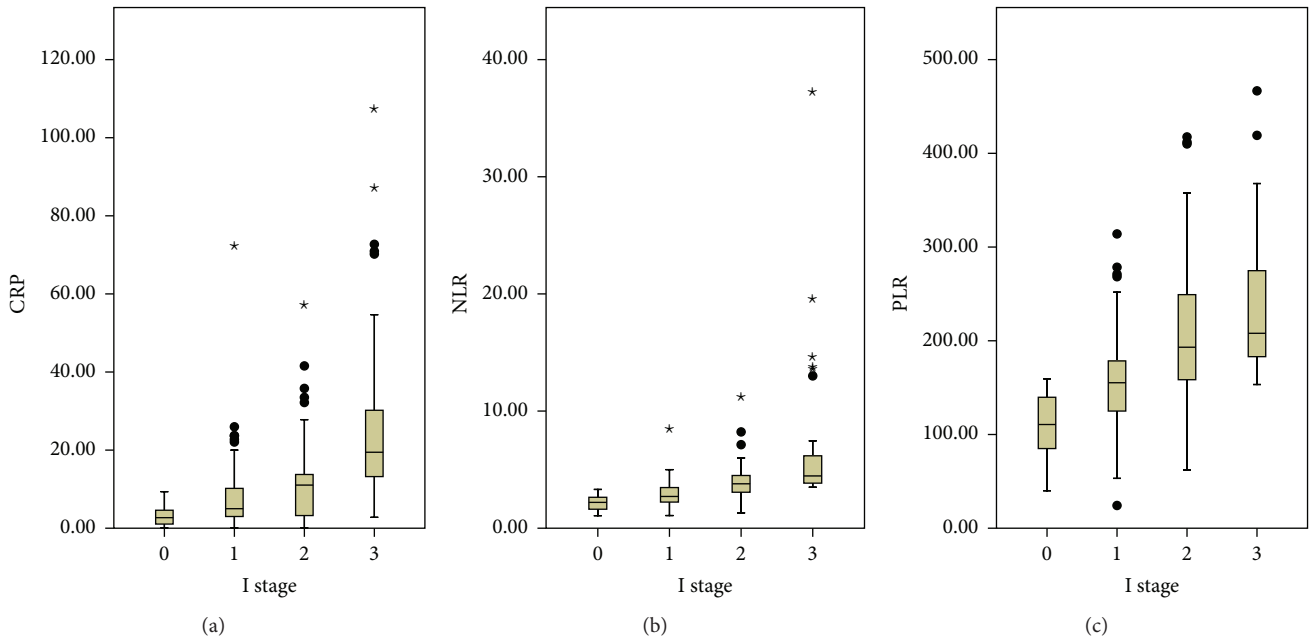

Figure 4: The CRP (a), NLR (b), and PLR (c) were significantly higher in patients with high I stage ($P < 0.001$). The "*" and "•" were created by SPSS statistical software.

and clinicopathological characteristics were shown in Table 2. Our study demonstrated that I stage was associated with tumor length ($P < 0.001$), perineural invasion ($P = 0.043$), T stage ($P < 0.001$), N stage ($P < 0.001$), and TNM stage ($P < 0.001$). In addition, our study revealed that CRP, NLR, and PLR were significantly higher in patients with high I stage ($P < 0.001$, Figure 4).

The 5-year CSS in patients with I0, I1, I2, and I3 was 50.0%, 30.9%, 18.2%, and 8.3%, respectively ($P < 0.001$, Figure 5) (I0 versus I1, $P = 0.002$; I1 versus I2, $P = 0.012$;

Table 3: Univariate analyses for patients with ESCC.

	5-year CSS (%)	*P* value	HR (95% CI)	*P* value
Age (years)		0.978		0.978
≤60	30.1		1.000	
>60	33.6		0.996 (0.762–1.302)	
Gender		0.322		0.327
Female	38.1		1.000	
Male	30.6		1.227 (0.815–1.848)	
Tumor length (cm)		0.003		0.004
≤3	41.6		1.000	
>3	27.8		1.580 (1.157–2.157)	
Tumor location		0.556		0.564
Upper	41.2		1.000	
Middle	33.1		0.735 (0.385–1.404)	0.351
Lower	29.0		0.908 (0.693–1.190)	0.483
Differentiation		0.198		0.207
Good	38.6		1.000	
Moderate	31.0		1.185 (0.786–1.786)	0.417
Poor	28.6		1.504 (0.933–2.424)	0.098
Vessel involvement		0.007		0.008
Negative	34.1		1.000	
Positive	18.9		1.577 (1.129–2.202)	
Perineural invasion		0.005		0.006
Negative	35.0		1.000	
Positive	17.5		1.551 (1.135–2.119)	
TNM stage		<0.001		<0.001
I	51.9		1.000	
II	32.7		1.878 (1.269–2.780)	0.002
III	18.8		2.943 (2.039–4.248)	<0.001
I stage		<0.001		<0.001
I0	50.0		1.000	
I1	30.9		1.696 (1.189–2.420)	0.004
I2	18.2		2.676 (1.837–3.900)	<0.001
I3	8.3		4.372 (2.924–6.536)	<0.001
Adjuvant therapy		0.398		0.402
No	32.0		1.000	
Yes	30.6		1.130 (0.849–1.504)	
CRP (mg/L)		<0.001		<0.001
≤10.0	39.2		1.000	
>10.0	17.1		2.217 (1.692–2.906)	
NLR		<0.001		<0.001
≤3.5	39.0		1.000	
>3.5	17.7		1.925 (1.471–2.519)	
PLR		<0.001		<0.001
≤150	43.9		1.000	
>150	17.3		2.260 (1.729–2.955)	

I2 versus I3, $P = 0.020$). In addition, our study revealed that patients with CRP (>10.0 mg/L), NLR (>3.5), or PLR (>150) were significantly associated with decreased CSS, respectively ($P < 0.001$). Then, we further stratified patients into different groups based on TNM stage. Our results demonstrated that I stage was also significantly correlated with CSS based on TNM stage (Figure 6).

Among the above variables, univariate analyses revealed that tumor length ($P = 0.004$), vessel involvement ($P = 0.008$), perineural invasion ($P = 0.006$), TNM stage

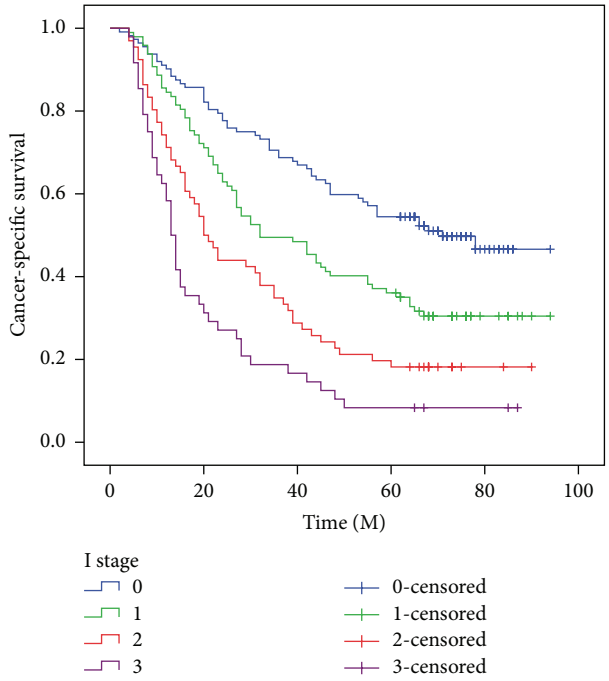

Figure 5: The 5-year CSS in patients with I0, I1, I2, and I3 was 50.0%, 30.9%, 18.2%, and 8.3%, respectively ($P < 0.001$) (I0 versus I1, $P = 0.002$; I1 versus I2, $P = 0.012$; I2 versus I3, $P = 0.020$).

($P < 0.001$), and I stage ($P < 0.001$) were predictive of CSS (Table 3). In multivariate analyses, we demonstrated that I stage was an independent prognostic factor in patients with resectable ESCC ($P < 0.001$) (Table 4).

Table 4: Multivariate analyses for patients with ESCC.

	HR (95% CI)	*P* value
Tumor length (cm)		0.603
≤3	1.000	
>3	1.075 (0.818–1.412)	
Vessel involvement		0.742
Negative	1.000	
Positive	1.060 (0.747–1.505)	
Perineural invasion		0.077
Negative	1.000	
Positive	1.341 (0.968–1.857)	
TNM stage		<0.001
I	1.000	
II	1.586 (1.048–2.400)	0.029
III	2.220 (1.456–3.384)	<0.001
I stage		<0.001
I0	1.000	
I1	1.543 (1.076–2.214)	0.018
I2	2.356 (1.602–3.466)	<0.001
I3	3.594 (2.363–5.467)	<0.001
CRP (mg/L)		0.493
≤10.0	1.000	
>10.0	1.151 (0.770–1.719)	
NLR		0.786
≤3.5	1.000	
>3.5	1.050 (0.740–1.488)	
PLR		0.065
≤150	1.000	
>150	1.440 (0.978–2.121)	

4. Discussion

In the current study, we initially proposed a novel inflammation-based prognostic system, named I stage (combination of CRP, NLR, and PLR), in patients with resectable ESCC. Our study revealed that I stage was associated with tumor length, perineural invasion, and TNM stage. In multivariate analyses, we revealed that I stage is a useful predictor of postoperative CSS in patients with resectable ESCC ($P < 0.001$).

Several hematological biomarkers have shown prognostic values in cancers. In particular, the CRP has been well validated. CRP is a representative acute-phase reactant for inflammation [19]. Recently, several previous studies have shown that CRP is associated with prognosis in several cancers, including ECs [6, 8–12]. In our study, patients with CRP ≤ 10.0 mg/L had a significantly better 5-year CSS than patients with CRP > 10.0 mg/L (39.2% versus 17.1%, $P < 0.001$). However, CRP was not an independent prognostic factor in multivariate analyses ($P = 0.493$).

The prognostic values of NLR and PLR in patients with EC remain uncertain. Several reports demonstrated that NLR is an independent prognostic factor in patients with EC [14, 15]. However, Rashid et al. [13] and Dutta et al. [16] revealed that NLR does not correlate with prognosis for patients with EC. Moreover, there have been few studies regarding PLR in EC patients. Dutta et al. [16] demonstrated that PLR does not correlate with prognosis in patients with EC. A retrospective study by Liu et al. [20] on 326 ESCC patients revealed PLR to be a potential prognostic factor. In our study, NLR and PLR were correlated with survival; however, NLR and PLN were not independent prognostic factors in multivariate analyses.

At present, the prognosis of cancer is commonly based on the TNM staging system [21, 22]. Inflammation plays an important role in cancer. Therefore, in our study, we proposed a novel inflammation-based prognostic system (I stage) in resectable ESCC patients. A significant association was found between the I stage and clinical characteristics. In multivariate analyses, we revealed that I stage is a useful predictor of postoperative CCS in patients with resectable ESCC ($P < 0.001$). It may well be that the influence of I stage on the subgroup with TNM stage is important for the understanding of its role in patients with ESCC. Our results demonstrated that I stage was also significantly correlated with CSS based on TNM stage.

Limitations should be acknowledged. Firstly, our study was a retrospective study. Secondly, we excluded patients with neoadjuvant treatment, which may have influenced the results. Neoadjuvant treatment will inevitably have an impact

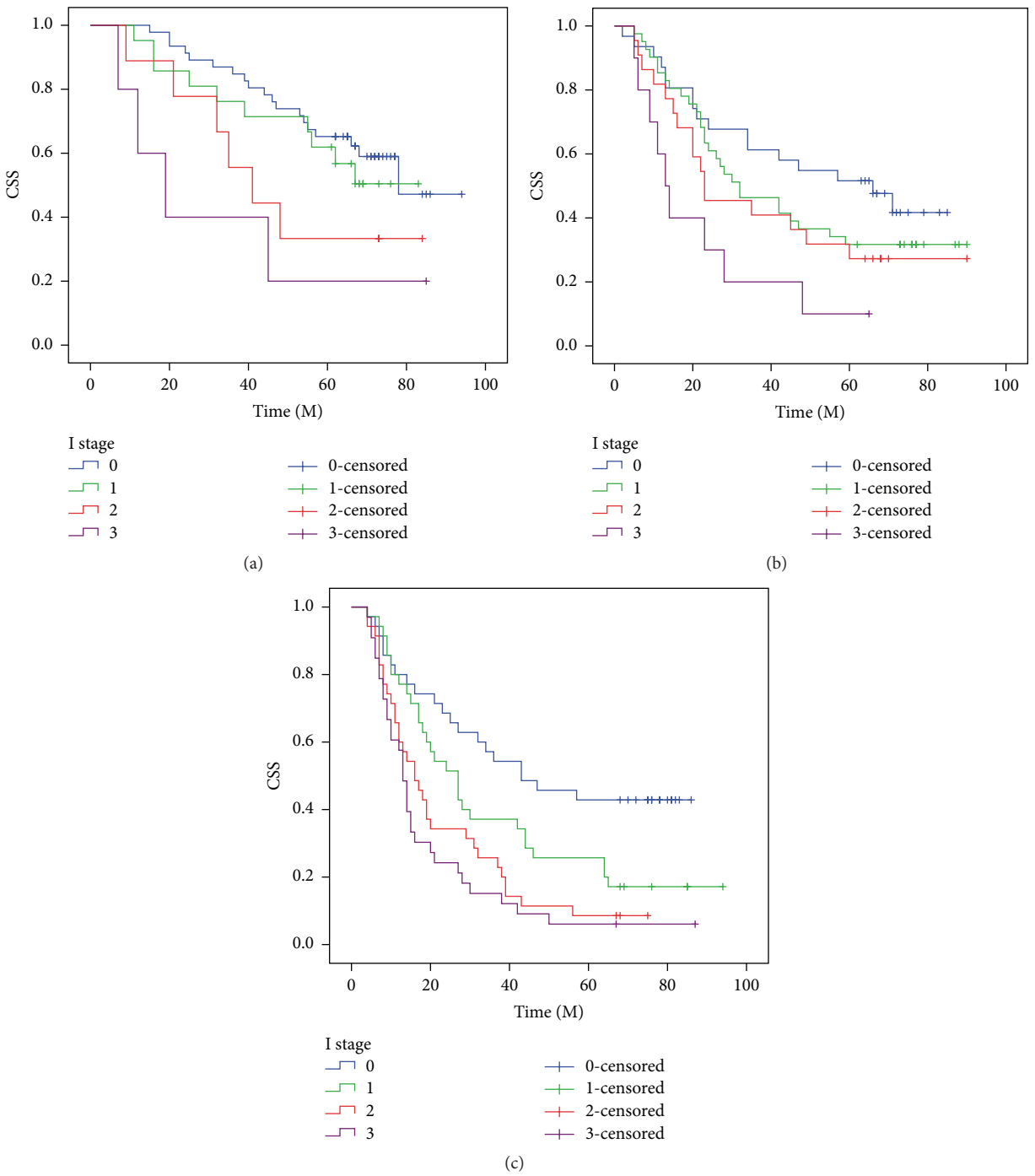

Figure 6: The predictive values of I stage were significant in patients based on TNM stage. TNM I stage ($P = 0.035$, (a)), TNM II stage ($P = 0.028$, (b)), and TNM III stage ($P < 0.001$, (c)).

on the systemic inflammation. Thus, evaluation of I stage in neoadjuvant therapy does not reflect the baseline impact of systemic inflammation for ESCC patients. Therefore, larger prospective studies will need to be performed to confirm these preliminary results.

In summary, there was a significant association between the I stage (combination of CRP, NLR, and PLR) and clinical characteristics. Based on the results of the current study, we believe that I stage is a novel and useful predictive factor for CSS in patients with resectable ESCC.

Competing Interests

The authors have no competing interests to disclose.

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