Ki-67 Expression in CRC Lymph Node Metastasis Does Not Predict Survival

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and a leading cause of cancer death worldwide [1–5]. European countries rank the highest in the global statistics, in terms of both CRC incidence and mortality [4, 6], although in recent years, a decline in CRC mortality rates has been observed, mostly due to improvement in earlier diagnosis and treatment [4, 7].

In Portugal, official data revealed that CRC is the second most common type of cancer, in both men and women, and in 2008 it was responsible for 18.7% and 15.1%, respectively, of all cancer in Portugal [8]. Regarding mortality, unlike European data [9], there was an average increase of 3% from 2000 to 2005 [10] and in 2012 incidence and mortality rates are higher than European rates [11].

For CRC, the pathologic clinical stage is currently the single most important prognostic factor [1–4, 12, 13], correlating with long-term survival [4, 14–17], although it does not fully predict individual clinical outcome [4, 17–19]. This is particularly true for those tumours with intermediate stage disease (T3–T4N0M0) [19], where one-third of patients with tumour-free lymph nodes have recurrences, and therefore adjuvant chemotherapy may be beneficial [20]. In this group, carcinoma cells are not detected in lymph nodes by conventional staging methods in 24% of patients. So, lymphatic staging is essential to improve treatment of these patients, indeed one-third of the patients submitted to curative intent...
surgery die of local and/or distant tumour recurrence [4, 15]. Abdominal lymph nodes (38%) are the second most frequent site of metastasis (38%), just after liver, that is, the organ most frequently involved (38–60% of cases) and followed by lung (38%) and peritoneum (28%) [4, 13].

A common feature of all cancers is the imbalance that exists between the proliferative activity and cell death; therefore, the evaluation of cell proliferation rate may be interesting in the study and characterization of tumours [21]. Some molecules, such as the Ki-67 protein, permit this assessment and are used as markers of proliferation because Ki67 expression is dependent of cell division rate; thus, overexpression of these markers may suggest a disruption in the proliferation mechanism leading to the appearance of tumours [21].

Ki-67 protein, when used to evaluate the percentage of dividing cells, allows us to determine neoplastic growth [21] and has been documented to correlate with neoplastic progression [22] showing different levels of expression between normal mucosa, adenoma, and adenocarcinoma [23], verifying a progressive increasing of positive Ki-67 expression from the first (normal mucosa) to the last part of the tissue (adenocarcinoma) [22, 24, 25].

Other studies correlate Ki-67 with the degree of malignancy, tumour invasiveness [25, 26], metastatic potential [21], patient survival, and the risk of relapse [27, 28]. Thus, a high Ki-67 expression in tumour cells is assumed to correlate with a poor tumour differentiation [24, 26] and an increased infiltration of the bowel wall (pT) [26]. Micev et al. [29] demonstrated that there is an association between Ki-67 expression and a less effective response in patients undergoing chemotherapy.

Other correlations with clinical and pathological data were also investigated and a correlation was detected between a high expression of this protein and the following variables: patient’s age [25], tumour size [30], tumour localization [28], dysplasia degree [30], the presence of lymph node metastasis [22, 25, 28], and TNM [25] and Dukes [28] classification. Thus, the younger is the patient, the greater is the cellular proliferation and the lower is the degree of differentiation; with increasing malignancy a increased frequency of invasion and metastasis are observed and thus poorer prognosis [25].

In CRC, the analysis of colon adenomas has shown a different pattern for Ki-67 expression between normal tissue, adenomas, and adenocarcinomas, being limited to the crypts in normal tissue and expressed both in the crypts and in the surface epithelium in adenomatous polyps (tubular, villous) [31] and distributed homogeneously in adenocarcinoma [32]. Nussrat et al. [30] also observed an increase in Ki-67 rates being associated with the growth and rise of dysplasia in adenomas.

Studies on CRC indicated Ki-67 as a prognostic marker as the survival rate for patients with high expression of Ki-67 is significantly lower compared to those with low expression [25, 33–35] and a predictor of CRC recurrence [36]. Also significant associations were found between higher index of Ki-67 and increased tumour penetration [35, 37], the presence of lymph node [22, 35] and distant [35] metastasis, advanced TNM stage [32, 35], highest degree of differentiation, and subtypes of adenocarcinoma other than mucinous [38].

However, not all studies are in agreement, and no correlations were observed with patient age, gender, tumour location [22, 30, 33, 39], and the type of adenoma [30] for some of them. Allegre et al. [40] described inverse associations, with a lower rate of Ki-67 to be associated with greater recurrence and worse overall survival and Jansson and Sun [39] did not find any associations between index Ki-67 and clinicopathological data or prognosis.

Regarding the use of Ki-67 in CRC lymph node metastasis, no information is available, and the only similar study found compares Ki-67 index in primary tumour with peritoneal metastasis and had observed a lower proliferative index in metastasis compared with the primary tumour [41]. However, in other types of cancer, in particular breast cancer, a higher Ki-67 index was found in lymph node metastasis than in primary tumours [42–44], suggesting greater aggressiveness of these [42] and that the use of Ki-67 in lymph node metastasis may be important in selecting the appropriate treatment for certain subgroups of patients [45].

Therefore, given the limited information concerning Ki-67 index in CRC lymph nodes metastasis and primary tumour, this study becomes relevant to determine Ki-67 index in the primary tumour and, respectively, lymph nodes metastasis whilst trying to establish correlations with this and clinicopathological data and the patient’s prognosis.

2. Materials and Methods

2.1. CRC Tumour Series. Tissue samples and data from 672 patients treated in Hospital de Braga, Portugal, between January 1, 2005, and January 1, 2010, with CRC diagnosis were collected prospectively. Tumour localization was recorded and classified as colon and rectum (between anal verge and 15 cm at rigid rectoscopy).

The histological type of CRC was classified by two experienced pathologists and tumour staging was graded according to the TNM classification, sixth edition [46]. Tissue microarrays (TMAs) were constructed with the CRC series of formalin-fixed, paraffin-embedded tissues and analyzed by immunohistochemistry. Prior to tumour construction, hematoxylin and eosin sections were reviewed to select representative areas of the tumour and normal-adjacent tissue. Each case was represented in the TMA by at least two cores of 0.6 mm.

2.2. Lymph Node Metastasis Series. From the same series of colorectal cancer, patients with the diagnosis of CRC lymph node metastasis were selected and a series of 210 patients were also collected.

Additionally, 35 patients, with the diagnosis of CRC but without lymph node metastasis, were also selected for control of protein lymph node expression (stages T1 and T2/N0). TMAs were constructed with the lymph node metastasis series of formalin-fixed, paraffin-embedded tissues and analyzed by immunohistochemistry. Each case was represented in the TMA by at least two cores of 0.6 mm.

The study protocol was approved by the Ethics Committee of Hospital de Braga and ICVS.
Figure 1: Immunohistochemical expression of Ki-67 in samples of skin: (a) original magnification ×40; (b) original magnification ×100; (c) original magnification ×200.

Table 1: Detailed aspects of the immunohistochemical procedure used to visualise the Ki-67.

| Protein marker | Antigen retrieval | Peroxidase inactivation | Detection system | Antibody | Dilution | Incubation period |
|----------------|-------------------|-------------------------|------------------|-----------|----------|-------------------|
| Ki-67          | CitrateBuffer     | 3% H₂O₂ in methanol, 10 min. | RTU Vectastain   | GenNova   | 1:200    | Overnight         |

2.3. Immunohistochemistry. CRC and lymph nodes TMAs protein expression was evaluated by immunohistochemistry. Detailed information is given in Table 1. After the immunohistochemical procedure, the slides were evaluated and then photographed under a microscope.

For positive control of the expression of Ki-67 a sample of the skin was used (Figure 1).

2.4. Immunohistochemical Evaluation. The percentage of immunoreactive cells was determined (which was named the Ki-67 index), counting a total of 100 cells per section at ×20 magnification, and each one was assigned a score from 0 to 3, as previously described by Pinheiro et al. [47]. Immunoreaction final score was defined as the sum of both parameters and grouped as negative (0-1) and positive (≥2). Evaluation of protein expression was performed by blind analysis by two observers and discordant cases were discussed in a double-head microscope in order to determine a final score.

2.5. Statistical Analysis. All data were analyzed using the Statistical Package for the Social Sciences, version 19.0 (SPSS Inc., Chicago, IL, USA). All comparisons were examined for statistical significance using Pearson’s chi-square (χ²) test and Fisher’s exact test (when n < 5), with the threshold for significance P < 0.05. Survival curves were determined for overall survival by the Kaplan-Meier method and log-rank test.

Expression differences between lymph node metastasis and primary CRC were tested with McNemar test, with the threshold for significance P < 0.05.

3. Results

3.1. Ki-67 Expressions in CRC Samples. A total of 672 samples were organized into TMAs, including tumour and normal adjacent epithelium (NAE). Sections were evaluated for immunoeexpression and the obtained results are given in Table 2, which summarizes the frequency of Ki-67 expression in tumour cells and NAE.
We observed that 68.2% (n = 345) of the samples of tumour tissue were positive for Ki-67, as compared to 24.3% (n = 34) of samples of the samples of NA E. Thus, it was concluded that the Ki-67 expression is significantly higher in tumour tissue (P < 0.05), such as is shown in Table 2.

Figure 2 shows representative cases of positive staining for Ki-67 in tumour cells and in NA E.

### 3.2. Associations between Ki-67 Expressions in CRC Tissues and Clinicopathological Data

The associations observed between the expression of Ki-67 in CRC and the clinicopathological data are described in Tables 3 and 4.

Analyzing the results in these tables, we found an association between the expression of Ki-67 and "tumour penetration" (P = 0.013) and "tumour differentiation" (P = 0.049).

For "tumour penetration," we observed a decreasing expression of Ki-67 from the pT1 (79.3%) to pT3 (68.1%) tumours and then a rise in expression for adenocarcinoma with invasion of other organs or structures (pT4) (73.1%).

Regarding "tumour differentiation," we observed an increasing expression of Ki-67 from the well-differentiated to the undifferentiated tumours, namely, well differentiated (64.6%), moderately differentiated (70.2%), and poorly differentiated (85.1%). Conversely, undifferentiated tumours showed lower expression of Ki-67 compared to the degree of differentiation mentioned above.

We did not find any statistically significant relationship between clinicopathological data and Ki-67 index in CRC for the remaining assessed data.

### 3.3. Overall Survival Curves according to Ki-67 Expressions in CRC Tissues

No statistically significant association was observed for Ki-67 expression in CRC tissues (P = 0.321 for CRC, and P = 0.213 and P = 0.874 for colon cancer and rectal cancer evaluated separately, resp.), as observed in Figure 4.

Relatively to CRC, survival of patients that are negative for Ki-67 is 65.6% with a medium of survival of 65.0 ± 2.8 months after diagnosis, while Ki-67 positive patients present a survival of 62.3% with a medium of survival of 62.1 ± 2.1 months after diagnosis, such as is shown in Table 5.

### 3.4. Ki-67 Expressions in Lymph Node Metastasis Samples

A total of 210 samples were organized into TMAs. Additionally 35 patients, with the diagnosis of CRC but without lymph node metastasis, were also selected for control of protein lymph node expression (stages T1 and T2 N0). Sections were evaluated for immunoexpression and the obtained results are given in Table 2, which summarizes the frequency of Ki-67 expression in "normal" lymph nodes and lymph node metastasis.

We observed that 55.5% (n = 60) of the samples of lymph node metastasis were positive for Ki-67, compared to 100% (n = 2) of samples of the samples of "normal" lymph nodes. No significant correlation was observed (P = 0.502), such as is shown in Table 2 and Figure 3.

### 3.5. Associations between Ki-67 Expressions in Lymph Node Metastasis and Clinicopathological Data

The associations observed between the expression of Ki-67 in lymph node metastasis of CRC and the clinicopathological data are described in Tables 3 and 4. Analyzing these tables, we did not find any statistically significant relationship between clinicopathological data and Ki-67 index in lymph node metastasis.

### 3.6. Overall Survival Curves according to Ki-67 Expressions in Lymph Node Metastasis

Relatively to overall survival, patients with negative Ki-67 index present a survival of 65.3% with a medium of survival of 63.4 ± 5.2 months after diagnosis, while Ki-67 index positive patients present a survival of 51.6% with a medium of survival of 50.0 ± 4.6 months after diagnosis, such as is shown in Table 5.

No statistically significant association was observed for Ki-67 expression in CRC lymph node metastasis tissues (P = 0.131 for CRC, P = 0.127 and P = 0.809 for colon cancer and rectal cancer evaluated separately, resp.); however, a tendency for the relationship between a positive Ki-67 index and a lower overall survival was observed, such as is shown in Figure 5.

### 3.7. Comparing Ki-67 Index Expressions in Lymph Node Metastasis and Primary Tumour

Table 6 represents a comparison between Ki-67 index in the primary tumour and the respective lymph node metastasis. Analyzing this table, it appears that 12 of the cases with negative Ki-67 index in the primary tumour have positive index in lymph node metastasis and that 29 of those with positive index in the primary tumour have a negative index in lymph node metastasis, for a total of 41 discordant cases. This means that there is a significant difference (P = 0.012) between the index of Ki-67 in primary tumour and the respective lymph node metastasis. A smaller number of cases of positive Ki-67 index were also observed in lymph node metastasis (n = 61; 57.5%) than in the primary tumour (n = 78; 73.6%) as is schematized in Figure 6.

### 4. Discussion

The mechanisms that culminate in CRC development, growth, and metastatization are still not fully understood.
However, common to all cancers are the loss of cellular differentiation and the imbalance between proliferation and cell death; these processes, involved in carcinogenesis, due to its significance, are increasingly being targeted for study.

Ki-67 protein has been widely used as a marker of tumour proliferation [21, 48, 49], and several studies compare Ki-67 index with clinicopathological data and follow-up in CCR [22, 32, 35–40]. With regard to Ki-67 index in lymph node metastasis, as far as we know, this is the first study realized in CRC and, the more similar that we found in literature is the study of Yamauchi et al. [41], which compares Ki-67 index in primary CRC tumours with the respective nodules of peritoneal metastization.

In this study, we determine immunohistochemical expression of Ki-67 protein in CRC samples and respective lymph node metastasis and intended to evaluate possible associations between these expressions and several clinicopathological parameters and patient survival. Further comparison was performed between Ki-67 index in CRC and respective lymph nodes metastasis.

Regarding Ki-67 expression in CRC samples and NA, we observed a significant expression of Ki-67 in the first over
Table 3: Assessment of correlation between Ki-67 expression and clinical data.

|                          | Ki-67 in CRC | Ki-67 in lymph node metastasis |   |
|--------------------------|--------------|-------------------------------|---|
|                          | n            | Positive n (%) | p    | n            | Positive n (%) | p |
| Gender                   |              |                  |      |              |                  |   |
| Male                     | 307          | 212 (69.1)       | 0.675| 71           | 41 (57.7)       | 0.438|
| Female                   | 180          | 121 (67.2)       |      | 38           | 19 (50.0)       |   |
| Age                      |              |                  |      |              |                  |   |
| ≤45                      | 23           | 14 (60.9)        | 0.491| 7            | 4 (57.1)        | 1.000*|
| >45                      | 464          | 319 (68.8)       |      | 102          | 56 (54.9)       |   |
| Presentation             |              |                  |      |              |                  |   |
| Asymptomatic             | 88           | 63 (71.6)        | 0.474| 19           | 8 (42.1)        | 0.212|
| Symptomatic              | 399          | 270 (67.7)       |      | 90           | 52 (57.8)       |   |
| Localization             |              |                  |      |              |                  |   |
| Colon                    | 353          | 240 (68.0)       | 0.764| 90           | 50 (55.5)       | 0.841|
| Rectum                   | 134          | 93 (69.4)        |      | 19           | 10 (52.6)       |   |
| Macroscopic cancer type  |              |                  |      |              |                  |   |
| Polypoid                 | 253          | 171 (67.6)       |      | 45           | 20 (44.4)       |   |
| Ulcerative               | 111          | 76 (68.5)        |      | 31           | 20 (64.5)       |   |
| Infiltrative             | 38           | 22 (57.9)        | 0.178| 11           | 6 (54.5)        | 0.373|
| Exophytic                | 39           | 32 (82.1)        |      | 12           | 7 (58.3)        |   |
| Villous                  | 2            | 2 (100.0)        |      | 1            | 0 (0)           |   |
| CEA (ng/mL)              |              |                  |      |              |                  |   |
| ≤10                      | 337          | 229 (68.0)       | 0.750| 67           | 34 (50.7)       | 0.757|
| >10                      | 73           | 51 (69.1)        |      | 22           | 12 (54.5)       |   |

*Comparisons were examined for statistical significance using Fisher’s exact test (when n < 5).

Increasing “tumour differentiation,” we observed an increasing expression of Ki-67 from the well-differentiated to the undifferentiated tumours; conversely, undifferentiated tumours showed lower expression of Ki-67 compared to the degree of differentiation mentioned above.

As was observed by some authors [38, 50], the more undifferentiated is the tumour, the higher is the rate of cell proliferation and therefore Ki-67 index. This is not consistent with our findings; however, since the lower expression in undifferentiated tumours may be explained by the small size of this sample, further studies with larger series of undifferentiated tumours are necessary.

When analyzing the correlation of Ki-67 expression in lymph node metastasis with pathological data, any statistically significant relationship was observed. In the literature, no other studies realized in lymph node metastasis of CRC were found, and similar results were observed by Jansson and Sun [39] on the primary tumour but contradict the other studies analyzed [22, 25, 28–30, 32, 35–40].

In our series, we have not observed association between Ki-67 expression and patient’s survival, for CRC (P = 0.321) and for lymph node metastasis (P = 0.131), series and the same was true when we considered separately colon cancer and rectal cancer. This was corroborated by the observations of Jansson and Sun [39]; however, these findings contradict, in part, the report of Valera et al. [35] that studied primary CRC tumours, and also contradict studies made with lymph node metastasis from breast cancer [43–45] and prostate cancer.
### Table 4: Assessment of correlation between Ki-67 expression and pathological data.

|                          | Ki-67 in CRC | Ki-67 in lymph node metastasis |
|--------------------------|--------------|--------------------------------|
|                          | n            | Positive n (%) | Positive n (%) | P  |
| Tumor size               |              |                |                |    |
| ≤4.5 cm                  | 279          | 190 (68.1)     | 65             | 39 (60.0) | 0.519 | 0.211 |
| >4.5 cm                  | 179          | 127 (70.9)     | 40             | 19 (47.5) |      |
| Histological type        |              |                |                |    |
| Adenocarcinoma           | 409          | 281 (68.7)     | 88             | 46 (52.3) | 0.665 | 0.483 |
| Mucinous adenocarcinoma  | 50           | 32 (64.0)      | 13             | 8 (61.3)  |      |
| Invasive adenocarcinoma  | 24           | 18 (75.0)      | 6              | 4 (66.7)  |      |
| Signet ring and mucinous | 4            | 2 (50.0)       | 2              | 2 (100.0) |      |
| Differentiation          |              |                |                |    |
| Well differentiated      | 209          | 135 (64.6)     | 38             | 19 (50.0) | 0.049 |
| Moderately differentiated| 208          | 146 (70.2)     | 46             | 26 (56.5) | 0.670 |
| Poorly differentiated    | 47           | 40 (85.1)      | 23             | 14 (60.9) |      |
| Undifferentiated         | 4            | 3 (75.0)       | 1              | 1 (100.0) |      |
| Tumour penetration       |              |                |                |    |
| pT1                      | 34           | 23 (79.3)      | 2              | 2 (100.0) | 0.013 |
| pT2                      | 57           | 39 (68.4)      | 4              | 2 (50.0)  |      |
| pT3                      | 370          | 252 (68.1)     | 96             | 53 (55.2) | 0.553 |
| pT4                      | 26           | 19 (73.1)      | 7              | 3 (42.9)  |      |
| Spread to lymph nodes    |              |                |                |    |
| Absent                   | 275          | 188 (68.4)     | 9              | 5 (55.6)  | 0.940 |
| Present                  | 198          | 136 (68.7)     | 89             | 50 (56.2) | 1.000 |
| Venous vessel invasion   |              |                |                |    |
| Absent                   | 264          | 179 (67.8)     | 29             | 13 (44.8) | 0.511 |
| Present                  | 201          | 142 (70.4)     | 74             | 46 (62.2) |      |
| TNM                      |              |                |                |    |
| Stage I                  | 75           | 54 (72.0)      | 74             | 46 (62.2) | 0.425 |
| Stage II                 | 181          | 121 (66.9)     | 78             | 43 (55.1) | 0.978 |
| Stage III                | 152          | 108 (71.1)     | 78             | 43 (55.1) |      |
| Stage IV                 | 70           | 45 (64.3)      | 31             | 17 (54.8) |      |

*Comparisons were examined for statistical significance using Fisher's exact test (when n ≤ 5).

### Table 5: Survival analysis: frequency and relative frequency for overall survival and medium of time to death.

| Ki-67         | Tissue         | n     | Deaths  | Median for survival time | Log-rank test P |
|---------------|----------------|-------|---------|--------------------------|-----------------|
|               |                |       | n (%)   | [95% confidence interval] |                 |
| Negative      | CRC            | 154   | 53 (65.6) | 65.00 [59.49–70.52] | 0.321 |
| Positive      |                | 332   | 125 (62.3) | 62.09 [58.06–66.12] |      |
| Negative      | Lymph node     | 49    | 17 (65.3) | 63.48 [53.18–73.79] | 0.131 |
| Positive      |                | 62    | 30 (51.6) | 50.07 [41.06–59.08] |      |

### Table 6: Comparison between Ki-67 index in primary tumor and respective lymph node metastasis.

| Ki-67 index in CRC | Ki-67 index in lymph node |
|--------------------|--------------------------|
| Negative           | Negative                 | Positive |
|                    | 16                       | 12       | 28 (26.4) |
| Positive           | 29                       | 49       | 78 (73.6) |
| Total n (%)        | 45 (42.5)                | 51 (57.5) | 106 (100) |

Comparisons were examined for statistical significance using McNemar test.
cancer [51], where a higher ki-67 index was associated with a worse patient survival.

Finally, we found association \( (P = 0.012) \) between Ki-67 index in primary tumour and the respective lymph node metastasis and also observed that Ki-67 index was more often positive in the primary tumour than in the respective lymph node metastasis. This result is consistent with the study carried out by Yamauchi et al. [41] to compare Ki-67 index in CRC primary tumour and respective nodules of peritoneal dissemination, which, as in the present study, present a greater proportion of proliferating cells in the primary tumour than in the nodules of peritoneal dissemination, not advancing; however, there is no explanation for this finding. Distinct results were observed for similar studies realized in breast cancer [42–44], where lymph node metastasis presents higher ki-67 index than primary tumour, but also no explanation was mentioned. Recently, Jo and colleagues have found a significant difference of higher Ki-67 proliferation in nodal metastasis and primary gastric cancer [52]. Most of the criticism of Ki-67 evaluation is related to the differences of antibodies, slide background, retrieval protocols applied in preparing the immunoreaction, and the scores used to evaluate the significance of Ki-67 proliferation rates. This concern is pertinent and most of the works that evaluate this premise did not reach a consensus. Interesting, automated evaluation of the Ki-67 labelled preparation has been adjudicated as superior than manual analyses. Moreover, subdividing the cases in low and high proliferative rate improve the kappa correlation. Besides these advisements, the lack of standard protocols among the laboratories limits the clinical relevance of the works [53].

This difference between Ki-67 index in primary CRC tumour and respective lymph node metastasis may explain the absence of correlation with clinicopathological data.

Figure 3: Immunohistochemical expression of Ki-67 in CRC lymph node metastasis samples: ((a) and (d)) original magnification \( \times 40 \); ((b) and (e)) original magnification \( \times 100 \); ((c) and (f)) original magnification \( \times 200 \).
Figure 4: Survival curve of patients with CRC according to Ki-67, assessed by the log-rank test: (a) colorectal cancer: $P = 0.321$; (b) colon cancer: $P = 0.213$; (c) rectal cancer: $P = 0.874$. 
Figure 5: Survival curve of patients with CRC lymph node metastasis according to Ki-67, assessed by the log-rank test: (a) colorectal cancer: $P = 0.131$; (b) colon cancer: $P = 0.127$; (c) rectal cancer: $P = 0.809$. 
A hypothesis was also stated by Cabibi et al. [44], relatively to the cell usage as a marker of cell proliferation, since Ki-67 expression may be due to of Ki-67 own proliferation or that Ki-67 might not be the more suitable proliferative marker for use in lymph node metastasis. This result also raises the possibility that tumour cells in lymph nodes can be more resistant to chemotherapy treatments, thus contributing to the poor prognosis of these patients.

**Conflict of Interests**

The authors declare that they have no conflict of interests.

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