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The clinicopathological potential of Ki67 labeling index in breast cancer

PhD thesis

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LIST OF ABBREVIATIONS

ASCO	American Society of Clinical Oncology
CCC	Concordance correlation coefficient
CK	Cytokeratin
DCIS	Ductal carcinoma in situ
DFS	Disease-free survival
DIA	Digital image analysis
DMFS	Distant metastases-free survival
ER	Estrogen receptor
ESMO	European Society for Medical Oncology
FDA	US Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded
FISH	Fluorescent in situ hybridization
FNAB	Fine-needle aspiration biopsy
HER2	Human epidermal growth factor 2
HR	Hormone receptor
ICC	Intra-class correlation coefficient
IHC	Immunohistochemistry
Ki67 LI	Ki67 labeling index
NAC	Neoadjuvant chemotherapy
NPI	Nottingham Prognostic Index
NQ	NuclearQuant
OS	Overall survival
pCR	Pathologic complete response (to neoadjuvant therapy)
PgR	Progesterone receptor
pNR	No response to neoadjuvant therapy
pPR	Partial response to neoadjuvant therapy
PQ	PatternQuant
ROC	Receiver operating characteristic
RT-qPCR	Real-time quantitative reverse-transcription analysis
SQ	Semi-quantitative evaluation
TMA	Tissue microarray

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TNBC	Triple-negative breast cancer
WHO	World Health Organization

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1. INTRODUCTION

1.1. Epidemiology of breast cancer

Breast cancer represents a major public health issue globally ranking as the 1st of the most frequently occurring cancers among women with over 1,600,000 new cases annually estimated from GLOBOCAN statistics in 2012 [1]. Each year, more than 500,000 women die of breast cancer, making it the first-leading cause of cancer related deaths among women worldwide (Figure 1A) [1].

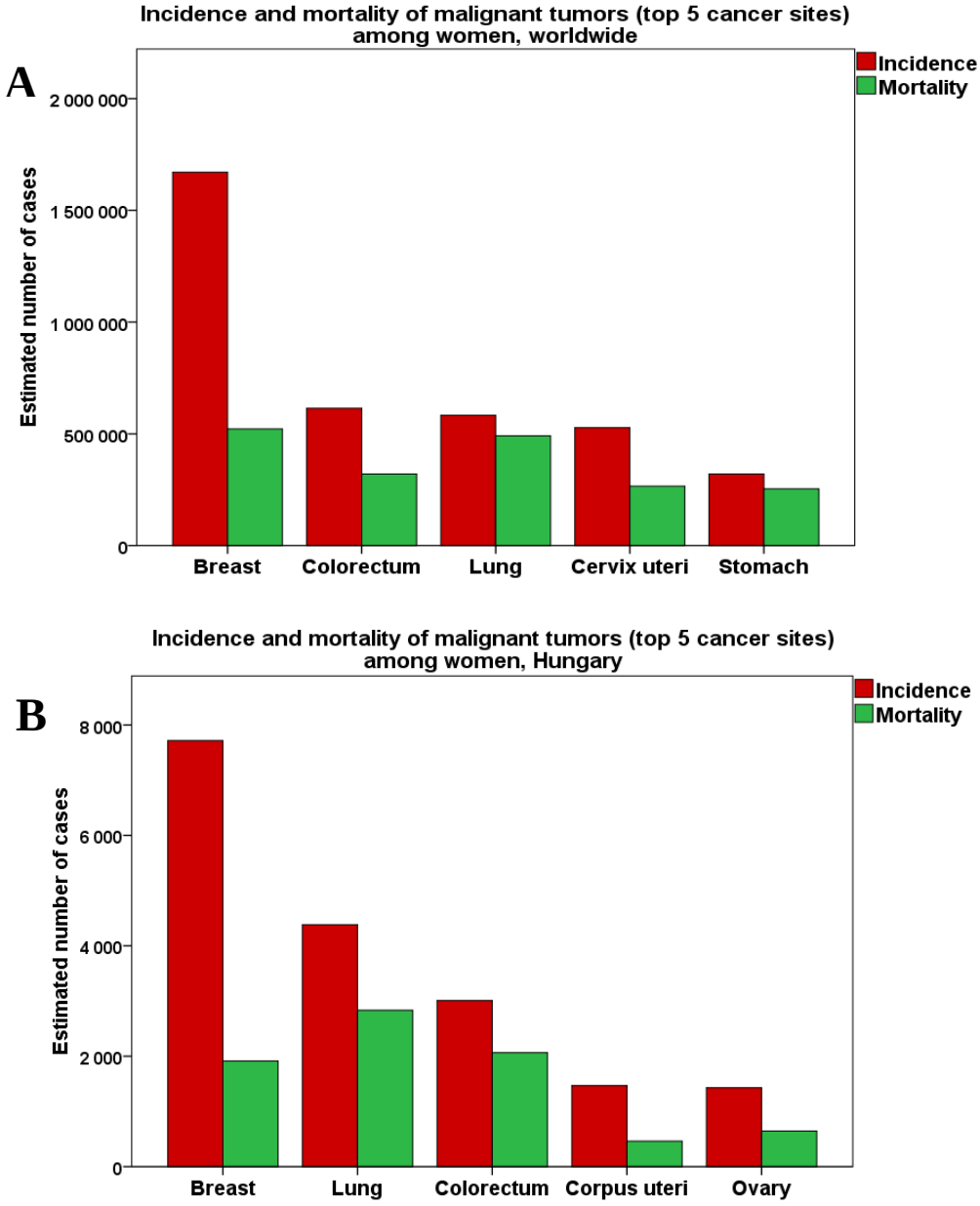

Figure 1: Incidence and mortality of the most common cancers among women worldwide (A) and in Hungary (B). Source of the data: GLOBOCAN [1] and National Cancer Registry Database [2] 2012.

In Hungary with over 7000 new cases and 1900 deaths per year, breast cancer is the most commonly diagnosed cancer and the third-leading cause of death after lung and colorectal malignancies among women (Figure 1B) [1,2]. The mortality of breast cancer is decreasing from the 1990's because of spreading of breast cancer screening and emerging new therapies. However, the morbidity of breast cancer shows an increasing trend as mean age rising as well as obesity, early menarche and childbearing in later age are getting more common in societies.

1.2. Diagnosis of breast cancer

Finding breast cancer early and getting appropriate cancer treatment are the most important strategies to decrease the mortality of breast cancer. Since many women with breast cancer have no symptoms, the diagnosis of breast cancer often begins with lesion found on x-ray mammography screening test. Breast cancer is sometimes found on a routine clinical – or self-examination when a new lump appears. This is followed by a breast ultrasound test, or when the clinical picture is ambiguous, a magnetic resonance imaging [3]. The only way to confirm a potentially malignant breast lesion detected on imaging test is performing biopsy. The most widely used biopsy techniques are core biopsy and fine-needle aspiration biopsy (FNAB). While only cells are obtained with FNAB, core biopsy allows to examine the histological structures as well. Furthermore, molecular tests are more commonly applied on core biopsy samples and thus used for clinical decision making. Based on the results of imaging and biopsy tests, the multidisciplinary team reviews the medical condition and sets up treatment options for each patient individually. The management of breast cancer requires close cooperation of different disciplines. Therefore, multidisciplinary oncoteam includes oncologist, surgeon, radiologist, radiation oncologist and pathologist.

1.3. Subtypes of breast cancer

It has long been known that breast cancer cannot be considered as a homogenous disease but rather a group of heterogeneous tumors in the same anatomical localization [4]. Breast tumors originate from the epithelial - or the mesenchymal tissue of the breast. Histologically, breast cancer classification is performed according to the regularly updated atlas of the World Health Organization (WHO) considering the

tumor's morphological characteristics [5]. The clear majority of malignant breast tumors arise from the epithelium. The most common is invasive breast carcinoma no special type (NST), then invasive lobular carcinoma, followed by many 'special types' such as tubular, medullary, mucinous, micropapillary, metaplastic carcinoma, etc. [6].

This heterogeneity has brought to the fore the concept, that gene defects and failure of gene regulatory networks driving to carcinogenesis and metastases might show stronger association with the phenotype and clinical behavior of tumors than the morphological classification. In the last decade, various molecular techniques have been used increasingly to help refine breast cancer classification. The pioneer studies have proposed a new description of the heterogeneous group of breast cancers at the molecular level by cDNA microarray analyses revealing several molecular/intrinsic subtypes beyond the traditional hormone receptor (HR)+ and HR – types [7,8]. The most reproducibly defined intrinsic subtypes among the HR+ cancers are the luminal A and luminal B subgroups [9]. The human epidermal growth factor 2 (HER2)-enriched and basal-like subgroups are the major intrinsic subtypes identified among HR- breast cancers [9]. The rationale underlying this classification is that these breast cancer molecular subtypes differ in their gene expression patterns, clinical features, prognosis and response to treatment (Table 1) [9,10].

Despite the discovery of these intrinsic subtypes, the immunohistochemical (IHC) expression of estrogen receptor (ER), progesterone receptor (PgR), Her2 and the HER2 gene amplification detected by fluorescent in situ hybridization (FISH) are used to assess breast cancers in clinical practice [9,11]. This approach is most widely used to approximate the intrinsic subtypes of breast cancer, as defined by gene expression profiling because of financial reason and because it clearly defines whether an actual breast cancer can be treated with targeted therapy [11]. In the surrogate definitions of intrinsic subtypes, the ER+ tumors can be divided into luminal A-like and luminal B-like groups based on the IHC expression of ER, PgR, Her2, Ki67 and/or HER2 gene amplification [9,11,12]. In ER- tumors, HER2 and Triple-negative (TNBC) subtypes are distinguished based on the IHC expression of Her2 and/or HER2 gene amplification. Both subgroups are characterized by negative IHC reactions of ER and PgR. Ki67 expression may vary in the ER- subgroups therefore has no relevance in therapy decision making (Table 1) [9,11,12].

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Table 1: Subtypes of breast cancer.

Intrinsic subtypes [9]	**Clinicopathologic surrogate definition (based on IHC and FISH tests) [12]**	**Notes**
Luminal A	Luminal A-like	ER + [a], HER2 –, PgR high [b], low Ki67 [c], low-risk molecular signature [d]
Luminal B	Luminal B-like HER2 –	ER + [a], HER2 –, PgR low [b] or high Ki67 [c], high-risk molecular signature [d]
	Luminal B-like HER2 +	ER + [a], HER2 +, any PgR, any Ki67
HER2-enriched	HR – and HER2 +	ER/PgR – and HER2 +
Basal-like	TNBC	ER/PgR – and HER2 –
[a]ER + ≥1% [b]Suggested threshold: 20% [c]Ki67 threshold varies between laboratories [d]if available		

1.4. Prognostic and predictive factors of breast cancer in daily practice

Breast cancer represents great diversity not only in its morphological and molecular features but also in its prognosis [13]. Several studies have shown that the histological subtypes have different prognosis [14-16]. Mucinous, tubular, medullary, invasive cribriform, infiltrating lobular, tubulo-lobular, adenoid cystic carcinoma, and low grade adenosquamous carcinoma have all been reported to have a more favorable prognosis than invasive carcinoma NST [14-22]. Primary soft tissue sarcoma of the breast is very rare and usually associated with poor outcome [23].

Age is one of the most important risk factor for breast cancer. As the age is rising, the probability of developing cancer is increasing [24]. However, many studies indicated the adverse risk related to younger age as breast cancer is rarer in young women, but if it occurs, it is often presenting with advanced stage resulting in poor prognosis [25].

It has long been known that the size of the tumor and the regional lymph node involvement are strongly linked to the prognosis of breast cancer [26,27]. Smaller tumor holds the promise of higher chance for complete recovery. However, smaller tumors with early lymph node metastases have more unfavorable prognosis compared to larger tumors without lymph node involvement [28].

The clinical and pathological stage (TNM) comprising the size of the tumor (T), lymph node status (N) and presence of metastasis (M) largely determines the course of breast cancer as well as the treatment options [29].

During routine microscopic examination of breast cancer, pathologists evaluate histologic grade (tubular differentiation + nuclear pleomorphism + mitotic count), lymphatic – and blood vessel invasion, Nottingham Prognostic Index (NPI = /0.2 x tumor size in cm/ + lymph node stage + tumor grade) that are strong and easily assessed prognostic factors [30-32].

IHC examinations may help breast cancer classification, as E-cadherin expression is absent in most lobular cancers distinguishing lobular and NST cancers in equivocal cases [33-35]. Cytokeratin (CK) 5/6, CK14 and p63 are used to reveal basal/ myoepithelial origin of breast cancer [36-38]. The IHC and/or FISH detection of ER, PgR and Her2 forms the backbone of the clinical management of breast cancer as they not just assign the surrogate intrinsic subtypes referring to prognosis but also basically define treatment options [12]. The IHC detection of p53 has prognostic potential as p53+ tumors have unfavorable prognosis, but they also show better response to chemotherapy [39,40]. However, p53 has not been included in the compulsory routine IHC tests. High Ki67 expression in tumor cells means higher proliferation capacity that has been linked to poor prognosis [41,42]. Various gene mutations have been linked to the genesis and prognosis of breast cancer, such as TP53, BRCA1 and 2, CDH1 etc. [43,44].

Although evaluation of individual prognostic and predictive factors has allowed the identification of clinically distinct subgroups of patients with breast cancer, there is urgent need to evolve a comprehensive profile of the biological and molecular characteristics of breast cancer that may improve assessment of prognosis and prediction of response to therapies in individual patients [9]. During the last few decades, several platforms of multigene classifiers have been developed to identify

patients with favorable prognosis, who can omit chemotherapy, and those with poor prognosis and higher risk of metastasis [9]. Several tests are now commercially available as follows: MammaPrint (Agendia, Amsterdam, Netherlands) is a microarray test approved by the US Food and Drug Administration (FDA) applying a set of 70 genes to accurately predict poor prognosis disease for patients with TNM stage 1 or 2, node-negative, invasive breast cancer of tumor size ≤5 cm [45,46]. The 76-gene signature test of Veridex investigating 60 genes for patients with ER-positive disease and 16 genes for ER-negative disease has a strong prognostic factor for 5 years' distant metastasis free survival [47]. MapQuant Dx (Ipsogen SA, Marseille, France) can stratify grade II breast carcinomas into grade I-like and grade III-like cancers in ER+ disease [48,49]. All three assays are based on DNA microarrays and require fresh or frozen samples implying a challenge in the daily practice and in prospective validation studies [10]. The technique of real-time quantitative reverse-transcription analysis (RT-qPCR) has also been used for developing prognostic assays extracting RNA from formalin-fixed paraffin-embedded (FFPE) tissue samples, thus relieving the sample procurement [10]. The simplified version of MapQuant Dx, Oncotype DX, and Theros platforms are using this method to predict prognosis [50-53]. The prognostic use of Oncotype DX is supported by level I evidence, and this test has been included in the National Comprehensive Cancer Network - and in the American Society of Clinical Oncology guidelines as a predictor of recurrence and a guide when making therapeutic decisions among early ER positive node negative breast cancers [54]. The largest criticism raised against these assays is that despite similar prognostic and/or predictive value, there is very little overlap in the genes included in these tests [55]. Furthermore, several studies demonstrate that a part of clinically diagnosed and validated breast cancers with Her2 + phenotype are not stratified into the HER2-enriched subtype by gene expression assays. Vice versa, a part of breast cancers characterized as HER2-enriched subtype do not occur as a Her2 + breast cancer in the daily practice [56-61]. Increasing evidence confirms that the expression of proliferation genes in luminal cancers does not form distinct subgroups (luminal A and luminal B) but a continuum. Thus, stratifying breast cancers into two luminal subgroups based on gene expression assays is artificial [56,62]. The prognostic information of these assays are almost exclusively based on the expression of the proliferation-related genes [54]. Considering the latter and that these

assays are too expensive to be widely applied, the question can be raised whether the use of cost-effective and widely available proliferation markers (e.g.: Ki67) should receive greater emphasis in daily practice.

1.5. Therapy of breast cancer

1.5.1. Surgery

The surgical treatment of primary breast cancer has been shifted to breast-conservation treatment. In some patients, mastectomy is implemented due to: Tumor size, multicentricity of the tumor, positive surgical margins after multiple resections, contraindications to radiation therapy, or patient choice. The biopsy of the sentinel lymph nodes is now the standard of care instead of full axillary nodal clearance, unless axillary node involvement is confirmed. However, axillary dissection can be omitted for patients with one or two metastatic sentinel lymph nodes [12,63].

1.5.2. Radiation therapy

Postoperative radiation therapy is indicated after breast-conservation surgery. Shorter fractionation schemes (15–16 fractions with 2.5–2.67 Gy single dose) have been confirmed in large studies and are generally indicated. Boost (tumor bed) irradiation further reduces the risk by 50% and it is recommended for patients with factors indicating high risk of local recurrence. Post-mastectomy radiation therapy is indicated for patients with involved axillary nodes and/or with T3–T4 tumors. Axillary irradiation is recommended for patients with involved lymph nodes [12,63].

1.5.3. Adjuvant therapy

The decision on systemic adjuvant therapies is based on the surrogate intrinsic phenotype determined by ER/PgR, HER2 and Ki67 assessment or on the genomic-based intrinsic subtype.

Endocrine therapy (anti-estrogens, selective estrogen receptor modulators, aromatase inhibitors) should be offered for all breast cancer patients with detectable ER expression (≥1% of invasive cancer cells).

Chemotherapy of breast cancer usually includes four to eight cycles of anthracycline- and/or taxane-based regimen. Sequential use of anthracyclines and taxanes is preferred

over concomitant use. Chemotherapy is indicated in most of triple-negative, HER2-positive breast cancers. For luminal B-like Her2− tumors, the indications for chemotherapy are based on the individual risk of relapse (e.g.: High Ki67). Luminal B-like Her2+ tumors should be treated with chemotherapy, endocrine therapy and Her2 targeted therapies. Most luminal A-like tumors, except those with T3/T4 size and extensive nodal involvement, require no chemotherapy. Her2 subtype (HR−) benefit from chemotherapy and Her2 targeted therapies. Triple-negative tumors should be treated with chemotherapy, with possible exclusion of low-risk subtypes such as adenoid cystic carcinomas [12,63].

1.5.4. Neoadjuvant therapy

In breast cancer cases with large tumor size and/or with locally advanced stage, neoadjuvant therapy may allow for achieving operability or decreasing the extent of surgery. All modalities (chemotherapy, endocrine therapy and targeted therapy) applied in adjuvant treatment may also be used in neoadjuvant setting. If chemotherapy is used, it is recommended to deliver all planned treatment without unnecessary breaks, irrespective of the magnitude of tumor response. In this case, the probability of achieving a pathologic complete remission is increased. Although neoadjuvant therapy has not been shown to improve survival superior to those of postoperative adjuvant therapy alone, there is increasing support for neoadjuvant cytotoxic therapy in Stage II triple-negative subtype. In HER2+ breast cancer especially with larger size, Her2 targeted therapy should be started in the neoadjuvant setting, combined with taxane-based chemotherapy. In patients with luminal breast cancer, neoadjuvant cytotoxic chemotherapy is not supported unless to achieve breast conservation surgery, since ER+ and Her2− tumors are usually less responsive to primary chemotherapy compared to Her2+ subtype. However, neoadjuvant endocrine therapy is generally given to post-menopausal women with breast cancer for 4 months preoperatively and continued adjuvant after surgery for 5 to 10 years [12,63].

1.6. Ki67: Gene, function, detection, role in breast cancer

1.6.1. Ki67 gene, structure, function

The Ki67 antigen was originally described by Gerdes et al. in the 1980s, by use of a mouse monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma-derived cell line L428 [64]. It was finally identified by the same group in 1991 and this non-histone protein was named after the researchers' location, Ki as Kiel University, Germany and the 67 label referring to the clone number on the 96-well plate [64]. The complete gene locus of the Ki67 protein, encompassing a 74 basepairs (bp) 5′ region and a 264 bp 3′ region, has been sequenced and aligned to a continuous sequence of 29 965 bp length located on the long arm of human chromosome 10 (10q25-ter). The gene comprises 15 exons with sizes from 67 to 6845 bp and 14 introns with sizes from 87 to 3569 bp. Three introns consist homologue copies of “Alu-repeats”. Exon 13 at the “center” of this gene is composed of 16 homologous segments with 366 bp (called Ki67 repeats), each including a highly-conserved motif of 66 bp (Ki67 motif). This is highly conserved between species and nine of the Ki67 motif regions contain a highly immunogenic five amino acid sequence that forms the epitope which is the target of several Ki67 antibodies like MIB1 and SP6. Two Ki67 protein isoforms with molecular weights of 345 and 395 kDa have been described [65-68]. The cellular location of Ki67 protein is strongly cell cycle-dependent: The protein is found primarily in the nucleolar cortex and in the dense fibrillar components of the nucleolus during interphase; during mitosis, it becomes linked with the periphery of the condensed chromosomes [69-71]. The half-life of the Ki67 protein has been found to be approximately 60-90 minutes, regardless of the cell position in the cell cycle making it a feasible marker of proliferating cells [72,73]. During the different cell-cycle phases, the expression of Ki67 varies: Its levels are low during the G1 and early S phase and rise to their peak level in mitosis. Later during mitotic phase (anaphase and telophase), a sharp decrease in Ki67 expression levels occurs. These differences seem to reflect variable de novo synthesis and not due to the accumulation of non-degraded proteins. It is not expressed during the resting phase G0 [74,75]. The exact function of Ki67 is not known. Studies have described the involvement of Ki67 in the early steps of polymerase I dependent rRNA synthesis and its important function in cell division [76,77]. Ki67 protein is

phosphorylated via serine and threonine and its inhibition results in the arrest of cell proliferation [70,72,78].

1.6.2. Detection of Ki67

There are two approaches for measuring Ki67 expression in breast cancer: (i) a quantitative analysis of the Ki67 (MKI67) mRNA content form frozen or FFPE samples; and (ii) determining the percentage of Ki67 positive cancer cells detected by IHC. Only few data are available about the comparison of RNA and IHC based Ki67 measurement in the same samples. A study reported weak correlation between Ki67 measurements of RT-qPCR and ICH detection in breast cancer [79]. Tan et al. has found significant correlation between Ki67 gene expression levels and IHC results of cases with Ki67 score of >10% [80]. Strong linear association was found between the recurrence score and Ki67 IHC results in a study investigating 53 breast cancer cases [81]. However, the IHC staining for Ki67 detection has the following advantages compared to RNA based method: Only cancer cells are considered when the pathologist assesses the IHC Ki67 score and inflammatory- or stromal cells showing positivity can be excluded. Besides this, the IHC method is widely available and relatively inexpensive [82]. Thus, in daily practice, Ki67 is most often measured on FFPE sections by IHC method.

In general, scoring systems of Ki67 IHC detection are based on the percentage of tumor cells stained by the antibody. In one method, the pathologist examines three to ten high-power fields (×40), counts at least 1000 tumor cells with a standard light microscope and the Ki67 labeling index (Ki67 LI) is defined as the percentage of total number of tumor cells with nuclear staining [83]. However, counting 1000 tumor cells is time-consuming and monotonous. Therefore, in the daily pathological practice this approach has limitations [84]. Thus, some pathologists estimate the percentage of positive tumor cells in different areas of the tumor giving an overall Ki67 LI. However, estimating the percentage of tumor cells has high chance of failure, which might lead to results with low reproducibility [84].

It is also important to note that throughout the cell cycle, the localization and the pattern of IHC Ki67 positivity varies. In early G1 phase the IHC reaction is granular/focal in the nucleus, while in late G1 phase the positivity is seen in the nucleolus. In G2 phase

the reaction is granular/focal or diffuse in the nucleus and in the S phase both the nucleolus and nucleus are positive. In the M prophase there is a fine net-like positive reaction in the nucleus, while in the M metaphase the positivity occurs on the surface of the chromosomes and following breakdown of the nuclear membrane some cytoplasmic positivity may also be detected [85]. Consequently, Ki67 LI should include all types of nuclear staining irrespective of the intranuclear localization (nuclear or nucleolar or nuclear membrane) and the distribution (granular or diffuse) and regardless of intensity [82].

One of the greatest challenges in Ki67 scoring is the selection of fields for evaluating because of the variations in cellular proliferation caused by intra-tumoral heterogeneity. In addition to spatial heterogeneity, a temporal heterogeneity may also occur because of neoadjuvant therapy [86-88]. Tumor heterogeneity is one of the causes for high inter-observer variability. Because of this, at least 500-1000 tumor cells should be evaluated when giving Ki67 LI to achieve an acceptable error rate [89].

1.6.3. Ki67 in breast tissues

Ki67 is expressed in normal breast tissue at low level (<3%) [90]. Numerous studies have reported that ER expression and Ki67 antigen are detected in separate cell populations in normal human breast epithelium. It is an important observation that Ki67 is expressed exclusively in ER-negative breast epithelial cells, which means that ER-positive luminal cells do not proliferate in normal human breast tissue. This separation between ER expression and proliferation does not exist in malignant breast tissue [91]. The association has been described between expression of Ki67 and breast density as well as with precancerous lesions [92,93]. Moreover, the continuous increase of Ki67 expression has been found from benign breast disease to ductal carcinoma in situ to invasive breast cancer [94-96].

It has also been shown that Ki67 expression decreased when aromatase inhibitor was given concomitantly in a study investigating high-risk women [97].

High levels of Ki67 expression were found in about 40% of ductal carcinoma in situ (DCIS). Increased Ki67 levels are associated with comedo necrosis, higher grade lesions, presence of microinvasion as well as the recurrence of DCIS [98,99].

Ki67 expression is one of the parameters that can help to distinguish several rare subtypes of breast cancers: The lipid-rich and sebaceous breast carcinomas typically express high Ki67 levels [100,101]. Invasive lobular cancer usually shows a low Ki67 index, and some researchers found low Ki67 level associated with the prognosis of lobular carcinoma [102].

1.6.4. Ki67 and its relationship with other markers of breast cancer

Many studies have demonstrated a strong correlation between Ki67 and histological grade [103-105]. However, this relation is not so surprising, since the mitotic index is one of the three components of histological grade [30]. Both Ki67 and mitotic index are widely applied in the daily practice to measure proliferation. However, some authors propone the use of Ki67 as prognostic marker superior to mitotic index because mitotic index is more subject to individual evaluation as distinguishing apoptotic- and mitotic figures is not always certain [84]. The association between lymph node status and Ki67 has also been intensively investigated and several studies involving large number of patients have revealed a positive correlation [106-108]. The relation between tumor size and Ki67 has also been demonstrated [109]. The HR status has been found to have an inverse relation with Ki67, so that ER and PgR positivity is mostly found in the least proliferating tumors [107,110,111]. The association between Ki67 and Her2 expression is controversial [112-114]. Mutation of p53 oncogene is mostly found in breast cancers expressing higher levels of Ki67 [115].

1.6.5. Ki67 in breast cancer: Prognostic and predictive potential

Several studies were published about the importance of proliferation measured by Ki67 expression in breast cancer, including the Oncotype DX that measures gene expression level of KI67 as one of the 16 genes [51,116,117]. Furthermore, the proliferation group is the mostly weighted in the algorithm of the Recurrence Score [51]. It has been long acknowledged - and more recently several studies have demonstrated - that the immunohistochemical detection of the Ki67 positive cells provides important prognostic information in breast cancer [118-120]. In one of the largest studies, Petrelli et al. [121] performed a systematic review of the literature which was followed by a meta-analysis of the involved studies. In total, 41 studies enrolling more than 64,000 patients were

investigated. Although different cut-off points in a range between 10% to >25% were applied, the study has shown that elevated levels of Ki67 are independently associated with adverse outcome in patients with breast cancer [121]. Moreover, breast pathologists had been undertaking retrospective studies for showing that Ki67 LI was almost as good as the Oncotype DX for the prediction of prognosis in ER + breast cancer cohorts [116,120,122]. However the optimal threshold of Ki67 LI is still uncertain: The St. Gallen Consensus Conference in 2013 recommended a 20% cut-off to distinguish between HER2 negative luminal B-like and luminal A-like breast carcinomas [123], while the majority of the panel in 2015 intended to accept a Ki67 LI threshold of 20-29% [63]. However, the panel also acknowledged that the threshold between Ki67 high and low breast cancers varies between laboratories [63]. Cserni G. et al suggested that different thresholds may be generated for different clinical purposes [124]. According to Denkert et.al. [89], an optimal threshold of Ki67 does not exist, because many cut-off points have a similar prognostic performance. Thus, they recommend to use Ki67 LI as a continuous marker, which reflects the biology of tumor proliferation [89]. In contrast to the guidelines of The St. Gallen Consensus Conference, the International Ki67 in Breast Cancer Working Group is more cautious about the recommendation of Ki67 in daily practice [116]. The European Society for Medical Oncology (ESMO) Clinical Practice Guidelines suggests that Ki67 may provide useful information, if the assay can be standardized [125]. The American Society of Clinical Oncology (ASCO) did not recommend the use of Ki67 for prognosis in newly diagnosed breast cancer patients because of lack of reproducibility across laboratories [126,127].

In addition to ongoing debate on its prognostic utility, Ki67 has also been investigated as a potential predictive marker in neoadjuvant and adjuvant settings. For neoadjuvant chemotherapy of breast cancer, Ki67 was significantly associated with clinical or pathological response in several studies [84,128]. However, in a recent research involving 506 breast cancer patients, Ki67 did not represent an independent predictive potential for neoadjuvant therapy [129]. In contrast to this, the systematic review by Luporsi et al. has determined a level of evidence of II-B for Ki67 regarding neoadjuvant treatment response [120]. The other setting for prediction of response to therapy is the evaluation of survival in adjuvant studies. In adjuvant setting, the predictive role of

Ki67 is even more uncertain. In the IBCSG 8/9 trial, no predictive potential of Ki67 for response to chemotherapy vs. no chemotherapy was found [130]. The elevated Ki67 was associated with a higher efficacy of docetaxel in PACS01 [131], but the evaluation of BCIRG001 did not confirm this [132]. The controversial predictive potential of Ki67 between neoadjuvant and adjuvant settings was addressed in the review by Denkert et al. expounding that Ki67 affects in opposite directions for assessment of prognosis and for assessment of response to neoadjuvant therapy [89].]. An elevated level of Ki67 is associated to unfavorable prognosis, as well as to better response to neoadjuvant therapy. It has also been shown that in some, but not all breast cancer subtypes, response to neoadjuvant therapy is linked to improved prognosis [89]. These two contrary effects cause an overlap in adjuvant studies. Thus, it is not possible to separate the negative prognostic effect of high Ki67 in non-responding cases from the positive prognostic effect of high Ki67 tumors that respond to therapy [89]. The authors also suggest distinguishing three groups of tumors in relation with Ki67 expression and responsiveness to therapy as follows: i) low Ki67 tumors that do not respond to chemotherapy but also have a good prognosis i.e.: Luminal A-like subtype (Low Ki67 associated with good outcome). ii) High Ki67 tumors with response to chemotherapy has better outcome (high Ki67 associated to favorable outcome) compared to iii) high Ki67 tumors that are chemotherapy-resistant (high Ki67 associated to poor outcome) [89].

1.6.6. Standardization efforts of the application of Ki67 in daily practice

Ki67 is currently one of the most promising yet controversial biomarker in breast cancer [133]. Despite the promise of Ki67 as a prognostic and/or predictive tool, controversy exists regarding its applied methodology in clinical practice. Therefore, there is an urgent need for reproducible methodology and consistent scoring methods of Ki67 LI. To overcome this struggle, the International Ki67 in Breast Cancer Working Group has introduced a recommendation for the application of Ki67 IHC in daily practice [116]. According to this, parameters that predominantly influence the IHC results of Ki67 include pre-analytical, analytical, interpretation and scoring, and data analysis steps [116].

Several pre-analytical issues might negatively affect Ki67 measurement as follows: Type of biopsy, time to fixation, type of fixative, time in fixative, and how the specimen is stored for long term [116]. Two studies have found that in general, Ki67 IHC has better tolerance in preanalytical variability than other IHC assays [134,135]. However, alterations in the appearance of stained nuclei were observed: The well-fixed core biopsies showed well-circumscribed, uniformly stained nuclei, while highly variable staining was found in nuclei of poorly-fixed specimens [116]. Tissue handling guidelines that are already established for ER (8–72 hours of neutral buffered formalin fixation) are adequate for Ki67 IHC [116].

The analytical issues of Ki67 IHC encompass the type of the used Ki67 antibody and IHC protocol. Ki67 IHC is most often performed using the MIB1 antibody and the International Ki67 in Breast Cancer Working Group has endorsed its use in daily practice [84,116]. However, little emphasis had been put so far on a very evident technical question, namely, are all commercially available Ki67 antibodies detecting the same amount of proliferating tumor cells in each case? Can we use the different antibodies interchangeably? Most published studies concluded that there are indeed differences between the protein expression levels of different Ki67 antibodies; however, the different results were not linked to the prognosis [136-139]. Regarding IHC protocol, positive and negative controls should be used in each group of Ki67 IHC; positive nuclei of non-malignant cells and mitotic figures provide the quality of a section. The best evidence supports the use of heat-induced antigen retrieval by microwave processing [116]. Chromogen development and counterstaining for Ki67 IHC do not differ from other antigen - antibody systems. The chromogenic staining needs optimization as negative nuclei represent usually the clear majority of overall cell population [116]. Thus, weak counterstaining can lead to overestimation of the Ki67 LI.

Difficulties in evaluating immunoreactions can also be responsible for discrepancies of Ki67 scoring reproducibility. Ki67 LI values are usually defined as the percentage of positive tumor cell nuclei, counted in 3-10 high-power fields by testing at least 500-1000 tumor cells [116]. Another method is to estimate the mean Ki67 LI in the entire lesion. Both methods are monotonous, time-consuming and exhausting with a chance of leading to controversial results and inaccurate reproducibility [84]. Although the counting method has been recommended by the International Ki67 in Breast Cancer

Working Group, other studies have demonstrated the counting method is not superior to visual estimation [124,140,141]. Biological heterogeneity of Ki67 staining can occur across the specimen and it has large impact on the Ki67 scoring. One approach is to evaluate Ki67 IHC in “hot-spot” fields that contain the most proliferating tumor cells. The other way is to give a representative score by averaging fields across the section [82]. This issue is currently being investigated to assess which method is more robust [116]. Although, recommendations published in 2011 provide a suitable landmark to improve pre-analytical and analytical validity, related protocols still show high variety and poor reproducibility linked with the context of different sampling, fixation, antigen retrieval, staining and scoring methods [116,120,142].

Rapid development of digital microscopy by now allows fast digitalization of histological slides at high-resolution, which can firmly support education, research and diagnostics in pathology [143,144]. The emergence of digital image analysis (DIA) platforms improved the capacity, precision and reproducibility of in situ biomarker evaluation [145]. However, these features alone may not be enough for diagnostic accuracy, which must be based on histological pattern recognition as the most relevant requirement of precise sample selection and assessment of immunoreactions [146]. DIA platforms are able to assess Ki67 LI, however it has not been clarified yet, if their results can meet the requirements of the daily diagnostic practice and reduce variability of Ki67 scoring [147].

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2. OBJECTIVES

In my PhD thesis, three aspects of clinical validity of Ki67 LI are investigated as follows: i) The comparison of different Ki67 antibodies used in daily practice. ii) The reproducibility between pathologists evaluating Ki67 LI and the potential of DIA in Ki67 scoring. iii) The role of Ki67 in neoadjuvant setting.

Therefore, in the breast cancer working group of 2nd Department of Pathology, Semmelweis University we aimed to:

1, Compare the semi-quantitatively defined Ki67 LI of five commercially available Ki67 IHC antibodies in a consecutive breast cancer patient population.

2, Correlate the prognosis prediction potential of each Ki67 antibodies with that of conventional clinicopathological factors in univariate and multivariate analyses.

3, Investigate the reproducibility of Ki67 LI among three pathologists, based on their conventional visual estimation.

4, Test the agreement of semi-quantitative and DIA Ki67 scoring.

5, Determine and compare the outcome prediction potential of each semi-quantitative and DIA assessments with that of conventional clinicopathological factors.

6, Find optimal cut-off values for Ki67 expression in neoadjuvant patient cohort that best correlates with response rates to neoadjuvant therapy and with distant metastasis-free survival as well as with overall survival.

7, Investigate the association between Ki67, subtype and pathological response.

8, Investigate the prognostic potential of Ki67 in neoadjuvant setting with multivariate analysis.

3. METHODS

3.1. Patients

Two distinct breast cancer patient cohorts were enrolled in the investigations encompassing 498 patients totally without any overlap: 1) 378 consecutive breast cancer cases from the Buda MÁV Hospital Pathology Unit, Budapest, Hungary diagnosed between 1999 and 2002 with 99.80 months median follow up (disease-free survival, DFS). All patients' breast cancers had been surgically removed. Pathological features were retrieved from the pathology reports or the original H&E stained slides were reviewed. Treatment data were retrieved from patients' medical records.

2) 120 patients diagnosed with invasive breast cancer and treated with neoadjuvant chemotherapy (NAC) at Semmelweis University, Budapest, Hungary between 2002 and 2013 were retrospectively recruited. Patients were enrolled only if they had completed NAC, thereafter underwent surgery. The median follow up time for overall survival (OS) and distant metastases-free survival (DMFS) was 60.5 and 59 months, respectively. Degree of response to NAC was categorized according to Pinder et al. (2007) [18] in the histological sections of the post-treatment surgical specimens as follows: Pathologic complete response (pCR) was defined as no residual invasive tumor and the absence of any residual invasive tumor in the lymph nodes. Partial response to therapy (pPR), either <10% of tumor remaining (pPRi), or 10-50% tumor remaining (pPRii), or >50% of tumor remaining but some evidence of response to therapy is present (pPRiii). Non-responders (pNR) were defined as no evidence of response to therapy.

The study was approved by the Institutional Review Board of Semmelweis University (TUKEB, #7-1/2008 and TUKEB 120/2013). Regarding the definition of surrogate molecular subtypes of breast cancer, we referred to the St. Gallen recommendations from 2013 that include five categories (luminal A, luminal B/HER2-, luminal B/HER2+, HER2+ and triple negative [123].

3.2. Tissue preparation

Tissue microarrays (TMA) were built from 10% neutrally buffered FFPE representative tissue blocks of the 378 consecutive cases. Tumor areas were selected by pathologists

based on hematoxylin & eosin stained slides. Duplicate cores (each 2 mm in diameter) were punched (TMA Master, 3DHISTECH Ltd., Budapest, Hungary) from each case, resulting 10 TMA blocks.

Regarding the neoadjuvant cohort involving 120 cases, the pre-treatment core biopsy specimens and in case of non pCR, the surgical specimens were investigated.

3.3. Immunohistochemistry

Paraffin sections of 3 μm thickness were cut from the TMA blocks for IHC. The following five antibodies (Table 2) were used for IHC detection of Ki67 on TMA blocks: SP6 (Histopathology), 30-9 (Ventana), N1574-poly (DAKO), B56 (Histopathology), MIB1 (Immunotech).

Table 2: Characteristics of the used Ki67 antibodies.

Clone	Manufacturer	Species	Clonality	Immunogenity	Epitope	Dilution
SP6	Histopathology	rabbit	mono	recognizes the same repeated Ki67 epitope as MIB1	c-terminus	1:50
30-9	Ventana	rabbit	mono	C-terminal portion of Ki-67	c-terminus	1:1 (Ready-To-Use)
poly (N1574)	DAKO	rabbit	poly	synthetic peptide from 62 base pair region of the human Ki-67	middle of Ki67 protein	1:1 (Ready-To-Use)
B56	Histopathology	mouse	mono	"immunodominant epitope of the Ki-67 protein"	repetitive 66 bp element	1:50
MIB1	Immunotech (acquisition by DAKO)	mouse	mono	1002 bp Ki-67 cDNA fragment	repetitive 66 bp element (FKEL and FKELF)	1:50

Furthermore, Ki67-MIB1 was investigated with immunofluorescent labeled (MIB1-IF) antibody (IR 626 DAKO) as well. The IHC reactions were performed in an automated immunostainer (Ventana Benchmark XT, Roche, Basel, Switzerland) according to the manufacturer's protocol (at 42 °C for 32 minutes) after antigen retrieval using the pH 9.0 CC1 buffer at 42 °C for 30 minutes. For antibody visualization, UltraView DAB Detection kit (Ventana, Tucson, USA) was applied. Immunofluorescent staining was performed manually.

To detect Ki67 in core biopsy and surgical specimens of the neoadjuvant breast cancer cohort MIB1 antibody was used with the same protocol.

Furthermore, ER, PgR and Her2 IHC were also performed using the following antibodies: 1:200 anti-ER (clone 6F11), 1:200 anti-PgR (clone 312) and 1:150 anti-HER2 (clone CB11) antibodies purchased from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK) with the same protocol. The cut-off value for ER and PgR positivity was 1% positive tumor cells with nuclear staining. Hormone receptor (HR) negativity was defined as being negative for both ER and PgR. HER2 IHC positivity was defined as score 3+ complete, strong membrane staining in >10 % of tumor cells. For IHC 2+ samples, FISH was performed to confirm gene amplification by using Ventana Benchmark automatic staining system with INFORM® Her-2/neu FISH test until 2008 and Zytovision® ERBB2/CEN17 dual FISH probe after 2008. HER2 status was defined according to the ASCO/CAP guideline valid at the time of diagnosis (ASCO/CAP guideline 2007 and ASCO/CAP guideline 2013) [148,149].

3.4. Semi-quantitative evaluation of Ki67 reactions

Semi-quantitative (SQ) evaluation of Ki67 IHC of 378 consecutive cases was performed on digital slides using the TMA Module software on the PannoramicViewer (v1.11.49.0) platform (all 3DHISTECH, Budapest, Hungary) as follows: Ki67 LI was defined as the percentage of positive tumor cell nuclei, estimated on average in 3-10 high-power fields, in each core. Any nuclear positivity was considered, including nuclear, nucleolar or nuclear membrane localization irrespective of the pattern (granular or diffuse) in a range of 100–500 cells, depending on the cellularity of the TMA cores. Duplicate cores were evaluated separately and their mean Ki67 LI was finally analyzed.

During the comparison of five Ki67 antibodies, the IHC reactions were evaluated by two pathologists independently and if any discrepancy occurred, the inconsistent cases were reassessed and a consensus Ki67 LI score was given.

When the reproducibility was investigated between observers, the IHC reactions of MIB1 antibody were evaluated by three pathologists (SQ-1, SQ-2, SQ-3) independently. The three pathologists have considerable but different level of experience in Ki67 scoring of breast cancer. SQ1 is the youngest with a pathology specialist status for a year only. SQ-2 and SQ-3 are consultant pathologists with substantial experience in diagnostic practice and special focus on breast pathology. Dichotomization of Ki67 LI values either at 14% or 20% and 30% thresholds was also performed [123,63].

Regarding the neoadjuvant cohort, the Ki67 IHC reactions were evaluated by two pathologists independently and if any discrepancy occurred, the inconsistent cases were reassessed and a consensus Ki67 LI score was given.

3.5. Digital image analysis of Ki67 reactions

TMA slides were digitized with Pannoramic Flash II slide scanner using x20 objective (NA=0.83), collecting sharp signals from 7 focal planes in “Extended-focus” mode through the 3 µm section thickness at 80 jpeg image quality factor. DIA was performed on the IHC reactions of MIB1 antibody using the PatternQuant (PQ) software of the QuantCenter package module enabling automated tissue pattern recognition by separating epithelial elements from stroma. All digital hardware and software tools were from 3DHISTECH Ltd. (Budapest, Hungary). Designation of training tissue patterns to be recognized and the calibration were done in co-operation by a pathologist and an IT expert to achieve the best recognition pattern (achieved at a PQ training magnification of 1.5x; a gamma level of 1; dilution of 3; a contour of 0). So, as the detection and quantification of tumor cell nuclei using NuclearQuant (NQ) at the following settings: Blur: 15; Radius minimum: 1.5; Radius maximum: 8; Area min: 15; Intensity minimum: 30; Contrast minimum 30 (Figure 2). The brown DAB and the hematoxylin counterstain were separated with digital color deconvolution [150]. Based on these settings of PQ and NQ, automated Ki67 evaluation was performed on each core (DIA-1 analysis). In the other DIA test, automated annotations were assessed by pathologists on each core, and when it was necessary, DIA settings were adjusted independently (from the Ki67 LI

results of DIA-1, SQ-1, SQ-2, SQ-3) to exclude artifacts, underestimation or overestimation of positive/negative cells and false detections (DIA-2 analysis).

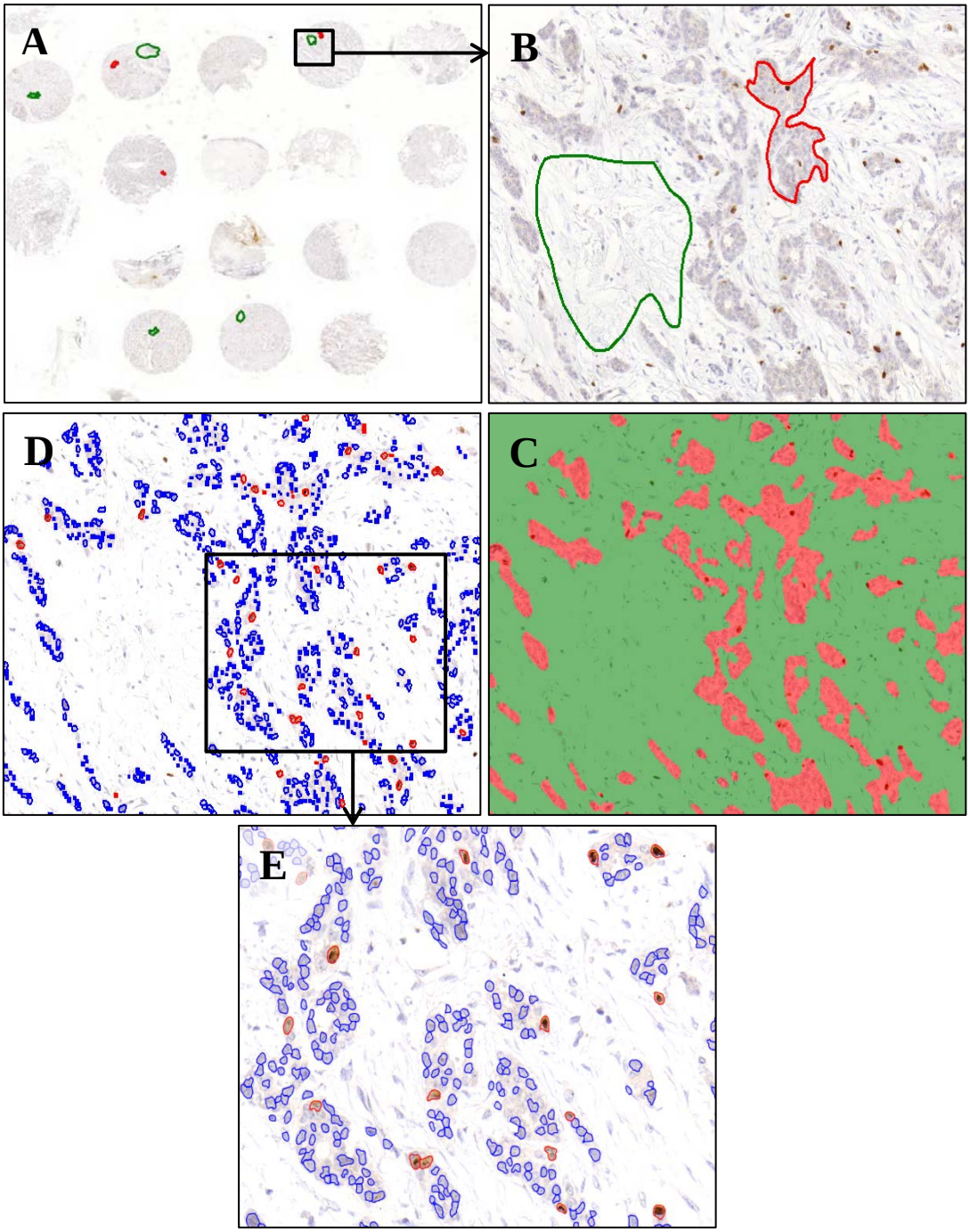

Figure 2: Workflow of 3DHistech DIA assessment. Examples of desired tissue patterns were given, demarcated with the red and green lines (red = epithel pattern, green = stroma pattern) [A,B], that we wanted to be recognized and distinguished by the software named PatternQuant [C]. Then the software named NuclearQuant counts the recognized negative (blue) and positive (red) cells only in the annotations designated by PatternQuant (red areas on picture C) [D,E].

3.6. Statistical analysis

For statistical analysis SPSS 22 software (IBM, Armonk, USA) and MedCalc 13.3.3.0 (MedCalc Software, Ostend, Belgium) software were used. Degree of agreement among different antibodies detecting Ki67 was evaluated by using intra-class correlation coefficient (ICC), concordance correlation coefficient (CCC), Cohen's kappa and Bland-Altman plot. To assess statistical differences between each antibody, Wilcoxon signed-rank and McNemar tests were applied, since our data were not normally-distributed, even after log-transformation (Shapiro-Wilk and Kolmogorov-Smirnov tests).

The reproducibility between pathologists was estimated with ICC and CCC. Altman's guideline was followed for the interpretation of ICC [151]. CCC was interpreted according to McBride [152]. Degree of agreement among different observers (SQ-1, SQ-2, SQ-3, DIA-1, and DIA-2) was evaluated by using Cohen's kappa and Bland-Altman Plot. To assess statistical differences between observers the Wilcoxon signed-rank and McNemar tests were applied, since our data were not normally-distributed, even after log-transformation (Shapiro-Wilk and Kolmogorov-Smirnov tests).

Differences in the distribution of characteristics between the parameters of patients with pCR or pPR and patients with pNR were evaluated using two-sided Fisher's Exact Test. Two-sided Mann-Whitney-Wilcoxon test was used to define age distributions in pCR vs. pNR and vs. pPR. The optimal cut-off value for Ki67 percentage to discriminate response to treatment was assessed by receiver operating characteristic (ROC) curve analysis. To identify the optimal Ki67 threshold for NAC, only pCR and pNR cases were involved in ROC analyses, because pPR status is considered as a soft endpoint. Kaplan-Meier analysis supported with log-rank test was executed to assess prognostic potential. To compare prognosis prediction potential, multivariate Cox-regression analysis was applied. OS was defined as the elapsed time from the date of diagnosis of the tumor by core biopsy to the date of death, or when patients were last censored if still alive. DMFS was defined as time from the date of primary diagnosis to the occurrence of first distant metastases. DFS was defined as time from the date of primary diagnosis to the occurrence of first relapse. In all statistical analysis, the level of significance was set at $p < 0.05$.

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4. RESULTS

Clinicopathological characteristics of the 378 breast carcinomas are shown in Table 3. Mean patient age was 59 years (range: 27-94 years). Most of the cases were pT1 and pT2, the majority with low mitotic index and histological grade of 1 or 2 and of luminal A - like subtype. Most patients had an axillary stage of pN0-1 (55.8%). In 92 cases (24.3%) axillary surgery was not performed due to clinical or patient related reason (see Table 3). More than half of the patients (57.7%) underwent postoperative breast irradiation, and slightly fewer patients (42.1%) received adjuvant chemotherapy in this cohort. All patients with ER positive breast cancer received endocrine treatment. Aggregate clinicopathological features of the 120 cases in the neoadjuvant cohort are displayed in Table 4. Mean patient age was 50.6 years (range: 29-74 years). Most patients (59.6 %) had node-positive disease and cT2 tumors (60.8 %). Tumors were ER-positive in 66.7 % of cases and presented PgR positivity >20.0 % in 41.2 % of the analyzed samples. In 34.2 % of cases HER2 positivity was detected. Of the 120 tumors, 12.5 % were of luminal A, 31.7 % of luminal B/HER2 negative, 22.5 % of luminal B/HER2 positive, 11.7 % of HER2+ and 21.7 % of TNBC subtype. Twenty three out of 120 patients (19.2 %) achieved pathologic complete remission (pCR), 73 (60.8 %) showed partial remission (pPR), whereas no response to NAC (pNR) was detected in 24 cases (20.0 %). In the group of patients who obtained pPR, residual tumor was detected in lymph nodes only in 7 patients (9.6 %), major response (>90 % tumor regression) to NAC was observed in 8 cases (11.0 %), a response rate between 50-90% was detected in 26 cases (35.6 %), whereas a response rate <50% was observed in 32 cases (43.8 %).

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Table 3: Clinicopathological data of the 378 breast carcinomas.

Patients		(n, %)	378	100%
Age	(mean ± SD, range)		58.90 ± 12.98	27-94
Tumor size (mm)	(mean ± SD)		23.58 ± 15.56	
Mitotic index (n/10HPF)	(mean ± SD)		9.07 ± 10.89	
Grade	1	(n, %)	146	38.6%
	2	(n, %)	143	37.9%
	3	(n, %)	89	23.5%
Subtype	LUMA	(n, %)	184	48.7%
	LUMB	(n, %)	124	32.8%
	HER2	(n, %)	20	5.2%
	TNBC	(n, %)	49	13.0%
	no data	(n, %)	1	0.3%
Lymph node status (TNM 7)	0	(n, %)	133	35.2%
	1	(n, %)	85	22.5%
	2	(n, %)	39	10.3%
	3	(n, %)	29	7.7%
	no data#	(n, %)	92	24.3%
Vascular Invasion	none	(n, %)	117	31.0%
	present	(n, %)	251	66.4%
	no data	(n, %)	10	2.6%
Necrosis	none	(n, %)	277	73.3%
	present	(n, %)	95	25.1%
	no data	(n, %)	6	1.6%
Chemotherapy	no	(n, %)	213	56.3%
	yes	(n, %)	159	42.1%
	no data	(n, %)	6	1.6%
Radiation therapy	no	(n, %)	154	40.7%
	yes	(n, %)	218	57.7%
	no data	(n, %)	6	1.6%
Follow-up time	(n, median, IQT*)		334, 99.80	57.93

*interquartile range. #29 cases were small, screen detected lesions before the nationwide screening was introduced. No sentinel lymph node technique was available at that time. Six patients developed second primary carcinoma in the same breast previously undergoing breast conserving surgery with axillary block dissection. In 2 cases, no lymph nodes were found in the removed axillary fat tissue. In 35 cases, due to co-morbidities or advanced age of patients axillary staging was omitted. In the remaining 20 cases, recurrent breast carcinoma was diagnosed (in these cases the primary tumors were not available).

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Table 4: Clinicopathological data of the 120 breast carcinomas.

Factors	Subgroups	Number of cases	Total %	Valid %
Age	*40 ≥*	29	24.2	24.2
	40 <	91	75.8	75.8
cT	*T1*	19	15.8	15.8
	T2	73	60.8	60.8
	T3	15	12.5	12.5
	T4	13	10.8	10.8
pT	*pT0*	20	16.7	18.7
	pT1	40	33.3	37.4
	pT2	34	28.3	31.8
	pT3	9	7.5	8.4
	pT4	4	3.3	3.7
	Unknown	13	10.8	
cN	*N0*	46	38.3	40.4
	N1	55	45.8	48.3
	N2	9	7.5	7.9
	N3	4	3.3	3.5
	Nx	6	5.0	
pN	*pN0*	51	42.5	48.1
	pN1	36	30	33.9
	pN2	13	10.8	12.3
	pN3	6	5	5.7
	Nx	14	11.7	
Grade	*1*	1	0.8	0.9
	2	46	38.3	41.8
	3	63	52.5	57.3
	Unknown	10	8.3	
ER status	*Positive*	80	66.7	66.7
	Negative	40	33.3	33.3
PgR status	*20% >*	8	6.7	6.7
	20% ≤	49	40.8	41.2
	Negative	62	51.7	52.1
	Unknown	1	0.8	
HER2 status	*Positive*	41	34.2	34.2
	Negative	79	65.8	65.8
Histological type	*Lobular*	6	5.0	5.1
	IBC NOS	112	93.3	94.9
	Other/Unknown	2	1.7	
Molecular subtype (surrogate definitions)	*Luminal A*	15	12.5	12.5
	Luminal B/HER2-	38	31.7	31.7
	Luminal B/HER2+	27	22.5	22.5
	HER2+	14	11.7	11.7
	Triple-negative	26	21.7	21.7

Response	*Complete*	23	19.2	19.2
	Partial	73	60.8	60.8
	Non-responder	24	20.0	20.0
Anthracyclines	*Yes*	88	73.3	73.3
	No	32	26.7	26.7
Taxanes	*Yes*	99	82.5	82.5
	No	21	17.5	17.5
Platinum	*Yes*	31	25.83	25.83
	No	89	74.16	74.16
Trastuzumab	*Yes*	12	10.0	10.0
	No	108	90.0	90.0

4.1. The validity of five Ki67 antibodies

4.1.1. Comparison of Ki67 LI score of the different antibodies

We investigated the Ki67 LI score of the 5 antibodies, and the following median values were observed: SP6 antibody: 8.00%, 30-9 antibody: 8.00%, poly antibody: 5.75%, MIB1 antibody: 3.50%, B56 antibody: 3.50%, MIB1-IF antibody: 3.50% (Figure 3).

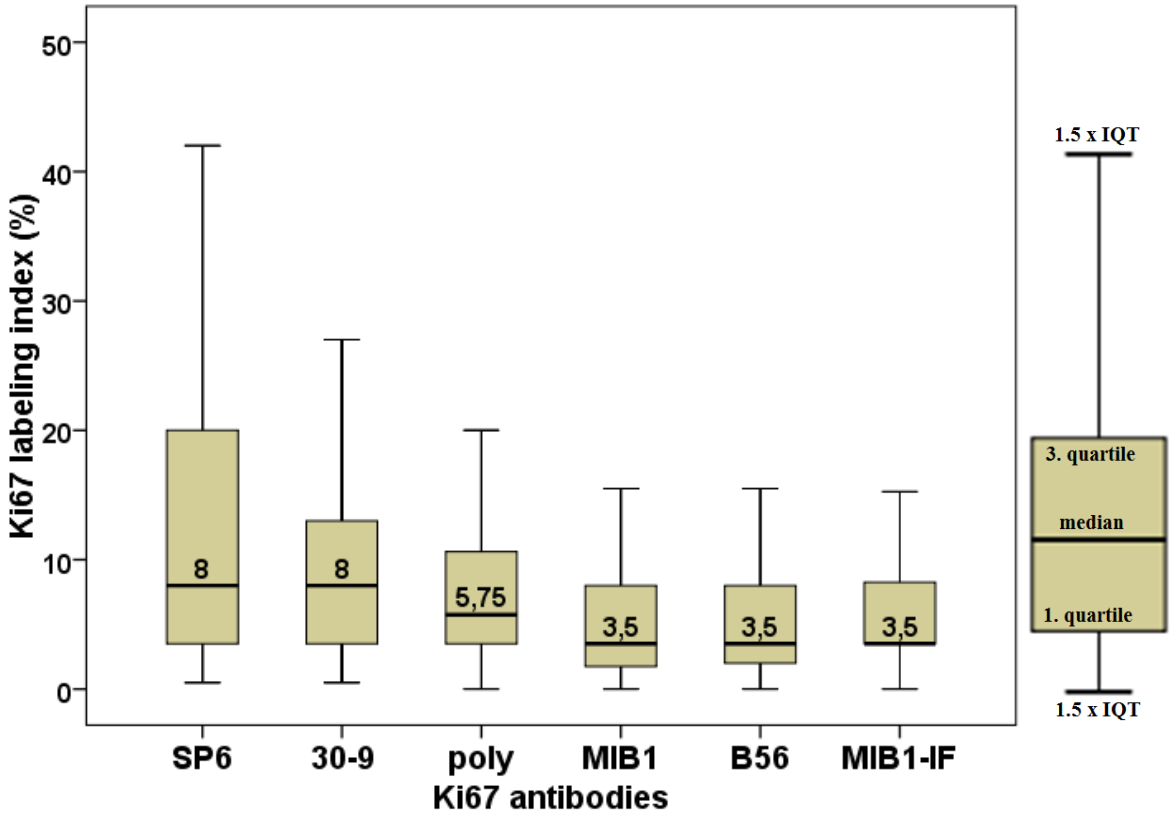

Figure 3: Boxplot of Ki67 LI of the five antibodies.

Significant difference occurred between all Ki67 LI assessments of the 5 antibodies (p values for all comparisons ≤ 0.005). Dichotomizing Ki67 LI scores at 20% threshold, we found no significant difference between MIB1, poly and MIB1-IF (MIB1 vs. poly p=0.052; MIB1vs. MIB1-IF p=0.230; poly vs. MIB1-IF p=0.405) (Table 5). At 30% cut-off score, no significant difference occurred between MIB1, poly and MIB1-IF (MIB1 vs. poly p=0.115; MIB1vs. MIB1-IF p=0.988; poly vs. MIB1-IF p=0.230), similarly to the results at 20% threshold. Furthermore, 30-9 and poly did not differ significantly at 30% cut-off score (p=0.096) (Table 5).

Table 5: Statistical comparisons of the five Ki67 antibodies.

Wilcoxon signed-rank test p	**SP6**	**30-9**	**poly**	**MIB1**	**B56**	**MIB1-IF**
SP6	-	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
30-9	≤ 0.001	-	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
poly	≤ 0.001	≤ 0.001	-	≤ 0.001	≤ 0.001	0.005
MIB1	≤ 0.001	≤ 0.001	≤ 0.001	-	0.002	0.002
B56	≤ 0.001	≤ 0.001	≤ 0.001	0.002	-	≤ 0.001
MIB1-IF	≤ 0.001	≤ 0.001	0.005	0.002	≤ 0.001	-
McNemar test p	**SP6 D20%**	**30-9 D20%**	**poly D20%**	**MIB1 D20%**	**B56 D20%**	**MIB1-IF D20%**
SP6 D20%	-	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
30-9 D20%	≤ 0.001	-	0.006	≤ 0.001	≤ 0.001	0.002
poly D20%	≤ 0.001	0.006	-	0.052	≤ 0.001	0.405
MIB1 D20%	≤ 0.001	≤ 0.001	0.052	-	≤ 0.001	0.230
B56 D20%	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	-	≤ 0.001
MIB1-IF D20%	≤ 0.001	0.002	0.405	0.230	≤ 0.001	-
McNemar test p	**SP6 D30%**	**30-9 D30%**	**poly D30%**	**MIB1 D30%**	**B56 D30%**	**MIB1-IF D30%**
SP6 D30%	-	0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
30-9 D30%	≤ 0.001	-	0.096	0.001	≤ 0.001	0.001
poly D30%	≤ 0.001	0.096	-	0.115	≤ 0.001	0.230
MIB1 D30%	≤ 0.001	0.001	0.115	-	≤ 0.001	0.988
B56 D30%	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	-	0.003
MIB1-IF D30%	≤ 0.001	0.001	0.230	0.988	0.003	-
D20% = dichotomized at 20% threshold D30% = dichotomized at 30% threshold						

4.1.2. Concordance of Ki67 LI score of the different antibodies

The Ki67 LI scores of the 5 antibodies showed a moderate agreement (ICC: 0.645, CI: 0.572-0.708, p<0.001). Highest concordance was observed between MIB1 and poly, 30-9 and poly, MIB1 and B56, 30-9 and SP6 as well as between MIB1 and 30-9 (CCC: 0.785, 0.780, 0.774, 0.762 and 0.745, respectively). Conversely, lowest agreement was found between SP6 and B56 as well as between SP6 and MIB1-IF (CCC: 0.448, 0.444, respectively) (Table 6).

Table 6: Concordance and agreement between the five Ki67 antibodies.

Intraclass correlation coefficient (CI)	**SP6**	**30-9**	**poly**	**MIB1**	**B56**	**MIB1-IF**
Between the five antibodies	0.645 (0.572-0.708)					
Concordance correlation coefficient	**SP6**	**30-9**	**poly**	**MIB1**	**B56**	**MIB1-IF**
SP6	-	0.762	0.640	0.604	0.448	0.444
30-9	0.762	-	0.780	0.745	0.603	0.560
poly	0.640	0.780	-	0.785	0.698	0.599
MIB1	0.604	0.745	0.785	-	0.774	0.668
B56	0.448	0.603	0.698	0.774	-	0.695
MIB1-IF	0.444	0.560	0.599	0.668	0.695	-
Cohen's kappa	**SP6 D20%**	**30-9 D20%**	**poly D20%**	**MIB1 D20%**	**B56 D20%**	**MIB1-IF D20%**
SP6 D20%	-	0.620	0.491	0.398	0.266	0.325
30-9 D20%	0.620	-	0.630	0.597	0.379	0.459
poly D20%	0.491	0.630	-	0.618	0.594	0.425
MIB1 D20%	0.398	0.597	0.618	-	0.603	0.573
B56 D20%	0.266	0.379	0.594	0.603	-	0.402
MIB1-IF D20%	0.325	0.459	0.425	0.573	0.402	-
Cohen's kappa	**SP6 D30%**	**30-9 D30%**	**poly D30%**	**MIB1 D30%**	**B56 D30%**	**MIB1-IF D30%**
SP6 D30%	-	0.608	0.544	0.400	0.187	0.353
30-9 D30%	0.608	-	0.650	0.454	0.321	0.460
poly D30%	0.544	0.650	-	0.626	0.535	0.428
MIB1 D30%	0.400	0.454	0.626	-	0.568	0.558
B56 D30%	0.187	0.321	0.535	0.568	-	0.501
MIB1-IF D30%	0.353	0.460	0.428	0.558	0.501	-
D20% = dichotomized at 20% threshold D30% = dichotomized at 30% threshold						

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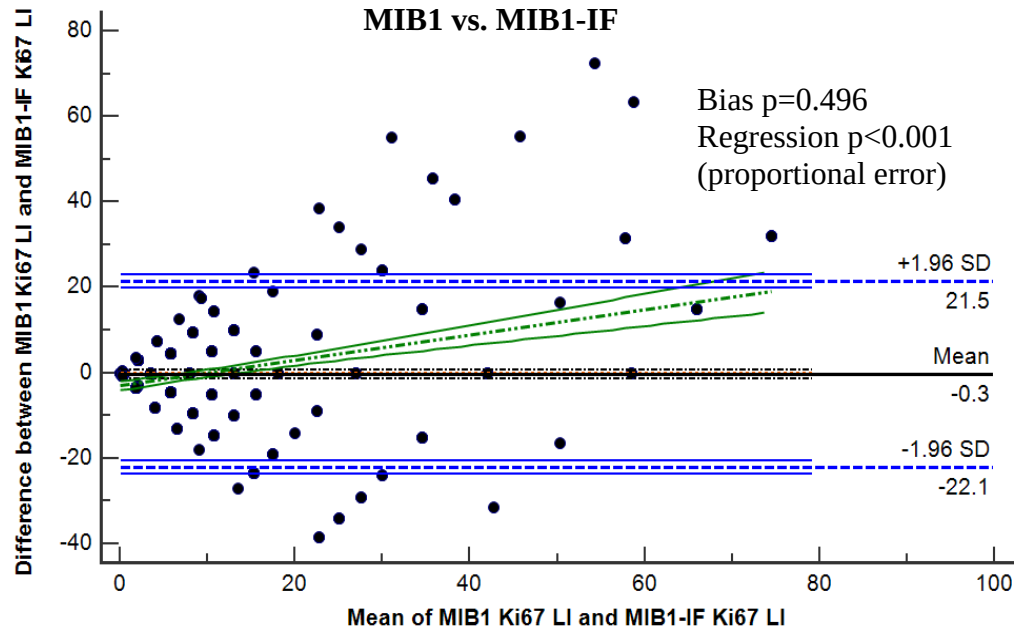
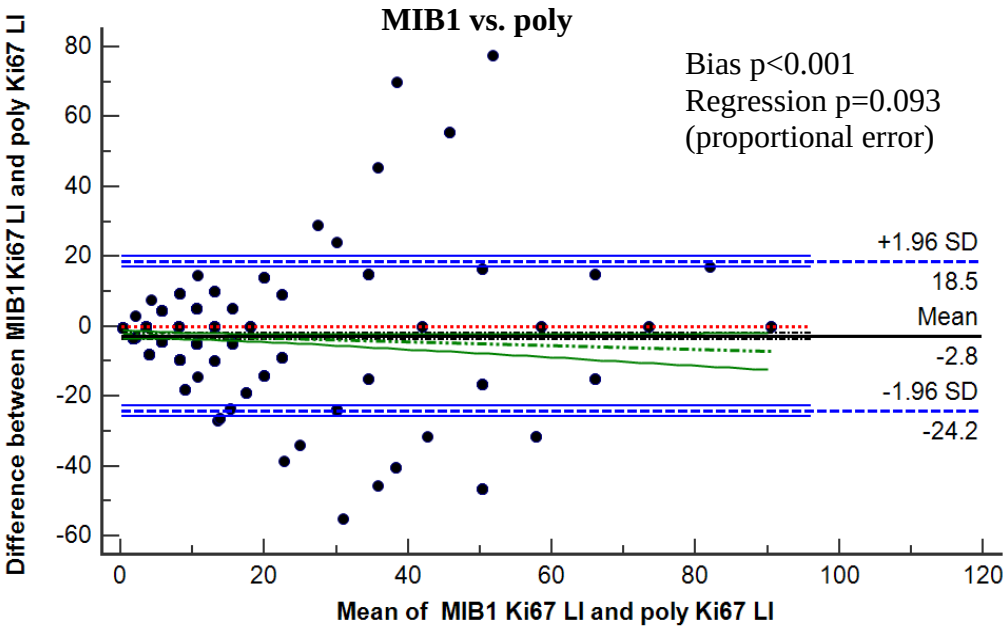
We also investigated the agreement of the 5 antibodies by Bland-Altman plot. Significant bias was observed in all comparisons except MIB1 vs. MIB1-IF (bias: -0.33 CI: 0.62-1.27 p=0.496) and the range of agreement was also wide (upper limit of agreement: +14.4-44.9; lower limit of agreement: -14.9-40.7). Furthermore, the variability of differences represented a systematic error between all the antibodies except between MIB1 and poly (p=0.093) (Figure 4). Although in the comparison of MIB1 and poly, the variability of differences showed an increasing trend, proportional to the magnitude of Ki67 LI.





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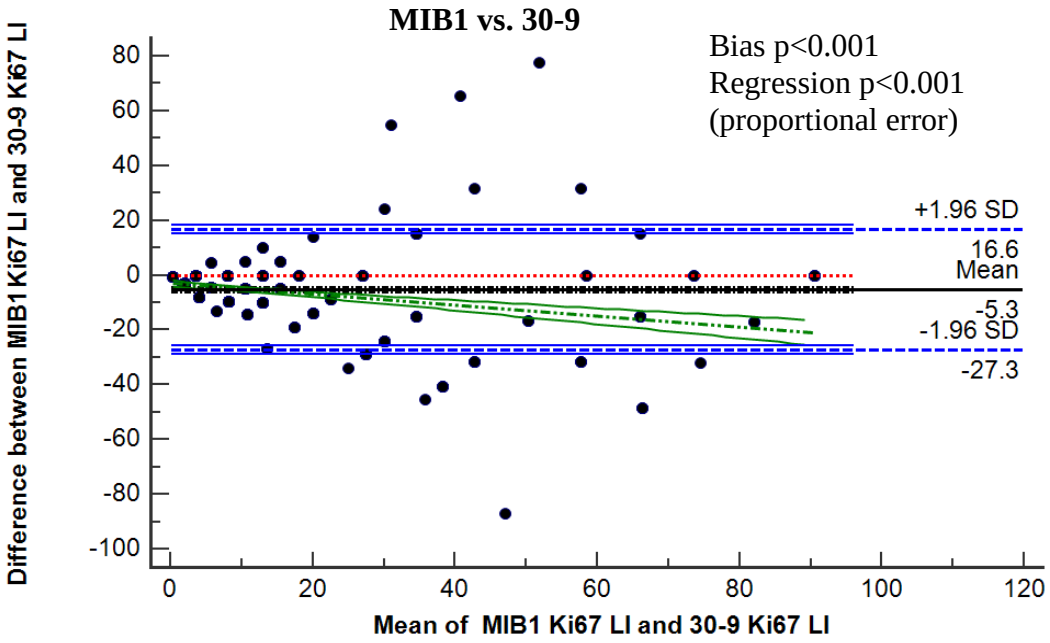

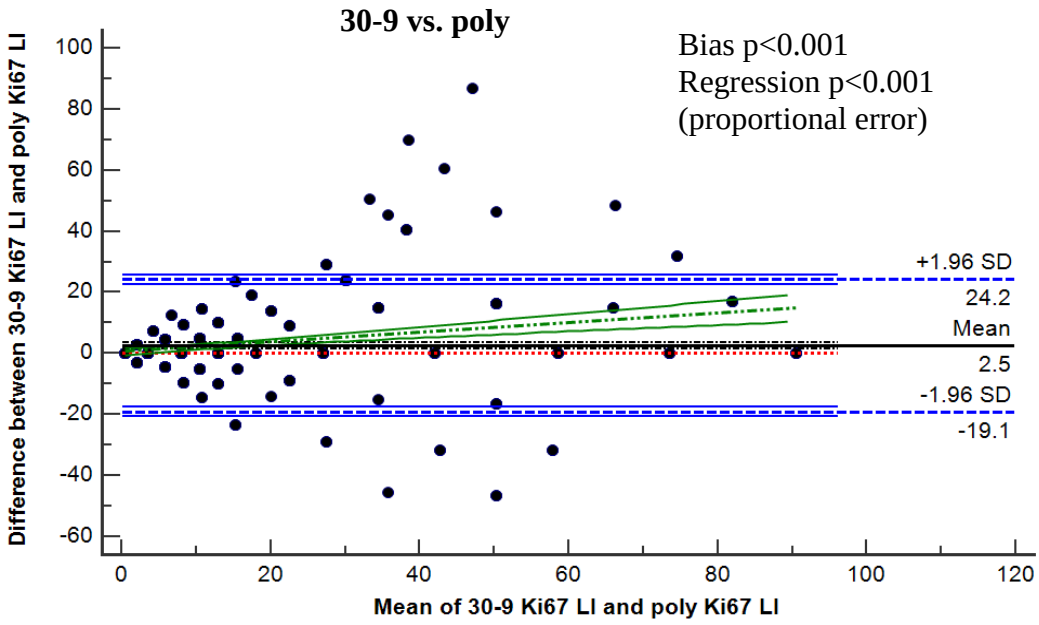

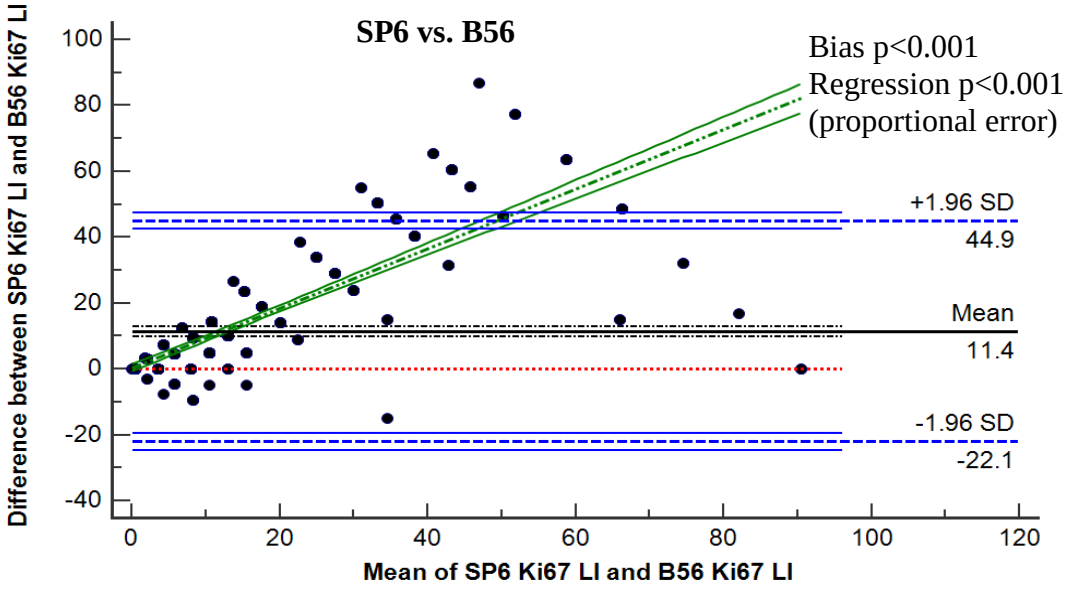

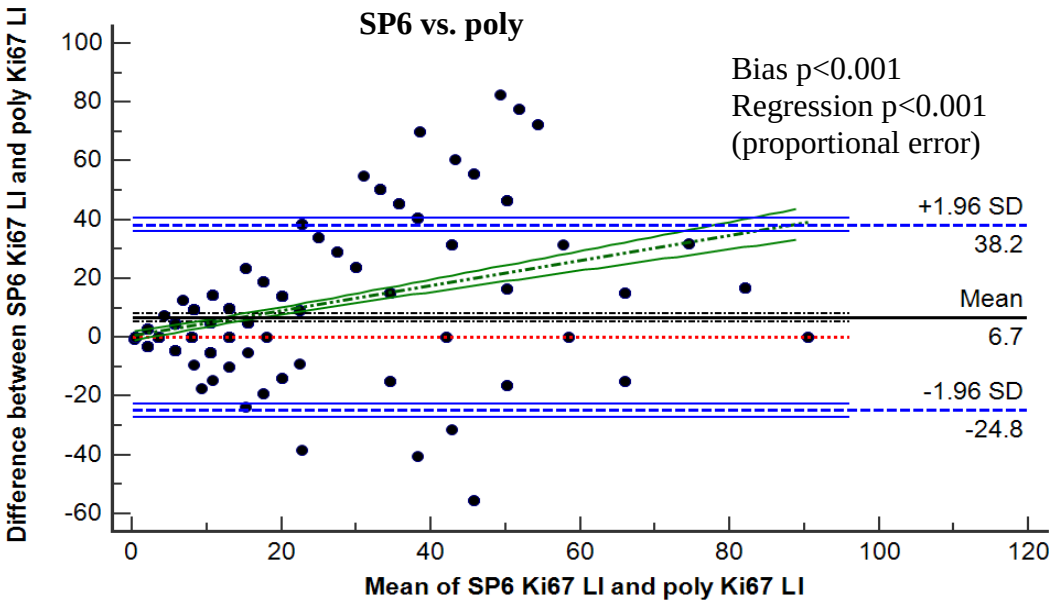

Figure 4: Bland-Altman plots comparing Ki67 LI scores of the antibodies. Red dashed line corresponds the expected mean zero difference between Ki67 LI scores of the antibodies. Black line represents the observed mean difference between Ki67 LI scores of the antibodies, namely the observed bias (black dashed lines are the CI of the observed mean difference). Blue dashed lines illustrate the range of agreement (lower and upper limit of agreement) based on 95% of differences (blue lines are the CI of the limits of agreement). Green dashed line is the fitted regression line to detect potential proportional difference (green lines are the CI of the regression line). Not all plots shown.

The agreement between dichotomized Ki67 LI scores vary between poor to good (κ=0.187-0.650) (Table 6). Highest agreement was found between poly and 30-9, MIB1 and poly, SP6 and 30-9 as well as between MIB1 and B56 (κ=0.650, 0.626, 0.620 and 0.603, respectively). Conversely, low agreement occurred between SP6 and B56, 30-9 and B56 as well as between SP6 and MIB1-IF (κ=0.187, 0.321, 0.325, respectively).

4.1.3. Capacity of the different Ki67 antibodies to predict disease-free survival

For prognosis, all the Ki67 antibodies (MIB1 p= 0.003, SP6 p= 0.014, 30-9 p= 0.004, poly p< 0.001, B56 p= 0.003) but the IF detection of MIB1 (p= 0.993) could perform statistically significant splitting of our cohort into 2 patients' groups with distinct DFS at 20% threshold (Figure 5). At 30% cut-off point, Ki67 LI of MIB1 (p= 0.005), SP6 (p= 0.023), 30-9 (p= 0.001) and poly (p< 0.001) could distinguish

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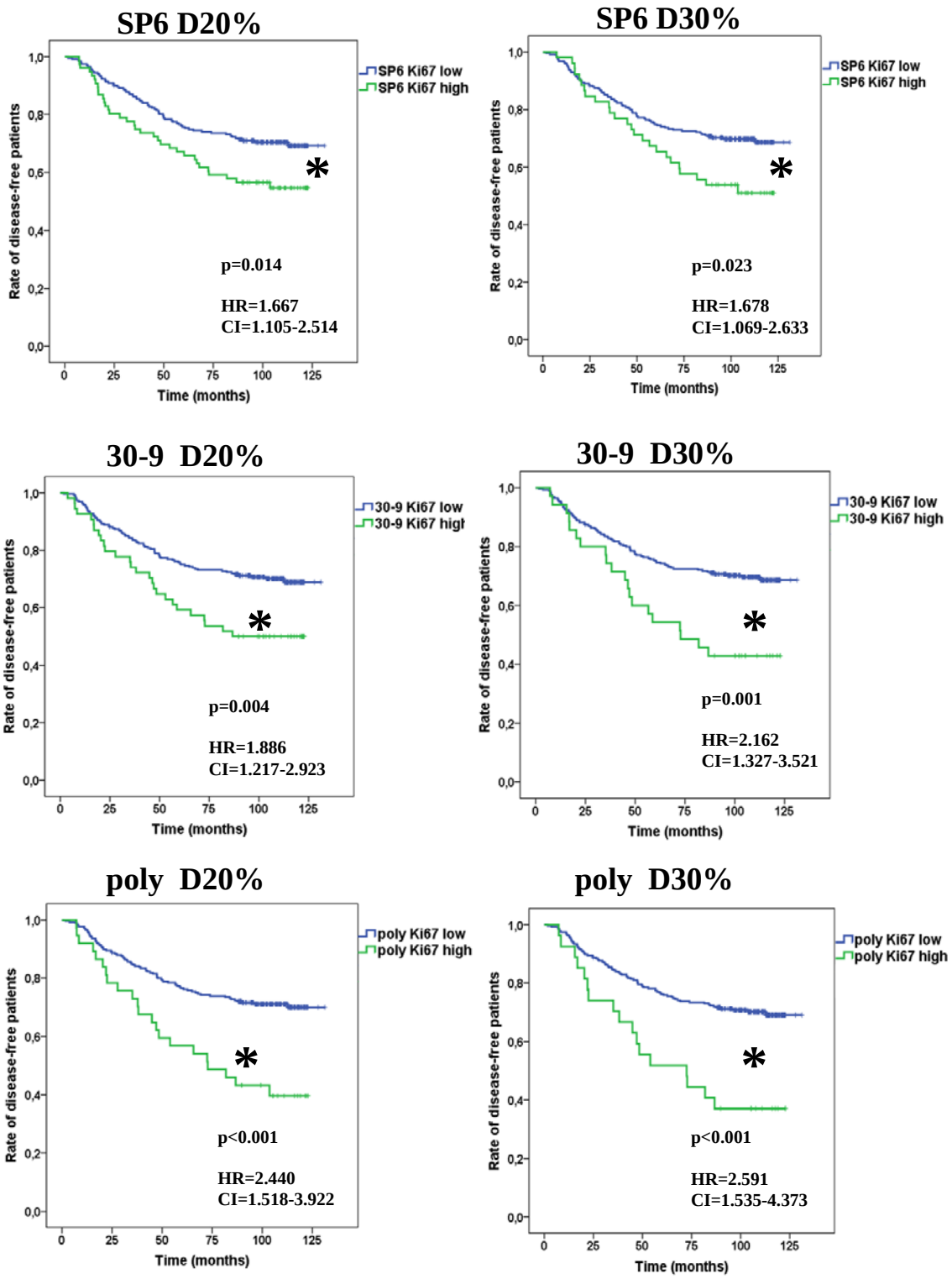

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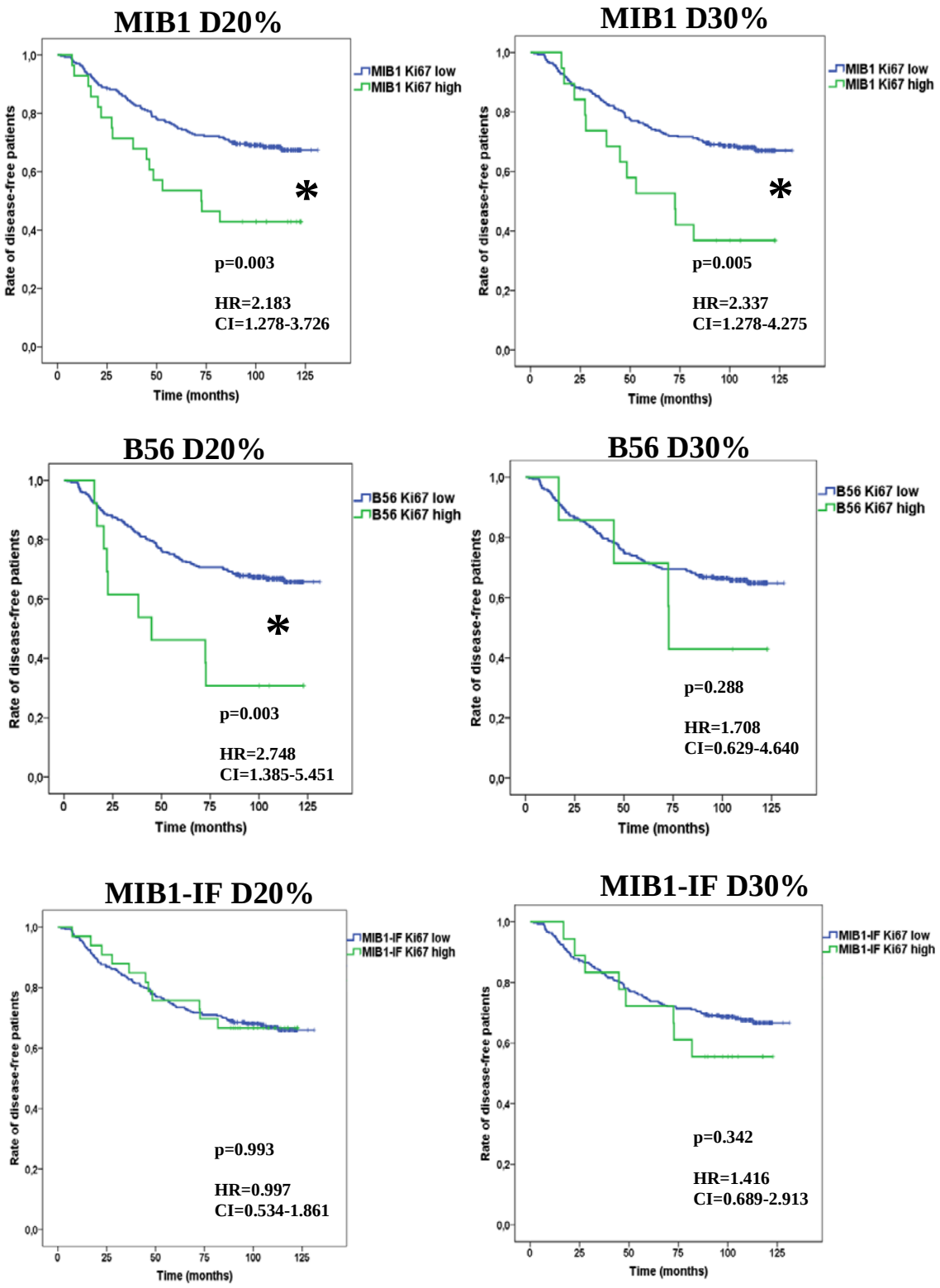

*Figure 5: Various Ki67 antibodies and their potential to predict disease-free survival at cut-off points 20% (D20%) and 30% (D30%), respectively. *Significant.*

good and unfavorable prognosis patients' cohorts. Meanwhile B56 (p= 0.288) and MIB1-IF (p= 0.342) did not represent any statistically significant prognosis predictor potential at 30% threshold (Figure 5). We had also investigated the utility of each Ki67 antibodies as potential independent predictors of DFS adjusted by age, IHC subtypes, lymph node and T status, histological grade, mitotic index, vascular invasion as well as necrosis at 20% and 30% thresholds. At 20% cut-off score, Ki67 LI of poly (p= 0.031) and lymph node status (p< 0.001) were significantly linked to DFS (Table 7). However, at 30% threshold, only lymph node status (p< 0.001) represented an independent association with survival (Table 7).

Table 7: Multivariate Cox regression analysis of the Ki67 antibodies and the clinicopathological factors.

Prognostic Factors	**Multivariate Cox regression analysis involving Ki67 LI scores of the five antibodies and the clinicopathological factors**		
	HR	**95% CI**	**p-value**
Age	0.884	0.716-1.090	0.249
Tumor size	0.976	0.652-1.460	0.905
IHC Subtype	1.111	0.876-1.409	0.384
Histological grade	0.867	0.564-1.335	0.518
Lymph node status (TNM 7)	1.552	1.211-1.988	<0.001
Mitotic index	1.152	0.769-1.725	0.493
Vascular invasion	0.774	0.427-1.404	0.399
Necrosis	1.481	0.814-2.694	0.199
SP6 D20%	1.109	0.589-2.087	0.749
30-9 D20%	1.341	0.697-2.577	0.379
poly D20%	2.100	1.068-4.129	0.031
MIB1 D20%	1.800	0.836-3.878	0.133
B56 D20%	2.284	0.856-6.093	0.099
MIB1-IF D20%	1.104	0.523-2.331	0.796
SP6 D30%	1.278	0.683-2.391	0.443
30-9 D30%	1.421	0.691-2.921	0.339
poly D30%	2.027	0.947-4.335	0.069
MIB1 D30%	1.870	0.816-4.282	0.139
B56 D30%	1.715	0.517-5.697	0.378
MIB1-IF D30%	1.332	0.558-3.178	0.519
D20% = dichotomized at 20% threshold D30% = dichotomized at 30% threshold			

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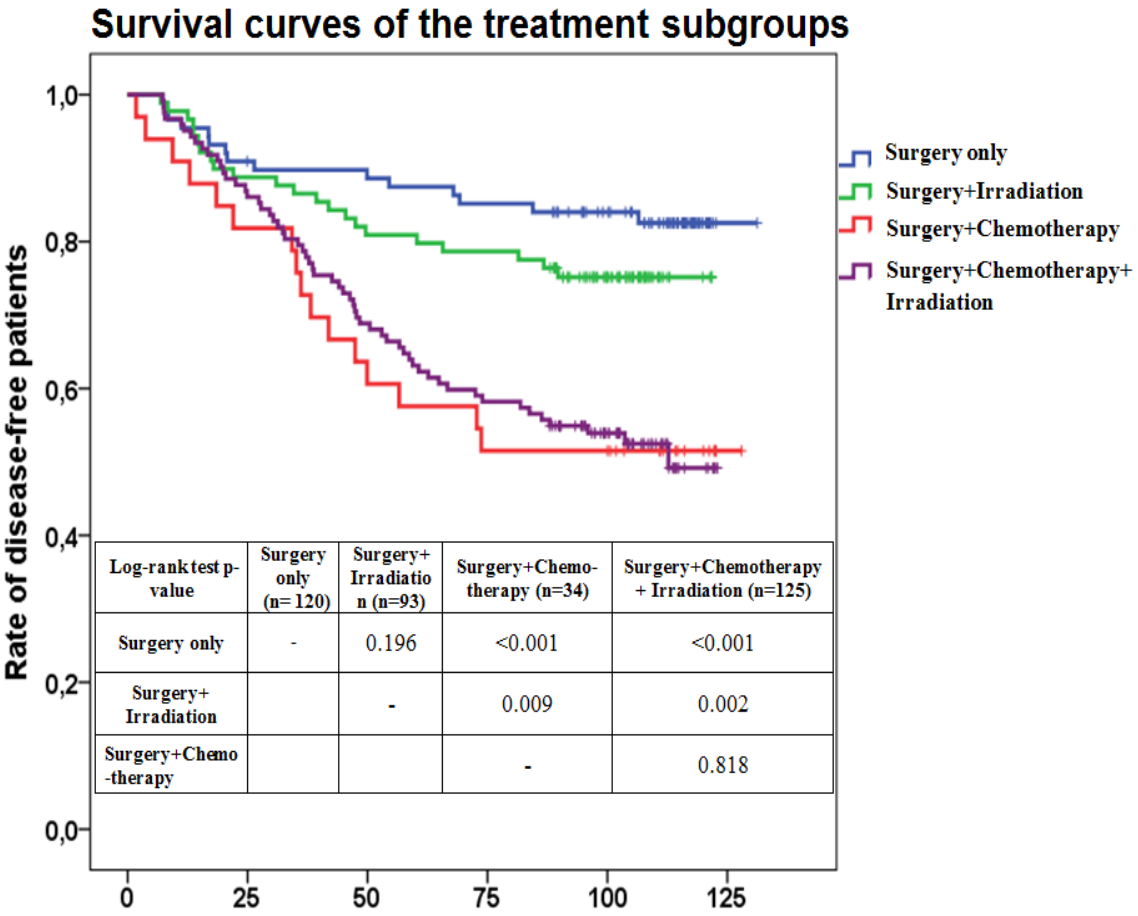
The effect of different treatment protocols on clinical outcome was also explored and significant difference occurred between patients' groups who received different treatment ($p<0.001$, Figure 6). Patients who underwent surgical intervention only had the longest DFS, while patients who received surgery+irradiation+chemotherapy combination had the most unfavorable prognosis (Figure 6).



Log-rank test p-value	Surgery only (n= 120)	Surgery+ Irradiation (n=93)	Surgery+Chemo-therapy (n=34)	Surgery+Chemotherapy + Irradiation (n=125)
Surgery only	-	0.196	<0.001	<0.001
Surgery+ Irradiation		-	0.009	0.002
Surgery+Chemo-therapy			-	0.818

Figure 6: Survival functions of the treatment subgroups.

None of the Ki67 antibodies represented a significant association with DFS adjusted by clinicopathological factors in the patient subgroup treated with surgery+irradiation, and in the patient subgroup treated with surgery+irradiation+chemotherapy combination ($p>0.250$ for all Ki67 antibodies in all comparisons). Multivariate analyses were not performed in patient subgroup with surgery only and in patient subgroup treated with surgery+chemotherapy due to the low number of cases and/or low event rate compared to the relatively numerous clinicopathological factors. In the patient subgroup with surgery only, Ki67 LI scores of all the antibodies ─ except SP6 at 20% threshold and MIB1-IF at all cut-off scores ─ could perform statistically significant splitting of our cohort into 2 patient groups with distinct DFS (Table 8). However, in the patient subgroup treated with surgery+chemotherapy, none of the Ki67 antibodies could distinguish good and unfavorable prognosis patients' cohorts.

*Table 8: Cox regression analysis of the Ki67 antibodies and the clinicopathological factors in the different treatment groups. Only significant factors shown. * Multivariate analyses were not performed due to the low event rate compared to relatively numerous clinicopathological factors.*

Treatment groups	**Prognostic factor**	**HR**	**95% CI**	**p-value**
Surgical treatment only (n=120)*	IHC Subtype	2.007	1.295-3.111	0.002
	30-9_D20%	4.091	1.295-12.925	0.016
	poly_D20%	4.129	1.268-13.446	0.019
	MIB1_D20%	6.580	2.046-21.168	0.002
	B56_D20%	6.788	2.189-22.004	0.001
	SP6_D30%	3.139	1.051-9.381	0.041
	30-9_D30%	5.959	1.777-19.982	0.004
	poly_D30%	5.944	1.820-19.415	0.003
	MIB1_D30%	6.369	2.067-22.789	0.009
	B56_D30%	6.411	2.340-22.495	0.007
Surgery+irradiation (n=93)	Age	0.174	0.041-0.745	0.018
	Tumor size	4.420	1.183-16.510	0.027
	IHC Subtype	1.720	1.021-2.899	0.042
	Lymph node status (TNM 7)	5.087	1.492-17.339	0.009
D20% = dichotomized at 20% threshold D30% = dichotomized at 30% threshold				

4.2. The reproducibility between different Ki67 evaluations

Since MIB1 is the most widely used antibody to detect Ki67 and showed the highest concordance and agreement with the poly antibody, it was used in the further investigations.

The total number of cases involved in the study investigating reproducibility between Ki67 scorings decreased to 347 out of 378 consecutive breast cancer cases, because in the previous study only those cases were included that showed evaluable reaction by at least two Ki67 antibodies out of the five. Furthermore, the pathologists evaluating Ki67 LI were not the same in the investigations detailed in 4.1 and in 4.2 chapters.

4.2.1. Comparison of semi-quantitative (SQ) evaluations

We examined the 3 SQ Ki67 LI evaluations (SQ-1, SQ-2, SQ-3), and the following median values were observed: 5 (SQ-1), 8 (SQ-2), 10 (SQ-3) (Figure 7).

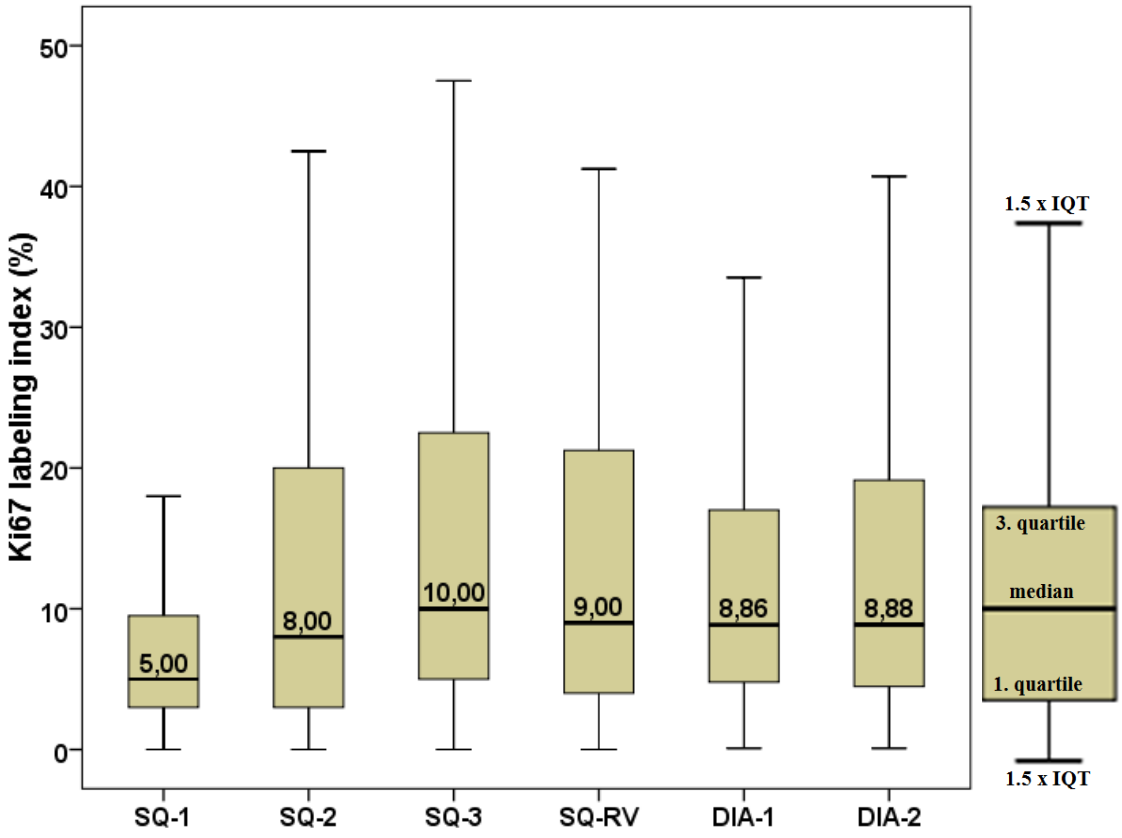


Figure 7: Boxplot of Ki67 LI evaluations.

Significant difference was found between all the 3 SQ Ki67 LI assessments expressed in percentage (p values for all comparisons ≤ 0.001, table 9). However, they showed a very good consistency (ICC= 0.853) concerning the relative difference between cases (Table 10). The best interobserver variability was found between SQ-2 and SQ-3 (CCC= 0.935), while SQ-1 showed poor concordance with SQ-2 and SQ-3 (CCC= 0.817, CCC= 0.827, respectively, table 10).

Table 9: Statistical comparisons of Ki67 LI assessments.

Wilcoxon signed-rank test p	**SQ-1**	**SQ-2**	**SQ-3**	**SQ-RV**	**DIA-1**	**DIA-2**
SQ-1	-	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
SQ-2	≤ 0.001	-	≤ 0.001	0.065	≤ 0.001	0.112
SQ-3	≤ 0.001	≤ 0.001	-	0.058	≤ 0.001	≤ 0.001
SQ-RV	≤ 0.001	0.065	0.058	-	≤ 0.001	0.754
DIA-1	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	-	≤ 0.001
DIA-2	≤ 0.001	0.112	≤ 0.001	0.754	≤ 0.001	-
McNemar test p	**SQ-1 D14%**	**SQ-2 D14%**	**SQ-3 D14%**	**SQ-RV D14%**	**DIA-1 D14%**	**DIA-2 D14%**
SQ-1 D14%	-	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
SQ-2 D14%	≤ 0.001	-	0.708	0.635	0.001	0.289
SQ-3 D14%	≤ 0.001	0.708	-	0.152	≤ 0.001	0.004
SQ-RV D14%	≤ 0.001	0.635	0.152	-	0.010	0.337
DIA-1 D14%	≤ 0.001	0.001	≤ 0.001	0.010	-	0.019
DIA-2 D14%	≤ 0.001	0.289	0.004	0.337	0.019	-
McNemar test p	**SQ-1 D20%**	**SQ-2 D20%**	**SQ-3 D20%**	**SQ-RV D20%**	**DIA-1 D20%**	**DIA-2 D20%**
SQ-1 D20%	-	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
SQ-2 D20%	≤ 0.001	-	0.082	0.044	≤ 0.001	0.770
SQ-3 D20%	≤ 0.001	0.082	-	0.503	0.864	≤ 0.001
SQ-RV D20%	≤ 0.001	0.044	0.503	-	≤ 0.001	0.701
DIA-1 D20%	≤ 0.001	≤ 0.001	0.864	≤ 0.001	-	≤ 0.001
DIA-2 D20%	≤ 0.001	0.770	≤ 0.001	0.701	≤ 0.001	-
D14% = dichotomized at 14% threshold D20% = dichotomized at 20% threshold						

Table 10: Interobserver variability between Ki67 LI assessments.

Comparison of Ki67 LI assessments	**Intra-class correlation coefficient (95% CI)**	**Concordance correlation coefficient (95% CI)**	**Cohen's Kappa**
SQ-1 vs. SQ-2 vs. SQ-3	0.853 (0.771-0.900)	-	-
SQ-1 vs. SQ-2	-	0.817 (0.777-0.850)	-
SQ-1 vs. SQ-3	-	0.827 (0.792-0.856)	-
SQ-2 vs. SQ-3	-	0.935 (0.907-0.953)	-
SQ-1_D14% vs. SQ-2_D14%	-	-	0.462
SQ-1_D14% vs. SQ-3_D14%	-	-	0.452
SQ-2_D14% vs. SQ-3_D14%	-	-	0.741
SQ-1_D20% vs. SQ-2_D20%	-	-	0.490
SQ-1_D20% vs. SQ-3_D20%	-	-	0.473
SQ-2_D20% vs. SQ-3_D20%	-	-	0.727
DIA-1 vs. SQ-RV	-	0.906 (0.887-0.922)	-
DIA-2 vs. SQ-RV	-	0.963 (0.954-0.969)	-
DIA-1 vs. DIA-2	-	0.943 (0.932-0.952)	-
DIA-1_D14% vs. SQ-RV_D14%	-	-	0.743
DIA-2_D14% vs. SQ-RV_D14%	-	-	0.849
DIA-1_D20% vs. SQ-RV_D20%	-	-	0.775
DIA-2_D20% vs. SQ-RV_D20%	-	-	0.868
DIA-1_D14% vs. DIA-2_D14%	-	-	0.894
DIA-1_D20% vs. DIA-2_D20%	-	-	0.852
SQ-1 vs. SQ-2 vs. SQ-3 vs. DIA-1 vs. DIA-2	0.886 (0.851-0.913)	-	-
D14% = dichotomized at 14% threshold D20% = dichotomized at 20% threshold			

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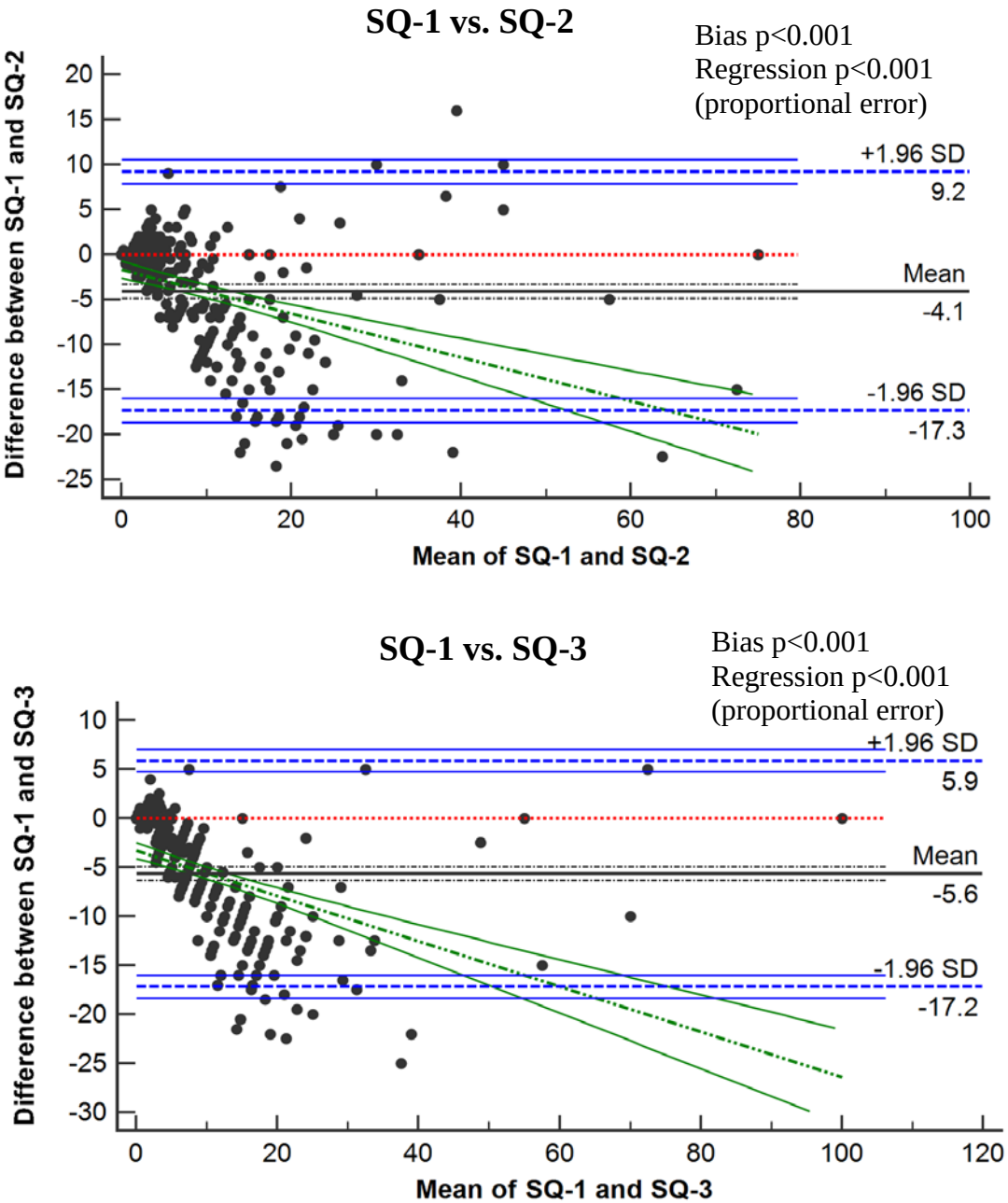
We also investigated the agreement of the three SQ evaluations using Bland-Altman plots (Figure 8). Significant bias was observed in all comparisons. The lowest bias and the narrowest range of agreement were found between SQ-2 and SQ-3 without a proportional error, however, the variability of differences still showed an increasing trend, proportional to the magnitude of Ki67 LI. Bland-Altman plots were also created for cases of <30% Ki67 LI values since these were overrepresented in our cohort, with still covering all clinically relevant thresholds. The same trends were observed between SQ evaluations with lower bias and narrower range of agreement.



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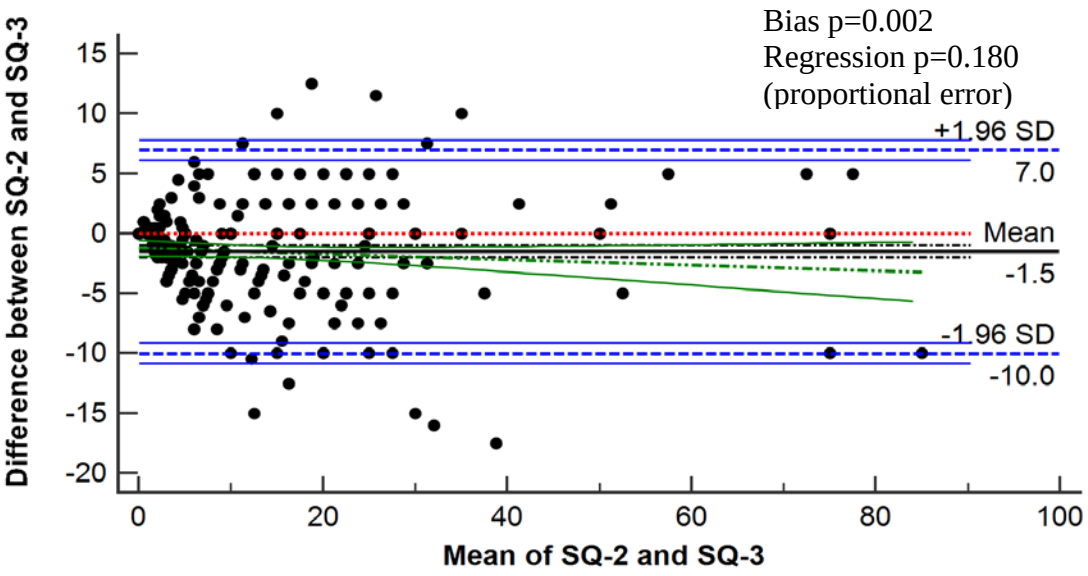
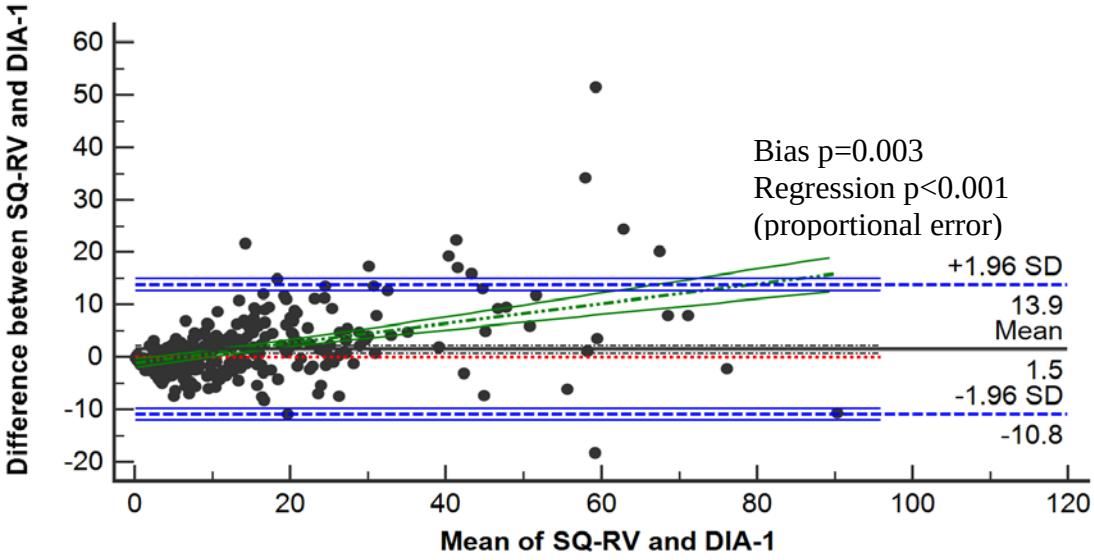
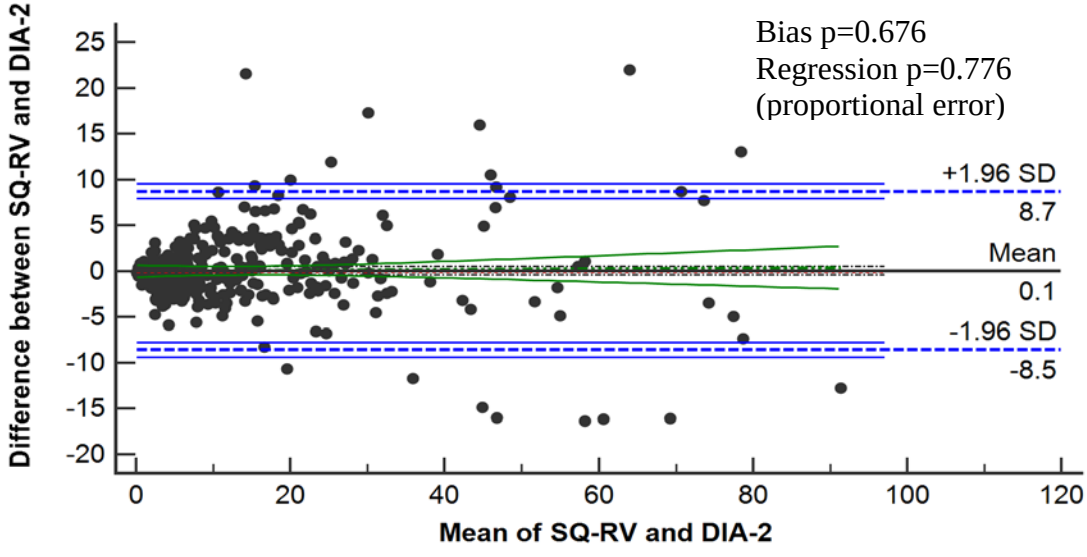
SQ-2 vs. SQ-3



SQ-RV vs. DIA-1



SQ-RV vs. DIA-2



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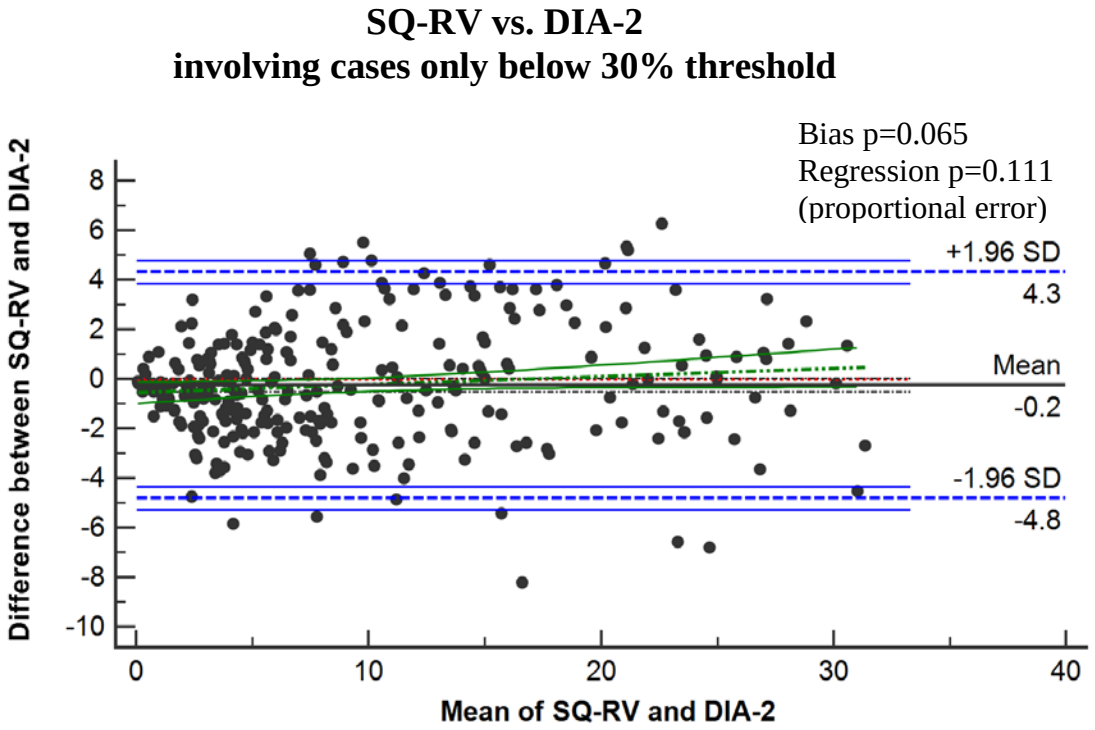

Figure 8: Bland-Altman plots comparing KIPI evaluations and Bland-Altman plots comparing KIPI evaluations involving cases only below 30% cut-off point. Red dashed line corresponds the expected mean zero difference between Ki67 LI scores of the antibodies. Black line represents the observed mean difference between Ki67 LI scores of the antibodies, namely the observed bias (black dashed lines are the CI of the observed mean difference). Blue dashed lines illustrate the range of agreement (lower and upper limit of agreement) based on 95% of differences (blue lines are the CI of the limits of agreement). Green dashed line is the fitted regression line to detect potential proportional error (green lines are the CI of the regression line). Not all plots shown.

Upon dichotomizing Ki67 LI values at 14% and 20% thresholds, SQ-1 still differed considerably from SQ-2 ($p \leq 0.001$, $p \leq 0.001$, respectively) and SQ-3 ($p \leq 0.001$, $p \leq 0.001$, respectively, table 9) with a moderate agreement (SQ-2 κ/14%/= 0.462, SQ-2 κ/20%/= 0.490, SQ-3 κ/14%/= 0.452, SQ-3 κ/20%/= 0.473, table 10). However, no significant difference ($p= 0.708$, $p= 0.082$, respectively, table 9), and substantial agreement (κ/14%/= 0.741, κ/20%/= 0.727, table 10) were found between SQ-2 and SQ-3, at these thresholds.

4.2.2. Comparison of digital image analyses (DIA) evaluations

The median values for DIA evaluations were the following: 8.86 (DIA-1) 8.88 (DIA-2) (Figure 7). For the comparison with DIA assessments, a reference SQ Ki67 LI value was generated (SQ-RV) as the mean of SQ-2 and SQ-3, since SQ-1 differed considerably from those. SQ-RV and automated DIA-1 differed ($p \leq 0.001$, table 9) and showed moderate concordance (CCC= 0.906, table 10). SQ-RV and adjustable DIA-2 showed no significant difference ($p= 0.754$, table 9), and represented a substantial concordance (CCC= 0.963, table 10). Significant difference ($p \leq 0.001$) but substantial concordance (CCC: 0.943) was found when DIA-1 was compared to DIA-2. Using Bland-Altman plots showed a significant bias and proportional error between SQ-RV and DIA-1 values, which was not seen between SQ-RV and DIA-2 values and the range of agreement was also superior in the latter case (Figure 8). Moreover, in the comparison of SQ-RV and DIA-2, the variability of differences did not show an increasing trend, proportional to the magnitude of Ki67 LI. The same results were found at 30% threshold between SQ-RV and DIA evaluations, but the range of agreement became narrower in all comparisons (Figure 8).

At 14% and 20% thresholds, though DIA-1 differed from SQ-RV significantly ($p= 0.010$, $p \leq 0.001$, respectively), DIA-2 and SQ-RV values did not ($p= 0.337$, $p= 0.701$, respectively, table 9). Both DIA methods showed substantial (DIA-1 $\kappa/14\%/= 0.743$, $\kappa/20\%/= 0.775$) or outstanding agreement (DIA-2 $\kappa/14\%/= 0.849$, $\kappa/20\%/= 0.868$) with SQ-RV (Table 10). Though significant difference occurred between DIA-1 and DIA-2 ($p/14\%/= 0.019$, $p/20\%/\leq 0.001$), agreements were high ($\kappa/14\%/ = 0.894$, $\kappa/20\%/ = 0.852$). Interobserver variability within DIA (DIA-1, DIA-2) and SQ (SQ-1, SQ-2, and SQ-3) evaluations referred to a very good consistency (ICC= 0.886).

4.2.3. Comparison of semi-quantitative (SQ) and digital image analyses (DIA) evaluations in prognosis prediction

For prognosis, all Ki67 evaluations (DIA-1 $p= 0.031$, DIA-2 $p= 0.018$, SQ-1 $p= 0.022$, SQ-2 $p= 0.008$) but SQ-3 ($p= 0.062$) could perform statistically significant splitting of our cohort into 2 patients' group with distinct DFS at 14% threshold (Figure 9). At 20% cut-off point, Ki67 evaluations of DIA-2 ($p= 0.004$), SQ-2 ($p \leq 0.001$) and

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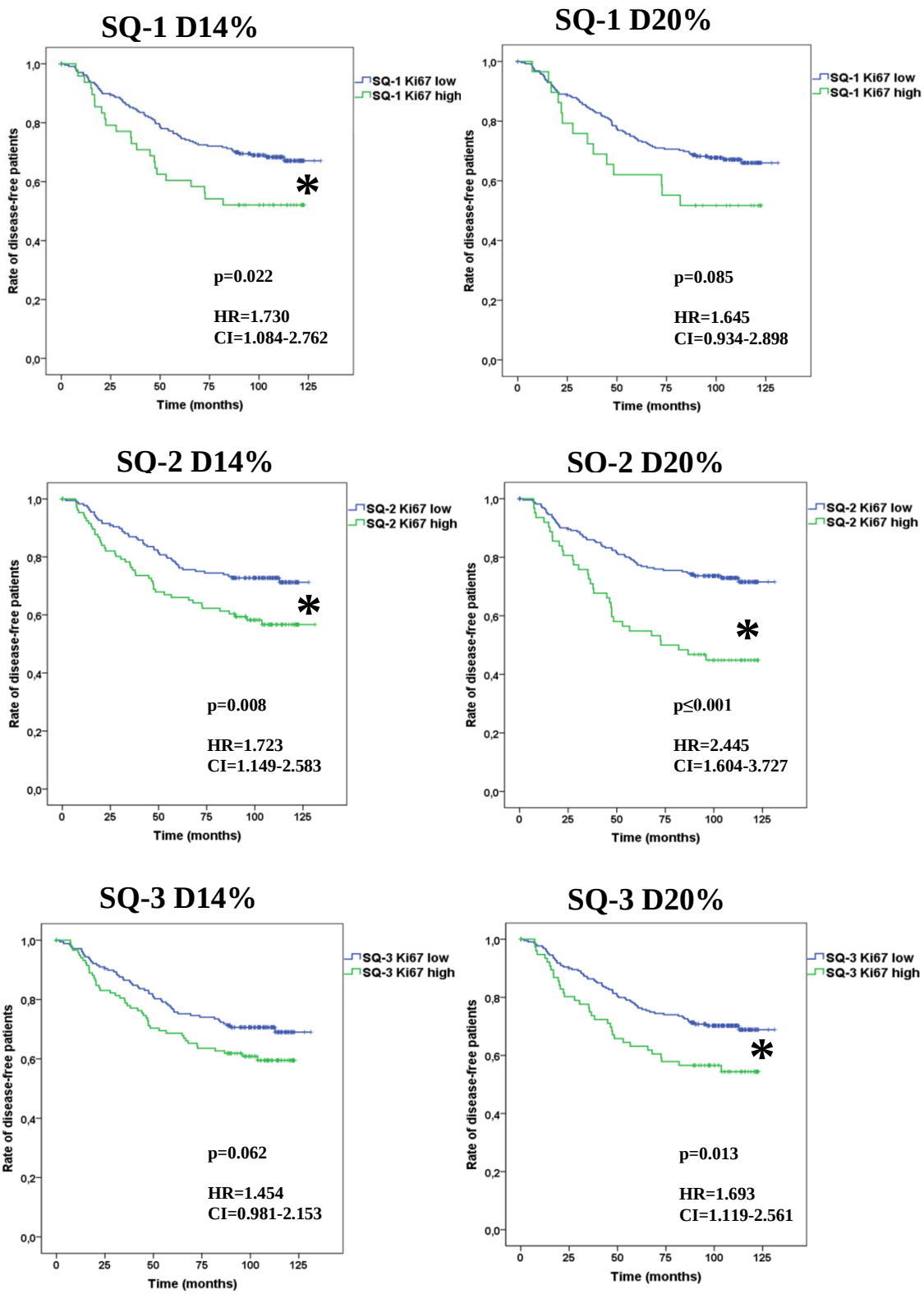

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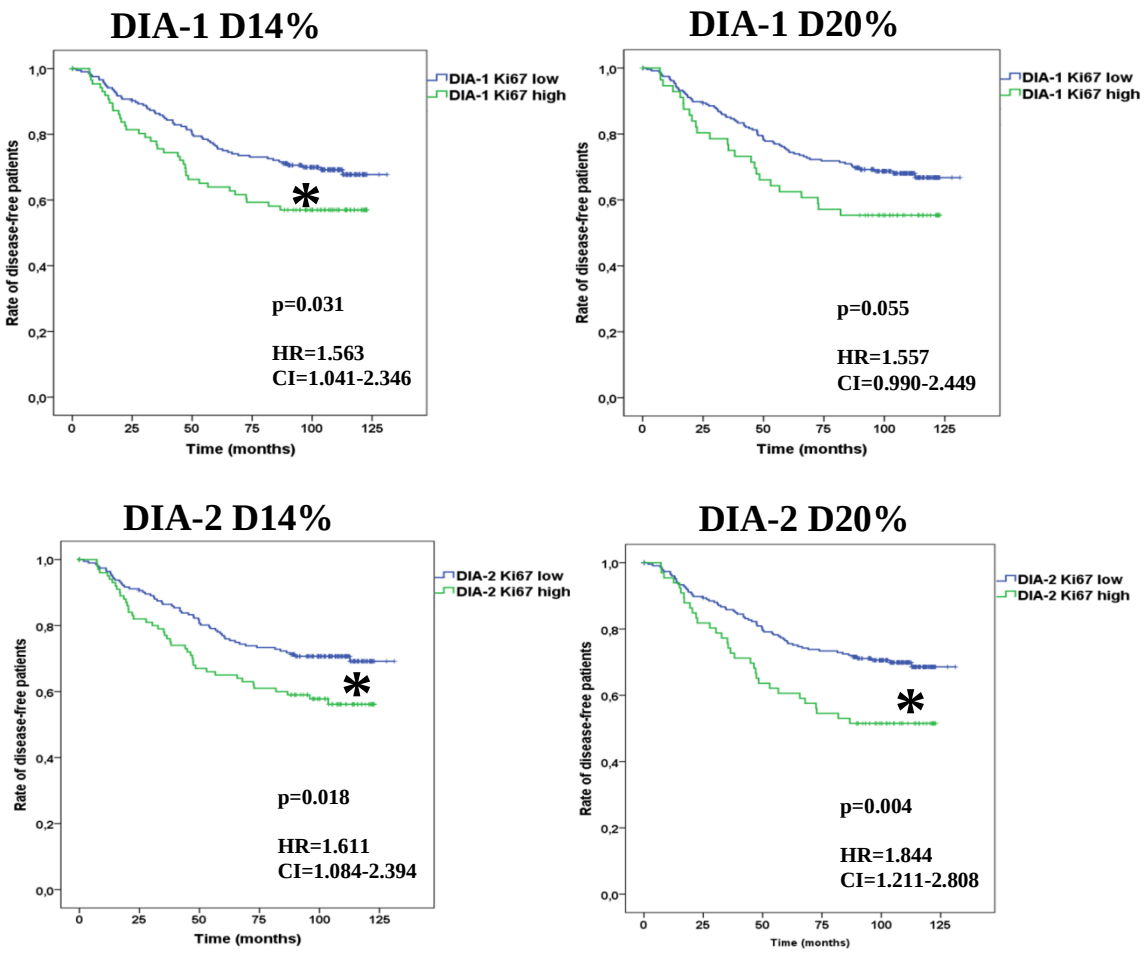

*Figure 9: Various KIPI evaluations and disease free survival. *Significant. D14% = dichotomized at 14% threshold. D20% = dichotomized at 20% threshold.*

SQ-3 (p= 0.013) could sort patients into good and unfavorable prognostic groups, while SQ-1 (p= 0.085) and DIA-1 (p= 0.055) did not (Figure 9).

Ki67 LI assessments were also tested as potential independent predictors of DFS adjusted by age, IHC subtypes, lymph node and T status, histological grade, mitotic index, vascular invasion as well as necrosis. At 14% cut-off, no Ki67 LI evaluation but only lymph node status (p= 0.001) showed independent association with DFS. However, at 20% threshold, both lymph node status and SQ-2 were significantly linked to DFS (p= 0.012, table 11).

Table 11: Multivariate Cox regression analysis of Ki67 LI assessments and pathological factors.

Prognostic Factors	**Multivariate Cox regression analysis involving Ki67 LI assessments and clinicopathological factors**		
	HR	**95% CI**	**p-value**
Age	0.915	0.596-1.406	0.685
Tumor size	1.245	0.796-1.945	0.337
IHC Subtype	1.078	0.910-1.277	0.385
Histological grade	1.064	0.699-1.620	0.771
Lymph node status (TNM 7)	1.435	1.133-1.817	0.001
Mitotic index	1.154	0.782-1.701	0.471
Vascular invasion	1.016	0.534-1.934	0.961
Necrosis	1.237	0.688-2.227	0.477
SQ-1 D14%	1.481	0.773-2.838	0.237
SQ-2 D14%	1.296	0.713-2.355	0.395
SQ-3 D14%	1.494	0.785-2.579	0.429
DIA-1 D14%	1.265	0.709-2.257	0.426
DIA-2 D14%	1.489	0.839-2.642	0.174
SQ-1 D20%	1.149	0.588-2.057	0.579
SQ-2 D20%	2.287	1.199-4.364	0.012
SQ-3 D20%	1.048	0.570-1.924	0.881
DIA-1 D20%	1.334	0.673-1.918	0.346
DIA-2 D20%	1.460	0.797-2.674	0.221
D14% = dichotomized at 14% threshold D20% = dichotomized at 20% threshold			

All Ki67 LI evaluations but SQ-1 could significantly distinguish good and unfavorable prognosis at 20% threshold in patients who underwent surgery only (SQ-1 p= 0.085, SQ-2 p<0.001, SQ-3 p= 0.020, DIA-1 p= 0.034, DIA-2 p= 0.010). In the group of patients treated with surgery+chemotherapy, statistically significant prognostic results were seen only with SQ-2 evaluation (p= 0.049, Table 12). Multivariate analyses of Ki67 LI assessments within treatment subgroups were not performed due to the low number of cases compared to relatively numerous clinicopathological factors.

Table 12: Univariate Cox regression analysis of Ki67 LI assessments and pathological factors in the different treatment groups. Only significant factors shown.

Treatment groups	Prognostic factor	Sub groups	HR	95% CI	p-value
Surgical treatment only (n=111)	IHC Subtype	TNBC	6.642	1.934-22.815	0.003
	Lymph node status (TNM 7)	1	2.652	1.072-6.562	0.035
	Mitotic index	>19	3.584	1.076-11.935	0.038
	SQ-1 D14%	-	4.975	1.619-15.287	0.005
	SQ-2 D20%	-	6.836	2.194-21.303	≤0.001
	SQ-3 D20%	-	3.514	1.217-10.144	0.020
	DIA-1 D20%	-	3.364	1.098-10.307	0.034
	DIA-2 D20%	-	4.181	1.402-12.467	0.010
Surgery+irradiation (n=83)	Tumor size	2-5cm	2.725	1.046-7.102	0.040
Surgery+chemotherapy (n=30)	IHC Subtype	Luminal-B like	6.529	1.147-37.165	0.034
	SQ-2 D14%	-	3.018	1.120-9.568	0.049
Surgery+chemotherapy + irradiation (n=117)	IHC Subtype	HER2	2.923	1.333-6.406	0.007
D14% = dichotomized at 14% threshold D20% = dichotomized at 20% threshold					

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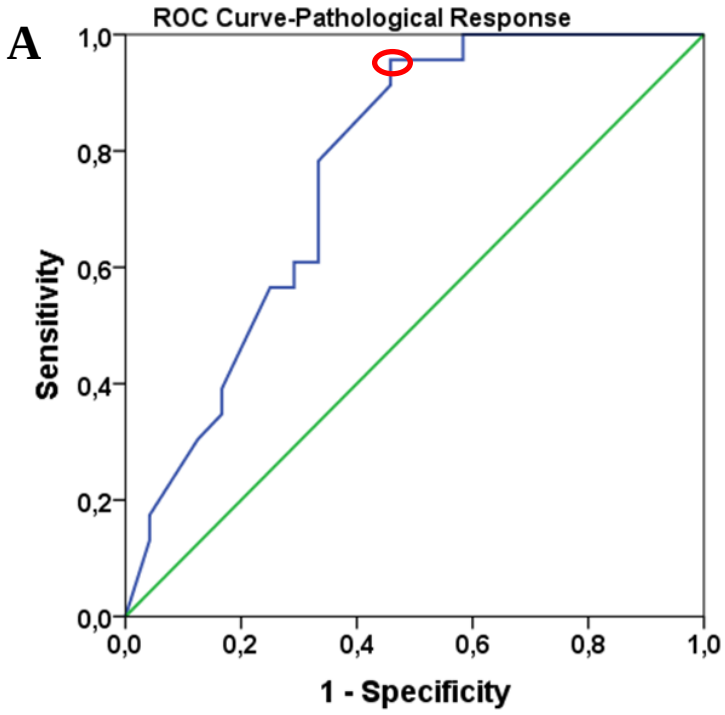
4.3. The role of Ki67 in neoadjuvant setting

4.3.1. Defining cut-off points for Ki67 LI in the pCR and pNR groups

ROC curve analysis was used to identify the optimal cut-off value of Ki67 LI that could best predict response to NAC (Figure 10 A). The optimal Ki67 cut-off value was 20% for distinguishing pCR from pNR patient cases (n= 47, AUC 0.767, sensitivity: 95.7%, specificity: 54.3%, p= 0.002). (Figure 10 A).

4.3.2. Defining cut-off points for Ki67 LI based on survival (DMFS and OS)

We also investigated the optimal threshold values for Ki67 LI regarding DMFS and OS. Based on DMFS, we were not able to detect a statistically significant cut-off value for Ki67 LI. The most relevant cut-off value was 20 % (n= 120, AUC 0.591, sensitivity: 82.2%, specificity 35.7, p = 0.208) (Figure 10 B). Based on OS data, the optimal cut-off point occurred at 15% for Ki67 LI (n= 120, AUC 0.708, sensitivity: 92.3%, specificity 29.6, p = 0.006) (Figure 10 C).



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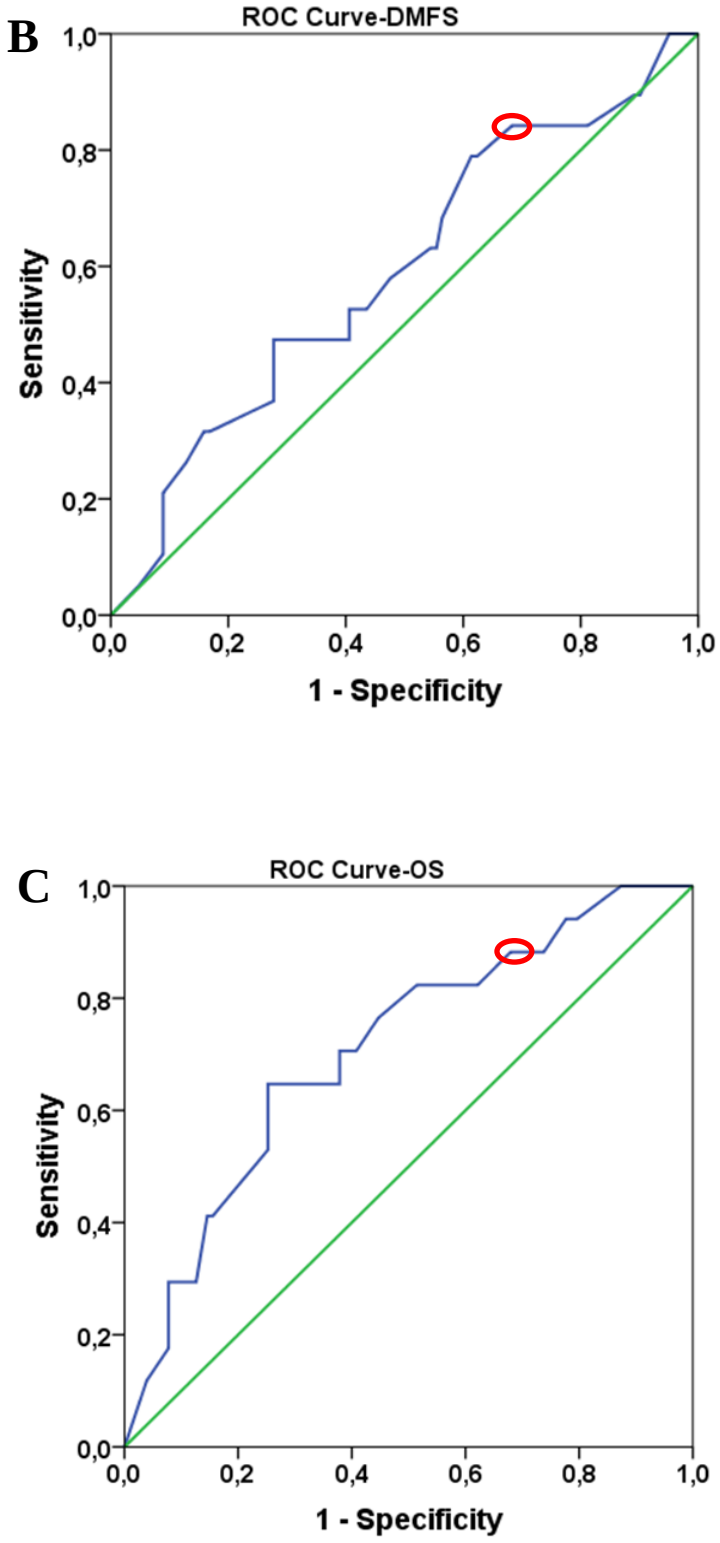

Figure 10: ROC curves to define optimal Ki-67 cut-off values for pathological response (A), DMFS (B), OS (C). Green line represents the diagonal reference line. Blue line corresponds to ROC curve. Red circles show the optimal cut-off values based on the ROC curves.

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4.3.3. Association between Ki67 LI, subtype and pathological response

Pathological response and Ki67 LI at investigated thresholds represented a significant association (Ki67 15% p= 0.001, Ki67 20% p= 0.010, Ki67 30% p= 0.018). The proportion of Ki67 low cases among non-responders was significantly higher compared to pPR and pCR cases (Table 13 A). The distribution of subtypes showed a significant difference in pathological response groups (p< 0.001). Most of the TNBC cases were represented in pCR group, while luminal A cases mainly occurred in pPR and pNR groups (Table 13 B). The Ki67 expression at any investigated cut-off points and subtypes also represented a significant correlation (p<0.001 for all comparisons). Luminal A subtype showed low Ki67, while TNBC and HER2+ cases mostly had high Ki67 (Table 13 C).

Table 13: Contingency tables of Ki-67 LI, subtype and pathological response.

A

Number of Cases	**Pathological Response**			
	pCR	**pPR**	**pNR**	**Total**
Ki-67 low (<15%)	0	19	10	**29**
Ki-67 high (≥15%)	23	54	14	**91**
Total	**23**	**73**	**24**	**120**
Number of Cases	**Pathological Response**			
	pCR	**pPR**	**pNR**	**Total**
Ki-67 low (<20%)	1	24	10	**35**
Ki-67 high (≥20%)	22	49	14	**85**
Total	**23**	**73**	**24**	**120**
Number of Cases	**Pathological Response**			
	pCR	**pPR**	**pNR**	**Total**
Ki-67 low (<30%)	6	31	16	**53**
Ki-67 high (≥30%)	17	42	8	**67**
Total	**23**	**73**	**24**	**120**

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B

Number of Cases	Pathological Response			
	pCR	**pPR**	**pNR**	**Total**
Luminal-A	0	9	6	**15**
Luminal-B	5	46	14	**65**
Her2	8	4	2	**14**
TNBC	10	14	2	**26**
Total	**23**	**73**	**24**	**120**

C

Number of Cases	Subtype				
	Luminal-A	**Luminal-B**	**Her2**	**TNBC**	**Total**
Ki-67 low (<15%)	15	11	1	2	**29**
Ki-67 high (≥15%)	0	54	13	24	**91**
Total	**15**	**65**	**14**	**26**	**120**
Number of Cases	**Subtype**				
	Luminal-A	**Luminal-B**	**Her2**	**TNBC**	**Total**
Ki-67 low (<20%)	15	16	2	2	**35**
Ki-67 high (≥20%)	0	49	12	24	**85**
Total	**15**	**65**	**14**	**26**	**120**
Number of Cases	**Subtype**				
	Luminal-A	**Luminal-B**	**Her2**	**TNBC**	**Total**
Ki-67 low (<30%)	15	28	4	6	**53**
Ki-67 high (≥30%)	0	37	10	20	**67**
Total	**15**	**65**	**14**	**26**	**120**

The association between Ki67 LI, subtype and pathological response was also investigated without luminal A cases, because NAC is not generally recommended in this subtype due to the high rate of pNR in contrast with the favorable prognosis. Excluding luminal A cases, Ki67 LI at any thresholds and pathological response did not show any significant association (Ki67 15% p= 0.068, Ki67 20% p= 0.122, Ki67 30% p= 0.140) (Table 14 A). Furthermore, Ki67 LI at any investigated cut-off points also did not represent any significant linkage with subtypes (Ki67 15% p= 0.410, Ki67 20% p= 0.158, Ki67 30% p= 0.173) (Table 14C). In contrast to this, subtypes were significantly linked to the pathological response groups (p<0.001). The clear majority of luminal B cases were in pPR and pNR groups, while TNBC cases mostly occurred in pCR subgroup (Table 14 B).

Table 14: Contingency tables of Ki-67 LI, subtype and pathological response without Luminal-A cases.

A

	Pathological Response			
	pCR	**pPR**	**pNR**	**Total**
Ki-67 low (<15%)	0	10	4	**14**
Ki-67 high (≥15%)	23	54	14	**91**
Total	**23**	**64**	**18**	**105**
Number of Cases	**Pathological Response**			
	pCR	**pPR**	**pNR**	**Total**
Ki-67 low (<20%)	1	15	4	**20**
Ki-67 high (≥20%)	22	49	14	**85**
Total	**23**	**64**	**18**	**105**
Number of Cases	**Pathological Response**			
	pCR	**pPR**	**pNR**	**Total**
Ki-67 low (<30%)	6	22	10	**38**
Ki-67 high (≥30%)	17	42	8	**67**
Total	**23**	**64**	**18**	**105**

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B

Number of Cases	Pathological Response			
	pCR	**pPR**	**pNR**	**Total**
Luminal-B	5	46	14	**65**
Her2	8	4	2	**14**
TNBC	10	14	2	**26**
Total	**23**	**64**	**18**	**105**

C

Number of Cases	Subtype			
	Luminal-B	**Her2**	**TNBC**	**Total**
Ki-67 low (<15%)	11	1	2	**14**
Ki-67 high (≥15%)	54	13	24	**91**
Total	**65**	**14**	**26**	**105**
Number of Cases	**Subtype**			
	Luminal-B	**Her2**	**TNBC**	**Total**
Ki-67 low (<20%)	16	2	2	**20**
Ki-67 high (≥20%)	49	12	24	**85**
Total	**65**	**14**	**26**	**105**
Number of Cases	**Subtype**			
	Luminal-B	**Her2**	**TNBC**	**Total**
Ki-67 low (<30%)	38	4	6	**38**
Ki-67 high (≥30%)	27	10	20	**67**
Total	**65**	**14**	**36**	**105**

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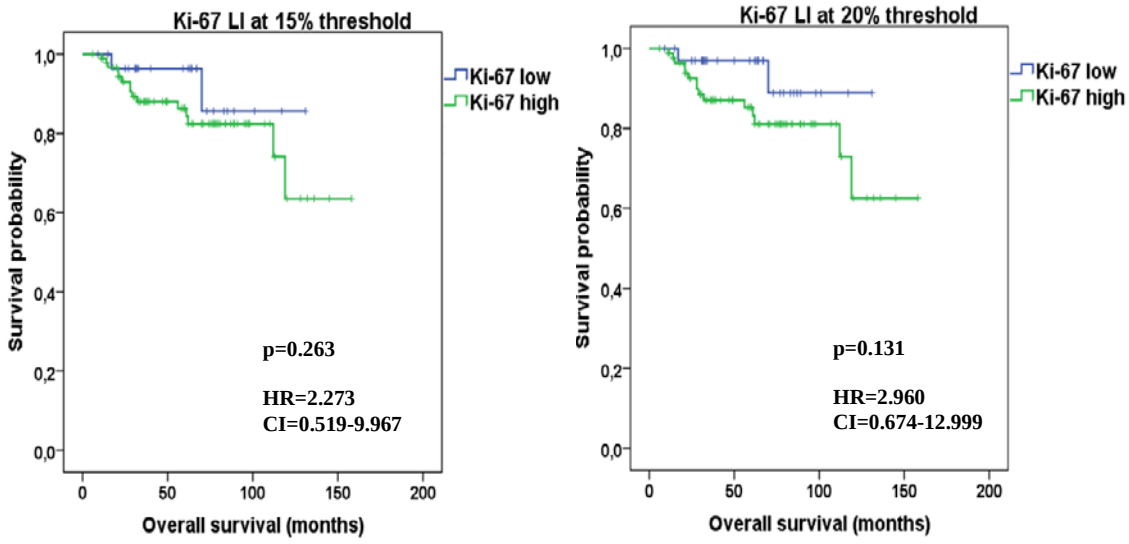
4.3.4. Prognostic potential of Ki67 LI, subtype and pathological response

Neither Ki67 LI at any thresholds nor subtype and not even pathological response were suitable to distinguish patient cohorts with different DMFS (Ki67 15% p= 0.391, Ki67 20% p= 0.185, Ki67 30% p= 0.566, subtype p= 0.771, pathological response p= 0.280). Regarding OS, Ki67 at 15% (p= 0.263) and at 20% threshold failed (p= 0.131), but Ki67 at 30% cut-off value (p= 0.040) furthermore subtype (p= 0.037) as well as pathological response (p=0.044) were suitable to separate patients into good and unfavorable prognosis cohorts (Figure 11). When luminal A cases were excluded, neither Ki67 LI at any cut-off points nor subtype not even pathological response were suitable to perform statistically significant splitting of our cohort into 2 patients' group with different DMFS (Ki67 15% p= 0.426, Ki67 20% p= 0.179, Ki67 30% p= 0.642, subtype p= 0.488, pathological response p= 0.222,) or with different OS (Ki67 15% p= 0.975, Ki67 20% p= 0.518, Ki67 30% p= 0.158, subtype p= 0.072, pathological response p= 0.058).



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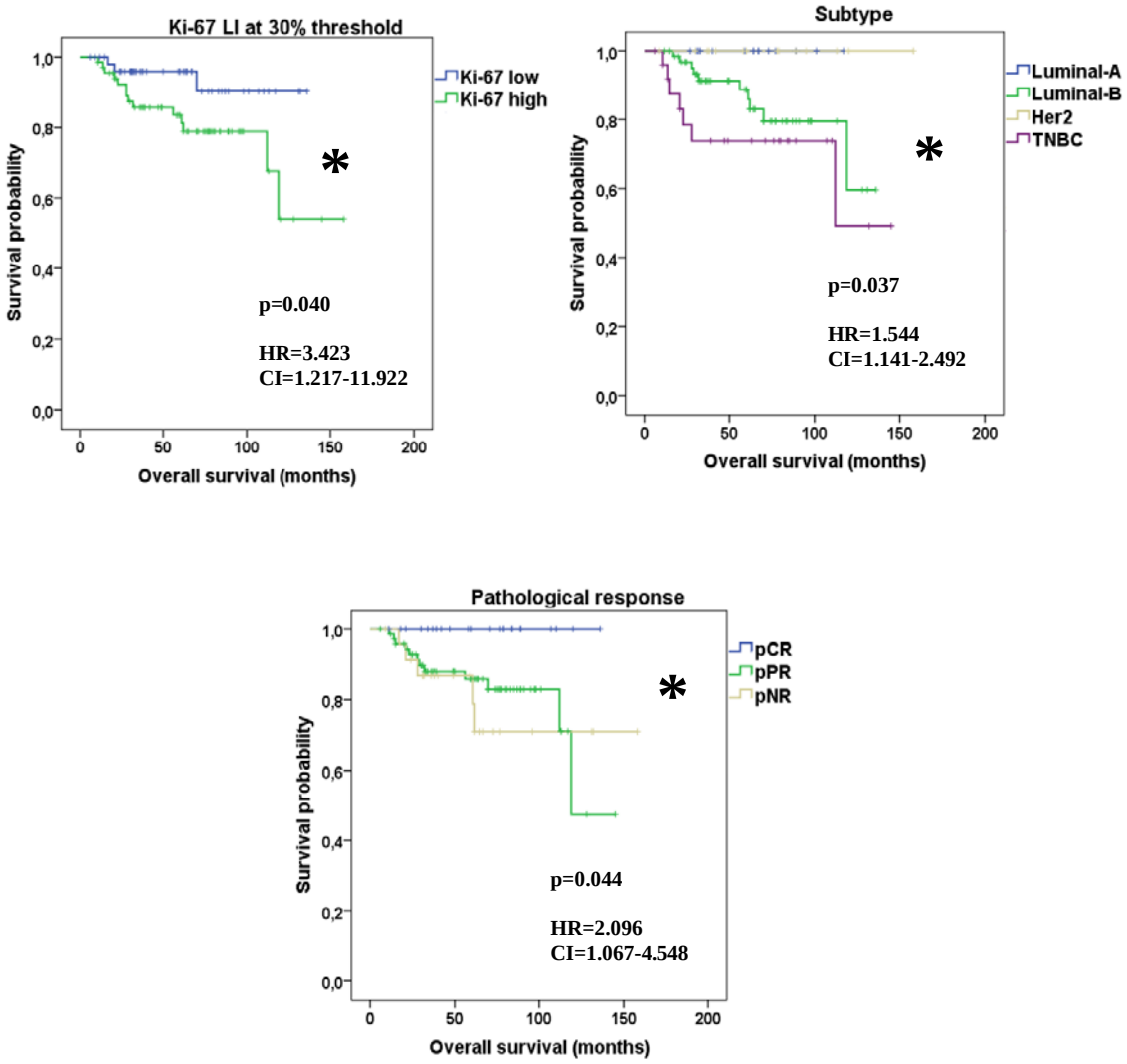

*Figure 11: Kaplan Meier plots of Ki-67, subtype and pathological response. *Significant.*

We also investigated the utility of Ki67 LI at 15%, 20% and 30% thresholds as potential independent predictor of DMFS and OS adjusted by age, pathological response, hormone receptor status, subtypes, histological grade, lymph node, cT and pT status. Neither Ki67 at any thresholds nor any other clinicopathological factors except pT status (p=0.029) showed an independent association with DMFS (Table 15). However, Ki67 LI at 30% threshold (p=0.029) and subtype (p=0.008) were independently linked to OS (Table 15). Without luminal A cases, Ki67 LI at 30% cut-off point (p=0.038) and subtype (p=0.009) represented also an independent association with OS (Table 15).

Table 15: Multivariate Cox regression analysis of the Ki-67 and the clinicopathological factors regarding distant metastasis-free survival and overall survival. Only significant factors shown.

Multivariate Cox regression	Prognostic factor	HR	95% CI	p-value
Distant metastasis-free survival (n=120)	**pT**	2.397	1.094-5.252	0.029
Overall survival (n=120)	**IHC Subtype**	2.230	1.231-4.043	0.008
	Ki-67 LI D30%	5.286	1.189-23.488	0.029
Overall survival without luminal A cases (n=105)	**IHC Subtype**	2.135	1.202-3.785	0.009
	Ki-67 LI D30%	4.850	1.089-18.379	0.038

4.3.5. Ki67 LI in the partial responder group (pPR)

The prognostic potential of Ki67 LI was also investigated in pPR subgroup that represents a heterogeneous group with a response rate to NAC between 10-90%. Attempting to find the most relevant threshold for Ki67 LI, we could conclude that, the best cut-off value in pPR group based on DMFS was 20% (n= 73, AUC 0.683, sensitivity: 82.4%, specificity 41.5%, p = 0.055, Figure 12 A), and 30% based on OS (n= 73, AUC 0.808, sensitivity: 92.2%, specificity 52.6, p = 0.001, Figure 12 B). No significant association was found between Ki67 LI and pPR subgroups (pPRi, pPRii, pPRiii; p=0.653)

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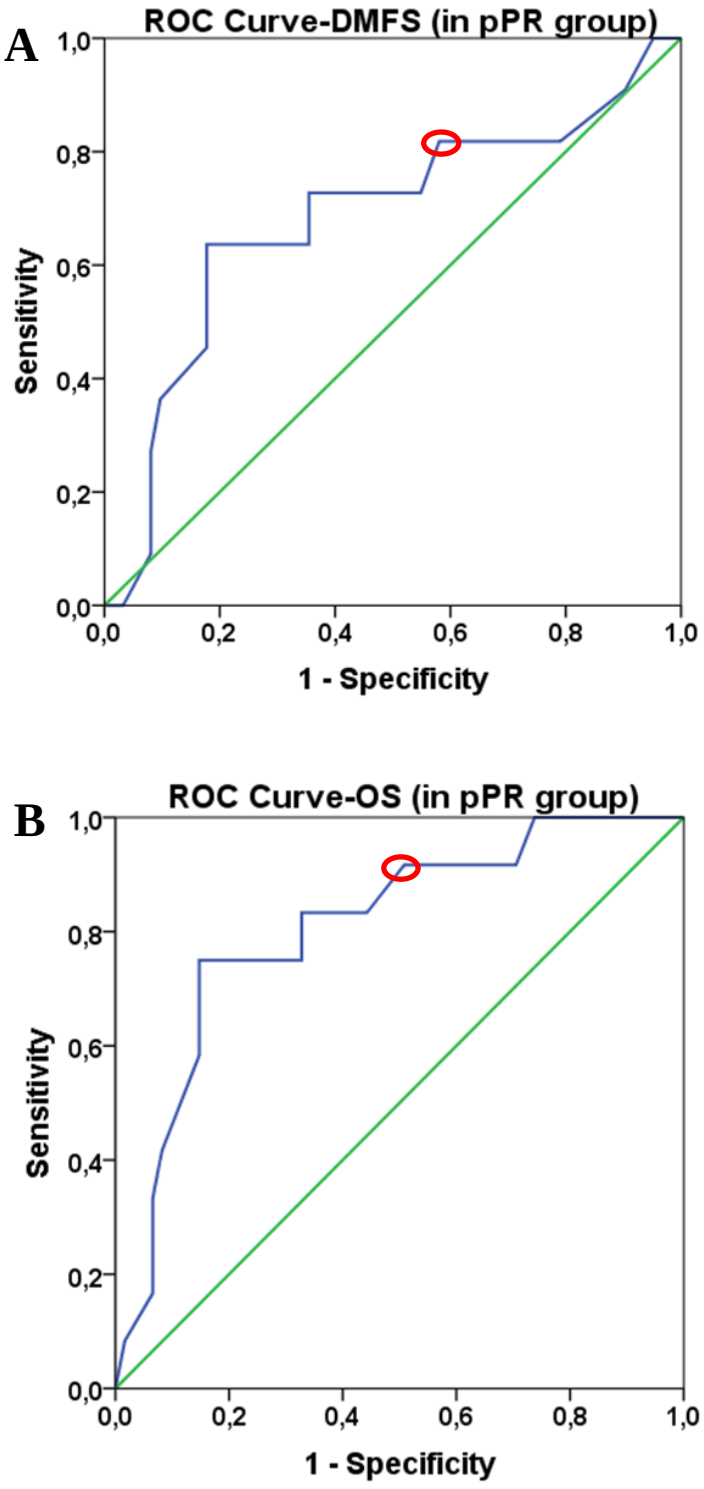

Figure 12: ROC curves to define optimal Ki-67 cut-off values for DMFS (A), OS (B) in pPR group. Green line represents the diagonal reference line. Blue line corresponds to ROC curve. Red circles show the optimal cut-off values based on the ROC curves.

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For prognosis prediction, neither Ki67 LI at any cut-off value (Ki67 20% p= 0.233, Ki67 30% p=0.336), nor subtype (p=0.218) not even pPR subgroups (p=0.669) were able to distinguish patient cohorts with different DMFS. Regarding OS, pPR subgroups (p=0.590) and Ki67 at 20% threshold failed (p=0.095), but Ki67 at 30% cut-off point (p=0.037) and subtype (0.015) were suitable to separate patients into good and unfavorable prognosis cohorts (Figure 13).

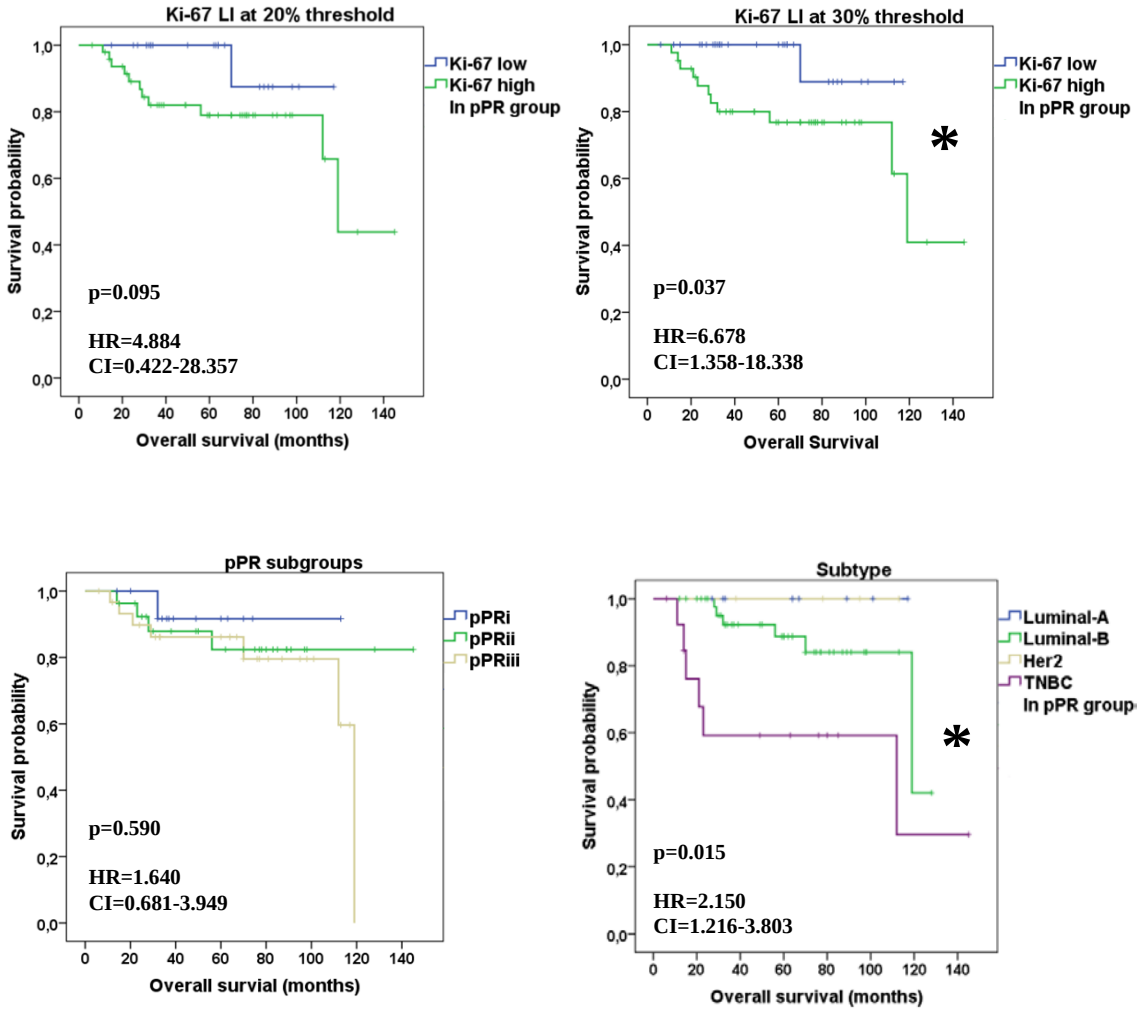

*Figure 13: Kaplan Meier plots of Ki-67, subtype and pathological response in pPR group. *Significant.*

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5. DISCUSSION

The ongoing debate and open questions regarding Ki67 immunohistochemistry in breast cancer pathology prompted us to perform a study with the aim to clarify whether various commercially available Ki67 antibodies perform similarly as well as to investigate whether with the generally suggested 20-30% positivity thresholds, these Ki67 antibodies could be meaningful with respect to prognosis as measured by duration of DFS.

Comparison of various Ki67 antibodies was performed earlier and differences in positivity rates were detected by different Ki67 antibodies [139,140,153]. In our study, we found that although MIB1, SP6, 30-9, poly, B56, and MIB1-IF represented a moderate concordance, statistically significant differences were noticed between the Ki67 LI scores of these antibodies. Highest agreement was found between MIB1 and poly, MIB1 and B56, poly and 30-9 as well as between 30-9 and SP6, while poor agreement was detected between SP6 and B56, 30-9 and B56 as well as between SP6 and MIB1-IF (Figure 14). Besides these findings, the variability of differences between the Ki67 LI scores of the antibodies represented an increasing trend, proportional to the magnitude of Ki67 LI measurements. Furthermore, a systematic error emerged in the variability of differences between the Ki67 LI scores of the antibodies except between MIB1 and poly. Although, the same microscopic fields were evaluated, the limits of agreement were wide between the antibodies compared to the acceptable range in pathological practice, resulting considerable differences in Ki67 LI values.

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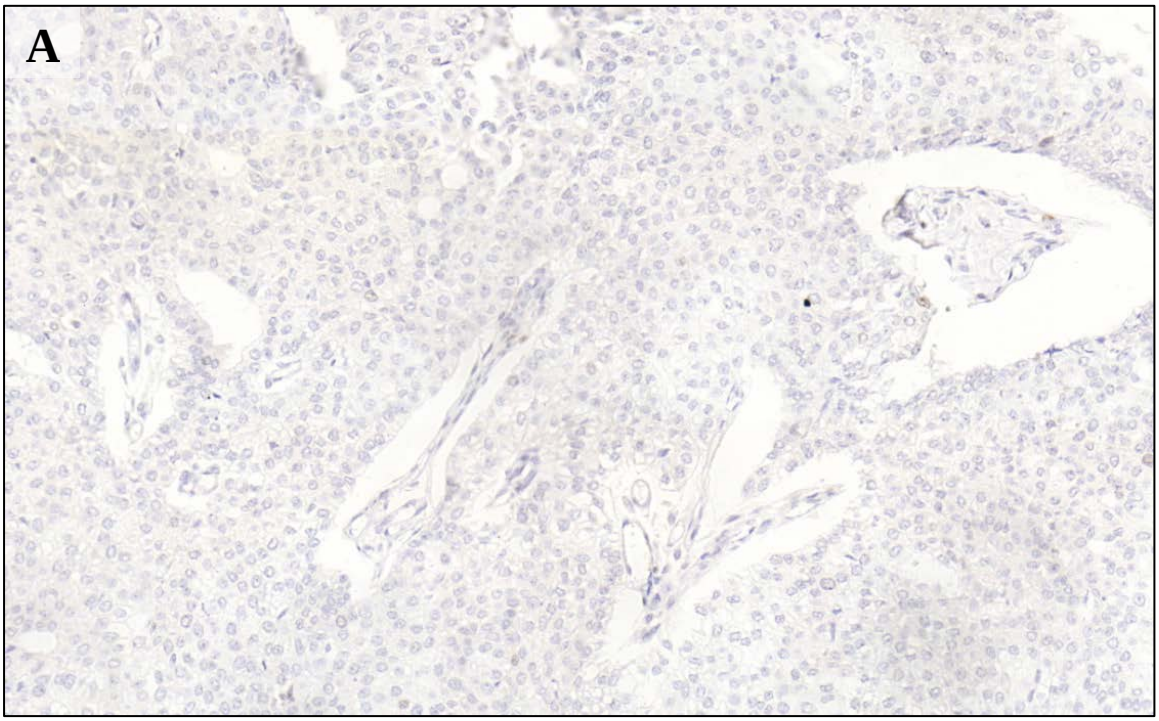

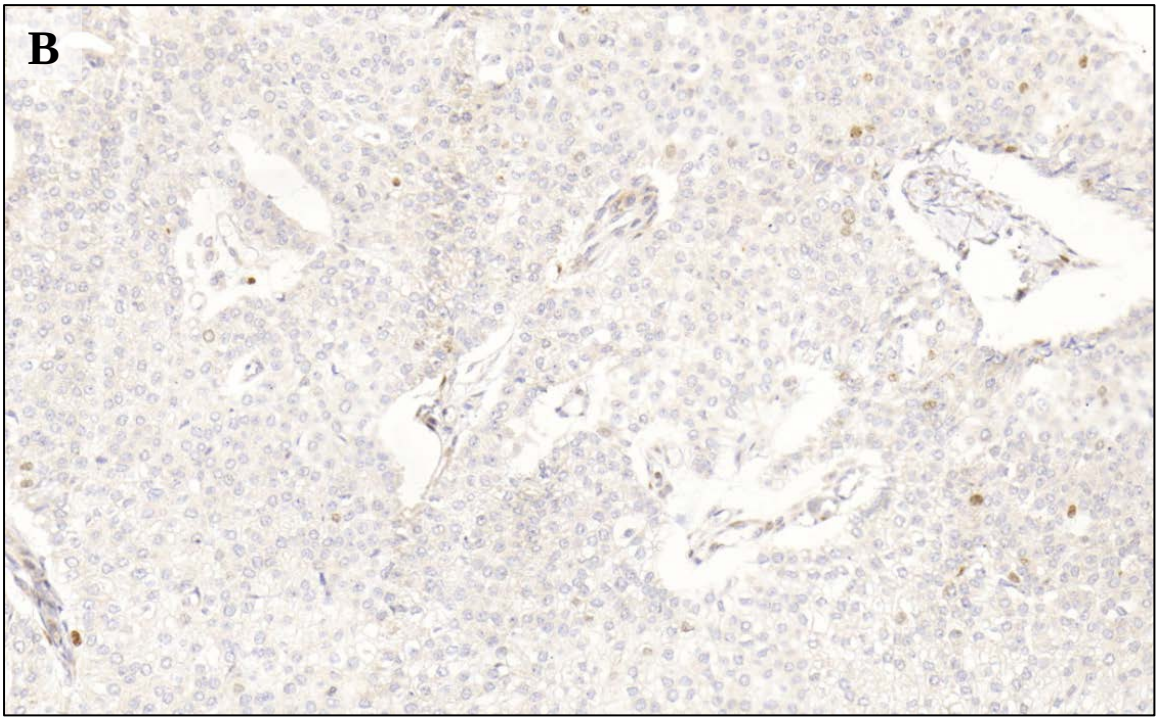

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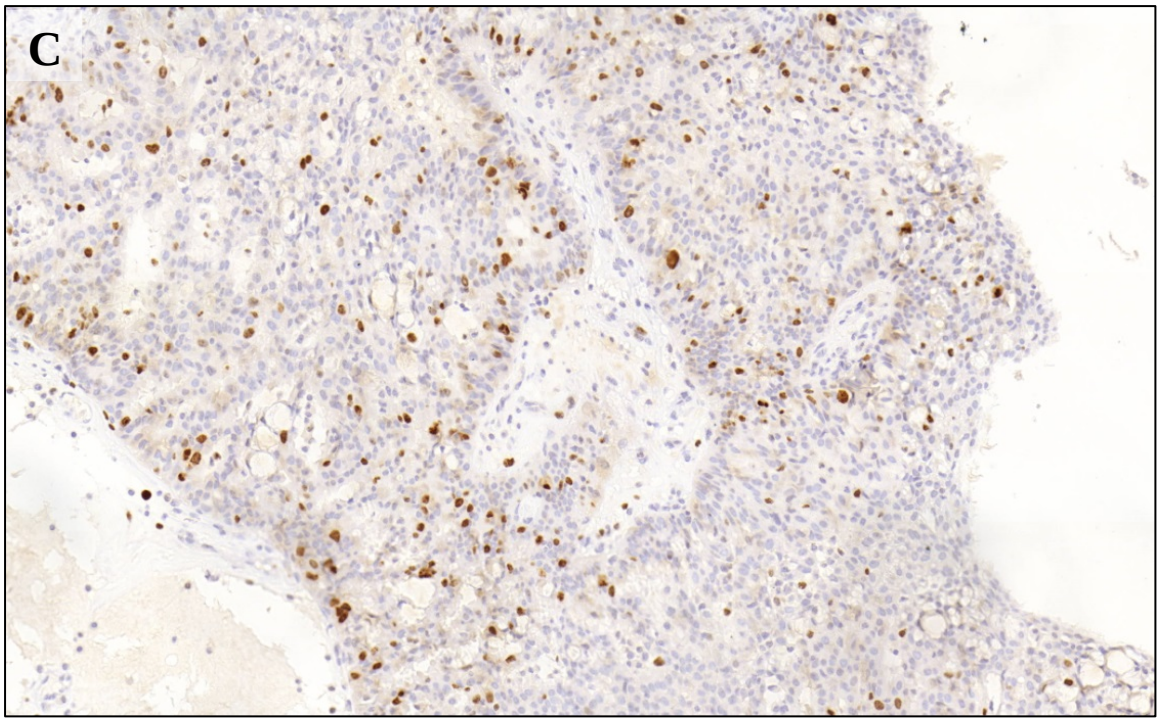

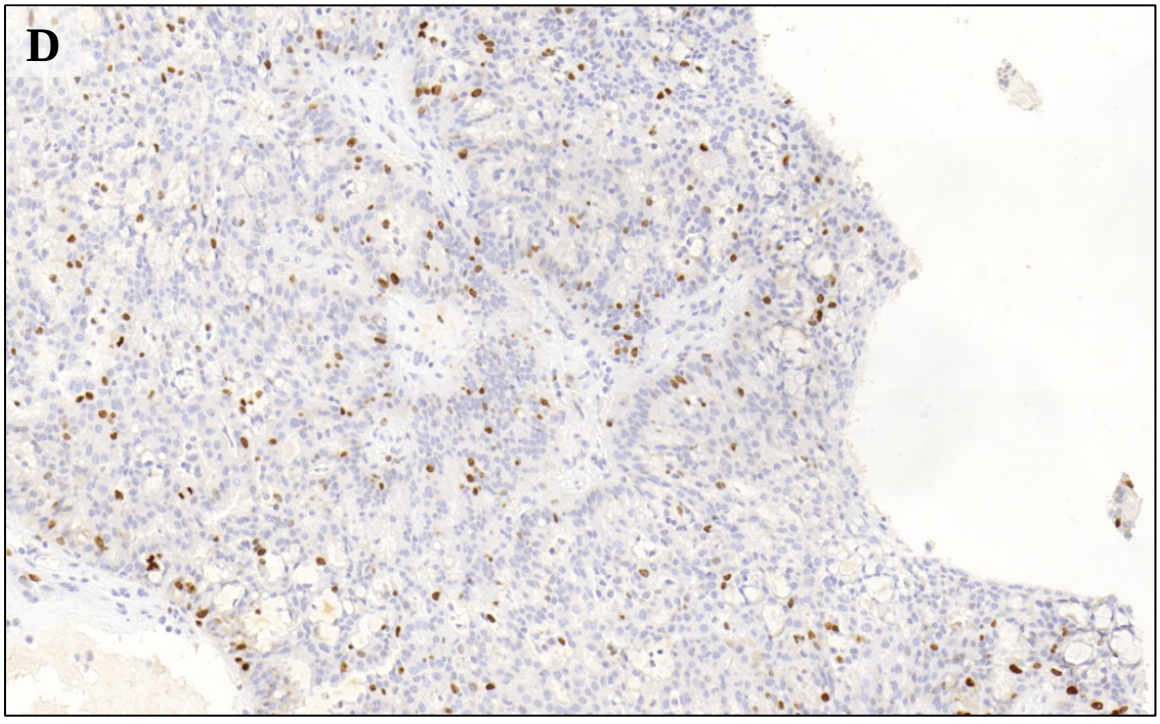

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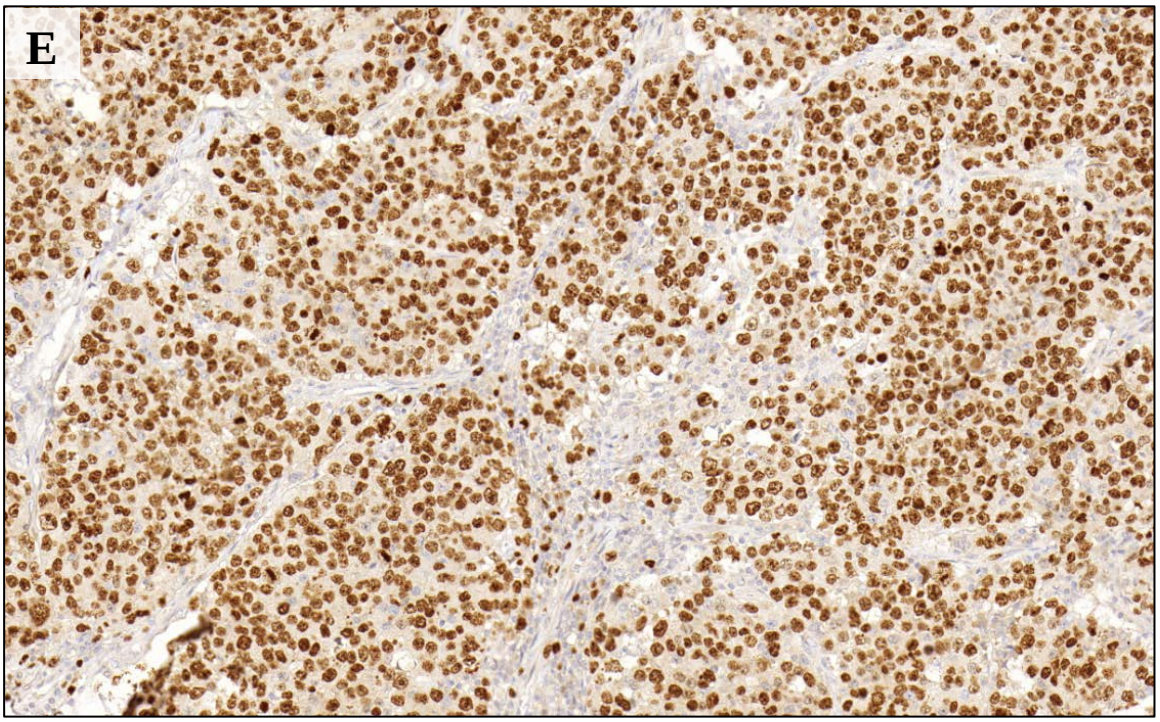

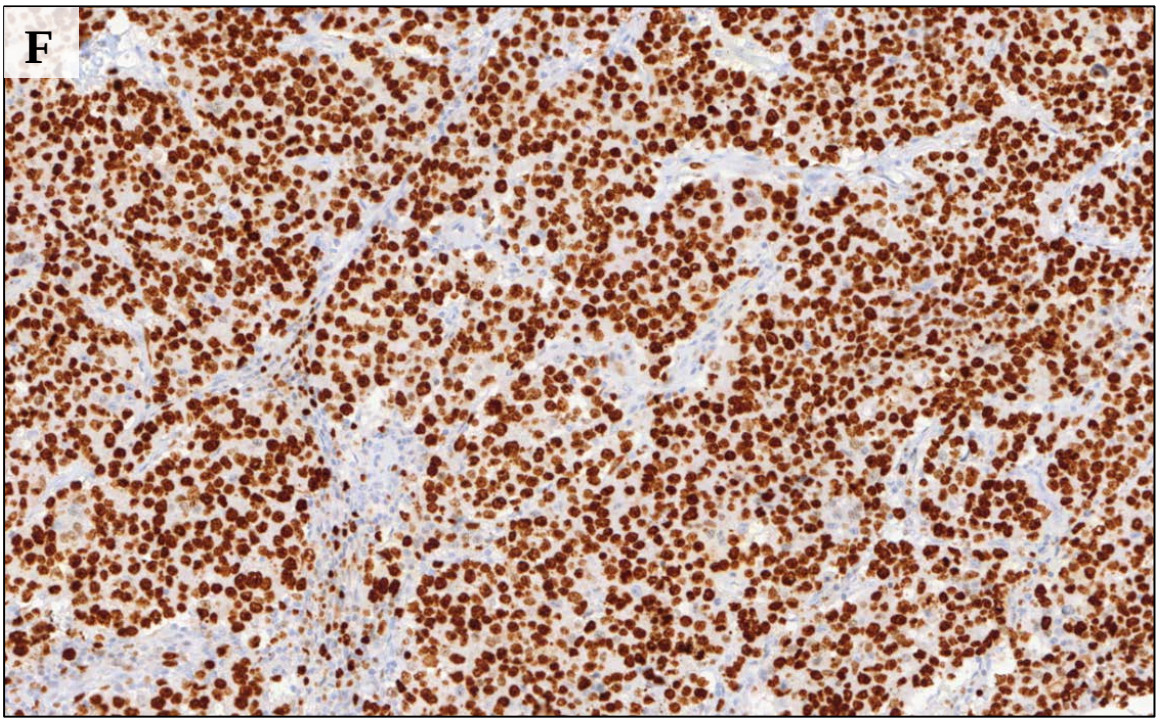

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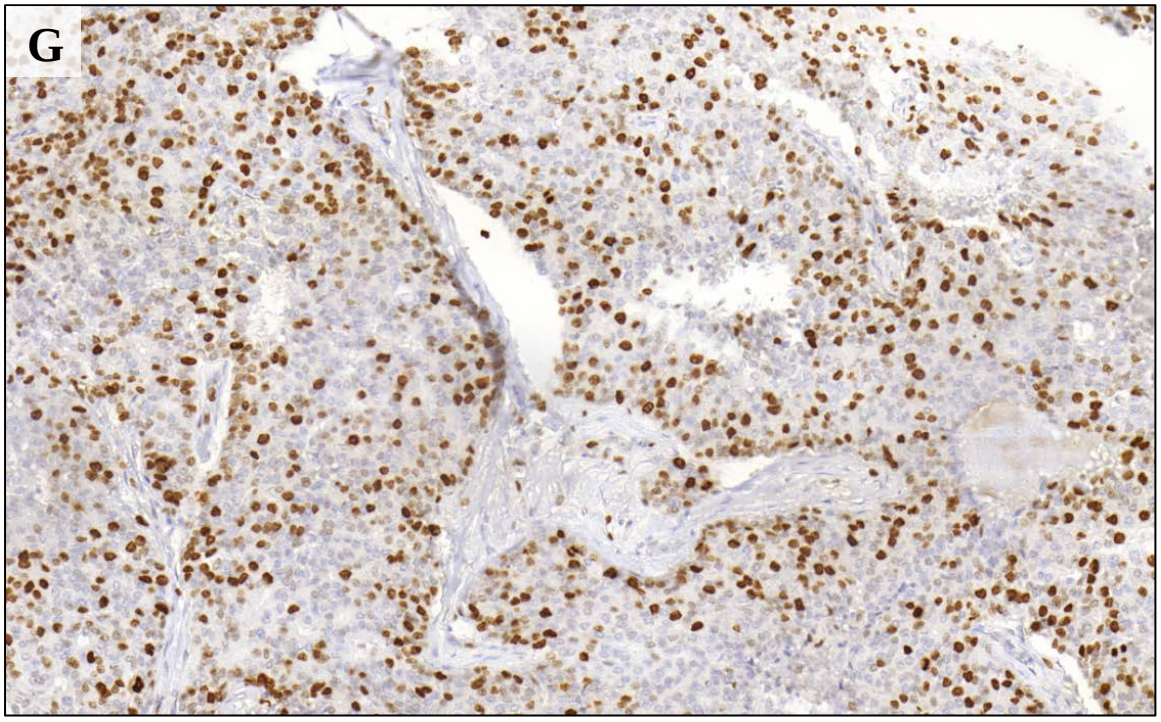

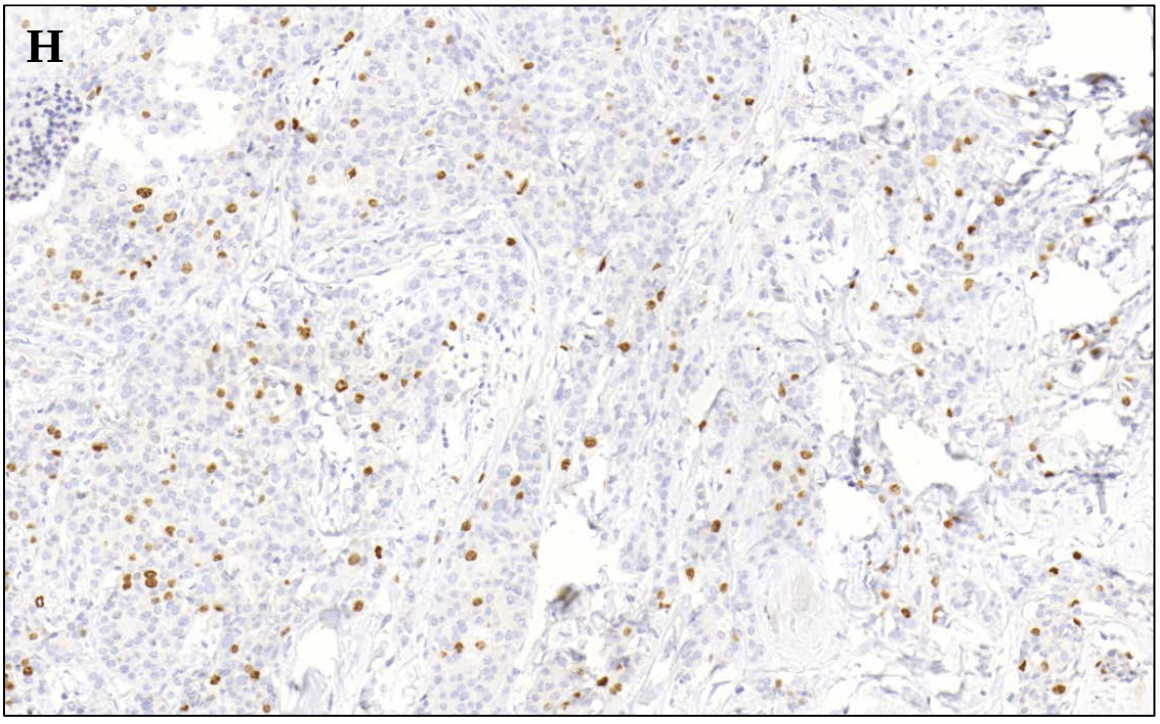

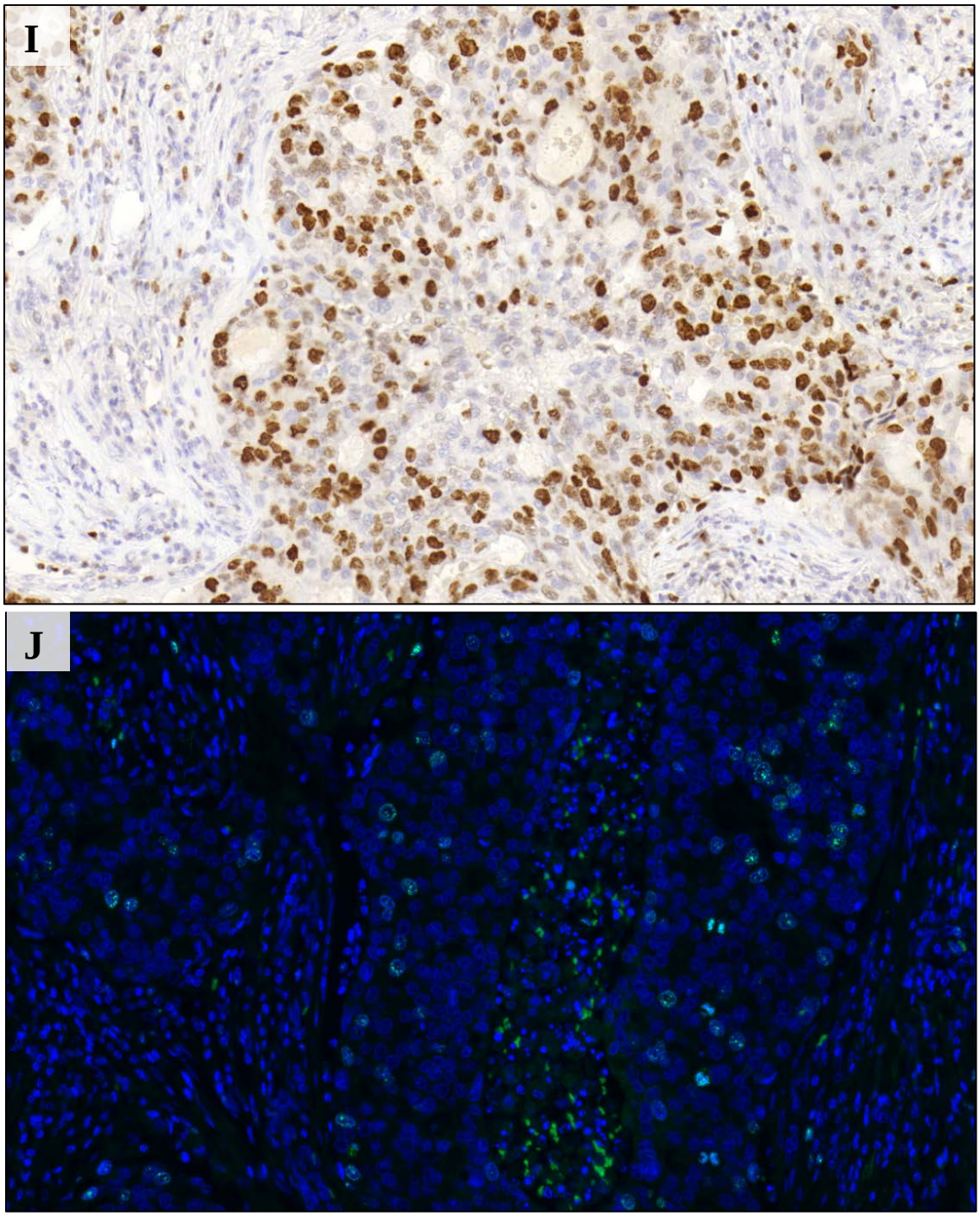

Figure 14: Immunohistochemical and immunofluorescent reactions of the five Ki67 antibodies. Highest agreement was found between MIB1 (A, magnification 15x, Ki67 LI: 0%) and poly (B, magnification 15x, Ki67 LI: 1%), MIB1(C, magnification 15x, Ki67 LI: 10%) and B56 (D, magnification 15x, Ki67 LI: 10%), poly (E, magnification 15x, Ki67 LI: 90%) and 30-9 (F, magnification 15x, Ki67 LI: 90%). Lowest agreement was represented between SP6 (G, magnification 15x, Ki67 LI: 40%) and B56 (H, magnification 15x, Ki67 LI: 5%), SP6 (I, magnification 20x, Ki67 LI: 50%) and MIB1-IF (J, magnification 20x, Ki67 LI: 5%). Pictures on the same page show Ki67 reactions from the same case.

Ki67 immunohistochemistry has been widely used in oncology decision-making even though the International Ki67 in Breast Cancer Working Group of the Breast International Group and North American Breast Cancer Group (BIG-NABCG) had been warning against its use in clinical practice [142,154,155]. The reason why this group of experts insists to prevent oncologists to use Ki67 IHC results in therapy decision making is manifold, but first and foremost the problems with its analytical validity have been emphasized. In their latest paper one of the take home messages is the following: „… we maintain that, unless and until preanalytical and analytical features for immunohistochemistry of Ki67 can be standardized, this assay platform should not be used to drive patient-care decisions in clinical practice" [154]. Our emphasis in this investigation was on an analytical issue: The selection of the Ki67 antibody. We feel that postanalytical issues (i.e. interpretation) didn't bias our results since we have used the same method (estimation or „eye-balling") with the same two observers for evaluating the Ki67 IHC slides, and in case of discordant scoring, scores were given following a consensus between the two evaluating pathologists. In our studies, it was agreed that all positivity pattern and intensity are to be considered.

The relevance of Ki67 as a prognostic factor was described earlier and its predictive power to chemotherapy response rate was discussed both in the adjuvant and the neoadjuvant settings [119,129,156]. In our study, all the Ki67 antibodies except MIB1-IF were suitable to subdivide our patient cohort into a better and a worse prognostic group at 20% cut-off. At 30% threshold, B56 and MIB1-IF failed, while MIB1, poly, SP6 and 30-9 could distinguish good and unfavorable outcome patients' cohorts. However, in multivariate analyses, only poly at 20% cut-off was significantly linked to DFS besides lymph node status, while at 30% threshold only lymph node status represented an independent association with survival.

In a larger study analyzing breast cancer samples and disease outcome in the GeparTrio trial with respect to Ki67 it was elegantly shown that defining one cut-point for Ki67 positivity is most probably not optimal and that this practice oversimplifies and does not reflect the heterogeneous biology of the disease [157]. Instead, low, intermediate and high Ki67 LI thresholds should be identified to achieve a better estimation regarding the expected therapy response. Another important finding in this study was that a single, universal Ki67 LI for the prediction of pathological complete response in the

neoadjuvant setting is not useful if we consider the different molecular (or surrogate) subtypes of breast cancer.

To exclude bias related to different treatment protocols, we have also investigated the prognosis prediction potential of the 5 antibodies in each treatment subgroup. By multivariate analyses, none of the 5 antibodies represented an independent association with DFS in the subgroup of patients who had irradiation only, and in the patient subgroup treated with the combination of irradiation and chemotherapy. By univariate analyses (due to the low number of cases and/or event rates), Ki67 LI scores of all the antibodies -except SP6 at 20% threshold and MIB1-IF at all cut-off scores- were suitable to distinguish good and unfavorable prognosis patients' cohorts in the patient subgroup with surgery only. However, in patient subgroup treated with chemotherapy, none of the Ki67 antibodies could perform statistically significant splitting of our cohort into 2 patient groups with distinct DFS.

To the best of our knowledge, similar study- where different 5+1 Ki67 antibodies were evaluated according to their capacity to predict DFS in operable breast cancer patients at the presently suggested 20-30% positivity ratio threshold- has not yet been performed.

The weakness of our retrospective study comparing 5+1 Ki67 antibodies is i.) the relatively low number of cases, for which reason we could not address the question of molecular/surrogate subtypes and the definition of optimal Ki67 LI thresholds for separating them, and ii-) only DFS data were available. Furthermore, iii) the clinical utility of Ki67 LI in breast cancer can be determined in whole slide analysis. However, the main purpose of this study was to compare the IHC expression of five different Ki67 antibodies in breast cancer in relation with DFS. Therefore, we did not exclude our HER2 positive and TNBC cases from this comparative study. We believe that the use of TMA to compare expression patterns of different Ki67 antibodies is appropriate.

We feel however, that the strengths override the weaknesses: We thoroughly evaluated five different Ki67 antibodies, including the most widely used MIB1 and the FDA approved Ventana 30-9 and we have correlated the results to disease prognosis (DFS). We could also evaluate the performance of each of the Ki67 antibodies in different treatment-stratified analyses. Our cases come from a single hospital, so fixation, tissue processing and other preanalytical issues influenced the immunohistochemical results uniformly.

Our results provide further evidence that the selection of the routinely used Ki67 antibody has great influence on the values of the Ki67 labeling index. Moreover, considerable differences occurred between the antibodies in detecting Ki67, even though the same microscopic fields were evaluated. According to our findings, only the immunofluorescent labeled MIB1 (at 20% and 30% thresholds) and B56 (at 30% threshold) failed to distinguish favorable and poor prognosis patients' cohorts (even if HER2 and TNBC cases were included). The widely used MIB1 LI was not proved to be an independent prognostic factor compared to that of poly antibody. However, MIB1, poly and 30-9 had the highest concordance among the five antibodies. Furthermore, none of the five antibodies had significant prognostic potential in patients treated with chemotherapy and/or irradiation.

The other reason besides preanalytical and analytical factors why the International Ki67 in Breast Cancer Working Group did not advise the application of Ki67 IHC results in therapy decision making is the high discrepancy between observers in Ki67 scorings resulting high interobserver variability [116,142]. Ring studies showed that moderate intraclass correlation (0.59-0.71) achieved between observers performing SQ evaluations, could be improved to 0.92, based on systematic training and following the guidelines [142,154]. We also performed a study with the aim to investigate the reproducibility between Ki67 evaluations. Although we found very good consistency between SQ evaluations, statistically significant difference and poor concordance also occurred between SQ-1 and SQ-2 as well as between SQ-1 and SQ-3. Besides this, the variability of differences between SQ-1 and SQ-2 as well as SQ-1 and SQ-3 represented a proportional error. The possible explanation for the discrepancy might be that SQ-1 has the least experience in daily diagnostic practice. This observation might emphasize the relevance of consecutive experience and training in breast pathology. In the 2013 Ki67 ring study of the Japan Breast Cancer Research Group intraclass correlation ranged from 0.57 to 0.66 when pathologists scored whole slides applying counting and visual estimate methods [158]. When they evaluated printed photographs of Ki67 stained slides to exclude variations by assessment of varied microscopic field, 0.82-0.94 of intraclass correlation was observed [158]. This study has claimed that the standardization of assessment area might be the essential point to evaluate Ki67 with high reproducibility [158]. We performed Ki67 evaluation on TMA slides to avoid

variation in scorings by different microscopic fields. Concerning the relative difference between cases, a very good intraclass correlation was observed between our pathologists, suggesting the area of interest to be assessed is essential regarding Ki67 LI. This conclusion was also implied in a recent study where the highest agreement between pathologists was observed when regions of interest were defined on whole slides to be assessed for Ki67 LI [141].

In the work of the Japan Breast Cancer Research Group, counting method was slightly superior to visual estimation [158]. In our studies, for SQ assessment the “eye balling” method was applied, because it was shown in numerous investigations that visual estimation could be just as good as the meticulous counting method of the ratio of positive tumor cell nuclei among all tumor cell nuclei [124,140,141]. Furthermore, visual estimation is less time-consuming and the possibility of miscalculation also persists as chance of error for the counting method.

Digital image analysis offers the opportunity to assess Ki67 LI more objectively and with increased reproducibility, but concordance compared to conventional evaluations is currently under examination [159,160]. In a recent study, an ICC of 0.885 was observed, when DIA Ki67 LI and conventional SQ Ki67 LI assessments were compared [161]. They performed Ki67 LI evaluations on whole slides of 50 cases of breast cancer, selecting 3-5 hot spots to be assessed, and both methods were performed on identical high-power fields [161]. Similarly, high concordance (ICC: 0.93) was found between the fully automated DIA assessment and SQ evaluation by Klauschen et al., who performed Ki67 IHC on whole core biopsies from 1,082 patients [162]. In our study, both automated DIA and adjustable DIA assessments represented substantial or outstanding agreement with SQ-RV evaluation. However, adjustable DIA seemed superior to automated DIA, since only the adjustable DIA assessments showed no proportional error compared to SQ-RV and the variability of their differences did not show an increasing trend, proportional to the magnitude of Ki67 LI. Furthermore, significant difference was observed between automated DIA and SQ-RV evaluations, while adjustable DIA and SQ-RV did not differ significantly. This result was also observed in the study by Laurinavicius et al. who found improvement in DIA evaluation when quality assessment was achieved on the default automated DIA evaluation [146]. In our study, significant difference was found between automated DIA and adjustable

DIA assessments. Basically, DIA method is more dependent on IHC staining quality, than conventional evaluation, since the human brain is able to compensate inadequate IHC quality [147]. Unequal tissue thickness and folds, cracks on glass slides, uneven coverglass glue layer might also lead to suboptimal quality in scanning slides and to false image analysis results [147]. In our opinion, significant discrepancies between our DIA methods were due to these features (Figure 15), which can be avoided by a pathologist's adjustment and by standardization of preanalytical and analytical steps of IHC. In our investigation, it has been also demonstrated, that the adjustable DIA is as robust as the visual estimation of Ki67 LI performed by well-trained and experienced pathologists.

Some authors have compared Ki67 LI assessment obtained by DIA to survival rates such as disease-free survival and overall survival [147]. To investigate the outcome prediction potential of Ki67 LI, dichotomizing is needed at a well-defined cut-off point. However, former guidelines have recommended different thresholds for such dichotomization; recent studies suggest, that an optimal cut-off score for Ki67 LI is not definable [63,89]. Thus, local laboratory specific cut-off points or Ki67 LI as a continuous marker should be applied to assess proliferation potential of the tumor [123]. In a recent study, the prognosis prediction of DIA Ki67 evaluation was significant in univariate analysis, although in multivariate analysis it has not remained significant compared to conventional clinicopathological factors [163]. In contrast with the results of this study, Klauschen reported that Ki6 LI obtained by automated DIA was significantly linked to prognosis in multivariate analysis adjusted by age, grade, ER, PgR and HER2 status as well as T status [162]. To ensure comparability between SQ's and DIA's prognosis prediction potential, we have utilized the widely applied 14% and 20% cut-offs for each assessment. In our hands, none of the Ki67 evaluations (regardless of DIA and SQ methods) were significantly linked to DFS at 14% threshold. However, at 20% threshold one of the three SQ assessments (SQ-2) was an independent prognostic factor besides lymph node status.

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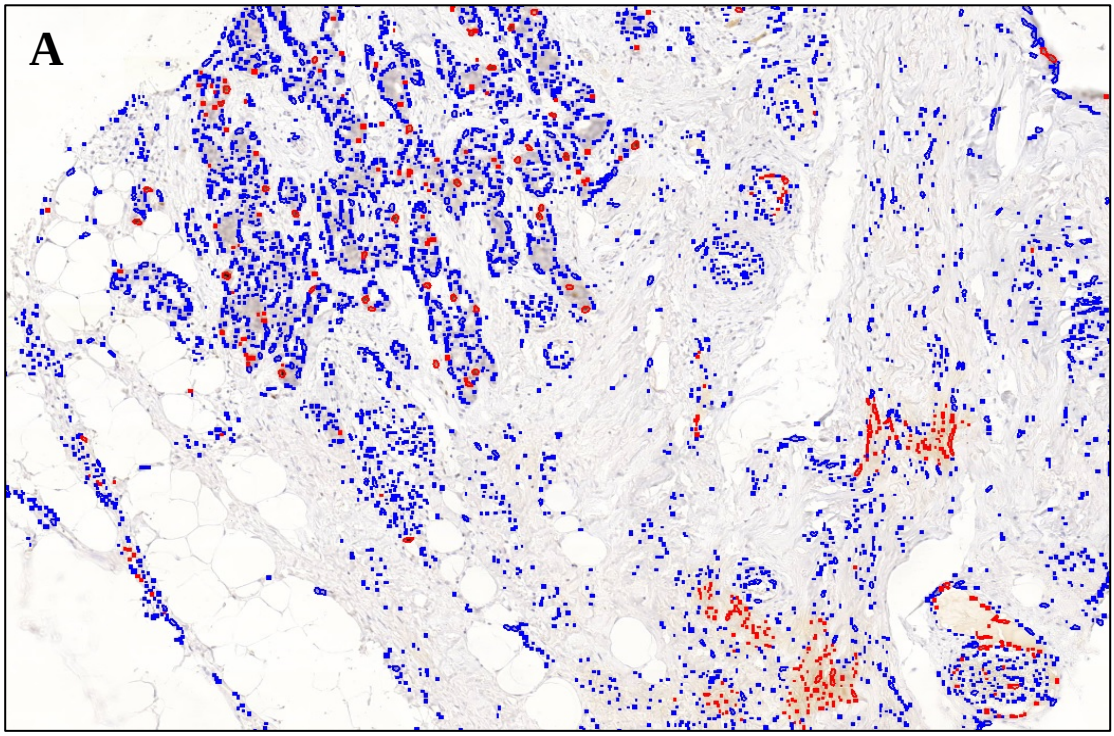

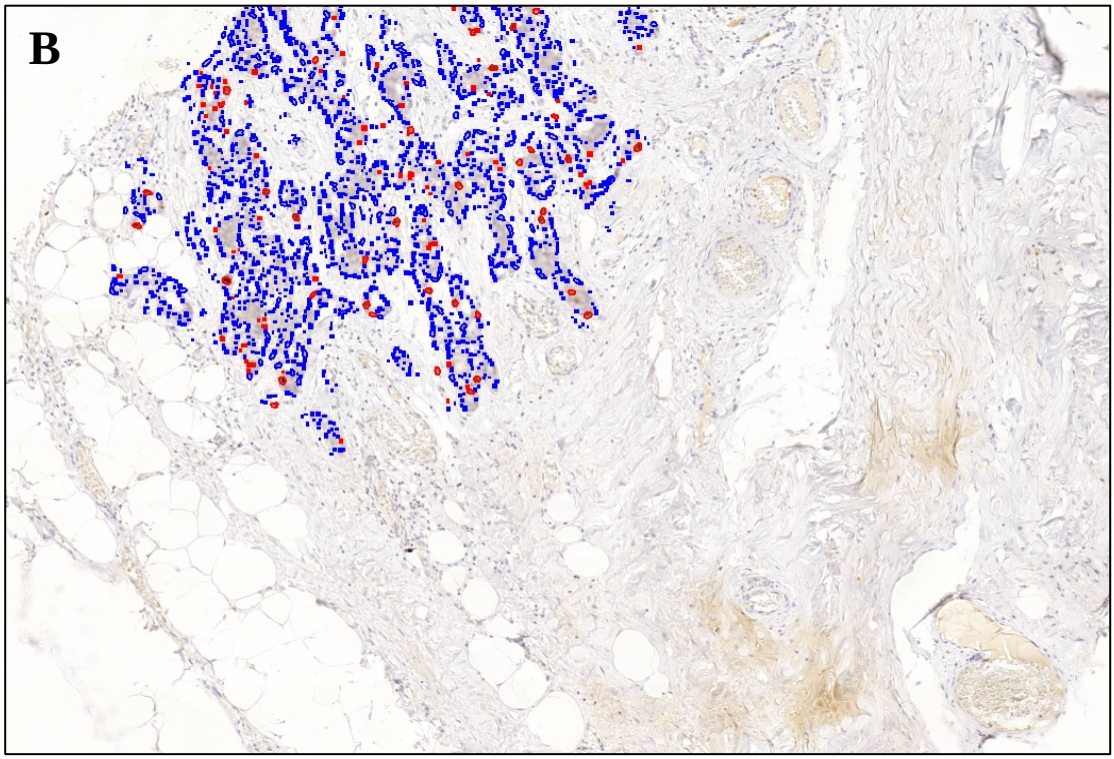

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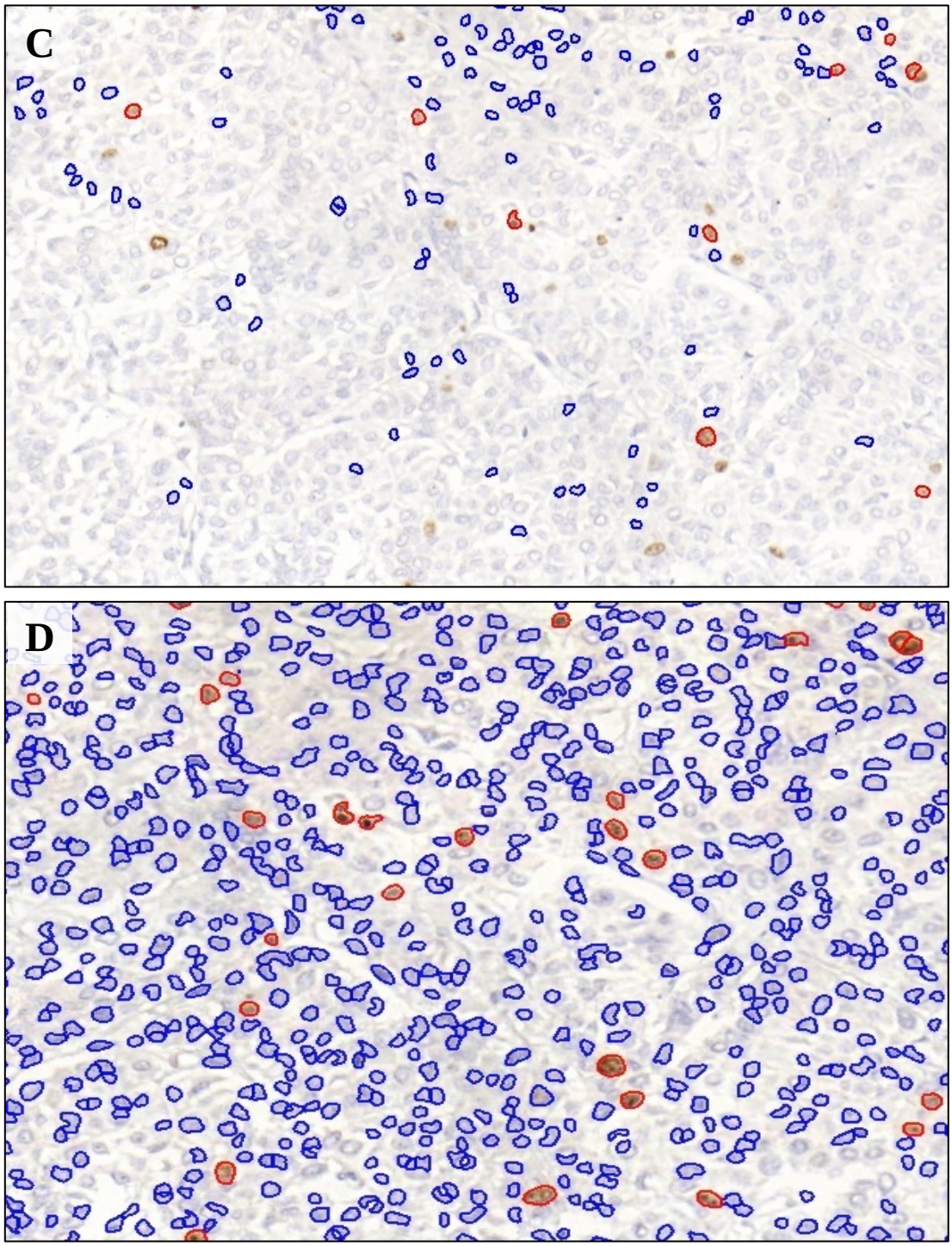

Figure 15: False detections were observed due to irrelevant Ki-67 staining [A] with automated DIA (DIA-1). These issues could be controlled by adjustable DIA method (DIA-2) with the presence of the pathologist [B]. Automated DIA (automated intensity threshold setting) was not able to recognize most of the tumor cells in some cases [C]. The reason for underestimated cell recognition is the inadequate quality of tissue processing. However, with adjustment of DIA (DIA-2 with adjustable intensity threshold), the vast majority of tumor cells were detected [D].

To exclude bias related to different treatment protocols, we have also investigated the prognosis prediction potential of Ki67 LI assessments in each treatment subgroup. All Ki67 evaluations but SQ-1 could distinguish good and unfavorable patient cohorts at 20% cut-off in the surgical treatment only subgroup, while in the patient subgroup treated with surgery+chemotherapy SQ-2 was able to perform statistically splitting the cohort. In treatment subgroups of surgery+irradiation and surgery+irradiation+ chemotherapy combination no prognosis prediction potential was observed for any Ki67 evaluation.

The limitation of our retrospective study comparing SQ and DIA evaluations is that Ki67 evaluations were performed on TMA slides, which might raise the possibility of underrepresented tumor areas related to prognosis prediction, even if we have used two cores from each case. Furthermore, we could retrieve DFS only from clinical data. Although preanalytical and analytical steps were not standardized in the contemporary terms, all the cases were collected from a single hospital resulting in uniform preanalytical conditions. Thus fixation, tissue processing and other preanalytical issues affected the immunohistochemical results uniformly. Therefore, we considered that preanalytical and analytical issues didn’t bias our results since all of our observers evaluated the same slides, thus discrepancies between final Ki67 LI values were derived from variability of each observer’s evaluations. Clinical data related to chemotherapy protocols were not available. Thus, we were not able to investigate predictive significance of each Ki67 evaluations for different chemotherapy regimens. In treatment stratified analyses, multivariate Cox regression was not performed due to low number of cases compared with events to relatively many clinicopathological factors.

Neoadjuvant systemic therapy is being increasingly used in the treatment of early stage breast cancer. Despite several classification systems developed for the assessment of pathologic response to NAC there is a current lack in uniformity regarding the definition of pathologic complete response [164,165]. Since pCR is considered as the primary endpoint for response to chemotherapy, most studies focus attention on pCR cases, while detailed analyses of partial responder or non-responder cases are relatively rare [129]. One of the hot topics in neoadjuvant therapy of breast cancer patients involves the question of reliable prognostic and predictive markers. Some of the questions about the performance of Ki67 LI as well as NAC in daily clinical practice

concern the issue of cut points for Ki67 LI and its use as prognostic or predictive marker. Different cut points are described, with values varying between 5% and 34% for OS [166], between 3%-94% for pCR, and between 6%-46% for DMFS [124,157]. The 2013 St. Gallen consensus recommended a Ki67 LI cut-off value of 14% for the separation of luminal A and - B tumors, but in the footnote of the respective table there was a note indicating 20% as cut-off for “high” Ki67 LI [123].

Our finding is in agreement with the results of Denkert et al. according to which Ki67 is a mixed prognostic and predictive marker with its effect differing in opposite directions as regards prognosis and prediction [89].

Our study revealed that a Ki67 LI cut-off value of about 20% distinguished pCR from pNR cases, whereas patients with Ki67 expression lower than 30% demonstrated a higher chance of better overall survival. Increased Ki67 LI was linked to worse OS, meaning that at least in some subgroups higher Ki67 expression was related to increased response to NAC and was also associated with worse prognosis. These data may suggest that if a tumor belongs to the group showing no response to NAC, increased Ki67 is a marker of poor prognosis.

Denkert et al. also suggest that based on Ki67 expression there are three different groups of tumors, such as a group with low Ki67 with good outcome, a group showing high Ki67 and good outcome and a third group with high Ki67 linked to poor outcome [89]. There are relatively few studies addressing the question of the role of Ki67 LI in non-responder or pPR groups, even if most cases treated with NAC show only partial response to chemotherapy.

In our study, most cases (60.83%) belonged to the pPR group. Based on Ki67 expression, this group represented a mixture of tumors showing Ki67 expression ranging from 1% to 100%. We analyzed whether the group of patients showing a near complete pathologic response (pPRi) showed higher Ki67 expression compared to pPRii and pPRiii. According to our findings, there were no significant differences between these groups regarding Ki67 expression. Based on the patients’ follow-up data and using ROC analysis, the most relevant prognostic cut-off value for the Ki67 LI in the pPR group was 20% based on DMFS and 30% based on OS.

Balmativola et al. analyzed markers of non-response to NAC. Using ROC analysis, they identified a cut-off value of 18% for Ki67 LI that performed well in differentiating the pNR and pCR + pPR categories [129].

In our study, Ki67 expression was found to be higher than 20% in all patients achieving pCR and we detected distant metastases in only one out of twenty-three pCR cases. In our study a Ki67 LI of 20% was found capable of significantly distinguishing between the pCR and pNR groups. Despite this finding, however, it cannot be concluded that this is the only or best threshold for Ki67 LI, since the question then arises as to why a significant number of tumors with a Ki67 value higher than 20% did not reach pCR.

Based on our results, both Ki67 and subtype showed significant association with pathological response. However, when luminal A cases were excluded, only subtype and pathological response were significantly linked, so we could conclude that subtype has a significant impact on the association between Ki67 LI and pathological response. In contrast to this, both Ki67 and subtype were independently associated with OS, while pathological response did not show significant relation with OS. Furthermore, both Ki67 and subtype were suitable to separate pPR patients into good and unfavorable prognosis cohorts.

The weakness of the retrospective study investigating the role of Ki67 in neoadjuvant setting is the relatively low number of cases, for which reason i.) we could not define the optimal Ki-67 cut-off point for each subtype. ii.) We could not investigate whether Ki-67 is suitable to predict pathological response in each subtype. Similarly, we could not address the question of the prognostic potential of Ki-67 for each subtype in breast cancer patients who underwent neoadjuvant chemotherapy. iii.) We could not perform treatment-stratified analyses.

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6. CONCLUSIONS

In the routine histopathological evaluation of breast cancer cases - due to the increasing importance and use of Ki67 labeling index - the selection and then the validation of Ki67 antibody requires great caution. Our results suggest that, as MIB1, poly, 30-9 antibodies showed the highest performance, they are suitable to detect Ki67 expression in the daily practice. We believe that this study provides a partial explanation to the various suggested Ki67 LI cut-off values in different published series of breast cancer cases.

The pathologists' experience is essential to control and adjust DIA and to avoid false detections. We also demonstrate that the adjustable DIA can be a feasible and reproducible tool to evaluate Ki67 LI in breast cancer which may support standardization efforts.

Neoadjuvant chemotherapy is more efficient in tumors presenting at least 20% Ki67 LI. A cut-off value of 20% distinguished pCR from pNR cases. Increased Ki67 LI was linked to worse OS, meaning that at least in some subgroups higher Ki67 expression is related to increased response to NAC and is also associated with worse prognosis. Additionally, our data also suggest that if a tumor is non-responder to NAC, increased Ki67 is a poor prognostic marker. Moreover, we provide further evidence that Ki67 LI is a significant and independent prognostic marker in breast cancer. Thus, we can conclude that Ki67 has potential utility in the clinical management of breast cancer. However, we can also state that Ki67 LI in itself is not suitable to decide whether a breast cancer patient should be treated with NAC or not.

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7. SUMMARY

Objectives: Three aspects of clinical validity of Ki67 labeling index (LI) were investigated in breast cancer as follows: i) The comparison of different Ki67 antibodies used in daily practice. ii) The reproducibility between pathologists evaluating Ki67 LI semi-quantitatively (SQ) and the potential of digital image-analysis (DIA) in Ki67 scoring. iii) The role of Ki67 in neoadjuvant setting.

Methods: Two breast cancer patient cohorts were enrolled in the investigations encompassing 498 patients totally: i) 378 consecutive breast cancer cases and ii) 120 patients diagnosed with invasive breast cancer and treated with neoadjuvant chemotherapy (NAC) were retrospectively recruited. Five antibodies were used to detect Ki-67 expression: MIB-1-using chromogenic detection and immunofluorescent labeling (IF), SP-6, 30-9, poly and B56. SQ evaluations were performed independently by three pathologists. DIA was completed using a fully automated histological pattern and cell recognition module for Ki67 LI detection (DIA-1) and an adjustable module (DIA-2) with the possibility of manual corrections.

Results: All the antibodies but MIB-1IF and B56 separated high and low risk patient groups. The highest concordance was found between MIB-1, poly and 30-9 antibodies. Significant difference and poor concordance occurred between SQ-1 and SQ-2 as well as between SQ-1 and SQ-3. Thus, the reference Ki67 LI value (SQ-RV) was generated from the mean values of SQ-2 and SQ-3. SQ-RV and DIA-2 results showed substantial concordance, while SQ-RV and DIA-1 values differed at only moderate concordance. The most relevant cut-off value for Ki-67 distinguishing complete remission cases from non-responders was 20%. Ki67 LI and partial responder subgroups were not significantly associated. In multivariate analyses, Ki67 LI were independently linked to survival.

Conclusions: Our results suggest that, MIB1, poly and 30-9 antibodies are suitable to detect Ki67 expression in the daily practice. The pathologists' experience is essential to control and adjust DIA and to avoid false detections. We demonstrate that the adjustable DIA can be a feasible and reproducible tool to evaluate Ki67 LI in breast cancer. We can conclude that Ki67 has utility in the clinical management of breast cancer. We can also state that Ki67 LI in itself is not suitable to decide whether a breast cancer patient should be treated with NAC or not.

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8. ÖSSZEFOGLALÁS

Célkitűzések: A Ki67 proliferációs index (LI) klinikai validitásának három aspektusát vizsgáltuk emlőrákban: i) a rutinban használt különböző antitestek összehasonlítása. ii) A patológusok által szemi-kvantitatívan (SQ) meghatározott Ki67 LI értékelések reprodukálhatósága és a digitális kép-elemzés (DIA) jelentősége a Ki67 LI értékelésben. iii) A Ki67 LI szerepe a neoadjuváns kezelés előrejelzésében.

Módszerek: Két emlődaganatos kohorszot, összesen 498 beteget vontunk be a kutatásainkba: i) 378 konszekutív emlődaganatos beteget és ii) 120 neoadjuváns kemoterápiával (NAC) kezelt emlőrákos beteget vizsgáltunk retrospektív módon. Öt antitestet használtunk a Ki67 kifejeződés detektálásra: MIB1 kromogén és immunfluoreszcens jelöléssel, SP-6, 30-9, poly és B56. A SQ értékeléseket három patológus végezte egymástól függetlenül. A DIA során egy teljesen automatikus szöveti mintázat- és sejtfelismerő modult (DIA-1) és egy manuálisan állítható adjusztálható modult (DIA-2) használtunk a Ki67 LI értékelésére.

Eredmények: A MIB1-IF és B56 antitestek kivételével valamennyi vizsgált antitest képes volt elkülöníteni az alacsony és magas kockázatú betegcsoportokat. A legmagasabb konkordanciát a MIB1, poly és 30-9 antitestek mutatták. Szignifikáns különbséget és alacsony konkordanciát tapasztaltunk az SQ-1 és SQ-2, illetve az SQ-1 és SQ-3 értékelések között, így Ki67 LI referencia értéknek (SQ-RV) az SQ-2 és SQ-3 értékelések átlagát jelöltük ki. Az SQ-RV és DIA-2 értékelések kiváló konkordanciát mutattak, míg az SQ-RV és DIA-1 értékelések összehasonlításakor szignifikáns különbséget és közepes konkordanciát tapasztaltunk. A neoadjuváns kezelésre nem reagáló – és a komplett remissziót mutató betegcsoportokat elkülönítő Ki67 LI határérték 20% volt. A parciális válasz mértéke és a Ki67 LI kifejeződés között nem találtunk összefüggést. A multivariáns elemzésekben a KI67 LI független prognosztikus markernek bizonyult.

Következtetések: A MIB1, 30-9 és poly antitestek alkalmasak a Ki67 kifejeződés meghatározására a patológiai rutinban. A patológus tapasztalata nélkülözhetetlen a DIA beállítására és kontrollálására a fals észlelések elkerülése végett. Az adjusztálható DIA hasznos és megbízható eszköz Ki67 LI értékelésére emlőrákban. Eredményeink szerint a Ki67 LI marker klinikai jelentőséggel bír az emlőrák ellátásában, ugyanakkor önmagában nem alkalmas a neoadjuváns kezelésre adott válasz előrejelzésére.

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10. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

10.1. Publications related to the PhD thesis

1. Balazs Acs, Lilla Madaras, Attila K. Kovacs, Tamas Micsik, Anna-Maria Tokes, Balazs Gyorffy, Janina Kulka, Marcell A. Szasz - **Reproducibility and Prognostic Potential of Ki-67 Proliferation Index when Comparing Digital-Image Analysis with Standard Semi-Quantitative Evaluation in Breast Cancer. –** Pathology & Oncology Research. 2017 Apr 11. doi: 10.1007/s12253-017-0220-8. – **Impact factor: 1.736**
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10.2. Publications not related to the PhD thesis

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2. Istvan A. Molnar, Bela A. Molnar, Laura Vizkeleti, Krisztina Fekete, Judit Tamas, Peter Deak, Judit Moldvay, Stefan Vari-Kakas, Csilla Szundi, Marcell A. Szasz, Balazs Acs, Janina Kulka, Anna-Maria Tokes - **Breast carcinoma subtypes show different patterns of metastatic behavior.** - Virchows Archiv. 2017 Jan 19. doi: 10.1007/s00428-017-2065-7. – **Impact factor: 2.848**
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