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Insulin and IGF-1 in Breast Cancer Patients are Associated with Tumor Cell Proliferation (Ki67) in their Breast Tumors

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Abstract

Introduction: Excess weight, insulin, insulin resistance and IGF-1 have been associated with breast cancer prognosis, but less is known about the association between these factors and Ki67 index, a marker of cell proliferation in malignant breast tumors.

Methods: Women with newly diagnosed stage I- II invasive breast cancer, aged 35-75 years, were invited to participate in a small clinical study, in order to study whether insulin, IGF-1 and insulin resistance [Homeostasis Model Assessment (HOMA)-score], were associated with Ki-67 index, a marker of cell proliferation in malignant breast tumors. Before surgery, body mass index [BMI, kg/m^2], waist circumference [cm], and total and truncal fat percentage [dual-emission X-ray absorptiometry], and fasting serum concentrations of insulin, IGF-1 and insulin resistance were assessed and determined. Ki67 index was determined as the percentage of Ki67-positive tumor cells according to national and international guidelines. Linear regression models were used to test for associations between Ki67 and selected patient characteristics.

Results: Among these 45 breast cancer patients included, mean age at diagnosis was 54.9 years, BMI was 24.8 kg/m^2 and mean waist circumference was 87.0 cm. Altogether, 91.1% of patients had estrogen receptor (ER)-positive tumors (> 1 % ER+), and 28.9% had axillary lymph node metastases. There was a negative association between Ki67 index and age (p=0.012), ER-positive tumors (p<0.0001), and progesterone receptor (PR)-positive tumors (p=0.001), and a positive association between Ki67 and histologic grade 3 *versus* 1 (p <0.0001). Among premenopausal patients, Ki67 was positively associated with insulin resistance, (p=0.097) and IGF-1 (p=0.025).

Conclusion: Our results, although small number of patients included, hypothesize an association between IGF-1, insulin resistance and Ki67 index. Our results should be focused in larger studies in where more detailed subgroup analysis can be performed.

Keywords: Excess weight, insulin, HOMA-score, IGF-1, Ki67 and breast cancer

Introduction

A positive energy balance over time may promote breast cancer growth (1-6). Potential mechanisms linking a positive energy balance to breast cancer risk and prognosis include elevated levels of circulating or tissue estrogens, insulin, insulin-like growth factor-1 (IGF-1) and low-grade chronic inflammation, as these factors are known to promote cell growth and inhibit apoptosis (1;2;2;3;3;5;7). Interestingly, studies support that IGF-1 plays a key role in the development and progression of breast cancer (4;6). Antiestrogens can reduce serum levels of IGF-

1, and the IGF-1 receptor is hyperactive and overexpressed in many breast cancers (1).

Furthermore, as Ki67 is found in proliferating cells, it can be a useful marker of cell proliferation (8-10). The fact that Ki67 is present during all active phases of the cell cycle [G(1), S, G(2), and mitosis], but is absent from resting cells [G(0)], makes it an excellent marker for determining the so-called growth fraction of a given cell population. Interestingly, high Ki67 expression has been associated with an increased risk of breast cancer recurrence and death (11;12), and recently it has been used as a marker to aid in predicting response to chemotherapy (13).

Thus, both insulin and IGF-1 and Ki67 have independently been associated with breast cancer development. However, less is known about whether excess weight, high levels of insulin and IGF-1, or insulin resistance are associated with Ki67. The main aim of this study was therefore to elucidate whether body weight, serum levels of insulin and IGF-1, or insulin resistance assessed in newly diagnosed breast cancer patients prior to surgery are associated with Ki67 index defined in malignant breast tumors.

Material and methods

Participants and study design

During June 1. 2011 to December 15. 2011, breast cancer patients aged 35-75 years who were newly diagnosed with histologically verified stage I or II invasive breast cancer at the Oslo University Hospital (OUS), Ullevål, were invited to participate in this small clinical study. Women with known severe illnesses (i.e. heart failure, diabetes), were excluded, leaving a total of 45 breast cancer patients included in the present analysis.

Assessment of variables

Baseline patient characteristics were assessed before treatment (surgery, radiation, chemotherapy) by trained study nurses at the Research Unit, OUS. We used a modified version of the Norwegian EBBA-I study questionnaire (14) (self-report and interview) to collect information on menstrual and reproductive history, birth weight, and on lifestyle habits. Recall and memory-probing aids were used to date reproductive history events, including a lifetime calendar, and a list of examples of milestones developed for use in the EBBA-I study (14).

Anthropometric measurements were performed with participants wearing light clothing and no footwear (3;14). Height was measured to the nearest 0.5 cm, and weight to the nearest 0.1 kg on an electronic scale. BMI (kg/m^2) was used to estimate relative weight. The percentage of total and truncal fat tissue was estimated using a dual-emission X-ray absorptiometry scan (Lunar PIXIMUS, Lunar, Madison, Wisconsin, USA). Waist circumference (cm) was measured in a horizontal line 2.5 cm above the umbilicus (3). Blood pressure was measured

three times (Dinamap-Pro Care 300, NY, USA), with the patient sitting in a resting position, and the mean of the final two measurements was used in the analysis.

Blood samples were drawn after overnight fasting. Concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, glucose and CRP were measured in fresh sera at the Department of Clinical Chemistry, OUS, Ullevål (Roche Diagnostics/Cobas Integra 800- Cobas 8000, Mannheim, www.roche.com). Cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. HDL cholesterol was quantified by a direct assay using polyethylene glycolmodified enzymes and dextran sulfat. Triglycerides were assayed by enzymatic hydrolysis with lipase. Insulin and IGF-1 were measured in fresh sera at the Hormone Laboratory, Aker University Hospital, Oslo, by an immunfluorometric assay (DELFIA kit, PerkinElmer Life Sciences, Wallac Oy, Turku, Finland) and an immunoluminometric assay (Immulite 2500, Siemens Healthcare Diagnostics, Los Angeles, California, USA), respectively. Serum concentrations of glucose were measured at the Department of Clinical Chemistry, OUS, Ullevål, by an enzymatic hexokinase method using kits and the analytical platform cobas 8000, c702 from Roche Diagnostics GmbH (Mannheim, Germany).

Tumor characteristics

Surgical specimens of all breast cancers were histologically and immunohistochemically examined, and subsequently classified according to histologic type (ductal, lobular, other) and histologic grade (1, 2, 3). Tumor diameter was measured both macro- and microscopically (mm). Lymph nodes were investigated using the sentinel lymph node biopsy technique to identify axillary macro- or micro-metastases (15). The tumor markers ER, PR, HER2 and Ki67 were investigated by immunohistochemistry. Sections of tumor biopsies were stained according to the manufacturer's protocols with primary antibodies against ER (clone SP1, Ventana, Roche Diagnostics, Oslo, Norway), PR (clone 1E2, Ventana, Roche Diagnostics), HER2 (Pathway anti-HER 2 kit, clone 4B5, Ventana, Roche Diagnostics), and Ki67 (MIB1 antibody, Dako, Oslo, Norway). ER, PR and HER2 expression were measured according to the international guidelines of the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP). ER+ was defined as $\geq$1%, and PR+ as $\geq$10%. Tumors were investigated with the HER2 Dual SISH *in situ* hybridization kit (Ventana, Roche Diagnostics, Oslo, Norway).

Ki67 index was determined as the percentage of Ki67-positive tumor cells according to national and international guidelines (10;13). To determine Ki67 index at least 500 tumor cells were counted in three representative high-power fields in the most proliferative area of the tumor ("hot spot"), as well as randomly selected high-power (x40 objective) fields, which were usually in the periphery. Ki67 index is defined as the percentage of positively stained cells among the total number of malignant cells scored (10). Cut-points used in the present study are

based on national and international guidelines: Ki67 ≤15%, >15%-30% and >30% (Figure 1) (10;13;16).

Statistical analyses

The distributions of patient and tumor characteristics are presented in Table 1, in the total study population and by menopausal status. Premenopausal status was defined as having regular menstruation. Postmenopausal status was defined as having no menstruation for 12 or more months. If there was uncertainty about postmenopausal status due to hormone replacement therapy, irregular menstruations within the latest 12 months or other circumstances, patients aged 50 years or over were considered postmenopausal. Comparisons of characteristics between pre- and postmenopausal patients were done using two-sample Student's t-test for continuous variables and Pearson's chi-squared test for categorical variables.

The World Health Organization (WHO)'s definitions of overweight (BMI >25 kg/m^2), obesity (BMI >30 kg/m^2) and central obesity (waist circumference >80 cm) (17) were used. Obesity is commonly associated with insulin resistance calculated by HOMA score (fasting glucose [mmol/l] x fasting insulin [μIU/ml] / 22.5) (18). In order to study the association between Ki67 and BMI, insulin, IGF-1 and insulin resistance, we divided BMI (kg/m2), insulin (pmol/l), IGF-1 (nmol/l) and insulin resistance (HOMA-score) by median split both in the total patient population and among postmenopausal breast cancer patients. However, it was not possible to stratify by median split of these factors among premenopausal patients, as these were too few. Analyses by median splits were done using two-sample Student's t-test for continuous variables and Pearson's chi-squared test for categorical variables.

Linear regression models were used to test associations between patient and tumor characteristics and Ki67. As levels of BMI, insulin, IGF-1 and insulin resistance showed large variations by menopausal status (Table 1), the association between these factors and Ki67 was studied by menopausal status (Figure 3). Statistical analyses were performed using Stata 12 (StataCorp LP, College Station, Texas). Results were considered statistically significant when the two-sided p-value was <0.05.

Ethical considerations

All participating breast cancer patients signed an informed consent form. The study protocol was reviewed and approved by the Norwegian Regional Committee for Medical Research Ethics.

Results

Mean values for patients were as follows: age at diagnosis, 54.9 years (range: 38.6-75.0 years); BMI, 24.8 kg/m^2; waist circumference, 87.0 cm, and age at

menarche, 13.3 years. Patients had mean fasting serum insulin of 36.5 pmol/l, mean IGF-1 of 19.1 nmol/l and mean insulin resistance (HOMA-score) of 1.30. Altogether, 28.9% of patients had axillary lymph node metastases, 91.1% had ER-positive tumors, and 4.4% had HER2-positive tumors.

When comparing premenopausal and postmenopausal breast cancer patients, premenopausal patients tended to have larger breast cancer tumors, and lower frequencies of ER-positive and lobular tumors compared to postmenopausal patients (Table 1).

Ki67 was negatively associated with age (β from linear regression= -0.92 [95% Confidence Interval CI: -1.63, -0.21], p=0.012), ER-positive tumors (β= -0.45 [95% CI: -0.63, -0.27], p<0.0001), and PR-positive tumors (β= -0.29 [95% CI: -0.45, -0.12], p=0.001), and positively associated with histologic grade (β=43.4 [95% CI: 32.0, 54.9], p <0.0001) (Figure 2) and IGF-1. Overall, we found no association between Ki67 and BMI, serum levels of insulin or insulin resistance. Among premenopausal, but not postmenopausal breast cancer patients, Ki67 was significantly associated with IGF-1 (β=1.73 [95% CI: 0.25, 3.21], p=0.025), and tended to be positively associated with insulin resistance (β=41.9 [95% CI: -8.75, 92.6], p=0.097) (Figure 3). When dividing by median split of BMI, serum levels of insulin and insulin resistance (median HOMA score), a pattern of higher Ki67 was observed among postmenopausal patients with higher serum levels of insulin and insulin resistance (not presented).

We studied whether overweight and serum levels of insulin in combination could predict level cell proliferation of breast cancer cells, Ki67 index. Among postmenopausal breast cancer patients with BMI ≥25 kg/m^2 and serum levels of insulin ≥40 pmol/l, compared with postmenopausal breast cancer patients with lower BMI (<25kg/m^2) and lower serum levels of insulin (<40 pmol/l), we observed that Ki67 was suggestively higher; 31.1% *versus* 25.0 %, respectively.

Discussion

In this small clinical study we observed that insulin resistance and IGF-1 were positively associated with Ki67, a marker of cell proliferation in malignant breast tumors, but only among premenopausal patients. Our observation is in part supported by laboratory data observing that insulin has mitotic and antiapoptotic effects in breast cancer cells (19). Moreover, hyperinsulinemia decreases sex hormone-binding globulin levels, thereby increasing the bioavailability of sex hormones (20;21). Moreover, animal models have demonstrated that increased insulin and IGF-1 signaling can enhance tumor growth, while inhibiting this signaling can reduce tumorigenesis (22).

Evans *et al.* showed that the use of the anti-diabetic drug metformin, which reduces insulin resistance, is associated with a reduction in breast cancer risk (23). Moreover, metformin treatment has recently been observed to induce pathological tumor response rates following neoadjuvant chemotherapy for breast cancer,

suggesting a potential use for metformin as an anti-cancer drug (24). Niraula *et.al* found short-term administration of metformin in non-diabetic women with early stage breast cancer to be associated with modest weight loss and changes in physiologic parameters (glucose, HOMA, and insulin) that are consistent with reduction in insulin resistance. Moreover, reductions in tumor cell proliferation, as reflected by Ki67 were also observed, and thus supporting our hypothesis (25).

Our findings that this marker of cell proliferation in malignant breast tumors, Ki67 was negatively associated with age, ER and PR status, but positively associated with histologic grade hypothesize that Ki67 index may reflect both characteristics of the tumor as well as being associated with characteristics of the patients, both influencing breast cancer development. Interestingly, the associations between Ki67 and insulin, IGF-1 and insulin resistance varied by menopausal status. In premenopausal patients, Ki67 was positively associated with insulin, IGF-1, and insulin resistance, whereas no such association was observed among postmenopausal patients overall. However, when performing analyses among postmenopausal patients by median split of BMI, insulin, and insulin resistance, a pattern of higher Ki67 was observed among postmenopausal patients with higher insulin and insulin resistance. No association was observed between Ki67 and IGF-1 in postmenopausal patients.

A limitation in our study is the small patient's number, but interestingly, our results hypothesize that specific patient and tumor characteristics should be further studied in combination. The nuclear antigen Ki67 is used as a marker of tumor cell proliferation. There is increasing evidence that Ki67 is a valuable prognostic marker, and recent data suggest that Ki67 above in particular 30%, and for some also above 10–14% defines a high-risk prognostic group (13;16). However, little is known about the association between Ki67 and an unfavorable metabolic profile, including excess weight, high levels of insulin and IGF-1, and insulin resistance. Importantly, our results are supported, in part, by others. Daling *et al.* found that in women aged below 45 years with invasive ductal breast cancer, excess weight was associated with an increased risk of breast cancer-specific mortality; furthermore, women in the highest quartile for BMI were more likely to have large tumors of high histologic grade, with a high mitotic cell count. Also, when tumors of 2 cm or more were taken from women in the highest BMI quartile and compared with tumors of the same size from women in the lowest quartile, they were found to have relatively high mitotic cell counts, a high S-phase fraction, and a high Ki67 expression (26). Moreover, Borgquist *et al.* examined tumors from 248 postmenopausal women, none of whom were using hormone replacement therapy (27). The women in the highest quartiles of body weight and BMI more often had breast cancers with low Ki67, supporting our findings of less aggressive tumors among postmenopausal overweight patients.

IGF-1 is a peptide that stimulates mitosis and inhibits apoptosis (6). Higher plasma concentrations of IGF-1 have been reported in women with breast cancer than in controls (28) in premenopausal, but not postmenopausal women (29), which supports our observation that IGF-1 was associated with Ki67 in

premenopausal, but not postmenopausal patients. Recently, IGF-1 receptor gene expression was associated with breast cancer survival (4). Moreover, estrogens are important in the etiology of breast cancer, and there is laboratory evidence for crosstalk in cells between the signaling pathways for estrogens and IGF-1 (30). Thus, our findings that especially younger breast cancer patients with high levels of insulin and IGF-1 have higher Ki67, indicating more aggressive breast cancer tumors, are indirectly supported by others, and may indicate that some younger women are more vulnerable to an unfavorable metabolic profile, which puts them at higher risk.

The biologic mechanisms underlying the relationship between energy balance and breast cancer risk and prognosis are not fully understood. In postmenopausal women, obesity is associated with higher estrogen levels due to increased peripheral aromatization of adrenal androgens in adipose tissue (31), as well as lower levels of sex-hormone-binding globulin, resulting in higher circulating levels of free estrogen (32). In premenopausal breast cancer patients, however, mechanisms other than estrogen may affect breast cancer risk and prognosis. Obesity, especially abdominal adiposity, is associated with higher levels of insulin and other metabolic hormones in both pre- and postmenopausal women. Elevated serum levels of insulin have been linked to an increased risk of breast cancer, and several recent reports have demonstrated that women with higher levels of insulin or related proteins at the time of breast cancer diagnosis are at increased risk of cancer recurrence and death (33-35). Interestingly, Goodwin *et al.* demonstrated a two-fold increase in the risk of breast cancer recurrence and a three-fold risk of death in patients with the highest *versus* the lowest quartile of fasting serum levels of insulin (33). Recently, women with high levels of C-peptide, a breakdown product of insulin production, at the time of breast cancer diagnosis were found to have a higher risk of breast cancer-specific and overall death *versus* women with lower levels of C-peptide (36).

In this population, 80% of postmenopausal breast cancer patients had a waist circumference over 80 cm. Interestingly, higher estrogen levels have been observed in overweight and obese women, and these higher levels have been suggested to stimulate dormant tumor cells with less aggressive features (33;37;38). These results support our findings that postmenopausal patients in the present study had a high level of ER- and PR-positive tumors.

The present study has several limitations and include a limited number of breast cancer tumors. Thus, our results need to be confirmed in larger study samples. In addition, the study was cross-sectional in design and not retrospective. However, there were also several strengths, including the comprehensive assessments of patient characteristics prior to surgery, and other treatment that could affect these measurements; the use of trained nurses for data collection; measurement of Ki67 in one laboratory by pathologists with specialized knowledge and experience in Ki67 assessment (13); and the use of one study site with standardized data collection procedures which minimizes potential for patient selection and information bias.

Conclusion

We hypothesize that patient characteristics related to energy balance, in particular insulin resistance and IGF-1, may be associated with Ki67, a marker of cell proliferation in malignant breast tumors. The small number of patient included is a limitation of our study, and these results need to be replicated in larger studies. However, our findings hypothesize that specific patient and tumor characteristics that might aid in predicting prognosis should be combined and included in future larger studies.

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Table 1. Characteristics of women (35-75 years), newly diagnosed with stage I or II invasive breast cancer, total and by menopausal status, mean (SD) or frequency (%), N=45[a]

Characteristics	Total n=45	Premenopausal n=15	Postmenopausal n=30	p-value
Clinical variables				
Age at diagnosis (years)	54.9 (8.7)	46.7(5.48)	58.9 (7.02)	<0.001|
Birth weight (g)	3416 (406.3)	3429 (374.4)	3408 (430.5)	0.88
Height (cm)	167.2 (6.1)	165.9 (5.06)	167.9 (6.54)	0.33
BMI (kg/m^2)	24.8 (3.4)	23.5 (3.32)	25.4 (3.28)	0.07
Truncal fat (DEXA) (%)	39.6 (8.6)	34.9 (11.9)	41.8 (5.67)	0.013
Waist circumference (cm)	87.0 (10.7)	81.7 (10.0)	89.7 (10.1)	0.016
Systolic blood pressure (mmHg)	129.3 (22.2)	116.9 (16.5)	135.5 (22.3)	0.007
Age at menarche (years)	13.3 (1.2)	12.8 (1.08)	13.6 (1.22)	0.056
Parity[b]	2.2 (0.9)	2.5 (1.1)	2.1 (0.8)	0.33
Fasting serum concentrations				
Total cholesterol (mmol/L)	5.70 (1.11)	5.29 (1.11)	5.91 (1.07)	0.08
Total cholesterol / HDL cholesterol	3.45 (1.10)	3.33 (1.35)	3.51 (0.97)	0.62
Triglycerides (mmol/L)	1.16 (0.59)	1.03 (0.71)	1.22 (0.53)	0.35
Glucose (mmol/l)	5.37 (0.63)	4.97 (0.50)	5.56 (0.60)	0.003
CRP (mg/l)	1.97 (2.57)	1.51 (1.29)	2.20 (3.01)	0.41
Insulin (pmol/l)	36.5 (14.9)	26.9 (6.35)	41.4 (15.6)	0.001
IGF-1 (nmol/l)	19.1 (6.27)	22.3 (8.23)	17.4 (4.26)	0.017
IGFBP3 (nmol/l)	92.6 (14.19)	95.1 (11.8)	91.7 (15.0)	0.55
HOMA-score[c]	1.30 (0.62)	0.88 (0.26)	1.50 (0.64)	0.001
Tumor characteristics				
Tumor diameter (cm)	1.59 (0.84)	1.8 (1.0)	1.5 (0.7)	0.26
Axillary lymph node metastases (%)	28.9	26.7	30.0	0.82
Histologic tumor type (%)				
Ductal	77.8	80.0	76.7	0.80
Lobular	13.3	6.7	16.7	0.35
Others	8.9	13..3	6.7	0.46
Histologic grade (%)				
Grade 1	24.4	20.0	26.7	0.62
Grade 2	48.9	53.3	46.7	0.67
Grade 3	26.7	26.7	26.7	1.0
Tumor markers				
Estrogen receptor-positive (%)	91.1	86.7	93.3	0.46
Progesterone receptor-positive (%)	84.4	80.0	86.7	0.56
HER2-positive (%)	4.4	6.7	3.3	0.61
Triple negative (%)	4.4	6.7	3.3	0.61

[a] Numbers of participants may vary as a result of missing information for certain variables.
[b] Among those who have given birth.
[c] HOMA score: As indicators of insulin resistance we used HOMA, calculated as the product of fasting glucose concentration (mmol/L) and fasting insulin concentration (μU/mL) divided by the constant 22.5.
SD=standard deviation; HDL=High-density lipoprotein; CRP=C-Reactive Protein, DEXA=dual-emission X-ray absorptiometry, HOMA= homeostasis model assessment.

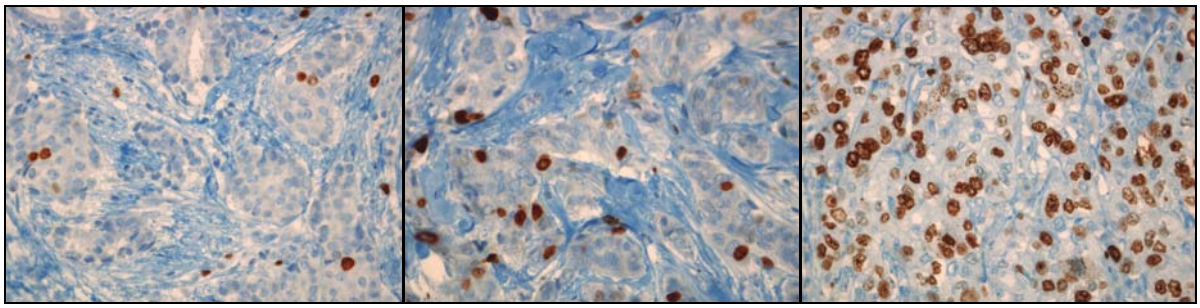

Figure 1. Ki67 staining in invasive malignant breast tumors (x40 objective). Tumor biopsies were fixed in neutral buffered formalin and sections stained for Ki67 with the MIB1 antibody (brown stain), and counterstained with mayer's hematoxylin (blue stain)

A: Ki67 ≤15%,
B: Ki67 >15%-30%
C: Ki67 >30%

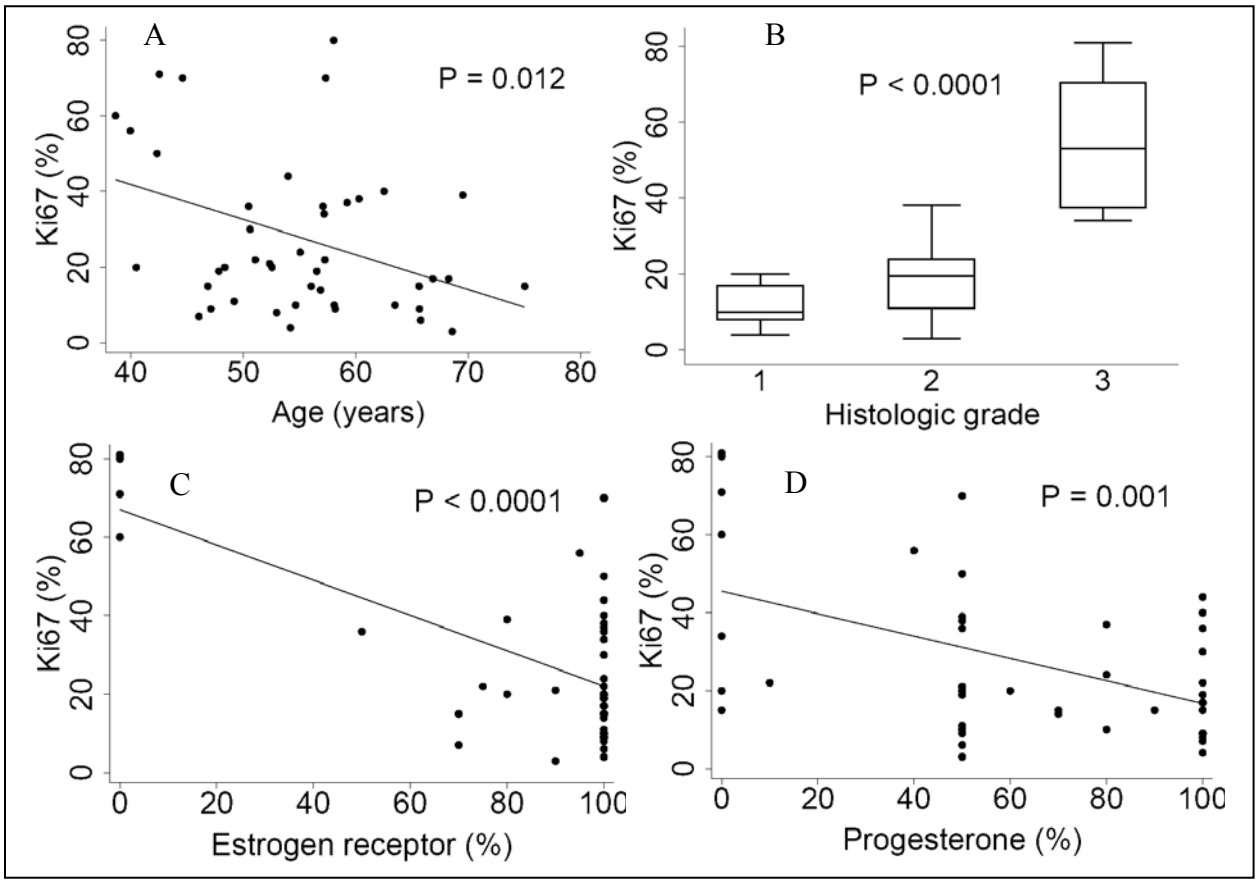

Figure 2. The association between age, histologic grade (I-III), estrogen receptor, progesterone receptor and tumor cell proliferation (Ki67) among women newly diagnosed with stage I or II invasive breast cancer N=45[a]

A. Age *versus* Ki67
B. Histologic grade (I,II,III) *versus* Ki67
C. ER % *versus* Ki67
D. PR % *versus* Ki67

[a]Numbers may vary due to missing values.

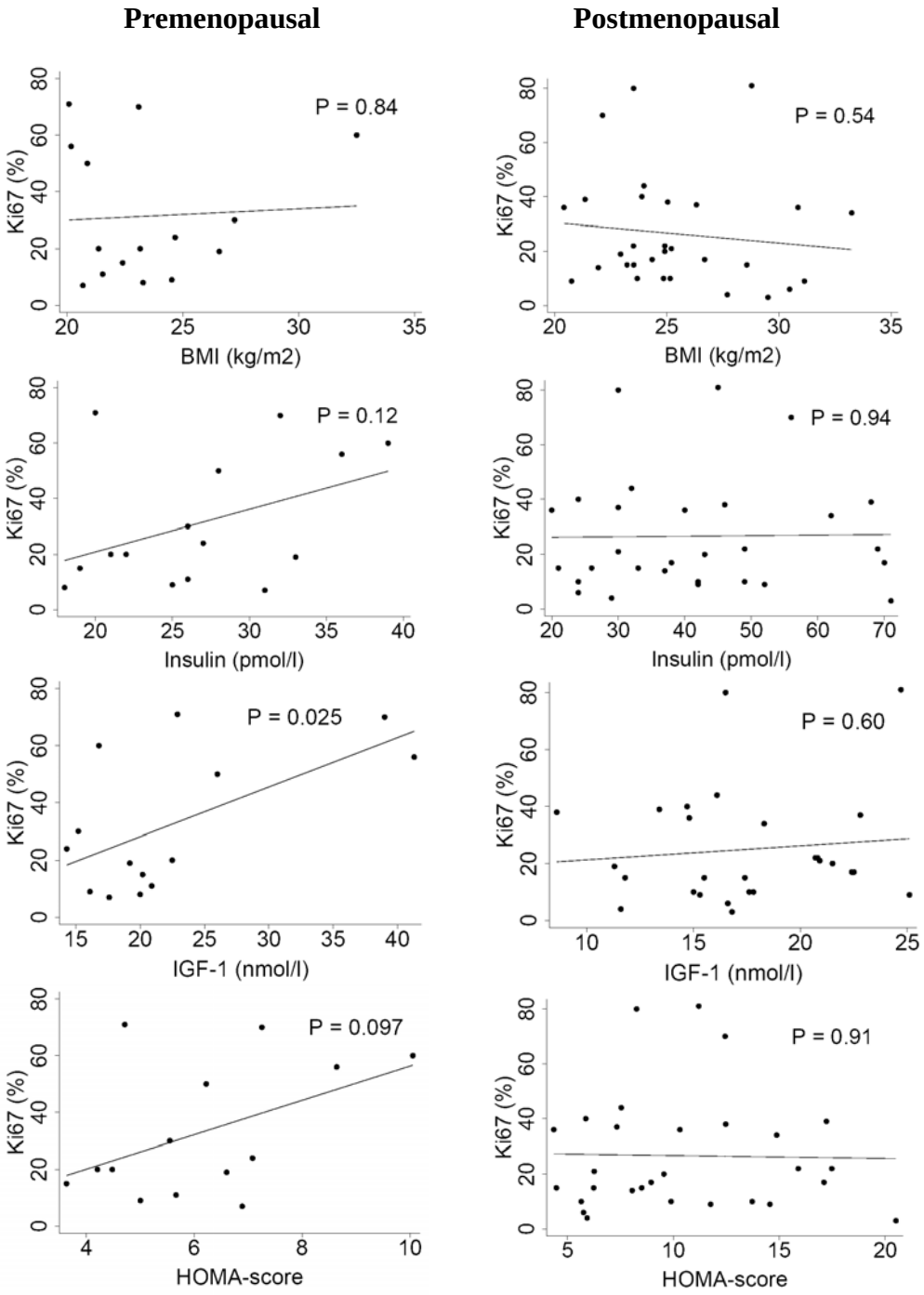

Figure 3. The association between body mass index (BMI, kg/m^2), insulin (pmol/l), insulin growth factor 1 (IGF-1, nmol/l) and insulin resistance (HOMA-score) by menopausal status and tumor cell proliferation (Ki67) among women newly diagnosed with stage I or II invasive breast cancer, N=45[a]
[a] Numbers may vary due to missing values.

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