

Differential effects of metformin on breast cancer proliferation according to markers of insulin resistance and tumor subtype in a randomized presurgical trial

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Abstract Treatment of diabetics with metformin is associated with decreased breast cancer risk in observational studies, but it remains unclear if this drug has clinical antineoplastic activity. In a recent presurgical trial, we found a heterogeneous effect of metformin on breast cancer proliferation (ki-67) depending upon insulin resistance (HOMA index). Here, we determined the associations of additional serum biomarkers of insulin resistance, tumor subtype, and drug concentration with ki-67 response to metformin. Two-hundred non-diabetic women were randomly allocated to metformin (850 mg/bid) or placebo for 4 weeks prior to breast cancer surgery. The ki-67 response to metformin was assessed comparing data obtained from baseline biopsy (ki-67 and tumor subtype) and serum markers (HOMA index, C-peptide, IGF-I, IGFBP-1,

IGFBP-3, free IGF-I, hs-CRP, adiponectin) with the same measurements at definitive surgery. For patients with a blood sample taken within 24 h from last drug intake, metformin level was measured. Compared with placebo, metformin significantly decreased ki-67 in women with HOMA > 2.8, those in the lowest IGFBP-1 quintile, those in the highest IGFBP-3 quartile, those with low free IGF-I, those in the top hs-CRP tertile, and those with HER2-positive tumors. In women with HOMA index > 2.8, drug levels were positively correlated with the ki-67 decrease, whereas no trend was noted in women with HOMA < 2.8 (p -interaction = 0.07). At conventional antidiabetic doses, the effect of metformin on tumor ki-67 of non-diabetic breast cancer patients varies with host and tumor characteristics. These findings are relevant to design breast cancer prevention and treatment trials with metformin.

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Introduction

The hypothesis that metformin, widely used in the treatment of type II diabetes, is useful in breast cancer prevention and/or treatment is receiving considerable attention. Both pharmaco-epidemiologic and laboratory studies have suggested anti-neoplastic activity of this compound, and plausible mechanisms of action have been proposed [1–4]. These include on one hand direct actions requiring exposure of target tissues to adequate drug concentrations, and on the other, effects on the endocrine environment at the whole organism level that result largely from actions of the drug on the liver, including reduction in gluconeogenesis and in circulating levels of insulin or cytokines. Metformin would be an appealing agent for use in oncology, as it is well tolerated and is available as a low-cost generic compound.

However, some of the pharmaco-epidemiologic data are negative [5] or controversial [6], and many laboratory studies demonstrating anti-neoplastic activity use drug concentrations higher than those achieved in serum with conventional metformin dosing. Furthermore, it is plausible that any effects of metformin on breast cancer risk or prognosis would be confined to subgroups of women, such as those with high BMI and/or high baseline levels of insulin, where metformin is known to have the largest effects on whole organism physiology [7]. Moreover, the effect of metformin on specific tumor molecular subtypes is unclear, although some studies have shown an effect on HER2 +ve tumors [8]. Therefore, pilot ‘window of opportunity’ presurgical studies with surrogate endpoints have been undertaken to generate clinical data to complement the epidemiologic and laboratory results. These studies involve sequential tissue sampling and biomarker measurements after relatively short-term exposure to metformin. Initial results of our randomized placebo-controlled study in 200 non-diabetic women with breast cancer [9] revealed that metformin exposure for 4 weeks was not associated with significant overall reduction in neither insulin levels nor tumor proliferation rate as estimated by ki-67 labeling index. However, we did detect a reduction of ki-67 by the metformin in subsets characterized by insulin resistance (HOMA index > 2.8) and high BMI (>27, the upper quartile), a finding consistent with the heterogeneous effect of metformin on diabetes development [10]. Similar studies conducted by other investigators involved smaller study populations. An early study of 47 patients documented a reduction in ki-67 level [11]. Data from a third study [12] of 39 patients without a control group revealed no significant effect on insulin levels, but weight loss, a reduction in ki-67 staining of 3 %, and a significant increase in apoptosis (TUNEL) staining following metformin administration. In our larger placebo-controlled study, we showed no effect of

metformin on apoptosis overall, but there was heterogeneity according to insulin resistance estimated by HOMA index [13]. Finally, a fourth uncontrolled study in 35 overweight/obese women with breast cancer found no change in ki-67 but a reduction of BMI, cholesterol, and leptin [14].

Here, we report further exploratory analysis of our window-of-opportunity trial addressing the effects of metformin according to biomarkers of insulin resistance, including HOMA index, BMI, C-peptide, IGF-I, IGFBP-1, IGFBP-3, free IGF-I, C-reactive protein, adiponectin, and by tumor subtype.

Methods

Study design and subjects

A detailed description of the main study characteristics and initial results of the clinical trial has previously been published [9]. Briefly, we conducted a randomized, phase II, double-blind, placebo-controlled trial in women with stage I-IIa breast cancer candidate to elective surgery who received either metformin or placebo for 4 weeks prior to surgery. Baseline core biopsies of tumor tissue and blood samples were obtained at study entry and before surgery for pre/post-treatment comparisons. Patients were randomly assigned to metformin, 850 mg tablets or placebo once daily on day 1–3 to adapt to gastrointestinal symptoms, followed by two 850 mg tablets after dinner from day 4 to 28 to minimize gastrointestinal symptoms during the daytime and to attain higher blood levels during morning blood sample. Treatment was stopped at least 48 h prior to anesthesia, in keeping with FDA and AIFA guidelines [15, 16], to avoid the risk of lactic acidosis [17]. The median (IQR range) time (h) elapsed from last drug intake to blood drawing for circulating biomarkers was 36 (14–43) versus 36 (19–43), for metformin and placebo arms, respectively. The median (IQR range) time (h) elapsed from last drug intake to surgery was 67 (21–74) versus 67 (21–75) in the metformin and placebo arms, respectively. Notably, this time gap might, if anything, dilute the metformin effects on ki-67 measurement toward the null hypothesis. Eligibility criteria were: age \geq 18 years; PS = 0; palpable, histologically confirmed breast cancer candidate to surgery and no prior treatment; signed informed consent.

Pathology

Pathological assessment included evaluation of histological type, grade, peritumoral vascular invasion, ER, progesterone receptor (PgR), HER2 and ki-67, as previously described [18]. Specifically, ki-67 was assessed by IHC according to recent international recommendations [19] using the Mib-1

monoclonal antibody (1:200 dilution; Dako, Denmark). Cut slides were stained using an automated Dako immunostainer. The percentage of cells showing definite nuclear immunoreactivity among 2,000 invasive neoplastic cells in randomly selected, high-power (40 \times) fields at the periphery of the tumor was recorded. If heterogeneity of ki-67 staining was present due to hot spots, the overall average score was adopted. In the core biopsy, all cells were counted regardless of their location in the tumor. Fluorescence in situ hybridization (FISH; PATHvision, Abbott, IL) was undertaken for tumors with a 2 + HER2 IHC score. Molecular tumor subtypes were classified by IHC in four categories according to 2011 St. Gallen criteria [20].

Circulating biomarkers of insulin resistance and serum drug concentrations

Morning fasting blood samples were collected between 8 and 10 am at baseline and before surgery. To obtain serum, blood was allowed to clot at room temperature for 30 min and then centrifuged at 1,500 g for 10 min. Serum aliquots were stored at -80°C until assayed. Serum glucose and total cholesterol concentrations were measured on fresh samples, whereas all other analytes were determined on frozen samples. We applied the homeostasis model assessment (HOMA) as a surrogate index of insulin sensitivity, obtained by the formula [fasting insulinemia (mU/L) \times glycaemia (mmol/L)]/22.5. We applied a cut-off of the HOMA index at 2.8 for insulin resistance based on a population study conducted in Northern Italy, as previously described [21]. Insulin was measured with an electro-chemo-luminescence immunoassay by the use of COBAS e411 (Roche Diagnostics, Mannheim, Germany). The analytical sensitivity was 0.20 uU/mL and intra- and inter-assay coefficients of variation 3.1 and 5.0 %, respectively for a control sample of 23.80 uU/ml. Serum concentrations of glucose and highly sensitive C-reactive protein (hs-CRP) were determined by the use of the automated instrument COBAS INTEGRA 800 (Roche Diagnostics, Mannheim, Germany). The sensitivity for the glucose was 0.24 mmol/L (4.32 mg/dL) and the intra- and inter-assay coefficients of variation never exceeded 1.5 %. The sensitivity for hs-CRP assay was 0.1 mg/L and intra- and inter-assay coefficients of variation were 4.1 and 6.4 % for hs-CRP (0.423 mg/L). Adiponectin was measured by an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). The minimum detectable dose was 0.25 ng/mL; the intra-assay CVs were 2.5 % (19.8 ng/mL) and the inter-assay precisions from 40 different assays of 3 samples of known concentration of 20.5 ng/mL was 6.8 %. Serum IGF-I and IGFBP-3 were measured by ELISA using reagents from Immunodiagnostic System, Ltd (Baldon, UK). IGFBP-1 was measured by ELISA (Abcam, Cambridge, UK). The sensitivity of the assay was 5 pg/ml,

and intra and inter-assay coefficients of variation were 3.9 and 6.3 %, respectively. The ratio of IGF-I over IGFBP-1 + IGFBP-3 was calculated as an estimate of free IGF-I.

Serum drug concentrations were measured at the end of the 4-week intervention in a subgroup of 41 subjects who had an interval <24 h from last drug intake to blood draw by HPLC, as previously described [22]. All biomarker measurements, including drug concentration levels, were done blinded to the allocated arm.

Statistical analysis

Main descriptive statistics were median and interquartile range (IQR). Spearman's rank correlation coefficients were used to test univariate correlations between continuous variables. The effect of treatment was tested using the standard approach for studies of biomarker changes, i.e., an analysis of covariance (ANCOVA) linear regression model with change in ki-67 as response variable, and baseline ki-67 and treatment arm as covariates [23]. Treatment effect modifications were statistically assessed testing the appropriate interaction term in the linear regression model described above, adjusting for age and BMI. We used the subpopulation treatment effect pattern plot (STEPP) methodology [24] to graphically explore the effect of the treatment on the change in ki-67 considering increasing values of the biomarkers considered, as previously described in the initial study finding [9]. While many comparisons may expose to false discovery, we did not apply any adjustment for multiple testing given the exploratory nature of the study, mainly based on secondary endpoints, aimed at generating further hypotheses. Median values and IQR of ki-67 changes according to identified thresholds and treatment arm are also presented. Except for HOMA and ER status, which were predefined in the study protocol, and HER2 status, defined by positivity, putative thresholds on other biomarkers were empirically anticipated looking at the STEPP. Linear regression model was used also to assess the association of ki-67 change with blood drug levels and to test the interaction with HOMA index. Post-treatment ki-67 was log-transformed ($\ln\text{ki-67}$) and the normal distribution of residuals of fully adjusted models was graphically checked. All analyses were conducted using STATA (version 11) and SAS (version 9.2). A two-tailed p value of 0.05 was considered as cut-off value to define the statistical significance.

Results

At baseline, there were significant correlations among markers of insulin resistance. For instance, BMI was positively related with hs-CRP ($r = 0.44$, $p < 0.0001$) and negatively related with IGFBP-1 ($r = -0.28$, $p < 0.0001$),

Table 1 Effect of metformin and placebo on biomarkers of insulin resistance and tumor proliferation (ki-67)

Biomarker	Metformin		Placebo		Treatment effect ^c	<i>p</i> [*]
	Pre-treatment <i>N</i> = 100	Post-treatment <i>N</i> = 97	Pre-treatment <i>N</i> = 100	Post-treatment <i>N</i> = 99		
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Mean (95 % CI)	
Weight (kg)	65(56; 73)	65 (56; 74)	62 (57; 71)	63 (57; 71)	−0.09 (−0.85; 0.67)	0.8
BMI (kg/m ²)	24.1 (21.3; 27.4)	24.1 (21.3; 27.5)	23.9 (21.8; 27.3)	24.1 (22.0; 27.3)	−0.05 (−0.35; 0.25)	0.7
Glucose (mmol/L)	87 (83; 93)	87 (81; 94)	91 (87; 98)	91 (83; 96)	−0.64 (−3.78; 2.49)	0.7
Insulin (mU/L)	9.1 (5.7; 12.4)	7.2 (5.6; 12.3)	8.9 (6.6; 12.7)	7.6 (5.2; 12.9)	−0.75 (−3.36; 1.85)	0.6
HOMA index ^a	1.9 (1.2; 2.8)	1.6 (1.1; 2.8)	2.0 (1.4; 3.0)	1.7 (1.2; 3.1)	−0.27 (−1.19; 0.65)	0.6
C-peptide (ng/mL)	1.5 (1.1; 2.2)	1.5 (1.1; 2.0)	1.6 (1.3; 2.0)	1.5 (1.1; 2.2)	−0.04 (−0.25; 0.17)	0.7
hs-CRP (mg/L)	1.4 (0.6; 3.0)	1.0 (0.5; 2.1)	1.0 (0.5; 2.1)	1.2 (0.5; 2.1)	−0.47 (−1.06; 0.12)	0.1
IGFBP-1 (ng/mL)	3.5 (2.1; 6.2)	3.6 (2.0; 6.1)	3.9 (2.5; 5.4)	3.6 (2.4; 5.9)	−0.10 (−0.98; 0.78)	0.8
IGFBP-3 (μg/mL)	4.1 (3.7; 4.6)	4.0 (3.6; 4.5)	4.3 (3.7; 4.7)	4.2 (3.6; 4.6)	0.03 (−0.10; 0.15)	0.7
IGF-I (ng/mL)	136 (111; 164)	134 (109; 164)	150 (123; 177)	147 (120; 184)	−3.1 (−10.0; 3.7)	0.4
Free IGF-I ^b	0.18 (0.15; 0.20)	0.18 (0.16; 0.21)	0.20 (0.17; 0.22)	0.20 (0.16; 0.23)	−0.006 (−0.01; 0.002)	0.1
Adiponectin (ng/ml)	9.6 (6.2; 12.6)	9.1 (5.9; 11.8)	8.2 (6.0;13.6)	8.4 (5.8; 12.6)	−0.64 (−1.17; −0.12)	0.02
ki-67 (%)	19 (14; 33)	21 (14; 32)	18 (12; 29)	20 (13; 31)	0.30 (−1.93; 2.53)	0.8

Data on HOMA index were available for 199 patients

IQR interquartile range, Δ post–pre treatment difference

* *p*-interaction between treatment and biomarker

^a Homeostasis model assessment (HOMA) formula: fasting blood glucose (mmol/L) X insulin (mU/L)/22.5

^b Free IGF-I = IGF-I/(IGFBP-1 + IGFBP-3)

^c Effect of metformin relative to placebo on the change (difference surgery-baseline) calculated from the linear regression model (dependent variable: change of biomarker, adjusted for the biomarker level at baseline, BMI and age). Interpretation for treatment effect: a positive value stands for an increase in the metformin arm relative to the placebo arm, a negative value for a decrease

and HOMA index was negatively related with IGFBP-1 ($r = -0.45$, $p < 0.0001$). Interestingly, baseline ki-67 levels exhibited a borderline significant positive correlation with HOMA index ($r = 0.13$, $p = 0.07$) and a negative correlation with IGFBP-1 ($r = -0.13$, $p = 0.07$) (all data not shown).

The effects of metformin and placebo on biomarkers of insulin resistance and ki-67 labeling index are illustrated in Table 1. Overall, there was no significant change compared with placebo on any biomarker except for adiponectin, which was decreased to a larger extent by metformin relative to placebo. However, the effect of metformin on IGFBP-1 and hs-CRP was significantly modified by BMI, since metformin increased IGFBP-1 and decreased hs-CRP levels only in women with BMI > 25 (Fig. 1).

Figure 2 illustrates the STEPP analyses of the change of ki-67 (the difference between endpoint surgery and baseline biopsy) in the metformin arm relative to the placebo arm according to the continuous scale for the following markers of insulin resistance: C-peptide, IGFBP-3, IGFBP-1, and free IGF-I. Metformin had a heterogeneous effect depending on markers of insulin resistance, which

manifested itself in a tendency to decrease ki-67 levels in women with insulin resistance (approximately a quarter), defined by high levels of HOMA, C-peptide, IGFBP-3, and by low levels of free IGF-I and IGFBP-1, whereas it showed a trend to an increase of ki-67 in the majority of the remaining women. Except for C-peptide (p -interaction = 0.3), all variables illustrated in Fig. 2 as well as hs-CRP significantly modified the effect of metformin on ki-67 at $p < 0.1$. Variables not exhibiting a significant interaction with metformin were: weight, BMI, glucose, insulin, adiponectin, and total IGF-I.

The differential effects of metformin on ki-67 changes according to the biomarker categories thresholds with p values for interactions are shown in Table 2. Approximately 25 % of our study population had biomarker levels of insulin resistance, whereas 11 % had HER2-positive tumors. In the placebo arm, a remarkable increase in median and IQR range of ki-67 between the baseline biopsy and the surgical sample was observed in women with HER2-positive tumors and in the highest hs-CRP tertile (>1.81 mg/L), and, to a lesser extent, in women in the lowest IGFBP-1 quintile (<2 ng/mL). A rise in ki-67

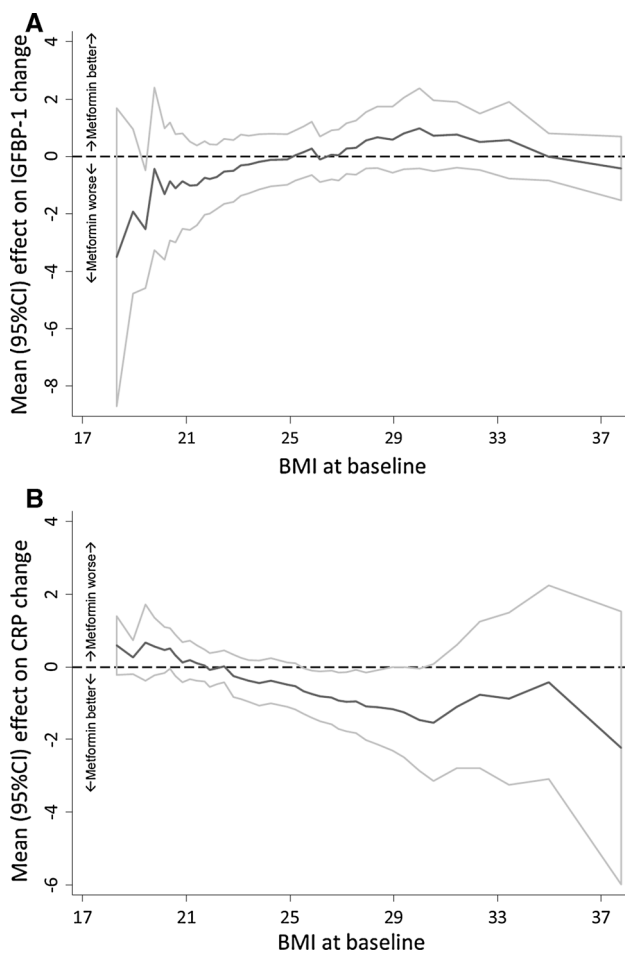


Fig. 1 Subpopulation Treatment Effect Pattern Plots (STEPP) of the change (difference between post- and pre-treatment level) of IGFBP-1 (**a**) and hs-CRP (**b**) according to body mass index (BMI). For IGFBP-1, positive change, metformin better; negative change, metformin worse; for hs-CRP, positive change, metformin worse; negative change, metformin better. *p* value for interaction treatment * BMI (threshold 25 kg/m²) = 0.09 and 0.05, respectively (from the linear regression model, adjusting for the biomarker levels at baseline and age)

between the time of biopsy and definitive surgery in patients with HER2-positive tumors receiving placebo has been well documented [25], although the basis of this phenomenon is not clear. It may relate to a true biological increase, but the possibilities of technical factors (such as difference in tissue procurement [biopsy versus surgery]) have not been excluded. In any case, these increases were significantly blunted by metformin. Also, in women with no insulin resistance as identified by HOMA < 2.8 or IGFBP-3 below the upper quartile (4.6 μg/mL), metformin exhibited a trend to an increase in ki-67 compared to placebo, although the IQR range included negative values. There was no significant interaction between metformin and BMI categorized at either 25 or 27 (*p* = 0.22).

The effect of metformin on ki-67 change in HER2-positive tumors is further illustrated in Fig. 3. Compared with placebo, metformin decreased ki-67 by a mean 5 % units in HER2-positive tumors, whereas there was a nearly 1 % mean unit increase under metformin in tumors not overexpressing HER2 (*p*-interaction = 0.076).

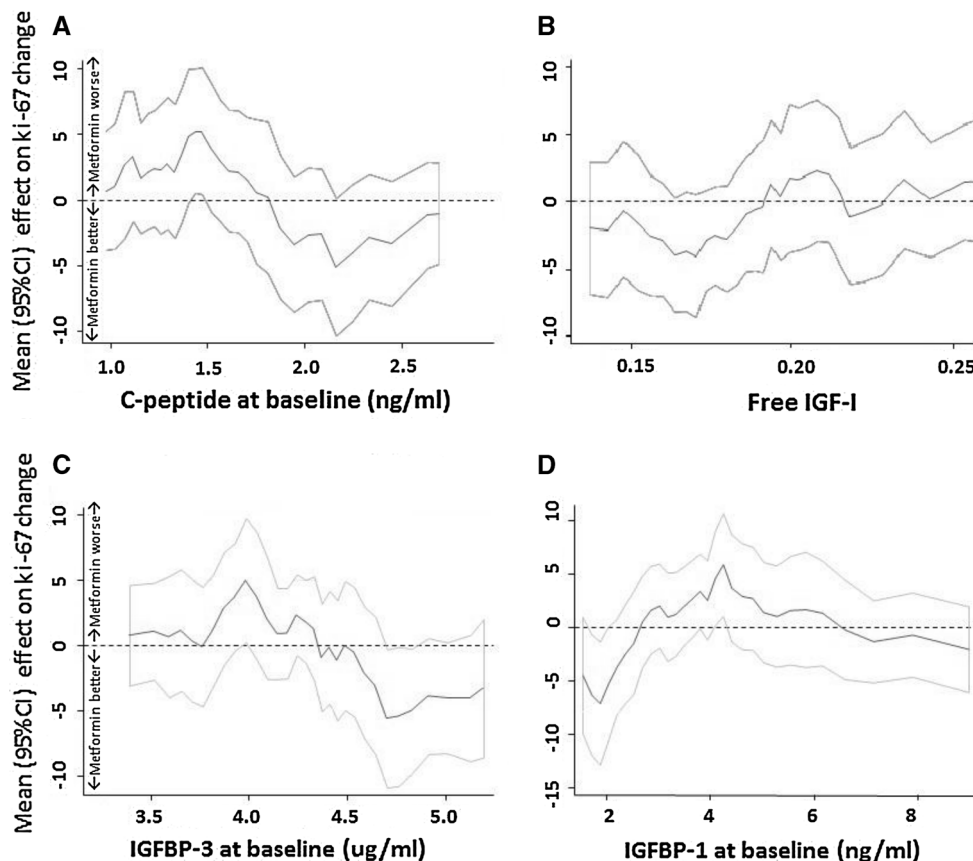
To further elucidate the effects of metformin on ki-67 according to HOMA index, serum drug concentrations were measured in a subgroup of 41 subjects in the metformin arm and in three random subjects in the placebo arm. Metformin was not detected in the placebo-treated patients. As expected, there was a significant negative correlation between serum drug levels and time (hours) since last drug intake (Spearman rho = -0.5, *p* = 0.001). However, time between drug intake was not associated with ki-67 change (coefficient = -0.27, *p* = 0.6, supplemental Table 1). Interestingly, there was a different association between serum drug levels and ki-67 change according to HOMA index (*p*-interaction = 0.07, Fig. 4). Specifically, in women with HOMA index > 2.8, higher drug concentrations were associated with a greater decrease of ki-67 (*p* = 0.04), whereas no association was noted in women with HOMA < 2.8 (*p* = 0.68). The association between drug levels and ki-67 change was independent of age, BMI, and interval from last drug intake. Likewise, no biomarker change was significantly affected by the time since last drug intake, including, blood glucose (Table 3).

Discussion

Our findings provide evidence for a complex heterogeneous effect of metformin on breast cancer proliferation in women without diabetes. Relative to placebo, metformin decreased ki-67 only in women with insulin resistance or HER2-positive tumors, whereas it showed a trend to an increase of ki-67 in the remaining women. In some subgroups, particularly in women with HER2-positive tumors and top hs-CRP levels, metformin significantly blunted the ki-67 rise observed in the placebo arm. This heterogeneous response to metformin also was influenced by serum drug measurements, inasmuch as high serum drug levels induced a greater ki-67 decrease in women with HOMA index > 2.8, whereas no effect was noted in women with HOMA < 2.8. All subgroup analyses, except for HOMA, are exploratory in nature and should, therefore, be considered hypothesis generating rather than definitive. However, given the epidemic of insulin resistance in western countries and the common association of insulin resistance and/or type II diabetes with breast cancer, our findings may have important clinical implications.

Our findings differ from those presented by Goodwin et al. in the first 500 patients enrolled in the NCIC CTG

Fig. 2 Subpopulation treatment effect pattern plots (STEPP) of the change (difference between endpoint surgery and baseline biopsy) of ki-67 according to the following covariates: C-peptide (panel A), free IGF-I (panel B), IGFBP-3 (panel C), IGFBP-1 (panel D). Positive change, metformin worse; negative change, metformin better. *p* values for interaction with treatment from the linear regression model (response variable: change of ki-67, adjusted for: ki-67 and BMI at baseline, age): C-peptide, *p* = 0.3 (threshold: median, 1.53 ng/mL); free IGF-I, *p* = 0.03 (continuous variable); IGFBP-3, *p* = 0.04 (median, 4.2 µg/mL); IGFBP-1, *p* = 0.016 (20th p.le, 1.91 ng/mL)



MA.32, a multicenter adjuvant trial in early-stage breast cancer, where favorable effects on metabolic markers were noted but no significant interaction between metformin and BMI or insulin was observed on insulin, glucose, leptin, and CRP at 6 months [26]. However, comparison between the two studies is difficult since the two populations differ substantially in mean BMI (24 in our study versus 28 in MA.32) and because we measured insulin resistance biomarkers which were not explored in the MA.32 study.

Our complex results are partly attributable to the increase of ki-67 from baseline biopsy to endpoint surgery in the placebo arm in several subgroups, particularly women with HER2-positive tumors and hs-CRP > 1.8 mg/L. Increases of ki-67 in the placebo arms have been noted in studies of a similar design addressing hypotheses unrelated to metformin [25, 27, 28]. We favor the interpretation that these findings do reflect a genuine biopsy or surgery-induced wound healing effect that stimulates cancer proliferation in specific tumor subtypes and host-related characteristics. In this regard, a previous study [29] has shown that by comparing histological sections of primary breast cancers overexpressing HER2 with residual tumors found in re-excision specimens, ki-67 increased by 10 % after a mean interval of 5 weeks. Moreover, drainage fluids collected from breast cancer patients shortly after surgery

were particularly active in stimulating HER2-positive cell lines, whereas wound-induced in vitro proliferation was blunted when these cell lines were treated with trastuzumab before drainage fluid was added. Our data show, with respect to this issue, that metformin is particularly effective in blunting the proliferative surge associated with HER2-positive tumors following surgery [30]. In vivo, metformin is able to induce downregulation of HER1 and HER2 and in vitro the drug inhibits self-renewal and proliferation of cancer stem cells in HER2 overexpressing breast cancer cell [31]. One implication of these data is that metformin administration may be of particular value if used in a neoadjuvant or perioperative context especially during the critical short period of metastasis formation which follows surgery, rather than postoperatively in an adjuvant fashion. A neoadjuvant clinical trial addressing the activity of metformin in HER2-positive breast tumors is underway [32].

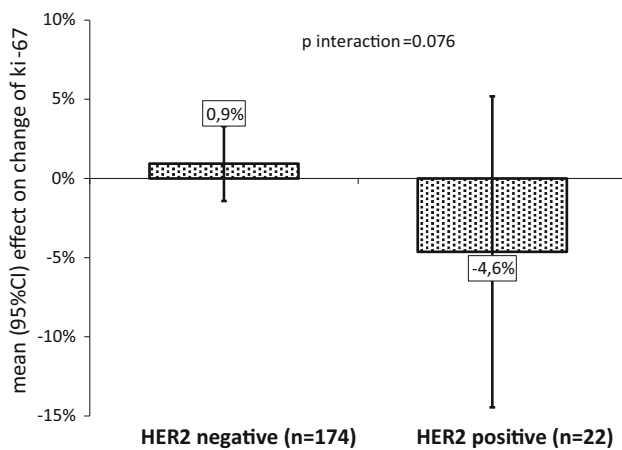
We previously reported [9] metformin-associated reductions of ki-67 labeling index in an insulin-resistant subset of patients defined by HOMA index, BMI, or hs-CRP. We now shows that IGFBP-1 levels at baseline are particularly useful to identify a subpopulation in which metformin is associated with reduced ki-67 labeling. IGFBP-1 is strongly downregulated by insulin. Thus, low

Table 2 Median (IQR) ki-67 changes by treatment arm and biomarker cutoff points

Risk biomarker threshold	N	Placebo	Metformin	p-interaction ^a
HOMA index > 2.8	53	0 (−2.0; 5.0)	0 (−5.0; 2.5)	0.03
HOMA index ≤ 2.8	142	0 (−2.0; 4.0)	1 (−2.0; 7.0)	
hs-CRP > 1.81 mg/L (3rdtertile)	65	2.5 (0;7)	0 (−3; 4)	0.02
hs-CRP ≤ 1.81 mg/L	131	0 (−3;5)	0.5 (−2; 8)	
IGFBP-3 > 4.6 µg/mL (4thquartile)	50	0 (−5.0; 7.0)	0 (−4.0; 2.0)	0.04
IGFBP-3 ≤ 4.6 µg/mL	146	0 (−1.5; 4.0)	1 (−3.0; 7.0)	
IGFBP-1 < 2 ng/mL (1st quintile)	40	1 (−5.0; 14.0)	0 (−4.0; 5.0)	0.02
IGFBP-1 ≥ 2 ng/mL	156	0 (−2.0; 4.0)	0.5 (−3.0; 7.0)	
HER2-positive	22	3.5 (0; 14.0)	0.5 (−4.0; 8.0)	0.076
HER2-negative	174	0 (−3.0; 4.0)	0 (−3.0; 7.0)	

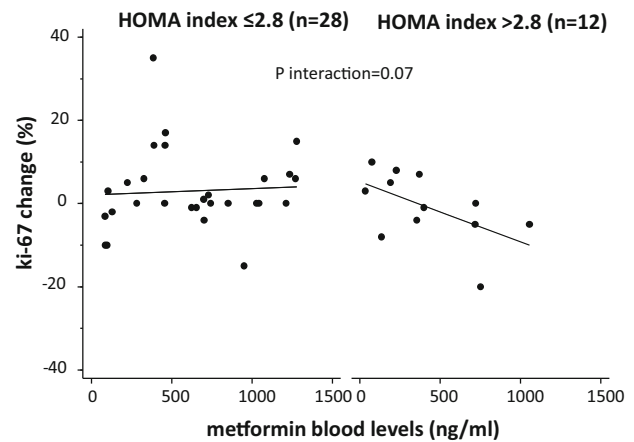
IQR interquartile range

^a p values for treatment, * covariate interaction on ki-67 change

**Fig. 3** Effect of metformin on ki-67 change (difference between endpoint surgery and baseline biopsy) by HER2 tumor status

IGFBP-1 represents a consequence of hyperinsulinemia. The consequences of reduced IGFBP-1 would be expected to include increased bioactivity of IGF-I and IGF-II in microenvironments where IGF bioactivity is probably constrained by IGFBP-1. As expected, we observed that women with low IGFBP-1 tend to have high BMI and high insulin levels. Interestingly, baseline ki-67 levels exhibited a borderline significant positive correlation with HOMA index and a negative correlation with IGFBP-1, suggesting that hyperinsulinemia is associated with tumor proliferation. Also, women with high IGFBP-3 and consequently low levels of free IGF-I tend to respond to metformin with a ki-67 decrease.

The trend to an increased proliferation in insulin sensitive women, albeit non-significant, suggests a complex

**Fig. 4** Scatterplot of ki-67 change (difference between endpoint surgery and baseline biopsy) and blood levels of metformin in a selected subgroup of women ($n = 40$) according to HOMA index levels (≤ 2.8 , $n = 28$, $p = 0.6$, and > 2.8 , $n = 12$, $p = 0.04$). p for treatment * HOMA index interaction = 0.07. Adjusted for age, BMI, and time from last drug intake. One subject was not assessable because ki-67 was missing at endpoint surgery**Table 3** Association between time since last metformin intake and change in biomarker

Biomarker	Coefficient	95 % CI	p
ki-67	−0.00202	−0.01752 ÷ 0.01349	0.8
Glucose	−0.00306	−0.02319 ÷ 0.01706	0.8
Insulin	−0.00801	−0.02609 ÷ 0.01006	0.4
HOMA index	−0.00231	−0.00862 ÷ 0.00401	0.5
C-peptide	−0.00046	−0.00189 ÷ 0.00098	0.5
hs-CRP	−0.00059	−0.00478 ÷ 0.00360	0.8
IGFBP-1	−0.00104	−0.00707 ÷ 0.00498	0.7
IGFBP-3	0.65105	−0.19487 ÷ 1.49696	0.1
IGF-I	0.00512	−0.04094 ÷ 0.05118	0.8
Free IGF-I	−0.00002	−0.00007 ÷ 0.00004	0.6
Adiponectin	−0.00016	−0.00371 ÷ 0.00339	0.9

From linear regression models (one for each biomarker) setting biomarker change as the response variable and time since last metformin intake as explanatory variable, adjusting for age, treatment arm, and biomarker level at baseline. The coefficient is estimated for the variable “time since last metformin intake” from each regression model

biological effect of metformin in these women. Animal studies have shown that metformin may have tumor suppressive effects where a metabolic phenotype of high caloric intake, metabolic syndrome, and diabetes exists, but no effect or even a growth promoting effect under normal energy intake [33, 34]. These data, together with laboratory studies that provide evidence that in certain contexts activation of AMPK may actually increase cell survival [35], and raise the possibility that metformin exposure may not be beneficial for all women in the context of breast cancer prevention and treatment.

It is clear that in breast cancer patients without diabetes, higher insulin or C-peptide levels are associated with poor prognosis [36, 37] a finding that extends also to colorectal and prostate cancer [38, 39]. However, it is not certain if insulin directly mediates this effect or acts as a surrogate for other effectors. The presence of insulin receptors in breast cancer tissue [40–42] certainly is in keeping with a direct mediating action of insulin, but recent data show that administration of insulin to diabetics (which leads to much higher circulating levels than are present in untreated type II diabetes) is not associated with an adverse effects on cancer endpoints [43, 44] emphasizing that caution is required in attempts to associate changes in insulin exposure with changes in breast cancer prognosis [45]. A prior phase III breast cancer adjuvant treatment trial used a somatostatin analog to lower insulin and IGF-I levels; statistically significant but small magnitude reductions in hormone levels were achieved, but treatment for two years was not associated with long-term clinical benefit [37].

Prior studies in different populations treated with metformin (for example [46] have not demonstrated the increase in adiponectin that would be expected with reduced insulin resistance. Indeed, we observed an unexpected modest decrease (0.64 ng/mL, 7 %) in adiponectin levels in women on metformin compared to those on placebo, but the magnitude of this change and its large statistical variability is of uncertain clinical significance.

Measurements of drug levels in a patient subgroup where blood draw was within 24 h of last drug intake reinforce the concept of heterogeneity of metformin effect on cancer proliferation depending upon insulin resistance. Although as expected the serum drug concentration was a function of the interval from the last drug intake, a positive relationship between drug levels and ki-67 response was evident only in women with HOMA > 2.8 and was independent of the interval from last drug intake. No relationship was noted between drug levels and ki-67 change in women without insulin resistance. These findings imply that drug levels achieved with conventional antidiabetic doses are sufficient to influence at least some tumors in patients with insulin resistance, likely by an “indirect” mechanism involving changes in the host hormonal milieu secondary to actions in the liver, where it is known that metformin at anti-diabetic doses has an impact on gluconeogenesis [1, 7]. “Direct” actions would be unlikely to vary with insulin resistance, while the hypothesized “indirect” mechanism would be expected to be more important when insulin resistance is high. Also in keeping with this interpretation is the fact that the measured serum concentration of metformin is lower than that used in most laboratory studies that demonstrate a “direct” effect. However, larger studies will be required to more formally

evaluate the relationship between peak and through metformin levels and the influence of the drug on tumorki-67, or ultimately on clinical endpoints related to efficacy of breast cancer treatment. It is possible that phenformin or other biguanides may be more effective than metformin in achieving “direct” effects [1]. A potential limitation is the variation in the duration of the interval from last drug intake to blood drawing (median 36 h, IQR, 14–43) and surgery (median 67 h, IQR 21–74), which is longer than the blood half-life of metformin (approximately 18 h, ref 15). However, we observed no significant association between any biomarker changes and the interval from treatment cessation, including, quite unexpectedly, blood glucose levels, suggesting that metformin effects may persist for several days from drug cessation [47, 48]. For instance, studies in diabetics have shown that the activation of AMPK α 2 by metformin persisted after an overnight withdrawal, when expected concentrations of the drug in blood would be low [49]. Finally, we gave metformin 1,700 mg once daily after dinner and not *bis in die* to minimize gastrointestinal symptoms during daytime and to attain higher blood levels in the subsequent morning blood sample. If anything, however, the wash-out time variability diluted our findings toward the null hypothesis without affecting overall conclusions.

In conclusion, our data describe effects of metformin on the hormonal/metabolic profile and tumor cell proliferation estimated by ki-67 labeling index in non-diabetic breast cancer patients, and show that there is considerable heterogeneity in these effects within the study population. We define novel biomarkers, such as HER2-positive tumors and serum IGFBP-1, which identify a subset of women for whom metformin given at conventional anti-diabetic doses reduces tumor cell proliferation. It is important to put the magnitude of effects of metformin on ki-67 into context relative to the effects of approved breast cancer drugs. Collectively, the metformin “window of opportunity trials”, including our data suggest an effect of metformin on ki-67-estimated proliferation in certain subgroups, but even within these subgroups the effect size is considerably less than that of tamoxifen [27] or aromatase inhibitors on ER-positive breast cancer [50]. These findings suggest that benefits of metformin in breast cancer may be confined to specific patient subgroups. In view of low toxicity and cost, metformin deserves study in breast cancer treatment and prevention, particularly peri-operatively and in combination with other agents [51].

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