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The effect of metformin on apoptosis in a breast cancer presurgical trial

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Background: Metformin has been associated with antitumour activity in breast cancer (BC) but its mechanism remains unclear. We determined whether metformin induced a modulation of apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) overall and by insulin resistance status in a presurgical trial.

Methods: Apoptosis was analysed in core biopsies and in surgical samples from 100 non-diabetic BC patients participating in a randomised trial of metformin *vs* placebo given for 4 weeks before surgery.

Results: Eighty-seven subjects (45 on metformin and 42 on placebo) were assessable for TUNEL measurement at both time points. TUNEL levels at surgery were higher than that at baseline core biopsy ($P<0.0001$), although no difference between arms was noted (metformin arm: median difference surgery-biopsy levels +4%, interquartile range (IQR): 2–12; placebo arm: +2%, IQR: 0–8, $P=0.2$). Ki67 labelling index and TUNEL levels were directly correlated both at baseline and surgery (Spearman's $r=0.51$, $P<0.0001$). In the 59 women without insulin resistance (HOMA index<2.8) ,there was a higher level of TUNEL at surgery on metformin *vs* placebo (median difference on metformin +4%, IQR: 2–14 *vs* +2%, IQR: 0–7 on placebo), whereas an opposite trend was found in the 28 women with insulin resistance (median difference on metformin +2%, IQR: 0–6, *vs* +5%, IQR: 0–15 on placebo, *P*-interaction = 0.1).

Conclusion: Overall, we found no significant modulation of apoptosis by metformin, although there was a trend to a different effect according to insulin resistance status, with a pattern resembling Ki67 changes. Apoptosis was significantly higher in the surgical specimens compared with baseline biopsy and was directly correlated with Ki67. Our findings provide additional evidence for a dual effect of metformin on BC growth according to insulin resistance status.

Recent evidence indicates a strong relationship between the presence of hyperinsulinemia, insulin resistance and breast cancer (BC). This condition partly explains the relationship between obesity and BC risk in postmenopausal women (Renehan *et al*, 2008; Gunter *et al*, 2009; Decensi and Gennari, 2011). There is a great interest in exploring the possibility that antidiabetic therapies lowering insulin levels may decrease BC incidence and mortality.

Metformin, an oral biguanide, has been used worldwide to treat type 2 diabetes and pre-diabetic condition for more than 40 years because of its good tolerability profile and low cost (Drugs.com, 2011 http://www.drugs.com/pro/metformin-html). Recent epidemiological and observational studies have shown an association between metformin use and reduced cancer incidence and cancer-related mortality, compared with other antidiabetic treatments in

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diabetic patients (Evans *et al*, 2005; Bowker *et al*, 2006; Decensi *et al*, 2010). However, the comparator groups are mostly formed by users of insulin and sulphanylureas, two drugs possibly associated with increased cancer risk (Johnson and Gale, 2010; Soranna, *et al*, 2012). Although the metformin mechanism of action is still under investigation, preclinical studies suggest a direct antineoplastic activity (Cazzaniga *et al*, 2009), entailing both insulin-dependent and -independent mechanisms (Zhou *et al*, 2001; Zakikhani *et al*, 2006; Mulligan *et al*, 2007; Goodwin *et al*, 2008, 2009; Gonzalez-Angulo and Meric-Bernstam, 2011). In humans, it is unclear whether these effects apply also to non-diabetic subjects or to subjects without insulin resistance. We have recently shown that a 4-week pre-surgical treatment with metformin in BC patients does not affect the proliferation antigen Ki67 labelling index (LI) overall, but does lower tumour proliferation in women with insulin resistance as measured by the homeostasis model assessment index (HOMAi, fasting blood glucose ($mmol\,l^{-1}$) $\times$ insulin ($mU\,l^{-1}$)/22.5 > 2.8) or with body mass index (BMI) > $27\,kg\,m^{-2}$ (Bonanni *et al*, 2012). These effects were particularly evident in the luminal B molecular subtype, suggesting a potential therapeutic effect of metformin in this tumour type.

In a recently completed window of opportunity, single-arm trial, Niraula *et al* (2012) have shown, in comparison with baseline, an increase in apoptosis or programmed cell death, as measured by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and a decrease of Ki67 LI after a median of 3 weeks of metformin before surgery. The aim of the current analysis was to determine whether metformin can modulate apoptosis overall and by HOMAi in a large group of subjects participating in a randomised presurgical trial.

PATIENTS AND METHODS

At the European Institute of Oncology (IEO), Milan, Italy, we conducted a randomised, phase II, double blind, placebo-controlled trial on women with stage I–IIIa BC who were candidates for elective surgery. The study was conducted with the approval of the European IEO ethical committee. Having provided written informed consent (for participation in the trial and publication of the data when appropriate), the patients received either metformin or placebo for 4 weeks before surgery (trial number S425/408, EudraCT 2008-004912-10). Baseline core biopsies of the tumour tissue and blood samples were obtained at study entry and before surgery to allow pre-/post-treatment comparisons. A detailed description of study design and primary end point Ki67 LI has recently been published (Bonanni *et al*, 2012). Briefly, 200 non-diabetic patients were randomly assigned to metformin, 850 mg tablets once daily for 3 days followed by 850 mg b.i.d. or placebo after dinner from day 4 to 28.

In the present analysis, we analysed the apoptotic cell nuclei by TUNEL from core biopsies and their paired surgical samples from the last 100 recruited subjects. On the basis of Niraula *et al* (2012), this number was sufficient to detect as significant with a power of at least 80% a 30% relative increase from baseline in TUNEL levels.

Immunohistochemistry. Ki67 LI, oestrogen and progesterone receptors and Her2/neu were assessed at the IEO Division of Pathology in all core biopsies and post-treatment surgical samples, as previously described (Bonanni *et al*, 2012).

Assessment of the AI. Apoptotic cell nuclei were identified by the TUNEL method, using an *in situ* apoptosis detection kit (TREVIGEN Inc, 8405 Helgerman Ct., Gaithersburg, MD, USA), according to the manufacturer's instructions. Briefly, after rehydration in ethanol and fixation in 3.7%-buffered formaldehyde, sections were digested with proteinase solution (23 min at 37 °C), immersed in TdT labelling buffer, covered with the labelling reaction mix (37 min–1 h), immersed in streptavidin solution and dyed with DAB. The apoptotic index (AI) was assessed by counting any nuclear brown staining in the cells from invasive carcinoma in 10 microscopic fields at $\times 40$ magnification (HPF). Cases of invasive carcinoma with an extent smaller than that obtained by summing the diameter of 10 HPF were deemed not to be assessable for AI.

Circulating biomarkers. Morning fasting blood samples were collected between 0800 and 1000 hours at baseline and at treatment completion. Serum aliquots for insulin were measured on frozen samples stored at $-80\,^{\circ}C$ until assayed, whereas glucose was measured on fresh samples. Insulin was measured with an electrochemiluminescence immunoassay by COBAS e411(Roche Diagnostics, Mannheim, Germany). Serum concentrations of glucose was determined by COBAS INTEGRA 800 (Roche Diagnostics). A HOMAi greater than 2.8 was the cutoff of insulin resistance based on the population study conducted in Northern Italy (Bonora *et al*, 1998).

Statistical analysis. Sample size calculation and statistical methods of the current clinical trial have been previously detailed (Bonanni *et al*, 2012). In this analysis, because of the highly skewed distribution of TUNEL, results were mainly reported as median and interquartile ranges (IQR) in tables and graphically represented by boxplots. The Wilcoxon rank-sum (the Mann–Whitney) test was used to test the difference between arms, and the Wilcoxon-matched pair signed-rank test was employed for the differences between biopsy and surgery differences within arms. To adjust for age, BMI and Ki67 LI at baseline, linear modelling was also adopted with TUNEL values, using post-treatment TUNEL (square root transformed because of the highly skewed distribution rich of 0 and 1 values) as the response variable, and baseline TUNEL and treatment arm as explanatory variables. Treatment $\times$ covariate interaction terms were tested for BMI and HOMA index, and a *P*-value for interaction <0.1 was considered as statistically significant in order to decrease the false-negative rate.

All analyses were performed using STATA software, version 11 (StataCorp., College Station, TX, USA). Two-tailed probabilities were reported at $P = 0.05$ significance level.

RESULTS

The main host and tumour characteristics of the 100 subjects included in this analysis are reported in Table 1. All variables were evenly distributed between arms and no significant difference with the original cohort was observed.

Analysis of the apoptotic cell nuclei by TUNEL was feasible in 87 core biopsies and their paired surgical samples (45 on metformin and 42 on placebo). The median TUNEL levels at surgery was 10%, IQR: 4–20, in the metformin arm and 8%, IQR: 3–15, in the placebo arm. These levels were significantly higher ($P < 0.0001$) when compared with baseline biopsy in both arms (median difference surgery-biopsy levels in metformin arm: +4%, IQR: 2–12; placebo arm: +2%, IQR: 0–8; Figure 1). The difference in TUNEL levels at surgery between arms was not significant ($P = 0.2$ from a linear model adjusted for TUNEL at baseline, age, BMI and Ki67 LI at baseline).

When we considered the interaction between treatment and the HOMAi on TUNEL, we found a borderline significant trend to a different metformin effect according to the HOMAi ($P = 0.1$, Figure 2). Specifically, median (IQR) TUNEL levels at surgery among the 59 women without insulin resistance (HOMAi < 2.8) was 10% (6–22) on metformin compared with 6% (3–12) on placebo ($P = 0.048$), with a difference between surgery and biopsy equal to +4%, IQR: 2–14 in the metformin arm *vs* +2%, IQR:

Table 1. Tumour characteristics by allocated arm

	Metformin ($n=50$)	Placebo ($n=50$)	P^a
Age (median, IQR)	50, 45–62	49, 45–57	0.5
Body mass index (median, IQR)	24.2, 20.8–26.8	24.7, 21.4–28.3	0.6
HOMA index (median, IQR)	2.18, 1.15–3.13	2.18, 1.72–3.15	0.4
Ki67 LI (median, IQR)	21, 12–38	20, 15–34	0.9
T stage (*n*, %)			
pT1	12 (24)	18 (36)	0.2
pT2	32 (64)	30 (60)	
pT3	6 (12)	2 (4)	
Nodal status(*n*, %)			
pN0	19 (38)	15 (30)	0.2
pN1	17 (34)	24 (48)	
pN2	6 (12)	8 (16)	
pN3	8 (16)	3 (6)	
Mastectomy(*n*, %)			
Yes	28 (56)	34 (68)	0.2
No	22 (44)	16 (32)	
Histology(*n*, %)			
Ductal	44 (88)	45 (90)	0.2
Lobular	5 (10)	1 (2)	
Mixed	0 (—)	2 (4)	
Other	1 (2)	2 (4)	
Molecular subtype by IHC(*n*, %)			
Luminal A	13 (26)	9 (18)	0.3
Luminal B	25 (50)	30 (60)	
HER 2+	8 (16)	6 (12)	
Triple negative	4 (8)	5 (10)	
Grade(*n*, %)			
1	4 (8)	3 (6)	0.8
2	23 (46)	26 (52)	
3	23 (46)	21 (42)	
Peritumoral vascular invasion(*n*, %)			
0	31 (62)	23 (46)	0.3
1	9 (18)	9 (18)	
2	9 (18)	16 (32)	
3	1 (2)	2 (4)	
HER2 overexpression/amplification(*n*, %)			
Yes	8 (16)	6 (12)	0.6
No	42 (84)	44 (88)	

Abbreviations: IHC = immunohistochemistry; IQR = interquartile range, LI = labelling index.
[a]Wilcoxon rank-sum or Pearson's χ^2-test.

0–7 in the placebo arm. Conversely, among the 28 women with insulin resistance (HOMAi $\geqslant$ 2.8), median and IQR TUNEL levels was 6% (4–10) on metformin *vs* 9% (6–18) on placebo ($P=0.3$), with a median difference pre-/post-treatment difference equal to +2%, IQR: 0–6 in the metformin arm *vs* +5%, IQR: 0–15 in the placebo arm.

Interestingly, we found a highly significant positive correlation between KI67 LI and TUNEL levels both at baseline (Figure 3) and surgery (Figure 4) (Spearman's $\rho=0.5$ and 0.6, respectively, $P<0.0001$).

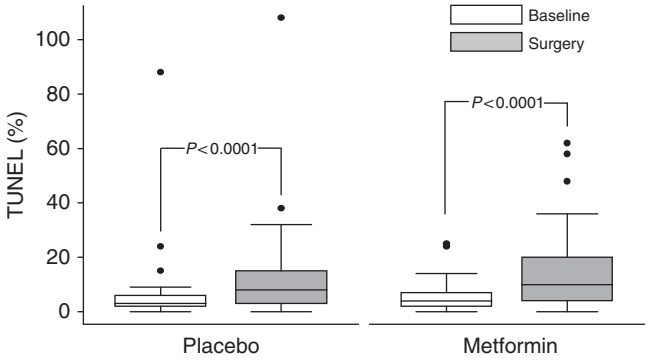


Figure 1. Boxplots of TUNEL levels by allocated arm at baseline and surgery. No difference between arms was noted ($P=0.2$, adjusted for age, BMI, baseline TUNEL and Ki67 LI). Median TUNEL levels were significantly higher at surgery compared with baseline biopsy in both arms ($P<0.0001$).

DISCUSSION

There is evidence to suggest that insulin resistance, hyperinsulinemia and the associated changes in IGFs, sex hormones, inflammatory and adipokine molecules levels are correlated with breast carcinogenesis (Calle and Kaaks, 2004; Pollak *et al*, 2004; Renehan *et al*, 2006; Yee, 2006). Abnormal tumour metabolism has therefore become a potentially new therapeutic target because several of these pathways are involved in carcinogenesis progression and potentially reversible by preventive and therapeutic interventions (Vander Heiden *et al*, 2009).

Antidiabetic therapies that lower or sensitise insulin effects may decrease BC incidence or its related mortality. Epidemiological (Decensi *et al*, 2010; Bosco *et al*, 2011, preclinical (Zakikhani *et al*, 2006; Sahra *et al*, 2008) and also clinical data by a recent retrospective analysis of patients receiving neoadjuvant chemotherapy and metformin (Jiralespong *et al*, 2009) suggest that this antidiabetic drug may exert antitumour activity through indirect (insulin mediated) and direct mechanisms of action, leading to a decreased proliferative activity of epithelial cells (Guppy *et al*, 2011).

Our recent window of opportunity trial had shown that a 4-week pre-surgical treatment with metformin did not affect proliferative activity overall as measured by Ki67 LI. However, there was a differential effect on Ki67 LI according to insulin resistance status. In the subgroup of patients with insulin resistance or who were overweight (BMI $\geqslant 27\,kg\,m^{-2}$), metformin therapy decreased Ki67 LI levels, particularly in the luminal B molecular subtype, whereas an opposite trend was noted in women with HOMAi $\leqslant$ 2.8 or BMI $< 27\,kg\,m^{-2}$.

In the present study based on the last 100 enrolled participants, we report the following three main findings: (1) apoptosis levels measured by TUNEL are higher in the surgical specimens compared with baseline core biopsy; (2) apoptosis is directly correlated with proliferation as measured by Ki67 LI, that is, TUNEL is high when Ki67 LI is high; and (3) the metformin effects on apoptosis mirrored those observed with Ki67, with a different effect according to HOMAi, that is, a trend to reduced TUNEL levels in insulin-resistant women and an increase in non-insulin-resistant women compared with placebo.

The higher TUNEL levels in the surgical specimen compared with baseline biopsy may be explained by a sampling effect in the first place. Given the scarcity of apoptosis in BC, the small tissue availability in the biopsy specimen may underestimate real levels that become evident in the surgical specimen.

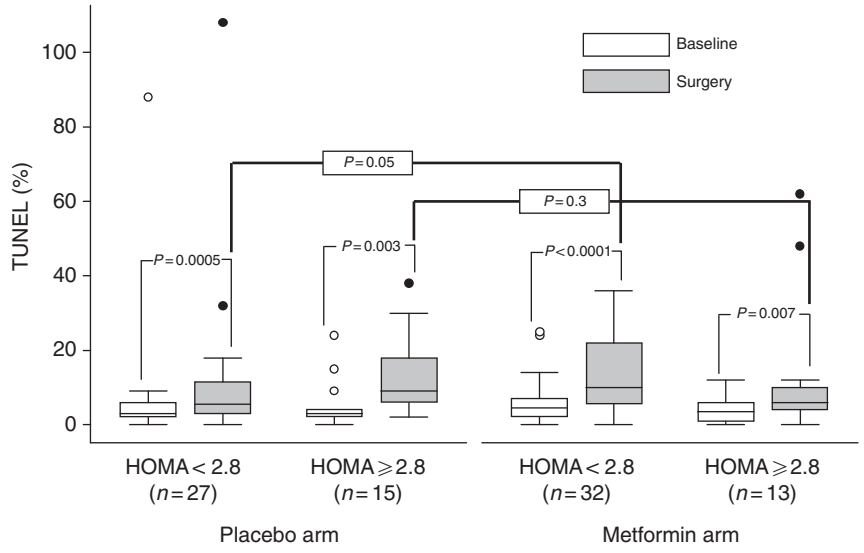


Figure 2. Boxplots of TUNEL levels by allocated arm and HOMAi according to a cutoff level of 2.8. The interaction between treatment and HOMAi was borderline significant ($P=0.1$).

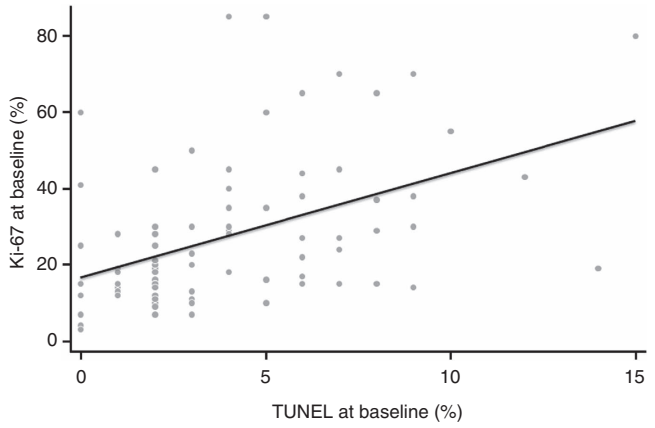


Figure 3. Correlation between Ki67 and TUNEL values at baseline in all study patients regardless of treatment arm (Spearman's $r=0.5$, $P<0.0001$).

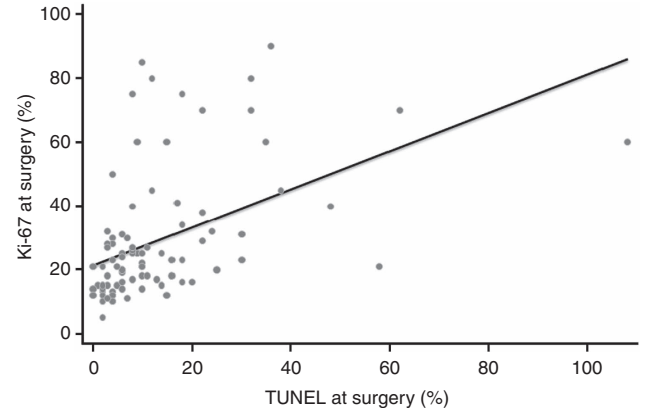


Figure 4. Correlation between Ki67 and TUNEL values at surgery in all study patients regardless of treatment arm (Spearman's $r=0.5$, $P<0.0001$).

The highly statistically significant direct correlation between Ki67 LI levels and TUNEL confirms the observation in several tumour types that cellular proliferation is accompanied by an increase of programmed cell death as a mechanism limiting a dimensional and faster abnormal growth of the neoplastic burden (Tan *et al*, 2005). Although metformin has been shown to promote cell death with an independent mechanism involving a direct activation of the apoptotic pathway in some studies (Zhuang and Miskimins, 2011), our findings suggest that an increased apoptosis is associated with an increased proliferation also in BC.

A close positive relationship between Ki67 LI and TUNEL has also been noted by the borderline significant interaction between metformin and HOMAi on TUNEL, resulting in a dual modulation according to insulin resistance status, similar to that which was noted in Ki67. We found a trend to a lower increase of TUNEL in insulin-resistant women under metformin relative to placebo and an opposite effect in non-insulin-resistant patients where TUNEL increased more under metformin compared with placebo. The decreasing trend in BC proliferation and apoptosis in women with high HOMAi under metformin suggests that indirect, insulin- and glucose-mediated effects are an important mechanism of the anticancer effect of metformin in human BC. Our results are consistent with the diabetes prevention trial (Knowler *et al*, 2002), where metformin had a significantly heterogeneous effect on diabetes onset according to baseline BMI and glucose levels, with a greater effect in obese and severely glucose-intolerant women.

Our findings strengthen the importance of a control arm in biomarker trials in order to adjust for the biological and technical variability of biomarkers. In the placebo arm of our trial, both Ki67 and TUNEL were significantly higher in the surgical sample compared with baseline. The increase in Ki67 LI within the placebo groups a few weeks apart has been associated with the most highly proliferating molecular subtypes (triple-negative and HER2-positive BC; Tagliabue *et al*, 2003; Gandini *et al,* 2013), suggesting a true biological increase in proliferation rather than a technical artifact due to different tissue sampling.

Our results differ from those obtained by Niraula *et al* (2012) who showed an increase in TUNEL along with a decrease in Ki67 LI after metformin treatment. Although we do not have a ready explanation for these differences, their study had a more limited sample (39 patients) and no control group. In addition, most patients were overweight or obese, in contrast to our study where only one quarter had those characteristics. Perhaps most importantly, different tumour characteristics and assay methods are likely, as our median Ki67 LI was nearly 50% lower and median TUNEL levels were ~10 times higher compared with those of Niraula *et al* (2012). Our laboratory was among those that

contributed to the recently published guidelines for the Ki67 measurement (Dowsett *et al*, 2011), which is known to be subject to a high variability. A potential limitation of our study is the drug cessation 24 h or longer before surgery, which was dictated by safety reasons (Drugs.com, 2011 http://www.drugs.com/pro/metformin-html). However, the finding of a selective effect of metformin only in insulin-resistant women weakens the criticism. Moreover, metformin can reach higher tissue:blood concentrations (Nestler, 2008), which should not decrease its biological effects up to several days from drug cessation. Finally, we had previously shown no significant association between any circulating biomarker changes and the interval from treatment cessation (Bonanni *et al*, 2012). If anything, the wash-out diluted our findings towards the null hypothesis without affecting overall conclusions.

CONCLUSIONS

These findings are hypothesis-generating and cannot have immediate clinical implications, but may have important public health implications in the near future. Indeed, the effects of metformin in insulin-resistant and/or obese women could be substantial and supportive of cancer therapeutic and preventive studies in women with these characteristics. This is especially important because the prevalence of obesity is rapidly increasing globally and has reached epidemic proportions in developed countries (Low *et al*, 2009). A phase III adjuvant trial is currently underway to determine the overall therapeutic effect of metformin and whether it differs according to obesity and insulin resistance (Goodwin *et al*, 2011). The results of this trial will shed light upon the cancer therapeutic effect of this fascinating drug.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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