Impact of baseline and on-treatment blood glucose levels on the efficacy of everolimus-exemestane in patients with advanced hormone-receptor positive breast cancer: the EVERMET study

running title: **Impact of plasma glucose levels on everolimus efficacy**

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ABSTRACT

The mTORC1 inhibitor everolimus (EVE) in combination with the aromatase inhibitor exemestane (EXE) is an effective treatment for patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative, advanced breast cancer (HR+/HER2- aBC). However, EVE can cause hyperglycemia and hyperinsulinemia, which could reactivate the PI3K/AKT/mTORC1 pathway and induce tumor resistance to EVE.

Here, we conducted a multicenter, retrospective, Italian study to investigate the impact of baseline and on-treatment (i.e. during first three months of therapy) blood glucose levels on progression-free survival (PFS) in 809 HR+/HER2- aBC patients treated with EVE-EXE. In a multivariable model accounting for clinically relevant patient- and tumor-related factors, we found that both baseline and on-treatment glycemia affect patient PFS, and this association is largely attributable to the interaction between the two variables. In particular, patients who are normoglycemic at baseline and experience on-treatment diabetes have an increased risk of tumor progression when compared with the remaining patients, and in particular when compared with patients who are already hyperglycemic at baseline and experience overt diabetes during EVE-EXE therapy.

Our results indicate that the impact of on-treatment glycemia on the efficacy of EVE-EXE therapy in HR+/HER2 aBC patients is affected by baseline glycemia. This study lays the foundations for investigating novel therapeutic approaches to target the glucose/insulin axis in combination with PI3K/AKT/mTORC1 inhibitors in HR+/HER2 aBC patients.

Commentato [32]: Treatment- related/ induced

Commentato [CG3]: Hyperglicemia?

Commentato [34]: Diabetes mellitus; For the definition of hyperglycemia used in EVERMET, this is essentially the treatment-related hyperglycemia. Same below.

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Commentato [36]: Treatment- related diabetes mellitus

Translational Relevance:

Everolimus (EVE) and other PI3K/AKT/mTORC1 pathway inhibitors are associated with metabolic adverse events, including hyperglycemia/diabetes and hyperinsulinemia. The impact of baseline and on-treatment blood glucose levels on the clinical efficacy of EVE-based combinations remains poorly

defined. Here we performed a large observational study, showing an interaction between baseline and on-treatment glycemia in affecting the risk of disease progression in advanced breast cancer patients treated with EVE-EXE combination. This study supports the use of early~~precocious~~ blood glucose alterations as an early biomarker of Everolimus treatment efficacy~~,~~ and provides the rationale for exploiting novel metabolic interventions as anticancer strategies in advanced breast cancer.

Commentato [37]: Early on-treatment glycemic variations

1. INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin complex 1 (mTORC1) pathway is the most commonly dysregulated oncogenic axis in hormone receptor-positive, HER2-negative breast cancer (HR+/HER2- BC) (1-4). In both preclinical and clinical studies, the PI3K/AKT/mTORC1 pathway has been crucially implicated in stimulating HR+/HER2- BC cell growth, proliferation and survival, as well as in causing primary or acquired tumor resistance to endocrine therapies (ETs) (5-7). In line with this preclinical evidence, randomized phase III trials showed that inhibiting different nodes of the PI3K/AKT/mTORC1 axis in combination with standard ETs results in a significant prolongation of progression-free survival (PFS) when compared to ET alone in HR+/HER2- advanced BC (aBC) patients (8,9). In particular, the BOLERO-2 trial demonstrated that the mTORC1 inhibitor everolimus (EVE) in combination with the steroidal aromatase inhibitor exemestane (EXE) improves PFS when compared to EXE alone in postmenopausal HR+/HER2- aBC patients progressing after/on prior non-steroidal aromatase inhibitor (NSAI) therapy (8). More recently, the PI3K inhibitor alpelisib in combination with the antiestrogen fulvestrant significantly prolonged PFS when compared with fulvestrant alone in patients with *PIK3CA*-mutated HR+/HER2- aBC progressing on previous AI therapy (9).

Metabolic adverse events (AEs), including hyperglycemia, hypercholesterolemia and hypertriglyceridemia, are common in patients treated with PI3K/AKT/mTORC1 inhibitors (8-11), and are considered a class effect of these drugs. In particular, hyperglycemia occurs in up to 17% of HR+/HER2- aBC patients treated with EVE (8,12), and results from a combination of impaired pancreatic β cell function, enhanced glycogen breakdown in the liver, and insulin resistance, which impairs glucose uptake in the skeletal muscle and adipose tissue (13-16). In turn, EVE-induced hyperglycemia can cause compensatory hyperinsulinemia, which could reactivate the insulin receptor (IR)/PI3K/AKT/mTORC1 pathway and make cancer cells resistant to EVE-EXE (17). In line with this hypothesis, a small

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retrospective Italian study showed that higher blood glucose levels during EVE-EXE therapy correlate with worse PFS in HR+/HER2- aBC patients (18). Moreover, one recent study demonstrated that PI3K inhibitor-induced increase of serum insulin concentration in cancer patients is sufficient to reactivate the PI3K/AKT/mTORC1 pathway, thus resulting in resistance to PI3K inhibition in HR+/HER2- BC cell lines and murine models (19).

Here, we performed a large, multicenter, retrospective study to investigate the impact of blood glucose levels on the efficacy of EVE-EXE treatment in HR+/HER2- aBC patients. We provide first evidence that both baseline and on-treatment glycemia are associated with EVE-EXE efficacy, and this effect is largely attributable to the interaction between these two variables.

Commentato [38]: Consider to omit the conclusions in the introduction part.

2. MATERIAL AND METHODS

2.1. Patient population and enrollment criteria

This was an observational, retrospective, multicenter study conducted in 20 Italian Cancer Centers [Fondazione IRCCS Istituto Nazionale dei Tumori di Milano (coordinating center); Istituto Oncologico Veneto di Padova; Policlinico Umberto I di Roma; Azienda Ospedaliero Universitaria Pisana; Azienda Ospedaliera Policlinico di Modena; Ospedale Policlinico San Martino di Genova; Ospedale Belcolle di Viterbo; Istituto Europeo di Oncologia, IRCCS - IEO di Milano; FPO-IRCCS Candiolo Cancer Institute; Humanitas Cancer Center di Milano; ASST di Cremona; Istituto Nazionale Tumori Regina Elena - IFO di Roma; Spedali Civili di Brescia; Ospedale "Vito Fazzi" di Lecce; Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS di Meldola; Università Federico II di Napoli; ASST Santi Paolo e Carlo di Milano; ASST Fatebenefratelli Sacco di Milano; IRCCS Centro di Riferimento Oncologico di Aviano; Azienda Sanitaria Universitaria Integrata di Udine]. Data were collected via electronic database.

The main enrollment criteria consisted in: 1) age ≥18 years; 2) histologically/cytologically confirmed diagnosis of HR+/HER2- advanced (inoperable locally advanced or metastatic) BC; 3) post-menopausal status, as defined as: a) patients of age equal to or higher than 60 years; b) patients of age lower than 60 years but with amenorrhea from at least 12 months that was not related to the administration of chemotherapy or LHRH analogs; c) pre/peri-menopausal patients receiving LHRH analogs in combination with EVE-EXE; 4) treatment for at least one month with daily EVE (initial dosage of 10 mg/day) plus EXE (25 mg/day) between January 2014 and December 2018 outside clinical trials sponsored by pharmaceutical companies; 5) disease recurrence or progression after/on prior therapy with NSAIs; 6) availability of at least one measurement of plasma glucose concentration at the initiation of EVE-EXE therapy or at 1, 2 or 3 months after treatment initiation); 7) any number of previous lines of treatment for advanced disease; 8) any prior therapy for localized disease, including (neo)adjuvant

Commentato [39]: Please to provide, thanks.

Commentato [310]: d) patients with ovarian ablation, either radiation therapy or bilateral ovariectomy

Commentato [311]: this use is off-label; we may prefer a phrasing like "*based on the local practice*".

Results of the MIRACLE/ LEO studies have been only recently reported, and not up-taken yet for LHRH-suppressed women.

Commentato [312]: "NSAI with or without CDK4/6" (this may be appropriate for the current treatment landscape)

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chemotherapy, surgery, ETs; patients with *de novo* metastatic disease at diagnosis were included as well. Prior NSAI therapy should not necessarily be the last treatment before EVE-EXE treatment. All patients were followed up until death, loss of contact, or time of data lock (31st July 2019). Written informed consent was obtained from all patients who were alive at the time of study conduction. The study was carried out in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. The study protocol was first approved by the Ethics Committee of the coordinating center (internal registration number of the study: INT 30/18), and then approved by Ethics Committees and/or Institutional Review Boards at each participating site.

2.2. Study objectives and statistical plan

The primary objective of the study was to investigate the association between the onset of precocious hyperglycemia and the PFS of HR+/HER2- aBC patients treated with EVE-EXE. Precocious hyperglycemia was defined as equal or higher than 126 mg/dl average fasting plasma glucose concentration during the first three months of EVE-EXE treatment (i.e. excluding baseline evaluation). PFS was defined as the time between EVE-EXE initiation and the detection of clinical/radiological disease progression or patient death from any cause, whichever came first. For sample size calculation, we assumed that 80% of patients had an average glycemia below 126 mg/dl during the first three months of EVE-EXE therapy and that normoglycemic patients had median PFS of 7 months. With these assumptions, in order to detect a hazard ratio (HR) of progressive disease (PD) of 1.43 in hyperglycemic versus normoglycemic patients with 90% statistical power and two-sided α error of 0.05, an accrual of approximately 800 patients was estimated. The HR threshold of 1.43 was chosen on the basis of a preliminary, monocentric evaluation performed in the first 110 patients treated with EVE-EXE at the coordinating center.

Secondary objectives of the study were: a) to investigate the association between baseline hyperglycemia (as defined as fasting blood glycemia ≥126 mg/dl measured within 28 days before the initiation of EVE-

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Commentato [318]: Based on the investigator's assessment. Both patient with measurable and non- measurable disease per RECIST were included, provided they were assessed for treatment response with imaging. CT scan, MRI, bone scan, US and PET were applicable.

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EXE) and patient PFS; b) to evaluate the association between the onset of precocious hypercholesterolemia and hypertriglyceridemia, as defined as average fasting plasma cholesterol and triglycerides ≥ 200 mg/dl and ≥ 170 mg/dl, respectively, during the first three months of EVE-EXE treatment, and patient PFS; c) to investigate the association between baseline hypercholesterolemia (≥ 200 mg/dl) or baseline hypertriglyceridemia (≥ 170 mg/dl) and patient PFS; d) to assess the impact of baseline and on-treatment glycemia, cholesterolemia and triglyceridemia, as evaluated as continuous variables, on patient PFS.

Patients who had not undergone disease progression or death at data cut off and analysis were censored at the time of last disease evaluation or last follow-up.

2.3. Glucose, cholesterol and triglyceride evaluation

Measurement of fasting (at least 8 hours after the last meal) plasma glucose, cholesterol and triglyceride concentration was performed at baseline and before initiating a new treatment cycle as per clinical practice; data regarding metabolite measurements at baseline and at 1, 2 and 3 months were collected whenever available. For the purpose of the study, metabolite measurements obtained during the first three months of EVE-EXE treatment (i.e. excluding baseline evaluations) were summarized as average, maximum and absolute differences with respect to baseline levels (delta). The average was defined as the arithmetic mean of metabolite concentrations during the study treatment (baseline excluded). The maximum (max) was defined as the highest value of metabolite measurement during the first three months of EVE-EXE therapy (baseline excluded). The delta was defined as the absolute difference between max and baseline values for each metabolic variable. Baseline, average and max values were analyzed both as dichotomous variables, with a cut off of 126 mg/dl, 200 mg/dl and 170 mg/dl for plasma glucose, cholesterol and triglycerides, respectively, and as continuous variables. On-treatment changes

of each metabolic parameter were evaluated by comparing baseline measurements with the average value of the same parameter during the first three months of treatment.

2.4. Statistical methods

Standard descriptive statistics was used to summarize clinical and biological patients' characteristics. Both paired and unpaired t-tests were used to compare baseline and on-treatment concentration of metabolic parameters. Median patient follow-up was quantified with the reverse Kaplan-Meier estimator (20).

Survival analysis methods were used to analyze PFS. Survival curves and related descriptive statistics were obtained with the Kaplan-Meier method and comparisons between curves were performed with the logrank test. Multivariable analyses were performed according to a two-step strategy. In the first step, we modeled covariates by resorting to a random forest method (21). This "ensemble" machine-learning approach was used to guide and benchmark the subsequent use of more conventional modeling methods according to the following endpoints: detection and exclusion of prognostically irrelevant covariates (based on minimal depth statistic); guidance on the presence of non-linear effects of continuous predictors or interactions among covariates; joint predictive performance. The second step relied on the use of Cox regression modeling, with the proportional hazard assumption checked by testing and plotting Schoenfeld residuals. For all continuous variables, non-linear effects were handled by means of restricted cubic splines. Cox model results were summarized using hazard ratios (HRs), together with the corresponding 95% confidence intervals (CI) and Wald's p values, while overall model performance was assessed in terms of discrimination with the bootstrap-adjusted Harrell's c index. Given the presence of missing data, we performed Cox model analyses both on complete datasets and after 10-fold multiple imputation (22). In addition, a landmark analysis was conducted to explore a possible bias introduced by the time-dependent assessment of metabolic parameters during the first three months of treatment; in this

landmark analysis, we investigated the impact of baseline and on-treatment glycemia on patient PFS after excluding patients undergoing disease progression during the first three months of therapy.

Statistical analyses were carried out with SAS (version 9.4, SAS Institute, Cary, NC) and R software (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at the conventional 5% two-sided threshold.

3. RESULTS

3.1. Patient population

We evaluated a total number of 848 patients. Of these, 35 patients were excluded due to the lack of at least one blood glucose evaluation at baseline or during the first three months of treatment, while 4 patients were excluded due to the unavailability of the date of last follow up. The study CONSORT diagram is shown in **Supplementary Figure 1**. Finally, 809 patients fulfilling all the enrollment criteria and treated with the EVE-EXE combination between January 2014 and December 2018 were included. Baseline patient and disease characteristics are displayed in **Table 1**. All patients had received prior therapy with NSAIs in the adjuvant or advanced treatment setting, while 54% of them received anti-estrogens (i.e. fulvestrant and/or tamoxifen) for the treatment of advanced disease. At data cut off and analysis, 775 patients had experienced disease progression during EVE-EXE treatment, and 435 patients had died. Median follow up time was 37.4 months (95% CIs: 35.5 - 41.0), with median PFS of 7.13 months (95% CIs: 6.60 - 7.82) and median OS of 32.1 months (95% CIs: 29.7 - 34.5).

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3.2. Effect of EVE-EXE on blood metabolic parameters

Details about baseline and on-treatment metabolic biomarkers are described in **Supplementary Table 1**. At baseline, fasting plasma glucose measurements were available for 722 (89.2%) patients; of these, 79 (10.2%) patients were hyperglycemic according to the pre-specified threshold (i.e. ≥126 mg/dl). At 1, 2 and 3 months after EVE-EXE initiation, plasma glucose measurements were available for 692 (85.5%), 643 (79.5%) and 537 (66.4%) patients, respectively. Consistent with the study assumptions, 185 (24.0%) out of 772 (95.4%) patients with at least one available on-treatment plasma glucose measurement were found to be hyperglycemic. Blood glucose, cholesterol and triglyceride concentration significantly increased after the first month of therapy (when compared to baseline values), and remained stable between the first and second month, with an initial reduction of blood glucose and cholesterol after three months (**Supplementary Table 1; Supplementary Figure 2**). Overall, average blood glucose,

cholesterol and triglyceride levels during the first three months of treatment were significantly higher when compared to baseline measurements (**Supplementary Figure 2**).

The distribution of patient and treatment characteristics according to on-treatment glycemic status are summarized in **Supplementary Table 2**. Overall, normoglycemic and hyperglycemic patients were well balanced with respect to these factors, with the only exception that hyperglycemic patients were significantly older and had higher body mass index (BMI).

Regarding cholesterol and triglycerides, baseline measurements were available for 536 (66.2%) and 500 (61.8%) patients, with a total number of 340 (63.4%) hypercholesterolemic (≥200 mg/dl) and 93 (18.6%) hypertriglyceridemic (≥170 mg/dl) patients. At 1, 2 and 3 months after treatment initiation, blood cholesterol measurements were available for 477 (59.0%), 421 (52.0%) and 387 (47.8%) patients, respectively, while data on triglyceride concentration were available for 440 (54.4%), 383 (47.3%), 351 (43.4%) patients, respectively. On-treatment hypercholesterolemia and hypertriglyceridemia were detected in 472 (78.9%) and 181 (32.3%) patients, respectively.

There was a moderate, positive correlation between baseline and on-treatment concentration of each of the three metabolites, while we found a strong, positive correlation between their average and maximum on-treatment concentration (**Supplementary Table 3**). Therefore, for subsequent evaluations we only considered the average concentration of each blood metabolite (rather than their maximum).

Commentato [321]: Any chance to report the correlation metric in the text?

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3.3. Primary analysis of the association between metabolic parameters and PFS

Patients who were hyperglycemic at baseline had non-statistically significantly different PFS when compared to normoglycemic patients (median PFS [mPFS], 6.14 vs. 7.26 months, respectively; unadjusted HR 1.13; 95% CI 0.95-1.33; p = 0.166) (**Figure 1A**). Similarly, there were no significant PFS differences between hyperglycemic and normoglycemic patients based on on-treatment glycemia (mPFS 6.97 vs 7.13 months; unadjusted HR 1.05; 95% CI 0.93-1.19; p = 0.385) (**Figure 1B**).

The impact of baseline and on-treatment cholesterol and triglyceride concentration according to the pre-specified thresholds was negligible as well. In particular, we did not find a significant association between baseline cholesterol or triglycerides levels and patient PFS (mPFS in hypercholesterolemic vs. normocholesterolemic patients: 7.95 vs 7.82 months; unadjusted HR 0.95; 95% CI 0.84-1.08; p = 0.472; mPFS in hypertriglyceridemic vs. normotriglyceridemic patients: 5.75 vs 7.95 months; unadjusted HR 1.08; 95% CI 0.92-1.27; p = 0.333) (**Supplementary Figure 3A-B**). Similarly, PFS was not statistically significantly different in hypercholesterolemic vs normocholesterolemic (mPFS of 7.59 vs. 6.51 months, respectively; unadjusted HR 0.95; 95% CI 0.82-1.10; p = 0.485) and in hypertriglyceridemic vs. normotriglyceridemic (mPFS: 7.95 vs 7.20 months, respectively; unadjusted HR 0.95; 95% CI 0.84-1.08; p = 0.466) patients when on-treatment metabolite levels were considered (**Supplementary Figure 3C-D**).

Commentato [323]: Non-significant

3.4. Impact of baseline and on-treatment glycemia on PFS

Then, we investigated in a multivariable model the impact of blood glucose concentration, as evaluated as a continuous variable, on patient PFS. To this aim, we first performed an exploratory analysis through machine-learning approach based on Random Forest algorithm (see Material and Methods) to exclude clinically irrelevant variables (i.e. variables not associated with PFS). Based on this analysis, the following covariates were excluded: presence of lung metastases, bone metastases, lymph node metastases, CNS metastases or soft tissue metastases; previous therapy with anthracyclines and/or taxanes; adjuvant chemotherapy; adjuvant ET; use of metformin (data not shown). The following predictors of PFS were instead selected for further evaluation in the multivariable model: patient age, body mass index (BMI), ECOG PS, line of EVE-EXE treatment, EVE dosages, presence of visceral disease, presence of liver metastases, disease-free interval, baseline and on-treatment glycemia, baseline and on-treatment cholesterol concentration, baseline and on-treatment triglyceride concentration. Of note, the effect of metabolic parameters on patient PFS was non-linear and, in the case of blood glucose,

Commentato [324]: This is curious, perhaps related to the low proportion of pts with brain mets

it was characterized by a pattern of interaction between baseline and on-treatment glycemia (data not shown).

After selecting potentially relevant variables, we fitted a Cox regression models to assess their independent impact on patient PFS. In a first model, among metabolic variables we only included baseline and on-treatment blood glucose levels, along with their interaction. Missing metabolic data were imputed (see Materials and Methods). This model revealed a negligible impact of baseline glycemia on PFS, while there was a moderate association between high on-treatment glycemia and worse PFS (**Table 2A**). Notably, the impact of both baseline and on-treatment glycemia on PFS was largely attributable to the interaction between these two factors, as demonstrated by hierarchical statistical testing of model coefficients (**Table 2A**). We found similar results when cholesterol and triglyceride concentration was also included in the Cox model (**Table 2B**). In both multivariable models, more advanced EVE-EXE treatment line, worse ECOG PS and the presence of liver metastases were associated with worse PFS, while a reduction of EVE dosage during the treatment course correlated with better PFS (**Tables 2A-2B**). To test the stability of the first model (**Table 2A**), we fitted another Cox model keeping the same structure, but only including complete data, i.e. after excluding missingness (643 patients). Of note, this analysis confirmed that the interaction between baseline and on-treatment glycemia is largely responsible for the observed association between blood glucose levels and patient PFS (**Supplementary Table 4**). To further confirm the robustness of these results, we performed a landmark analysis, in which we excluded patients undergoing disease progression during the first three months of EVE-EXE treatment (i.e. when on-treatment glycemia is evaluated). Also this analysis confirmed an impact of baseline and on-treatment glycemia on patient PFS (**Supplementary Table 5**).

3.5 Role of the interaction between baseline and on-treatment glycemia on PFS

The presence of an interaction between baseline and on-treatment glycemia makes results of Cox models poorly interpretable, in particular with respect to the HRs that summarize the impact of individual

variables on clinical outcomes. To dissect the pattern of interaction between baseline and on-treatment glycemia, we plotted log-relative hazards according to on-treatment blood glucose concentrations (80-270 mg/dl range) at three different levels of baseline blood glycemia, namely 85 mg/dl, 95 mg/dl and 125 mg/dl, which correspond to the 10^{th}, 50^{th} and 90^{th} distribution quantiles, respectively. For baseline glycemia of 85 mg/dl, we found a 4-fold increase in log-Relative hazard for increasing on-treatment blood glucose levels (**Figure 2A**). At a level of baseline glycemia of 95 mg/dl, we observed a similar pattern, with a 2-fold increase in log-Relative hazard for increasing on-treatment blood glucose levels (**Figure 2B**). Finally, the log-Relative hazard curve was flat at the level of 125 mg/dl baseline glycemia (**Figure 2C**). These data indicate that an increase of blood glucose levels during EVE-EXE therapy might be associated with an increased risk of disease progression in patients with normal glycemia at baseline, but not in patients who are already hyperglycemic before treatment initiation.

Since the log-Relative hazard metric does not have immediate clinical translation, we used a contour plot to illustrate the predicted 1-year PFS as a joint effect of baseline and on-treatment blood glucose concentration, while keeping the remaining factors at their average level. As shown in **Figure 2D**, most points - each point representing and individual patient - lied in a wide yellow area of the plot, which corresponds to approximately 30% one-year PFS probability (i.e. the average PFS in the whole patient population). Of note, point-patients with lower baseline glycemia and undergoing an increase of their glycemia during the EVE-EXE treatment, corresponding to the red area in the lower-right corner of the plot (roughly delimited by the 25% level curve), were associated with the lowest PFS, while point-patients with higher baseline and lower on-treatment glycemia (upper-left corner) corresponded to the best PFS.

To illustrate the impact of the interaction between baseline and on-treatment glycemia in a more intuitive way, we compared PFS Kaplan Meier curves of patients who were normoglycemic at baseline (< 100 mg/dl) and became diabetic (≥ 126 mg/dl) during EVE-EXE therapy with PFS Kaplan Meier curves of

Commentato [325]: Based on how the "during treatment" values were estimated (averaged), I am not sure we should conclude that these patients are "diabetic", unless these patients had received a diagnosis of. Definitely they fall in "average" in the hyperglycemic range of diabetes mellitus.

One possible criticism may be raised on the use of antidiabetic drugs, especially insulin, in these patients. I would assume they were mostly treated with metformin. If this data is available, it may be worthy to report (e.g., *hyperglycaemia was mostly managed with metformin, N*=x), to avoid comments on the artificial pharmacological rise of the insulin counteracting action.

other patient subsets. As shown in **Figures 3A-3B**, patients with normal baseline blood glucose levels who became diabetic during the treatment had significantly worse PFS when compared to the remaining patients (**Figure 3A**). Among patients who experienced precocious diabetes during EVE-EXE therapy, we also compared the PFS of patients with normal baseline glycemia and patients who were already hyperglycemic at baseline; interestingly, the former had significantly worse PFS when compared to the latter patients (**Figure 3B**).

Commentato [326]: Consider to add "(n=36)"

Commentato [327]: Consider to add "(n=68)"

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i.e., 6.34 months Vs 10.32 months

4. DISCUSSION

The mTORC1 inhibitor EVE in combination with EXE is an effective treatment for HR+/HER2- aBC patients progressing on/after prior NSAI therapy (8). Hyperglycemia/diabetes and hyperinsulinemia are common AEs in patients treated with EVE or other PI3K/AKT/mTORC1 axis inhibitors (8-10), and could reduce the efficacy of these agents by reactivating the IR/PI3K/AKT/mTORC1 pathway (19). Here, we conducted a large, multicenter study, namely EVERMET, to investigate the impact of baseline and on-treatment blood glucose concentration on PFS in HR+/HER2- aBC patients treated with EVE-EXE.

We found that both baseline and on-treatment glycemia, as evaluated as continuous variables, are associated with patient PFS, and this association is mainly attributable to their interaction. In detail, patients with normal baseline glycemia who became diabetic during EVE-EXE treatment had significantly worse PFS when compared to the remaining patients, and in particular when compared to patients who were already hyperglycemic at baseline and experienced overt diabetes during EVE-EXE therapy. The robustness of the study results was confirmed by a parallel multivariable model in which we also included other important metabolic parameters that are modulated by EVE-EXE therapy, i.e. triglycerides and cholesterol, as well as by a landmark analysis that excluded patients undergoing disease progression during the first three months of EVE-EXE treatment.

As per clinical protocol, we initially evaluated the potential impact of baseline or on-treatment hyperglycemia, as defined as blood fasting glucose concentration ≥ 126 mg/dl, on patient PFS. In the primary analysis, we did

not find a significant association between hyperglycemia and the risk of disease progression. When interpreting these results in the light of the final study findings, we should consider that: 1) in the primary analysis we only evaluated the effect of metabolic variables at one time point (baseline or on-treatment glycemia), while we did not take into account the impact of their interaction on PFS; 2) both baseline and on-treatment glycemia are continuous variables, while in the primary analysis we evaluated them as dichotomous. In clinical studies, dichotomizing continuous variables is a common tool that is used to identify parameter thresholds that can be used to allocate patients in different classes of risk, thus favoring decision processes by physicians. However, dichotomization of continuous variables can be misleading for several reasons: a) commonly used thresholds may not be appropriate for the specific clinical context; for instance, the 126 mg/dl threshold, which is used for the diagnosis of diabetes mellitus, might fail to distinguish between cancer patients more or less likely to benefit from a specific antitumor therapy; b) even if appropriate thresholds are found for specific clinical contexts, dichotomization may be misleading in the case of non-monotonic or non-linear relationships between metabolite concentration and clinical outcomes, as was the case of the association between blood glucose levels and patient PFS in our study. For these reasons, the impact of metabolic factors on clinical outcomes could be more reliably assessed when these variables are evaluated as continuous rather than dichotomous variables, and by using interactive, longitudinal models.

To explain the interaction between baseline and on-treatment glycemia in affecting patient PFS, we hypothesize that higher baseline blood glucose and insulin levels could be associated with higher baseline activation of the PI3K/AKT/mTORC1 axis in cancer cells and, potentially, with higher tumor cell sensitivity to EVE-induced inhibition of mTORC1 regardless of on-treatment glycemia/insulinemia. On the other hand, tumors arising in patients with normal baseline glycemia/insulinemia might display lower baseline activation of the PI3K/AKT/mTORC1 axis; in conditions of normal extracellular blood glucose/insulin concentration, these tumors could maintain some sensitivity to EVE-EXE, while EVE-induced hyperglycemia and hyperinsulinemia could result in a rapid boost of PI3K/AKT/mTORC1 activity, and in cancer cell resistance to the treatment. While

this hypothesis needs to be confirmed by preclinical studies and prospective clinical studies, our findings indicate that blood glucose and, potentially, insulin concentration does not affect HR+/HER2- BC cell response to pharmacological mTORC1 inhibition *per se*, but their effect could be strongly influenced by the metabolic environment in which the tumor grew before the treatment, and in particular by baseline blood glucose/insulin concentration.

If confirmed by future prospective studies, our findings could have relevant clinical implications. Indeed, in the subgroup of patients with normal baseline glycemia, preventing or promptly reversing EVE-induced hyperglycemia or diabetes could improve EVE-EXE efficacy. To this aim, specific dietary and/or pharmacologic interventions capable of preventing EVE-induced hyperglycemia/diabetes should be considered in HR+/HER2- aBC patients treated with EVE-EXE, especially if they are normoglycemic at baseline. Regarding dietary approaches, a low intake of refined carbohydrates and sugars should be recommended to patients initiating EVE-EXE treatment. As for pharmacological approaches, metformin or other antidiabetic medications should be promptly initiated if dietary interventions are insufficient to keep blood glycemia below the diabetic threshold during the first months of treatment. Of note, since EVE-induced hyperglycemia tends to spontaneously resolve during the course of the treatment (23), blood glucose levels should be more intensively monitored to prevent (

Commentato [329]: Could be?

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to promptly manage EVE-induced hyperglycemia/diabetes during the first three months of therapy, when a non-irrelevant proportion of disease progression events occur (15.1% of patients in the EVERMET study). At the same time, our results indicate that patients who are hyperglycemic at the time of EVE-EXE initiation could achieve poor, if any benefit from blood glucose reduction during EVE-EXE treatment; in these patients, a tight control of patient glycemia and, in case, the reversal of EVE-induced diabetes could be potentially less impactful on tumor-related outcomes, while antidiabetic treatments should be primarily used to prevent diabetes-induced symptoms and complications.

Since hyperglycemia and hyperinsulinemia are class effects of PI3K/AKT/mTORC1 axis inhibitors, results of our study could also apply to other clinical contexts in which these compounds are used. For instance, the PI3K

inhibitor alpelisib has been recently approved by the FDA and EMA in combination with fulvestrant for the treatment of patients with HR+/HER2- aBC progressing on/after prior AI therapy (9). Similar to EVE, alpelisi

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can cause hyperglycemia and hyperinsulinemia, which could reduce its efficacy (19). Since the alpelisib-fulvestrant combination is potentially used in approximately 40% of all HR+/HER2- aBC patients - i.e. those bearing *PIK3CA*-mutated tumors -, and since the incidence of severe (grade 3 or 4) hyperglycemia is much more common with alpelisib than with EVE despite the precocious use of metformin in the SOLAR-1 study (24), exploring strategies to prevent or promptly manage alpelisib-induced hyperglycemia/diabetes is a clinically relevant issue, especially for patients with normal baseline blood glucose levels.

In recent years, metformin has been extensively investigated in both preclinical and clinical setting for its potential direct (cell-autonomous) or indirect (through its impact on systemic metabolism) antitumor effects (25-27). Since metformin acts by reducing glucose production in the liver and at the same time by sensitizing peripheral tissues to the effects of insulin, it has been considered a good candidate drug to be combined with EVE-EXE for the treatment of HR+/HER2- aBC patients. Quite disappointingly, one recent prospective study showed modest clinical efficacy of upfront EVE-EXE plus metformin combination in overweight/obese postmenopausal women with HR+/HER2- aBC (27), and similarly negative results emerged from a preclinical study in which metformin was used to improve the efficacy of Pi3K inhibitors in mouse models of HR+/Her2- BC (19). These data, together with the potential pharmacokinetic interactions between EVE and metformin in patients with advanced cancers (28) and the risk of increasing the incidence of diarrhea, indicate that metformin might be not an ideal drug to be used in combination with EVE. Conversely, specific dietary interventions, such as ketogenic diets or cyclic calorie-restricted, low-carbohydrate, low-protein diets, collectively referred to as fasting-mimicking diets (FMDs), which reduce blood glucose/insulin concentrations and do not have overlapping toxicities with EVE, have been shown to inhibit the PI3K/AKT/mTORC1 pathway synergistically with ETs or PI3K inhibitors in preclinical *in vivo* experiments (29,30). In one study, high-fat ketogenic diets were found to be more effective than metformin in reducing PI3K inhibitor-induced hyperglycemia and hyperinsulinemia, and

demonstrated additive or synergistic *in vitro* and *in vivo* antitumor activity in combination with PI3K inhibitors (19). More recently, cyclic FMDs showed synergistic antitumor activity with standard ETs plus/minus cyclin-dependent kinase 4/6 (CDK 4/6) inhibitors in preclinical models of HR+/HER2- BC, with initial promising results also in cancer patients (29). Of note, the synergistic activity between ET and FMD was mediated by FMD-induced reduction of insulin/IGF-1 levels, which results in increased PTEN expression and consequent inhibition of the PI3K/AKT/mTORC1 pathway in cancer cells. Since ketogenic diets and FMD are potentially safe and feasible interventions in well-selected cancer patient populations, combining them with EVE or other inhibitors of the PI3K/AKT/mTORC1 pathway could produce highly synergistic antitumor effects, while at the same time improving the tolerability of these drugs.

The following are major strengths of this study: a) this was the first, large multicenter study to reveal an interaction between baseline and on-treatment blood glucose concentration in affecting the PFS of HR+/HER2- aBC patients treated with the EVE-EXE combination; b) the large sample size and the multicenter nature of the study render our data robust; in this respect, PFS data in the whole population of patients enrolled in the EVERMET study are consistent with data reported in the experimental arm of the BOLERO-2 trial and in previous real world data studies (8,31,32); c) we enrolled a high number of patients receiving the same treatment in a relatively short-time interval (5 years), thus excluding a significant role of relevant changes in clinical practice of HR+ BC treatment; d) at least one blood glucose measurement at baseline and during the first three months of EVE-EXE therapy was available for the majority of patients; e) the main study findings were confirmed in different multivariable models and also by a landmark analysis.

The main limitation of this study consists in the retrospective design and the consequent missing data, which could in part limit the reliability of our findings; nonetheless, metabolic data were imputed in only 10.7% of all patients, and the main study findings were confirmed after patients with incomplete data were removed, thus adding robustness to our results. Moreover, the study was negative as for its primary endpoint, and the lack of a

control arm does not allow establish definitive causal associations between metabolic toxicities and treatment efficacy.

In conclusion, patients with normal baseline blood glucose levels are at higher risk for disease progression if the experience precocious diabetes during EVE-EXE treatment. Prospective clinical trials are needed to investigate the impact of dietary or pharmacologic interventions aimed at preventing or precociously reversing EVE-induced increase of blood glucose concentration on the clinical outcomes of HR+/HER2- aBC patients.

Commentato [332]: For the nature of the finding, and the missed primary endpoint, "might be" or "could be" fits better.

Abbreviations and Acronyms

BC	Breast Cancer
CI	Confidence Interval
ECOG PS	Eastern Cooperative Oncology Group Performance Status
ET	Endocrine Therapies
EVE	Everolimus
EXE	Exemestane
HR	Hazard Ratio
HR+	Hormone Receptor-Positive
IR	Insulin Receptor
NSAI	Non-Steroidal Aromatase Inhibitor
OS	Overall Survival
PD	Progressive Disease
PFS	Progression-Free Survival
PI3K/AKT/mTORC 1	Phosphatidylinositol 3-Kinase/Protein Kinase B/Mechanistic Target of Rapamycin Complex 1

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