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

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

Claudio Vernieri[1,2*#], Federico Nichetti[1*#], Luca Lalli[3], Luca Moscetti[4], Carlo Alberto Giorgi[5], Gaia Griguolo[5], Antonio Marra[6], Giovanni Randon[1], Carmen G. Rea[1], Francesca Ligorio[1], Simone Scagnoli[7], Claudia De Angelis[8], Chiara Molinelli[9], Agnese Fabbri[10], Emanuela Ferraro[6], Dario Trapani[6], Andrea Milani[11], Elisa Agostinetto[12], Ottavia Bernocchi[13], Giovanna Catania[14], Amelia Vantaggiato[15], Michela Palleschi[16], Anna Moretti[17], Debora Basile[18], Marika Cinausero[19], Arta Ajazi[2], Lorenzo Castagnoli[20], Salvatore Lo Vullo[3], Lorenzo Gerratana[18,21], Fabio Puglisi[18,21], Nicla La Verde[22], Grazia Arpino[23], Andrea Rocca[16], Mariangela Ciccarese[15], Rebecca Pedersini[24], Alessandra Fabi[14], Daniele Generali[13,25], Agnese Losurdo[12], Filippo Montemurro[11], Giuseppe Curigliano[6,26], Lucia Del Mastro[9,27], Andrea Michelotti[8], Enrico Cortesi[7], Valentina Guarneri[5], Giancarlo Pruneri[26,28], Luigi Mariani[3*] & Filippo de Braud[1,26*]

Affiliations:

[1] Department of Medical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

[2] IFOM, the FIRC Institute of Molecular Oncology, Milan, Italy

[3] Unit of Clinical Epidemiology and Trial Organization, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

[4] Division of Medical Oncology, Department of Oncology and Hematology, University Hospital of Modena, Modena, Italy

[5] Department of Surgery, Oncology and Gastroenterology, University of Padova, Medical Oncology 2, Veneto Institute of Oncology IOV - IRCCS, Padua, Italy

[6] Division of Early Drug Development for Innovative Therapies, IEO, European Institute of Oncology IRCCS, Milan, Italy

[7] Department of Medico-Surgical Sciences and Translational Medicine, "Sapienza" University of Rome, Rome, Italy

[8] UO Oncologia Medica 2, Ospedale S. Chiara, Dipartimento di Oncologia, Dei Trapianti e Delle Nuove Tecnologie, Azienda Ospedaliera Universitaria Pisana, Pisa, Italy

[9] IRCCS Ospedale Policlinico San Martino, U.O.S.D. Breast Unit, Genova, Italy

[10] Medical Oncology Unit, Belcolle Hospital, Viterbo, Italy

[11] Multidisciplinary Oncology Outpatient Clinic, Candiolo Cancer Institute, FPO-IRCCS, Turin, Italy

[12] Medical Oncology and Hematology Unit, Humanitas Clinical and Research Center – IRCCS, Rozzano, Milan, Italy

[13] Department of Medical, Surgery and Health Sciences, University of Trieste, Trieste, Italy

[14] Division of Medical Oncology, IRCCS Regina Elena National Cancer Institute, Rome, Italy

[15] Medical Oncology & Breast Unit, "Vito Fazzi" Hospital, Lecce, Italy

[16] Department of Medical Oncology, IRCCS Istituto Romagnolo per lo Studio dei Tumori "Dino Amadori" – IRST, Meldola, Italy

[17] Department of Oncology, ASST Fatebenefratelli Sacco - PO Fatebenefratelli, Milan, Italy

[18] Department of Medical Oncology, IRCCS Centro di Riferimento Oncologico di Aviano (CRO), Aviano, Italy

[19] Department of Oncology, ASUFC University Hospital of Udine, Udine, Italy

[20] Molecular Targeting Unit, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

[21] Department of Medicine, University of Udine, Udine, Italy

[22] Department of Oncology, ASST Fatebenefratelli Sacco - PO Luigi Sacco, Milan, Italy

[23] Department of Clinical Medicine and Surgery, University of Naples Federico II, Naples, Italy

[24] Department of Medical & Surgical Specialties, Radiological Sciences, & Public Health, Medical Oncology, University of Brescia at ASST Spedali Civili, Brescia, Italy

[25] Breast Cancer Unit & Translational Research Unit, ASST Cremona, Cremona, Italy

[26] Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy

[27] Dipartimento di Medicina Interna e Specialità Mediche (DiMI), School of Medicine, University of Genova, Genova, Italy

[28] Department of Diagnostic Pathology and Laboratory Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

* these authors contributed equally

[#] corresponding authors

Correspondence:

Claudio Vernieri, M.D., P.h.D.,
Department of Medical Oncology
Fondazione IRCCS Istituto Nazionale dei Tumori
Via Venezian 1, 20133 Milan, Italy
Phone: + 39-0223903066
E-mail: claudio.vernieri@istitutotumori.mi.it

Metabolic Reprogramming of Solid Tumors Unit
IFOM, the FIRC Institute of Molecular Oncology
Via Adamello 16, 20139, Milan, Italy
E-mail: claudio.vernieri@ifom.eu

Federico Nichetti, M.D.
Department of Medical Oncology
Fondazione IRCCS Istituto Nazionale dei Tumori
Via Venezian 1, 20133 Milan, Italy
Phone: + 39-0223903066
E-mail: federico.nichetti@istitutotumori.mi.it

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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. In particular, patients with normal baseline glycemia have significantly worse clinical outcomes if they experience on-treatment hyperglycemia. This study supports the use of early alterations in blood glucose concentration as a biomarker of EVE-EXE efficacy and provides the rationale for exploiting novel metabolic interventions as anticancer strategies in advanced breast cancer.

ABSTRACT

Purpose: 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.

Experimental Design: 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 HR+/HER2- aBC patients treated with EVE-EXE.

Results: We evaluated 809 HR+/HER2- aBC patients treated with EVE-EXE as any-line of therapy for advanced disease. When evaluated as dichotomous variables, baseline and on-treatment glycemia were not significantly associated with PFS. However, when blood glucose concentration was evaluated as a continuous variable, a multivariable model accounting for clinically relevant patient- and tumor-related variables revealed that both baseline and on-treatment glycemia are associated with PFS, and this association is largely attributable to their interaction. In particular, patients who are normoglycemic at baseline and experience on-treatment diabetes have lower PFS compared to patients who are already hyperglycemic at baseline and experience diabetes during EVE-EXE therapy (mPFS 6.34 vs. 10.32 months; HR 1.76; 95% CI 1.15-2.69; p=0.008).

Conclusions: The impact of on-treatment glycemia on the efficacy of EVE-EXE therapy in HR+/HER2 aBC patients depends on 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.

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 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 preclinical study indicated that PI3K inhibitor-induced increase of serum insulin concentration in cancer patients might be 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.

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 Clinical and Research Center - IRCCS 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 Friuli Centrale, Udine]. Data were collected through an 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; d) patients with ovarian ablation, either through radiation therapy or bilateral ovariectomy; 4) treatment for at least one month with daily EVE (initial dosage of 10 mg/day) plus EXE (25 mg/day) between October 2012 and July 2019 outside clinical trials sponsored by pharmaceutical companies; 5) disease recurrence or progression after/on prior therapy with NSAIs plus/minus Cyclin-Dependent Kinase 4/6 (CDK 4/6) inhibitors; 6) availability of at least one measurement of plasma glucose concentration at the initiation of EVE-EXE therapy or at 1, 2 or 3

185 months after treatment initiation); 7) any number of previous lines of treatment for advanced disease;
186 8) any prior therapy for localized disease, including (neo)adjuvant chemotherapy, surgery, ETs;
187 patients with *de novo* metastatic disease at diagnosis were included as well. Prior NSAI therapy should
188 not necessarily be the last treatment before EVE-EXE therapy. All patients were followed up until
189 death, loss of contact, or time of data lock (31st July 2019). Written informed consent was obtained
190 from all patients who were alive at the time of study conduction. The study was carried out in
191 accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. The study
192 protocol was first approved by the Ethics Committee of the coordinating center (internal registration
193 number of the study: INT 30/18), and then approved by Ethics Committees and/or Institutional Review
194 Boards at each participating site.

195 ***2.2. Study objectives and statistical plan***

196 The primary objective of the study was to investigate the association between the onset of early
197 hyperglycemia and the PFS of HR+/HER2- aBC patients treated with EVE-EXE. Early hyperglycemia
198 was defined as equal or higher than 126 mg/dL average fasting plasma glucose concentration during
199 the first three months of EVE-EXE treatment (i.e., excluding baseline evaluation). PFS was defined as
200 the time between EVE-EXE initiation and the detection of clinical/radiological disease progression
201 (according to RECIST v1.1 criteria) or patient death from any cause, whichever occurred first. For
202 sample size calculation, we assumed that 80% of patients had an average glycemia below 126 mg/dL
203 during the first three months of EVE-EXE therapy and that normoglycemic patients had median PFS of
204 7 months (8). With these assumptions, in order to detect a hazard ratio (HR) of progressive disease
205 (PD) of 1.43 in hyperglycemic versus normoglycemic patients with 90% statistical power and two-
206 sided α error of 0.05, an accrual of approximately 800 patients was estimated. The HR threshold of
207 1.43 was chosen on the basis of a preliminary, monocentric evaluation performed in the first 110
208 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-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 PFS; d) to assess the impact of baseline and on-treatment glycemia, cholesterolemia and triglyceridemia, as evaluated as continuous variables, on PFS. Patients who had not experienced 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-

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

234 ***2.4. Statistical methods***

235 Standard descriptive statistics were used to summarize clinical and biological patients' characteristics.
236 Both paired and unpaired t-tests were used to compare baseline and on-treatment concentration of
237 metabolic parameters, adjusting p values for multiple comparisons through the Benjamini-Hochberg
238 procedure. Median patient follow-up was quantified with the reverse Kaplan-Meier estimator (20).
239 Survival analysis methods were used to analyze PFS. Survival curves and related descriptive statistics
240 were obtained with the Kaplan-Meier method and comparisons between curves were performed with
241 the logrank test. Multivariable analyses were performed according to a two-step strategy. In the first
242 step, we modeled covariates by resorting to a random forest method (21). This approach was used to
243 guide and benchmark the subsequent use of more conventional modeling methods according to the
244 following endpoints: detection and exclusion of prognostically irrelevant covariates (based on minimal
245 depth statistic); guidance on the presence of non-linear effects of continuous predictors or interactions
246 among covariates; joint predictive performance. The second step relied on the use of Cox regression
247 modeling, with the proportional hazard assumption checked by testing and plotting Schoenfeld
248 residuals. For all continuous variables, non-linear effects were handled by means of restricted cubic
249 splines. Cox model results were summarized using hazard ratios (HRs), together with the
250 corresponding 95% confidence intervals (CI) and Wald's p values, while overall model performance
251 was assessed in terms of discrimination with the bootstrap-adjusted Harrell's c index. In Cox models,
252 the HR for continuous variables was reported as the HR related to the interquartile range (interval
253 between the 75^{th} and 25^{th} quantiles). Given the presence of missing data, we performed Cox model
254 analyses both on complete datasets and after 10-fold multiple imputation (22). In addition, a landmark
255 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 measurement 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 October 2012 and July 2019 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 [interquartile range (IQR): 22.8 - 56.4], with median PFS of 7.13 months (IQR: 3.8 - 12.9) and median OS of 32.1 months (IQR: 15.9 - 54.8).

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 (10.9%) 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, 186 (24.1%) out of 772 patients with at least one available on-treatment plasma glucose measurement were found to be hyperglycemic (i.e., average plasma glucose concentration ≥ 126 mg/dL).

Blood glucose, cholesterol and triglyceride concentration significantly increased after the first month on therapy (when compared to baseline values), and remained stable between the first and second month, with an initial reduction of blood glucose and cholesterol levels after three months (**Supplementary Table 1**; **Supplementary Figure 2**). Overall, average blood glucose, cholesterol and triglyceride concentration during the first three months of treatment was significantly higher when compared to baseline measurements (**Supplementary Figure 2**).

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 exception that hyperglycemic patients were significantly older and had higher body mass index (BMI). In addition, hyperglycemic patients were more likely to receive metformin as an antidiabetic medication, started either before or during EVE-EXE treatment.

Regarding plasma cholesterol and triglyceride concentration, baseline measurements of these parameters were available for 536 (66.3%) and 500 (61.8%) patients, respectively, 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 EVE-EXE 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%), and 351 (43.4%) patients, respectively. Average 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).

3.3. Association between dichotomized 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 1.18; 95% CI 0.93-1.50; p = 0.168) (**Figure 1A**). Similarly, there were no significant PFS differences between hyperglycemic and normoglycemic patients according to on-treatment glycemia (mPFS 6.97 vs. 7.13 months; unadjusted HR 1.08; 95% CI 0.91-1.28; p = 0.371) (**Figure 1B**).

The impact of baseline and on-treatment cholesterol and triglyceride concentration according to the pre-specified thresholds was non-statistically significant 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 0.94; 95% CI 0.78-1.12; p = 0.479; mPFS in hypertriglyceridemic vs. normotriglyceridemic patients: 5.75 vs. 7.95 months; unadjusted HR 1.12; 95% CI 0.89-1.41; p = 0.342) (**Supplementary Figure 3A-B**). Similarly, PFS was not statistically significantly different in hypercholesterolemic vs. normocholesterolemic (mPFS of 7.59 vs. 6.21 months, respectively; unadjusted HR 0.92; 95% CI 0.75-1.12; p = 0.403) and in hypertriglyceridemic vs. normotriglyceridemic (mPFS: 7.95 vs. 7.20 months, respectively; unadjusted HR 0.93; 95% CI 0.78-1.12; p = 0.479) patients when on-treatment metabolite levels were considered (**Supplementary Figure 3C-D**).

3.4. Impact of baseline and on-treatment glycemia as continuous variables 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 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, central nervous system (CNS) metastases or soft tissue metastases; prior therapy with anthracyclines and/or taxanes; adjuvant chemotherapy; adjuvant ET. The use of metformin was also excluded as a covariate for subsequent analyses (**Supplementary Figure 4A**). The following predictors of PFS were instead selected for further evaluation in the multivariable model: patient age, body mass index (BMI), Eastern Cooperative Oncology Group Performance Status (ECOG PS), line of EVE-EXE treatment, EVE dosages, presence of visceral disease, presence of liver metastases, disease-free interval (as defined as the time between surgery of the primary tumor and tumor recurrence as metastatic disease), baseline and on-treatment glycemia, baseline and on-treatment cholesterolemia, baseline and on-treatment triglyceridemia (**Supplementary Table 4**). Of note, the effect of metabolic parameters on patient PFS was non-linear and, in the case of blood glucose, it was characterized by a pattern of interaction between baseline and on-treatment glycemia (**Supplementary Figure 5A-B**).

After selecting potentially relevant variables, we fitted a Cox regression model to assess the independent impact of these variables 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 (**Supplementary Table 5**). 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

359 treatment course correlated with better PFS (**Tables 2A-2B**). To test the stability of the first model
360 (**Table 2A**), we fitted another Cox model keeping the same structure, but only including complete data,
361 i.e., after excluding missingness, for a total number of 643 patients included. Of note, this analysis
362 confirmed that the interaction between baseline and on-treatment glycemia is largely responsible for
363 the observed association between blood glucose levels and patient PFS (**Supplementary Table 6**). To
364 further confirm the robustness of these results, we performed a landmark analysis, in which we
365 excluded patients experiencing disease progression during the first three months of EVE-EXE
366 treatment (i.e., when on-treatment glycemia is evaluated). This analysis confirmed an impact of
367 baseline and on-treatment glycemia on patient PFS (**Supplementary Table 7**). In all these models,
368 patients undergoing precocious EVE interruption or dose reduction had a lower risk of undergoing
369 disease progression when compared to patients continuing EVE until disease progression
370 (**Supplementary Figure 6A**). We asked if this finding could be explained by a different duration of
371 EVE exposure (time to EVE treatment interruption, TTI) in different patient subsets. Interestingly,
372 patients undergoing EVE dose reduction were exposed to EVE for longer time intervals when
373 compared to patients who received standard EVE dosages until disease progression; by contrast, the
374 length of EVE exposure was significantly lower in patients undergoing precocious treatment
375 interruption when compared to patients who did not interrupt EVE, as well as when compared to
376 patients undergoing EVE dose reduction (**Supplementary Figure 6B**). As expected, EVE-induced
377 grade 1/2 (G1/G2) or G3/G4 adverse events were significantly more common in patients undergoing
378 treatment interruption/dose reduction (**Supplementary Table 8**). Removing the variable "EVE
379 interruption/dose variations" from the multivariable model confirmed the main study findings,
380 including the interaction between baseline and on-treatment glycemia in affecting patient PFS
381 (**Supplementary Table 9**).
382

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 PFS. 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 concentration 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 an 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, which correspond 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 glycemia 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 other patient subsets. Patients with normal baseline blood glucose levels who became diabetic during the treatment (Group A) had significantly worse PFS when compared to the remaining patients (Group B) (mPFS 6.34 vs. 7.33 months; unadjusted HR 1.42; 95% CI 1.01-1.99; p= 0.040) (**Figure 3A**). Also within these two different cohorts, metformin use was not associated with significantly different PFS (**Supplementary Figure 4B-C**).

Among patients who experienced early diabetes during EVE-EXE therapy, we also compared the PFS of patients with normal baseline glycemia (Group A) and patients who were already hyperglycemic at baseline (i.e., plasma glucose concentration in the 100-125 mg/dL range, Group B); interestingly, the former had significantly worse PFS when compared to the latter patients (mPFS 6.34 vs. 10.32 months; unadjusted HR 1.76; 95% CI 1.15-2.69; p=0.008) (**Figure 3B**).

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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, when 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 experienced hyperglycemia/diabetes 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 on-treatment hyperglycemia/diabetes. 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.

Among variables that were consistently associated with better patient PFS in multivariable models was the precocious interruption or dose reduction of EVE, which were both associated with an increased incidence of treatment-induced adverse events, as expected. To explain this association, we hypothesized that patients undergoing EVE interruption/dose reduction had been exposed to longer duration of EVE treatment which, in turn, might have conditioned interruption/dose reduction on the one hand, and longer clinical benefit on the other hand. To test this hypothesis, we compared the duration of EVE therapy in patients undergoing/not undergoing EVE interruption or dose reduction. Of

note, EVE treatment exposure was significantly longer in patients undergoing EVE dose reduction when compared to patients continuing EVE at full dosage until disease progression, while it was significantly lower in patients who precociously interrupted EVE therapy. Based on results of these analyses, we conclude that EVE dose reduction might have contributed to longer drug exposure which, in turn, might have resulted in higher clinical benefit from EVE. On the other hand, the observed PFS prolongation in patients undergoing precocious EVE interruption could reflect higher systemic and intratumor exposure to the drug during the first months of treatment, which could justify an increased incidence of treatment-related adverse events on the one hand, and higher treatment efficacy and longer PFS on the other hand.

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 statistically 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 the occurrence of precocious EVE-induced hyperglycemia and hyperinsulinemia could result in a boost of PI3K/AKT/mTORC1 activation, and in cancer cell resistance to the treatment. While this hypothesis needs to be confirmed by preclinical 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 could 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 or 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 postmenopausal women and men with HR+/HER2- aBC progressing on/after prior AI therapy (9). Similar to EVE, alpelisib can cause hyperglycemia and hyperinsulinemia, which could reduce its efficacy (19). Although the widespread use of alpelisib in the daily treatment of HR+/HER2- aBC patients bearing *PIK3CA*-mutated tumors might be limited by several factors, including the lack of an extensive tumor genomic profiling in several cancer centers, the suboptimal safety profile of alpelisib and recent labels limiting alpelisib use in Europe and Italy to patients previously treated with single-agent endocrine therapy (which has now been replaced by

endocrine therapy plus CDK 4/6 inhibitor-based combinations as a standard-of-care first-line therapy), the alpelisib-fulvestrant combination remains a potentially useful therapy that could be used in up to 40% of all HR+/HER2- aBC patients. Therefore, since the incidence of severe (grade 3 or 4) hyperglycemia is common with alpelisib (actually more common 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 in combination with PI3K inhibitors in mouse models of HR+/HER2- BC (19). In line with these data, in our study we did not find a significant association between metformin use and PFS in HR+/HER2- aBC patients treated with EVE-EXE. This evidence, 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 blood 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 show 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 make 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, the main study findings were confirmed after removing patients with incomplete data from the analysis, 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 concentration are at higher risk for disease progression if they experience precocious hyperglycemia/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.

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/mTORC1	Phosphatidylinositol 3-Kinase/Protein Kinase B/Mechanistic Target of Rapamycin Complex 1
TTI	Time to EVE Treatment Interruption

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TABLES

Table 1. Patient and tumor characteristics.

Characteristic	Total N of patients = 809
	N (%)
ECOG PS	
0	567 (70.2)
1	227 (28.1)
2	14 (1.7)
NA	1
Use of metformin	
Started before EVE-EXE	62 (7.8)
Started during EVE-EXE	31 (3.9)
NA	15
Sites of metastatic disease	
Lymph nodes	307 (38.0)
NA	2
Bones	590 (73.2)
NA	3
Liver	258 (32.0)
NA	2
Lungs	229 (28.4)
NA	2
CNS	21 (2.6)
NA	2
Soft tissues	86 (10.7)
NA	2
Others	68 (8.4)
NA	2
Visceral disease	450 (55.8)
NA	2
Prior antineoplastic therapies	
Prior adjuvant ET	569 (70.9)
NA	6
Prior adjuvant ChT	453 (56.6)
NA	9
Prior Anthracycline Treatment	499 (61.9)
NA	3
Prior Taxane Treatment	418 (51.9)
NA	3
Prior anti-estrogens	434 (53.6)
NA	-
EVE dose variations	
Full dose	428 (52.9)
Reduction (5 mg)	325 (40.2)
Interruption***	56 (6.9)
NA	-
	Median (IQR)
Age, years	63 (56 - 70)
NA	2
BMI	24.7 (22.2 - 27.8)
NA	36
Disease Free Interval, months*	54 (19 - 106)
NA	23

Line of Everolimus treatment	
ET + ChT**	3 (2 - 4)
ET only**	2 (2 - 4)
NA	1

Data are presented as N (%) unless otherwise specified.
* defined as the time between surgery for the primary tumor and diagnosis of distant relapse.
** defined as the EVE-EXE treatment line for advanced disease considering both previous ET and ChT, and ET only, respectively.
*** Everolimus precocious interruption was defined as treatment suspension at least 3 months before disease progression.
Abbreviations: BMI: Body Mass Index; ChT: chemotherapy CNS: central nervous system; ECOG PS: Eastern Cooperative Oncology Group Performance Status; ET: endocrine treatment; EVE-EXE: everolimus plus exemestane; IQR: interquartile range; NA: not available.

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Table 2A-B. Multivariable Cox proportional hazards models for Progression Free Survival when considering only baseline and on-treatment blood glucose concentration as a metabolic variable (A) or after also including cholesterol and triglyceride levels (B). In both models, missing blood glucose measurements were imputed.

A. Imputed data / Blood glucose only

Variables		HR	95% CI	p
Baseline glycemia*	Continuous	0.94	0.78 - 1.13	**<.001**
On-treatment glycemia*	Continuous	1.19	0.98 - 1.44	**<.001**
Line of EVE-EXE treatment	Continuous	1.23	1.12 - 1.35	**<.001**
Age	Continuous	1.15	0.94 - 1.41	0.157
Disease Free Interval	Continuous	0.89	0.71 - 1.12	0.733
BMI	Continuous	1.04	0.84 - 1.27	0.251
EVE interruption/dose reduction	Reduction vs full dose Interruption vs full dose	0.78 0.40	0.67 - 0.91 0.30 - 0.53	**<.001**
ECOG PS	1 vs 0 2 vs 0	1.31 1.46	1.11 - 1.55 0.65 - 3.25	**0.005**
Visceral Disease	Yes vs No	1.18	0.97 - 1.42	0.093
Presence of liver metastases	Yes vs No	1.32	1.07 - 1.63	**0.010**

* including non-linear and interaction terms.
The HR for continuous variables is expressed as the HR of disease progression related to the interquartile range (interval between the 75th and 25th quantiles). Abbreviations: BMI: Body Mass Index; CI: confidence interval; EVE-EXE: everolimus plus exemestane; ECOG PS: Eastern Cooperative Oncology Group Performance Status; HR: Hazard Ratio.

B. Imputed data / all metabolic parameters

Variables		HR	95% CI	p
Baseline glycemia*	Continuous	0.92	0.77 - 1.10	**<.001**
On-treatment glycemia*	Continuous	1.19	0.98 - 1.45	**<.001**
Baseline cholesterol	Continuous	1.10	0.91 - 1.32	0.398
Average cholesterol	Continuous	0.89	0.74 - 1.07	0.206
Baseline triglycerides	Continuous	1.16	0.95 - 1.40	0.323
Average triglycerides	Continuous	0.95	0.77 - 1.18	0.901
Line of EVE-EXE treatment	Continuous	1.24	1.13 - 1.36	**<.001**
Age	Continuous	1.15	0.93 - 1.40	0.185
Disease Free Interval	Continuous	0.91	0.72 - 1.14	0.811
BMI	Continuous	1.01	0.82 - 1.24	0.212
EVE interruption/dose reduction	Reduction vs full dose Interruption vs full dose	0.78 0.38	0.67 - 0.91 0.28 - 0.52	**<.001**
ECOG PS	1 vs 0 2 vs 0	1.31 1.46	1.11 - 1.54 0.65 - 3.27	**0.006**
Visceral Disease	Yes vs No	1.20	0.99 - 1.45	0.059
Presence of liver metastases	Yes vs No	1.31	1.05 - 1.62	**0.015**

* including non-linear and interaction terms
The HR for continuous variables is expressed as the HR of disease progression related to the interquartile range (interval between the 75th and 25th quantiles). Abbreviations: BMI: Body Mass Index; CI: confidence interval; EVE-EXE: everolimus plus exemestane; ECOG PS: Eastern Cooperative Oncology Group Performance Status; HR: Hazard Ratio.

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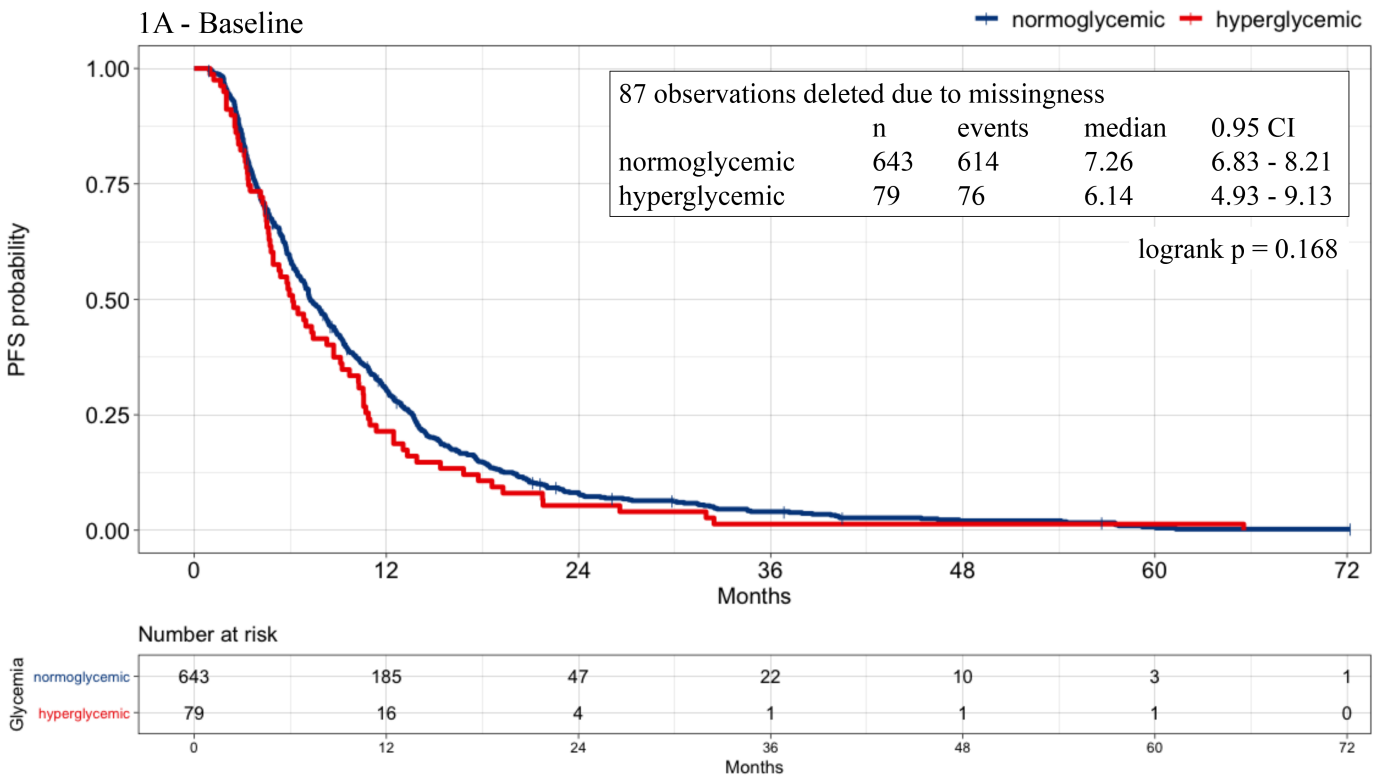
FIGURE LEGENDS:

Figure 1. Progression Free Survival represented through Kaplan Meier curves according to baseline (A) and on-treatment (average) blood glucose (B) concentration.

Figure 2. A-C) Curves showing the impact of on-treatment glycemia on hazard for disease progression, according to baseline glycemia. Curves were drawn at the 10^{th} (A), 50^{th} (B) and 90^{th} (C) percentile of the baseline (85, 95, 125). **D)** Contour plot model describing how the impact of baseline glycemia (y axis), on-treatment glycemia (x axis) and predicted patient PFS (z axis, corresponding to the color scale).

Figure 3. Kaplan Meier curves representing patient progression-free survival (PFS) according to baseline glycemia (normal *vs.* high) and on-treatment diabetic status (yes *vs.* no).

Figure 1A-B



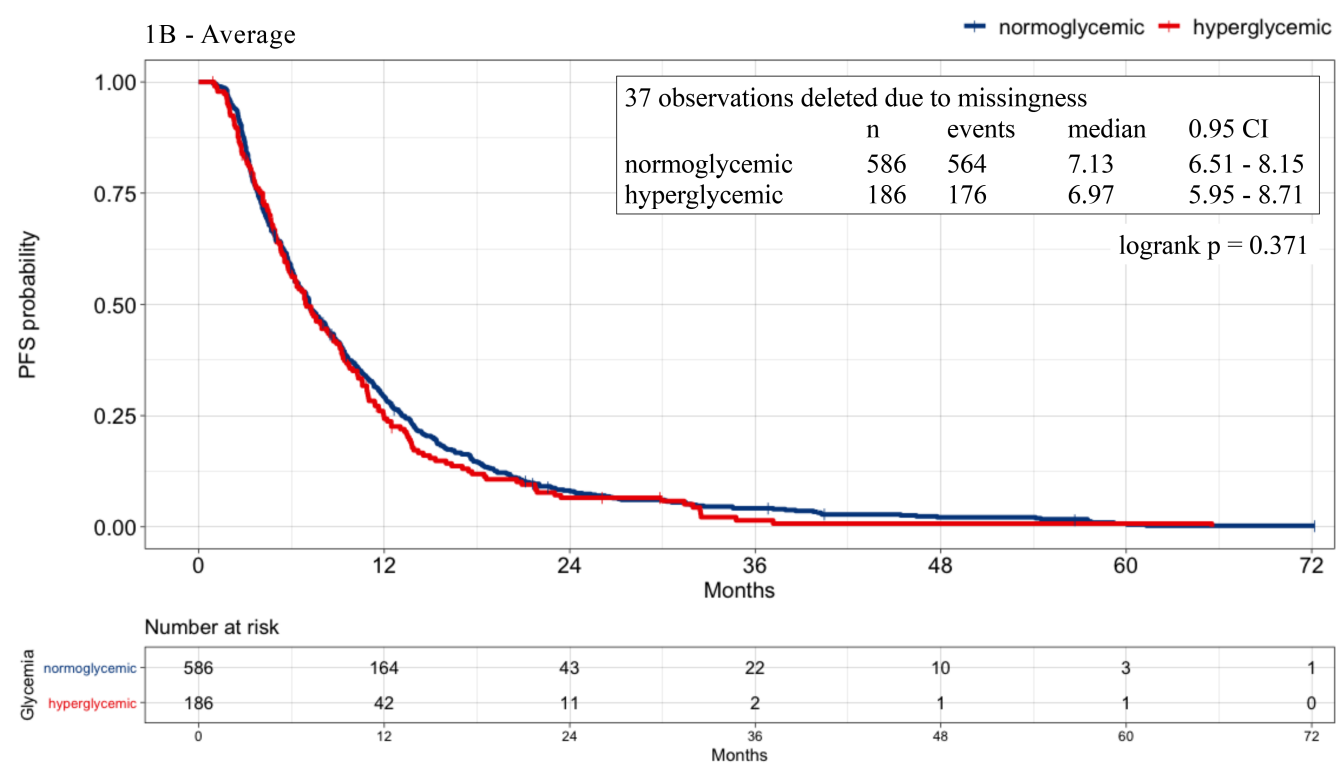
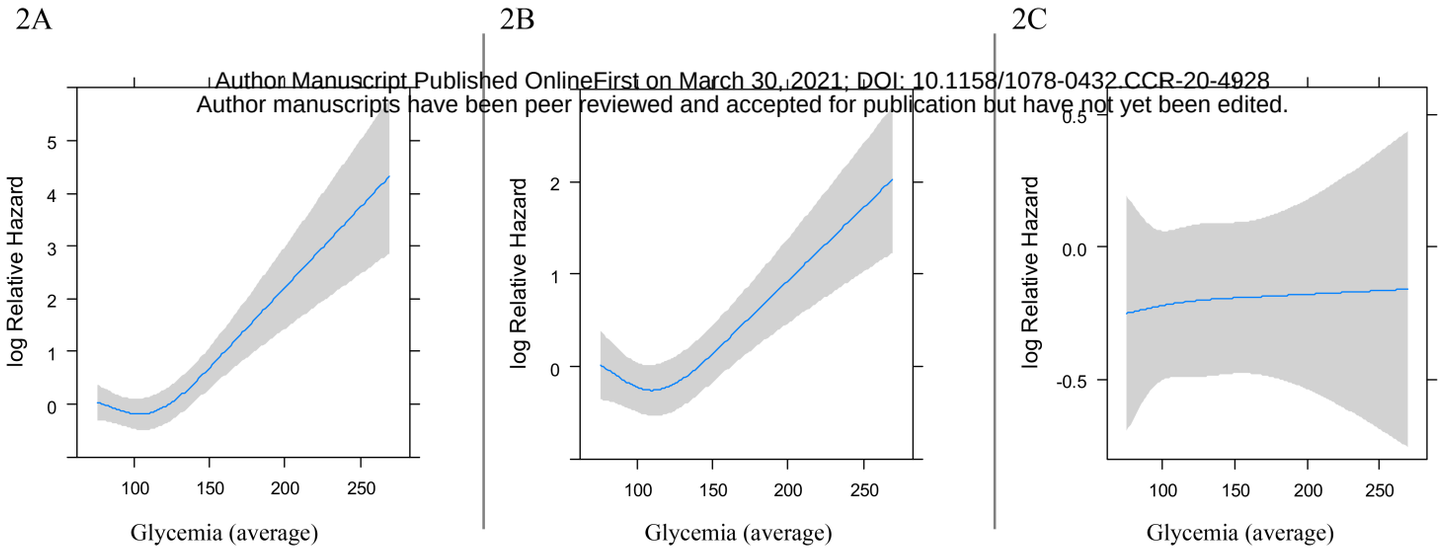


Figure 2A-D



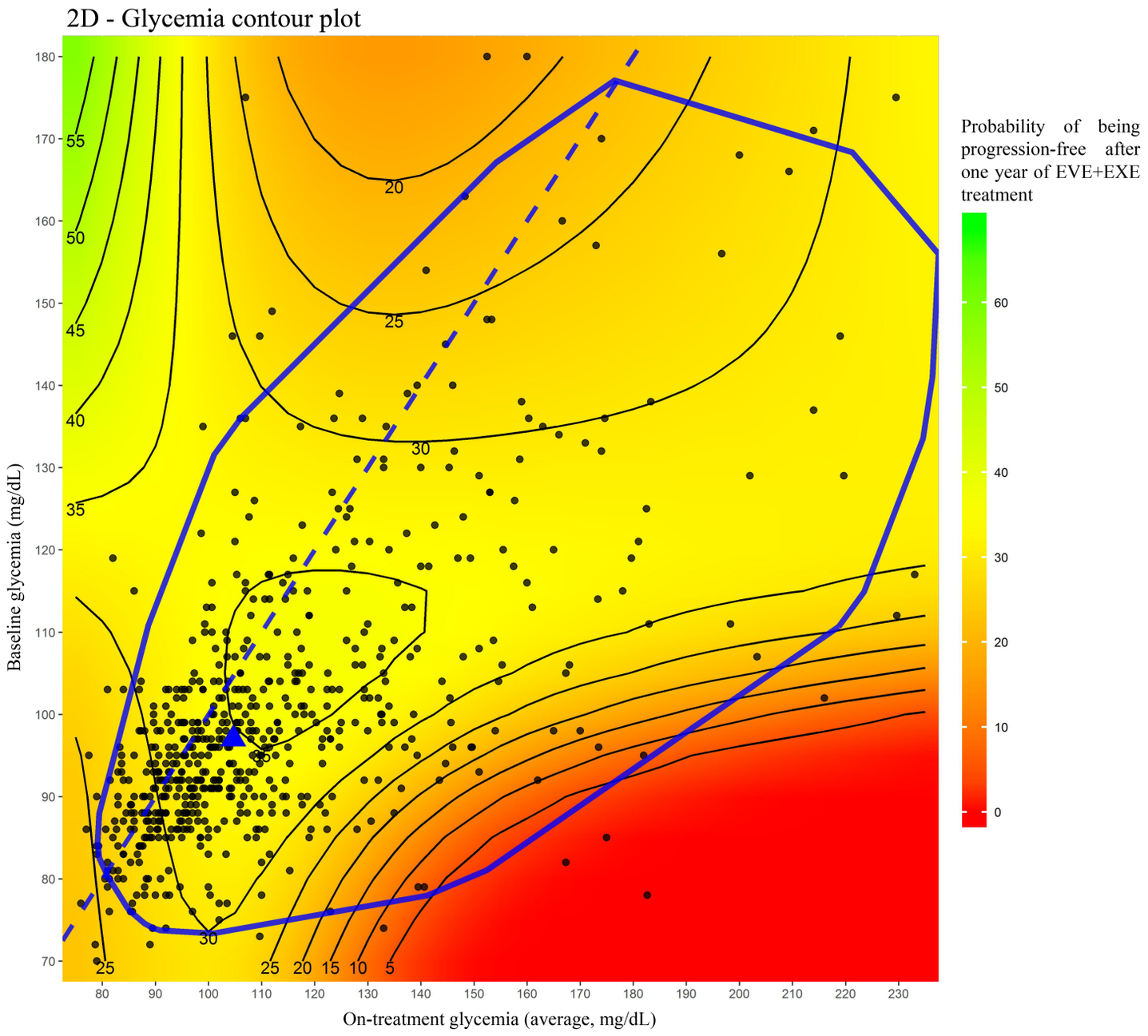


Figure 3A-B

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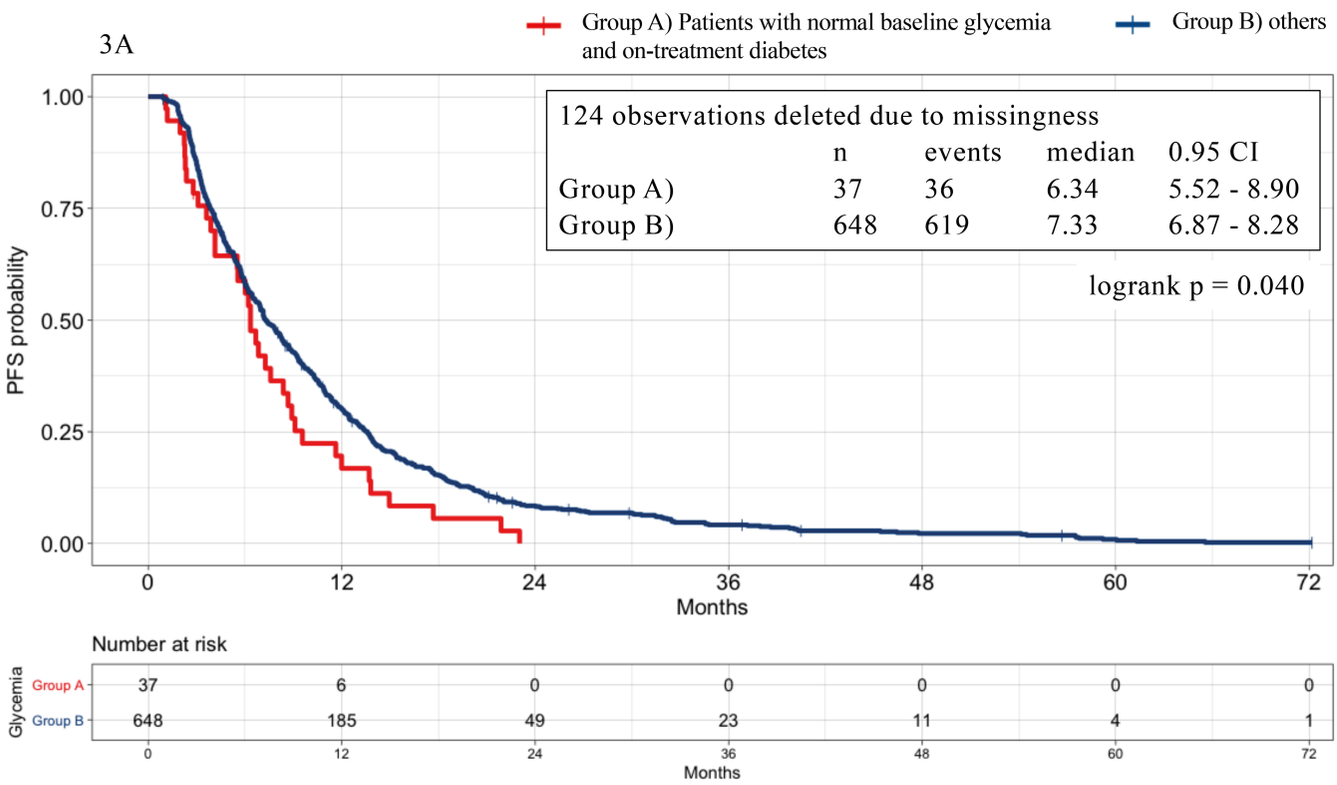

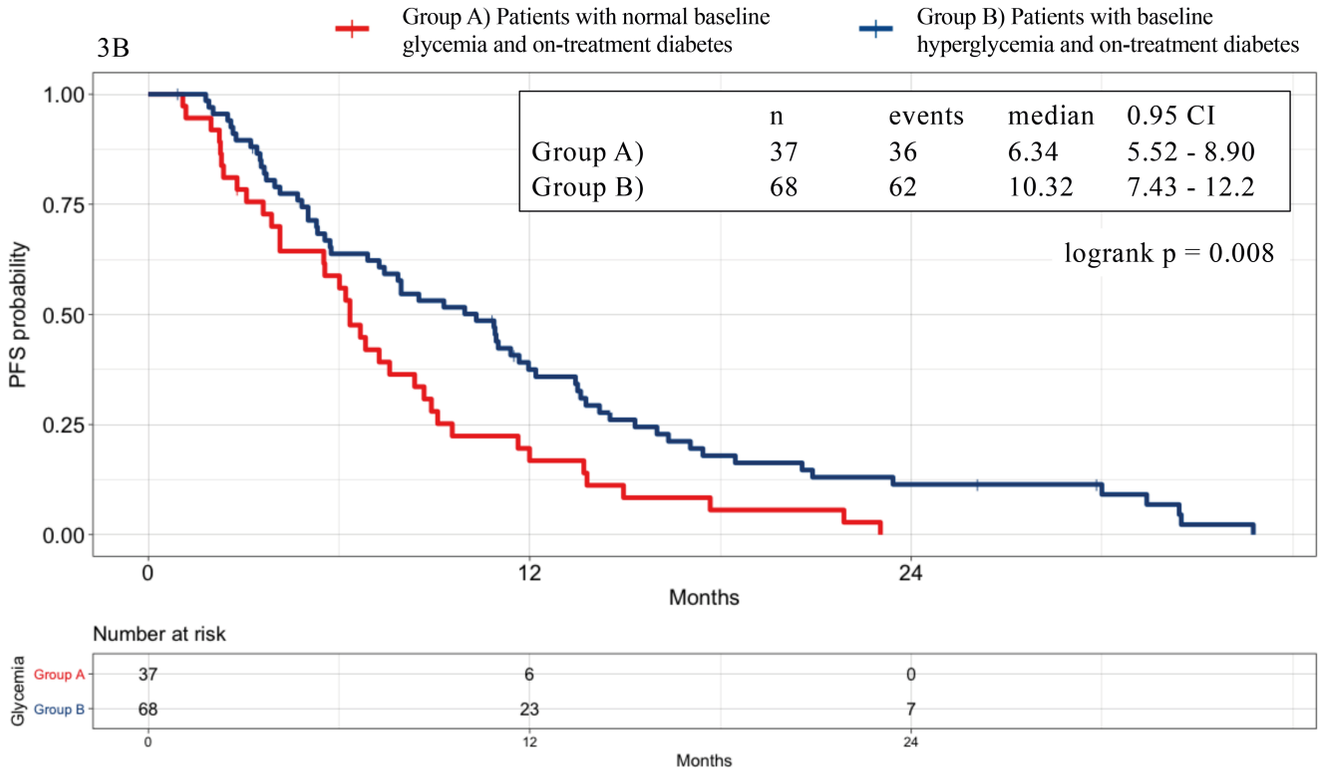

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Impact of baseline and on-treatment glycemia on everolimus-exemestane efficacy in patients with hormone receptor-positive advanced breast cancer (EVERMET)

Claudio Vernieri, Federico Nichetti, Luca Lalli, et al.

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