

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

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

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94 **Translational Relevance:**
95 Everolimus (EVE) and other PI3K/AKT/mTORC1 pathway inhibitors are associated with metabolic
96 adverse events, including hyperglycemia/diabetes and hyperinsulinemia. The impact of baseline and
97 on-treatment blood glucose levels on the clinical efficacy of EVE-based combinations remains poorly
98 defined. Here we performed a large observational study, showing an interaction between baseline and
99 on-treatment glycemia in affecting the risk of disease progression in advanced breast cancer patients
100 treated with EVE-EXE combination. In particular, patients with normal baseline glycemia have
101 significantly worse clinical outcomes if they experience on-treatment hyperglycemia. This study
102 supports the use of early alterations in blood glucose concentration as a biomarker of EVE-EXE
103 efficacy and provides the rationale for exploiting novel metabolic interventions as anticancer strategies
104 in advanced breast cancer.

105

106 **ABSTRACT**

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

112 **Experimental Design:** We conducted a multicenter, retrospective, Italian study to investigate the
113 impact of baseline and on-treatment (i.e., during first three months of therapy) blood glucose levels on
114 progression-free survival (PFS) in HR+/HER2- aBC patients treated with EVE-EXE.

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

124 **Conclusions:** The impact of on-treatment glycemia on the efficacy of EVE-EXE therapy in
125 HR+/HER2 aBC patients depends on baseline glycemia. This study lays the foundations for
126 investigating novel therapeutic approaches to target the glucose/insulin axis in combination with
127 PI3K/AKT/mTORC1 inhibitors in HR+/HER2 aBC patients.

128 1. INTRODUCTION

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

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

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

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

161 2. MATERIAL AND METHODS

162 2.1. Patient population and enrollment criteria

163 This was an observational, retrospective, multicenter study conducted in 20 Italian Cancer Centers
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173 Centrale, Udine]. Data were collected through an electronic database.

174 The main enrollment criteria consisted in: 1) age ≥ 18 years; 2) histologically/cytologically confirmed
175 diagnosis of HR+/HER2- advanced (inoperable locally advanced or metastatic) BC; 3) post-
176 menopausal status, as defined as: a) patients of age equal to or higher than 60 years; b) patients of age
177 lower than 60 years but with amenorrhea from at least 12 months that was not related to the
178 administration of chemotherapy or LHRH analogs; c) pre/peri-menopausal patients receiving LHRH
179 analogs in combination with EVE-EXE; d) patients with ovarian ablation, either through radiation
180 therapy or bilateral ovariectomy; 4) treatment for at least one month with daily EVE (initial dosage of
181 10 mg/day) plus EXE (25 mg/day) between October 2012 and July 2019 outside clinical trials
182 sponsored by pharmaceutical companies; 5) disease recurrence or progression after/on prior therapy
183 with NSAIs plus/minus Cyclin-Dependent Kinase 4/6 (CDK 4/6) inhibitors; 6) availability of at least
184 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.

209 Secondary objectives of the study were: a) to investigate the association between baseline
210 hyperglycemia (as defined as fasting blood glycemia ≥ 126 mg/dL measured within 28 days before the
211 initiation of EVE-EXE) and patient PFS; b) to evaluate the association between the onset of precocious
212 hypercholesterolemia and hypertriglyceridemia, as defined as average fasting plasma cholesterol and
213 triglycerides ≥ 200 mg/dL and ≥ 170 mg/dL, respectively, during the first three months of EVE-EXE
214 treatment, and patient PFS; c) to investigate the association between baseline hypercholesterolemia (\geq
215 200 mg/dL) or baseline hypertriglyceridemia (≥ 170 mg/dL) and PFS; d) to assess the impact of
216 baseline and on-treatment glycemia, cholesterolemia and triglyceridemia, as evaluated as continuous
217 variables, on PFS. Patients who had not experienced disease progression or death at data cut off and
218 analysis were censored at the time of last disease evaluation or last follow-up.

219 ***2.3. Glucose, cholesterol and triglyceride evaluation***

220 Measurement of fasting (at least 8 hours after the last meal) plasma glucose, cholesterol and
221 triglyceride concentration was performed at baseline and before initiating a new treatment cycle as per
222 clinical practice; data regarding metabolite measurements at baseline and at 1, 2 and 3 months were
223 collected whenever available. For the purpose of the study, metabolite measurements obtained during
224 the first three months of EVE-EXE treatment (i.e., excluding baseline evaluations) were summarized as
225 average, maximum and absolute differences with respect to baseline levels (delta). The average was
226 defined as the arithmetic mean of metabolite concentrations during the study treatment (baseline
227 excluded). The maximum (max) was defined as the highest value of metabolite measurement during
228 the first three months of EVE-EXE therapy (baseline excluded). The delta was defined as the absolute
229 difference between max and baseline values for each metabolic variable. Baseline, average and max
230 values were analyzed both as dichotomous variables, with a cut off of 126 mg/dL, 200 mg/dL and 170
231 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 75th and 25th 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

256 metabolic parameters during the first three months of treatment; in this landmark analysis, we
257 investigated the impact of baseline and on-treatment glycemia on patient PFS after excluding patients
258 undergoing disease progression during the first three months of therapy.

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

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265 3. **RESULTS**

266 **3.1. Patient population**

267 We evaluated a total number of 848 patients. Of these, 35 patients were excluded due to the lack of at
268 least one blood glucose measurement at baseline or during the first three months of treatment, while 4
269 patients were excluded due to the unavailability of the date of last follow up. The study CONSORT
270 diagram is shown in **Supplementary Figure 1**. Finally, 809 patients fulfilling all the enrollment
271 criteria and treated with the EVE-EXE combination between October 2012 and July 2019 were
272 included. Baseline patient and disease characteristics are displayed in **Table 1**. All patients had
273 received prior therapy with NSAIs in the adjuvant or advanced treatment setting, while 54% of them
274 received anti-estrogens (i.e., fulvestrant and/or tamoxifen) for the treatment of advanced disease. At
275 data cut off and analysis, 775 patients had experienced disease progression during EVE-EXE treatment,
276 and 435 patients had died. Median follow up time was 37.4 months [interquartile range (IQR): 22.8 -
277 56.4], with median PFS of 7.13 months (IQR: 3.8 - 12.9) and median OS of 32.1 months (IQR: 15.9 -
278 54.8).

279

280 **3.2. Effect of EVE-EXE on blood metabolic parameters**

281 Details about baseline and on-treatment metabolic biomarkers are described in **Supplementary Table**
282 **1**. At baseline, fasting plasma glucose measurements were available for 722 (89.2%) patients; of these,
283 79 (10.9%) patients were hyperglycemic according to the pre-specified threshold (i.e., ≥ 126 mg/dL).
284 At 1, 2 and 3 months after EVE-EXE initiation, plasma glucose measurements were available for 692
285 (85.5%), 643 (79.5%) and 537 (66.4%) patients, respectively. Consistent with the study assumptions,
286 186 (24.1%) out of 772 patients with at least one available on-treatment plasma glucose measurement
287 were found to be hyperglycemic (i.e., average plasma glucose concentration ≥ 126 mg/dL).

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

294 Patient and treatment characteristics according to on-treatment glycemic status are summarized in
295 **Supplementary Table 2**. Overall, normoglycemic and hyperglycemic patients were well balanced with
296 respect to these factors, with the exception that hyperglycemic patients were significantly older and had
297 higher body mass index (BMI). In addition, hyperglycemic patients were more likely to receive
298 metformin as an antidiabetic medication, started either before or during EVE-EXE treatment.

299 Regarding plasma cholesterol and triglyceride concentration, baseline measurements of these
300 parameters were available for 536 (66.3%) and 500 (61.8%) patients, respectively, with a total number
301 of 340 (63.4%) hypercholesterolemic (≥ 200 mg/dL) and 93 (18.6%) hypertriglyceridemic (≥ 170
302 mg/dL) patients. At 1, 2 and 3 months after EVE-EXE initiation, blood cholesterol measurements were
303 available for 477 (59.0%), 421 (52.0%) and 387 (47.8%) patients, respectively, while data on
304 triglyceride concentration were available for 440 (54.4%), 383 (47.3%), and 351 (43.4%) patients,
305 respectively. Average on-treatment hypercholesterolemia and hypertriglyceridemia were detected in
306 472 (78.9%) and 181 (32.3%) patients, respectively.

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

312

313 **3.3. Association between dichotomized metabolic parameters and PFS**

314 Patients who were hyperglycemic at baseline had non-statistically significantly different PFS when
315 compared to normoglycemic patients (median PFS [mPFS], 6.14 vs. 7.26 months, respectively;
316 unadjusted HR 1.18; 95% CI 0.93-1.50; $p = 0.168$) (**Figure 1A**). Similarly, there were no significant
317 PFS differences between hyperglycemic and normoglycemic patients according to on-treatment
318 glycemia (mPFS 6.97 vs. 7.13 months; unadjusted HR 1.08; 95% CI 0.91-1.28; $p = 0.371$) (**Figure**
319 **1B**).

320 The impact of baseline and on-treatment cholesterol and triglyceride concentration according to the
321 pre-specified thresholds was non-statistically significant as well. In particular, we did not find a
322 significant association between baseline cholesterol or triglycerides levels and patient PFS (mPFS in
323 hypercholesterolemic vs. normocholesterolemic patients: 7.95 vs. 7.82 months; unadjusted HR 0.94;
324 95% CI 0.78-1.12; $p = 0.479$; mPFS in hypertriglyceridemic vs. normotriglyceridemic patients: 5.75 vs.
325 7.95 months; unadjusted HR 1.12; 95% CI 0.89-1.41; $p = 0.342$) (**Supplementary Figure 3A-B**).
326 Similarly, PFS was not statistically significantly different in hypercholesterolemic vs.
327 normocholesterolemic (mPFS of 7.59 vs. 6.21 months, respectively; unadjusted HR 0.92; 95% CI 0.75-
328 1.12; $p = 0.403$) and in hypertriglyceridemic vs. normotriglyceridemic (mPFS: 7.95 vs. 7.20 months,
329 respectively; unadjusted HR 0.93; 95% CI 0.78-1.12; $p = 0.479$) patients when on-treatment metabolite
330 levels were considered (**Supplementary Figure 3C-D**).

331 **3.4. Impact of baseline and on-treatment glycemia as continuous variables on PFS**

332 Then, we investigated in a multivariable model the impact of blood glucose concentration, as evaluated
333 as a continuous variable, on patient PFS. To this aim, we first performed an exploratory analysis based
334 on Random Forest algorithm (see Material and Methods) to exclude clinically irrelevant variables (i.e.,

335 variables not associated with PFS). Based on this analysis, the following covariates were excluded:
336 presence of lung metastases, bone metastases, lymph node metastases, central nervous system (CNS)
337 metastases or soft tissue metastases; prior therapy with anthracyclines and/or taxanes; adjuvant
338 chemotherapy; adjuvant ET. The use of metformin was also excluded as a covariate for subsequent
339 analyses (**Supplementary Figure 4A**). The following predictors of PFS were instead selected for
340 further evaluation in the multivariable model: patient age, body mass index (BMI), Eastern Cooperative
341 Oncology Group Performance Status (ECOG PS), line of EVE-EXE treatment, EVE dosages, presence
342 of visceral disease, presence of liver metastases, disease-free interval (as defined as the time between
343 surgery of the primary tumor and tumor recurrence as metastatic disease), baseline and on-treatment
344 glycemia, baseline and on-treatment cholesterolemia, baseline and on-treatment triglyceridemia
345 (**Supplementary Table 4**). Of note, the effect of metabolic parameters on patient PFS was non-linear
346 and, in the case of blood glucose, it was characterized by a pattern of interaction between baseline and
347 on-treatment glycemia (**Supplementary Figure 5A-B**).

348 After selecting potentially relevant variables, we fitted a Cox regression model to assess the
349 independent impact of these variables on patient PFS. In a first model, among metabolic variables we
350 only included baseline and on-treatment blood glucose levels, along with their interaction. Missing
351 metabolic data were imputed (see Materials and Methods). This model revealed a negligible impact of
352 baseline glycemia on PFS, while there was a moderate association between high on-treatment glycemia
353 and worse PFS (**Table 2A**). Notably, the impact of both baseline and on-treatment glycemia on PFS
354 was largely attributable to the interaction between these two factors, as demonstrated by hierarchical
355 statistical testing of model coefficients (**Supplementary Table 5**). We found similar results when
356 cholesterol and triglyceride concentration was also included in the Cox model (**Table 2B**). In both
357 multivariable models, more advanced EVE-EXE treatment line, worse ECOG PS and the presence of
358 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

383 ***3.5 Role of the interaction between baseline and on-treatment glycemia on PFS***

384 The presence of an interaction between baseline and on-treatment glycemia makes results of Cox
385 models poorly interpretable, in particular with respect to the HRs that summarize the impact of
386 individual variables on PFS. To dissect the pattern of interaction between baseline and on-treatment
387 glycemia, we plotted log-relative hazards according to on-treatment blood glucose concentrations
388 (80-270 mg/dL range) at three different levels of baseline blood glycemia, namely 85 mg/dL, 95 mg/dL
389 and 125 mg/dL, which correspond to the 10th, 50th and 90th distribution quantiles, respectively. For
390 baseline glycemia of 85 mg/dL, we found a 4-fold increase in log-Relative hazard for increasing
391 on-treatment blood glucose levels (**Figure 2A**). At a level of baseline glycemia of 95 mg/dL, we
392 observed a similar pattern, with a 2-fold increase in log-Relative hazard for increasing on-treatment
393 blood glucose levels (**Figure 2B**). Finally, the log-Relative hazard curve was flat at the level of 125
394 mg/dL baseline glycemia (**Figure 2C**). These data indicate that an increase of blood glucose
395 concentration during EVE-EXE therapy might be associated with an increased risk of disease
396 progression in patients with normal glycemia at baseline, but not in patients who are already
397 hyperglycemic before treatment initiation.

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

406 patients with higher baseline glycemia and lower on-treatment glycemia (upper-left corner)
407 corresponded to the best PFS.

408 To illustrate the impact of the interaction between baseline and on-treatment glycemia in a more
409 intuitive way, we compared PFS Kaplan-Meier curves of patients who were normoglycemic at baseline
410 (< 100 mg/dL) and became diabetic (≥ 126 mg/dL) during EVE-EXE therapy with PFS Kaplan-Meier
411 curves of other patient subsets. Patients with normal baseline blood glucose levels who became diabetic
412 during the treatment (Group A) had significantly worse PFS when compared to the remaining patients
413 (Group B) (mPFS 6.34 vs. 7.33 months; unadjusted HR 1.42; 95% CI 1.01-1.99; $p= 0.040$) (**Figure**
414 **3A**). Also within these two different cohorts, metformin use was not associated with significantly
415 different PFS (**Supplementary Figure 4B-C**).

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

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431 **4. DISCUSSION**

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

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

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

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

464 As per clinical protocol, we initially evaluated the potential impact of baseline or on-treatment
465 hyperglycemia, as defined as blood fasting glucose concentration ≥ 126 mg/dL, on patient PFS. In the
466 primary analysis, we did not find a statistically significant association between hyperglycemia and the
467 risk of disease progression. When interpreting these results in the light of the final study findings, we
468 should consider that: 1) in the primary analysis we only evaluated the effect of metabolic variables at
469 one time point (baseline or on-treatment glycemia), while we did not take into account the impact of
470 their interaction on PFS; 2) both baseline and on-treatment glycemia are continuous variables, while in
471 the primary analysis we evaluated them as dichotomous. In clinical studies, dichotomizing continuous
472 variables is a common tool that is used to identify parameter thresholds that can be used to allocate
473 patients in different classes of risk, thus favoring decision processes by physicians. However,
474 dichotomization of continuous variables can be misleading for several reasons: a) commonly used
475 thresholds may not be appropriate for the specific clinical context; for instance, the 126 mg/dL
476 threshold, which is used for the diagnosis of diabetes mellitus, might fail to distinguish between cancer
477 patients more or less likely to benefit from a specific antitumor therapy; b) even if appropriate
478 thresholds are found for specific clinical contexts, dichotomization may be misleading in the case of

479 non-monotonic or non-linear relationships between metabolite concentration and clinical outcomes, as
480 was the case of the association between blood glucose levels and patient PFS in our study. For these
481 reasons, the impact of metabolic factors on clinical outcomes could be more reliably assessed when
482 these variables are evaluated as continuous rather than dichotomous variables, and by using
483 interactive, longitudinal models.

484 To explain the interaction between baseline and on-treatment glycemia in affecting patient PFS, we
485 hypothesize that higher baseline blood glucose and insulin levels could be associated with higher
486 baseline activation of the PI3K/AKT/mTORC1 axis in cancer cells and, potentially, with higher tumor
487 cell sensitivity to EVE-induced inhibition of mTORC1 regardless of on-treatment
488 glycemia/insulinemia. On the other hand, tumors arising in patients with normal baseline
489 glycemia/insulinemia might display lower baseline activation of the PI3K/AKT/mTORC1 axis; in
490 conditions of normal extracellular blood glucose/insulin concentration, these tumors could maintain
491 some sensitivity to EVE-EXE, while the occurrence of precocious EVE-induced hyperglycemia and
492 hyperinsulinemia could result in a boost of PI3K/AKT/mTORC1 activation, and in cancer cell
493 resistance to the treatment. While this hypothesis needs to be confirmed by preclinical and prospective
494 clinical studies, our findings indicate that blood glucose and, potentially, insulin concentration does not
495 affect HR+/HER2- BC cell response to pharmacological mTORC1 inhibition *per se*, but their effect
496 could be strongly influenced by the metabolic environment in which the tumor grew before the
497 treatment, and in particular by baseline blood glucose/insulin concentration.

498 If confirmed by future prospective studies, our findings could have relevant clinical implications.
499 Indeed, in the subgroup of patients with normal baseline glycemia, preventing or promptly reversing
500 EVE-induced hyperglycemia or diabetes could improve EVE-EXE efficacy. To this aim, specific
501 dietary and/or pharmacologic interventions capable of preventing EVE-induced
502 hyperglycemia/diabetes should be considered in HR+/HER2- aBC patients treated with EVE-EXE,

503 especially if they are normoglycemic at baseline. Regarding dietary approaches, a low intake of refined
504 carbohydrates and sugars could be recommended to patients initiating EVE-EXE treatment. As for
505 pharmacological approaches, metformin or other antidiabetic medications should be promptly initiated
506 if dietary interventions are insufficient to keep blood glycemia below the diabetic threshold during the
507 first months of treatment. Of note, since EVE-induced hyperglycemia tends to spontaneously resolve
508 during the course of the treatment (23), blood glucose levels should be more intensively monitored to
509 prevent or to promptly manage EVE-induced hyperglycemia/diabetes during the first three months of
510 therapy, when a non-irrelevant proportion of disease progression events occur (15.1% of patients in the
511 EVERMET study). At the same time, our results indicate that patients who are hyperglycemic at the
512 time of EVE-EXE initiation could achieve poor, if any benefit from blood glucose reduction during
513 EVE-EXE treatment; in these patients, a tight control of patient glycemia and, in case, the reversal of
514 EVE-induced diabetes could be potentially less impactful on tumor-related outcomes, while
515 antidiabetic treatments should be primarily used to prevent diabetes-induced symptoms and
516 complications.

517 Since hyperglycemia and hyperinsulinemia are class effects of PI3K/AKT/mTORC1 axis inhibitors,
518 results of our study could also apply to other clinical contexts in which these compounds are used. For
519 instance, the PI3K inhibitor alpelisib has been recently approved by the FDA and EMA in combination
520 with fulvestrant for the treatment of postmenopausal women and men with HR+/HER2- aBC
521 progressing on/after prior AI therapy (9). Similar to EVE, alpelisib can cause hyperglycemia and
522 hyperinsulinemia, which could reduce its efficacy (19). Although the widespread use of alpelisib in the
523 daily treatment of HR+/HER2- aBC patients bearing *PIK3CA*-mutated tumors might be limited by
524 several factors, including the lack of an extensive tumor genomic profiling in several cancer centers,
525 the suboptimal safety profile of alpelisib and recent labels limiting alpelisib use in Europe and Italy to
526 patients previously treated with single-agent endocrine therapy (which has now been replaced by

527 endocrine therapy plus CDK 4/6 inhibitor-based combinations as a standard-of-care first-line therapy),
528 the alpelisib-fulvestrant combination remains a potentially useful therapy that could be used in up to
529 40% of all HR+/HER2- aBC patients. Therefore, since the incidence of severe (grade 3 or 4)
530 hyperglycemia is common with alpelisib (actually more common than with EVE) despite the
531 precocious use of metformin in the SOLAR-1 study (24), exploring strategies to prevent or promptly
532 manage alpelisib-induced hyperglycemia/diabetes is a clinically relevant issue, especially for patients
533 with normal baseline blood glucose levels.

534 In recent years, metformin has been extensively investigated in both preclinical and clinical setting for
535 its potential direct (cell-autonomous) or indirect (through its impact on systemic metabolism)
536 antitumor effects (25-27). Since metformin acts by reducing glucose production in the liver and at the
537 same time by sensitizing peripheral tissues to the effects of insulin, it has been considered a good
538 candidate drug to be combined with EVE-EXE for the treatment of HR+/HER2- aBC patients. Quite
539 disappointingly, one recent prospective study showed modest clinical efficacy of upfront EVE-EXE
540 plus metformin combination in overweight/obese postmenopausal women with HR+/HER2- aBC (27),
541 and similarly negative results emerged from a preclinical study in which metformin was used in
542 combination with PI3K inhibitors in mouse models of HR+/HER2- BC (19). In line with these data, in
543 our study we did not find a significant association between metformin use and PFS in HR+/HER2-
544 aBC patients treated with EVE-EXE. This evidence, together with the potential pharmacokinetic
545 interactions between EVE and metformin in patients with advanced cancers (28) and the risk of
546 increasing the incidence of diarrhea, indicate that metformin might be not an ideal drug to be used in
547 combination with EVE.

548 Conversely, specific dietary interventions, such as ketogenic diets or cyclic calorie-restricted, low-
549 carbohydrate, low-protein diets, collectively referred to as fasting-mimicking diets (FMDs), which
550 reduce blood glucose/insulin concentrations and do not have overlapping toxicities with EVE, have

551 been shown to inhibit the PI3K/AKT/mTORC1 pathway synergistically with ETs or PI3K inhibitors in
552 preclinical *in vivo* experiments (29,30). In one study, high-fat ketogenic diets were found to be more
553 effective than metformin in reducing PI3K inhibitor-induced hyperglycemia and hyperinsulinemia, and
554 demonstrated additive or synergistic *in vitro* and *in vivo* antitumor activity in combination with PI3K
555 inhibitors (19). More recently, cyclic FMDs showed synergistic antitumor activity with standard ETs
556 plus/minus cyclin-dependent kinase 4/6 (CDK 4/6) inhibitors in preclinical models of HR+/HER2-
557 BC, with initial promising results also in cancer patients (29). Of note, the synergistic activity between
558 ET and FMD was mediated by FMD-induced reduction of blood insulin/IGF-1 levels, which results in
559 increased PTEN expression and consequent inhibition of the PI3K/AKT/mTORC1 pathway in cancer
560 cells. Since ketogenic diets and FMD are potentially safe and feasible interventions in well-selected
561 cancer patient populations, combining them with EVE or other inhibitors of the PI3K/AKT/mTORC1
562 pathway could produce highly synergistic antitumor effects, while at the same time improving the
563 tolerability of these drugs.

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

575 The main limitation of this study consists in the retrospective design and the consequent missing data,
576 which could in part limit the reliability of our findings; nonetheless, the main study findings were
577 confirmed after removing patients with incomplete data from the analysis, thus adding robustness to
578 our results. Moreover, the study was negative as for its primary endpoint, and the lack of a control arm
579 does not allow establish definitive causal associations between metabolic toxicities and treatment
580 efficacy.

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

586 **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

587

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592

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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.

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

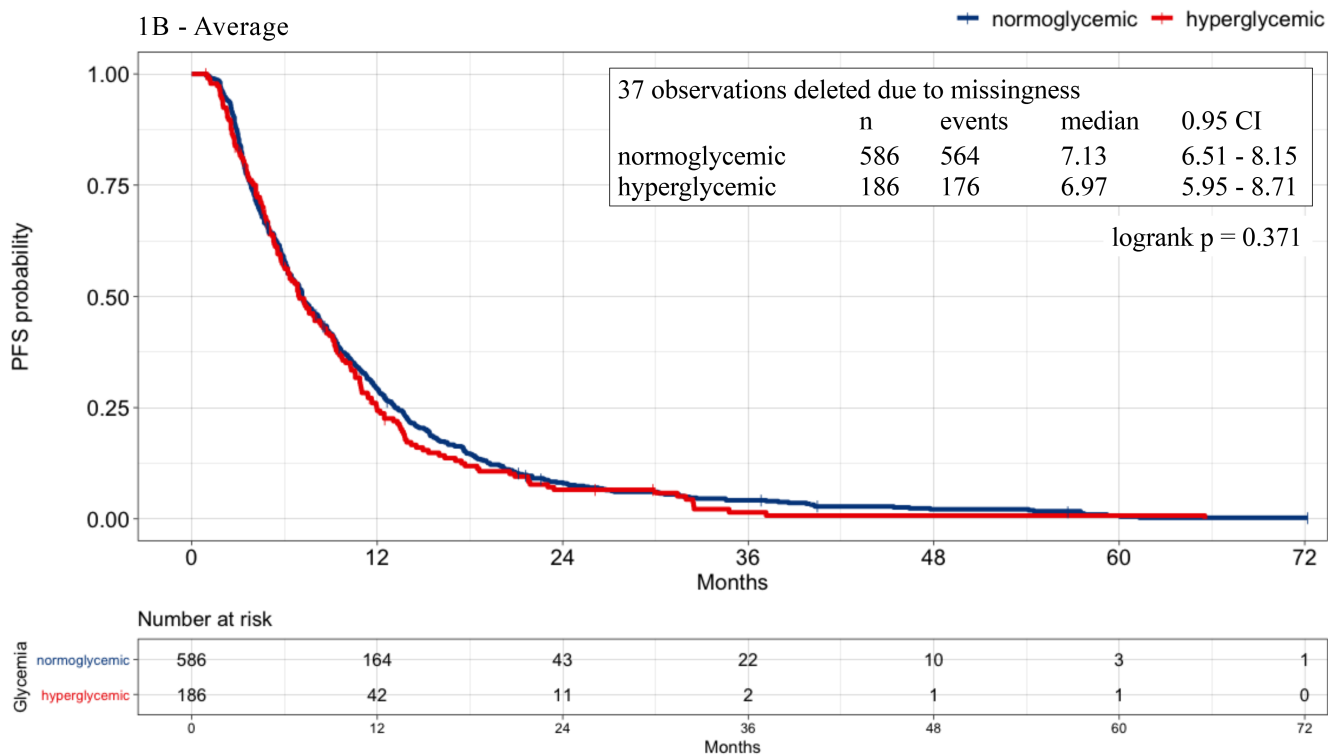
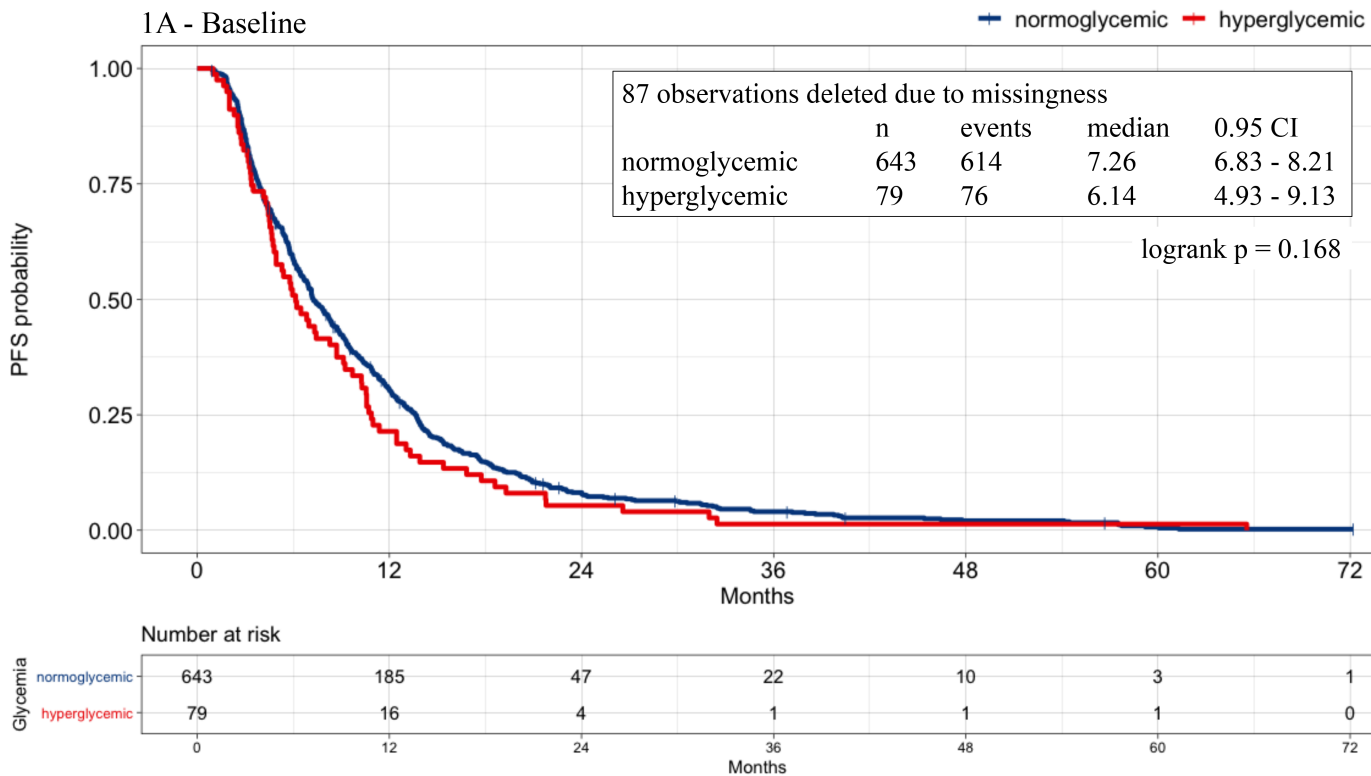
The HR for continuous variables is expressed as the HR of disease progression related to the interquartile range (interval between the 75th and 25th quantiles). Abbreviations: BMI: Body Mass Index; CI: confidence interval; EVE-EXE: everolimus plus exemestane; ECOG PS: Eastern Cooperative Oncology Group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

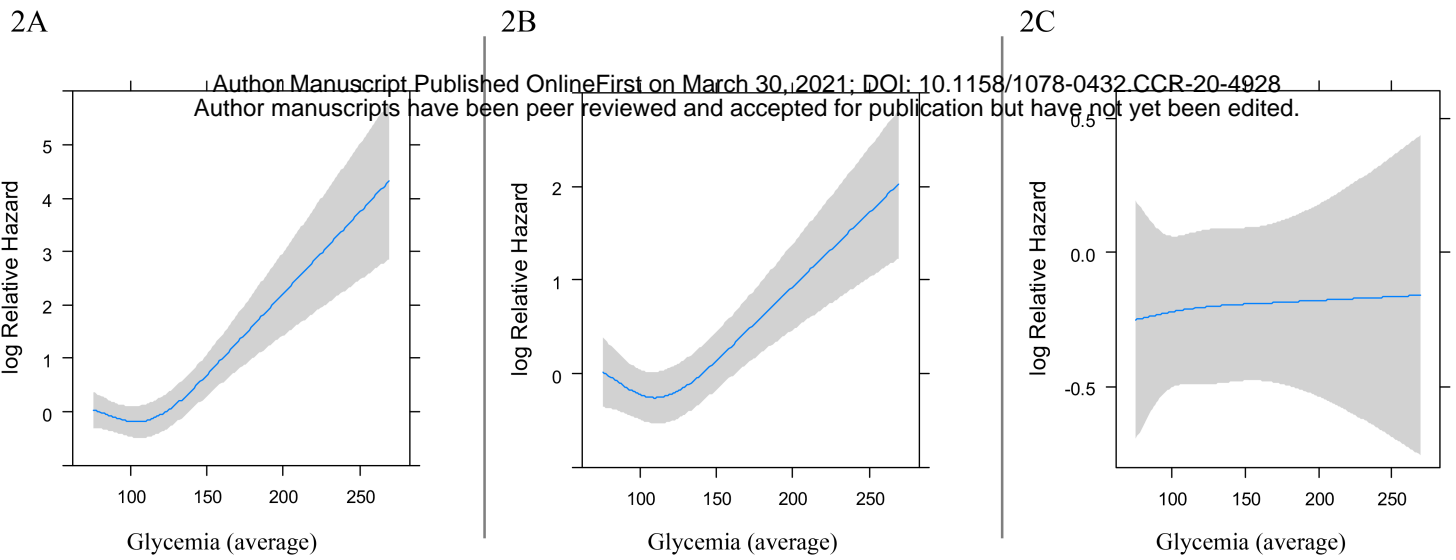
FIGURE LEGENDS:

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

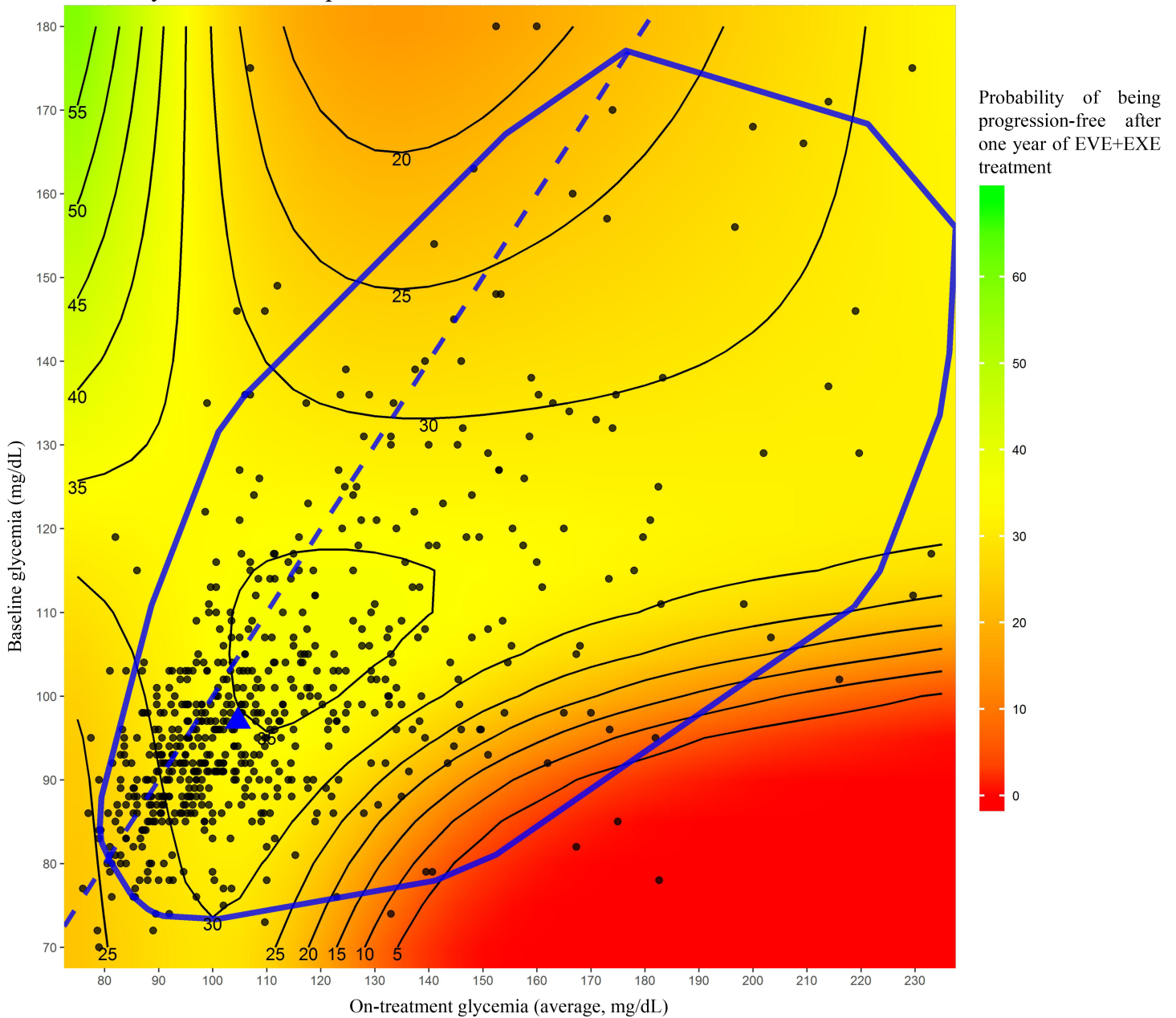
Figure 2. A-C) Curves showing the impact of on-treatment glycemia on hazard for disease progression, according to baseline glycemia. Curves were drawn at the 10th (A), 50th (B) and 90th (C) percentile of the baseline (85, 95, 125). **D)** Contour plot model describing how the impact of baseline glycemia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

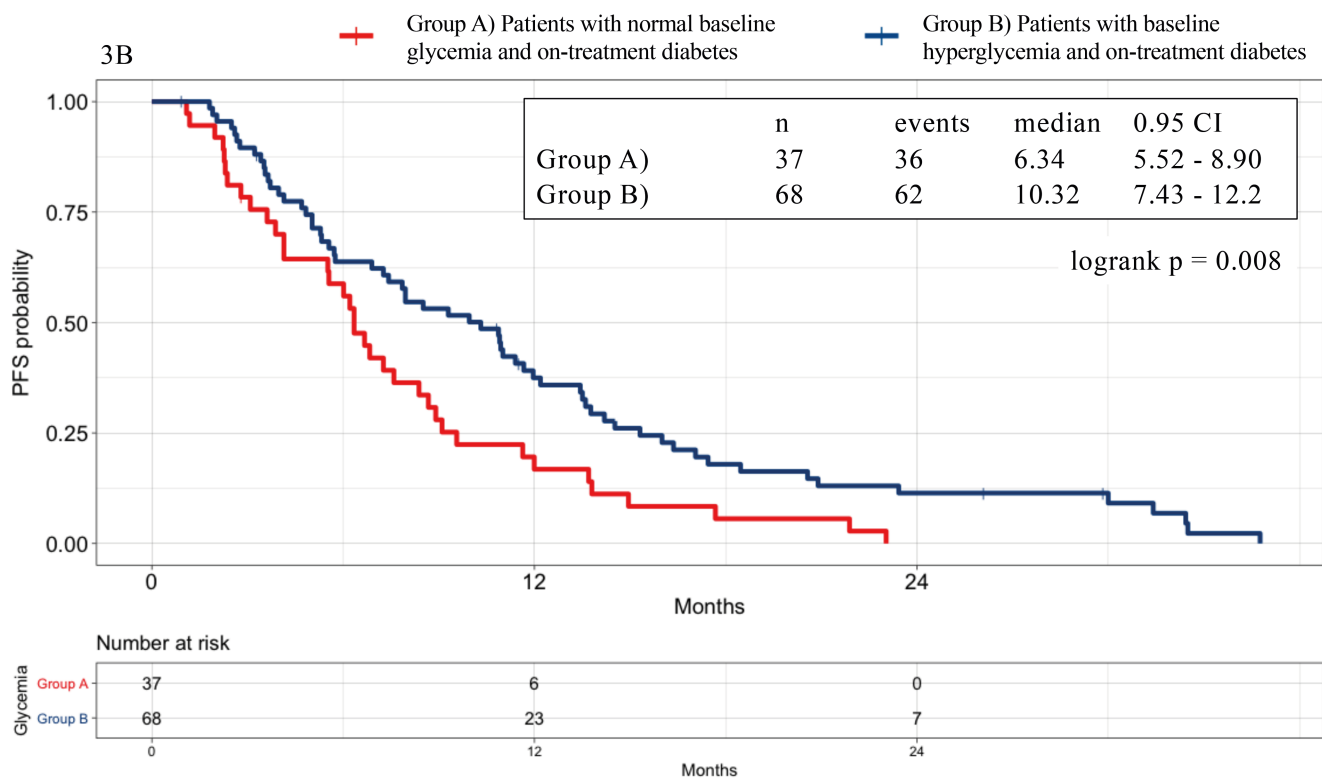
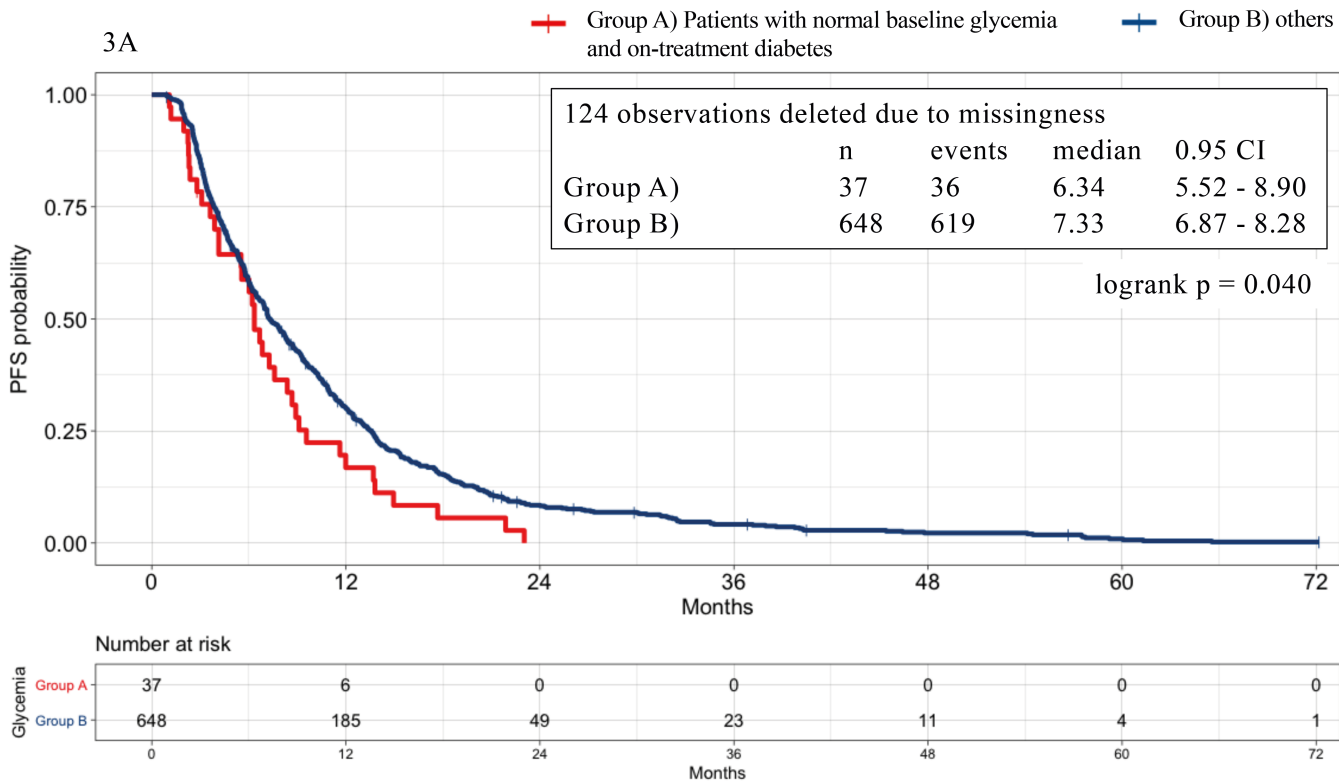
Figure 3. Kaplan Meier curves representing patient progression-free survival (PFS) according to baseline glycemia (normal vs. high) and on-treatment diabetic status (yes vs. no).





2D - Glycemia contour plot





Clinical Cancer Research

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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