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CLINICAL TRIAL

Insulin-like growth factor-1 receptor (IGF-1R) expression on circulating tumor cells (CTCs) and metastatic breast cancer outcome: results from the TransMYME trial

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Abstract

Purpose To evaluate the prognostic value of IGF-1R expression on circulating tumor cells (CTCs) in a prospective randomized clinical trial comparing chemotherapy plus metformin with chemotherapy alone in metastatic breast cancer (MBC) patients.

Methods CTCs were collected at baseline and at the end of chemotherapy. An automated sample preparation and analysis system (CellSearch) were customized for detecting IGF-1R expression. The prognostic role of CTC count and IGF-1R was assessed for PFS and OS by univariate and multivariate analyses.

Results Seventy-two out of 126 randomized patients were evaluated: 57% had ≥ 1 IGF-1R positive CTC and 37.5% ≥ 4 IGF-1R negative cells; 42% had CTC count ≥ 5/7.5 ml. At univariate analysis, the number of IGF-1R negative CTCs was strongly associated with risk of progression and death: HR 1.93 ($P = 0.013$) and 3.65 ($P = 0.001$), respectively; no association was detected between number of IGF-1R positive CTCs and PFS or OS ($P = 0.322$ and $P = 0.840$). The prognostic role of CTC count was confirmed: HR 1.69, $P = 0.042$ for PFS and HR 2.80 for OS, $P = 0.002$. By multivariate analysis, the prognostic role of the number of IGF-1R negative CTCs was maintained, while no residual prognostic role of CTC count or number of IGF-1R positive cells was found.

Conclusion Loss of IGF-1R in CTCs is associated with a significantly worse outcome in MBC patients. This finding supports further evaluation for the role of IGF-1R on CTCs to improve patient stratification and to implement new targeted strategies. Clinical trial registration: Clinicaltrials.gov (NCT01885013); European Clinical Trials Database (EudraCT No.2009-014,662-26).

Keywords Circulating tumor cells (CTCs) · Breast cancer (BC) · Liquid biopsy · Clinical trials · Invasion · Metastasis

Abbreviations

IGF-1R	Insulin-like growth factor-1 receptor
CTCs	Circulating tumor cells
MBC	Metastatic breast cancer
BC	Breast cancer
IRS-2	Insulin receptor substrate-2

Introduction

In the past 15 years, a substantial body of evidence has developed regarding the role of insulin and the insulin-like growth factor (IGF) family in breast cancer (BC) [1, 2]. Along with the metabolic effects of insulin on glucose balance, insulin has also been shown to induce cancer cell proliferation: this effect might explain the adverse prognostic effect of hyperinsulinemia in BC patients, observed in epidemiological studies [3, 4].

The pathways downstream of the insulin/IGF system are well defined: IGF-1 and insulin activate the tyrosine kinase receptor pathway, i.e., the insulin, IGF-1 and hybrid IGF-1/insulin receptors, all of which are overexpressed in BC cells. Activation of these receptors results in upregulation of the

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Insulin Receptor Substrate-2 (IRS2), leading to downstream activation of the MAPKinase and PI3K-Akt pathways. Moreover, insulin itself may also modulate circulating levels of IGFs and their binding proteins [5]. These data suggest that the insulin pathway plays a major role in BC prognosis and may represent a therapeutic target, especially in those patients exposed to high insulin plasmatic level. It has been observed that in women with early BC, hyperinsulinemia is associated with the presence of insulin resistance, hence, the development of therapies targeting hyperinsulinemia is currently one of the most intriguing fields of research in BC as well as in other types of cancer [6].

Recent interest has focused on metformin, an antidiabetic drug widely prescribed for the treatment of hyperglycemia and hyperinsulinemia. The main systemic effect of metformin in reducing the circulating level of IGF-1/insulin has been associated with anticancer action [7]. However, so far, metabolic targeting in patients with solid tumors, did not translate into a measurable clinical benefit [8, 9]. Possible reasons for failure include the complexity of the IGF-1R/insulin receptor system and the presence of parallel pathways of growth and survival, as well as the lack of appropriate patient selection markers [10].

Effective tools in the identification of patients likely to benefit from targeted therapies are needed to optimize patient selection and treatment effectiveness. Circulating tumor cell (CTC) isolation and characterization has been proposed as a tool for patient selection in MBC, being associated with prognosis and response to treatment [11, 12]. CTCs can be easily evaluated in peripheral blood by "liquid biopsy", with a minimally invasive procedure. The molecular features of CTCs can also be profiled by fluorescence in situ hybridization or immunofluorescence, supporting their use as a noninvasive approach for patient selection. The analysis of IGF-1R expression on CTCs, previously described by de Bono, is particularly appealing due to the role of the insulin/IGF pathway in tumor metastasis, cancer cell proliferation, invasion, and angiogenesis [13, 14].

To evaluate the potential impact of metabolic targeting in advanced BC, we have conducted a phase II randomized clinical trial, comparing the association of metformin plus chemotherapy (CT) with CT alone, as first line treatment in HER2 negative, non-diabetic, MBC patients (MYME trial) [15]. We present here the final results of the TransMyme study, nested in the MYME clinical trial and aimed at evaluating the prognostic role of IGF-1R expression on CTCs.

Patients and methods

Eligibility criteria

The TransMyme study was nested in MYME trial, a phase II comparative trial, of AC (non-pegylated liposomal doxorubicin 60 mg/m^2 + cyclofosfamide 600 mg/m^2, × 6/8 cycles Q21) versus AC plus Metformin (2000 mg pos daily until disease progression), that enrolled 126 HER2-negative, non-diabetic, MBC patients, at first evidence of disease relapse, between April 2010 and May 2015. The study was approved by the Ethics Committees of all the participating centers. Written informed consent was obtained from all patients before study entry. Eligibility criteria included: stage IV histologically confirmed MBC; measurable and/or non-measurable disease, availability of HOMA index calculated according to Matthews' formula [16], prior endocrine therapy was allowed in the adjuvant and/or metastatic setting; prior chemotherapy was allowed in the adjuvant setting, including anthracyclines; patients with known diabetes (type 1 or 2) were excluded. Staging procedures for disease evaluation were performed at baseline and every two months afterwards.

A HOMA index ≥ 2.5 was chosen as the cut-off value for insulin resistance based on the results from an Italian-based population study [17]. Progression-free survival (PFS) was the primary outcome measure and it was calculated from the date of randomization to the date of disease progression, death from any cause, or loss to follow-up, whichever came first. One hundred twenty-two patients were evaluable for PFS. At a median follow-up of 39.6 months (interquartile range [IQR] 24.6–50.7 months), 112 disease progressions and 71 deaths have been registered. Median PFS was 9.4 months (95% CI 7.8–10.4) for patients treated with AC plus metformin and 9.9 (95% CI 7.4–11.5) for patients treated with AC ($P = 0.651$). In patients with HOMA index < 2.5, median PFS was 10.4 months (95% CI 9.6–11.7) versus 8.5 (95% CI 5.8–9.7) in those with HOMA index ≥ 2.5 ($P = 0.034$). The effect of metformin was similar in patients with HOMA index < 2.5 and ≥ 2.5, in terms of PFS and OS.

The study was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice norms and local and national regulatory requirements.

CTC assessment

For CTC evaluation, blood samples were collected at baseline, at the end of CT administration (cycle 6 or 8) and at evidence of disease progression. All blood samples were collected into CellSave® Preservative Tubes and centrally

processed within 96 h after blood draw, at the CTC laboratory of IOV-IRCCS. CTC results were blinded to the reference physician. The CellSearch System (Menarini Silicon Biosystems, LLC) was used to count CTCs in peripheral blood, according to the manufacturer's instructions and users' guidelines [18, 19]. An event was classified as a CTC when its morphological features were consistent with that of a cell and it exhibited the phenotype EpCAM +, Cytokeratin 8, 18, 19 + (CK +), DAPI + and CD45.

Phenotypic profiling of CTC

To clarify the mechanism of putative modulation of insulin/IGF pathway throughout the treatment with metformin, IGF-1R positive CTCs were detected integrating CTC assay with specific monoclonal antibody, as previously reported for other customized tests [13, 19], by integrating the CXC kit (Menarini) with a specific mAb for detecting IGF-1R expression (Clone 33,255, #MAB391, R&D Systems, Minneapolis, MN, USA); the anti-IGF-1R was conjugated with Phycoerythrin (PE), (AcZon, Nano Biotech, Bologna, BO, Italy).

Results were expressed as the total number of CTCs, IGF-1R positive, and IGf-1R-negative CTCs per 7.5 ml of blood at each time-point.

IGF-1R CTC assay in house re-editing

An online staining procedure based on the CellSearch platform was used to obtain with a single test tube both the CTC count and the IGF-1R expression' level. Advantages of this approach are that anti-IGF-1R is added and processed simultaneously with the CK-FITC and CD45-APC antibodies, thus minimizing cell loss or disruption during permeabilization and staining steps, performed by the automated platform. To this purpose, the test firstly exploited by de Bono et al. [13] was re-edited in house. At first, the IGF-1R PE was used in conjunction with the CXC kit, to specifically quantify IGF-1R expression on the MCF7 cell line (a human BC cell line) that express this antigen in 80% of the population at low fluorescence intensity, but with a good resolution of signal to background (data not shown).

MCF7 cells were then spiked into whole blood sample of healthy donors, at numbers like those observed in vivo in cancer patients (200–1000 cells/7.5 ml peripheral blood) to be finally processed by CellSearch System. The IGF-1R-integrated CTC assay was then fully developed in blood samples obtained from healthy donors and cancer patients.

The integrated CTC assay clearly showed to discriminate IGF-1R-positive and IGF-1R-negative cells (Fig. 1), according to morphological features included in CTC definition by users' guidelines (clear visible nucleus of at least 4-micron diameter, cytoplasmic/nuclear areas overlapping more than 50%, uniformly CK staining of cytoplasm) and on the basis of staining profile (sufficient resolution of signal to background in the fourth "customized" filter).

Statistical analysis

Categorical variables were expressed as counts (%) for categorical variables and median and interquartile range (IQR) for continuous variables. Time-to-event data (PFS, OS) were described using the Kaplan–Meier curves and compared with the log-rank test. Ninety-five percent confidence intervals (95% CI) were calculated by non-parametric methods. Estimated HRs with 95% CI were calculated using univariate

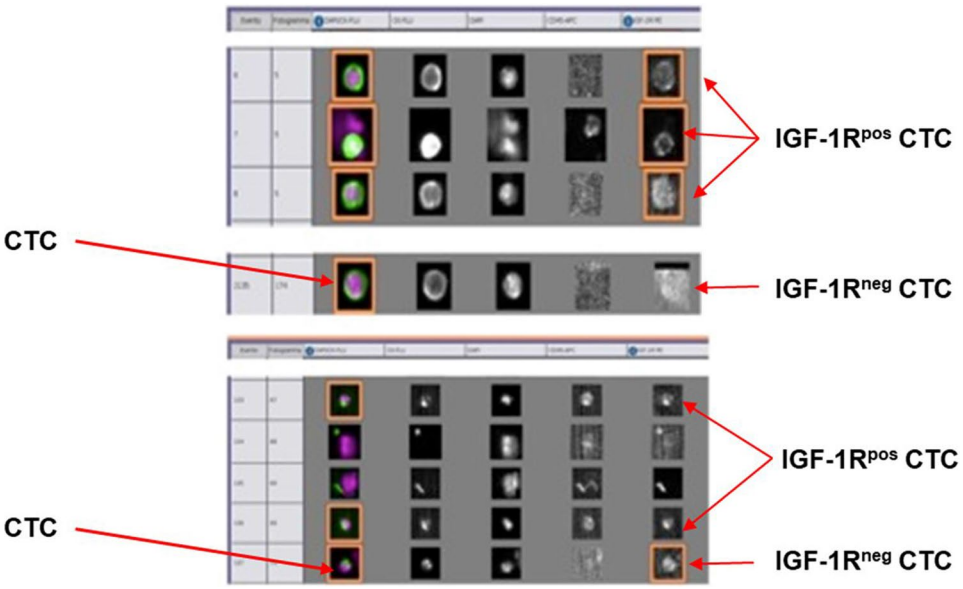

Fig. 1 IGF-1R CTC assays

and multivariate Cox proportional hazard models. PFS and OS were measured as the time between date of study entry and the disease progression or death. Patients alive and free from disease progression at the time of analysis were censored, using the time between the baseline CTC count and the most recent follow-up evaluation. A receiver operating characteristics (ROC) analysis was conducted to identify the best IGF-1R cut-off able to divide patients in two classes according to PFS. Univariate and multivariate analyses by CTC number and number of IGF-1R negative CTC were performed. The differential effect of metformin on PFS and OS was evaluated by testing the interaction between treatment arm and CTC number and IGF-1R expression. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using STATA/MP 15.0 for Windows (StataCorpLP, College Station, TX, USA). No correction for multiple testing was applied.

Results

Seventy-nine out of 126 patients enrolled into the MYME trial (62.7%) were prospectively included in the TransMyme study on CTCs; of these, 72 are included in the present analysis. Figure 2 illustrates the patient selection process in the TransMyme study. At baseline, 30 out of 72 patients (42.0%) had > 5 CTCs/7.5 ml of blood and 41 (57.0%) had at least 1 IGF-1R-positive CTC; 27 patients (37.5%) had ≥ 4 IGF-1R negative cells. Patient characteristics are reported in Table 1.

Median PFS in patients with CTC count < 5/7.5 ml was 10.8 months (95% CI 8.1–11.7) versus 8.3 months (95% CI 6.3–9.7) in patients with CTC count ≥ 5/7.5 ml (HR 1.69 (95% CI 1.02–2.79), $P = 0.042$, Fig. 3a). Similarly, a significantly longer OS was observed: 37.0 months (95% CI 26.8-NE) versus 17.9 (95% CI 13.7–22.2); HR 2.80 (95% CI 1.47–5.30), $P = 0.002$, Fig. 3b.

When CTCs were characterized according to IGF-1R expression level, i.e., IGF-1R positive ($n = 41$) and IGF-1R negative ($n = 31$), a striking difference was observed in their prognostic effect: median PFS was 10.1 months (95% CI 7.4–11.5) in patients with < 1 IGF-1R positive CTCs and 9.6 months (95% CI 7.3–11.5) in patients with ≥ 1 IGF-1R positive CTC (HR = 0.78; 95% CI 0.48–1.27, $P = 0.322$). Conversely, median PFS was 7.9 months (95% CI 3.4–9.7) in patients with ≥ 4 IGF-1R-negative CTCs versus 10.7 (95% CI 9.2–11.7) in patients with < 4 IGF-1R negative CTCs (HR 1.93; 95% CI 1.15–3.23, $P = 0.013$) (Fig. 3c).

Similarly, no association between the number of IGF-1R positive CTCs and OS was found: median OS was 22.2 months (95% CI 16.6-NE) in patients with < 1 IGF-1R positive CTCs and 30.8 months (95% CI 17.9–37.2) in patients with ≥ 1 IGF-1R positive CTCs (HR 0.94; 95% CI 0.49–1.78, $P = 0.840$). Median OS was 37 months (95% CI 28.6-NE) in patients with < 4 IGF-1R negative CTCs versus

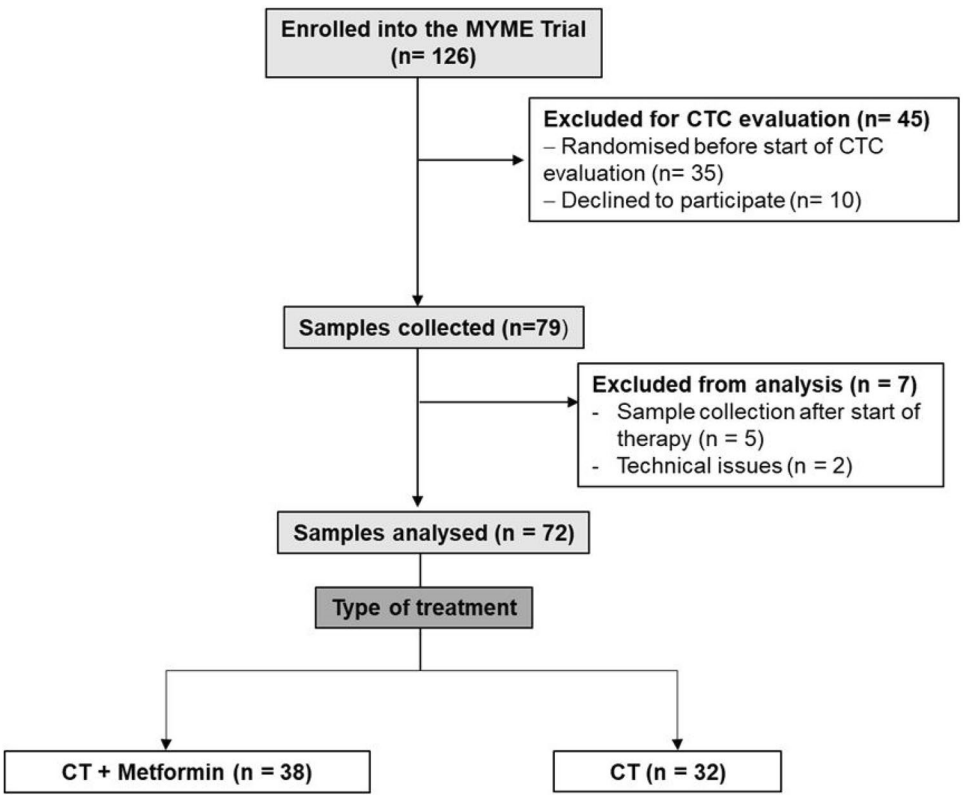

Fig. 2 TransMyme study flow diagram

Table 1 Patients' characteristics

	TransMyme study population ($n=72$)	MYME study population ($n=122$)
Median age, years (IQR range)	59 (50–67)	60 (51–66)
Post-menopausal	57 (79.2%)	100 (82.0%)
ER-positive	61 (84.7%)	106 (86.9%)
ECOG performance status		
0	57 (79.2%)	94 (77.1%)
1	14 (19.4%)	26 (21.3%)
2	1 (1.4%)	2 (1.6%)
Prior adjuvant chemotherapy	43 (59.7%)	73 (59.8%)
Prior adjuvant endocrine therapy	42 (58.3)	75 (61.5%)
Prior endocrine therapy for MBC	26 (36.1)	44 (36.1%)
Dominant metastatic site		
Bone only	12 (16.7%)	19 (15.6%)
Viscera	49 (68.1%)	78 (63.9%)
Soft tissue	11 (15.2%)	25 (20.5%)
No. of metastatic sites		
1	24 (33.3%)	39 (31.9%)
2	20 (27.8%)	40 (32.8%)
>2	28 (38.9%)	43 (35.3%)
Body mass index (BMI)		
<25	30 (41.7%)	51 (41.8%)
≥25 and <30	29 (40.3%)	50 (41.0%)
>30	13 (18.0%)	21 (17.2%)
HOMA index		
<2.5	41 (56.9%)	65 (53.3%)
≥2.5	31 (43.1%)	57 (46.7%)
Metformin treatment	38 (52.8%)	57 (46.7%)
Median insulin value (IQR range)	8.70 (7–14.2)	9.75 (7.0–14.8)

16.8 (95% CI 12.5–19.0, $P=<0.001$) in patients with ≥4 IGF-1R-negative CTCs (HR = 3.65; 95% CI 1.88–7.09, $P<0.001$) (Fig. 3d).

At multivariate analysis, the prognostic role of the number of IGF-1R negative CTCs was confirmed in terms of OS, while no residual prognostic effect of the total number of CTCs was detected; results are shown in Table 2. Similar data were obtained, when HOMA index and BMI were added to the model.

In an exploratory analysis, the role of IGF-1R expression and CTC count was evaluated by treatment arm: no evidence of interaction between IGF-1R expression or CTC count and metformin was detected, either in terms of PFS or OS.

Treatment induced modification in IGF-1R expression and CTC count was investigated in 55 out of 72 patients (76.4%) with paired blood samples collected at baseline and after the end of treatment; of these, 31 (56.4%) received chemotherapy alone and 24 (43.6%) the combination of chemotherapy and metformin. Overall, in terms of PFS no effect due to modification in the number of IGF-1R negative CTC or CTC count was observed ($P=0.10$ and 0.13, respectively). In terms of OS, a striking difference was detected: median OS was 41.4 months (95% CI 30.8 – NE) in 36 patients with no change in IGF-1R negative CTCs (<4 IGF-1R), 20.1 months (95% CI 15.2-NE) in 14 patients with ≥4 IGF-1R negative CTC at baseline and <4 after treatment, and 12.4 months (95% CI 10.1-NE) in 5 patients with ≥4 IGF-1R negative CTCs and no change with treatment ($P=<0.0001$).

Similarly, median OS was 37 months (95% CI 30.8-NE) in 31 patients with no change in CTC count (<5/7.5 ml), 22.2 months (95%CI 15.2-NE) in 15 patients with a baseline CTC count ≥5/7.5 ml and <5/7.5 ml after treatment and 12.4 months (95% CI 10.1-NE) in 9 patients with no change in CTC count (≥5/7.5 ml) or an increase to ≥5/7.5 ml CTCs ($P<0.0001$).

By multivariate analysis, no effect of metformin on modifications in IGF-1R expression or CTC count was observed ($P=0.63$).

Discussion

The TransMyme study was nested in the MYME trial comparing first line CT with first line CT plus metformin in MBC. Overall, results excluded any beneficial effect of metformin in combination with CT either in terms of PFS or OS. The primary objective of the TransMyme study was to evaluate the potential prognostic and predictive role of IGF-1R expression on CTCs. IGFs are endocrine mediators of growth hormones and also act in paracrine and autocrine fashion to regulate cell growth, differentiation, apoptosis, and transformation in many tissues including the breast [20]. Activation of these receptors by IGFs or insulin has been shown to induce the downstream activation of the MAPKinase and PI3K-Akt pathways, thus enhancing cancer cell proliferation and survival, as well as resistance to CT in BC [21]. In this regard, our results indicate a strong adverse prognostic effect of the lack of IGF-1R expression on CTCs, with an almost doubled risk of progression and death for those patients with ≥4 IGF-1R negative CTCs. At the same time, the adverse prognostic effect of CTC count was confirmed at univariate analysis, with a significantly worse PFS and OS in patients with a baseline value of ≥5 CTCs/7.5 ml of blood, in line with what previously observed in larger cohorts [22]. However, when a multivariate model, including CTC characterization and CTC count was applied, the adverse prognostic value of number of IGF-1R negative CTCs was confirmed, whereas the prognostic value

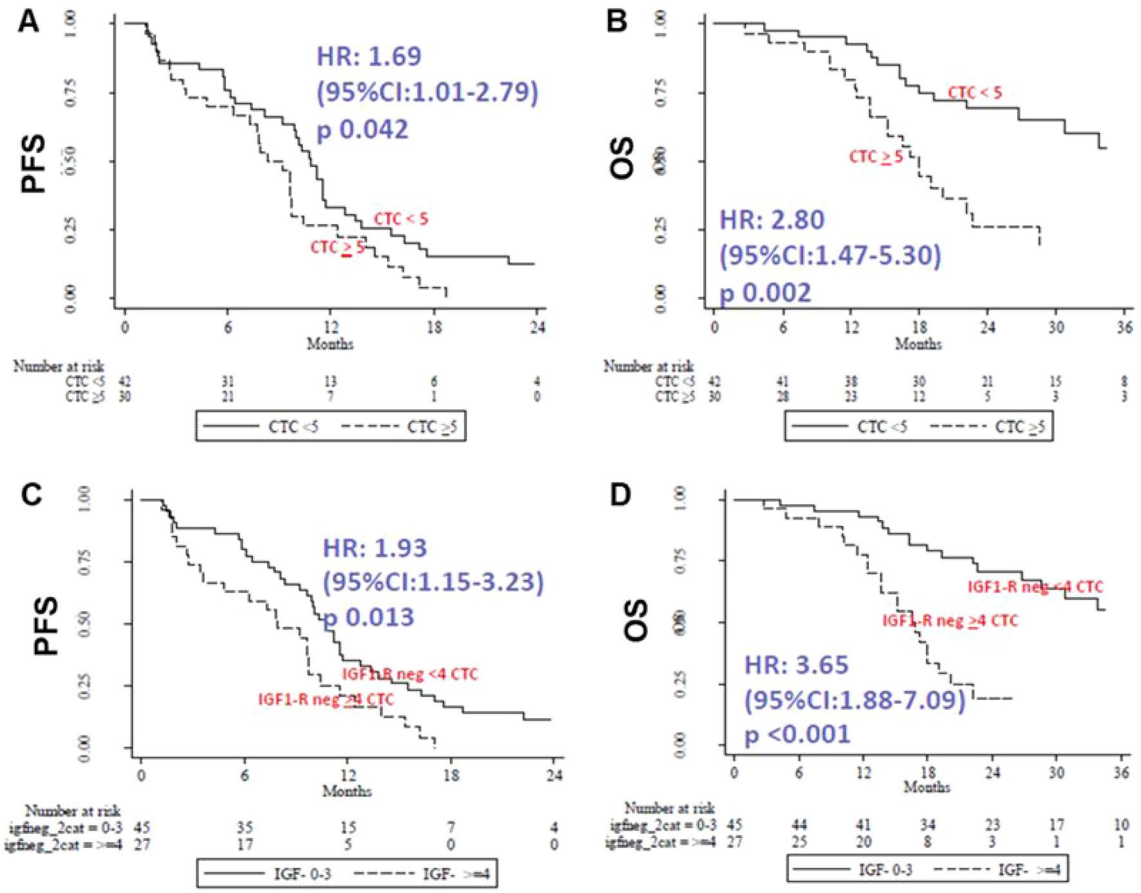

Fig. 3 Kaplan–Meier curves of PFS and OS: Kaplan–Meier curves for PFS (**a**) and OS (**b**) of MBC patients with CTC count < 5/7.5 ml versus ≥ 5/7.5 ml and Kaplan–Meier curves for PFS (**c**) and OS (**d**) of MBC patients with ≥ 4 IGF-1R negative CTCs versus < 4 IGF-1R negative CTCs

Table 2 Prognostic role of number of IGF-1R negative CTCs and CTC count: univariate and multivariate analyses

	Univariate analysis		Multivariate analysis	
	PFS HR (95% CI)	OS HR (95% CI)	PFS HR (95% CI)	OS HR (95% CI)
IGF-1R neg < 4	1	1	1	1
IGF-1R neg ≥ 4	1.93	3.65	1.79	2.83
	(1.15–3.23)	(1.88–7.09)	(0.78–4.16)	(1.09–7.39)
CTC < 5/7.5 mL	1	1	1	1
CTC ≥ 5/7.5 mL	1.69	2.80	1.09	1.41
	(1.02–2.79)	(1.47–5.30)	(0.48–2.48)	(0.55–3.59)

of CTC count was not maintained. The possible predictive role of IGF-1R expression on CTCs could not be demonstrated, since any interaction between IGF-1R expression and metformin was detected. Finally, an exploratory analysis on treatment induced modification in IGF-1R CTC expression suggested that the acquisition of a "bad phenotype" (i.e., ≥ 4 IGF-1R negative CTCs) resulted in a significantly worse overall survival. However, these data on paired blood samples, were achieved in a smaller number of patients, and should be interpreted with caution.

The phenotypic characterization of CTCs has been proposed as a non invasive prognostic and predictive tool to address treatment selection. In particular, IGF-1R expression on CTCs has been previously demonstrated in different tumor types. In 2007, Bono et al. [13] showed that IGF-1R is frequently expressed in CTCs of patients with metastatic tumors and, a few years later, Pizon et al [23]. reported that 84% of BC patients harbored IGF-1R positive CTCs. More recently, the expression of IGF-1R was evaluated in 85 CTC-positive patients with early ($n = 28$)

and advanced ($n = 57$) BC [24]. In this study, IGF-1R expression was detected in 79% of CTC-positive MBC; conversely, 21% of the patients had exclusively IGF-1R negative CTCs. A reduction in the frequency of IGF-1R positive CTCs was observed with transition from early to the metastatic stage, suggesting a potential association between IGF-1R expression and BC aggressiveness, as hypothesized by pre-clinical data showing a correlation between IGF-1R expression level and metastatic potential [25, 26]. In this study, we prospectively evaluated for the first time, the prognostic effect of IGF-1R expression on CTCs in MBC patients randomized into a clinical trial of first line CT. In conclusion, the TransMyme study is the largest prospective study to date that has evaluated the prognostic and predictive role of the expression IGF-1R on CTCs, in MBC patients treated with first line CT. Our results indicate that the lack of IGF-1R expression on CTCs is significantly correlated with an adverse prognosis in this patient population. This IGF-1R dependent effect is maintained at multivariate analysis, whereas the effect of CTC count is lost. These data should also be considered in the multivariate landscape of the "metabolic approach" to breast cancer. In the past 10 years, several studies have evaluated the impact of targeting the insulin-IGF axis as a treatment strategy in early and advanced BC. In our experience, we performed two randomized clinical trials in BC: in the MYME study [15], no evidence of an improvement in terms of PFS was shown with the addition of metformin to conventional chemotherapy in MBC. In the second window of opportunity study [9], we evaluated the effect of metformin single agent at standard antidiabetic dosages, on tumor cell proliferation (ki67) as compared to matching placebo, in the preoperative setting of early BC: again no significant effect was detected.

Conversely, a number of clinical studies evaluating anti-IGF drugs (either monoclonal antibodies or TKI inhibitors) reported inconsistent results or were complicated by excessive metabolic toxicity (hyperglycemia). Other trials with metformin and anti-IGF agents are ongoing. In these perspective, our data, showing a clear prognostic role of IGF-1R expression on CTCs, together with the moderate impact of a non-invasive liquid biopsy approach, could improve patient stratification to better address a strategy of "metabolic targeting" in BC.

Author Contributions AG, DA, ON, and PB designed and supervised the trial. AG, AR, ADC, AB, LC, LG, LS were responsible for patient recruitment and data collection. FF, ON and PB analyzed the data. AF, ER, and RZ performed CTC tests. The first draft of the manuscript was written by AG, VM, FF, and ON, and the remaining co-authors subsequently provided valuable input. All the authors read and approved the final version of the article. The corresponding author assumes responsibility for the completeness and integrity of data, the study fidelity to the protocol, and the statistical analysis. She had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Compliance with ethical standards

Conflict of interests The authors declare no potential conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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