Table 4. Association between each polymorphism and progression-free survival in patients treated with paclitaxel and bevacizumab (cont.).

Polymorphisms	Genes	Carriers	n	HR	95% CI	p-value
rs4145836 (cont.)		AA	3	0.07	0.01–0.53	0.01
rs4073	*IL-8*	AA	20	1		
		AT	62	1.39	0.77–2.51	0.28
		TT	30	1.18	0.61–2.29	0.62

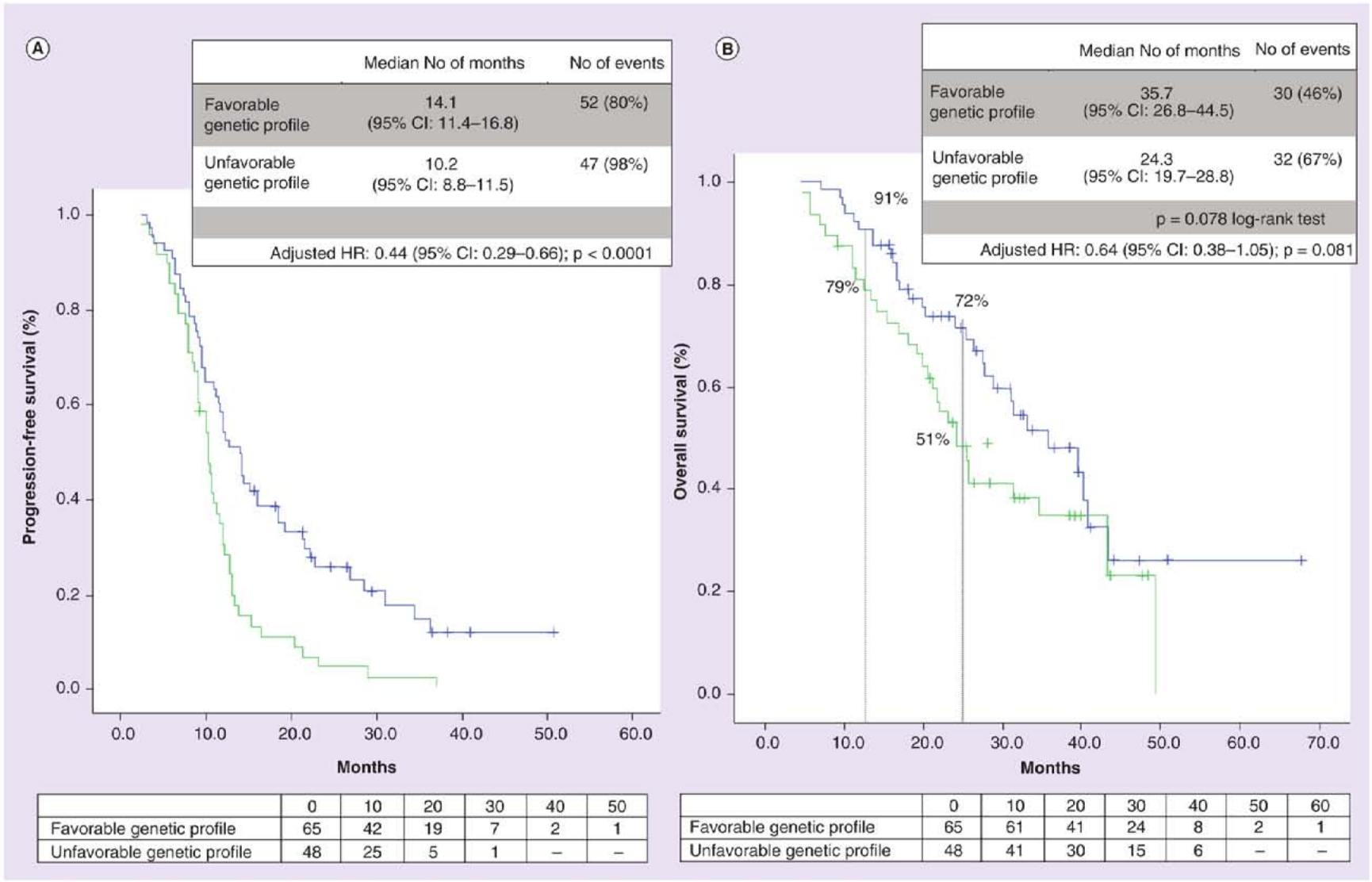
Hormonal receptor status, bevacizumab maintenance and number of sites involvement are the covariates used for the Cox proportional hazards analysis.
A p-value <0.00357 was defined as statistically significant (Bonferroni's correction).
HR: Hazard ratio.

population of MBC patients revealed a genetic interaction profile, consisting of the combination between specific genotypes of *VEGFR-2* rs11133360 and *IL-8* rs4073, associated with PFS. Particularly, two genetic profiles were identified in patients, as reported in Table 5. The first one was associated with a greater PFS benefit and the second one with a lower PFS, respectively. In some reports, both *VEGFR-2* rs11133360 and *IL-8* rs4073 were singularly linked to the response in advanced-stage cancer patients treated with bevacizumab [40,45].

Because of the connectivity within biological networks, the effects of a single mutation or variation can spread through thousands of gene–gene interactions, resulting in multiple phenotypes [50]. Gene–gene interactions are well established as essential to gene regulation, signal transduction, biochemical and physiological pathways [51]. In our study we demonstrated, through the MDR methodology, a statistical interaction between *IL-8* and *VEGFR-2* gene SNPs that potentially relates to bevacizumab efficacy on PFS. It is the physical interactions among proteins and other biomolecules and their impact on phenotype that constitute biological epistasis. However, the relationship between biological and statistical epistasis can be difficult to assess. Biological epistasis occurs at the level of the individual and involves DNA sequence variations, biomolecules and their physical interactions at a particular point in time and space [52]. Statistical epistasis is a population phenomenon that is made possible by interindividual variability in genotypes, biomolecules and their physical interactions (e.g., the mechanism by which bevacizumab is effective in our patients) [52]. Clearly, making hypotheses or conclusions about biological function and causation from statistical results will always be a challenge if the relevant biomolecular information has not been measured [53]. However, MDR methodology has been successfully applied to detecting gene–gene interactions for several clinical phenotypes and it may provide means to find new hypotheses for further testing about epistatic interactions in pharmacogenetic data. While it is difficult to dissect the biological meaning of our statistical epistatic data, it should be noted that several lines of evi-

Table 5. Results of the genetic interaction analysis to translate the genotype combinations of the *VEGFR-2* rs11133360 and *IL-8* rs4073 polymorphisms into favorable or unfavorable genetic profiles for progression-free survival in patients treated with paclitaxel and bevacizumab.

VEGFR-2 SNP	Genotypes	*IL-8* SNP	Genotypes
Favorable genetic profile			
VEGFR-2 rs11133360	TT	*IL-8* rs4073	TT
VEGFR-2 rs11133360	CC	*IL-8* rs4073	AA
VEGFR-2 rs11133360	CC	*IL-8* rs4073	AT
VEGFR-2 rs11133360	CT	*IL-8* rs4073	AA
VEGFR-2 rs11133360	CT	*IL-8* rs4073	AT
Unfavorable genetic profile			
VEGFR-2 rs11133360	TT	*IL-8* rs4073	AA
VEGFR-2 rs11133360	TT	*IL-8* rs4073	AT
VEGFR-2 rs11133360	CC	*IL-8* rs4073	TT
VEGFR-2 rs11133360	CT	*IL-8* rs4073	TT



	0	10	20	30	40	50
Favorable genetic profile	65	42	19	7	2	1
Unfavorable genetic profile	48	25	5	1	–	–

	0	10	20	30	40	50	60
Favorable genetic profile	65	61	41	24	8	2	1
Unfavorable genetic profile	48	41	30	15	6	–	–

Figure 1. Progression free and overall survival curves in patients treated with paclitaxel and bevacizumab. **(A)** Progression-free survival and **(B)** overall survival curves calculated by the Kaplan–Meier method according to the favorable and unfavorable genetic profiles, with the adjusted HR.
HR: Hazard ratio.

Table 6. Multivariable Cox regression model, including significant variables in the univariate analysis of patients treated with paclitaxel and bevacizumab.

Characteristics		Progression-free survival (n = 113)		
		HR	95% CI	p-value
Hormone receptor	Negative	1	0.20–0.61	0.0002
	Positive	0.34		
Bevacizumab maintenance	No	1	0.33–0.84	0.0073
	Yes	0.53		
Sites involvement	<3	1	1.07–2.50	0.023
	≥3	1.64		
Favorable genetic profile	No	1	0.32–0.72	0.0004
	Yes	0.48		
Age	<65	1	0.52–1.27	0.359
	≥65	0.81		

HR: Hazard ratio.

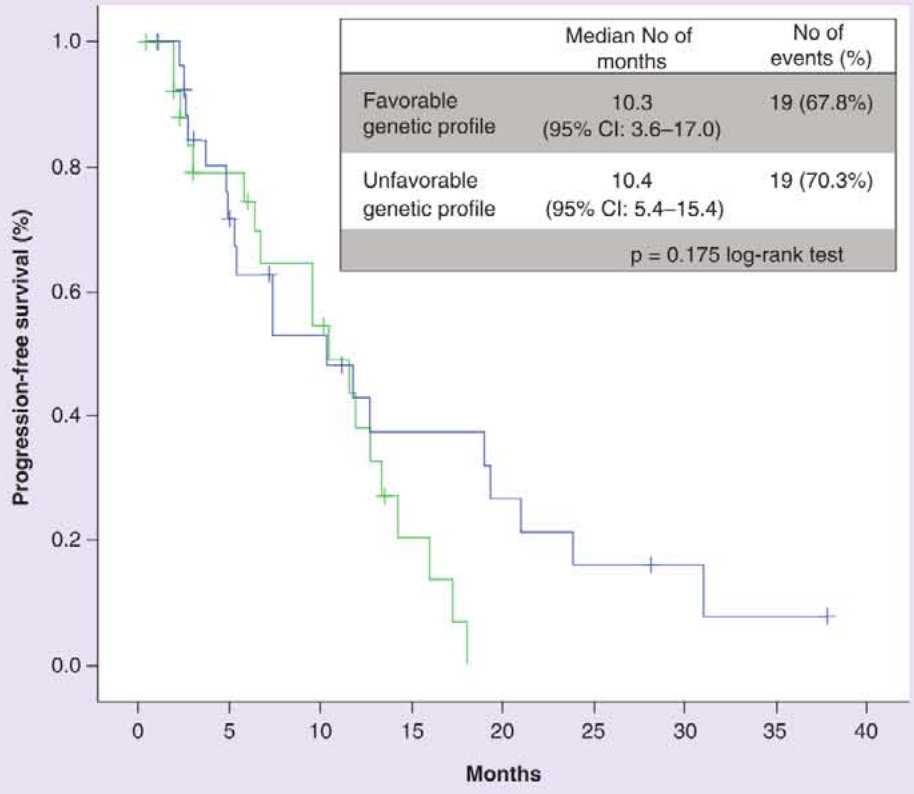
dence support a possible role of IL-8 and VEGFR-2 in the clinical activity of bevacizumab. Although at a first glance the two genes, and, consequently, the two proteins seem to belong to two different signaling pathways (apparently not connected to each other), it is not surprising that IL-8 and VEGFR-2 have more than a simple biological link. In fact, it has been clearly demonstrated that IL-8 stimulates VEGFR-2 phosphorylation in a VEGF-independent manner [54–56]. The VEGFR-2 transactivation by IL-8 is probably due to physical interactions between VEGFR-2 and the IL-8 receptors [54]. Activation of VEGFR-2 signaling is critical for tumor angiogenesis [57,58] and the published data suggest that IL-8 transactivates VEGFR-2 also in the presence of neutralizing VEGF antibody [56] or VEGF inhibitors, such as CBO-P11 [54], thus independently of extracellular VEGF.

Based on these premises, it is conceivable to hypothesize that, in patients carrying the unfavorable genetic profile, the tumor angiogenesis is not blocked (with a graduality due to the various combinations of genotypes) in spite of the presence of bevacizumab that inhibits the biologically active VEGF. The preservation of the angiogenic process could be due to an increase of the production of IL-8 (e.g., due to the presence of *IL-8* rs4370 A allele; [45]) which may continue to transactivate the VEGFR-2. Depending on the *VEGFR-2* rs11133360 genotype, this receptor may be upregulated or not modified in its structure and thus able to completely transduce its signal and sustains the angiogenic process. Unfortunately, the phenotypes corresponding to the *VEGFR-2* rs11133360 genotypes are still unknown [40]. Therefore, it might be plausible that the genetic background may be responsible, in part, for the lack of effect of bevacizumab maintenance therapy in these MBC patients. Conversely, in patients with a favorable genetic profile, the microenvironment conditions due to the different genotype combinations may result in a reduction of the IL-8 production and in the presence of fewer VEGFR-2 or of their lower activity, on tumor endothelial cells which are not capable to proliferate, migrate or survive because no replacement for the VEGF action – blocked by bevacizumab – is present.

The lack of any benefit in terms of efficacy in the group of patients treated with only chemotherapy, and the above biological considerations could also suggest a possible predictive role of the favorable genetic profile for bevacizumab response, but final considerations are limited by the exploratory nature of this retrospective study. Indeed, these results should be only considered as generating an hypothesis and only a well-designed prospective clinical trial may eventually confirm the suggested innovative role for the MDR. Understanding the reasons why the singular genotypes of both *VEGFR-2* rs11133360 and *IL-8* rs4073 were not associated to the greater benefit in terms of PFS in our study, in contrast to what reported when combined in the interaction analysis, remains a challenge.

In spite of these observations, the main and original finding of our analyses suggests the hypothesis that a genetic profile may, early, identify a group of patients with a higher PFS, translating in a trend toward an OS benefit.

The gold standard assessment to evaluate the efficacy of a new cancer drug in cancer patients with advanced disease should reasonably be the evidence of a statistically significant and clinically meaningful improvement in OS and/or in quality of life. For these reasons, the US FDA revoked the initial approval



	0	5	10	15	20	25	30	35
Favorable genetic profile	28	16	10	7	5	3	2	1
Unfavorable genetic profile	27	17	11	3	–	–	–	–

Figure 2. Progression-free survival curves calculated by the Kaplan–Meier method, according to the favorable and unfavorable genetic profiles, with the adjusted hazard ratio in patients treated with paclitaxel alone.

of bevacizumab for the first-line treatment of MBC patients, because of the lack of any benefit in terms of OS when bevacizumab plus chemotherapy was compared with chemotherapy alone in three randomized clinical trials [7]. However, many argued that the lack of an observed OS benefit in patients treated with bevacizumab could not mean a lack of improvement in OS because the trials were not powered to demonstrated this hypothesis. To evaluate the impact of new drugs in solid tumors considering OS as primary end point, larger sample sizes and longer follow-up should be necessary. For these reasons, PFS as surrogate for OS has been helpful in addressing these limitations and in the last years, many of the drug approval indications were based on trials with PFS as primary end point [59]. However, the debate is still open and the issue is far from being solved [60].

A rational approach to validate the PFS as a true surrogate of OS, might be the identification of validated predictive biomarkers for selecting those patients with the best chance of response to drug in terms of PFS. The aim should be to identify a subgroup of patients, within the population that has obtained the best response to the treatment in terms of PFS, with an efficacy even greater, such to be translated into detectable OS benefit. Therefore, the PFS is generally the primary end point in the studies that have evaluated possible predictive factors of response to drugs in cancers, as well as for bevacizumab [14]. For this reason we decide to choose the PFS as primary end point for the our analysis.

Thus, the main question remains if what observed has a prognostic value or, although the small sample size, the absence of a difference in terms of PFS in the group treated without bevacizumab could suggest a possible predictive role of response to bevacizumab of the favorable genetic profile.

Therefore, in consideration of these preliminary but encouraging results, we have planned a prospective study in MBC patients being treated with bevacizumab combined with first-line paclitaxel, in accordance with the formula described by Schoenfeld, to

confirm the reduction in the risk of progression by the 50% for the favorable genetic profile [61]. In addition, assuming a possible role of the favorable genetic profile to predict bevacizumab response, the impact of the two genetic profiles on PFS will also be assessed in a control group of patients (with the same sample size) treated with chemotherapy alone. Finally, if positive results will confirm what has been observed with the present analysis, also in terms of OS, further analysis could be performed in this setting of patients to establish the possible corresponding phenotype, looking at the IL-8 or soluble VEGFR-2 plasma levels or gene expression. Indeed, the favorable genetic profile could help to identify a subgroup of patients with the best probability of bevacizumab response in terms of OS as compared with chemotherapy alone.

Conclusion

The MDR methodology has been applied in this unselected MBC patients to investigate the role of an interaction between *VEGFR-2* and *IL-8* gene polymorphisms in identifying a genetic profile associated with the greater probability of PFS. The final results have confirmed the relevance of MDR analyses, as already described by other authors in metastatic colorectal cancer [48], suggesting a more rational approach when SNPs are investigated as possible predictors of response.

Future perspective

New pharmacogenetic favorable determinants of antiangiogenic therapies could be found from a genetic analysis of the interaction among SNPs rather than from the investigation of a SNP of a single gene. In the next 5–10 years antiangiogenic therapy could be chosen based on the particular favorable or unfavorable genetic profiles of the patients (e.g., the use of bevacizumab in combination with paclitaxel in metastatic breast cancer) based on gene–gene interaction analyses.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Although many attempts have been made to identify *VEGF-A* SNPs associated with bevacizumab response, in advanced cancer patients, the results are still inconclusive.
- Recently, many authors have rightly pointed out the unlikeness that just a SNP can predict the bevacizumab response, mainly due to the complexity of the involved biological systems.
- Therefore, the current approach of correlating the bevacizumab response to a SNP should be replaced by a genetic analysis of the interaction among SNPs.

Patients & methods

- On the basis of this hypothesis, we conducted a study to assess the ability of the multifactor dimensionality reduction (MDR) methodology to identify a pharmacogenetic profile of various polymorphisms related to bevacizumab response for progression-free survival in a population of 113 metastatic breast cancer patients treated with bevacizumab and paclitaxel and on 56 metastatic breast cancer patients treated with paclitaxel alone.

Results

- The MDR software provided two pharmacogenetic interaction profiles consisting of the combination between specific *VEGFR-2* rs11133360 and *IL-8* rs4073 genotypes.
- The median progression-free survival was 14.1 months (95% CI: 11.4–16.8) and 10.2 months (95% CI: 8.8–11.5) for the favorable and the unfavorable genetic profile, respectively (HR: 0.44, 95% CI: 0.29–0–66, $p < 0.0001$).

Conclusion

- The final results have confirmed the relevance of MDR analyses, as already described in metastatic colorectal cancer, suggesting a more rational approach when SNPs are investigated as possible predictors of response.

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