Author's response to reviews

Title: Anti-HER2 (erbB-2) oncogene effects of phenolic compounds directly isolated from commercial Extra-Virgin Olive Oil (EVOO)

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Author's response to reviews: see over
Dearest Editor-in-Chief,

Thank you very much for reviewing our manuscript entitled “Anti-HER2 (erbB-2) oncogene effects of phenolic compounds directly isolated from commercial Extra-Virgin Olive Oil (EVOO)” (MS: 1289208520566679). Since we understand that the Editorial Board of BMC CANCER must ensure the rapid publication of manuscripts of exceptional importance, with a broad scientific audience, and that their consideration is based upon a comparative pool and therefore highly selective, we truly appreciate this opportunity to address the reviewer’s comments.

We have incorporated all the reviewers’ suggestions and are enclosing a complete set of new figures in the revised version of our manuscript. Additionally, we are enclosing detailed responses to each of the reviewers’ concerns (see below).

We have taken a complex experimental approach including chemical isolation, characterization and purification of EVOO phenolics, retroviral generation of experimental breast cancer in vitro models mimicking HER2 oncogene-driven breast cancer disease, and cellular/molecular characterization of the anti-HER2 effects of EVOO phenolics. Because of the biological and clinical relevance of this original approach we therefore urge you to evaluate this manuscript for publication in BMC CANCER.

All authors of this manuscript have directly participated in the planning, execution, and analysis of the study. All authors are aware of and agree to the content of the manuscript, and all authors have approved the final version submitted and their being listed as an author on the manuscript. The contents of this manuscript have not been copyrighted or published previously. There are no directly related manuscripts or abstracts, published or unpublished, by one or more authors of this manuscript. The contents of this manuscript are not now under consideration for publication elsewhere. Neither the submitted manuscript nor any similar manuscript, in whole or in part, will be copyrighted, submitted, or published elsewhere while the Journal is under consideration.
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Thank you in advance for reevaluating this manuscript for publication in your influential journal. Please do not hesitate to contact me at your earliest convenience.

We look forward to seeing our work published in **BMC CANCER**.

Yours sincerely,

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1) The data presented are original and interesting. However, there is a problem in the interpretation and discussion of the data: most of the strength of the data relies on the experiments investigating the potential interaction of the polyphenolic compounds examined in this paper with HER-2. Whether these polyphenolic compounds inhibit the expression of HER-2 is clearly shown, since they modify several cell functions involving HER2. What is not convincingly addressed is whether HER-2 is their sole target, as suggested by the title of the ms., or is simply an innocent bystander of other important effects triggered by these compounds. Most of their demonstration is based on Figures 7-10-13, concerning respectively siRNA, lapatinib and trastuzumab testing. Figure 7 is supposed to present the effects of polyphenolic compounds on cell viability, proliferation or apoptosis in the presence or absence (suppressed by siRNA) of HER-2. It does not since the authors have chosen to provide a pharmacologic-like interpretation of their results, i.e.: synergism, protection or antagonism, instead of providing the raw data, which are never available. Likewise, data presented in figures 10 and 13 are pivotal to stress the type of interaction of the polyphenolic compounds with HER-2, i.e.: extracellular domain investigated through the effect of Trastuzumab, or tyrosine kinase activity of the receptor investigated through the effect of Lapatinib. Again, it is impossible to appreciate the scientific strength of these results since the data are transformed into a similar interpretation. This makes the strength of the original data implicit while they could easily be explicated (in a presentation similar to that used in Figure 12). Therefore, if the authors are willing to put a high emphasis on these particular data, then they should provide the raw data on which they built their interpretation. If they believe that this is not needed, then they should weaken the emphasis they put on to this part of the manuscript.

We thank this reviewer for his constructive criticism. We agree that in the original version of this manuscript we putted an excessive emphasis on the biochemical interpretation arising from the mathematical analysis of the nature of interaction (i.e. antagonism, addition or synergism) that occurs when sequentially culturing breast carcinoma cells with HER2-targeting siRNA or HER2 tyrosine kinase inhibitors prior exposure to EVOO-derived polyphenols. We decided to employ interaction index graphs as they are quite easy to understand for the readers instead of showing a group of graphs just showing that HER2-targeting siRNA or HER2 tyrosine kinase inhibitor such as lapatinib completely prevent the cytotoxic effects (i.e. antagonize or protect) of EVOO phenolics. We still believe, in agree with Reviewer #2, that the appearance of antagonistic and protective interactions when employing a highly specific technique such as siRNA is good enough to suggest that HER2 overexpression is a key molecular hallmark through which EVOO phenolics decrease cell viability (we did not tested the effects of HER2-targeted siRNA and lapatinib on EVOO phenolics-induced breast cancer cell growth).

On the other hand, HER2 is unlikely to be an innocent bystander of other molecular effects triggered by EVOO phenolics. In this revised version of the manuscript we mention our earlier studies regarding this issue:

Page 16:

[...] The fact that depletion of endogenous HER2 significantly prevented the growth-inhibitory effects of EVOO polyphenols together with our earlier findings demonstrating that the down-regulatory effects of the secoiridoid oleuropein aglycone were specifically restricted to HER2 without affecting other key members of the oncogenic HER network such as HER1 (EGFR)
[20], suggested that secoiridoids- and lignans-induced changes in cell viability might relate, at least in part, to changes in the expression of HER2 oncoprotein.

[…] Neither secoiridoids nor lignans treatments caused detectable changes in HER1 (EGFR) expression in breast cancer cells (data not shown) […].

We honestly believe that the fact that EVOO phenolics exhibit a high degree of specificity against HER2 among other (structurally and functionally) related members of the HER network together with the protective interactions occurring when combining siRNA-targeted HER2 or lapatinib with EVOO phenolics, provide a good evidence that EVOO-derived secoiridoids and lignans can induce changes in cell viability, at least in part, through changes in the expression and activity of the tyrosine kinase receptor HER2.

Of course, we have weakened the emphasis of these findings in the results section as well as in the discussion section.

2) Other data are generally sound, with the exception of the results presented in the electronic supplement in figures I and II. The data documenting the effects of NAC or Trolox on EVOO polyphenolic compounds actions on cancer or control cell are not provided. Therefore, the hypothesis that the effects of polyphenolic compounds on cells are mediated through an oxidative stress mechanism is not substantiated.

We believe that this appreciation is a misunderstanding. We did not combine NAC or Trolox with EVOO polyphenols because our hypothesis was as follows:

Page 18:

[...] Since all the main polyphenols that occur at high levels in EVOO have demonstrated antioxidant activity and antioxidants are believed to be responsible for a number of EVOO’s biological activities, we envisioned that EVOO polyphenols-induced depletion of HER2 might represent a general response of HER2-overexpressing cancer cells growing upon anti-oxidant conditions. To test this hypothesis MCF-7/HER2 cells and MCF-7/pBABE matched control cells were treated with graded concentrations of the well-established anti-oxidants 6-hydroxy-N-acetylcysteine (NAC, a glutathione precursor and scavenger of reactive oxygen species) and 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue). Interestingly, MCF-7/HER2 cells were more sensitive to the anti-proliferative effects of NAC (Figure III; Electronic Supplementary Material,) and Trolox (Figure IV; Electronic Supplementary Material) as they exhibited IC₅₀ values significantly lower (3 to 4-times) than those found in MCF-7/pBABE cells. However, treatment with either Trolox or NAC failed to modulate HER2 protein levels in MCF-7/HER2 cells (Figures III and IV; Electronic Supplementary Material). Overall, these results suggest that anti-oxidant agents preferentially suppress the growth of HER2-overexpressing cancer cells, whereas promoting an antioxidant status in HER2-positive breast cancer cells is not sufficient to down-regulate HER2 expression in human breast cancer cells.

Given these findings we cannot exclude that EVOO phenolics may exert, to some extent, anti-HER2 effects through a redox-related mechanism. Although we did not assess the effects of pro-oxidants or anti-oxidants on the anti-HER2 effects of EVOO phenolics, it is clear that changes in the oxidative status of HER2-positive breast cancer cells fails to significantly modulate HER2 expression. Therefore, it is unlikely that the sole anti-oxidant activity of EVOO phenolics will account for their strong HER2 inhibitory effects.
3) Limitations of the work are not clearly stated. The experimental system used (i.e.: transformed cell lines) is not appropriate to investigate polyphenolic compounds effects on HER-2 positive breast cancer incidence. Thus, considering the results, this proposition is an overstatement.

We thank this reviewer for his constructive criticism. Indeed, we now clearly state the limitations of our work:

Page 22, 2nd paragraph:

[...]

4) Title and abstract have to be modified according to previous comments.

Both the title and the abstract have been modified according to previous comments. Moreover, the conclusions section has been entirely modified to clearly state that our findings should be viewed in terms of a previously unrecognized relevance of these phenolics as accessible (and cheap) chemical platforms to further develop new anti-breast cancer drugs:

Page 23, 2nd paragraph:

[...] This study reveals for the first time that all the major families of EVOO polyphenols (i.e. secoiridoids and lignans) represent previously unrecognized phytochemicals that significantly affect breast cancer cell proliferation and survival through a molecular mechanism involving, at least in part, a significant down-regulation of HER2 expression and activity (Figure 14). These findings, together with the fact that humans have safely been ingesting significant amounts of lignans and secoiridoids as long as they have been consuming olives and EVOO, strongly suggest that the stereochemistry of these polyphenols might provide an excellent and safe platform for the design of new anti-breast cancer drugs. Although EVOO-rich has been linked with reduced breast cancer risk, and experimental studies begin to support the hypothesis of EVOO phenolics as inhibiting compounds of HER2-related breast cancer growth, important issues such as the accessibility of EVOO-derived secoiridoids and lignans to tumor tissues should be carefully addressed in animal models and human pilot studies. Only then an appropriate dietary intervention aimed to reproduce the prominent anti-oncogenic features of EVOO phytochemicals could be viewed as a new molecular approach in the management of HER2-positive breast cancer disease.

Concerning the other parts of the ms. there are few minor essential revisions to underline:
5) Methods are appropriate and well described, with the exception of the methods used to investigate the role of the oxidative stress and that of the proteasome, which are lacking.

The methods used to investigate the role of the oxidative stress and that of the proteasome are just a part of the “Metabolic status assessment (MTT-based cell viability assays)” and “HER2-specific Enzyme-Linked Immunosorbent Assay” sub-sections. However, we acknowledge that NAC, Trolox and MG-132 were not mentioned as experimental agents in the original version of this manuscript.

6) Several criticisms relate to the style and organization of the manuscript. The amount of data provided is abundant and the data are diluted by repetitions (as an example, the same information is provided under different forms both in the Material and Methods section and the Results section). A more concise style will improve the quality of the paper.

All the repetitive information has been removed in this new version of the manuscript. Most of these repetitions related to methodological approaches and, as such, they are now exclusively mentioned in the “Material and methods” section.

Other comments:

Figures labelling:

- “Figure x “ is repeated twice in all figures presented. This should be corrected.

Done

- Information on polyphenolic compounds concentrations used is lacking in figures 5-6-12-13.

Done

- Cell type is lacking in figures 3-4-8-12.

Done

- Since results presented in figure 7 left panel and in figure 10 upper panel are experiments quality control, they could be presented in the electronic supplement to help make the ms. more concise.

Done

- Statistics in figure 12: “Fraction x (+) / MG-132 (-)” has to be compared to “Fraction x (+) / MG-132 (+)”. Legends: “3.) 1µM MG-132 (2h) # EVOO...” is wrong and should read “3.) 1Mm MG-132 (2h) # EtOH...”.

Done

Discretionary Revisions:

Some abbreviations are lacking (such as ELISA, IMEM, FSB, ATCC, OD...).

All the abbreviations have been included in this new version of the manuscript.
Other comments:

The authors clearly acknowledge any work upon which they are building, both published and unpublished. In conclusion, this article is of importance in the field of breast cancer biology. However, the authors have to respond to the “Minor Essential Revisions” comments and modify their manuscript accordingly to make it convincing.

REVIEWER: Luigi Ricciardiello

Major Compulsory Revisions: none
Minor Essential Revisions: none

The authors report a new mechanism of HER2 blockage induced by phenolic compounds obtained from phenols-rich EVOO. The findings are important and convincing, indicating a possible role of these compounds in chemotheraphy. The employed methods are appropriate and the data is strengthened by the use of several HER2 inhibitors (siRNA, Lapatinib and MG132) clearly demonstrating a direct effect of the phenolic compounds in a dose/dependent manner. The manuscript is very well written and the data are strong to support the conclusions.

The only comment that I would like the authors to address is on the mechanism of apoptosis induced by polyphenols after blockade of HER2. Is this mechanism similar to the mechanism obtained after blockade with Herceptin (trastuzumab)? In the report by Brodowicz et al. (BJC 2001), the authors report 97% of apoptotic cells in SKBR3 cells treated with Trastuzumab for 96 hours. Have the authors tested any synergistic effect among the phenolic compounds on apoptosis and cell proliferation?

We here demonstrate that simultaneous exposure of SKBR3 cells to the anti-HER2 monoclonal antibody trastuzumab with sub-optimal doses of the 1-(+)-acetoxypinoresinol-rich fraction 6 or the DAOA-rich fraction 4 likewise demonstrated greater decreases in cell viability that did each agent alone (Interaction Indexes < 1.0). This synergistic interaction occurring between EVOO-derived lignans and secoiridoids further extends our previous findings that demonstrated a supra-additive cytotoxic effect when combining trastuzumab with the EVOO secoiridoid oleuropein aglycone. Indeed, our earlier studies described that the EVOO polyphenols oleuropein aglycone was capable to completely reverse breast cancer acquired autoreistance to trastuzumab. Preliminary results in our laboratory clearly show that EVOO-derived lignans and secoiridoids strongly inhibit cell growth of HER2-overexpressing breast cancer cell in vitro models exhibiting intrinsic or acquired autoreistance to trastuzumab (JIMT-1 and SKBR3/TzbR cells, respectively; data not shown), thus supporting the notion that the anti-HER2 EVOO polyphenols’ mechanism of action (i.e. HER2 proteasomal degradation) should not be affected by the mechanisms of resistance recently described for trastuzumab-based anti-HER2 immunotherapy. We and other groups have failed to achieve the extremely high levels of apoptotic cell death reported by Brodowicz et al (BJC 2001). Indeed, the level of apoptosis induced by long-term exposure to trastuzumab (96 hours), at least in our laboratory, does not reach 30%. We are currently investigating if other non-apoptotic mechanisms of cell death are involved in the growth inhibitory effects of trastuzumab and the molecular mechanisms underlying the sensitizing effects of EVOO phenolics. Our preliminary findings suggest that changes in the basal status of cellular autophagy might account for the synergistic effects of trastuzumab and EVOO phenolics. We believe that these findings, if finally correct, certainly merit being published independently as they are beyond the scope of our current study.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.