Author’s response to reviews

Title: Intratumor cholesteryl ester accumulation is associated with human breast cancer proliferation and aggressive potential: a molecular and clinicopathological study

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Author’s response to reviews:

REVIEWER 1

We would like to thank this Reviewer for his/her critical comments that have highly improved the quality of the manuscript.

1 This study used 3 representative classes of breast cancer (Luminal A (ER+/PR+/Her-2-), Her-2, and triple negative) but provided no explanation for why triple positive breast cancers (ER+/PR+/Her-2+) were not included in the present study. Interestingly, few studies have shown that 27-hydroxycholesterol (27HC) mimics estrogen and that it has been implicated in the proliferation and metastasis of ER+ tumors. Because this study found that tumors with the highest CE levels were exclusively composed of Her-2 and TN carcinomas, the authors need to elaborate on the mechanism that explains how high CE is associated with extensive cell proliferation in ER-negative tumors.

As the Reviewer states, we have not included ER+/PR+/Her2+ (luminal B) tumors in this study. The reason is that the frequency of luminal B tumors is much lower than that of luminal A tumors (33% vs 67%). The inclusion of luminal A tumors was facilitated by their availability in the Tumor Tissue Bank. We are now performing a specific study comparing intratumoral lipid levels and their relation with molecular characteristics in luminal A and luminal B groups.

Concerning the association between high intratumoral CE concentration and cell proliferation in ER- negative tumors, there are huge amounts of evidences supporting an impact of cholesterol in tumor phenotype and malignancy independently of the presence of ER (Mulas et al., 2011; Paillasse et al., 2009;
Tumor cells uptake lipoproteins rich in CE more efficiently than normal tissues (Nygren et al., 1997; Tosi and Tugnoli, 2005) and the inhibition of the esterification process has anticancer and chemopreventive effects (de Medina et al., 2006; de Medina et al., 2010). Stored CE reduces the energetically costly de novo lipid synthesis, favor membrane biogenesis, induce lipid raft formation and alter tumor cell signaling, essential processes for tumor proliferation, invasiveness and survival (Danilo and Frank, 2012; Danilo et al., 2013; Paillasse et al., 2009; Tosi and Tugnoli, 2005). Indeed, robust evidence has demonstrated that tumor cells and solid tumors have an altered lipid homeostasis towards an increased accumulation of CE (Dessi et al., 1997; Swarnakar et al., 1998; Tosi et al., 2003). We have included this explanation in the Discussion section of the revised ms version.

2. The concluding figure (Figure 5) indicates that intratumor CE accumulation is associated with aggressiveness, but the study (lines 305-308) could not detect a relationship between intratumor CE accumulation and migration and invasion. As Grade III breast cancers are regarded to be invasive, it is not entirely clear if high CE content in breast tumors with “aggressive” behavior mean extensive proliferation without the ability to invade. This point will need to be clarified for the readers.

We completely agree with the Reviewer’s suggestion. An unexpected finding from this study is the lack of relation between intratumor CE content, migration and invasion markers (MMP9, MMP2, TIMP and CTSS) and invasion clinicopathological characteristics (TNM stage, lymph node affected or vascular invasion). Previous in vitro studies showed that modified lipoproteins induce breast tumor proliferation, migration and invasiveness (Pan et al., 2012a; Pan et al., 2012b; Soto-Guzman et al., 2010). Blockade of CE entry and biosynthesis led to decreased cell migration (Antalis et al., 2011; Danilo et al., 2013). In contrast with data reported by these groups, previous results from our group showed that intracellular CE accumulation decreased human vascular smooth muscle cell migration even in the presence of the hypoxic stimulus (Otero-Viñas et al., 2007; Revuelta-López et al., 2013). In line with the negative effect of intracellular CE content on the vascular cell migration, results from this study do not support a positive effect of intratumor CE accumulation on invasiveness of breast carcinoma. We have included the need of further studies to study why CE-rich tumors are not associated with higher invasiveness. Since CE-rich tumors are associated with higher histologic grade and tumor necrosis, we have included the term “aggressive potential”, instead aggressiveness, in the title and through the text of revised ms version.

3. This manuscript, at numerous places (lines 246, 258, 311, and 320), stated that “intratumor CE accumulation is a good indicator of breast cancer proliferation and dedifferentiation.” This study has used Ki-67 staining for measuring cell proliferation index but marker for differentiation (specific cytokeratins) was not assessed. These statements need to be corrected.

As suggested by the Reviewer, we have removed the term dedifferentiation from the title and through all the revised ms. version.
4. Lines 55 - 57: The statement that “Plasma level of ...are frequently altered in patients with breast cancer” requires some clarification as to the nature of the alteration in these patients (are the levels of those metabolites high or low?). We have changed this statement in the revised ms. version.

5. Line 62: I am not sure about the term “Plasmatic lipoporteins...”; do the authors mean plasma lipoproteins...”
We have corrected the expression in the revised ms. version.

6. Line 81 - 82: The statement is confusing. What is the relationship between the data in Table 1 and Spain.
This typographic mistake has been corrected in the revised ms. version.

7. The statement presented on lines 103 -104 in the Materials and Methods section is repeated on lines 109 - 110, 135 - 136, and 151 – 152. There is no need to repeat this statement in the Results section.
We have deleted the repetitions of this statement in the Methods section.

8. The statement on lines 189 -190 needs clarification. How is high CE associated with the cytoplasmic vacuoles in Her-2 and TN breast tumors? Why are data for luminal A tumors not presented in Fig. 1.
We have not intended to study the association between intratumor CE content and cytoplasmic vacuoles because vacuoles were not immnohistochemically detected in luminal A tumors. According to our results, Luminal A contain similar levels than triglycerides than the other two groups (Fig. 1A & Fig. 1C). However, luminal A contain significantly lower cholesteryl ester levels than the other two groups (Fig. 1A & Fig. 1C). Further studies are required to know whether cholesteryl esters help to conform stable and big vacuoles easily detectable by immunohistochemistry.

9. Lines 207 – 208: It is true that tumors with higher CE tertile results were exclusively composed of Her-2 and TN tumors, but a greater number of Her-2 tumors (6 out of 10) had low CE levels. Similarly it is also true that all tumors with high CE levels were grade III (10 out of 30 total tumors), but a similar number of tumors (10 out of total 30) also had low CE. Calculating the percentage using the number of tumors in a subgroup as compared to the total number of tumors is overestimating the % difference in data reported in Table 4. These points need clarification.
Our intention was to divide the sample of tumors according to CE tertile. Using this arbitrary method we designed two groups: Control group (N=20) with CE-less rich or -poor breast tumors, and CE-rich group (N=10), with those tumors with higher CE concentration. Interestingly, we observed that all tumors in CE-rich group were Her-2 or TN tumors (10/10, 100%) and Grade III (10/10 100%). These results were different that those observed in control group. Indeed, only the 50 % of control group were Grade III (10/20). This made us to speculate about an association between CE-rich tumors and histological grade that was
confirmed using logistic regression analysis (Table 6).

REVIEWER 2

We would like to thank this Reviewer for his/her critical comments that have highly improved the quality of the manuscript.

Major criticism: I am unclear as to what the authors mean by "dedifferentiation" since no clear evidence of a loss of phenotype is provided. Perhaps that expression could be removed from the title since it is, in my opinion, somewhat misleading.

As proposed by the Reviewer, we have removed this expression from the title and through all the revised ms. manuscript.

Minor point: the inclusion of lines to indicate the standard deviations would improve the clarity of the plots shown throughout the manuscript.

Results are analyzed as non-parametric data. Therefore, results should be expressed as median and interquartile range. Interquartile range is the appropriate dispersion measure for data analyzed using non-parametric tests. This is the reason why we showed interquartile ranges instead standard deviation.

References


