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Adipocyte extracellular vesicles carry enzymes and fatty acids that stimulate mitochondrial metabolism and remodeling in tumor cells.

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 9th July 2019

Thank you for submitting your manuscript entitled" Adipocyte vesicles: 'all-in-one' packages that stimulate tumor mitochondrial metabolism and dynamics" to The EMBO Journal. Please accept my apologies for the unusual length of the review process, due to the delayed arrival of some reports. Your study has been sent to three referees for evaluation, whose reports are enclosed below.

As you can see, the referees concur with us on the overall interest of your findings. However, they also raise a few points that need to be addressed before they can support publication in The EMBO Journal. In particular, referee #1 requests you to analyze and interpret the data in a more thorough manner. Importantly, this reviewer is concerned about the protocols employed for purifying EVs and asks you to determine the contribution of EVs and non-EVs to the observed phenotypes. Also, s/he invites you to test melanoma cell migration in response to growth factors or cytokines. Referee #2 asks you to better clarify the link between obesity and melanoma in the introduction, and to describe the adipocyte differentiation protocol in more detail. Furthermore, s/he would like you to extend the discussion on i) other EV-factors that can induce melanoma aggressiveness, ii) the translational significance of the findings, and iii) the differences between murine and human EVs/melanoma cells. Also, referee #2 finds that cell aggressiveness has to be measured using additional parameters (e.g. cell invasiveness, survival etc.).
Referee #3 requests you to address the role of canonical lipolysis in your system and to analyze the effects of EV treatment on the transcription of endogenous Fatty Acid Oxidation genes. Finally, this reviewer asks you to discuss the role of EV non-protein factors on recipient cells.

Given the overall interest of your study, I would like to invite you to revise the manuscript in response to the referee reports. I should also note that addressing these issues and all the minor points by the referees is essential for publication in The EMBO Journal.

REFEREE REPORTS

Referee #1:

The article by Clement et al describes the transfer from adipocytes to melanoma cells of both enzymes involved in fatty acid oxidation and fatty acids themselves. The authors develop elegant lipidomic and SILAC-based proteomic approaches and conclude that transfer of the enzymes is not responsible for the effects observed in melanoma (increased migration, alteration of the mitochondrial network), but transfer of the fatty acids themselves is. They show a role of autophagy-based lipolysis of the captured fatty acid, and modification of mitochondrial pattern, required for these migration effect. They also show that obese-derived adipocyte EVs display enhanced fatty acid transfer ability, and finally correlate expression of the relevant enzymes and poor survival in a database of melanoma patients.

The article is very interesting, and the results novel, with strong potential for developments for clinical situations. The data, however, could be analysed and interpreted in a more thorough way, and could provide some much stronger conclusions, including on the actual involvment of EVs, rather than other lipidic co-isolated structures, in the effects described.

Major points
In particular, my major concern is that the protocol of ultracentrifugation used by the authors to isolate EVs likely co-isolates other lipidic components, such as lipoproteins or exomeres (Zhang et al, Nat Cell Bio 2018: #29459780), and thus the fatty acid transfer observed here may not be due to EVs, but to co-isolated lipid-containing structures. It is thus crucial for this study to determine the contribution of EVs and non-EVs in the observed effect. The authors could find interesting suggestions in the most recent guidelines of ISEV, (MISEV2018, J Extracell Vesicles: # 30637094), which they seem not to be aware of, since they quote only articles of 2013, 2014 and 2016 when referring to the ISEV guidelines.
Most importantly, to determine the contribution of EVs and co-isolated non-EV components, the authors must further purify the material recovered in the 100K pellet, possibly by Size Exclusion Chromatography (Boing et al 2014: # 25279113), which is likely to separate EVs (coming out in the first fractions), from smaller lipid-containing structures that are eluted later: the authors have all the tools to quantify proteins AND lipids in all the fractions of a SEC column, to determine where the fatty acids elute, and use these fractions (or pools of fractions containing EVs and of fractions containing soluble components) in some of their functional assays, to determine if non-EV or EV components are the actual vector. In this case, density gradients alone will most likely not allow separation of EVs and liproproteins or others (Yuana et al, 2014, # 25018865; Karimi et al 2018 # 29441425).

In addition, the authors must provide a very important additional panel in figure 1 (or a panel replacing current 1C), showing the full list of proteins transferred versus non-transferred, and if possible comparing this list with those of the Zhang et al proteomics comparing exomeres to 2 populations of small EVS (called small exosomes and large exosomes by Zhang et al): could it be that only one out of the 2 subpopulations of small EVs and exomeres is the one transferring proteins? Even if the authors do not provide the comparison with Zhang's data, other readers may find important hints in the full list of transferred and of un-transferred proteins.

Another note of caution is on the specificity of intracellular mechanisms induced by the transferred fatty acids: to convincingly show the involvement of lipolysis and mitochondrial transfer in the migratory effect on melanoma cells and that these intracellular effects are all specific of fatty acid transfer (even if this fatty acid transfer is due to non-EV components), the authors should use another non-lipid containing stimulus able to induce melanoma cell migration (a growth factor or cytokine for instance), and show that mitochondria are not affected nor redirected to membrane extensions. As such figure 6 is not very convincing nor relevant to the message of the article since migration is likely to require energy and thus be dependent on an activation of mitochondria, whatever the stimulus that induced it.

Minor points:
The article should describe more clearly the protocols used for EV isolation, according to the MISEV2018 guidelines, especially since the M&M indicates "as before" referring to the 2016 article where the protocol was also indicated "as before", referring to a 2015 article where the authors isolated tumor-derived, and not adipocyte-derived vesicles. In addition, a recent paper quoted by the authors (Flaherty et al, Science 2019) stated that adipocyte-derived EVs had to be isolated by a different protocol involving filtration and size fractionation, because the "standard purification strategies" proved inefficient ... It seems contradictory with the current results where authors used a classical differential centrifugation protocol.

The use of mouse adipocyte-derived vesicles on human melanoma cells is not entirely satisfying, and it is in fact not always easy to understand if mouse or human EVs are used in the different figures (it seems that only mouse EVs are used). The authors should clarify better in each figure, they should justify this choice, and if possible, they should use at least for some functional assays a species-homogenous experimental system, ie mouse lean/obese adipocytes on a mouse melanoma cell line (B16-F10 or B16-F0 are the most classically used), which is probably easier to set up than using human lean/obese adipocyte-derived EVs on the human melanoma cell lines.

All bar graphs should be replaced by graphs showing position of individual biological replicates, to display clearly reproducibility of the experiments, according to Weissgerber et al. (2015) Beyond Bar and Line Graphs: Time for a New Data Presentation Paradigm. PLoS Biol 13(4): e1002128.

some inaccuracies in the text should be corrected:
p5 describing figure 2 mentions obese adipocyte EV whereas the figure displays ND/HFB-EV : please homogenize words and abbreviations
p5 interpretation of figure 2D as a transfer of FAO enzymes is not correct: the increased expression as compared to control samples could be due to increased expression of the endogenous enzymes: the SILAC experiments of figure 12 convincingly show actual transfer of FAO enzymes, but does not determine the respective contribution of such transfer as compared to endogenous expression. Unless the antibodies used for Figure 2D are specific of the mouse proteins (carried by EVs) and do not recognize the human ones (expressed by target cells, interpretation must be reworded.
p6 describes figure 3F as showing "increase in lipid storage within cytoplasmic droplets", which is not substantiated by demonstration that the bodipy-containing structures are droplets, rather than endocytic compartments or others.
legend of figure 3E indicates right and left panels, whereas the figures has top and bottom panels
figure 6 shows nice images, but with no quantifications, it would be useful to quantify the redistribution of bodipy-containing structures (% of cells displaying this phenotypes) and the number or size of lipid droplets observed in EM pictures.

Referee #2:

Intercellular communication that involves important, yet frequently overlooked, non-malignant cells of the tumor microenvironment, particularly adipocytes, has emerged as a critical mechanism that may drive tumor progression in diverse cancers. The idea that metabolites, adipokines or other factors, such as phosphoproteins and microRNAs packaged in secreted extracellular vesicles (EV) communicate with nearby tumor cells to instruct the tumors into more aggressive behaviors, has been explored by a few groups recently. The Muller group in particular has reported (JCI Insight, 2017) that adipocyte lipids can be transferred to nearby breast tumor cells and promote their aggressiveness through metabolic reprogramming. Here, this team investigates how these adipocyte-origin EVs promote aggressiveness in melanoma models. The overall hypothesis is that adipocytes provide both metabolic fuel, in the form of fatty acids, and enzymatic machinery, in the form of enzymes to promote fatty acid oxidation, that recipient melanoma cells use to become more migratory and aggressive.

In general, the experiments are elegant, well designed, technically sound and well controlled, and the data are convincing. This work is highly innovative and has major significance for our rapidly evolving understanding of the microenvironment of diverse cancer types. I recommend publication, contingent on completion of minor revisions as detailed below.

Major points

The conceptual links between obesity and melanoma presented in the introduction are not sufficiently clear. Several population studies and meta-analyses have not uncovered a significant relationship between obesity and melanoma incidence or progression. Opinion is mixed in this area, with some groups reporting that obesity improves survival in metastatic melanoma. Nevertheless, adipocytes are obviously present in the skin microenvironment of patients with incident or progressing melanoma, whether the melanoma patient is obese or lean, so all of the mechanisms proposed in the manuscript may be relevant, however it is not necessary to cite obesity as a significant driving factor.

The investigators have performed an important and technically challenging characterization of the proteins carried in the EV, and used SILAC labeling to identify 2111 proteins in the vesicles that account for 85% of the proteins carried, of which 587 were transferred to nearby melanoma cells. This approach greatly helps establish the scope of the problem. Enzymes important for fatty acid oxidation were included among these proteins.

The experiments were carried out with murine models (mature adipocytes derived from 3T3-F442A murine pre-adipocytes). Please describe the adipocyte differentiation protocol in more detail. Normally, murine 3T3-F442A cells are treated with insulin, dexamethasone and isobutylmethylxanthine to differentiate, but the methods section only refers to insulin. The 3T3-L1 model additionally requires a glitazone to differentiate; what justifies the choice of 3T3-F442A over the more widely used 3T3-L1 model? Do control experiments establish that the biochemical and transcriptional profile of the differentiated 3T3-F442A adipocytes is consistent will a fully differentiated state (expected high expression of FABP4, GLUT4, etc)?

Many humans with obesity and metabolic disease have been treated with glitazone drugs to improve adipogenesis (rosiglitazone, pioglitazone), often in combination with metformin, thought to activate AMPK in these patients (and relevant to this team's previous publication in breast cancer). How might these medications alter the mechanisms explored here? No new experiments are needed to address this question, but additional discussion of translational significance would be helpful.

Many cell line models of melanoma are available. Here, the investigators used human SKMEL28 and human-origin 1205Lu melanoma cells passaged in mouse xenografts, yet more discussion is required to justify these choices of models. Why were murine melanoma cell lines such as B16 not used for experiments with murine adipocytes? Possible incompatibilities or confounders for interpretation based on species differences should be discussed.

In the volcano plot of Figure 2B, many highly significant differentially expressed proteins are shown, yet the investigation only focuses on a small subset of these, most of which are not as strongly indicated in the plot. Was this choice made to pursue these specific candidates because they supported the overall hypothesis? Unbiased analysis of other possible signaling networks (such as survival) should be considered.

The major dependent variable used to define 'aggressiveness' appears to be only cellular migration. Why were other measures (invasiveness, survival/induction of anti-apoptotic proteins, proliferation, EMT) of the melanoma target cells not included?

Adipocyte-origin EV carry a very broad range of biochemical factors that might be important to increase the aggressiveness of nearby melanoma cells (fatty acids, micro RNAs, phosphoproteins, adipokines, enzymes), so a serious challenge for any research program focused on this area will be to resolve the more important factors from the less important ones. The Discussion does not fully consider all the factors: for example, the experimental design does not rule out the possibility that miRNAs or non-enzymatic adipokines (leptin?) carried in the EV are important for the results reported here; leptin has been implicated in the growth of melanoma cells. Please discuss.

Minor points

The term 'normoponderal' is obscure jargon and not widely used in scientific English. Please just simply state 'normal weight' or 'non-obese'. Please label figures more clearly (e.g. Fig 2) to specify

when murine-origin adipocytes are used, vs human-origin.

'Independant' should be spelled 'independent'. Please use SpellCheck in the main manuscript and supplementary text to catch any typographical errors that may persist.

Some statements in the Discussion are not justified by the data presented and are highly speculative, including: "Adipocyte EV may also accompany circulating tumor cells to provide them with the nutrients they require to reach and colonize metastatic niches..." and "...adipocyte EV can regulate energy metabolism, for example by providing muscles with the necessary molecules for FAO during physical activity". Please be more conservative in making claims, or qualify the statements, noting them clearly as speculation.

Referee #3:

This manuscript provides insights into the roles of adipocyte extracellular vesicles (EV) in stimulating the mitochondrial metabolism in tumor cells, which is required for tumor cell migration and motility. Using a comprehensive isotope-based proteomics, the authors identify interesting and unexpected protein candidates that are secreted into the extracellular space via EV and taken up by tumor recipient cells. These include machinery proteins (fatty acid oxidation [FAO] enzymes and regulators of mitochondrial dynamics) which lead recipient cells to enhance FAO. The adipocyte EV also transfers energy substrates (fatty acids) to tumor cells. This study also compares the impact of adipocyte EV from obese murine samples or human individual (versus their lean controls) on tumor cell migratory capacity, demonstrating a physically relevant significance in this study.
This is a timely and interesting paper. Several concerns need to be addressed to further support some of the conclusions and interpretations.
Major concerns:
1. Fig.1. EV contains non-protein molecules including microRNA, which can transcriptionally regulate recipient cells. Although the authors revealed a clear transfer of adipocyte-derived EV proteins to the tumor cells, the non-protein factors within EV (and whether these factors could also impact on protein expression) in recipient cells are not explored and/or discussed. Are the levels of unlabeled proteins involved in FAO/OXPHPOS also change in tumor cells because of exposure to adipocyte EV?

2. Fig.2. it should be addressed whether the transcriptional expression of endogenous FAO genes (eg: CPT1) in tumor cells is regulated upon treatment with EV from lean or obese samples. According to the authors' statement, the increased FAO induced by obese adipocyte EV is not dependent on FAO enzyme transfer. This again raises the question of whether other factors, which are also delivered by EV, could transcriptionally up-regulate FAO in tumor cells. Does FAO remain up-regulated in tumor cells in response to adipocyte EVs when tumor cells are treated with cycloheximide? This could provide important data on the possible regulation of transcriptional/translation by adipocyte-EV in the FAO of recipient cells.

3. Fig.3. Is EV-delivered FA content affected by lipolysis in adipocytes. Does exposure to tumor cells increase FA content in EV? Compared to uptake via fatty acid transporter FABP2 or CD36 by tumor cells, is the EV-mediated FA transfer more efficient and predominant?

4. Fig.4. Is lipophagy the predominant mechanism whereby transferred FA is released from lipid droplets? How about the canonical lipolysis pathway mediated by the hydrolase HSL and ATGL? check p-HSL levels and the influence of its inhibition on lipid accumulation and cell migration in tumor cells.

5. Does the mitochondrial redistribution toward protrusions (Fig.5A) affect total mitochondrial content and activity? 3T3-EV stimulates cell motility whereas knockdown of DRP1 abolish this effect (Fig.5D), does the adipocyte-EV stimulate DRP1 expression and mitochondrial fission?

6. According to the authors, FA stored in lipid droplets is released by lipophagy. Are lysosomes also redistributed toward cell protrusions as lipid droplets (Fig.6A)?

7. More aggressive tumor cells harbor enhanced FAO capacity (Fig.7C), but it is hard to appreciate that these cells also have an increase in lipid accumulation but not a reduction (Fig. 7B).
8. Moreover, does FAO capacity correlate with cell motility in these five tumor cell types of distinct aggressiveness?

Minor concern:
1. Fig.5A, A quantification of mitochondrial distance from nuclei will be informative.

2. Mdivi-1 treatment in tumor cells (Fig.5A) is not described in the text.

1st Revision - authors' response 16th October 2019

We thank referees for their comprehensive overview of our work and positive feedback. All points have been addressed and our responses can be found below. We have adapted our manuscript accordingly and references to all changes are indicated in blue in the manuscript.

Referee #1:

The article by Clement et al describes the transfer from adipocytes to melanoma cells of both enzymes involved in fatty acid oxidation and fatty acids themselves. The authors develop elegant lipidomic and SILAC-based proteomic approaches and conclude that transfer of the enzymes is not responsible for the effects observed in melanoma (increased migration, alteration of the mitochondrial network), but transfer of the fatty acids themselves is. They show a role of autophagy-based lipolysis of the captured fatty acid, and modification of mitochondrial pattern, required for these migration effect. They also show that obese-derived adipocyte EVs display enhanced fatty acid transfer ability, and finally correlate expression of the relevant enzymes and poor survival in a database of melanoma patients. The article is very interesting, and the results novel, with strong potential for developments for clinical situations. The data, however, could be analysed and interpreted in a more thorough way, and could provide some much stronger conclusions, including on the actual involvment of EVs, rather than other lipidic co-isolated structures, in the effects described.

Before we address the referee's concerns, we would like to address an important point. It seems that one of the key messages of our present study may have been misunderstood, most probably due to a lack of clarity on our behalf. Indeed, our work shows that adipocyte extracellular vesicles (EV) transfer both the machinery (enzymes) and the substrate [fatty acids (FA)] required for fatty acid oxidation (FAO) to melanoma cells. Our results suggest that it is the transfer of these 'all-in-one' packages that leads to the global remodeling of melanoma FA metabolism and subsequent increase in migration in melanoma cells. In obesity, adipocyte EV have a stronger effect and we found that this is not due to increased protein transfer but rather to increased levels of FA in these vesicles. Therefore, our conclusion is not 'that transfer of the enzymes is not responsible for the effects observed in melanoma' but rather that transfer of enzymes is not responsible for the heightened effect of EV in obesity. In order to improve clarity for readers concerning this point, we have modified our manuscript accordingly (page 4, lines 23-26, page 6, lines 3-4).

Major points

1. In particular, my major concern is that the protocol of ultracentrifugation used by the authors to isolate EVs likely co-isolates other lipidic components, such as lipoproteins or exomeres, and thus the fatty acid transfer observed here may not be due to EVs, but to co-isolated lipid-containing structures. It is thus crucial for this study to determine the contribution of EVs and non-EVs in the observed effect. The authors could find interesting suggestions in the most recent guidelines of ISEV, (MISEV2018, J Extracell Vesicles: # 30637094), which they seem not to be aware of, since they quote only articles of 2013, 2014 and 2016 when referring to the ISEV guidelines.

Most importantly, to determine the contribution of EVs and co-isolated non-EV components, the authors must further purify the material recovered in the 100K pellet, possibly by Size Exclusion Chromatography (Boing et al 2014: # 25279113), which is likely to separate EVs (coming out in the first fractions), from smaller lipid-containing structures that are eluted later: the authors have all the tools to quantify proteins AND lipids in all the fractions of a SEC column, to determine where the fatty acids elute, and use these fractions (or pools of fractions containing EVs and of fractions containing soluble components) in some of their functional assays, to determine if non-EV or EV components are the actual vector. In this case, density gradients alone will most likely not allow separation of EVs and liproproteins or others (Yuana et al, 2014, # 25018865; Karimi et al 2018 # 29441425).

Referee 1 raises an important point often confronted by those studying EV. Ultracentrifugation-based protocols can indeed lead to the isolation of non-EV structures. We agree with the fact that, in this article, we have not sufficiently described the method of purification and the characterization of the vesicles we obtain. We are also aware that this protocol is not sufficient to purify small EV, but rather enrich them. Nevertheless, in our first article, which studied the effect of adipocyte EV on tumor cells, we characterized the structures isolated using our ultracentrifugation-based technique (Lazar et al, 2016). This characterization followed most of the recommendations of "MISEV2018" to identify small EV (Thery et al, 2018), in particular, transmission electron microscopy, gradient density, WB targeting Alix, TSG101, FLOT1, CD81, and proteomic analysis revealing the presence of a large number of other exosomal/EV markers.

Nevertheless, these data alone do not allow us to rule out the presence of exomeres or lipoproteins in addition to small EV. However, new data, taken alongside our previous findings, show that EV are indeed the major component in our 100,000 g pellet and that it is only these structures that contain FAO enzymes and FA.

First, EV size typically ranges from 60-120nm whereas exomeres are less than 50nm (Zhang et al, 2018a) and lipoprotein range from 8 to 50nm depending on their subclass (Phillips & Perry, 2015). Transmission electron microscopy images (negative staining) show that most particles recovered in our 100,000 g pellet are within the 60-120nm size range and have a cup-shaped morphology [Figure 1A and supplementary S8A in (Lazar et al, 2016)] resembling the exosomal populations observed by Zhang et al rather than exomeres (Zhang et al, 2018a) or lipoproteins (Zhang et al, 2011). Also, few lipoprotein-specific proteins, based on Zhang's proteomic study, were found in the proteome of our 100,000 g pellet (only 9 proteins/41, whereas 11/41 were found in Zhang's small EV purified by asymmetric flow field-flow fractionation). Moreover, only 1.3% of proteins found

in our analysis were specific to exomeres in Zhang's dataset, whereas an average of 3.5% (range 1.32 to 4.83%) were identified in their small EV populations.
To further prove that small EV are responsible for the effects observed in our study, we performed Size Exclusion Chromatography (SEC), as suggested by referee 1. The technique used, based on Boing et al's publication (Boing et al, 2014) can be found in our revised supplemental material and methods section (page 18, lines 6-15). In accordance with Boing's study, the majority of particles recovered in the 100,000g pellet are eluted in fractions 6 to 13 (Appendix Figure S4A-E), as expected for EV, whereas triglycerides and proteins are also present in fractions 16-23, fractions that could contain lipoproteins. Of note, a similar profile was obtained with the control (ultracentrifugated medium, processed in the same way), indicating that most of these larger particles come from bovine serum.
Importantly, high levels of FA were detected in fractions 6-13, while much less or no FA were present in other fractions (Appendix Figure S4C). Moreover, only fractions 6-13, corresponding to small EV, can reproduce the biological effect of the total 100,000 g pellet on melanoma cells in terms of lipid accumulation and migration (Appendix Figure S4F-G). All together, these results support the notion that the processes described in this study are EV dependent. This point has been included in the manuscript (page 6, lines 16-22).

3. In addition, the authors must provide a very important additional panel in figure 1 (or a panel replacing current 1C), showing the full list of proteins transferred versus non-transferred, and if possible comparing this list with those of the Zhang et al proteomics comparing exomeres to 2 populations of small EVS (called small exosomes and large exosomes by Zhang et al): could it be that only one out of the 2 subpopulations of small EVs and exomeres is the one transferring proteins? Even if the authors do not provide the comparison with Zhang's data, other readers may find important hints in the full list of transferred and of un-transferred proteins.

All results from our SILAC experiment can be found in Table EV1. This table provides an exhaustive list of proteins identified in melanoma cells, including those that are transferred from adipocytes by EV, which are listed in the first part of the table. More than 580 proteins are transferred by EV, so presenting this data in a figure would not be readable. It is for that reason that we chose to only present transferred FAO proteins in Figure 1.
When comparing our findings to those published by Zhang et al (Zhang et al, 2018a), although the proteome of the different types of vesicles is likely to differ, owing to their different cellular origins (37% proteins within adipocyte EV are specific when compared to the particles analyzed from Zhang's cell lines), we found a stronger similarity between our EV population and the populations of exosomes described by Zhang et al, when compared to exomeres or lipoproteins (see point 1).
If we consider proteins specific for each type of vesicle, exomeres seems to be rather less transferred than other EV. Indeed, when comparing Zhang et al's dataset to the proteins identified as transferred to melanoma cells by adipocyte EV in our SILAC experiment, only 0.7% of exomere-specific proteins were found, although these exomere-specific proteins represent 1.3% of total proteins in our 100,000 g pellet. In contrast, 33% of exosome-specific proteins were found to be transferred, and these proteins represent 30% of total proteins within our 100,000 g pellet. Similar results were obtained when considering Zhang et al's small and large exosomes separately.
However, despite these conclusions that strengthen the fact that our 100,000 g pellet is highly enriched in EV that are transferred to melanoma cells, we believe these analyses are beyond the scope of our study, so we have not presented them in our revised manuscript. If the referee wishes, we can make these data available to them.

4. Another note of caution is on the specificity of intracellular mechanisms induced by the transferred fatty acids: to convincingly show the involvement of lipolysis and mitochondrial transfer in the migratory effect on melanoma cells and that these intracellular effects are all specific of fatty acid transfer (even if this fatty acid transfer is due to non-EV components), the authors should use another non-lipid containing stimulus able to induce melanoma cell migration (a growth factor or cytokine for instance), and show that mitochondria are not affected nor redirected to membrane extensions.

To investigate this point, we treated melanoma cells with the cytokine TNF-α, known to increase melanoma migration (Katerinaki et al, 2003; Zhu et al, 2004). As expected, in response to TNF-α stimulus, melanoma cell migration was increased (Appendix Figure S11A), but there was no effect on melanoma lipid accumulation or redistribution (Appendix Figure S11B). Nevertheless, mitochondria were located within membrane protrusions in cells treated with TNF-α, which is unsurprising, as mitochondrial fission and redistribution have been clearly shown to be required for tumor cell migration in a number of models (Cunniff et al, 2016; Zhao et al, 2013). It is plausible that migration induced by this stimulus may still depend on mitochondrial metabolism but use other energy sources to fuel this. However, the novelty of our results is that FAO (here, induced by adipocyte EV) can be responsible for this process, alongside a redistribution of lipid droplets (Figure 5, Figure EV2B and Figure 6). These results support the hypothesis that the remodeling of melanoma FA metabolism and the downstream processes that promote melanoma migration are specific to adipocyte EV transfer. This point has been included in the manuscript (page 8, lines 26-29).

5. As such figure 6 is not very convincing nor relevant to the message of the article since migration is likely to require energy and thus be dependent on an activation of mitochondria, whatever the stimulus that induced it.

To address this point, first, we have added the quantification of lipid droplets within membrane protrusions to strengthen our findings (Figure 6A-B, right panel, Figure 6E-F and Figure EV3C-D). This data reinforces that lipid droplets are clearly redistributed to these areas after treatment with adipocyte EV. Moreover, our new data shows that treatment with TNF-α does not induce the redistribution of lipid droplets towards membrane protrusions. Therefore, adipocyte EV induce specific mechanisms within melanoma cells to promote migration that depend on lipid uptake and trafficking. For this reason, we believe that figure 6 presents important and relevant findings with regards to the specificity of the mechanisms induced by adipocyte EV in melanoma cells to promote aggressiveness, particularly in obesity.

Minor points:
1. The article should describe more clearly the protocols used for EV isolation, according to the MISEV2018 guidelines, especially since the M&M indicates "as before" referring to the 2016 article where the protocol was also indicated "as before", referring to a 2015 article where the authors isolated tumor-derived, and not adipocyte-derived vesicles.

This point has been addressed (Material and methods section, page 14, lines 23-32, paragraph *Preparation of ultracentrifugated DMEM, EV isolation and Nanoparticle Tracking Analysis*) and our previous data concerning EV characterization has been more clearly cited.

2. In addition, a recent paper quoted by the authors (Flaherty et al, Science 2019) stated that adipocyte-derived EVs had to be isolated by a different protocol involving filtration and size fractionation, because the "standard purification strategies" proved inefficient ... It seems contradictory with the current results where authors used a classical differential centrifugation protocol.

We agree that these results are contradictory with not only our results, but also with all the results previously published concerning adipocyte EV (dozens of articles). It is difficult to explain these conflicting results because: i) the "standard purification strategies for exosome purification" used in this study are not described in the published manuscript; ii) results obtained using this "standard protocol" are not shown. Furthermore, it is unclear whether this population is mainly EV or other particles.

3. *The use of mouse adipocyte-derived vesicles on human melanoma cells is not entirely satisfying, and it is in fact not always easy to understand if mouse or human EVs are used in the different figures (it seems that only mouse EVs are used). The authors should clarify better in each figure, they should justify this choice, and if possible, they should use at least for some functional assays a species-homogenous experimental system, ie mouse lean/obese adipocytes on a mouse melanoma cell line (B16-F10 or B16-F0 are the most classically used), which is probably easier to set up than using human lean/obese adipocyte-derived EVs on the human melanoma cell lines.*

To address this point, first, figures and/or legends have been altered to improve clarity concerning the species used in each experiment. Moreover, in order to ensure the validity of our results obtained in human melanoma cells treated with murine adipocyte EV, we set up a species-homogenous experimental system. Indeed, we have now performed key experiments using the murine melanoma cell line B16-BL6 and murine adipocyte EV (Clonogenicity, Appendix Figure 1; FAO: Figure 2A; FLC16 transfer by 3T3F442A adipocyte EV: Figure EV1B, lipid droplet accumulation in melanoma cells treated with EV from normal and obese mice: Appendix Figure S5; effect of Lalistat 2 on FLC16 accumulation: Figure EV1C; effect of Bafilomycin on melanoma migration: Appendix Figure S7; effect of Mdivi on melanoma migration: Figure EV2A). These new data show that the effects of adipocyte EV on murine cells are much alike to those found using the murine-human system. This is in accord with many previous studies performed by our team that used similar species-heterogeneous experimental set ups to decipher mechanisms, which were then confirmed in murine models and human tumors (Dirat et al, 2011; Laurent et al, 2016; Wang et al, 2017). Moreover, in our previous work on adipocyte EV (Lazar et al, 2016), we had performed experiments using human adipocyte EV with human melanoma cell lines and our findings were, again, similar to those obtained when using murine adipocyte EV. Thus, the results found with our murine-human system are representative of the processes that take place in species-homogenous experimental setups.

4. All bar graphs should be replaced by graphs showing position of individual biological replicates, to display clearly reproducibility of the experiments, according to Weissgerber et al. (2015) Beyond Bar and Line Graphs: Time for a New Data Presentation Paradigm. PLoS Biol 13(4): e1002128.

This point has been addressed and all bar graphs have now been changed to graphs representing individual replicates with mean and SEM.

some inaccuracies in the text should be corrected:
5. p5 describing figure 2 mentions obese adipocyte EV whereas the figure displays ND/HFB-EV: please homogenize words and abbreviations

The text has been modified accordingly.

6. p5 interpretation of figure 2D as a transfer of FAO enzymes is not correct: the increased expression as compared to control samples could be due to increased expression of the endogenous enzymes: the SILAC experiments of figure 12 convincingly show actual transfer of FAO enzymes, but does not determine the respective contribution of such transfer as compared to endogenous expression. Unless the antibodies used for Figure 2D are specific of the mouse proteins (carried by EVs) and do not recognize the human ones (expressed by target cells, interpretation must be reworded.

We agree with the referee that the increased protein levels of FAO enzymes taken alone cannot be interpreted as a proof of transfer. However, this data taken into account alongside our SILAC experiment data and data from our previous paper showing that RNA levels of these FAO enzymes are unaffected by adipocyte EV (Lazar et al, 2016), strongly supports the hypothesis that adipocyte EV provide melanoma cells with these enzymes. Moreover, we have also now included new findings that show that inhibition of protein synthesis with cycloheximide treatment has no effect on EV-induced FAO (see point 4 referee 3, Figure 2F) and that RNA levels of FAO enzymes are not increased in response to primary adipocyte EV from lean and obese animals (Figure 2E). We have, however, reworded our interpretation to be more cautious (page 5, lines 34-37; page 6, lines 1-4).

7. p6 describes figure 3F as showing "increase in lipid storage within cytoplasmic droplets", which is not substantiated by demonstration that the bodipy-containing structures are droplets, rather than endocytic compartments or others.

We have now included new data showing that BODIPY-positive structures are surrounded by perilipins 2 and 3, specific lipid droplet-coating proteins (PLIN2/3, Appendix Figure S6), confirming these are indeed lipid droplets.

8. *Legend of figure 3E indicates right and left panels, whereas the figures has top and bottom panels*

The text has been modified accordingly.

9. *Figure 6 shows nice images, but with no quantifications, it would be useful to quantify the redistribution of bodipy-containing structures (% of cells displaying this phenotypes) and the number or size of lipid droplets observed in EM pictures.*

These quantifications have now been included in Figure 6A-B, E-F and Figure EV3C-D.

Referee #2:
Intercellular communication that involves important, yet frequently overlooked, non-malignant cells of the tumor microenvironment, particularly adipocytes, has

emerged as a critical mechanism that may drive tumor progression in diverse cancers. The idea that metabolites, adipokines or other factors, such as phosphoproteins and microRNAs packaged in secreted extracellular vesicles (EV) communicate with nearby tumor cells to instruct the tumors into more aggressive behaviors, has been explored by a few groups recently. The Muller group in particular has reported (JCI Insight, 2017) that adipocyte lipids can be transferred to nearby breast tumor cells and promote their aggressiveness through metabolic reprogramming. Here, this team investigates how these adipocyte-origin EVs promote aggressiveness in melanoma models. The overall hypothesis is that adipocytes provide both metabolic fuel, in the form of fatty acids, and enzymatic machinery, in the form of enzymes to promote fatty acid oxidation, that recipient melanoma cells use to become more migratory and aggressive. In general, the experiments are elegant, well designed, technically sound and well controlled, and the data are convincing. This work is highly innovative and has major significance for our rapidly evolving understanding of the microenvironment of diverse cancer types. I recommend publication, contingent on completion of minor revisions as detailed below.

Major points

1. The conceptual links between obesity and melanoma presented in the introduction are not sufficiently clear. Several population studies and meta-analyses have not uncovered a significant relationship between obesity and melanoma incidence or progression. Opinion is mixed in this area, with some groups reporting that obesity improves survival in metastatic melanoma. Nevertheless, adipocytes are obviously present in the skin microenvironment of patients with incident or progressing melanoma, whether the melanoma patient is obese or lean, so all of the mechanisms proposed in the manuscript may be relevant, however it is not necessary to cite obesity as a significant driving factor.

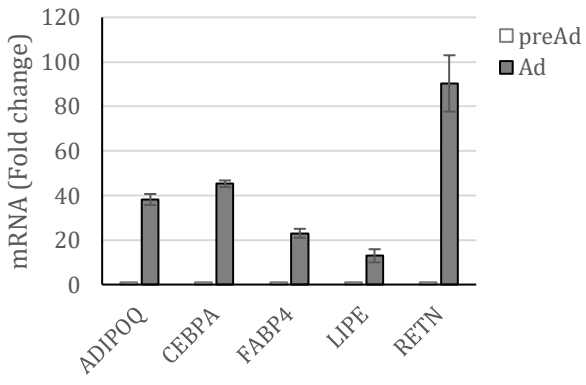
We agree that studies concerning this point sometimes present conflicting results. However, meta-analysis show that, overall, obesity is associated with an increased risk of developing melanoma (Sergentanis et al, 2013), particularly when correcting for confounding factors (Gallus et al, 2006; Rousseau et al, 2005; Shors et al, 2001), and with disease aggressiveness (de Giorgi et al, 2013; Skowron et al, 2015; Stenehjem et al, 2018), for a recent review, (Clement et al, 2017). As stated by the referee, some seemingly contradictory results were published by McQuade and collaborators in 2018 showing that high BMI is associated with increased progression-free and overall survival in male patients with metastatic melanoma (McQuade et al, 2018). However, it is important to note that this association was only observed for patients treated with immunotherapy or targeted therapy, but not chemotherapy. This indicates that obesity favors response to specific treatments, rather than affecting the aggressiveness of the disease itself. Interestingly, recent studies show that heightened FAO is in fact involved in the adaptation of BRAF-mutated melanoma to MAPK inhibitors (Aloia et al, 2019) and is positively correlated to response to immunotherapies (Harel et al, 2019). These data, taken alongside McQuade and collaborators study and our results, could explain the protective effect of obesity in patients treated with targeted therapy or immunotherapy. This point is now discussed in the introduction of the manuscript (page 3, lines 11-24).

Regarding the mechanisms induced by adipocyte EV, we show here that obesity significantly increases FA storage and utilization in melanoma cells in response to

EV and all of the downstream processes leading to increased melanoma aggressiveness. Thus, we believe that citing obesity as an important factor is imperative for our paper.

2. The investigators have performed an important and technically challenging characterization of the proteins carried in the EV, and used SILAC labeling to identify 2111 proteins in the vesicles that account for 85% of the proteins carried, of which 587 were transferred to nearby melanoma cells. This approach greatly helps establish the scope of the problem. Enzymes important for fatty acid oxidation were included among these proteins. The experiments were carried out with murine models (mature adipocytes derived from 3T3-F442A murine pre-adipocytes). Please describe the adipocyte differentiation protocol in more detail. Normally, murine 3T3-F442A cells are treated with insulin, dexamethasone and isobutylmethylxanthine to differentiate, but the methods section only refers to insulin. The 3T3-L1 model additionally requires a glitazone to differentiate; what justifies the choice of 3T3-F442A over the more widely used 3T3-L1 model? Do control experiments establish that the biochemical and transcriptional profile of the differentiated 3T3-F442A adipocytes is consistent will a fully differentiated state (expected high expression of FABP4, GLUT4, etc)?

3T3-L1 and 3T3-F442A are both extensively used preadipocyte cell lines that, when cultured under appropriate conditions, undergo adipogenesis. Our choice to use the 3T3-F442A cell line was based on the fact that these cells are more advanced in their commitment to the adipocyte lineage than 3T3-L1 cells (Sarjeant & Stephens, 2012). For example, 3T3-F442A cells, but not 3T3-L1, generate fatty tissue when implanted in mice that is equivalent to endogenous adipose tissue (Green & Kehinde, 1979; Mandrup et al, 1997). Moreover, as oppose to 3T3-L1 cells, 3T3-F442A require only stimulation with insulin once they reach confluence to induce differentiation (Moustaid et al, 1990; Rubin et al, 1978; Spiegelman & Ginty, 1983). This model is routinely used in our lab and we have previously described our differentiation protocol (Meulle et al, 2008), which is now more detailed in the material and methods section of our manuscript (page 13, lines 16-20). Using such a protocol, we obtain cells that express adipocyte markers (see the figure below) and accumulate neutral lipid stores [supplementary figure 2A in (Lazar et al, 2016)]. These results have not been included in the manuscript, as these results have previously been described (Djian et al, 1985; MacDougald & Lane, 1995).



RT-qPCR analysis of adipocyte markers in undifferentiated and differentiated 3T3F442A cells *3T3F442A preadipocytes (preAd) are grown for 14 days in the presence of insulin to reach the differentiated state of adipocytes (Ad), then mRNA was extracted and RT-qPCR was performed to analyze the expression of adipocyte markers. For each gene, results are expressed relative to the*

corresponding value for preadipocytes.

3. Many humans with obesity and metabolic disease have been treated with glitazone drugs to improve adipogenesis (rosiglitazone, pioglitazone), often in combination with metformin, thought to activate AMPK in these patients (and relevant to this team's previous publication in breast cancer). How might these medications alter the mechanisms explored here? No new experiments are needed to address this question, but additional discussion of translational significance would be helpful.

This point is now discussed in the manuscript (page 12, lines 3-13).

4. Many cell line models of melanoma are available. Here, the investigators used human SKMEL28 and human-origin 1205Lu melanoma cells passaged in mouse xenografts, yet more discussion is required to justify these choices of models. Why were murine melanoma cell lines such as B16 not used for experiments with murine adipocytes? Possible incompatibilities or confounders for interpretation based on species differences should be discussed.

In this study, we chose to use human melanoma cell lines in order to best mimic the processes that take place in human melanoma patients. Amongst the commercially available lines, we chose SKMEL28 and 1205Lu as they are commonly used cells (in google scholar, a search for 'SKMEL28' renders around 11900 results and '1205Lu' and 'Lu1205', around 1750) and they present different levels of aggressiveness [both *in vitro* and *in vivo*, (Lazar et al, 2015), (Goodall et al, 2008)]. In our previous paper, we also showed that adipocyte EV also increase melanoma migration in 501Mel, Lyse and A375M cells [supplementary Figure 1 of (Lazar et al, 2016)].
Concerning the use of a species-homogenous experimental setup to confirm our findings, this important point was also raised by referee 1, minor point 3, and we have now addressed this.

5. In the volcano plot of Figure 2B, many highly significant differentially expressed proteins are shown, yet the investigation only focuses on a small subset of these, most of which are not as strongly indicated in the plot. Was this choice made to pursue these specific candidates because they supported the overall hypothesis? Unbiased analysis of other possible signaling networks (such as survival) should be considered.

Our results revealed that adipocyte EV promote melanoma aggressiveness through FAO, with a heightened effect in obesity. In order to understand the processes orchestrating the heightened effect in obesity, we have performed comparative proteomics of adipocyte EV from lean and obese mice. Unexpectedly, FAO protein levels were equivalent in both samples (and this is the conclusion of the results illustrated in Figure 2B). On the other hand, in melanoma cells treated by adipocyte EV from obese mice when compared to those treated with EV from lean mice, FAO is increased whereas mRNA and protein levels of FAO enzymes are not (respectively Figure 2E and 2D). These results led us to conclude that transfer of FAO enzymes was not responsible for the observed effect and to continue on to the FA transfer hypothesis.

However, a number of other proteins were found to be deregulated in adipocyte EV in obesity. The analysis of the pathways associated with the proteins over-represented in either lean or obese samples (biological process in GO, KEGG pathways or REACTOME) do not reveal any signaling networks involved in survival, proliferation or migration. As we believe this data does not enrich our study, we have not included this analysis in our revised manuscript. If the referee wishes, we can make our analyses available to them.

6. The major dependent variable used to define 'aggressiveness' appears to be only cellular migration. Why were other measures (invasiveness, survival/induction of anti-apoptotic proteins, proliferation, EMT) of the melanoma target cells not included?

Although, in this work, we focused on cell migration as a marker of cell aggressiveness, our previous work shows that adipocyte EV favor melanoma migration and invasion *in vitro* and metastatic potential *in vivo* in a tail vein assay, but have little effect on cell number *in* vitro (Lazar et al, 2016). Moreover, we have added new data showing that adipocyte EV increase the clonogenic capability of melanoma cells (Appendix Figure S1, page 4, lines 33-35).

7. *Adipocyte-origin EV carry a very broad range of biochemical factors that might be important to increase the aggressiveness of nearby melanoma cells (fatty acids, micro RNAs, phosphoproteins, adipokines, enzymes), so a serious challenge for any research program focused on this area will be to resolve the more important factors from the less important ones. The Discussion does not fully consider all the factors: for example, the experimental design does not rule out the possibility that miRNAs or non-enzymatic adipokines (leptin?) carried in the EV are important for the results reported here; leptin has been implicated in the growth of melanoma cells. Please discuss.*

This point is now discussed in the text (page 10, lines 15-21).

Minor points
1. The term 'normoponderal' is obscure jargon and not widely used in scientific English. Please just simply state 'normal weight' or 'non-obese'.

This point has been rectified.

2. Please label figures more clearly (e.g. Fig 2) to specify when murine-origin adipocytes are used, vs human-origin.

We have modified the manuscript, figures and/or legends to improve clarity.

3; 'Independant' should be spelled 'independent'. Please use SpellCheck in the main manuscript and supplementary text to catch any typographical errors that may persist.

This mistake has been corrected.

4. Some statements in the Discussion are not justified by the data presented and are highly speculative, including: "Adipocyte EV may also accompany circulating tumor cells to provide them with the nutrients they require to reach and colonize metastatic niches..." and "...adipocyte EV can regulate energy metabolism, for example by providing muscles with the necessary molecules for FAO during physical activity". Please be more conservative in making claims, or qualify the statements, noting them clearly as speculation.

We have modified our statements according to the referee's comment (page 11, lines 3-11).

Referee #3:

This manuscript provides insights into the roles of adipocyte extracellular vesicles (EV) in stimulating the mitochondrial metabolism in tumor cells, which is required for tumor cell migration and motility. Using a comprehensive isotope-based proteomics, the authors identify interesting and unexpected protein candidates that are secreted into the extracellular space via EV and taken up by tumor recipient cells. These include machinery proteins (fatty acid oxidation [FAO] enzymes and regulators of mitochondrial dynamics) which lead recipient cells to enhance FAO. The adipocyte EV also transfers energy substrates (fatty acids) to tumor cells. This study also compares the impact of adipocyte EV from obese murine samples or human individual (versus their lean controls) on tumor cell migratory capacity, demonstrating a physically relevant significance in this study. This is a timely and interesting paper. Several concerns need to be addressed to further support some of the conclusions and interpretations.

Major concerns:

1. Fig.1. EV contains non-protein molecules including microRNA, which can transcriptionally regulate recipient cells. Although the authors revealed a clear transfer of adipocyte-derived EV proteins to the tumor cells, the non-protein factors within EV (and whether these factors could also impact on protein expression) in recipient cells are not explored and/or discussed.

A potential role of microRNA or other EV molecules is possible, in addition to the processes described here, but we believe such a study is beyond the scope of this work. However, this point is now discussed in the manuscript (page 10, lines 15-21).

2. Are the levels of unlabeled proteins involved in FAO/OXPHPOS also change in tumor cells because of exposure to adipocyte EV?

This point could not be addressed based solely on the data from our SILAC experiment as the treatment time with adipocyte EV that we used (12h) is shorter than in other functional assays (48h for metabolism experiments) and insufficient to correctly study transcriptional/translational remodeling. The 12h time point used for this assay was chosen based on EV internalization kinetics to choose the earliest time point at which cells had taken up EV, in order to identify transferred proteins before degradation could take place (and release labelled amino acids that could go on to be used for protein synthesis). It is possible that increased FA metabolism due to adipocyte EV factors could, in turn, lead to increased endogenous expression of proteins involved in this process to support this metabolic remodeling. However, our current and past data strongly suggest that adipocyte EV derived enzymes are responsible, at least in part, for this remodeling as FAO enzymes are transferred to melanoma cells (Table EV1, Fig 1C) and total protein levels of such enzymes are increased when compared to control cells [Figure 2D and (Lazar et al, 2016)] despite unchanged mRNA levels [Figure 2E and (Lazar et al, 2016)]. Moreover, cycloheximide treatment has no effect on the EV-induced phenotype (detailed below, point 4).

3. Fig.2. it should be addressed whether the transcriptional expression of endogenous FAO genes (eg : CPT1) in tumor cells is regulated upon treatment with EV from lean or obese samples. According to the authors' statement, the increased FAO induced by obese adipocyte EV is not dependent on FAO enzyme transfer. This again raises the question of whether other factors, which are also delivered by EV, could transcriptionally up-regulate FAO in tumor cells.

We agree that evaluating the transcriptional expression of endogenous FAO enzymes in melanoma cells after treatment with lean and obese adipocyte EV is important, as, in our previous work (Lazar et al, 2016), we had only addressed this point using 3T3-F442A adipocyte EV. Therefore, we have added new data showing that primary adipocyte EV from both lean and obese animals, have no effect on the RNA levels of key FAO enzymes HADH and ECHA (Figure 2E). Moreover, we have studied CPT1A, B and C expression by RT-qPCR in 1205Lu treated with EV from ND and HFD mice. Our results show that CPT1A mRNA expression is not modified by this treatment (Figure 2E) and, in our hands, CPT1B and C were undetected in 1250Lu melanoma cells. Overall, these results suggest that EV do not promote transcriptional activation of key FAO genes, page 5, lines 34-37.

4. Does FAO remain up-regulated in tumor cells in response to adipocyte EVs when tumor cells are treated with cycloheximide? This could provide important data on the possible regulation of transcriptional/translation by adipocyte-EV in the FAO of recipient cells.

Following on from the referee's prior points, treatment with cycloheximide does not alter FAO levels in the presence of these vesicles (Figure 2F), further attesting that transcription/translation does not seem to be involved in the effect of adipocyte EV on melanoma cell metabolism. These results have been included in the revised manuscript (page 5, lines 37 and page 6, lines 1-2).

5. Fig.3. Is EV-delivered FA content affected by lipolysis in adipocytes.

To answer this question, adipocytes were stimulated with isoproterenol to induce lipolysis. This treatment stimulated EV secretion and increased their FA content. These results have been included in the revised manuscript (Appendix Figure S12, page 10, lines 10-12).

6. Does exposure to tumor cells increase FA content in EV?

We agree with referee 3 that studying the role of adipocyte EV in response to tumor secretions is a very interesting and important area of study that should follow on from this work. Indeed, many studies, including twp published by our team, show that tumor-derived secretions induce adipocyte lipolysis and increase FA transfer from these cells to tumors (Laurent et al, 2019; Nieman et al, 2011; Wang et al, 2017; Zhang et al, 2018b). In light of our findings, understanding the role of EV in such a process is important, but beyond the scope of our current work. Here, we aim to study the role of EV from 'naïve' adipocytes, unmodified by tumor secretions. We, and others, have shown that tumor-induced adipocyte lipolysis requires a close proximity between tumor cells and adipocytes (Dirat et al, 2011; Laurent et al, 2019; Zhang et al, 2018b) and therefore only occurs in advanced diseases, once tumors become invasive and penetrate local adipose tissue. So, our current results show that naïve adipocyte EV can also influence tumor FA metabolism, suggesting this process could occur at the early stages of disease progression before tumors become invasive due to the ability of EV to diffuse through tissues and circulate in bodily fluids (Shah et al, 2018). This point is now addressed in the discussion (page 10, lines 12-14).

7. Compared to uptake via fatty acid transporter FABP2 or CD36 by tumor cells, is the EV-mediated FA transfer more efficient and predominant?

A number of previous studies have underlined the importance of FA transporters in tumor progression. For example, FA uptake via CD36 has been shown to promote epithelial-mesenchymal transition (Nath et al, 2015) and CD36 has also been shown to be a marker for metastasis-initiating cells (Pascual et al, 2017). To our knowledge, FABP2 has not been associated with FA uptake in cancer cells, but other FABPs have been (McKillop et al, 2019), in particular FABP4 (Nieman et al, 2011).
However, several studies have evaluated the role of FA transporters in the FA transfer that takes place between adipocytes and tumor cells. First, Nieman and collaborators have demonstrated in metastatic ovarian cancer that FABP4 is overexpressed in tumor cells, and this overexpression is involved for lipid accumulation in tumor cells, and, consequently adipocyte-mediated invasion (*in vitro*) or metastasis (*in vivo*). This finding was also shown in bone metastasis from prostate cancer (Herroon et al, 2013). Of note, this overexpression is only observed in ovarian cancer cells cocultivated with adipocytes or, *in vivo*, at the invasive front of tumors where adipocytes and cancer cells are in close proximity (Nieman et al, 2011). The second study by Zhang and collaborators, demonstrates that adipocytes provide melanoma cells with FA, a process that stimulates their aggressiveness (Zhang et al, 2018b). The authors show that addition of lipofermata (an FATP inhibitor) to co-cultures decreases FA transfer and the effect of adipocytes on melanoma aggressiveness. However, this inhibitor only decreases, but does not totally abolish, the transfer of FA from adipocytes to melanoma cells, showing that other transport systems probably exist. Moreover, as lipofermata is added directly to co-cultures between adipocytes and melanoma cells, the question as to the effect of this compound on adipocytes and, in particular, on their FA uptake, but also release (it is highly probable a retro-control exists), was not investigated in this study. Finally, both studies only concern cancer-associated adipocytes that have not been modified by the presence of tumor cells, and not naïve adipocytes.
Our findings demonstrate that EV are also responsible for FA transfer from adipocytes to melanoma cells. Although other FA transport systems are most probably also involved, in particular FA membrane transporters that uptake free FA released by adipocytes, we show that FA from EV alone are sufficient to remodel melanoma metabolism and favor aggressiveness. However, EV-mediated transfer and transport by other systems likely act in synergy to increase the effects of adipocyte-derived lipids on melanoma progression, especially at the invasive front. This point is now discussed in the revised manuscript (page 10, lines 2-8).

8. Fig.4. Is lipophagy the predominant mechanism whereby transferred FA is released from lipid droplets? How about the canonical lipolysis pathway mediated by the hydrolase HSL and ATGL? check p-HSL levels and the influence of its inhibition on lipid accumulation and cell migration in tumor cells.

To answer this question, we have studied the effect of 2-(5,5-Dimethyl-1,3,2-dioxaborinan-2-yl)benzoic Acid Ethyl Ester (HSLi), an HSL inhibitor, on lipid accumulation and migration in melanoma cells in the presence of adipocyte EV. Our results show that neither process is impacted by this treatment. Accordingly, in melanoma cells treated with adipocyte EV, the protein levels of the cytosolic lipases MAGL, HSL and ATGL are unchanged, as is the level of activated p-HSL.

These results have been included in the manuscript (Appendix S8, page 7, lines 15-20).

9. Does the mitochondrial redistribution toward protrusions (Fig.5A) affect total mitochondrial content and activity?

In a previous study (Lazar et al, 2016), we have shown that mitochondrial number is increased after adipocyte EV treatment. In response to referee 3's comment, we also analyzed mitochondrial activity using a Seahorse assay. This technique allows one to measure cellular oxygen consumption rates (OCR) in the presence of different stimuli. The experimental protocol we used can be found in our revised additional material and methods section (page 18, lines 34-37 and page 19, lines 1-8). Our results show that adipocyte EV increase melanoma basal and maximal respiration, as well as respiration coupled to ATP production. Furthermore, cell respiration is more dependent on FAO after treatment with adipocyte EV as shown by readings in the presence of the FAO inhibitor, Etomoxir. Therefore, after treatment with adipocyte EV, mitochondrial redistribution is associated with an increase in mitochondrial respiration dependent on FAO. These results have been included in the revised manuscript (Appendix S10, page 8, lines 13-17).

10. 3T3-EV stimulates cell motility whereas knockdown of DRP1 abolish this effect (Fig.5D), does the adipocyte-EV stimulate DRP1 expression and mitochondrial fission?

To address this question, we performed a WB targeting DRP1 but also FIS1 in melanoma cells treated, or not, with adipocyte EV. Although DRP1 (DNM1L) protein levels were unchanged, FIS1 is increased in 1205Lu cells and slightly increased in SKMEL28 cells after treatment. The modest increase in SKMEL28 cells may be due to higher amounts of endogenous FIS1 when compared to 1205Lu cells. Moreover, we have compared the size of mitochondria in melanoma cells treated or not with adipocyte EV and have shown that this size is decreased. Both results have been included in the revised manuscript (respectively Figure 5B and Appendix S9, page 8, lines 2-6).

11. According to the authors, FA stored in lipid droplets is released by lipophagy. Are lysosomes also redistributed toward cell protrusions as lipid droplets (Fig.6A)?

To address this important point, we have now quantified the percentage of cells presenting lysosomes within membrane protrusions after treatment with adipocyte EV. Our new results show that adipocyte EV increase the presence of lysosomes in membrane protrusions when compared to untreated cells (Figure 6G and Figure EV3E), as is the case for lipid droplets. These findings reinforce the importance of lysosomal degradation of lipids in our model.

12. More aggressive tumor cells harbor enhanced FAO capacity (Fig.7C), but it is hard to appreciate that these cells also have an increase in lipid accumulation but not a reduction (Fig. 7B).

When FA enter a cell, they can be transported towards lipid droplets for esterification into triglycerides for storage and/or mitochondria for oxidation. Indeed, these two processes are not mutually exclusive. In fact, triglycerides can be simultaneously synthesized and hydrolyzed for oxidation in both normal and

pathological conditions (Banke et al, 2010). Indeed, in a cardiac myocyte model, the majority of lipids oxidized within mitochondria are first esterified to form triglycerides before they undergo hydrolysis (Banke et al, 2010). In tumor cells, Nomura et al show that newly synthesized FA are immediately esterified into triglycerides before their release to fuel cell metabolism (Nomura et al, 2010). Moreover, when FA uptake is increased in cells, if maximal oxidative capacity is reached, the excess lipids must be stored to avoid lipotoxicity. Therefore, when FA input is strongly increased, such is the case when adipocyte EV are added to melanoma cells, this can result in an increase in FAO but also in lipid storage. Our study supports this concept as, in Figure 3C-D, we have shown that the FA transferred from adipocytes to melanoma cells are stored in lipid droplets (LD), but are also used by FAO since lipid droplets are increased in presence of Etomoxir.

13. Moreover, does FAO capacity correlate with cell motility in these five tumor cell types of distinct aggressiveness?

In a previous study, migration of these cell lines has been evaluated *in cellulo* [Figure S5 in (Lazar et al, 2015)], as well as their metastatic potential *in vivo* [Figure 1A in (Lazar et al, 2015)]. The cell lines presenting highest migratory and metastatic potentials (Lazar et al, 2015) are those that also present the highest FAO levels (Figure 7).

Minor concern:

1. Fig.5A, A quantification of mitochondrial distance from nuclei will be informative.

This analysis has now been performed (Figure 5A).

2. Mdivi-1 treatment in tumor cells (Fig.5A) is not described in the text.

Mdivi-1 treatment is described page 13, line 27 (Material and methods, paragraph *Cell lines, culture and treatments*).

References

Aloia A, Mullhaupt D, Chabbert CD, Eberhart T, Fluckiger-Mangual S, Vukolic A, et al (2019) A Fatty Acid Oxidation-dependent Metabolic Shift Regulates the Adaptation of BRAF-mutated Melanoma to MAPK Inhibitors. *Clinical cancer research : an official journal of the American Association for Cancer Research*

Banke NH, Wende AR, Leone TC, O'Donnell JM, Abel ED, Kelly DP, Lewandowski ED (2010) Preferential oxidation of triacylglyceride-derived fatty acids in heart is augmented by the nuclear receptor PPARalpha. *Circulation research* **107:** 233-241

Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R (2014) Single-step isolation of extracellular vesicles by size-exclusion chromatography. *Journal of extracellular vesicles* **3**

Clement E, Lazar I, Muller C, Nieto L (2017) Obesity and melanoma: could fat be fueling malignancy? *Pigment cell & melanoma research* **30:** 294-306

Cunniff B, McKenzie AJ, Heintz NH, Howe AK (2016) AMPK activity regulates trafficking of mitochondria to the leading edge during cell migration and matrix invasion. *Molecular biology of the cell* **27:** 2662-2674

de Giorgi V, Gori A, Papi F, Grazzini M, Rossari S, Verdelli A, et al (2013) Excess body weight and increased Breslow thickness in melanoma patients: a retrospective study. *Eur J Cancer Prev* **22:** 480-485

Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al (2011) Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer research* **71:** 2455-2465

Djian P, Phillips M, Green H (1985) The activation of specific gene transcription in the adipose conversion of 3T3 cells. *Journal of cellular physiology* **124:** 554-556

Gallus S, Naldi L, Martin L, Martinelli M, La Vecchia C (2006) Anthropometric measures and risk of cutaneous malignant melanoma: a case-control study from Italy. *Melanoma research* **16:** 83-87

Goodall J, Carreira S, Denat L, Kobi D, Davidson I, Nuciforo P, S, et al (2008) Brn-2 represses microphthalmia-associated transcription factor expression and marks a distinct subpopulation of microphthalmia-associated transcription factor-negative melanoma cells. *Cancer research* **68:** 7788-7794

Green H, Kehinde O (1979) Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *Journal of cellular physiology* **101:** 169-171

Harel M, Ortenberg R, Varanasi SK, Mangalhara KC, Mardamshina M, Markovits E, B et al, (2019) Proteomics of Melanoma Response to Immunotherapy Reveals Mitochondrial Dependence. *Cell* **179:** 236-250 e218

Herroon MK, Rajagurubandara E, Hardaway AL, Powell K, Turchick A, Feldmann D, Podgorski I (2013) Bone marrow adipocytes promote tumor growth in bone via FABP4-dependent mechanisms. *Oncotarget* **4:** 2108-2123

Katerinaki E, Evans GS, Lorigan PC, MacNeil S (2003) TNF-alpha increases human melanoma cell invasion and migration in vitro: the role of proteolytic enzymes. *British journal of cancer* **89:** 1123-1129

Laurent V, Guerard A, Mazerolles C, Le Gonidec S, Toulet A, Nieto L, et al (2016) Periprostatic adipocytes act as a driving force for prostate cancer progression in obesity. *Nature communications* **7:** 10230

Laurent V, Toulet A, Attane C, Milhas D, Dauvillier S, Zaidi F, et al (2019) Periprostatic Adipose Tissue Favors Prostate Cancer Cell Invasion in an Obesity-Dependent Manner: Role of Oxidative Stress. *Molecular cancer research : MCR* **17:** 821-835

Lazar I, Clement E, Dauvillier S, Milhas D, Ducoux-Petit M, LeGonidec S, et al (2016) Adipocyte Exosomes Promote Melanoma Aggressiveness through Fatty Acid Oxidation: A Novel Mechanism Linking Obesity and Cancer. *Cancer research* **76:** 4051-4057

Lazar I, Clement E, Ducoux-Petit M, Denat L, Soldan V, Dauvillier S, et al (2015) Proteome characterization of melanoma exosomes reveals a specific signature for metastatic cell lines. *Pigment cell & melanoma research* **28:** 464-475

MacDougald OA, Lane MD (1995) Transcriptional regulation of gene expression during adipocyte differentiation. *Annual review of biochemistry* **64:** 345-373

Mandrup S, Loftus TM, MacDougald OA, Kuhajda FP, Lane MD (1997) Obese gene expression at in vivo levels by fat pads derived from s.c. implanted 3T3-F442A preadipocytes. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 4300-4305

McKillop IH, Girardi CA, Thompson KJ (2019) Role of fatty acid binding proteins (FABPs) in cancer development and progression. *Cellular signalling* **62:** 109336

McQuade JL, Daniel CR, Hess KR, Mak C, Wang DY, Rai RR, et al (2018) Association of body-mass index and outcomes in patients with metastatic melanoma treated with targeted therapy, immunotherapy, or chemotherapy: a retrospective, multicohort analysis. *The Lancet Oncology* **19:** 310-322

Meulle A, Salles B, Daviaud D, Valet P, Muller C (2008) Positive regulation of DNA double strand break repair activity during differentiation of long life span cells: the example of adipogenesis. *PloS one* **3:** e3345

Moustaid N, Lasnier F, Hainque B, Quignard-Boulange A, Pairault J (1990) Analysis of gene expression during adipogenesis in 3T3-F442A preadipocytes: insulin and dexamethasone control. *Journal of cellular biochemistry* **42:** 243-254

Nath A, Li I, Roberts LR, Chan C (2015) Elevated free fatty acid uptake via CD36 promotes epithelial-mesenchymal transition in hepatocellular carcinoma. *Scientific reports* **5:** 14752

Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al (2011) Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature medicine* **17:** 1498-1503

Nomura DK, Long JZ, Niessen S, Hoover HS, Ng SW, Cravatt BF (2010) Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. *Cell* **140:** 49-61

Pascual G, Avgustinova A, Mejetta S, Martin M, Castellanos A, Attolini CS, et al (2017) Targeting metastasis-initiating cells through the fatty acid receptor CD36. *Nature* **541:** 41-45

Phillips CM, Perry IJ (2015) Lipoprotein particle subclass profiles among metabolically healthy and unhealthy obese and non-obese adults: does size matter? *Atherosclerosis* **242:** 399-406

Rousseau MC, Parent ME, Siemiatycki J (2005) Comparison of self-reported height and weight by cancer type among men from Montreal, Canada. *Eur J Cancer Prev* **14:** 431-438

Rubin CS, Hirsch A, Fung C, Rosen OM (1978) Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. *The Journal of biological chemistry* **253:** 7570-7578

Sarjeant K, Stephens JM (2012) Adipogenesis. *Cold Spring Harbor perspectives in biology* **4:** a008417

Sergentanis TN, Antoniadis AG, Gogas HJ, Antonopoulos CN, Adami HO, Ekbom A, Petridou ET (2013) Obesity and risk of malignant melanoma: a meta-analysis of cohort and case-control studies. *Eur J Cancer* **49:** 642-657

Shah R, Patel T, Freedman JE (2018) Circulating Extracellular Vesicles in Human Disease. *The New England journal of medicine* **379:** 958-966

Shors AR, Solomon C, McTiernan A, White E (2001) Melanoma risk in relation to height, weight, and exercise (United States). *Cancer causes & control : CCC* **12:** 599-606

Skowron F, Berard F, Balme B, Maucort-Boulch D (2015) Role of obesity on the thickness of primary cutaneous melanoma. *Journal of the European Academy of Dermatology and Venereology : JEADV* **29:** 262-269

Spiegelman BM, Ginty CA (1983) Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell* **35:** 657-666

Stenehjem JS, Veierod MB, Nilsen LT, Ghiasvand R, Johnsen B, Grimsrud TK, et al (2018) Anthropometric factors and Breslow thickness: prospective data on 2570 cases of cutaneous melanoma in the population-based Janus Cohort. *The British journal of dermatology* **179:** 632-641

Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of extracellular vesicles* **7:** 1535750

Wang YY, Attane C, Milhas D, Dirat B, Dauvillier S, Guerard A, et al (2017) Mammary adipocytes stimulate breast cancer invasion through metabolic remodeling of tumor cells. *JCI insight* **2:** e87489

Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, et al (2018a) Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nature cell biology* **20:** 332-343

Zhang L, Song J, Cavigiolio G, Ishida BY, Zhang S, Kane JP, et al (2011) Morphology and structure of lipoproteins revealed by an optimized negative-staining protocol of electron microscopy. *Journal of lipid research* **52:** 175-184

Zhang M, Di Martino JS, Bowman RL, Campbell NR, Baksh SC, Simon-Vermot T, et al (2018b) Adipocyte-Derived Lipids Mediate Melanoma Progression via FATP Proteins. *Cancer discovery* **8:** 1006-1025

Zhao J, Zhang J, Yu M, Xie Y, Huang Y, Wolff DW, et al (2013) Mitochondrial dynamics regulates migration and invasion of breast cancer cells. *Oncogene* **32:** 4814-4824

Zhu N, Lalla R, Eves P, Brown TL, King A, Kemp EH, et al (2004) Melanoma cell migration is upregulated by tumour necrosis factor-alpha and suppressed by alpha-melanocyte-stimulating hormone. *British journal of cancer* **90:** 1457-1463

2nd Editorial Decision 13th November 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by the original referees whose comments are shown below.

As you will see, they find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, there are a few editorial issues about text and figures that I need you to address before we can officially accept the manuscript.

-> Address the remaining minor points from referee #2.

REFEREE REPORTS

Referee #1:

In this revised version, the authors, did a great job to answer all the reviewers comments. The article is now suitable for publication

Referee #2:

The authors have been highly responsive to concerns of reviewers and satisfactorily addressed the outstanding concerns. The revised manuscript includes significant new data and rigorous characterization of EVs and biochemistry, which respond well to prior critique and increase the impact of the report. This work is original and provocative, and should have a strong influence on thinking in the EV-cancer field. I recommend acceptance upon completion of two minor corrections detailed below.

Arguments are now clearer. In particular, new framing has clarified the question of the EV payload; the authors state more cleanly that the adipocyte EVs transfer both enzymatic machinery important for fatty acid oxidation, and the fatty acid substrates themselves, noting that in obesity increased delivery of fatty acid contained in the EVs further promotes the effect. This point on pages 4 and 6 is now very clear.

Discussion and data concerning the rigor of purification of EVs, and resolving their biochemical activity in the assay compared to exomeres or lipoprotein aggregates, is also thorough and satisfactory. New data in Suppl Fig 4 convincingly show that the biochemically active fractions (6-13) of size-fractionated EVs (where the assay is melanoma cell migration) contain most of the particles, fatty acids and neutral lipids of the prep.

The previous version of the manuscript contained some ambiguities about when human-origin or murine-origin EVs were used. In several experiments, murine and human factors were mixed as though this should not matter, which was not acceptable. Here, new experiments establish species specificity, with murine 3T3F442A adipocyte EVs tested against murine melanoma B16 cells in a clonogenicity assay, for example (Suppl Fig 1). Clonogenicity in addition to migration assays help to establish the functionally significant endpoints of the EV transfer. Ultimately, other different kinds of cancer-relevant functional assays, (survival assays, induction of anti-apoptotic proteins, EMT in the target cells) should inform future investigation.

In my original review, I noted "...glitazone drugs to improve adipogenesis (rosiglitazone, pioglitazone), often in combination with metformin, thought to activate AMPK..." The authors provide new discussion on Page 12, however their new language is misleading, with the statement "glitazone drugs activate AMPK in adipocytes". The accepted view is that metformin activates AMPK, while glitazone drugs activate PPARgamma. Potential roles for pioglitazone or rosiglitazone in activation of AMPK are much less direct, and not how these drugs are considered in clinical practice (Cf. Kim et al. Exp Mol Med 2016;48: e224). Please correct. Also, the paper cited (Page 12 line 21) to support an anti-cancer role for metformin (Shafiei-Irannejad et al 2017) is obscure, please cite a better paper from this well established literature.

The inclusion in EVs of biochemically active factors other than FA and FAO enzymes (miRNA, leptin) are now mentioned (Page 10 lines 24-30) as requested.

Many of the minor points appear also to have been addressed satisfactorily. Figure legends and labels are now clearer.

Referee #3:

The authors have reasonably responded to my critiques.

2nd Revision - authors' response — 20th November 2019

Point-by-point response:

We thank you for the quality of the review process and for your positive feedback on our work. We have addressed all of the editorial issues in the text and figures, as well as the two minor corrections requested by referee 2. Below, you will find a point-by-point response to these queries. All changes in new version of our manuscript are in red.

Response to Referee reports.

Referee #1:

In this revised version, the authors, did a great job to answer all the reviewers comments. The article is now suitable for publication.

We thank the referee for their positive feedback on our revised manuscript.

Referee #2:

The authors have been highly responsive to concerns of reviewers and satisfactorily addressed the outstanding concerns. The revised manuscript includes significant new data and rigorous characterization of EVs and biochemistry, which respond well to prior critique and increase the impact of the report. This work is original and provocative, and should have a strong influence on thinking in the EV-cancer field. I recommend acceptance upon completion of two minor corrections detailed below.
Arguments are now clearer. In particular, new framing has clarified the question of the EV payload; the authors state more cleanly that the adipocyte EVs transfer both enzymatic machinery important for fatty acid oxidation,

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The previous version of the manuscript contained some ambiguities about when human-origin or murine-origin EVs were used. In several experiments, murine and human factors were mixed as though this should not matter, which was not acceptable. Here, new experiments establish species specificity, with murine 3T3F442A adipocyte EVs tested against murine melanoma B16 cells in a clonogenicity assay, for example (Suppl Fig 1). Clonogenicity in addition to migration assays help to establish the functionally significant endpoints of the EV transfer. Ultimately, other different kinds of cancer-relevant functional assays, (survival assays, induction of anti-apoptotic proteins, EMT in the target cells) should inform future investigation.
In my original review, I noted "...glitazone drugs to improve adipogenesis (rosiglitazone, pioglitazone), often in combination with metformin, thought to activate AMPK..." The authors provide new discussion on Page 12, however their new language is misleading, with the statement "glitazone drugs activate AMPK in adipocytes". The accepted view is that metformin activates AMPK, while glitazone drugs activate PPARgamma. Potential roles for pioglitazone or rosiglitazone in activation of AMPK are much less direct, and not how these drugs are considered in clinical practice (Cf. Kim et al. Exp Mol Med 2016;48: e224). Please correct. Also, the paper cited (Page 12 line 21) to support an anti-cancer role for metformin (Shafiei-Irannejad et al 2017) is obscure, please cite a better paper from this well established literature.
The inclusion in EVs of biochemically active factors other than FA and FAO enzymes (miRNA, leptin) are now mentioned (Page 10 lines 24-30) as requested.
Many of the minor points appear also to have been addressed satisfactorily. Figure legends and labels are now clearer.
We thank the referee for their positive feedback on our revised manuscript. We have corrected our discussion on glitazone drugs (page 12, lines 5-8) and we have replaced the paper we had originally cited on the anti-tumoral properties of metformin (page 12, line 12).

Referee #3:
The authors have reasonably responded to my critiques.
We thank the referee for their positive feedback on our revised manuscript.

Accepted 2nd December 2019

I am pleased to inform you that your study has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Pr Laurence Nieto
Journal Submitted to: Embo J
Manuscript Number: 102525

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen when designing experiments in order to follow the field's best practice and obtain an adequate amount of independant data points to perform accurte statistical analyses. Indeed, all experiments were repeated at least three times, which allowed us to detect any significant effects.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A - No animal studies, per se, are included in this work. However, we did use primary cells and tissues from mice to obtain conditioned media from which EV were purified. For every experiment using these primary EV, at least 3 independant batches were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Cells were randomly assigned to treatment and control groups.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Different investigators performed main experiments and data obtained by each were equivalent. All microscopy experiments were quantified using semi-automatic macros with identical settings for all groups, and images were chosen to best reflect the results of this analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	The statistical significance of differences between the means (of at least three independent assays) was evaluated using Student's t-tests, if 2 groups are compared, or one-way ANOVA, if more than 2 groups are compared, with the indicated associated post hoc tests using GraphPad Prism software.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The normality of samples was determined using a Kolmogorov–Smirnov test, which allowed us to then determine the appropriate post hoc test to apply depending on whether data met the assumption of normality.
Is there an estimate of variation within each group of data?	The standard error of the mean was calculated and plotted for all binding data.

USEFUL LINKS FOR COMPLETING THIS FORM

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http://1degreebio.org
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http://ClinicalTrials.gov
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http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

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http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	See Appendix Table S1
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All of this information can be found in the material and methods section of our manuscript.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Eight week old C57BL/6J male mice (Janvier, Le Genest St Isle, France) were used in this study. Mice were housed according to national and institutional guidelines for animals, in a controlled and enriched environment with a 12:12-h light-dark cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Mice were handled in accordance with National Institute of Medical Research (INSERM) principles and guidelines. All experiments were approved by the local committee on ethics of animal experimentation.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Human AT samples were collected from abdominal dermolipectomies in accordance with the recommendations of the ethics committee of the Rangueil Hospital (Toulouse, France).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All patients gave their informed consent in accordance with the Declaration of Helsinki Principles as revised in 2000.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Done
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A