TLCR TRANSLATIONAL LUNG CANCER RESEARCH
AN OPEN ACCESS JOURNAL FOCUSING ON CLOSING THE GAP BETWEEN "BENCH AND BEDSIDE"
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Peer Review File

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Reviewer A

The original research paper entitled “Increased Plasma Levels of Damage-Associated Molecular Patterns During Systemic Anticancer Therapy in Patients with Advanced Lung Cancer”, by Inoue et al is clinically relevant and well conducted. In this manuscript, authors found an increase in the maximum levels of HMGB1, CRT, HSP70, or annexin A1 in relation to baseline in lung cancer patients exposed to various therapies. Single-agent chemotherapies and platinum-based combinations increased the levels of HMGB1 and CRT in serum. Other findings showed a 'tendency' of difference, not actual differences. The work is well conducted and clinically relevant, since immunotherapies have been proposed as promising alternatives in lung cancer, alone or in combination with chemotherapy. The work is well conducted and clinically relevant, since immunotherapies have been proposed as promising alternatives in lung cancer, alone or in combination with chemotherapy. However, there are several critical aspects that should be clarified or better explored in the study, as detailed below.

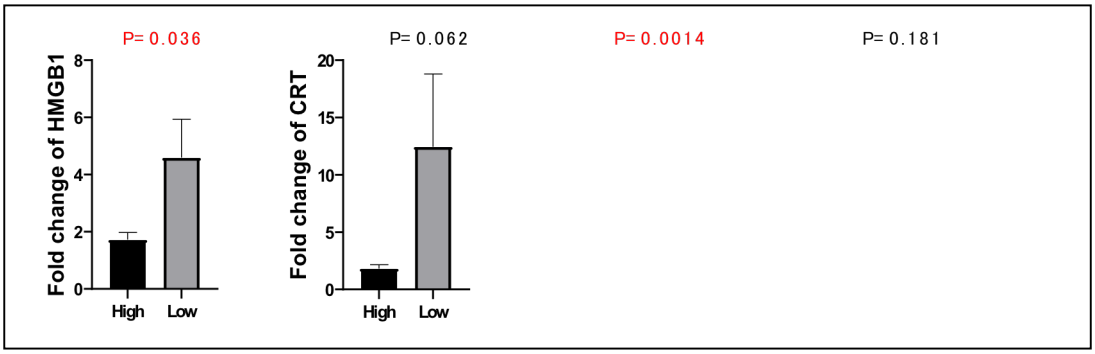
MAJOR POINTS

1. Authors included patients treated with cytotoxic chemotherapy, radiotherapy, or molecularly targeted therapy with an EGFR-TKI or ALK-TKI. However, these strategies trigger a multitude of cellular responses in cancer cells. Authors should segregate patients according to treatments, separating either by type or by class of therapy and assessing the variation of DAMPs in each condition.

Reply 1: We have already compared the maximum fold changes in plasma DAMP levels according to treatment modality for patients showing a complete or partial response. Treatment modalities included platinum doublet chemotherapy (n = 22), single-agent chemotherapy (n = 3), CCRT (n = 11), and EGFR- or ALK-TKIs (n = 8). The mean values for the maximum fold change in HMGB1 levels were numerically greater in patients receiving platinum-based combination or single-agent chemotherapy than in those receiving CCRT or TKIs (*Figure 3A*). The maximum fold change in plasma CRT levels was greatest in patients treated with platinum-based combination chemotherapy (*Figure 3B*). There were no robust differences in the maximum changes in HSP70 or annexin A1 levels among treatment modalities (*Figure 3C and D*).

2. Another strategy that authors could use is to segregate according to baseline values, independently of the therapy of choice. This could allow authors to investigate if baseline values could be predictive of DAMP increase in response to therapy in general or to specific therapies.

Reply 2: As suggested, we classified patients according to baseline values, independently of administered therapy. As shown below, the maximum fold changes in HMGB1 and HSP70 levels for patients with a high baseline of these DAMPs were significantly smaller than those for patients with a low baseline, whereas there were no corresponding significant differences for CRT or annexin A1. These data suggest that the baseline values of HMGB1 and HSP70 may be predictive of the treatment-induced increases. However, we did not include these data in the revised manuscript because it does not seem surprising that lower initial values are associated with higher relative increases.



3a. Recently, Solari et al (doi: 10.1186/s12885-020-06964-5) suggested an integration between DAMPs in an Index. I suggest that authors use a similar strategy, generating an Index of Immunogenicity to each patient and associating this Index with clinical parameters (e.g. tumor size, tumor infiltration, tumor necrosis, lymphatic or systemic metastasis, age, tumor stage, histological subtype, among others).

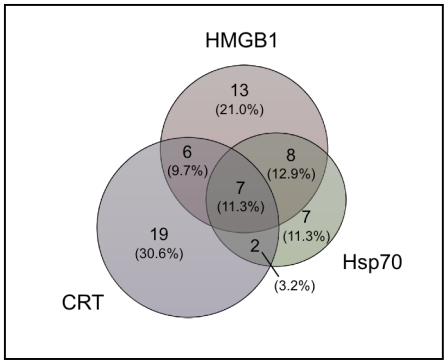
Reply 3a: We appreciate the reviewer's suggestion, but we did not pursue the development of such an index because the previous study performed an integrative analysis with data obtained for ICD-associated DAMPs, cell number, apoptosis, and autophagy in the A549 lung cancer cell line, and because it would be difficult to examine the relation between plasma DAMP levels and the extent of apoptosis or autophagy in lung tumor tissue, which is not readily available. Instead, we have now addressed the study by Solari *et al*. in the Discussion section of the revised manuscript.

Changes in the text: "Among various chemotherapeutic agents recently tested either alone or in combination, cisplatin alone was found to be most effective at inducing the release of ICD-associated DAMPs from A549 lung cancer cells *in vitro*, suggesting that the pairing of cisplatin with immunotherapy may be a promising treatment strategy for lung cancer (23). This previous study also suggested that derivation of a DAMP index of immunogenicity by mathematical integration might prove useful as a measure of the extent of ICD for comparison with clinical parameters such as the response to anticancer therapies (23). Our results suggest that a DAMP index based on the treatment-induced increases in the plasma levels of HMGB1, CRT, HSP70, and

annexin A1 may prove helpful in this regard, although a study with a larger patient population will be necessary to evaluate this notion." (page 13, lines 261–270)

3b. Authors could also calculate a rate of qualitative concordance among markers, rather than correlation (i.e. in X% of patients, CRT and HMGB1 increased; in Y% of patients, CRT and Annex increased; and etc).

Reply 3b: As suggested, we generated a VENN diagram showing such concordance for patients manifesting a >2-fold increase in HMGB1 (n = 34), CRT (n = 34), or HSP70 (n = 24) levels. We did not assess the concordance for annexin A1 and histone H3 because the numbers of patients showing a >2-fold increase were low (n = 5 and 3, respectively). As is now presented in the new *Figure 4G*, described in the Results section, and shown below, the rates of concordance for HMGB1, CRT, and HSP70; for HMGB1 and CRT; for HMGB1 and HSP70; and for CRT and HSP70 were 11.3%, 21.0%, 24.2%, and 14.5%, respectively.
Changes in the text: "Rates of concordance were examined for patients who showed a >2-fold increase in HMGB1 (n = 34), CRT (n = 34), or HSP70 (n = 24) levels (*Figure 4G*), with this analysis not being performed for annexin A1 because only five patients showed such an increase. The rates of concordance for HMGB1, CRT, and HSP70; for HMGB1 and CRT; for HMGB1 and HSP70; and for CRT and HSP70 were 11.3%, 21.0%, 24.2%, and 14.5%, respectively (23)." (page 10, lines 203–207)
<Figure 4G>



4a. Figure 1 is quite difficult to read. I strongly suggest that the authors compare the levels of DAMPs before and after treatment for individual patients, since DAMP levels are quite heterogeneous already at baseline. Thus, I suggest that authors transform the initial values for each individual to 1 in Figure 1, and then calculate the delta between the intervals. These deltas could be used to segregate patients according to the median, for instance.

Reply 4a: As suggested, we transformed the initial values for each individual to 1 as shown in *Figure 1A*, and we then compared the mean fold changes between baseline and each time point (*Figure 1B*) as well as the maximum fold changes and baseline (*Figure 1C*). We modified the text of the Results section accordingly.
Changes in the text: "We measured the plasma levels of five DAMPs (HMGB1, CRT, HSP70, annexin A1, and histone H3) at four serial time points including

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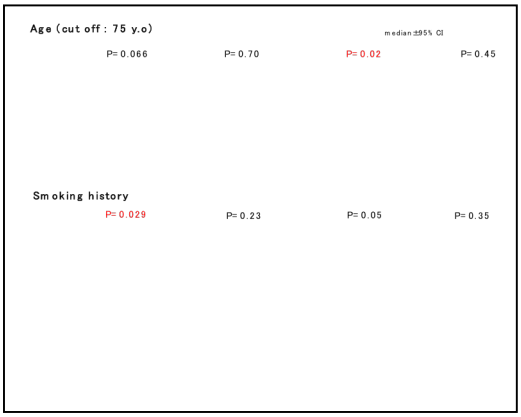
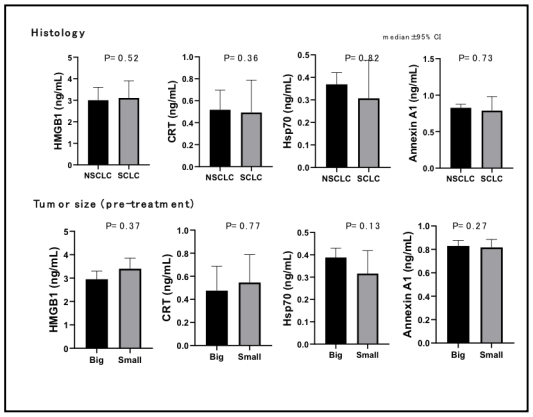
baseline (the day before the first cycle of systemic anticancer therapy), days 3 and 8 of the first cycle, and the day before the second cycle of treatment (*Supplementary Figure 1*). The fold changes in the concentrations of the five DAMPs during the first treatment cycle relative to the baseline value were determined (*Figure 1A*), with the fold changes in both HMGB1 and CRT levels at the three time points after treatment onset being substantially higher than the baseline value but those in HSP70 and annexin A1 being only slightly higher (*Figure 1B*). The mean of the maximum fold change in HMGB1, HSP70, or annexin A1 apparent after the onset of systemic anticancer therapy was significantly higher than that of the corresponding baseline value [1.00 vs. 3.15 ($P = 0.002$), 1.00 vs. 1.57 ($P < 0.0001$), and 1.00 vs. 1.18 ($P < 0.0001$), respectively] (*Figure 1C*). No corresponding significant difference was apparent for CRT and histone H3 levels [1.00 vs. 6.52 ($P = 0.053$) and 1.00 vs. 1.26 ($P = 0.60$), respectively]." (page 8, line 159–page 9, line 170)

4b. With this, the authors could even associate the baseline levels with the histopathological and clinical characteristics of the tumors and response to therapy (in other words, authors could check whether the baseline levels of DAMPs may predict the response to therapy). Actually, the manuscript is poor in associating basal levels of DAMPs with all clinical data. Authors should segregate according to age, histological type and other variables cited in item 3.

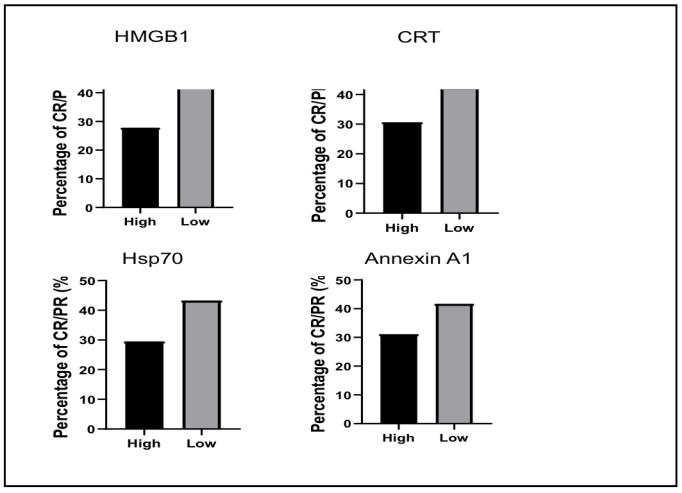
Reply 4b: As suggested, we attempted to evaluate the relation of baseline levels of DAMPs to clinical characteristics and response to therapy. We first assessed the baseline levels of four DAMPs (HMGB1, CRT, HSP70, and annexin A1) and clinical characteristics including age (young vs. old with a cutoff of 75 years), smoking history (smoker vs. nonsmoker), histology (NSCLC vs. SCLC), and tumor size before therapy (small vs. large as calculated based on RECIST criteria). We could not perform such analysis for histone H3 given that it was undetectable at baseline in most (97 of 121) patients. As shown in the figures below, the baseline level of HSP70 was significantly higher in young patients than in old patients ($P = 0.02$), whereas that of HMGB1 was significantly higher in smokers than in nonsmokers ($P = 0.029$). None of the other comparisons showed a significant difference.





We then examined the relation between baseline DAMP levels and clinical

response. As shown in the figures below, patients with low baseline levels of the four DAMPs showed a higher percentage of complete or partial responses compared with those with high baseline levels. These data suggested that baseline levels of these DAMPs may predict the clinical response to therapy in general and may reflect the extent of pathology or malignancy.



We decided not to include these data relating to baseline DAMP levels in the revised manuscript because the number of enrolled patients was too small for robust statistical comparisons and because we are currently performing a prospective observational study to investigate the relation between the changes in plasma DAMP levels and the clinical benefit of chemotherapy combined with immune checkpoint inhibitors.

5. I strongly suggest that the authors assess the levels of inflammatory cytokines in the patients' serum, associating these levels with serum DAMPs.

Reply 5: We are planning to measure the serum levels of inflammatory cytokines such as CCL2, CXCL1, and type I IFN, which are also considered to be DAMPs according to the latest ICD guidelines, and to assess their relation to clinical response in the near future. However, it is not possible for us to perform these assessments in the present study because the approved study plan does not include measurement of these cytokines. We have now addressed this issue in the Conclusions section of the revised manuscript.
Changes in the text: "We are currently performing a prospective observational study to investigate the relation between the changes in plasma levels of DAMPs including inflammatory cytokines and the clinical benefit of chemotherapy combined with immune checkpoint inhibitors." (page 14, lines 292–294)

6. Despite assessing the levels of individual DAMPs is important, it is fundamental to evaluate if these levels are sufficient to activate immune cells. The authors should expose lymphocytes in vitro to the patients' serum and investigate a possible activation of these cells. This, and not only the presence of DAMPs, is necessary to

affirm that a treatment induce Immunogenic Cell Death.

Reply 6: We agree with the reviewer on this point, and we are now planning to evaluate if DAMPs in plasma obtained from patients are able to activate antigen presenting cells such as dendritic cells in vitro. Unfortunately, it is also not possible for us to perform these experiments in the present study because the approved study plan does not allow it. We have now addressed this issue in the Discussion section of the revised manuscript.
Changes in the text: "Although assessment of the circulating levels of individual DAMPs is important, it will also be essential to investigate whether these levels are sufficient to activate antigen presenting cells such as dendritic cells in cancer patients during anticancer therapy." (page 13, lines 270–272)

7. In material and methods Authors cite that "the concentration of DAMPs were measured immediately before the second treatment cycle". However, considering that authors included patients treated with various therapies, it is plausible to assume that the 'second treatment cycle' was different among patients. What drugs or treatments were used in the first cycle? How long were these treatments? What 'immediately' means?

Reply 7: The first and second treatment cycles refer to the same treatment protocol. In general, each treatment cycle ranges from 3 to 4 weeks. As mentioned in the Results section (page 8, line 161), "immediately" in the context of before the second treatment cycle means the day before.

MINOR POINTS
1. Authors should mention the study by Solari et al, which described the release of DAMPs by lung cancer cells exposed to several chemotherapeutics in vitro, both alone and in combination. doi: 10.1186/s12885-020-06964-5

Reply 1: As suggested, we have now addressed this study in the Discussion section of the revised manuscript.
Changes in the text: "Among various chemotherapeutic agents recently tested either alone or in combination, cisplatin alone was found to be most effective at inducing the release of ICD-associated DAMPs from A549 lung cancer cells in vitro, suggesting that the pairing of cisplatin with immunotherapy may be a promising treatment strategy for lung cancer (23). This previous study also suggested that derivation of a DAMP index of immunogenicity by mathematical integration might prove useful as a measure of the extent of ICD for comparison with clinical parameters such as the response to anticancer therapies (23). Our results suggest that a DAMP index based on the treatment-induced increases in the plasma levels of HMGB1, CRT, HSP70, and annexin A1 may prove helpful in this regard, although a study with a larger patient population will be necessary to evaluate this notion." (page 13, lines 261–270)

2. In the introduction (p.3 l 75-78): references 8-10 do not consider Annexin A1 and H3 as DAMPs. Authors may add the consensus guidelines for the definition, detection and interpretation of immunogenic cell death (doi: 10.1136/jitc-2019-000337), which describes all DAMPs except H3.

Reply 2: As suggested, we have now cited the consensus guidelines for immunogenic cell death (ICD) in the Introduction section of the revised manuscript and modified the text accordingly.
Changes in the text: "ICD has been characterized by the release or exposure on the cell surface of a defined set of molecules that are known as damage-associated molecular patterns (DAMPs) (8,9) and which include high-mobility group box 1 (HMGB1), calreticulin (CRT), heat shock protein 70 (HSP70), and annexin A1 (8,10). Histone H3 has also been shown to function as a DAMP, or alarmin, in inflammatory conditions (11,12)." (page 5, lines 82–87)

3. In the material and methods and/or in the results section, I suggest mentioning the name of the main chemotherapeutic drugs used.

Reply 3: As suggested, we have now mentioned the names of the main chemotherapeutic drugs administered to the study patients in the Methods and Results sections of the revised manuscript.
Changes in the text: "… including cytotoxic chemotherapy [such as platinum (cisplatin or carboplatin) doublet chemotherapy and single-agent chemotherapy (including docetaxel, pemetrexed, and S-1 among others)], CCRT (cisplatin- or carboplatin-based)…" (page 7, lines 119–122)

"With regard to treatment modalities, 59 (49%) patients received platinum (cisplatin or carboplatin) doublet chemotherapy, 28 (23%) single-agent chemotherapy (docetaxel, pemetrexed, or S-1 among others), 23 (19%) CCRT (cisplatin- or carboplatin-based), and 11 (9%) an EGFR-TKI (erlotinib or osimertinib) or ALK-TKI (alectinib, crizotinib, or brigatinib)." (page 8, lines 152–156)

4. In introduction (p.3, l67), Ref. 1 (2017); I suggest to replace by this (doi: 10.3322/caac.21590) as it is the most current.

Reply 4: We replaced the reference as suggested.
Changes in the text: Reference (1) was updated as suggested.

5. In introduction (p.3, l68), this sentence is also true to small cell lung cancer. Please include this information.

Reply 5: As suggested, we modified the sentence to include small cell lung cancer.
Changes in the text: "The standard treatments for advanced non–small cell lung cancer and small cell lung cancer have changed markedly over the last decade,

however (2).” (page 5, lines 74–76)

6. In introduction (p.3, l69), which platinum compound?

Reply 6: We have now clarified the platinum compound.
Changes in the text: “Several recent phase 3 studies have revealed that platinum (cisplatin or carboplatin)–based combination chemotherapy…” (page 5, line 76).

7. In introduction (p.3, l82). Reference 14, please replace by the current guidelines (doi: 10.1136/jitc-2019-000337).

Reply 7: As suggested, we have replaced the original reference with the current guidelines.
Changes in the text: The reference was updated (new ref. 9).

8. In results (p.5, l141), authors mentioned that “69% of patients were in stage IV”. Which histological subtype of lung cancer?

Reply 8: Stage IV disease (69% of enrolled patients) included adenocarcinoma, squamous cell carcinoma, NOS (not otherwise specified), and small cell lung cancer (61.4 %, 13.3%, 6.0%, and 19.3%, respectively).

9. In results (p.5, l 142), which platinum compound (cisplatin, carboplatin or oxaliplatin)?

Reply 9: See our response to minor point 3 above.

10. In results (p.5,141-142), Identify the chemotherapy and histological subtype used for treatment.

Reply 10: Platinum (cisplatin or carboplatin) doublet chemotherapy was used for NSCLC or SCLC patients as standard first-line chemotherapy. Single-agent chemotherapy including docetaxel, pemetrexed, and S-1 was used for patients with NSCLC such as adenocarcinoma and squamous cell carcinoma, predominantly as standard second-line chemotherapy. We have now included the therapeutic agents in the Results section as outlined in our responses to minor points 3 and 9.

11. In results (p.4, 143), specify which compound was used in patients.

Reply 11: Please see our responses to minor points 3, 9, and 10.

Reviewer B

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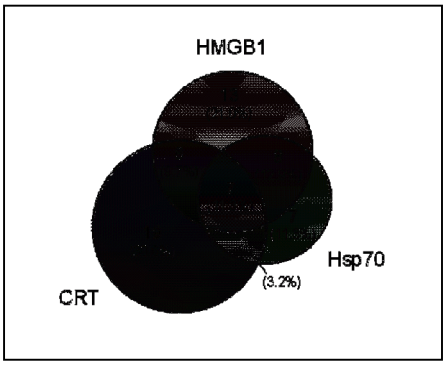
The manuscript by Inoue et al. presents an interesting paper investigating the DAMP release of patients with lung cancer.

1. So far, the authors investigated the individual DAMPS separately. Could the authors consider combining DAMP increases for each patient? By cumulating the increases of the different DAMPS, the authors could generate a "DAMP index".

Reply 1: We now present in the new *Figure 4G* a VENN diagram showing concordance for the increases in DAMP levels in patients showing >2-fold increases in HMGB1 (n = 34), CRT (n = 34), or HSP70 (n = 24). Among all study patients, however, the rates of concordance for HMGB1, CRT, and HSP70; for HMGB1 and CRT; for HMGB1 and HSP70; and for CRT and HSP70 were low (5.7%, 10.5%, 12.1%, and 7.3%, respectively). We therefore did not pursue development of such a DAMP index, as the statistical power would likely not be sufficient to obtain significant results. We have now addressed this issue in the Discussion section of the revised manuscript.
Changes in the text: "Our results suggest that a DAMP index based on the treatment-induced increases in the plasma levels of HMGB1, CRT, HSP70, and annexin A1 may prove helpful in this regard, although a study with a larger patient population will be necessary to evaluate this notion." (page 13, lines 267–270)

2. Figure 1B: it is not clear whether the patients that show increases in HMGB1 (in red) are in part the same as the patients that show increases in any of the other DAMPS? Please clarify.

Reply 2: As mentioned in Reply 1, we analyzed concordance between increases in DAMP levels in patients showing >2-fold increases in HMGB1 (n = 34), CRT (n = 34), or HSP70 (n = 24) with the use of a Venn diagram (new *Figure 1G*). The rates of concordance for HMGB1, CRT, and HSP70; for HMGB1 and CRT; for HMGB1 and HSP70; and for CRT and HSP70 were 11.3%, 21.0%, 24.2%, and 14.5%, respectively. We have now described these findings in the Results section of the revised manuscript.
Changes in the text: "Rates of concordance were examined for patients who showed a >2-fold increase in HMGB1 (n = 34), CRT (n = 34), or HSP70 (n = 24) levels (*Figure 4G*), with this analysis not being performed for annexin A1 because only five patients showed such an increase. The rates of concordance for HMGB1, CRT, and HSP70; for HMGB1 and CRT; for HMGB1 and HSP70; and for CRT and HSP70 were 11.3%, 21.0%, 24.2%, and 14.5%, respectively (23)." (page 10, lines 203–207)



3. Subsequently, would the authors have the possibility to correlate this cumulated DAMP index to the overall or disease-free survival etc. for patients presenting increased levels of multiple DAMPS. Could the authors compare the fate of patients presenting DAMP release in the upper vs. lower quartile?

Reply 3: We appreciate the reviewer's suggestion, but, as mentioned in Reply 1, we did not pursue the development of a DAMP index because we feel it would be premature and the statistical power conferred by the relatively small number of enrolled patients would likely be insufficient. We are currently collecting more clinical samples and information relating to prognosis such as OS and PFS in order to evaluate the relation between increases in DAMP levels and outcome.
Changes in the text: See Reply 1.