

Pesaro (Italy), 08-April-2020

To Editor-in-Chief

World Journal of Clinical Oncology

Dear Chief Editor,

we wish to submit to your journal for consideration the invited manuscript **ESPS Manuscript Number ID: 03535487, entitled Immune response after hyperthermic intraperitoneal chemotherapy (HIPEC) of peritoneal carcinomatosis: a pilot study.**by Giammaria Fiorentini, Donatella Sarti, Alberto Patriti, Emilio Eugeni, Francesco Guerra, Stefano Guadagni

1. **Reviewer ID 3769068** Conclusion: Major revision

Scientific Quality: Grade D (Fair)

Language Quality: Grade B (Minor language polishing)

The study is interesting, despite the small number of patients.

1) I don't think it's appropriate to use the term immune reaction.

**Reply: done, Abstract, page 2, line 3 "to investigate whether an immune response".**

2) The results of the abstract need to be better described.

**Reply: done, Abstract, page 2, line 11 "The total numbers of CD3+, CD3+/CD4+ T-Helper, CD3+/CD8+ cytotoxic T, CD3+/CD56+ NK and CD19+ B lymphocytes, and CD4+:CD8+ lymphocyte ratios were increased in all but one patient 30 days following the CRS-HIPEC procedure, and these increases were significant ( $p \leq 0.05$ ) for CD3+/CD4+ T Helper and CD3+/CD8+ cytotoxic T lymphocyte numbers."**

3) I believe that in the conclusion (abstract) the authors should mention that more studies need to be done.

**Reply: done, Abstract, page 2, line 20 "to be fully characterised in future studies."**

4) The flow cytometry methodology needs to be detailed.

**Reply: done, Methods, page 5, line 3 "Single-cell suspensions were labelled with fluorochrome-conjugated anti-human CD3, CD4, CD8, CD19 and CD56 monoclonal**

antibodies or matched isotypes, and CD3+ total, CD3+/CD4+ T-Helper, CD3+/CD8+ cytotoxic T, CD3+/CD56+ NK and CD19+ B lymphocytes, quantified by fluorescence-activated cell sorting, an antibody-based cell sorting method for heterogeneous mixtures based upon the specific light scattering and fluorescent characteristics of each antibody-labelled cell-type [12]. Additional immunological parameters included the CD4+: CD8+ T lymphocyte ratio. Immunological populations were also analyzed in stained samples, after exclusion of debris and doublets, as previously described [13]."

5) Was a normality test performed to apply the t test?

Reply: done, Methods, Statistical analysis, page 5, line 17 "Unilateral paired Students' t-tests were used"; Results, page 6, line 11 "(unilateral paired Student's t test,  $p < 0.05$ )"; Table 2.

6) I believe that the results deserve to be better presented.

Reply: done, Results "The 6 patients in this study were comprised of 3 males (50%) and 3 females (50%), with a median age of 55 years (range 48-71 years). Two patients (33%) presented with PM from colorectal cancer and the other 4 (77%) with PMP (Table 1).

CT scans, performed 3, 6 and 12 months following CRS-HIPEC, demonstrated complete responses in all 6 patients, associated with a median PFS of 12 months. No complications were observed and the mean hospitalization-time, following CRS-HIPEC, was  $10 \pm 2$  days. One patient however, was hospitalized for 58 days with severe oxaliplatin toxicity, characterized by grade 4 hematopoietic toxicity, according to National Cancer Institute, Common Terminology Criteria for Adverse Events (CTCAE v4.03), and grade 3 hematomas in the abdominal wall and pelvis. The other patients did not exhibit any HIPEC drug-associated adverse events.

Evaluation of system lymphocyte populations in patient PB samples (Table 2), revealed increases in systemic CD3+ total, CD3+/CD4+ T Helper, CD3+/CD8+ cytotoxic T, CD3-/CD56+ NK, CD19+ B lymphocyte numbers and increased CD4+: CD8 + T lymphocyte ratios, statistically significant for CD3+/CD4+ T Helper and CD3+/CD8+ Cytotoxic T lymphocyte populations (unilateral paired Student's t test,  $p < 0.05$ ) (Figure 2). These increases were observed in all but one patient, who exhibited reduced systemic lymphocyte counts post-HIPEC, consistent with severe oxaliplatin toxicity."

7) The authors in the discussion need to assume the limitations of the study.

**Reply: done, Discussion** “The limitation of this pilot study, however, is that it only provides preliminary evidence that this methodological approach is appropriate for evaluating immunological changes following CRS and HIPEC, and is, therefore, a forerunner to a future confirmatory large cohort study. Furthermore, this study was not designed to evaluate treatment safety, efficacy or effectiveness [20], justifying the small sample size. The data should also be interpreted with some care, since HIPEC is performed immediately following tumour debulking, implicating surgical trauma as an alternative source of physiological and immunological alteration. Indeed, the normal physiological response to tissue injury involves a complex integration of inflammatory, immunological, neuroendocrine and metabolic mechanisms, and incision, dissection, organ manipulation and vascular alterations all induce acute inflammation for the purpose of host defense and tissue repair [10], with negative immunosuppressive feedback activated to avert an exaggerated immunological/inflammatory response [21]. Notwithstanding this, the difference in the characteristics of acute inflammation induced by surgical stress-induced and HIPEC-induced long-lived increases in systemic CD3+, CD3+/CD4+ T Helper, CD3+/CD8+ cytotoxic T, CD3-/CD56+ NK, CD19+ B lymphocytes, and CD4+ : CD8+ T lymphocyte ratio, statistical significant for CD3+/CD4+ T Helper and CD3+/CD8+ cytotoxic T lymphocyte populations, supports the hypothesis that HIPEC activates the immune system, with differences consistent with generalized activation of an adaptive immune response.”

8) I think it's important that the authors put images resulting from flow cytometry.

**Reply: done, Figure 1 has been added.**

9) The use of graphics would be interesting.

**Reply: done by Figure 2**

10) The purposes of the statistical tests employed are not clear.

**Reply: done, Methods, Statistical analysis** “Unilateral paired Students’ t-tests were used to demonstrate intra-patient and intra-cohort treatment response variability, trend, and statistical significance associated with a probability (p) of  $\leq 0,05$ .”

2. **Reviewer ID 02974589** Conclusion: Minor revision

Scientific Quality: Grade C (Good)

Language Quality: Grade C (A great deal of language polishing)

1: Abstract: I would change "the immune reaction" to "an immune reaction" since there are many different types of immune responses.

**Reply: done, Abstract, page 2, line 3 "to investigate whether an immune response".**

2: Many people in the field advocate for changing from peritoneal carcinomatosis to peritoneal metastases. Carcinomatosis means "cancer transformation" of the peritoneum and we hope to change this view of the peritoneum. To see peritoneal surface disease as a locoregional metastatic disease in much the same way as the liver is. My suggestion is to change this term throughout the article and use peritoneal metastases or peritoneal metastatic disease or something likewise instead.

**Reply: done, Title, page 1, line 2 "peritoneal metastases"; Abstract, page 2, line 2 "peritoneal metastases (PM)"; Abstract, page 2, line 4 "PM"; "; Abstract, page 2, line 16 "activity in PM"; Keywords "peritoneal metastases"; Core tips, page 3, line 5 "peritoneal metastases"; Introduction, page 3, line 11 "peritoneal metastases (PM)"; Introduction, page 4, line 9 "PM"; "; Material, page 4, line 12 "PM"; Results, page 5, line 22 "PM"; Discussion, page 7, line 7 "peritoneal metastases (PM)"; Discussion, page 6, line 16 "PM"; Discussion, page 6, line 18 "PM"; Discussion, page 6, line 24 "PM"; Discussion, page 7, line 3 "PM"; Discussion, page 8, line 5 "PM".**

3: Abstract – take away the first instance of "on" in the first sentence of the abstract.

**Reply: done, Abstract, page 2, line 2 "HIPEC for peritoneal metastases (PM)".**

4: Introduction – page 4 – in the beginning of page 4 a sentence ends abruptly "systemic bloodstream of ...." Please complete sentence.

**Reply: done...the sentence (Introduction – page 4) has been changed at line 2 "characterized by ablated tissue transitional zone infiltration by immune and inflammatory cell populations, including: dendritic cells, neutrophils, macrophages, B and T lymphocytes and natural killer (NK) cells [10], with a systemic increase in immune cells also detected [8].".**

5: Introduction – page 4 – RFA is not explained in the introduction. Please write out the first instance of acronyms.

**Reply: done...the sentence (Introduction – page 4) has been changed at line 6 "The effect of HIPEC is similar to that of radio frequency ablation (RFA)".**

6: Methods – The abstract and methods sections don't say the same thing. As I read the abstract, I interpret the method section there to mean that blood samples were taken 1 time

preoperatively and then 3 times postoperative on postop day 1, 3, and 30. That means 4 samples per patient. However, in the main text methods, samples were taken day 0 and day 30 which isn't the same. Day 0 is that the same day as the HIPEC procedure then? Please clarify very specifically when the preop sample was taken and exactly when the postop samples were taken. The methods in the abstract needs to match the methods in the main text file.

**Reply: done, Abstract, page 2, line 6 "Peripheral blood samples were obtained from each patient prior to (day 0) and post-procedure (day 30), and used to evaluate the number of CD3+ total..."; Methods, page 5, line 1 "Peripheral blood (PB) samples were collected in ethylenediaminetetraacetic acid (EDTA), prior to (day 0) and following (day 30) CRS-HIPEC procedures, and immunophenotypic analysis was performed within 24 hours (Figure 1).".**

7: Please indicate in table two which tests that were statistically increased from baseline to post-HIPEC.

**Reply: done in Table 2.**

8: Please indicate which sample time was used in the post HIPEC results (was it 30 days).

**Reply: done in Table 2 and in Figure 2.**

9: In the discussion, a recommendation of at least 60 minutes of hyperthermia was given. However, this study does not provide the basis for such a recommendation (there was no comparison between different hyperthermia times). Please remove this recommendation and only make conclusions on the data that has been acquired.

**Reply: done, ....the sentence (Discussion, previous page 6) has been changed at page 7, line 7 "In our opinion, therefore, HIPEC is not only a useful chemotherapeutic procedure but also stimulates the immune-system. In fact, a 60-minutes HIPEC appears to promote not only efficacious cytotoxicity but also sufficient release of tumour debris, antigens and damage-associated molecules to activate the immune system and promote cytotoxic T lymphocyte maturation.".**

10: In general the language needs editing. There are a number of incorrect spellings such as "cytoreduction" and "oxalyplatin". Also a number of grammatical errors that need language editing.

**Reply: done. Complete English language revision has been performed by a new co-author (Mackay AR) born and graduated in UK.**

11: The discussion has a short paragraph touching on the issue of surgical trauma. Are there any good articles to reference for this. Because this study only looked at the combination CRS+HIPEC. It would have been nice to have a few patients with only CRS without HIPEC in order to be able to see if there is an actual HIPEC related immune response. As the study is set up now, you can only evaluate the combination of CRS+HIPEC. Thus, it is difficult to say for sure that this immune stimulation is HIPEC related. It may actually be CRS related. Please comment a little more on this. Perhaps seeing if there is any relevant literature that has looked into the immune response after just surgical trauma.

**Reply: We fully agree. The paragraph touching on the issue of surgical trauma has been changed "The data should also be interpreted with some care, since HIPEC is performed immediately following tumour debulking, implicating surgical trauma as an alternative source of physiological and immunological alteration. Indeed, the normal physiological response to tissue injury involves a complex integration of inflammatory, immunological, neuroendocrine and metabolic mechanisms, and incision, dissection, organ manipulation and vascular alterations all induce acute inflammation for the purpose of host defense and tissue repair [10], with negative immunosuppressive feedback activated to avert an exaggerated immunological/inflammatory response [21]. Notwithstanding this, the difference in the characteristics of acute inflammation induced by surgical stress-induced and HIPEC-induced long-lived increases in systemic CD3+, CD3+/CD4+ T Helper, CD3+/CD8+ cytotoxic T, CD3-/CD56+ NK, CD19+ B lymphocytes, and CD4+ : CD8+ T lymphocyte ratio, statistical significant for CD3+/CD4+ T Helper and CD3+/CD8+ cytotoxic T lymphocyte populations, supports the hypothesis that HIPEC activates the immune system, with differences consistent with generalized activation of an adaptive immune response."**

**Thank you very much for your suggestion. In the future we will evaluate the same parameters also in patients submitted for CRS without HIPEC.**

12: The overall conclusion is good with the need of a larger study to really evaluate the HIPEC effect and not the CRS effect on the immune response.

**Reply: done, we changed Conclusion including "In conclusion, this pilot study provides the first evidence that HIPEC activates the immune response in PM patients,**

supporting an additional immunomodulatory function for this procedure. Of course, results from this small patient cohort pilot study must await confirmation in a larger patient cohort, which could also benefit from comparing the CRS-HIPEC-induced immune response activation to that induced by HIPEC combined with minimally-invasive surgical procedures.”

3. **Reviewer ID 00009760** Conclusion: Accept (General priority)

Scientific Quality: Grade B (Very good)

Language Quality: Grade B (Minor language polishing)

This paper describes positive changes in immunological function post CRS + HIPEC. This is interesting. A small number of patients.

**Reply: your consideration is very appreciated. The small number of patients was indicated as one of the main limitation of the manuscript.**

4. **Reviewer ID 02942954** Conclusion: Major revision

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

This manuscript compared the differences in the absolute number of several immune cells between pre- and post-operation, in order to investigate the effects of hyperthermic intraperitoneal chemotherapy on immune responses. It is interesting, but some questions remained to be solved.

1. For the title, the abbreviation “HIPEC” should be avoided.

**Reply: done, Title, page 1 “hyperthermic intraperitoneal chemotherapy (HIPEC)”**

2. Paired chi-square or t tests may be more suitable for your data.

**Reply: done, Methods, Statistical analysis, page 5, line 17 “Unilateral paired Students’ t-tests were used”; Results, page 6, line 11 “(unilateral paired Student’s t test,  $p < 0.05$ )”; Table 2.**

3. If convenient, please provide the representative dot plots of flow cytometry and the frequency of various cell types in lymphocyte.

**Reply: done, Figure 1 has been added.**

Best Regards,

The corresponding author on behalf of all authors

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