Cross-sectional Study

Evaluation and comparison of intratumoural and intrastromal infiltrating lymphocytes with clinicopathological features in breast carcinoma patients who have received neoadjuvant chemotherapy - A cross-sectional study

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A B S T R A C T

Background: The microenvironment of breast cancer plays a significant role in determining the prognosis of the disease. With the shifting paradigm on the predictive factors post-Neoadjuvant Chemotherapy (NAC), it was sought out that Tumour infiltrating lymphocytes (TILs) are of valuable use for the same. Yet, the delineation of the two types - Intrastromal and Intratumoural has seldom been facilitated. This study, therefore, aimed to evaluate, analyse and compare the two - to gauge the importance of the treatment outcome and clinicopathological features.

Materials and methods: 180 breast cancer patients were included in this study who underwent NAC, and their post-surgically resected tumour specimens were sectioned and stained using routine Haematoxylin and Eosin techniques. The evaluation of TILs in the stroma and tumour was done based on the standardised guidelines.

Results: Out of the 180 patients, 55 (i.e. 30.56%) displayed pathological complete resolution (pCR). Furthermore, Intratumoural TILs had a slight association with the pCR (p = 0.0335) whereas Intrastromal TILs had a significantly large association with pCR (p < 0.0001) dependent on the lymphocytic response. Backward regression revealed that - the age at operation, pCR, lymph node involvement and menopause highly contributed to predicting 68.2% of the total cases correctly with a sensitivity of 93.0% and specificity of 24.6% for Intratumoral TILs. Age at operation, pCR, lymph node involvement and tumour emboli highly contributed to predicting 71.5% of the total cases correctly with sensitivity of 71.6% and specificity of 71.4% for Intrastromal TILs.

Conclusion: TILs and the prediction of NAC and pCR should be made standardised and reproducible so that they can be universally available to all patients with breast cancer. Through this study, further avenues of research have opened up for their relations with clinicopathological features mainly age at operation and menopausal status.

1. Introduction

Breast cancer is known for its heterogeneous histopathological, gross and immunohistochemical features. As mentioned by Walter E. Sistrunk and William C. MacCarty [1], “It is impossible to foretell the duration of life of all patients with carcinoma of the breast, because the degree of malignancy varies widely, and persons react differently to the disease”. Consequently, there are striking differences in the prognosis of breast cancer according to the tumour characteristics and immune response.

The body’s immune system has proven to be significantly influential in the prognosis of breast cancer [2] and by observing this immunological response of the body to cancer in the microenvironment of the tumour we can find that the body plays a vital role in predicting the treatment response and outcome of the patient.

The varying immunohistochemical subtypes of breast cancer form the basis of multiple research modules specifically designed to assess the Tumour infiltrating lymphocytes in triple-negative breast cancer (TNBC), human epidermal growth factor receptor 2(HER2) - enriched

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breast cancer and hormone-receptor breast cancer (HRBC) [3,4]. Recent studies have gone to show the relevance of tumour infiltrating lymphocytes (TILs) as a potential biomarker for breast cancer [5] and have showcased the importance of neoadjuvant chemotherapy (NAC) employed in breast cancer patients to achieve pathological complete response (pCR) and increase the breast conservation rate thereby improving the surgical outcome and reducing the risk of distant metastases.

TILs have proven to be a predictor of outcome and recurrence in colorectal cancer [6] and ovarian cancer [7] and tumour infiltrating lymphocyte therapy has been also employed in cancers with bleak prognoses such as metastatic melanoma [8]. Though TILs have been employed as a biomarker for the aforementioned cancers, over the years there has been a shifting emphasis on its use in invasive breast carcinoma and therefore by characterising the breast carcinoma by subtype and immune environment it will help in providing insight into effective therapy and a larger population of breast cancer patients will benefit from the targeted immune therapy.

2. Material and methods

The given study was registered and certified under the Indian Council of Medical Research (ICMR) Short Term Studentship-2019 (STS-2019) (Ref. No. 2019–02057). Institutional Ethics Committee approval was obtained from The Institutional Ethics Committee of Krishna Institute of Medical Sciences, Karad (Reference number - KIMSDU/IEC/02/ 2019) following protocol approval (can be made available from the corresponding author on reasonable request). The study was reported following STROCSS 2021 guidelines [9]. The observational study was conducted on Archival Sections. The minimum number of subjects to be included was obtained according to Asano Y. et al. [4].

\[ n = \frac{(p_H q_L + p_L q_H)^2 (Z_{1-\alpha/2} + Z_{1-\beta})^2}{\alpha^2 (p_H - p_L)^2} \]

Where:

- \(p_H\): Is the frequency of High TIL in pathological complete response HRBC (17.4%)
- \(q_H\): Is the frequency of High TIL in non-pathological complete response HRBC (82.6%)
- \(p_L\): Is the frequency of Low TIL in pathological complete response HRBC (29.8%)
- \(q_L\): Is the frequency of Low TIL in non-pathological complete response HRBC (70.2%)

Thereby, 180 patients who completed neoadjuvant chemotherapy were included for this study whose post-surgically resected tumour specimens were sectioned and stained using routine Haematoxylin and Eosin techniques.

FNAC/Core Needle Biopsy confirmed breast cancer patients who had undergone neoadjuvant chemotherapy followed by surgical resection of the tumour were included while patients having inflammatory or benign lesions of the breast were excluded.

2.1. Procedure

Pathological complete response was defined as there being no invasive and no in situ residuals in breast and nodes according to Gunter von Minckwitz et al. [10]. Pathological complete response for each subtype was determined by the means of the above definition.

The evaluation and analysis of the Intratumoural and Intrastromal Infiltrating Lymphocytes were done according to the guidelines put forward by Salgado et al. [11].

The method used for evaluation of Stromal Tumour infiltrating Lymphocytes:

1. The stromal area for TIL evaluation was identified. TIlTs immediately adjacent to the tumour border were included and TILs closely related to the tumour cells, invasive margin, and the residual tumour bed were excluded. Areas of crush artefacts, necrosis, inflammation, and hyalination were not considered.
2. Inflammatory infiltrate was determined. Only mononuclear cells - lymphocytes and plasma cells were included. Polymorphonuclear leukocytes were excluded.
3. The percentage of stromal TILs was assessed. It was measured semiquantitatively as a continuous variable. The average TIL from the different microscopic fields was taken and expressed in percentage. Stromal TIL % was the area occupied by mononuclear inflammatory cells over the total stromal area.
4. Depending on the percentage, the evaluation was done as follows:
   - High grade TIL: 50–90% stromal TILs.
   - Low Grade TIL: 0–10% stromal TILs.

The method used for evaluation of Intratumoural infiltrating Lymphocytes:

1. The tumour area for TIL evaluation was identified. TILs closely related to the tumour cells were included and TILs closely related to the invasive margin were excluded. Areas of crush artefacts, necrosis, inflammation, and hyalination were not considered.
2. Inflammatory infiltrate was determined. Only mononuclear cells - lymphocytes and plasma cells will be included. Polymorphonuclear leukocytes were excluded.
3. The percentage of intratumoral TILs was assessed. It was measured semiquantitatively as a continuous variable. The average TIL from the different microscopic fields was taken and expressed in percentage. Intratumoral TIL % was the area occupied by mononuclear inflammatory cells over the total tumour area.
4. Depending on the percentage, the evaluation was done as follows:
   - High grade TIL: 50–90% tumour TILs.
   - Low Grade TIL: 0–10% tumour TILs.

   (Intermediate grade TIL: 20–40% stromal TILs. For the intermediate group different areas were evaluated at higher magnification.)

2.2. Statistical analysis

Data was collected manually on a pro forma and entered into a secure spreadsheet and categorised according to subtype and features. Thereafter, univariate analyses were conducted by chi-squared tests on a 2x2 contingency table. Data was analysed on InStat software where \(p\) values < 0.05 were considered significant with a 95% confidence interval. Multivariate analyses were carried out on SPSS software by performing logistical regression.

2.3. Observations and results

Out of the 180 breast cancer patients taken in this study 179 patients were female and 1 patient was male. 55 patients (i.e. 30.56%) out of 180 displayed pathological complete resolution (pCR). Further, the intratumoral and intrastromal TIL count was calculated for all 180 patients.

Intratumoral Group: There were a total of 180 patients out of which 66 patients (i.e. 36.67%) were categorised into the high intratumoral TIL group (since the intratumoral count was between 50% and 90%) and...
114 patients (i.e. 63.33%) were categorised into the low intratumoral TIL group (since the intratumoral count was between 0% and 10%).

Intrastromal group: There were a total of 180 patients out of which 105 patients (i.e. 58.33%) were categorised into the high intrastromal TIL group (since the intrastromal count was found to be between 50% and 90%) and 75 patients (i.e. 41.67%) were categorised into the low intrastromal TIL group (since the intrastromal count was found to be between 0% and 10%).

The IT and IS TIL scores were then compared to the clinicopathological features and the following was found:

On comparison of Intratumoural TIL with the clinicopathological features, the following was established at a 95% Confidence Interval as shown in Table 1 - There was a very strong association of age (p = 0.0045) with the Intratumoural TIL count followed by a significant association of Menopause (p = 0.0156). An extremely significant linkage was found with the HRBC subtype (p = 0.0008) and a notable relation with the TNBC (p = 0.0121) subtype. A significant association was found between the pathological complete response (p = 0.0335) and the Intratumoural TIL count. No correlation was found between any other clinicopathological feature or breast cancer subtype.

On comparison of Intrastromal TIL with the clinicopathological features, it was recognised that at the 95% Confidence Interval as shown in Table 2 - There was a significant association of age (p = 0.0147) with the intrastromal TIL count followed by a highly significant association found with the HRBC subtype (p < 0.0001), TNBC subtype (p < 0.0001) and HER2BC subtype (p = 0.0025). A noteworthy association was found between the pathological complete response (p < 0.0001) and the Intrastromal TIL count. No correlation was found between any other clinicopathological feature or breast cancer subtype.

As depicted in Fig. 1, it is evident that the Intratumoural TIL count has a higher concentration and is skewed upwards in pCR patients with a high TIL score while the plot is observed to be evenly distributed in the Non-pCR patients. The median is also found to be higher in pCR patients compared to Non-pCR patients and the interquartile range is much larger in Non-pCR.

Through Fig. 2, it is clear that the Intratumoural TIL count is evenly distributed in both the pCR and Non-pCR patients. The median is slightly higher in pCR patients compared to Non-pCR with both in the intermediate and low TIL score range and the interquartile ranges for pCR and Non-pCR were similar.

On comparison between the Intratumoural and Intrastromal TIL plots, it is apparent that the pCR patients have a higher median and smaller interquartile range focused in the high Intratumoural TIL score area as opposed to the Intratumoural TILs which have a lower median and larger interquartile range distributed in the lower score range area of the graph. This therefore could be the reason for the stronger association of high Intratumoural TIL scores and pCR in comparison to the high Intrastromal TIL score and pCR rate.

For Multivariate Analysis, One male patient was excluded. Therefore the sample size considered was n = 179.

2.4. In the intratumoral group

Logistic regression was performed to predict Intratumoural TIL by introducing 7 variables as independent parameters as found in Tables 3-5. Backward regression revealed that four (age at operation, pathological complete response, lymph node involvement and menopause) of those parameters highly contributed to predicting 68.2% of the total cases correctly with sensitivity of 93.0% and specificity of 24.6% when the cut off probability was 0.5.

2.5. In the intrastromal group

Logistic regression was performed to predict Intrastromal TIL by introducing 7 variables as independent parameters as depicted in Tables 6-8. Backward regression revealed that four (age at operation, pathological complete response, lymph node involvement and tumour emboli) of those parameters highly contributed to predicting 71.5% of the total cases correctly with sensitivity of 71.6% and specificity of 71.4% when the cut off probability was 0.5.

3. Discussion

TILs have been found to play an important role in the microenvironment of the tumour and can help predict the behaviour of the tumour [5]. Not only restricting the prediction to the tumour environment, but the neoadjuvant chemotherapy employed also contributes to improving the surgical outcome to achieve pathological complete resolution.

### Table 1

Univariate analysis of Intratumoural TIL with Clinicopathological features in patients with Breast Cancer.

| Sr. No. | Parameter                      | Intratumoural TIL | chi square | p - value | Odds Ratio | Confidence Interval |
|---------|--------------------------------|-------------------|------------|-----------|------------|---------------------|
| 1       | Age at Operation               |                   |            |           |            |                     |
|         | ≤52 years                      | 47 (71.21%)       | 55 (48.25%)| 8.068     | 0.0045     | 2.654              |
|         | >52 years                      | 19 (28.79%)       | 59 (51.75%)|           |            | 1.389-5.070        |
| 2       | Menopause (n = 179; One Patient is male) |               |            |           |            |                     |
|         | Premenopausal                  | 37 (56.06%)       | 40 (35.40%)| 6.438     | 0.0112     | 2.328              |
|         | Postmenopausal                 | 29 (43.94%)       | 73 (64.60%)|           |            | 1.252-4.331        |
| 3       | Tumour Size                    |                   |            |           |            |                     |
|         | ≤4.5 cm                        | 42 (63.64%)       | 74 (63.91%)| 0.0001160 | 0.9914     | 0.9459             |
|         | >4.5 cm                        | 24 (36.36%)       | 40 (35.09%)|           |            | 0.5027-1.780       |
| 4       | Lymph Node Status              |                   |            |           |            |                     |
|         | Not Involved                   | 29 (43.94%)       | 57 (50.00%)| 0.3964    | 0.5289     | 0.7838             |
|         | Involved                       | 37 (56.06%)       | 57 (50.00%)|           |            | 0.4262-1.442       |
| 5       | Periordial Spill               |                   |            |           |            |                     |
|         | Negative                       | 54 (81.82%)       | 90 (78.95%)| 0.07327   | 0.7866     | 1.2000             |
|         | Positive                       | 12 (18.18%)       | 24 (21.05%)|           |            | 0.5551-2.594       |
| 6       | Tumour Emboli                  |                   |            |           |            |                     |
|         | Negative                       | 44 (66.67%)       | 74 (64.91%)| 0.00576   | 0.9395     | 1.081              |
|         | Positive                       | 22 (33.33%)       | 40 (35.09%)|           |            | 0.5698-2.051       |
| 7       | Subtype HRBC                   |                   |            |           |            |                     |
|         | HRBC                           | 17 (25.76%)       | 60 (52.63%)| 11.259    | 0.0008     | 0.3122             |
|         | Non-HRBC                       | 49 (74.24%)       | 54 (47.36%)|           |            | 0.1609-0.6060      |
| 8       | Subtype TNBC                   |                   |            |           |            |                     |
|         | TNBC                           | 36 (54.55%)       | 39 (34.21%)| 6.299     | 0.0121     | 2.308              |
|         | Non-TNBC                       | 30 (45.45%)       | 75 (65.79%)|           |            | 1.241-4.291        |
| 9       | Subtype HER2BC                 |                   |            |           |            |                     |
|         | HER2BC                         | 10 (15.15%)       | 15 (13.16%)| 0.02223   | 0.8815     | 1.179              |
|         | Non-HER2BC                     | 56 (84.85%)       | 99 (86.84%)|           |            | 0.4963-2.799       |
| 10      | Subtype TPBC                   |                   |            |           |            |                     |
|         | TPBC                           | 02 (03.03%)       | 00 (00.00%)| 1.280     | 0.2579     | 8.876              |
|         | Non-TPBC                       | 64 (96.97%)       | 114 (100.00%)|          |            | 0.4193-187.7       |
| 11      | Subtype Luminal B              |                   |            |           |            |                     |
|         | Luminal B                      | 01 (01.52%)       | 00 (00.00%)| 0.07698   | 0.7814     | 5.244              |
|         | Non-Luminal B                  | 65 (98.48%)       | 114 (100.00%)|          |            | 0.2104-130.07      |
| 12      | Pathological Complete Response  |                   |            |           |            |                     |
|         | pCR                            | 27 (40.91%)       | 28 (24.56%)| 4.522     | 0.0335     | 2.126              |
|         | Non-pCR                        | 39 (59.09%)       | 86 (75.44%)|           |            | 1.110-4.074        |
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Multiple studies have been carried out on Intrastromal TILs and their significance in measuring the disease-free survival rates and the likelihood of recurrence [3,12,13], while the use of Intratumoural TILs to forecast the response is seldom performed.

Guidelines put forward by Salgado et al. [11] throw light upon recent studies having found stromal TILs to be of a higher calibre and more reproducible when compared to its counterpart. The main reason for this is that Intratumoral TILs are scarce and the process of delineating them from tumour cells on H&E-stained slides is rather tedious and the growth pattern of these lymphocytes could be disturbed by the nest of tumour cells. Therefore, this study was solely focused on comparing and contrasting the above two to come to a consensus on their relative effectiveness.

On comparing TIL with the Clinicopathological features, the Intratumoral TILs proved to be highly significant with age ($p = 0.0045$) where 59 out of 78 members with age greater than 52 years were found to garner a Low IT TIL score. This can be pinned down to the fact that as age increases there is a steady decline in the immunogenicity of an individual [14] and therefore the lymphocytic response is reduced in the microenvironment of the tumour and this low response can be indicative of a bad prognosis.

| Sr. No. | Parameter                                      | Stromal TIL | chi - square | p - value | Odds Ratio | Confidence Interval |
|---------|-----------------------------------------------|-------------|--------------|-----------|------------|---------------------|
| 1       | Age at Operation                              | ≤52 years   | 68 (64.76%)  | 34 (45.33%)| 5.957      | 0.0147              | 2.216 | 1.209–4.062        |
|         | >52 years                                     | 37 (35.24%) | 41 (54.67%)  |           |            |                     |       |                   |
| 2       | Menopause (n = 179; One Patient is male)      | Premenopausal| 50 (47.62%)  | 27 (36.49%)| 1.764      | 0.1841              | 1.582 | 0.8607–2.910       |
|         | Postmenopausal                                | 55 (52.38%) | 47 (63.51%)  |           |            |                     |       |                   |
| 3       | Tumour Size                                   | ≤4.5 cm     | 65 (61.90%)  | 51 (68.00%)| 0.4683     | 0.4938              | 0.7647 | 0.4093–1.429       |
|         | >4.5 cm                                       | 40 (38.10%) | 24 (32.00%)  |           |            |                     |       |                   |
| 4       | Lymph Node Status                             | Not Involved | 48 (45.71%)  | 38 (50.67%)| 0.2545     | 0.6139              | 0.8199 | 0.4528–1.485       |
|         | Involved                                      | 57 (54.29%) | 37 (49.33%)  |           |            |                     |       |                   |
| 5       | Periodal Spill                                | Negative    | 83 (79.05%)  | 61 (81.33%)| 0.03571    | 0.8501              | 0.8659 | 0.4101–1.828       |
|         | Positive                                      | 22 (20.95%) | 14 (18.67%)  |           |            |                     |       |                   |
| 6       | Tumour Emboli                                 | Negative    | 71 (67.62%)  | 47 (62.67%)| 0.2812     | 0.5959              | 1.244  | 0.6683–2.316       |
|         | Positive                                      | 34 (32.38%) | 28 (37.33%)  |           |            |                     |       |                   |
| 7       | Subtype HRBC                                   | HRBC        | 23 (21.90%)  | 54 (72.00%)| 42.829     | <0.0001             | 0.1091 | 0.05503–0.2162     |
|         | Non-HRBC                                      | 82 (78.10%) | 21 (28%)     |           |            |                     |       |                   |
| 8       | Subtype TNBC                                   | TNBC        | 57 (54.29%)  | 18 (24.00%)| 15.288     | <0.0001             | 3.760  | 1.954–7.236        |
|         | Non-TNBC                                      | 48 (45.71%) | 57 (76.00%)  |           |            |                     |       |                   |
| 9       | Subtype HER2BC                                 | HER2BC      | 22 (20.95%)  | 03 (04.00%)| 9.143      | 0.0025              | 6.361  | 1.828–22.142       |
|         | Non-HER2BC                                    | 83 (79.05%) | 72 (96.00%)  |           |            |                     |       |                   |
| 10      | Subtype TPBC                                   | TPBC        | 02 (01.90%)  | 00 (00.00%)| 0.2311     | 0.6307              | 3.647  | 0.1725–77.136      |
|         | Non-TPBC                                      | 103 (98.10%)| 75 (100.00%) |           |            |                     |       |                   |
| 11      | Subtype Luminal B                             | Luminal B   | 01 (00.95%)  | 00 (00.00%)| 0.7183     | 0.3967              | 2.167  | 0.08703–53.981     |
|         | Non-Luminal B                                 | 104 (99.05%)| 75 (100.00%) |           |            |                     |       |                   |
| 12      | Pathological Complete Response                | pCR         | 48 (45.71%)  | 7 (09.33%)  | 25.602     | <0.0001             | 8.180  | 3.435–19.483       |
|         | Non-pCR                                       | 57 (54.29%) | 68 (90.67%)  |           |            |                     |       |                   |

Fig. 1. Intrastromal TIL score association with pCR.

Fig. 2. Intrastromal TIL score association with pCR.
Logistic Regression model to predict High and Low IT TIL.

### Table 5

| Sr. No. | Parameter               | B     | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I. for Exp(B) |
|---------|-------------------------|-------|------|------|----|------|--------|---------------------|
| 1       | Age at Operation        | 0.709 | 0.403| 3.009| 1  | 0.083| 2.013  | 0.913 - 4.438       |
| 2       | Pathological Complete Response | 0.790 | 0.355| 4.950| 1  | 0.026| 2.204  | 1.099 - 4.420       |
| 3       | Lymph Node Involved     | -0.413| 0.239| 1.403| 1  | 0.236| 0.663  | 0.341 - 1.290       |
| 4       | Menopause               | 0.465 | 0.393| 1.403| 1  | 0.236| 1.592  | 0.737 - 3.437       |
| Constant|                         | -0.259| 0.475| 0.296| 1  | 0.586| 0.772  |                     |

Provenance and peer review

Not commissioned, externally peer-reviewed.

Ethical approval

The study was approved by the Institutional Ethics Committee of Krishna Institute of Medical Sciences, Karad (Reference number - KIMSDU/IEC/02/2019).

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Table 8
Logistic Regression model to predict High and Low IS TIL.

| Sr. No. | Parameter                        | B       | S.E.    | Wald  | dF | Sig.  | Exp(B) | 95% C.I. for Exp(B) |
|---------|----------------------------------|---------|---------|-------|----|-------|--------|---------------------|
| 1       | Age at Operation                 | 0.946   | 0.345   | 7.495 | 1  | 0.006 | 2.575  | 1.308 - 5.067       |
| 2       | Pathological Complete Response   | 2.260   | 0.467   | 23.453| 1  | 0.000 | 9.585  | 3.840 - 23.924      |
| 3       | Tumour Emboli                    | 0.207   | 0.364   | 0.323 | 1  | 0.570 | 1.230  | 0.603 - 2.509       |
| 4       | Lymph Node Involvement           | -0.575  | 0.358   | 2.580 | 1  | 0.108 | 0.563  | 0.279 - 1.135       |
| Constant|                                  | -2.251  | 0.476   | 22.317| 1  | 0.000 | 0.105  |                     |

Author contribution

RR was the major contributor in writing the entire article and analysing the results. SRK was the guide and imparted knowledge related to how to analyse the TILs and was the overseer of the analysis of the project. SJB was the Onco-surgeon in charge whose post-surgically resected breast tumour specimens were employed for the project. SVK helped in multivariate analysis. NJP facilitated the IHC marking for the project. AG was the oncologist who oversaw the neoadjuvant chemotherapy for the patients. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Guarantor

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Trial registry number

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors have declared no conflicts of interest.

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Abbreviations

TNBC  Triple-negative breast cancer

TPBC  Triple positive breast cancer
HER2  human epidermal growth factor receptor 2
HRBC  Hormone-receptor breast cancer
TILs  Tumour infiltrating lymphocytes
NAC  Neoadjuvant chemotherapy
pCR  Pathological complete response

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amsu.2022.104308.

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