Chemotherapy is the standard first-line treatment for most metastatic triple-negative breast cancer (mTNBC) patients. However, treatment outcomes are poor. Immune checkpoint inhibitors (ICIs), anti–programmed death 1 (PD-1), and anti–programmed death ligand 1 (PD-L1) have demonstrated antitumor activity across various indications. In particular, pembrolizumab (Keytruda®) is approved for high-risk, early-stage TNBC in combination with chemotherapy in the neoadjuvant setting and in the recurrent unresectable or metastatic setting.

Previously, we reported that delivering ultra-high concentrations of nitric oxide (UNO) to mouse colon tumors (CT26) stimulated innate and adaptive immune responses leading to the rejection of secondary-induced tumors. Moreover, adding a murine anti-PD-1 antibody (anti-PD-1) to UNO treatment resulted in primary tumor regression in 53% of the mice, the rejection of distant tumors in all mice, and prolonged survival compared to control and anti-PD-1 arms.

In this study, we utilize the aggressive murine breast cancer model (4T1) to investigate single and repeat dosing of UNO as monotherapy and in combination with checkpoint inhibitors.

**Methods**

**Method A**

Day 0: 50 ppm NO 0.2 LPM 2 minutes intranasal

Day 5: 50 ppm NO 0.2 LPM 10 minutes intranasal (optional)

Follow-up:
- Tumor progression
- Tumor-free mice
- Survival

At day 15 post the 1st NO treatment the primary tumors of ~50% of the mice were resected.

**Method B**

Day 0: 100K ppm NO 0.2 LPM 2 minutes intranasal

Day 3: 50 ppm NO 0.2 LPM 10 minutes intranasal

Follow-up:
- Tumor progression
- Tumor-free mice
- Survival

All in vivo experimental procedures were carried out in accordance with the protocol approved by the Ethics Committee on the Use and Care of Animals of the IMOH (Israel).

**Results**

**Primary tumor volume following 50K ppm NO gNO dosing combined with anti-mPD-1 (method A)**

![Graph 1](attachment:image1.png)

**Table 1**: Average tumor volume (A) gNO+anti-mPD-1-treated tumors was significantly smaller compared to untreated and treated with only anti-mPD-1 tumors. N=10 mice per arm. (B) gNO+untreated tumors with or without anti-mPD-1 was significantly smaller compared to untreated or treated with only anti-mPD-1 tumors. N=15-17 mice per arm. Statistical analysis for (A) and (B) Mixed model for repeated measures (MMMR) with Kenward-Roger’s method ***P<0.001 ***P<0.0001

**Survival following 50K ppm NO gNO repeat dosing in combination with anti-mPD-1 (method A)**

![Graph 2](attachment:image2.png)

**Conclusions**

Single and repeat dosing of UNO as monotherapy improved outcomes compared to checkpoint inhibitor monotherapy. Average tumor volume was 13.2% smaller after single gNO treatment and 21.0%/12.9% smaller with repeat gNO treatment. Repeat dosing of UNO monotherapy significantly prolonged mice survival compared to anti-mCTLA-4 monotherapy. Repeat dosing of UNO in combination with anti-mPD-1 prolonged mice survival compared to anti-mPD-1 monotherapy. These findings suggest that local administration of UNO, either alone or in combination with ICIs, can be a viable treatment option for patients not responding to immune checkpoint inhibitors.