

1 Title: **Effects of physiological deficits in pineal melatonin on Triple Negative Breast**  
2 **Cancer.**

3

4 Short title: **Effects of melatonin on Triple Negative Breast Cancer.**

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26 **Abstract**

27

28 **Background.** Triple negative breast cancer (TNBC) is aggressive and treatment resistant.  
29 Evidence suggests that deficits in melatonin signaling increase TNBC risk: conditions that  
30 suppress melatonin increased incidence, low melatonin receptor expression correlates with  
31 worse prognosis, and high-dose melatonin can inhibit TNBC. Together this suggests that  
32 normalizing pineal melatonin could reduce TNBC incidence and/or mortality. The goal of this  
33 study was to determine whether small physiological deficits in melatonin alone, can increase  
34 risk for TNBC, and how 'normal' melatonin would be protective.

35 **Methods.** The effect of melatonin treatment on 4t1 cells *in vitro* was measured using the MTT  
36 cell viability assay, and gene expression of breast cancer and melatonin signaling markers. The  
37 effect of pineal gland status on 4t1 cell allografts was tested in C3Sn mice (*Mus Musculus*) with  
38 either an intact pineal (control) or surgical removal of the pineal causing a ~50% deficit in  
39 plasma melatonin. Orthotopic tumors were assessed by histopathology and metastasis by strain  
40 specific qPCR against 4t1 cell and host gDNA.

41 **Results.** Melatonin treatment induced significant changes in gene expression, with a significant  
42 reduction in derived PAM50 Risk of Recurrence score (ROR in Not treated =  $65.5 \pm 10.6$  Mean  
43 SEM; Treated with 25pg/ml of melatonin  $20.8 \pm 8.3$ ;  $P = 0.008$ ), suggesting melatonin treatment  
44 would improve prognosis. A ~50% reduction in plasma melatonin increased orthotopic tumors,  
45 but this was non-significant, and had no effect on metastasis from tail vein allograft.

46 **Conclusions.** Physiological deficits in melatonin do alter the oncogenic status of 4t1 tumor cells  
47 but this has only a limited effect on growth and metastasis *in vivo*. Lack of significance in  
48 orthotopic tumor formation may be due to small sample size, and it is possible that any  
49 protective effect of melatonin occurs earlier in tumor development than we have tested.

50

51

## 52 **Introduction**

53

54 Breast cancer is a leading cause of death among women in industrialized countries, with  
55 ~250,000 new cases and ~40,000 deaths each year in the United States [1]. The etiology of  
56 breast cancer is multifactorial and can result in a spectrum of presentations that have divergent  
57 prognoses. Notably, hormone receptor deficient breast cancers are typically aggressive and  
58 treatment resistant, with triple negative breast cancer (TNBC) accounting for 12-17% of cases,  
59 but having a higher proportion of total mortality [2].

60

61 Because TNBC is often treatment resistant and life-threatening, factors that affect incidence  
62 and/or severity can therefore be effective in limiting cancer morbidity and mortality [3]. One  
63 surprising risk factor for breast cancer is exposure to artificial light-at-night (ALAN). ALAN  
64 correlates with increased breast cancer incidence, and night shift work that would expose  
65 individuals to ALAN correlates with a marked increase in estrogen receptor negative breast  
66 cancer [4–7]. Consistent with this correlation, a reduced response to light in blind women has a  
67 protective effect against breast cancer [8,9].

68

69 Light acts on multiple aspects of physiology and behavior and the mediating mechanisms of  
70 these effects may be complex [10]. Current evidence does suggest that the suppression of  
71 pineal melatonin by light is a major contributor to these effects [11]. Pineal melatonin is  
72 suppressed by light at night in a dose dependent manner [12,13]. Additionally, melatonin  
73 receptors are expressed in breast tissue and MT1 melatonin receptor expression correlates with  
74 positive outcomes in breast cancer [14–16]. Finally, high dose exogenous melatonin inhibits  
75 mammary cell division *in vitro*, and mammary tumor growth *in vivo* [17–20].

76

77 Although most research points to an anti-estrogenic protective effect of melatonin, in patients  
78 with TNBC, melatonin receptor expression does correlate with survival, and polymorphisms of  
79 melatonin signaling correlate with incidence [16,21]. Further, an effect of melatonin on TNBC  
80 cells is also supported experimentally. *In vitro*, melatonin has an oncostatic effect on TNBC cell  
81 invasiveness and proliferation [22,23]. *In vivo*, extremely high doses of exogenous melatonin  
82 inhibit TNBC xenograft growth, and have oncostatic effects on tumor microenvironment,  
83 especially immune and angiogenic markers [24,25].

84

85 Based on this evidence, we might hypothesize that maintaining optimal plasma melatonin levels  
86 would reduce the burden of TNBC [11,16]. However, these studies do not show whether  
87 physiological deficits in endogenous melatonin levels, such as would occur with exposure to  
88 ALAN, are sufficient to increase risk for hormone receptor deficient breast cancer. Nor do they  
89 adequately demonstrate which stages in neoplastic development are affected by melatonin:  
90 initiation, promotion, progression and/or metastasis [26].

91

92 The goal of this study was to determine whether physiologically realistic deficits in endogenous  
93 melatonin impact the behavior of a hormone receptor deficient breast cancer, using the mouse  
94 4t1 TNBC cell line [27–29]. We first assessed effects of melatonin *in vitro* on cell viability and  
95 then on expression of genes that give a prognostic prediction and assess potential mechanisms  
96 of melatonin action [30,31]. We then tested the effects of pineal melatonin on 4t1 cells *in vivo*.  
97 Pineal melatonin competent C3Sn mice were sham operated (Intact-control), or had the pineal  
98 gland tip surgically removed (PinealX) to generate a model of ~50% melatonin deficiency  
99 [12,32–34]. First, an orthotopic 4t1 cell allograft was used to assess the effect of pineal  
100 melatonin on viability and formation of solid tumors [35]. Second, a tail-vein 4t1 cell allograft was  
101 used to assess the effect of pineal melatonin on metastasis [36].

102

103 **Materials and methods**

104

105 Cells. 4t1 cells are a mouse mammary tumor cell line with features of TNBC: low *Esr1*, absent  
106 *Pgr*, and normal or low *Erbb2/Her2* [28,29]. Cells were sourced commercially (ATCC, Manassas,  
107 VA), and grown in Roswell Park Memorial Institute medium (RPMI; ATCC, Manassas, VA), with  
108 10% fetal bovine serum (FBS; MidSci, Valley Park, MO) and 1% penicillin/streptomycin  
109 (Pen/Strep; HyClone, Logan, UT). There was a maximum of five passages before experimental  
110 use.

111

112 Animals. Animal care and use was conducted in accordance with U.S. Public Health Service  
113 Policy on Humane Care and Use of Laboratory Animals and approved by the New Mexico Tech  
114 Institutional Animal Care and Use Committee. C3Sn.BLiA-*Pde6b*<sup>+</sup>/DnJ (C3Sn) mice were  
115 selected for use in this study because they produce pineal melatonin, are highly susceptible to  
116 breast cancer, and do not suffer from retinal degeneration like the background C3H/He mouse  
117 [32,33,37,38]. C3Sn were commercially sourced (Jackson Laboratories, Bar Harbor, ME) and  
118 then bred on site. Food and water were provided *ad libitum* throughout the study.

119

120 Experiment 1. Effect of melatonin on 4t1 cells *in vitro*. To test whether melatonin treatment  
121 affects 4t1 cells *in vitro* we designed a custom gene expression assay, then tested how a  
122 physiologically relevant concentration of melatonin affected gene expression.

123

124 Reliable measurements of *in vivo* plasma melatonin in C3H background mice show a circadian  
125 rhythm with low levels during the day of  $\leq 5$  pg/mL and a 6 to 8 hour elevation at night of  $\sim 25$   
126 pg/mL [33]. Our test concentration of no treatment and  $\sim 25$  pg/mL was intended to represent  
127 the maximal physiological range of plasma melatonin concentration.

128

129 Our goal with gene expression assessment was to reliably identify effects of melatonin on tumor  
130 status and to probe mechanisms of melatonin effect. Gene expression is an effective tool in  
131 prognosis prediction for human breast cancer. To more directly relate our data to modern  
132 practice in human cancer assessment, we included mouse homologs of the panel of targets  
133 used on the Prosigna® PAM-50 test [30,31]. Critically, all fifty targets have a homolog in the  
134 mouse. To that panel, we added known and plausible mediators of melatonin effects on  
135 oncogenic status and transcripts associated with tumor initiation, development, and metastasis  
136 to specific tissues (**Supplementary materials S1**).

137

138 We first tested whether melatonin treatment would affect the number of viable cells available for  
139 mRNA extraction. Cells were grown as described above, treated with melatonin, then assayed  
140 in a standard MTT assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
141 (ThermoFisher, Waltham, MA) [39]. MTT is a colorimetric assay designed to test the metabolic  
142 activity of a culture. To prepare these assays, melatonin was dissolved in Dimethyl sulfoxide  
143 (DMSO) at a concentration of 1 mg/ml. The cells were plated at 4,000 cell/well in a 96-well  
144 microtiter plate and treated at concentrations ranging from 200 pg/ml down to ~0.5 pg/ml. The  
145 cells were incubated for 48 h in 200 µl of RPMI media with 1% Pen/Strep and 10% FBS. Twenty  
146 percent v/v of MTT reagent in 1X PBS (5 mg/mL) was added to each well and incubated further  
147 for 2 h. Media was removed and replaced by 100 µl of DMSO. Absorbance at 595 nm was  
148 measured using a Thermomax Molecular Device plate reader. The experiments were performed  
149 in quadruplicate. A 0.1% DMSO was used as a vehicle control and 10 µM phenyl arsine oxide  
150 (PAO) was used as a positive killing control. Statistical analysis was limited to the dose  
151 response data for melatonin treated groups with Not Treated control assumed to be 0.0 pg/ml;  
152 assessment was by Welch's ANOVA in Prism (GraphPad, San Diego, CA).

153

154 Then to determine how melatonin affects gene expression in 4t1 cells, we applied melatonin to  
155 cells *in vitro*, isolated RNA, and assessed expression of a custom panel of target genes. Cells  
156 were grown to confluence. Melatonin was added to the media in flasks at 25.0 pg/mL (N = 6  
157 flasks) with no treatment controls (N = 6). After 4 hours, a sample of media was frozen for  
158 melatonin content quantification, then cells were dissociated using trypsin, stabilized in  
159 RNAprotect cell reagent (Qiagen, Germantown, MD) and frozen for later mRNA isolation.

160

161 For control tissue, 16-week old virgin female C3Sn mice were euthanized by anesthetic  
162 overdose (200 mg/kg Ketamine, 20 mg/kg Xylazine) at the circadian phase of peak melatonin  
163 production (6 to 9 hours after lights off) under far red light (720 nm). Blood was drawn and  
164 serum isolated for melatonin quantification, protecting melatonin content by minimizing  
165 exposure of the sample to light, and by storing at -80 °C after serum separation. The thoracic  
166 and abdominal lobes of the right breast were pooled and snap frozen for RNA isolation.

167

168 RNA was isolated using an RNAeasy Protect Cell Mini Kit (Qiagen) for *in vitro* cell samples, and  
169 an RNAeasy Mini Kit (Qiagen) for C3Sn mouse normal mammary tissue. All samples were  
170 reverse transcribed using an RT2 First Strand kit (Qiagen). Our panel of 88 target genes was  
171 prepared on a 96-well plate format with housekeeping genes and controls (RT2 PCR array,  
172 Qiagen). Expression was quantified on an ABI7500 real-time PCR machine (Applied  
173 Biosystems, Beverly, MA) using RT2 SYBR Green ROX qPCR mastermix (Qiagen). One non-  
174 treated sample was excluded for failing quality controls.

175

176 Gene expression data for the PAM50 panel genes was used to generate a breast cancer Risk of  
177 Recurrence (ROR) prognosis score [30]. The “*rors*” algorithm in geneFu version 2.23.0  
178 01/31/2020 was applied [40]. The derived score is on a scale of 0–100: a *low* score (<40)  
179 indicates a 10-year ROR less than 10%, an *intermediate* score (40 to 60) indicates a 10-year

180 ROR of 10–20%, and a *high* score (>60) indicates a 10-year ROR of >20%. Comparison of  
181 ROR scores in not-treated controls and cells treated with 25pg/ml of melatonin was by unpaired  
182 parametric 2-tailed t-test in Prism (GraphPad).

183

184 Then, data was normalized by CT of Symplakin (Sympk), which had the most consistent  
185 expression across samples (Mean CT 25.5 SD 0.7), and has been identified as an optimal  
186 housekeeping gene for breast cancer gene expression [41]. Percent change was calculated  
187 relative to mean expression in ‘not treated’ controls using a power of 2. Statistical significance of  
188 change in gene expression was determined by a two-sample equal variance two-way t-test.

189

190 *Experiment 2. Orthotopic allograft of 4t1 cells in C3Sn mice.* A preliminary study of orthotopic  
191 allograft was conducted to demonstrate that BALB/c 4t1 cells could form viable tumor cell  
192 colonies in our melatonin competent C3Sn strain host [12]. This small sample size experiment  
193 also provided a preliminary assessment of whether pineal melatonin affected the formation of  
194 primary tumors and metastasis from those primary tumors.

195

196 *Pineal surgery.* Between 7 -10 weeks of age, C3Sn mice were surgically pinealectomized  
197 (PinealX) or were sham operated leaving the pineal gland intact (Intact-control) [34]. To prevent  
198 selection and treatment bias, we evenly and randomly assigned animals in a litter to pinealX or  
199 sham surgery, then housed those animals together irrespective of their pineal status and  
200 masked experimenters to pineal status. Analgesia and anesthesia protocols included 100 mg/kg  
201 Ketamine; 10 mg/kg Xylazine; 2.5 mg/kg Acepromazine; Buprenorphine HCL at 0.1 mg/kg; and  
202 post-surgical access to an oral tablet containing 2mg of the NSAID, carprofen (Bio-Serv,  
203 Flemington, NJ). After surgical site preparation, mice were placed on a heat mat and mounted  
204 into a stereotaxic frame (Kent Scientific, Torrington, CT). A 1.5 cm midline vertical incision and  
205 blunt dissection was used to expose the scalp. A section of skull, centered on the intersection of



206 the sagittal and occipital fissures of the calvarium was removed using a hand drill and 2.3 mm $\varnothing$   
207 trephine drill bit (Fine Science Tools, Foster City, CA). This approach removes the tip of the  
208 pineal gland with the cap of skull that is removed but should leave part of the pineal intact.  
209 Removal of the pineal gland was confirmed using a SZ-745 dissecting microscope (McBain,  
210 Westlake Village, CA), and the incision closed with non-absorbable 5-0 sutures (Ethicon, San  
211 Angelo, TX).

212

213 *Allograft.* After 2 weeks of surgical recovery, mice had an allograft of 4t1 cells to the fat pad of  
214 the left 4th mammary gland using a previously described approach [42]. Cells and mice were  
215 prepared in parallel to minimize time between cell preparation and allograft (N for each group =  
216 8, 4 male and 4 virgin female). 4t1 cells were dissociated with trypsin in RPMI media, then  
217 suspended in RPMI at a concentration of  $4 \times 10^7$  cells/ml. Within 60 minutes, the 4t1 cell  
218 preparation was injected orthotopically to the 4th left mammary fat pad of mice. Mice were  
219 lightly anesthetized with 50 mg/kg Ketamine; 5 mg/kg Xylazine; 1.25 mg/kg acepromazine. The  
220 surgical site was prepared and a small incision made approximately 1.5 mm above the 4<sup>th</sup>  
221 nipple. 50  $\mu$ l of 4T1 cells totaling  $2 \times 10^6$  cells was then injected into the fat pad using a gas-tight  
222 micro-syringe (Hamilton, Reno, NV). Because the incision was small and shallow, no suture was  
223 used to close it.

224

225 *Tumor development and assessment.* After recovery from allograft anesthesia, mice were  
226 housed in environment control cabinets under a daily cycle of 12-hours light ( $20 \mu\text{Wcm}^{-2}$ ), and  
227 12-hours dark. At 16 weeks post cell injection, the mice were euthanized by anesthetic  
228 overdose followed by cervical dislocation. Hair was removed from the abdomen of the mouse  
229 using depilatory cream (Nair, Ewing, NJ). Left and right mammary chains and the lungs of each  
230 animal were collected and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO)  
231 for 6 hours, then transferred to 1% phosphate buffered saline (PBS) and stored at 4 °C. Tissue

232 was embedded in Tissue Freezing Medium (General Data, Cincinnati, OH) sectioned at 10-16  
233  $\mu\text{m}$  on a Shandon FE Cryostat (Thermo Fisher scientific, Waltham, MA). Slides were stained  
234 with Hematoxylin and Eosin (all reagents from VWR, Radnor, PA). Number of sections was  
235 recorded to allow size calculation. Images were recorded on a Leica ICC50 HD and processed  
236 with Microsoft Image Composite Editor.

237  
238 Larger tumors were defined as a cluster of dense cells with a defined border demarcating it from  
239 surrounding normal adipose tissue. We assumed a solid tumor was a single mass unless there  
240 were three sequential sections with no tumor between two masses. After assessments, animal  
241 pineal status was unmasked and total tumor load compared between experimental PinealX and  
242 Intact-control groups using Welch's *t*-test.

243  
244 Single epithelial cells in the fat mass of the mammary pad might be invasive 4t1 cells. To  
245 assess differences in this potential marker of local invasiveness, we quantified the extent of  
246 triple negative tumor presence in the left breast away from the introduction site at the abdominal  
247 or 4<sup>th</sup> mammary gland using a percentage-based severity grade. Invasiveness was defined as a  
248 small dense cluster of cells that displayed branching into surrounding tissue. The invasive  
249 burden was measured by counting invasive clusters of cells in a section (0-7, 7 being the most  
250 severe). Because this scoring approach is subjective, scorers were masked to animal ID and  
251 analysis by three separate scorers was averaged. After assessments, animal pineal status was  
252 unmasked and total tumor load compared between experimental PinealX and Intact-control  
253 groups using Welch's *t*-test.

254  
255 *Experiment 3. Effects of pineal melatonin on metastasis of 4t1 cells from tail-vein allograft.* It has  
256 been demonstrated that tail-vein injection allograft of 4t1 cells establishes metastases [36]. We  
257 therefore adopted this model to determine whether melatonin affects the extent and sites of

258 metastases of 4t1 TNBC cells. We focused on common metastatic sites for breast cancer:  
259 breast, liver, and lymph nodes. However, there was potential for low levels of metastatic burden  
260 in these tissues, so we developed a gDNA based quantification host and donor content in  
261 tissues using strain selective qPCR (**see supplementary material S2**). This is similar to the  
262 approach used in quantifying xenografts by others [43–45].

263  
264 *Pineal surgery.* Mice were Intact-control (n=10, 6 female and 4 male) or PinealX (n=13, 6 female  
265 and 7 male) operated between 7 and 10 weeks old as described for experiment 3.

266  
267 *Tail-vein injection.* After 2 weeks of surgical recovery, mice had an allograft of 4t1 cells to the  
268 tail-vein using a previously described approach [27,36]. Cells and mice were prepared in parallel  
269 to minimize time between cell preparation and allograft. 4t1 cells were dissociated with trypsin in  
270 RPMI media, then suspended in RPMI at a concentration of  $5 \times 10^7$  cells/ml. Within 60 minutes,  
271 0.1 ml (500,000 cells) were injected into the tail vein of mice lightly anesthetized with 50 mg/kg  
272 Ketamine; 5 mg/kg Xylazine; 1.25 mg/kg acepromazine. After cell injection, mice were housed  
273 in a light tight environment control cabinet with a 12-hour light, 12-hour dark daily cycle.

274  
275 *Tissue collection.* At 14 days post allograft, mice were euthanized by anesthetic overdose (200  
276 mg/kg Ketamine, 20 mg/kg Xylazine) at the circadian phase of peak melatonin production (6 to  
277 9 hours after lights off) under far red light (720 nm). Blood was drawn and serum isolated for  
278 melatonin quantification, protecting melatonin content by minimizing exposure of the sample to  
279 light, and by storing at  $-80^\circ\text{C}$  after serum separation. Tissue samples of common metastatic  
280 sites were then collected into dry tubes for snap-freezing. Each lobe of the lung was collected  
281 separately: right cranial, middle, caudal, accessory and left lung. Both inguinal lymph nodes  
282 were pooled. The thoracic and abdominal sections of the right breast were pooled. Liver  
283 collection was limited to a ventral biopsy.

284

285 *Melatonin status confirmation.* A direct melatonin immunoassay was used to measure melatonin  
286 concentration in serum at the time cells were harvested (MEL31-K01 direct melatonin  
287 serum/plasma EIA kit, Eagle Biosciences, Nashua, NH). Samples were tested according to  
288 manufacturer guidelines and absorbance at 450nm measured on an Infinite MPlex 96-well plate  
289 reader (Tecan, Baldwin Park, CA). A standard curve was plotted using a 4-parameter sigmoid  
290 function, and concentrations interpolated from the curve, in Prism (GraphPad).

291

292 *DNA isolation.* DNA was isolated using a DNeasy Blood and Tissue kit (Qiagen). After adding a  
293 lysis buffer, tissue was mechanically disrupted using a Tissuerruptor II with disposable probes  
294 (Qiagen). Further lysis was promoted with Proteinase K step for 2 hours before finishing DNA  
295 isolation. The exception to this protocol was for bone, where the Proteinase K step was 40  
296 hours, and then mechanical disruption was completed with a Tissuerruptor II. Quality of DNA  
297 was tested by 260/280nm or 260/230nm ratios on a Nanodrop spectrophotometer (Thermo  
298 Fisher). Quantity of DNA was measured by the 260nm adjusted absorbance.

299

300 *gDNA quantification.* Isolated gDNA was amplified in triplicate with strain-selective BALB/c and  
301 C3H primers (IDT, Coralville, IA) and a qPCR and Go SYBR® Hi-ROX Kit (MP Biomedicals,  
302 Irvine, CA) on an ABI7500 real-time PCR machine (Applied Biosystems). Threshold cycle ( $C_T$ )  
303 values were converted to percent of total gDNA using a power of 2. After assessment, animal  
304 pineal status was unmasked and total tumor load compared between Intact-control and PinealX  
305 groups using a two-tailed equal variance *t*-test.

306

307

308 **Results**

309

310 Experiment 1. Effect of melatonin on 4t1 cells *in vitro*. There were viable 4t1 cells under all  
311 concentrations of melatonin treatment, so we were able to select a concentration of  
312 melatonin for testing gene expression that was a maximal physiological concentration  
313 (**Figure 1**). Melatonin had no identifiable effects on the gross morphology of 4t1 cells. In  
314 addition, there was no effect on proliferation/survival of 4t1 cells in an MTT assay (Welch's  
315 ANOVA of melatonin treatment dose response curve  $P = 0.29$ ;  $W = 1.4$ ,  $DFn = 9.0$ ,  $DFd = 11.3$ ).  
316 Comparison of no treatment control confirmed that there was no difference to DMSO vehicle  
317 control (Mann Whitney test  $P = 0.20$ ), and that cells were effectively killed by PAO positive  
318 control.

319

320 **Figure 1. Effects of melatonin on 4t1 cell morphology and viability. (A)** An example image  
321 of 4t1 cells with melatonin treatment at a concentration much higher than physiological levels.  
322 **(B)** In the MTT assay, viable cells breakdown of tetrazolium dye to formazan, which has  
323 absorbance at 595nm, so absorbance = metabolic activity = viable cells. Absorbance for 4t1  
324 cells is shown with melatonin treatment at a range of concentrations. Melatonin concentration  
325 gradient is implied by shading of bars on the graph. PAO positive killing control and DMSO  
326 vehicle control are also shown. Data are expressed as mean  $\pm$  SEM.

327

328 Gene expression confirmed the TNBC status of 4t1 cells, with significantly reduced expression  
329 of *Pgr* (<0.01% of normal breast expression,  $P < 0.00001$ ), *Esr1* (1.2% of normal breast,  $P <$   
330  $0.00001$ ), and *Erbb2/Her2* (10.4% of normal breast,  $P < 0.0001$ ) (**Figure 2A, Table S1**). These  
331 data also identified significantly lower expression of melatonin receptor *Mtnr1a* (3.0% of normal  
332 breast,  $P < 0.001$ ), and the melatonin receptor regulated transcription factor *Rora* (0.05% of  
333 normal breast,  $P < 0.00001$ ).

334

335 **Figure 2. Gene expression of 4t1 cells *in vitro*.** Expression of selected genes was determined  
336 by qPCR for 4t1 cells grown in cell culture media. **(A)** *Esr1*, *Pgr* and *Erbb2/Her2* are the  
337 hormone receptors used to identify breast cancer as triple negative. Gene expression is shown  
338 as percentage of the expression in normal mammary tissue from C3Sn mice, after normalizing  
339 samples to a housekeeping control *Symplekin*, with bars showing Mean and SEM. **(B)**  
340 Expression levels of the PAM50 panel of genes allowed a Risk-Of-Recurrence score (ROR) to  
341 be calculated for experimental replicates of 4t1 cells grown in untreated cell culture media (NT)  
342 and in media treated with 25pg/ml of melatonin for 4-hours, which approximates peak plasma  
343 melatonin in mice. **(C)** Melatonin treatment significantly changed gene expression of multiple  
344 targets in our assay. Shading shows genes that are part of the PAM50 panel, non-shaded  
345 genes were our melatonin mechanism focused additions to the panel. Mean and SEM percent  
346 change was calculated against expression in cells with no melatonin pretreatment. Significance  
347 in all panels is indicated by \* P<0.05, \*\* P<0.01, and \*\*\* P<0.001.

348  
349 There was an effect of melatonin treatment within the physiological range (25pg/ml) on gene  
350 expression of 4t1 cells in culture (**Figure 2B**). Assessment of the genes that constitute the  
351 PAM50 human breast cancer Risk-Of-Recurrence (ROR) prognosis score calculator identified a  
352 significant decrease in oncostatic status with melatonin treatment (P = 0.008; F = 1.61). Non-  
353 treated cells had a *high* ROR score (Mean 65.5 ± 10.6 SEM), which in human patients identifies  
354 10-year ROR as greater than 20%. Cells treated with 25pg/ml of melatonin had a *low* score  
355 (Mean 20.8 ± 8.3 SEM), which in human patients identifies 10-year ROR as less than 10%.

356  
357 Among the PAM50 panel and our added targets, fifteen genes showed a significant increase in  
358 expression with melatonin pretreatment (**Figure 2C**). These included genes that: anchor cells  
359 into tissue (*Cdh2*, *Nrp1* and *Sema3f*), enable invasion and metastasis (*Krt14*, *Mlph*, *Mmp1a*,  
360 *Mmp2*, *Mmp11*, *St6galnac5*), and that affect the rate of differentiation, proliferation and/or

361 apoptosis (*Mapt*, *Mdm2*, *Mybl2*, *Myc*, *Nanog*, *Tfap2c*). Note: when identifying changes caused  
362 by melatonin treatment, we did not correct for multiple measures. This approach is likely to  
363 include type 1 errors (false positives), but with Bonferroni correction, type 2 errors are likely  
364 (false negatives), and due to the large number of targets in our panel, with correction none of  
365 the changes were significant.

366

367 Experiment 2. Effect of pineal melatonin on a 4t1 cell orthotopic allograft into C3Sn mice.

368 Orthotopic allograft was intended to test whether 4t1 cells were viable in a C3Sn mouse, and to  
369 allow us to assess whether pineal melatonin had any effect on 4t1 tumor burden, invasiveness  
370 and metastasis from an orthotopic placement.

371

372 A tumor cell burden was present in the majority of mice, demonstrating that 4t1 tumors will  
373 establish themselves successfully in a C3Sn host mouse, regardless of sex or pineal/melatonin  
374 status (**Figure 3**). There was a non-significant increase in the incidence of solid tumors in  
375 female PinealX mice ( $P = 0.38$ ,  $F = 8.0$ ). There were also single epithelial cells in the fat mass of  
376 the mammary pad that might be invasive 4t1 cells. If these are invasive 4t1 cells, there was no  
377 difference in the severity of local invasion between female PinealX and Intact-control ( $P = 0.68$ ,  
378  $F = 15.5$ ).

379

380 **Figure 3. Effect of pineal melatonin on orthotopic allograft viability. (A)** Example of a solid  
381 tumor at the allograft site in the lower left breast chain. **(B)** Example of single epithelial cells in  
382 mammary fat pad that might be invasive 4t1 cells. **(C)** The number of solid tumors in the lower  
383 left mammary chain of Intact-Pineal control and PinealX mice is expressed as Mean  $\pm$  SEM.  
384 Tumor numbers are shown for female mice, male mice, and combined groups of male and  
385 female mice.

386

387 Experiment 3. Effect of melatonin on metastasis of 4t1 cells from tail-vein allograft. There was  
388 no effect of pineal status on metastases to lung, breast, lymph or liver (**Figure 4**). Pineal surgery  
389 significantly reduced serum melatonin (Mean  $\pm$  SD serum melatonin pg/ml: Intact-Control  
390 32.2pg/ml  $\pm$  4.7; PinealX 16.2  $\pm$  2.1; P < 0.0001; F = 5.11). There was no sex difference in mid-  
391 dark-phase serum melatonin (Intact-Control Male 31.0  $\pm$  5.8; Intact-Control Female 33.5  $\pm$  4.0;  
392 P = 0.40).

393

394 **Figure 4. Effect of pineal status on metastasis.** 4t1 cell burden is shown as a percentage of  
395 the total tissue calculated by strain specific qPCR: 4t1 cells have a BALB/c origin so the  
396 proportion of BALB/c and C3Sn host gDNA provided a measure of 4t1 cell content. Mean and  
397 SEM of data are shown for Intact-Control and PinealX mice for: **(A)** breast, **(B)** lung, **(C)** lymph,  
398 and **(D)** liver. Comparison is Intact-Control versus PinealX for male mice, female mice, and then  
399 combined male and female data.

400

401 However, there was a significant difference in metastases of 4t1 cells to the breast of male and  
402 female mice (Mean and SD females = 16.2  $\pm$  4.6, males = 10.4  $\pm$  3.0, P = 0.002; two-tailed,  
403 equal variance t-test, significance threshold Bonferroni corrected for multiple measures from  
404 0.05 to 0.0127) (**Figure 5**). There was also no effect of sex on metastases to lung, lymph or  
405 liver.

406

407 **Figure 5.** Effect of sex on metastasis. 4t1 cell burden is shown as a percentage of the total  
408 tissue calculated by strain specific qPCR. Mean and SD of data separated by sex are shown for:  
409 **(A)** breast, **(B)** lung, **(C)** lymph, and **(D)** liver.

410

411 **Discussion**

412



413 There is compelling evidence that reduced melatonin signaling correlates with increased  
414 incidence and poor prognosis in TNBC patients [5,9,22,24,25,29]. However, studies of  
415 melatonin effects on TNBC have typically been limited to very high, non-physiological doses of  
416 melatonin, and/or used strains of mice that do not make endogenous melatonin so are chronic  
417 deficits in melatonin with unspecified developmental consequences of that deficit. The goal of  
418 this study was to determine whether the relatively small reductions in pineal melatonin that  
419 might occur with use of artificial light-at-night (ALAN), have any effect on a hormone receptor  
420 deficient breast cancer.

421  
422 Assessment of the effect of melatonin on 4t1 cells *in vitro* was intended to identify any effect on  
423 tumor cell status (ROR score) and suggest mechanisms mediating effects of melatonin. The  
424 lack of melatonin effect on survival of 4t1 cells *in vitro*, was consistent with one study of MDA-  
425 MB-231 and HCC-70 TNBC cells, but different from a later study of MDA-MB-231 cells [22,23].  
426 However, there was a clear effect of melatonin on gene expression. Gene expression also  
427 confirmed that 4t1 cells are valid as a model of TNBC: *Pgr* was undetectable, and *Esr1* and  
428 *Erb2/Her2* were considerably reduced. This is consistent with the finding that 4t1 cells lack  
429 *Esr1* protein and an estradiol growth response [27]. However, this is different from the observed  
430 expression of *Esr1* and *Erb2* in another recent study, which likely reflects our use of C3Sn  
431 mouse to provide normal breast gene expression data [29].

432  
433 Notably, the significant reduction in PAM50 ROR prognosis score with melatonin treatment  
434 suggested that melatonin has the potential to dramatically reduce the aggressiveness of 4t1  
435 TNBC [30,31]. The caveat with the ROR observation is that the PAM50 panel and ROR  
436 prognosis score are developed and clinically validated for assessment of human breast cancer.  
437 However, we felt this panel would make both typing and prognosis assessment more  
438 translationally relevant in this and future studies. For example, the PAM50 panel was developed

439 for a variety of breast cancer types (Luminal A, Luminal B, HER2-enriched, basal-like and  
440 normal-like), and tools to derive the clinically validated prognosis score as a 10-year risk of  
441 recurrence (ROR) are freely available [40].

442

443 Of the melatonin induced changes in specific genes, patterns of interest included an increase in  
444 genes associated with tissue remodeling (*Mmp1a*, *Mmp2*, *Mmp11*), but also an increase in cell-  
445 cell anchoring genes (*Cdh2*, *Sema3f*, *Nrp1*). The sum of these changes may be negative,  
446 positive or neutral, which emphasizes the value of a clinically validated prognosis predictor such  
447 as the PAM50 ROR.

448

449 Given the effect of gene expression, the limited effect of deficits in melatonin *in vivo*, was  
450 unexpected. We had successfully developed a surgical model of partial pineal gland removal  
451 that reduces plasma melatonin by ~50%. In our preliminary study to test allograft viability, we  
452 saw a non-significant increase in burden of solid tumors in female mice from 4t1 cells introduced  
453 orthotopically. However, in a larger cohort with 4t1 cells introduced by tail-vein injection, we saw  
454 no effect of pineal status on metastasis. Interestingly, the burden of 4t1 cells from tail-vein  
455 allograft was significantly higher in the breast of female mice. This measurement was  
456 proportional to total mammary tissue collected so could result from a sex-hormone effect on 4t1  
457 cells or a functional difference in the mammary tissue of virgin female mice [46].

458

459 While we saw no effect of pineal melatonin on metastasis, our approach of quantifying  
460 metastases by gDNA was successful. Metastases of 4t1 cells from tail vein injection form  
461 disseminated colonies, which would make quantification difficult [36]. Others have developed  
462 methods for quantification of submicroscopic xenograft metastases using DNA quantification  
463 [45]. The genetic differences between BALB/c 4t1 cells and our C3Sn host allowed us to design  
464 strain specific qPCR primers, which allowed us to quantify dispersed metastases from a

465 transplant to a member of the same species [47]. To our knowledge, this approach has not been  
466 applied to an allograft.

467

468 Our gene expression data suggests that melatonin does have a meaningful effect on TNBC.

469 The lack of melatonin effect in an MTT assay and on metastasis from tail-vein injection,

470 suggests the effect is not on proliferation or invasion of tissues from the vasculature. In that

471 context, the trend for increased tumor burden from orthotopic allograft in females with deficits in

472 pineal melatonin could suggest any protective effects occur earlier in stages of neoplastic

473 development. For example, the increased expression of *Cdh2*, *Sema3f* and *Nrp1* would reduce

474 shedding of cells from a tumor. This indicates a need to more extensively study *in vitro*

475 properties (colony formation, wound healing, and trans-well cell invasion) and the time course of

476 orthotopic allograft tumor development and metastasis, as well as incidence of TNBC induced

477 by chemical carcinogens in our model.

478

479 *Clinical relevance.* Exposure to LAN will reduce the duration and/or amount of melatonin action.

480 Currently our data finds no *in vivo* effect of melatonin deficits, but *in vitro* gene expression does

481 suggest melatonin could affect TNBC pathogenesis. If melatonin acts earlier in oncogenesis

482 than invasion of circulating cells into tissue, such as reducing the prior shedding of cells from a

483 tumor, then effective risk reduction would be best achieved with prophylactic reductions in ALAN

484 exposure or use of melatonin supplements or agonists. Even if the risk reduction is modest, the

485 prevalence and mortality of TNBC would make that effect meaningful.

486

487

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489

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493 - approach development and paper preparation. SDW - pinealectomy development. ST-  
494 conceived and led the study, conducted experiments and analysis, and led paper preparation.  
495

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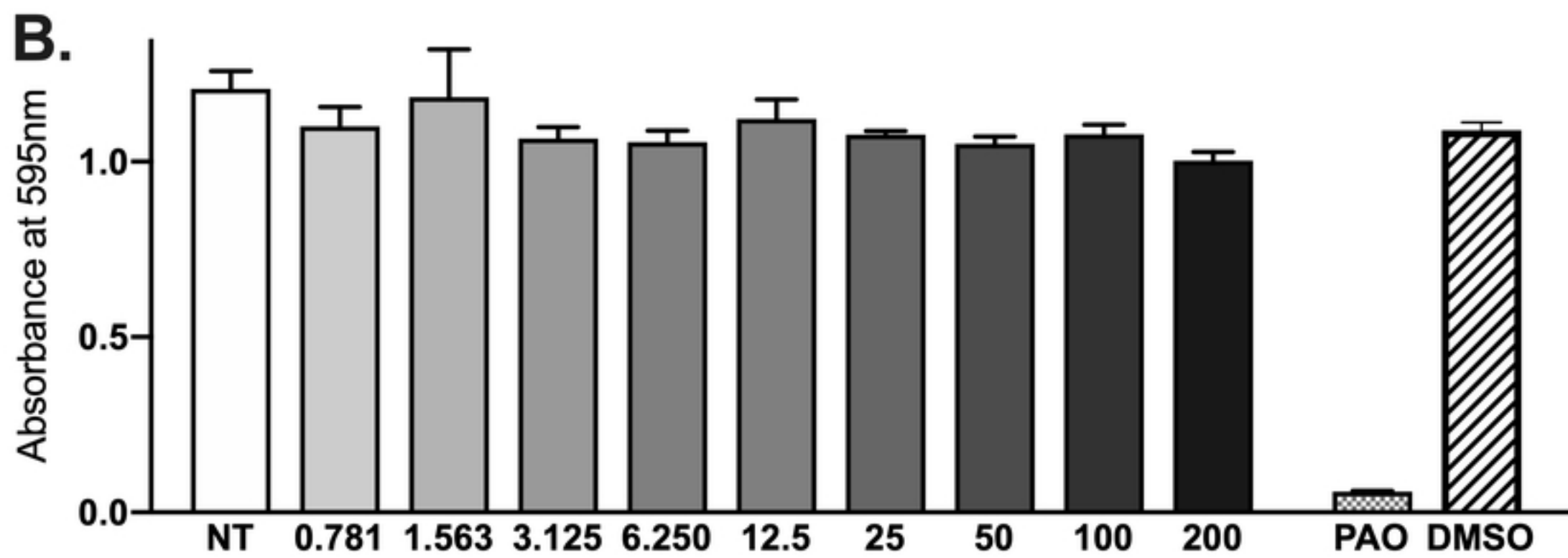
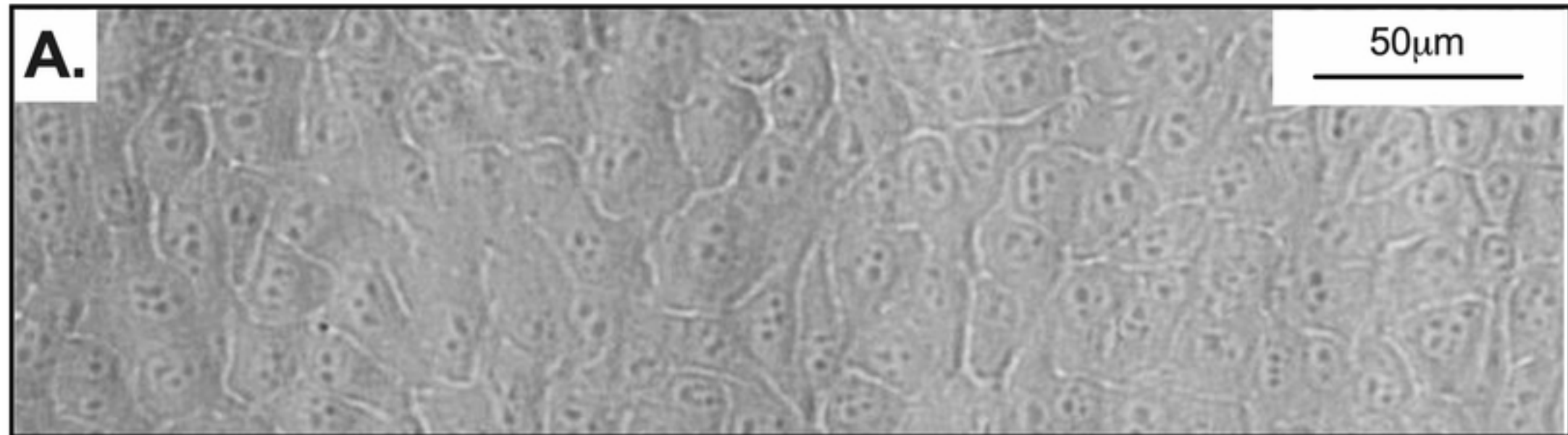


Figure 1

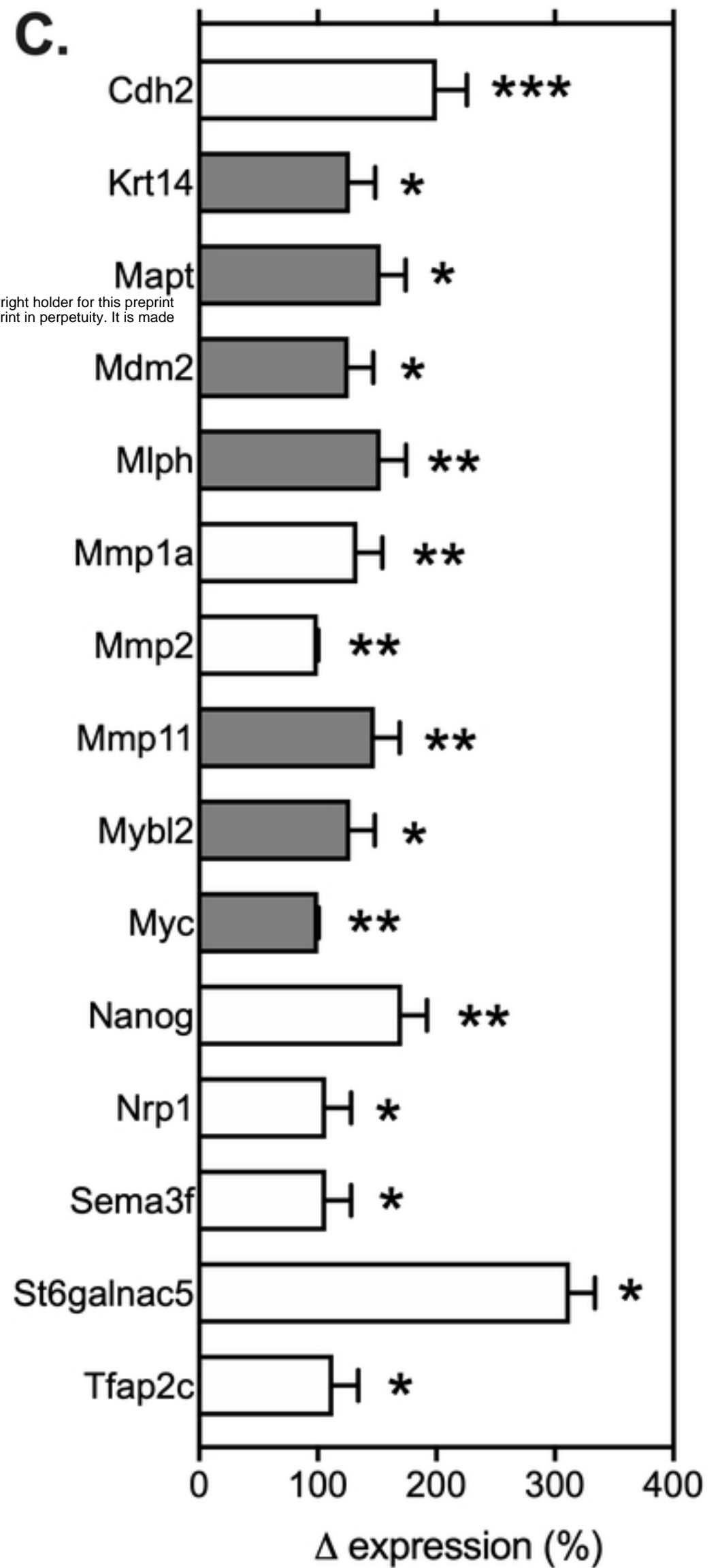
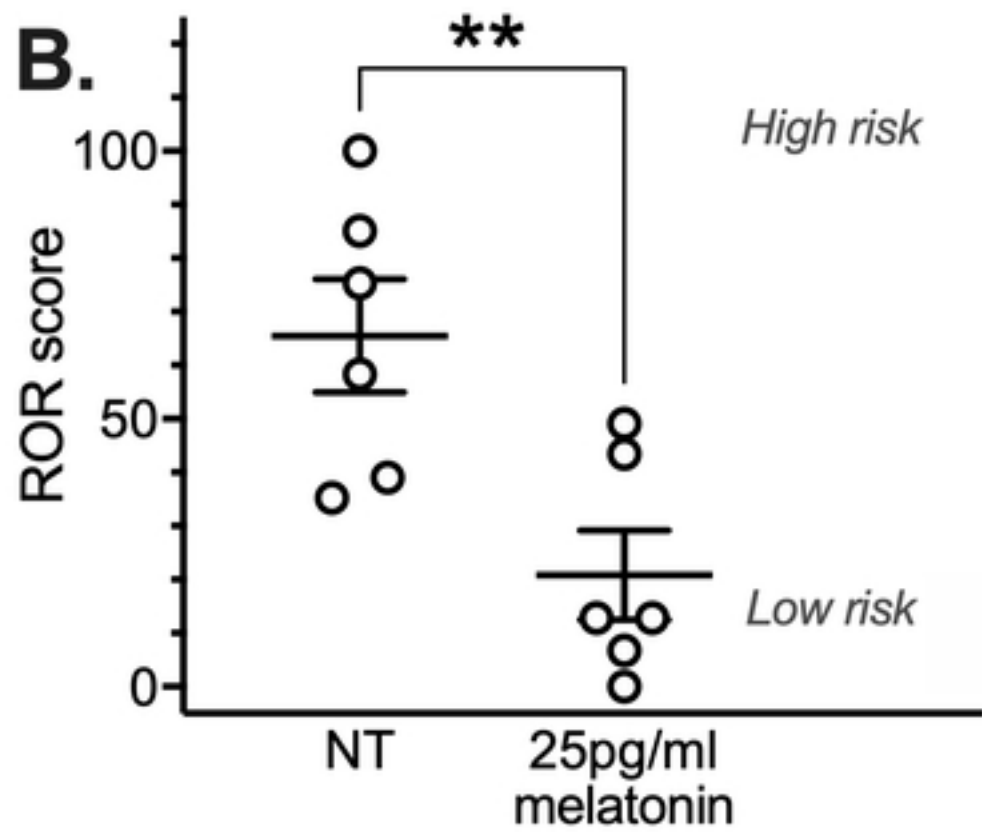
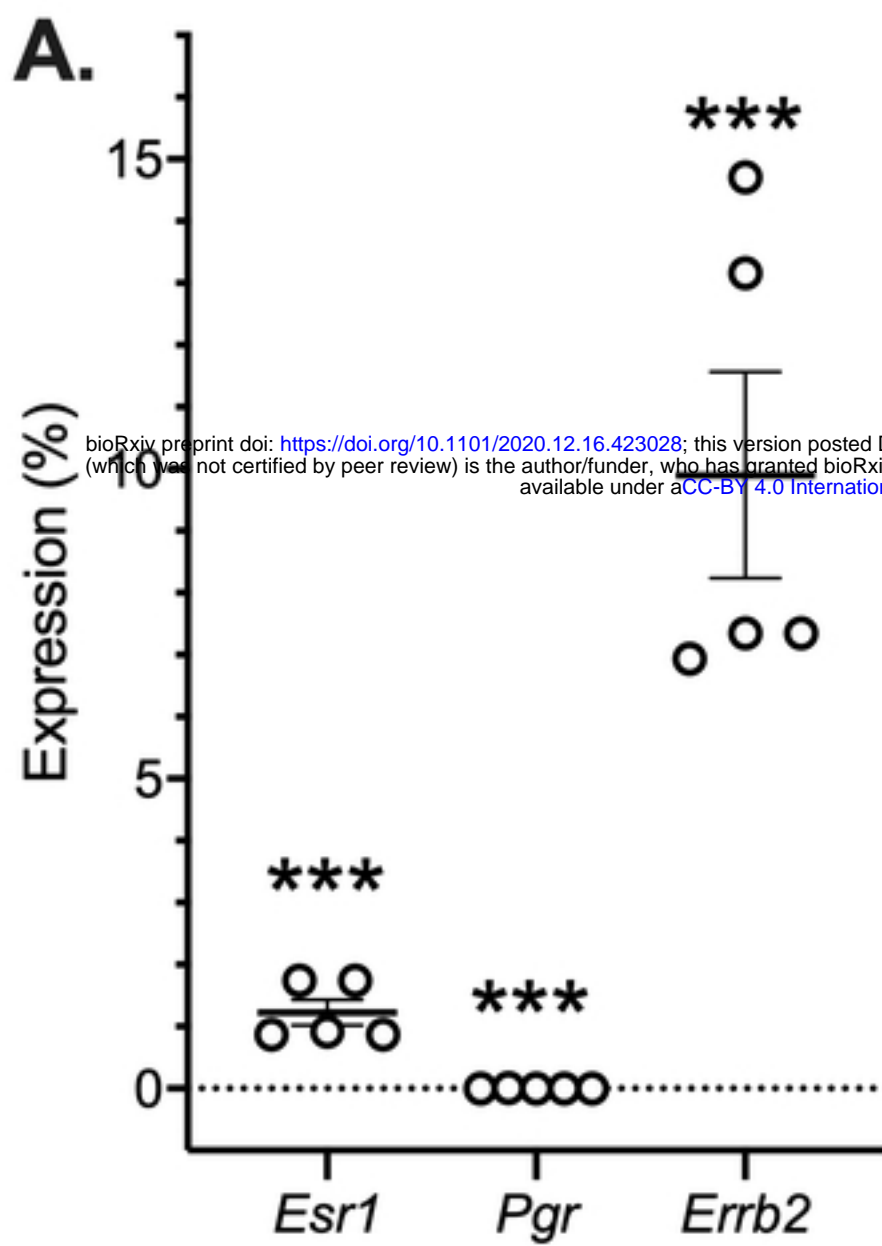


Figure2

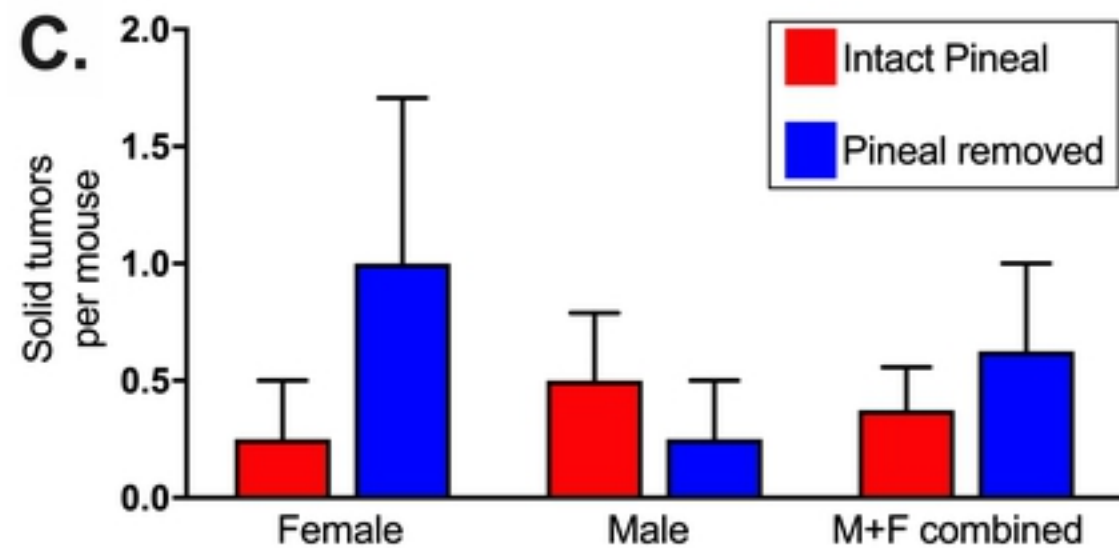
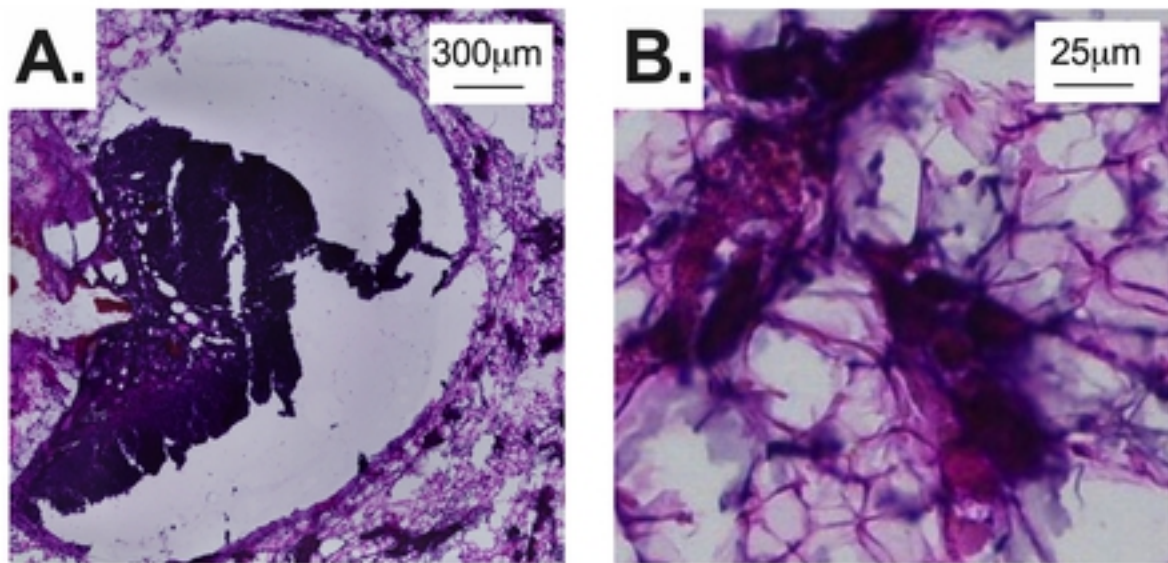
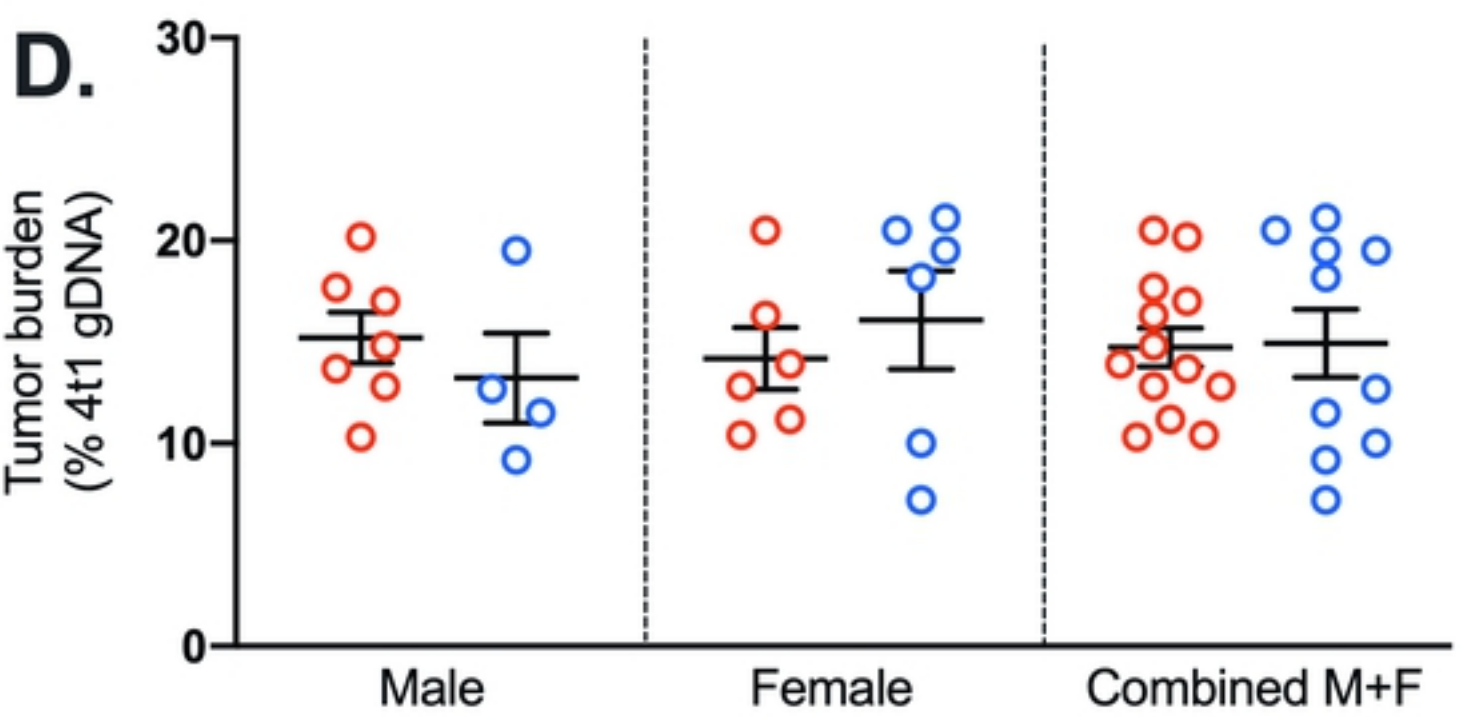
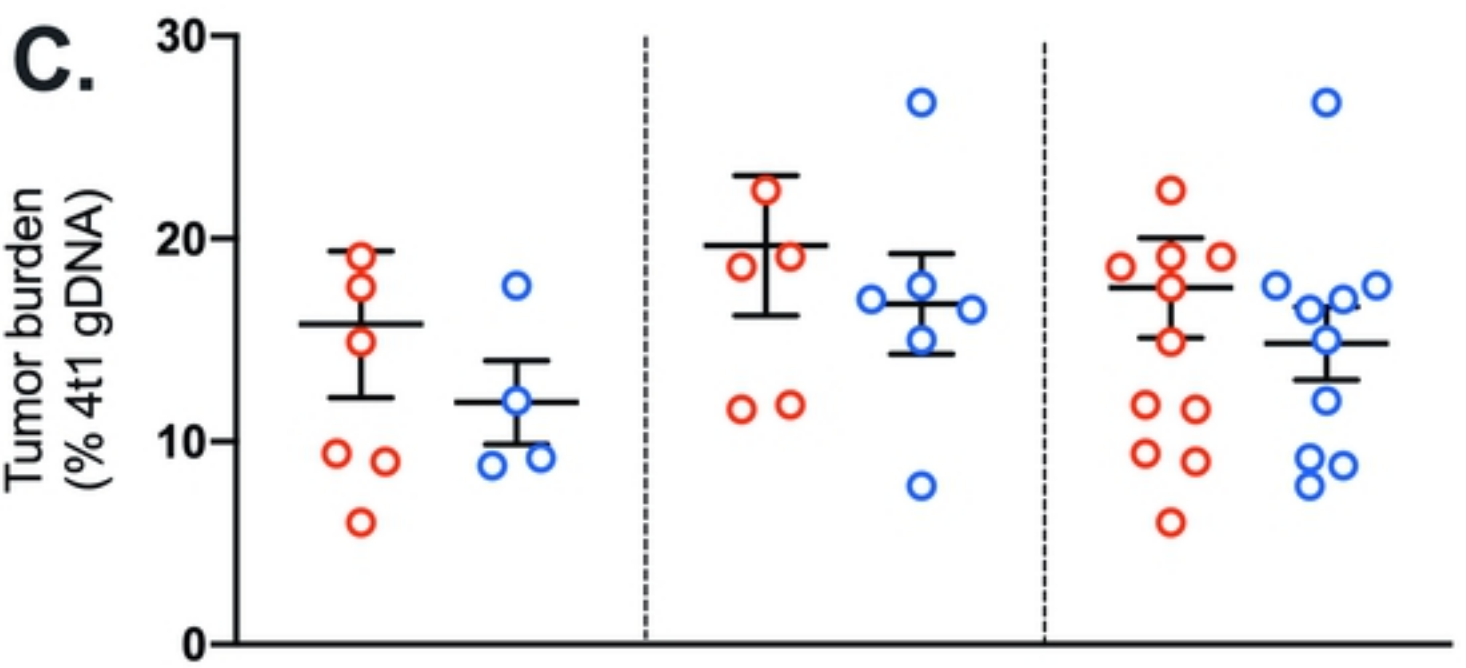
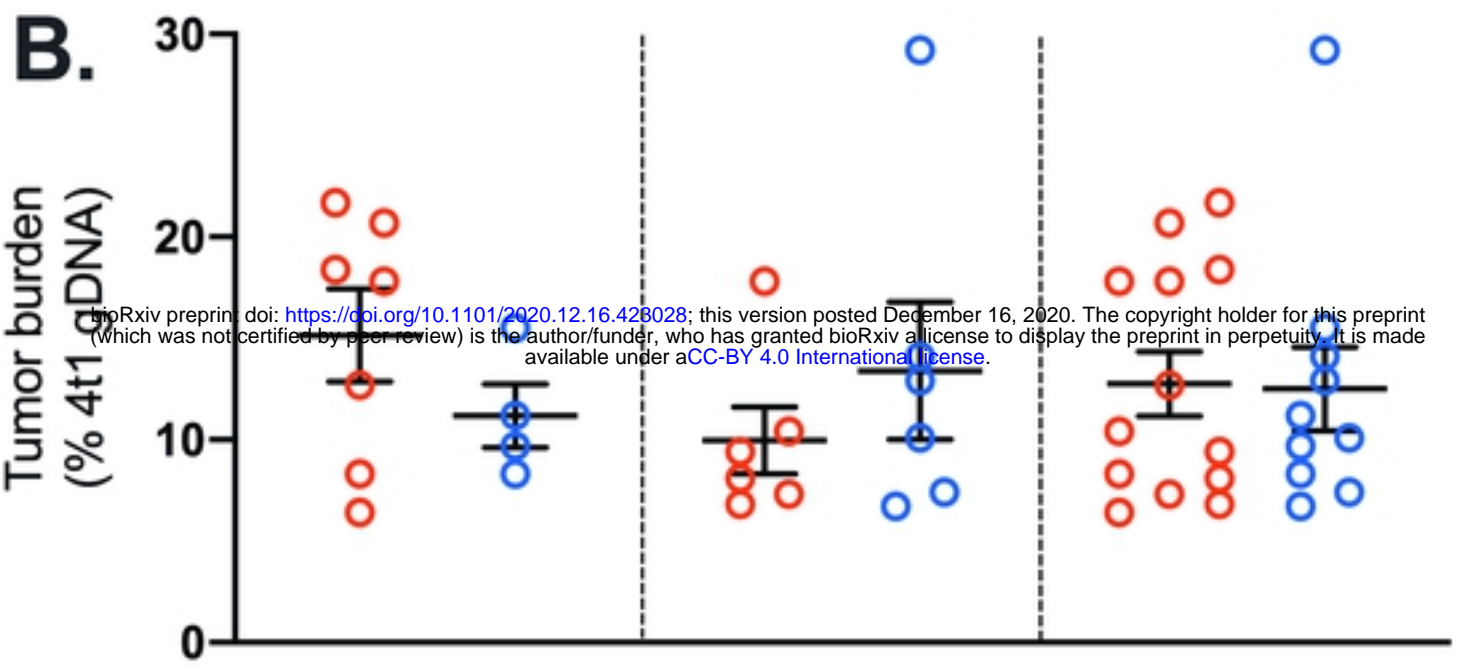
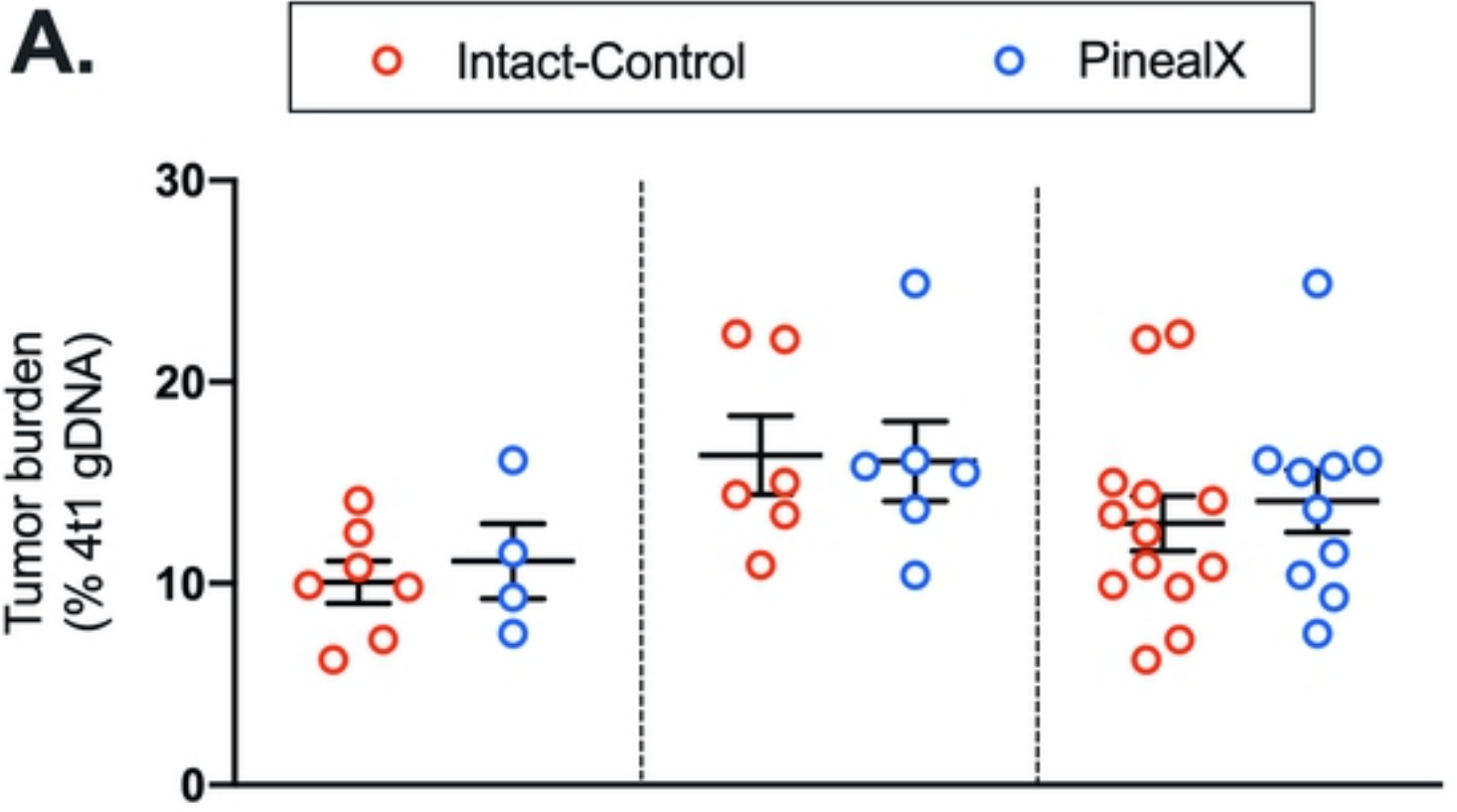


Figure3



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Figure4



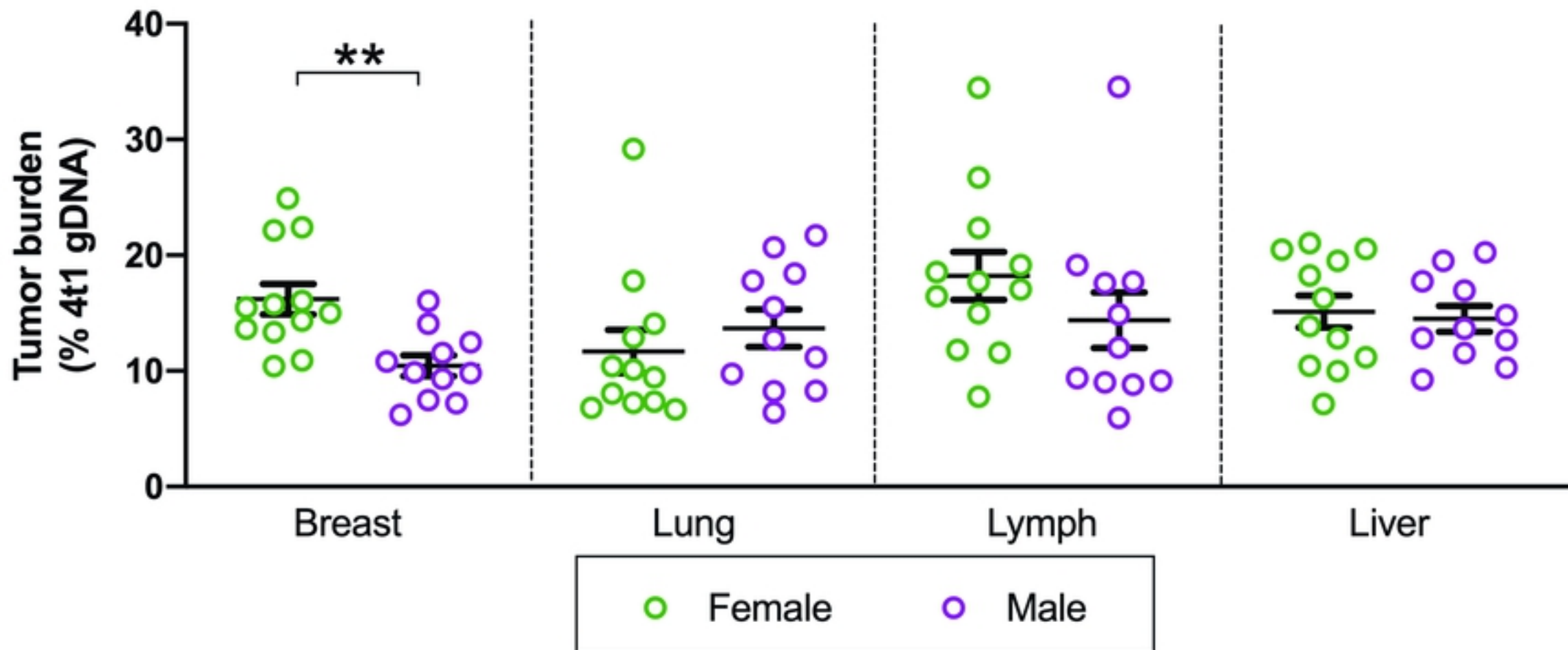


Figure5