View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repository@Hull - CRIS

Journal of
Cellular and Molecular Medicine
Open Access

# The toxic effect of cytostatics on primary cilia frequency and multiciliation.

| | |
|---|---|
| Journal: | *Journal of Cellular and Molecular Medicine* |
| Manuscript ID | JCMM-03-2019-024.R1 |
| Wiley - Manuscript type: | Original Article |
| Date Submitted by the Author: | 30-Apr-2019 |
| Complete List of Authors: | Filipova, Alzbeta; University of Defense Faculty of Military Health Sciences, Department of Radiobiology<br>Diaz Garcia, Daniel; University Hospital Hradec Kralove, Biomedical Research Center<br>Bezrouk, Ales; Charles University Faculty of Medicine in Hradec Kralove, Department of Medical Biophysics<br>Cizkova, Dana; Charles University Faculty of Medicine in Hradec Kralove, Department of Histology and Embryology<br>Dvorak, Josef; Thomayer Hospital, Department of Oncology<br>Filip, Stanislav;<br>Sturge, Justin; University of Hull, Faculty of Health Sciences<br>Sinkorova, Zuzana; University of Defense Faculty of Military Health Sciences, Department of Radiobiology |
| Keywords: | Primary cilium, multiple cilia, cytostatics, toxicity, cell line |
| | |

SCHOLARONE™
Manuscripts

# ~~Primary cilia - an *in vitro* model to evaluate~~ T~~t~~he toxic effect of cytostatics ~~on~~ on primary cilia frequency and multiciliation~~cytoskeletal stability~~.

~~Therefore, the increased primary cilia frequency induced by cytostatics could be used in other studies trying to assess the toxicity of these drugs.~~

**Alžběta Filipová[1*], Daniel Diaz Garcia[2], Aleš Bezrouk[3], Dana Čížková[4], Josef Dvořák[5], Stanislav Filip[6*], Justin Sturge[7], Zuzana Šinkorová[1]**

[1]Department of Radiobiology, Faculty of Military Health Sciences in Hradec Králové, University of Defense, Hradec Králové, Czech Republic;
[2]University Hospital, Department of Clinical Biochemistry, Hradec Králové, Czech Republic.
[3]Charles University, Faculty of Medicine, Department of Medical Biophysics, Hradec Králové, Czech Republic;
[4]Charles University, Faculty of Medicine, Department of Histology and Embryology, Hradec Králové, Czech Republic;
[5]Charles University, Charles University and Thomayer Hospital, Department of Oncology, Prague, Czech Republic;
[6]Charles University, Faculty of Medicine, Department of Oncology and Radiotherapy, Hradec Králové, Czech Republic;
[7]University of Hull, School of Biological, Biomedical and Environmental Sciences, UK;

* Correspondence:

Alžběta Filipová, MSc, PhD.
Department of Radiobiology, Faculty of Military Health Sciences in Hradec Králové, University of Defense,
Třebešská 1575,
Hradec Kralove
500 01
Czech Republic
alzbeta.filipova@unob.cz

Prof. Stanislav Filip, MD, PhD, DSc.
Charles University, Medical Faculty and University Hospital, Department of Oncology and Radiotherapy
Sokolská 548
500 05 Hradec Králové
Czech Republic
stanislav.filip@fnhk.cz

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

# Abstract

The primary cilium is considered as a key component of morphological cellular stability. However, cancer cells are notorious for lacking primary cilia in most cases, depending upon the tumour type. Previous reports have shown the effect of starvation and cytostatics on ciliogenesis in normal and cancer cells although with limited success, especially when concerning the latter. In this study we evaluated the presence and frequency of primary cilia in breast fibroblasts and in triple negative breast cancer cells after treatment with cytostatics finding that, in the case of breast fibroblasts, primary cilia were detected at their highest incidence 72 hours after treatment with 120 nM doxorubicin. Further, multiciliated cells were also detected after treatment with 80 nM doxorubicin. On the other hand, treatment with taxol increased the number of ciliated cells only at low concentrations (1.25 and 3.25 nM) and did not induce multiciliation. Interestingly, triple negative breast cancer cells did not present primary cilia after treatment with either doxorubicin or taxol. This is the first study reporting presence of multiple primary cilia in breast fibroblasts induced by Doxorubicin. However, the null effect of these cytostatics on primary cilia incidence in the evaluated TNBC cell lines requires further research.

~~These results suggest that primary cilia could be involved in specific cell signalling events and cytoskeletal stability after chemotherapy, although further research is needed in this regard.~~


For Peer Review

**Key words:** Primary cilium, multiple cilia, cytostatics, toxicity, cell line ~~triple negative breast cancer~~.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

# 1 Introduction

Primary cilium, an organelle found on nearly every cell in the human body, typically serves as a mechanical sensory tool; further, it is also involved in cell proliferation and embryonic development. This organelle is dynamically regulated during the cell cycle, appearing during the G0/G1 phases and resorbed prior to mitosis [1] . The exact solubilization moment of primary cilia is determined by cell type and the expression of genes affecting resorption, such as Aurora A, Plk1, TcTex-1 [2, 3] . Usually, a cell has only one primary cilium which, as previously mentioned, is involved in morphogenesis, cell proliferation and differentiation signalling [4, 5] . Should there be a multiplication of centrosomes, a higher number of primary cilia will appear on the surface of cells as well, often bearing the same length and construction design, and spawning from the same ciliary pocket; however, the presence of multiple cilia has been mostly recorded in solid tumours [6, 7] after exposure to ionizing irradiation [8, 9] or in ciliopathies [10] . Typically, primary cilia are always observed in myoepithelial cells and fibroblasts and with low incidence in luminal epithelium cells. Regarding cancer cells, a study of 26 breast cancer biopsy samples revealed the presence of primary cilia only on exceptional cases, especially in epithelial cells [11] .

For Peer Review

Basal subtype B tumours, a classification of triple negative breast carcinomas (TNBC), is characterized by the absence of oestrogen, progesterone, and Her2/neu receptors [12], as well as by the rare presence of primary cilia. Among breast cancer tumours, TNBC has an estimated incidence of 10-20%, although from a histological point of view TNBCs are little differentiated and are often included in basal-like subgroups. From a clinical standpoint, these tumours are frequently resistant to treatment, have quick progression, low 5-year survival rate, increased local recurrence, and are highly metastatic. This kind of tumours can be observed at any age; however, they mostly occur accompanied by BRCA1 mutations in younger women (>40 years of age) [13]. Chemotherapy is the treatment of choice for triple negative breast cancer patients, of which Doxorubicin and Taxol are the standard chemotherapeutic agents used as anticancer therapy in combination with α-HER2/neu receptor targeted therapy. Doxorubicin belongs to the anthracyclines group, whereas Taxol is considered as a taxane. The former is an effective intercalating cytotoxic agent used in the treatment of various tumour types and commonly used in combination with the latter in adjuvant and neoadjuvant therapeutic strategies for breast cancer patients [14] .

Previous research on the effect of cytostatics on primary cilia has focused in the effects of Taxol over the elongation and shortening of primary cilia. In a study by Sharma et al., Taxol was shown to block the emergence of primary cilia in mammalian cell cultures [15]. However, low concentrations result in an increased quantity of free tubulin subunits in the cytosol, leading to enlarged primary cilia [16, 17]. Ongoing research highlights two important questions in the cell biology of cancer and primary cilia: 1) the significance of having primary cilia in normal cells, and 2) the loss of primary cilia in cancer cells and its relation to drug resistance [18] . Therefore, the increased primary cilia frequency induced by cytostatics could be used in other studies trying to assess the toxicity of these drugs.

# 2 Material and methods

## 2.1 Cell culture

Unless otherwise stated, all standard chemicals and antibodies were purchased from Sigma-Aldrich, Czech Republic. In this study, TNBC cell lines BT-549 (ATCC, USA) and MDA-MB-231 were used (kindly supplied by Mgr. Jaroslav Truksa, Ph.D., Laboratory of Tumor Resistance, Institute of Biotechnology CAS, Prague), as well as skin fibroblasts. BT-549 cells were cultured in DMEM 10% FBS (PAA, USA), 2% glutamine (Gibco, UK), 1% penicillin/streptomycin (Gibco, UK), 0.023 IU/ml insulin and incubated in a 5% $CO_2$ atmosphere at 37°C. MDA-MB-231 and fibroblasts were cultured in DMEM, 10% FBS (PAA, USA), 2% glutamine (Gibco, UK), 1% penicillin/streptomycin (Gibco, UK) and incubated in a 5% $CO_2$ atmosphere at 37°C. All cell lines were used until $10^{th}$ to $12^{th}$ passage and medium was replaced every two days for all experiments.

For Peer Review

## 2.2 Skin fibroblasts

Human fibroblasts were isolated from skin biopsies obtained in accordance and approved by the Ethics Committee of the University Hospital Hradec Kralove, Czech Republic and the European Ethics committee under the directive approved on $10^{th}$ July 2014 (Reference/license number: 201407 S12P). Donor patients signed an informed consent allowing us to work with the obtained samples. To isolate the fibroblasts, skin biopsies were kept in a basic solution (30 ml/2g of tissue) after surgery and transported to the laboratory, where the biopsies were washed in PBS and the subcutaneous tissue was removed. Each individual tissue sample was incubated in 5 ml of a 2U/ml dispase solution (Gibco, UK) for 18 hours at 4°C. After

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

incubation, the samples were washed 3 times in PBS at room temperature (22°C). The dermis was separated from the epidermis using tweezers, washed in PBS and cut into small sections (<5mm$^2$). Afterwards, the tissue was incubated in 10 ml of digestion solution (1g tissue/10 ml) in a rotating incubator at 37°C/6 *g*. After 4 hours, the samples were filtered through a 40 µm strainer into a new sterile 50 ml tube. The cell number was determined from this suspension and centrifuged for 10 min/150 *g*. The supernatant was decanted and the cell pellet was resuspended in culture media, plated in a T-75 flask (1x10$^5$ cells per flask) and incubated in a 5% $CO_2$ atmosphere at 37°C for 14 days.

## 2.3 Cytostatic drugs

Doxorubicin and Taxol were dissolved in 0.5% DMSO and kept in 1 mM stock solutions. Doxorubicin and Taxol were diluted in culture media before use at a ratio of 1:100 and 1:1000, respectively.

## 2.4 Cell treatment and Immunofluorescence

MDA-MB-231, BT-549, and fibroblasts cells were cultured in 6-well plates at a density of 3 x 10$^5$ cells per well and incubated at 37°C/5% $CO_2$ for 24h, each well contained a gelatine coated coverslip. After this period, the cells were treated with Doxorubicin (10, 20, 40, 80 and 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25, and 12.5 nM) for 72 hours. Control cells were kept under the same conditions in culture media with or without DMSO (0.5%). After treatment, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed three times with PBS. Immunostaining was performed as follows: cells were blocked with goat serum (Jackson Immunoresearch, USA) 1:20 for 30 minutes; anti-acetylated tubulin 1:800 for 1 hour; anti-gamma tubulin 1:500 for 1 hour; Cy3-conjugated donkey anti-mouse secondary antibody 1:300 for 45 minutes in the dark; Alexa 488 conjugated donkey anti-rabbit secondary antibody (JacksonImmunoresearch, USA) 1:300 for 45 min in the dark. Imaging analysis was performed using a Nikon Eclipse fluorescence microscope (Prague, Czech Republic) observed with an oil-immersion 60X objective. All experiments were performed in triplicate.

## 2.5 Cytotoxicity test

A WST-1 test (Roche; Basel, Switzerland) was used to determine cell viability after treatment with Doxorubicin (10, 20, 40, 80, 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25, 12.5 nM). All cell lines were plated and treated in a 96-well-plate (1 x 10$^3$ cells per well), and incubated at 37°C/5%$CO_2$ for 24h. After 72 h, 10µL of WST-1 reagent was added and incubated for 3 h at

For Peer Review

37°C/5% $CO_2$ and analysed in a Tecan SpectraFluor Plus spectrometer (Tecan Austria GmbH; Grödig, Austria) at a wavelength of 440 nm. All experiments were performed in triplicate.

### 2.6 Transmission electron microscopy (TEM)

Fibroblasts were fixed in 3% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.2) for 5 min. at 37°C and then for 3 h at room temperature, washed in cacodylate buffer (0.1 M, pH 7.2) and post-fixed in 1% OsO4 (in 0.1 M cacodylate buffer, pH 7.2) for 1 h at room temperature. After rinsing in cacodylate buffer (0.1 M, pH 7.2), the cells were dehydrated in graded alcohols (50%, 75%, 96%, 100%), cleared in propylene oxide and embedded in a mixture of Epon 812 and Durcupan (Sigma; polymerization for 3 days at 60°C). Semi-thin sections were stained with toluidine blue. Ultra-thin sections were cut on an Ultrotome Nova (LKB, Sweden), mounted into formvar carbon-coated copper grids, counterstained with uranyl acetate and lead citrate and examined in a JEOL JEM-1400Plus transmission electron microscope (at 120 kV JEOL, Japan). Images were captured with integrated 8Mpix CCD camera and software (JEOL, Japan).

### 2.7 Statistical analysis

Graphs were made using the GraphPad Prism 6 biostatistics software (GraphPad Software, USA). The statistical analysis between the evaluated groups was performed using one-way ANOVA followed by a post-hoc Tukey test ($p<0.05$).

## 3 Results

### 3.1 Proliferation and viability of BT-549, MDA-MB-231, and fibroblasts after treatment with Doxorubicin and Taxol

Cytostatics are known inhibitors of cell viability and proliferation, therefore we had to determine a suitable concentration in which the cells would stop proliferating without losing viability. To achieve this purpose, the MDA-MB-231, BT-549 and fibroblast cells were maintained with Doxorubicin or Taxol at various concentrations (10, 20, 40, 80 and 120 nM and 1.25, 3.25, 5.25, 6.25 and 12.5 nM, respectively), followed by a WST-1 test after 72 hours of treatment. The number of living cells was significantly lower after Doxorubicin (Figure 1A-C) and Taxol (Figure 1D-F) treatment across all cell lines.

For Peer Review

## 3.2 Primary cilia incidence and multi-ciliation induced by the cytostatics Doxorubicin and Taxol

Regarding Doxorubicin, the number of fibroblasts ~~cells~~ with primary cilia increased to ~70%,~~,~~ ~~when compared~~in comparison with ~~to~~ control untreated cells, ~~within~~ after 72 hours of treatment with various concentrations of this cytostatic (10, 20, 40, 80 and 120 nM), observing a higher incidence after treatment with 120 nM Doxorubicin. Overall, this effect could be observed evenly across the entire ~~dosage~~ dose range used in this study (Figure 2A). After 72 hours of treatment, primary cilia were detected by immunostaining (Figure 2B – control cells; Figure 2C – 120nM Doxorubicin) and electron microscopy (Figure 2D – control; Figure 2E – 120 nM Doxorubicin). Interestingly, ~~Approximately~~ ~20 ~~to~~ -40% of fibroblasts treated with 20-120 nM Doxorubicin showed two or more cilia after 72 hours of treatment, observing a higher number of multi-ciliated cells at a dose of 80 nM Doxorubicin (~40%; Figure 3A). However, no multi-ciliated cells could be observed after treatment with 10 nM Doxorubicin or in ~~the control~~ untreated cells (Figure 3a). Multiple primary cilia were detected by immunostaining (Figure 3B) and electron microscopy (Figure 3C) after 72 hours of treatment.

Concerning Taxol, the treatment of fibroblasts with various concentrations (1.25, 3.25, 5.25, 6.25 and 12.5 nM) for 72 hours revealed a significantly higher incidence of primary cilia after treatment with 1.25 and 3.25 nM Taxol (~80%) (Figure 4A), as detected by immunostaining (Figure 4B) and electron microscopy (Figure 4C). However, this incidence rate was slightly decreased in the cells within the higher concentration range (5.25, 6.25 and 12.5 nM)~~ in comparison with the lower concentration range (1.25 and 3.25 nM)~~, although without change when compared to the untreated cells.

Interestingly, a complete absence of primary cilia was noted in MDA-MB-231 cells after treatment with either Doxorubicin (10, 20, 40, 80 and 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25 and 12.5 nM) for 72 hours, an effect that was repeated in BT-549 cells (Figure 5).

# 4 Discussion

In the present work we sought to ~~assess~~ determine the effect that the cytostatics Doxorubicin and Taxol could have on ~~cytoskeletal stability~~primary cilia incidence in triple negative breast cancer cells. We chose these cytostatics because they are commonly used in the treatment of breast cancer. On one hand, Doxorubicin (DOX) acts by intercalating into the DNA strands inhibiting topoisomerase II activity and thus inducing strand breaks when DNA is being

For Peer Review

replicated; in addition, it also promotes the formation of reactive oxygen species (ROS), which are highly toxic. Taxol, on the other hand, inhibits microtubule depolymerization [19]~~-~~, resulting in shorter primary cilia and affecting their frequency in the exposed cells [15] ; therefore, it was included as a negative control drug in our experiments.

Solid ~~tumors~~tumours possess a characteristic absence or low incidence of primary cilia which, when present, may also have a compromised structure and function. However, some ~~tumor~~tumour types that are dependent upon the Hedgehog (Hh) ~~signaling~~signalling pathway often have an increased frequency of primary cilia [20, 21]~~–~~. Further, several types of ~~tumors~~tumours have been associated with altered Hh, Wnt, NOTCH and Hippo ~~signaling~~signalling pathways, which are related to primary cilia; therefore, compromised signal transduction could also be caused by defects in the formation, structure or function of primary cilia [22] .

Primary cilia normally occur in approximately 70% of fibroblasts and from 7 to 19% of epithelial cells from healthy breast tissue [11] . However, the study of 11 breast cancer cell lines revealed that primary cilia were only present in 4 of these and at the very low frequency of 0.3% - 4%; curiously, these cell lines had the shared characteristic of being basal B subtypes, which are analogous to triple negative breast cancer cells [11]~~-~~. Regardless, primary cilia have been found in some cases of TNBC, which hints at the existence of several TNBC subtypes in which the reason for this exclusive presence of primary cilia is yet unclear. A possible explanation could be that these ~~tumor~~tumour subtypes are dependent upon the Hh ~~signaling~~signalling pathway and hence upon primary cilia, as it has been observed in most basal cell carcinomas and medulloblastomas [22] .

In the present study, the chemosensitive triple negative breast cancer cell lines BT-549 and MDA-MB-231 were used, in addition to normal skin fibroblasts as a comparative control, to test the effect that these cytostatics could have on primary cilia incidence. The cell lines were treated with 10, 20, 40, 80 and 120 nM Doxorubicin and 1.25, 3.25, 5.25, 6.25 and 12.5 nM Taxol for 72 hours to determine the dose ~~at~~in which the cells would become affected by the cytostatic drug without compromising viability or inducing cell death. ~~Consistently~~Accordingly, the number of viable cells decreased with an ~~increasing~~ increased concentration of cytostatics~~, which also correlated with the increased percentage of dead cells (data not shown)~~.

For Peer Review

Regarding Taxol, the included cell lines showed consistently decreasing proliferation and viability as the dosage of Taxol increased; therefore, low doses of 1.25, 3.25, 6.25 and 12.5 nM Taxol were chosen based on previous experiments [23] .

In our study, fibroblasts were treated with the mentioned dose range of Doxorubicin and Taxol ~~T~~to establish ~~a~~ their baseline effect ~~of the cytostatics~~on ~~Doxorubicin and Taxol on~~ ciliogenesis~~, fibroblasts were treated first with the before mentioned dose range of Doxorubicin and Taxol~~. Concerning Doxorubicin, an even increment in primary cilia frequency was observed across the full dose range (10, 20, 40, 80 and 120 nM) after 72h of treatment, reaching a maximum value of 70% after ~~a~~ under a 120 nM dose. ~~Further~~Interestingly, 20 to 40% of fibroblasts showed two or more cilia after treatment with doses of 20-120 nM, reaching the maximum value of 40% multi-ciliated cells after treatment with 80 nM. It must be mentioned that multi-ciliated cells also were in possession of multiple centrosomes, an effect that has also been observed in some ~~tumor~~tumour cells after exposure to cytostatics or ionizing radiation [7, 9]~~–~~. The treatment ~~of fibroblasts~~ with Taxol also resulted in increased primary cilia frequency, reaching a value of 80% after 72h of treatment with 1.25 and 3.25 nM; however, exposure to ~~the~~ higher doses~~ range~~ (5.25, 6.25 and 12.5 nM) resulted in a slightly decreased primary cilia frequency in the treated fibroblasts when compared ~~with~~ to the lower dose range (1.25 and 3.25 nM)~~,~~ but not when compared against the untreated control. It must be highlighted that, unlike the treatment with Doxorubicin, the treatment ~~of fibroblasts~~ with Taxol did not result in multi-ciliated cells.

Once the baseline effect of Doxorubicin and Taxol was determined on healthy cells, we proceeded to test their effect on primary cilia frequency in the triple negative breast cancer cell lines MDA-MB-231 and BT-549.

Unlike fibroblasts, however, the treatment of these cells ~~lines MDA-MB-231 and BT-549~~ with either Doxorubicin (10, 20, 40, 80, and 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25, and 12.5 nM) ~~resulted in the complete absence of~~did not increase the frequency of primary cilia, which was somewhat intriguing.

Based on previous results we were aware that primary cilia are absent or in very low percentage in the triple negative breast cancer cell lines BT-549 and MDA-MB-231 [11, 24, 25]~~-~~. This absence of primary cilia has~~; further, the reason for this absence has~~ been associated with a loss of function mutation in the ~~tumor~~tumour suppressor gene p53 [26]~~-~~. However, the effect that Doxorubicin could have on primary cilia frequency in these cell lines has been heretofore unreported.

For Peer Review

A particular issue in this regard is that multiple primary cilia are associated with an abnormal number of centrosomes in the cell and, according to other unpublished results from our group, it appears that this is a relevant issue in understanding the relation between ciliogenesis and carcinogenesis. Elaborating in this regard, several ~~tumors~~tumours and some ciliopathies [6, 7, 10]~~,~~ possess a characteristic and aberrant number of centrosomes. Multiple centrosomes occur in ~~tumor~~tumour cells in which mutations of the p53, BRCA1 and BRCA2 genes are present [27–29]~~-~~. Often, the surface of cells with multiple centrosomes display~~s~~ an increased number of primary cilia (2-6) with the same structure and similar length. Curiously, the total number of Smo receptors, serotonin type 6 receptors, fibrocystin protein, and Arl13b protein (ADP-ribosylation factor-like protein 13b) present on these multiple cilia remains the same as in single primary cilia. This suggests that since this number remains the same regardless of the number of cilia, the receptor and protein content along the total length of the primary cilia is greater, meaning that the amount of these proteins per unit of length in the primary cilia is smaller in multi-ciliated cases, thus resulting in a lower receptor density. Such dilution in receptor density can lead to weaker ~~signaling~~signalling stimuli [30]~~-~~, which has been observed in the alternative Wnt ~~signaling~~signalling pathways of cells with two or more primary cilia [31]~~-~~.

Primary cilia play an important role in cell growth and tissue homeostasis (Schneider et al. 2005) and, in normal cells, the development of primary cilia is a dynamic process whose formation can occur in either G0/G1 or, more commonly, during the S/G2 phases; however, and almost without exception, the cilium is absorbed before entering mitosis and reappears once again in post-cytokinetic phases of the cell cycle. This periodic cilium absorption is therefore related to the cell cycle and affects cell sensitivity to external signals associated with cilia receptors [32]~~-~~. TNBC cells, however, are notorious for their lack of primary cilia, although they can sometimes occur with extremely low frequency [33]~~-~~. Regardless, this low primary cilium frequency cannot be increased under serum starvation conditions in TNBC cells, which has been a proven method in healthy cells [11]~~-~~.

Our study reports the null effect of Doxorubicin or Taxol on the incidence of primary cilia in triple negative breast cancer cell lines BT-549 and MDA-MB-231. Previous reports had only addressed the use of taxanes and their effect on ciliary length in breast cancer cell lines but not of Doxorubicin, which has not been reported elsewhere. Further, we also report the presence of multiple primary cilia in breast fibroblasts induced by Doxorubicin which, to the best of our knowledge, is now reported for the first time. The null effect of these

For Peer Review

1
2
3 cytostatics on primary cilia incidence in the evaluated TNBC cell lines, as opposed to their
4
5 effect on healthy cells, hints at some larger mechanism at play that could be involved with the
6
7 inherent characteristics of malignant cells; Under the observed circumstances, we could
8
9 suggest that primary cilia are responsible for specific cell signaling events and cytoskeletal
10
11 stability after chemotherapy, and could therefore be involved in the survival of TNBC cells;
12 however, these considerations must be addressed further and more in depth before an accurate
13
14 conclusion can be reached. We suggest that this increased primary cilia frequency could be
15
16 used as a tool in other toxicological studies evaluating the toxicity of cytostatic drugs.
17
18
19 **Acknowledgements and Funding**
20
21 This work was supported by the Ministry of Defense of the Czech Republic - long-term
22 organization development plan Medical Aspects of Weapons of Mass Destruction of the
23
24 Faculty of Military Health Sciences, University of Defense; the Ministry of Education, Youth
25
26 and Sport, Czech Republic (Specific Research Project No: SV/FVZ201703) and Progress,
27
28 Q40/06, Q40/09. Thanks also to Daniel Diaz, Ph.D. for his kind assistance in English
29 language revision.
30
31
32
33 **Conflict of Interest**
34 The authors declare no conflicts of interest.
35
36 **AUTHOR CONTRIBUTIONS**
37
38 Alžběta Filipová and Daniel Diaz Garcia wrote the article. Alžběta Filipová performed
39
40 experiments and analyzed the data. Daniel Diaz Garcia edited the article. Aleš Bezrouk and
41
42 Dana Čížková performed experiments and analyzed the data from TEM. Stanislav Filip
43 designed the research. Josef Dvořák, Justin Sturge and Zuzana Šinkorová assisted with the
44
45 design of experiments. All authors read and approved the final manuscript.
46
47
48 **References**
49
50
51 [1] **Pugacheva EN, Jablonski SA, Hartman TR, et al.** HEF1-dependent Aurora A
52 activation induces disassembly of the primary cilium. *Cell* 2007; 129; 1351–63.
53 [2] **Li A, Saito M, Chuang J-Z, et al.** Ciliary transition zone activation of phosphorylated
54 Tctex-1 controls ciliary resorption, S-phase entry and fate of neural progenitors. *Nature*
55 *Cell Biology* 2011; 13; 402–11.
56
57 [3] **Spalluto C, Wilson DI, Hearn T**. Evidence for reciliation of RPE1 cells in late G1
58 phase, and ciliary localisation of cyclin B1. *FEBS Open Bio* 2013; 3; 334–40.
59
60

For Peer Review

[4] **Luesma MJ, Cantarero I, Castiella T, et al.** Enteric neurons show a primary cilium. *J. Cell. Mol. Med.* 2013; 17; 147–53.

[5] **Malone AMD, Anderson CT, Tummala P, et al.** Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104; 13325–30.

[6] **Nigg EA**. Origins and consequences of centrosome aberrations in human cancers. *Int. J. Cancer* 2006; 119; 2717–23.

[7] **Nigg EA, Čajánek L, Arquint C**. The centrosome duplication cycle in health and disease. *FEBS Lett.* 2014; 588; 2366–72.

[8] **Conroy PC, Saladino C, Dantas TJ, et al.** C-NAP1 and rootletin restrain DNA damage-induced centriole splitting and facilitate ciliogenesis. *Cell Cycle* 2012; 11; 3769–78.

[9] **Filipová A, Diaz-Garcia D, Bezrouk A, et al.** Ionizing radiation increases primary cilia incidence and induces multiciliation in C2C12 myoblasts: Induction of primary cilia by irradiation. *Cell Biology International* 2015; 39; 943–53.

[10] **Lee K, Battini L, Gusella GL**. Cilium, centrosome and cell cycle regulation in polycystic kidney disease. *Biochim. Biophys. Acta* 2011; 1812; 1263–71.

[11] **Yuan K, Frolova N, Xie Y, et al.** Primary cilia are decreased in breast cancer: analysis of a collection of human breast cancer cell lines and tissues. *J. Histochem. Cytochem.* 2010; 58; 857–70.

[12] **Habib JG, O'Shaughnessy JA**. The hedgehog pathway in triple-negative breast cancer. *Cancer Medicine* 2016; 5; 2989–3006.

[13] **Dubuc C, Savard M, Bovenzi V, et al.** Targeting intracellular B2 receptors using novel cell-penetrating antagonists to arrest growth and induce apoptosis in human triple-negative breast cancer. *Oncotarget* 2018; 9; 9885–906.

[14] **Hofman J, Skarka A, Havrankova J, et al.** Pharmacokinetic interactions of breast cancer chemotherapeutics with human doxorubicin reductases. *Biochemical Pharmacology* 2015; 96; 168–78.

[15] **Sharma N, Kosan ZA, Stallworth JE, et al.** Soluble levels of cytosolic tubulin regulate ciliary length control. *Mol. Biol. Cell* 2011; 22; 806–16.

[16] **Bershteyn M, Atwood SX, Woo W-M, et al.** MIM and Cortactin Antagonism Regulates Ciliogenesis and Hedgehog Signaling. *Developmental Cell* 2010; 19; 270–83.

[17] **Kim J, Lee JE, Heynen-Genel S, et al.** Functional genomic screen for modulators of ciliogenesis and cilium length. *Nature* 2010; 464; 1048–51.

[18] **Goranci-Buzhala G, Gabriel E, Mariappan A, et al.** Losers of Primary Cilia Gain the Benefit of Survival. *Cancer Discov* 2017; 7; 1374–5.

[19] **Stinchcombe JC, Salio M, Cerundolo V, et al.** Centriole polarisation to the immunological synapse directs secretion from cytolytic cells of both the innate and adaptive immune systems. *BMC Biology* 2011; 9; 45.

[20] **Dvořák J, Sitorová V, Nikolov HD, et al.** Primární řasinky a jejich biologické funkce 2011; 5; 234–8.

[21] **Liu A, Wang B, Niswander LA**. Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development* 2005; 132; 3103–11.

[22] **Gerhardt C, Leu T, Lier JM, et al.** The cilia-regulated proteasome and its role in the development of ciliopathies and cancer. *Cilia* 2016; 5; 14.

[23] **Giannakakou P, Robey R, Fojo T, et al.** Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. *Oncogene* 2001; 20; 3806–13.


For Peer Review

[24] **Menzl I, Lebeau L, Pandey R, et al.** Loss of primary cilia occurs early in breast cancer development. *Cilia* 2014; 3; 7.
[25] **Hassounah NB, Nunez M, Fordyce C, et al.** Inhibition of Ciliogenesis Promotes Hedgehog Signaling, Tumorigenesis, and Metastasis in Breast Cancer. *Mol. Cancer Res.* 2017; 15; 1421–30.
[26] **Alli E, Yang J-M, Hait WN**. Silencing of stathmin induces tumor-suppressor function in breast cancer cell lines harboring mutant p53. *Oncogene* 2007; 26; 1003–12.
[27] **Fukasawa K, Choi T, Kuriyama R, et al.** Abnormal Centrosome Amplification in the Absence of p53. *Science* 1996; 271; 1744–7.
[28] **Nigg EA**. Centrosome aberrations: cause or consequence of cancer progression? *Nat. Rev. Cancer* 2002; 2; 815–25.
[29] **Tutt A, Gabriel A, Bertwistle D, et al.** Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification. *Curr. Biol.* 1999; 9; 1107–10.
[30] **Mahjoub MR, Stearns T**. Supernumerary centrosomes nucleate extra cilia and compromise primary cilium signaling. *Curr. Biol.* 2012; 22; 1628–34.
[31] **Lancaster MA, Schroth J, Gleeson JG**. Subcellular spatial regulation of canonical Wnt signalling at the primary cilium. *Nat. Cell Biol.* 2011; 13; 700–7.
[32] **Plotnikova OV, Pugacheva EN, Golemis EA**. Primary cilia and the cell cycle. *Methods Cell Biol.* 2009; 94; 137–60.
[33] **Nobutani K, Shimono Y, Yoshida M, et al.** Absence of primary cilia in cell cycle-arrested human breast cancer cells. *Genes Cells* 2014; 19; 141–52.

For Peer Review

**Figure Legends**

**Figure Legends**

**Figure 1.** WST-1 test after 72 hours of treatment with Doxorubicin (10, 20, 40, 80, and 120 nM) in BT-549 (A), MDA-MB-231 (B), and fibroblasts (C). WST-1 test after 72 hours of treatment with Taxol (1.25, 3.25, 5.25, 6.25, and 12.5 nM) in BT-549 (D), MDA-MB-231 (e), and fibroblasts (f) (60x). $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ *vs.* control group.

**Figure 2.** Panel (A) shows the percentage ciliated fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Control cells with primary cilia were observed after 72 hours of culture. (C) Primary cilia were observed 72 hours after treatment with 120 nM Doxorubicin. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (d) Longitudinal section of a primary cilium (arrow) in fibroblast control cells. The cilium emerges from a basal body (open arrows). The proximal region of the cilium is situated within

an invagination of the plasma membrane, a ciliary pocket (arrowheads). n – nucleus (Bar 500 nm). (e) Transverse section of a primary cilium in fibroblasts after treatment with 40 nM Doxorubicin, showing the structure of its axoneme (arrows): nine microtubule doublets arranged at the periphery, an undeveloped central microtubule doublet. Outer plasma membrane of the ciliary pocket surrounding the cilium shaft (arrowheads). n – nucleus (Bar 200 nm). $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ *vs.* control group (60x).

**Figure 3.** Panel (A) shows the percentage of multi-ciliated fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Multiple cilia were observed 72 hours after treatment with 80 nM Doxorubicin. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (C) Transverse section of multiple cilia in fibroblasts after treatment with 80 nM of Doxorubicin reveals the structure of its axoneme (arrows). The outer plasma membrane of the ciliary pocket surrounds the cilium shaft (arrowheads). n – nucleus (Bar 1.5 nm). $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ *vs.* control group. Magnification (60x).

For Peer Review

**Figure 4.** Panel (A) shows the percentage of cells with primary cilia in fibroblasts after treatment with various concentrations of Taxol (1.25, 3.25, 5.25, 6.25 and 12.5 nM). (B) Primary cilia were observed after 72 hours of treatment with 1.25 nM Taxol. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme) and gamma-tubulin (green, basal body), nuclei (blue). (C) TEM image of fibroblasts after treatment with 1.25 nM Taxol shows the transverse section of a primary cilium (arrow), it contains six microtubule doublets arranged at the periphery and one centrally located microtubule doublet. The open arrows depict the outer plasma membrane of the ciliary pocket surrounding the cilium shaft (Bar 0.25 nm) (60x). $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ *vs.* control group.

**Figure 5.** Representative immunofluorescence images of BT-549 and MDA-MB-231 cells: acetylated tubulin (red), nuclei (blue). Primary cilia are absent after 72 hours of treatment with Doxorubicin and Taxol in BT-549 and MDA-MB-231 (60x).

**Figure 1** WST-1 test after 72 hours of treatment with Doxorubicin (10, 20, 40, 80, 120 nM) in BT-549 (A), MDA-MB-231 (B), and fibroblasts (C). WST-1 test after 72 hours of treatment with Taxol (1.25, 3.25, 5.25, 6.25, 12.5 nM) in BT-549 (D), MDA-MB-231 (e), and fibroblasts (f) (60x). $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ *vs.* control group.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

**Figure 2** Panel (A) shows the percentage of ciliated cells in fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Control cells with primary cilia were observed 72 hours of culture. (C) Primary cilia were observed 72 hours after treatment with 120 nM Doxorubicin. Representative images of primary cilia detected by immunofluorescence: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (d) Longitudinal section of a primary cilium (arrow) in fibroblast control cells. The cilium emerges from a basal body (open arrows). The proximal region of the cilium is situated within an invagination of the plasma membrane, a ciliary pocket (arrowheads). n – nucleus (Bar 500 nm). (e) Transverse section of a primary cilium in fibroblasts after treatment with 40 nM Doxorubicin, showing the structure of its axoneme (arrows): nine microtubule doublets arranged at the periphery, an undeveloped central microtubule doublet. Outer plasma membrane of the ciliary pocket surrounding the cilium shaft (arrowheads). n – nucleus (Bar 200 nm). $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ *vs.* control group (60x).

**Figure 3** Panel (A) shows the percentage of multiple cilia in fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Multiple cilia were observed 72 hours after treatment with 80 nM Doxorubicin. Representative images of primary cilia detected by immunofluorescence: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (C) Transverse section of multiple cilia in fibroblasts after treatment with 80 nM of Doxorubicin reveals the structure of its axoneme (arrows). The outer plasma membrane of the ciliary pocket surrounds the cilium shaft (arrowheads). n – nucleus (Bar 1.5 nm). $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ *vs.* control group. Magnification (60x).

**Figure 4** Panel (A) shows the percentage of cells with primary cilia in fibroblasts after treatment with various concentrations of Taxol (1.25, 3.25, 5.25, 6.25 and 12.5 nM). (B) Primary cilia were observed 72 hours after treatment with 1.25 nM Taxol. Representative images of primary cilia detected by immunofluorescence: acetylated tubulin (red, axoneme) and gamma-tubulin (green, basal body), nuclei (blue). (C) TEM image of fibroblasts after treatment with 1.25 nM Taxol shows the transverse section of a primary cilium (arrow), it contains six microtubule doublets arranged at the periphery and one centrally located microtubule doublet. The open arrows depict the outer plasma membrane of the ciliary pocket surrounding the cilium shaft (Bar 0.25 nm) (60x). $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ *vs.* control group.



For Peer Review

1
2
3
4
5
6
7
8

~~**Figure 5** Representative images of immunofluorescent staining of BT-549 and MDA-MB-231 cells: acetylated tubulin (red), nuclei (blue). Primary cilia are absent after 72 hours treatment with Doxorubicin and Taxol in BT-549 and MDA-MB-231 (60x).~~

9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

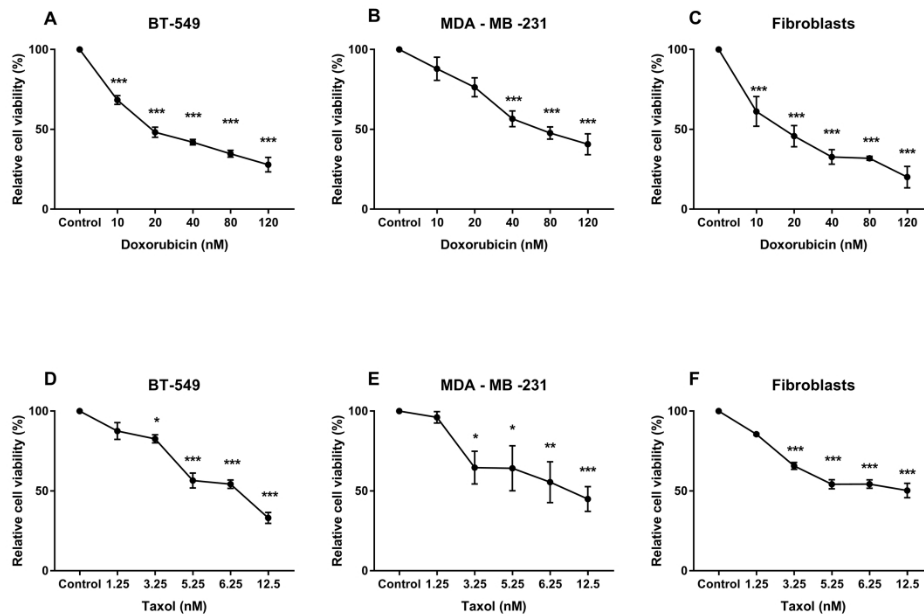
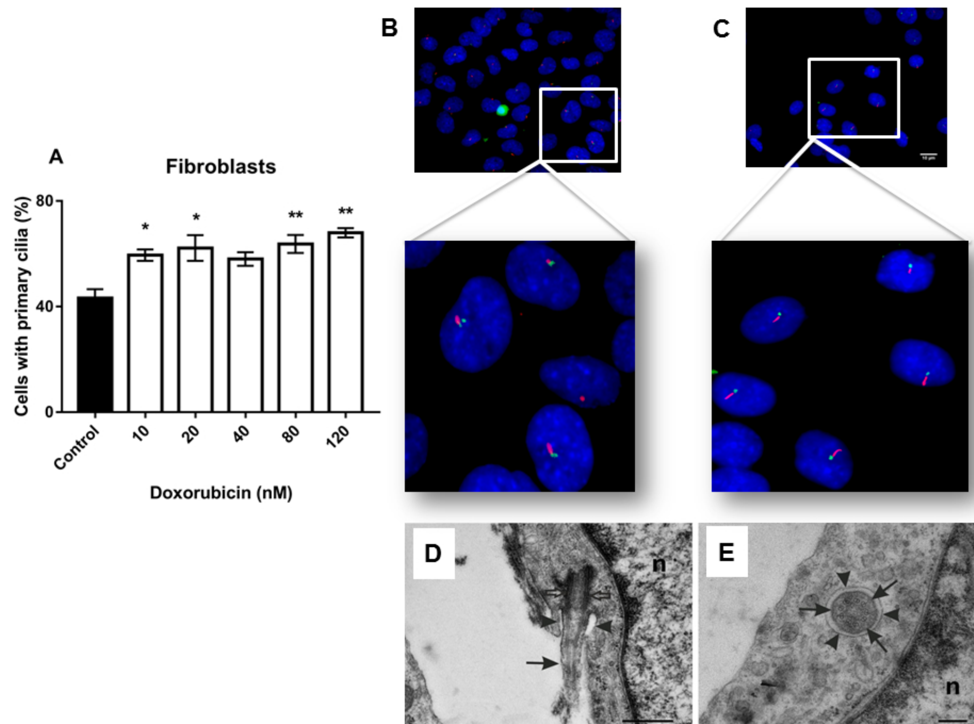



Figure 1 WST-1 test after 72 hours of treatment with Doxorubicin (10, 20, 40, 80, 120 nM) in BT-549 (A), MDA-MB-231 (B), and fibroblasts (C). WST-1 test after 72 hours of treatment with Taxol (1.25, 3.25, 5.25, 6.25, 12.5 nM) in BT-549 (D), MDA-MB-231 (e), and fibroblasts (f) (60x). *P < 0.05 **P < 0.01 ***P < 0.001 *vs*. control group.

266x173mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30




31 Figure 2 Panel (A) shows the percentage of ciliated cells in fibroblasts after treatment with various
32 concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Control cells with primary cilia were
33 observed 72 hours of culture. (C) Primary cilia were observed 72 hours after treatment with 120 nM
34 Doxorubicin. Representative images of primary cilia detected by immunofluorescence: acetylated tubulin
35 (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (d) Longitudinal section of a primary
36 cilium (arrow) in fibroblast control cells. The cilium emerges from a basal body (open arrows). The proximal
37 region of the cilium is situated within an invagination of the plasma membrane, a ciliary pocket
38 (arrowheads). n – nucleus (Bar 500 nm). (e) Transverse section of a primary cilium in fibroblasts after
39 treatment with 40 nM Doxorubicin, showing the structure of its axoneme (arrows): nine microtubule
40 doublets arranged at the periphery, an undeveloped central microtubule doublet. Outer plasma membrane
of the ciliary pocket surrounding the cilium shaft (arrowheads). n – nucleus (Bar 200 nm). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group (60x).

41
42 79x59mm (300 x 300 DPI)

43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

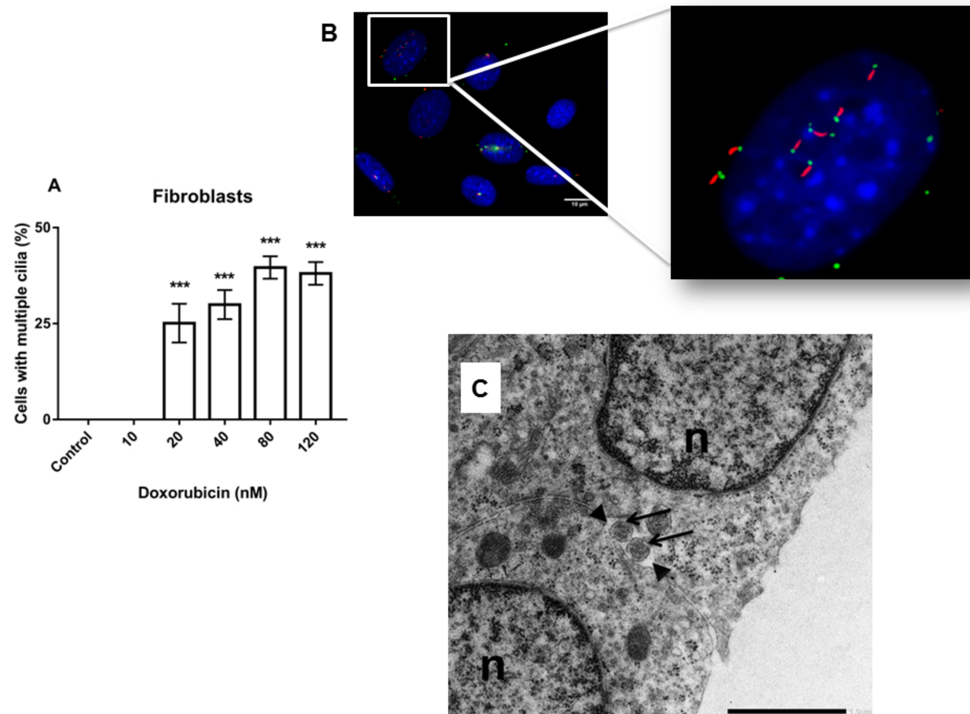
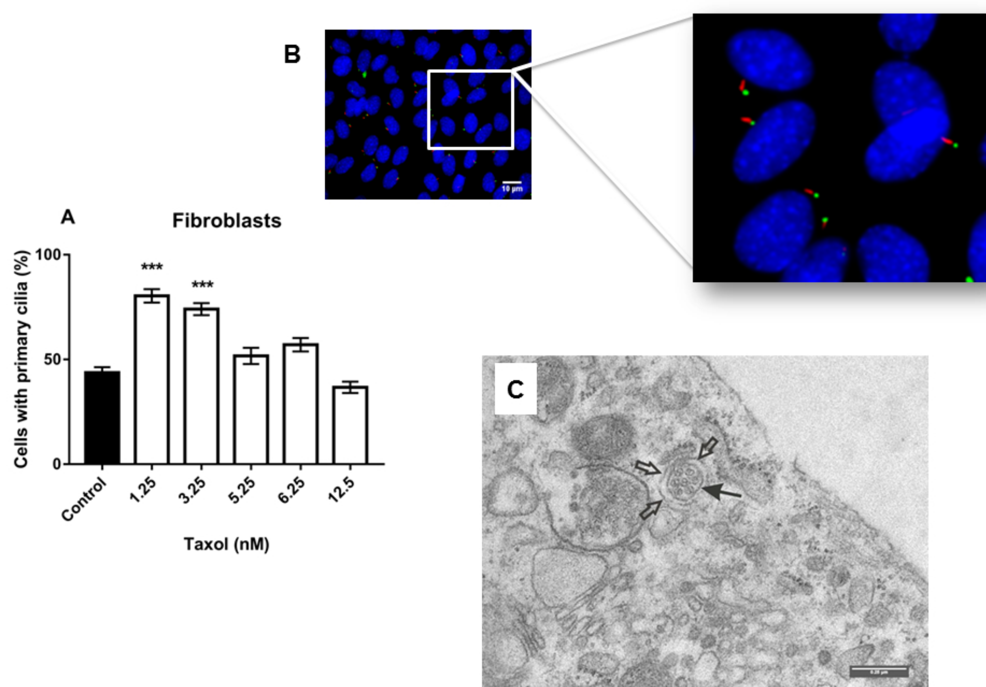



Figure 3 Panel (A) shows the percentage of multiple cilia in fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Multiple cilia were observed 72 hours after treatment with 80 nM Doxorubicin. Representative images of primary cilia detected by immunofluorescence: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (C) Transverse section of multiple cilia in fibroblasts after treatment with 80 nM of Doxorubicin reveals the structure of its axoneme (arrows). The outer plasma membrane of the ciliary pocket surrounds the cilium shaft (arrowheads). n – nucleus (Bar 1.5 nm). *P < 0.05 **P < 0.01 ***P < 0.001 vs. control group. Magnification (60x).

80x60mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29




30 Figure 4 Panel (A) shows the percentage of cells with primary cilia in fibroblasts after treatment with various
31 concentrations of Taxol (1.25, 3.25, 5.25, 6.25 and 12.5 nM). (B) Primary cilia were observed 72 hours after
32 treatment with 1.25 nM Taxol. Representative images of primary cilia detected by immunofluorescence:
33 acetylated tubulin (red, axoneme) and gamma-tubulin (green, basal body), nuclei (blue). (C) TEM image of
34 fibroblasts after treatment with 1.25 nM Taxol shows the transverse section of a primary cilium (arrow), it
35 contains six microtubule doublets arranged at the periphery and one centrally located microtubule doublet.
36 The open arrows depict the outer plasma membrane of the ciliary pocket surrounding the cilium shaft (Bar 0.25 nm) (60x). *P < 0.05 **P < 0.01 ***P < 0.001 vs. control group.
37
38 78x56mm (300 x 300 DPI)
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

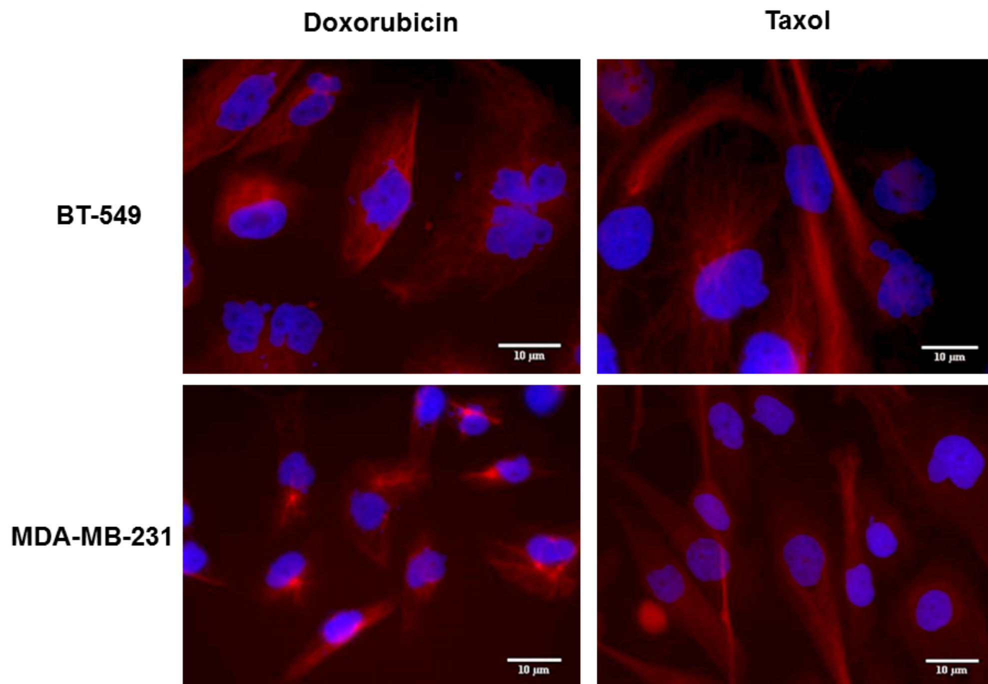



Figure 5 Representative images of immunofluorescent staining of BT-549 and MDA-MB-231 cells: acetylated tubulin (red), nuclei (blue). Primary cilia are absent after 72 hours treatment with Doxorubicin and Taxol in BT-549 and MDA-MB-231 (60x).

76x52mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Author's Response to Decision Letter for (JCMM-03-2019-024)

Primary cilia - an in vitro model to evaluate the toxic effect of cytostatics on cytoskeletal stability.

April 29th, 2019
Hradec Králové, Czech Republic

Dear Editor;

We thank you for your consideration and the reviewers for their input and valuable comments. We have addressed the shortcoming of our manuscript "Primary cilia - an in vitro model to evaluate the toxic effect of cytostatics on cytoskeletal stability" as follows:

Reviewer: 1
Comments to the Author
In the submitted manuscript, the authors claim to use the incidence of ciliated cells as a marker for the effectiveness of cytostatic drugs in the context of chemotherapy, specifically for triple-negative forms of breast cancer.
Unfortunately, the authors do not yet provide sufficient evidence to substantiate or refute their claim. There are at least two major areas of confusion that must be clarified prior to acceptance:

We agree with the reviewer on this point and have modified the text of the manuscript as well as its title so it reflects its content more accurately; the new title of the manuscript is: "The toxic effect of cytostatics on primary cilia frequency and multiciliation". We added that this is the first time that doxorubicin has been used in this context and the first time that multiciliation induced by doxorubicin is reported; also, we clarify that the lack of primary cilia after treatment with cytostatics is rather an interesting observation in TNBC cells which might, or might not, be relevant to their malignant characteristics and needs to be researched further.

1) The connection between ciliogenesis and the cell cycle potentially interferes with the author's conclusions. This shortcoming appears in various places throughout the manuscript, for example a) The section on cell culture has an unclear timeline: how long does ciliogenesis 'normally' take? When was the serum concentration reduced (to promote differentiation and ciliogenesis) if it was reduced? When was the taxol/doxorubicin introduced in the context of ciliogenesis?
There is an undeniable and tight relation between the cell cycle and ciliogenesis that has been described extensively elsewhere. Elaborating a bit further, ciliogenesis usually occurs during the G0/G1 stages of the cell cycle, and sometimes during G2, to be later resorbed during mitosis. To the best of our knowledge, there are no available reports describing the time evolution of primary cilia; further, such

timing was not the aim of the present study but rather to measure the incidence of ciliated cells after treatment with cytostatics. We modified the text so that the experimental treatment of the cells (24 hours after plating) for treatment with cytostatics is clear. Although serum starvation has been the standard in primary cilia study, we did not use it in this study. Also, we are not inducing cell differentiation.

2) Confusion between the cell survival rate and ciliation rate. For example, the authors claim that after treatment, the number of cells that survive *decrease* and the fraction of ciliated cells *increase*, but does that increased fraction account for the overall decrease in cell number? Also, the exclusive use of fixation prior to cilia counting is problematic, as cilia often break during the fixation step- live cell imaging is superior. The authors also state that primary cilia are absent in the cell lines *without* treatment (lines 34-35, page 9) and then make claims about the low fraction of ciliated cells after treatment, which doesn't make sense.

The reviewer is right to some extent in this regard, as it could be confusing; however, this increment in primary cilia incidence is measured in percentage, that is, its numerical value is relative not arithmetic. Regarding fixation, there are previous reports indicating that the use of PFA as a fixative is acceptable in the detection of primary cilia allowing the good resolution of immunofluorescent staining (Biology Open 5:1001-9; 2016) and is not different from other fixation methods when immunolabeling Acetylated-α-tubulin for primary cilia detection like we did in this study (Cilia 6:5; 2017). Regarding the low-fraction of ciliated cells; cancer cells are widely heterogenous and one of their characteristics is that they can be either ciliated or not, depending of the cancer cell type. In this study we used the cell lines BT-549 and MDA-MB-231, which are similar to triple negative breast cancer cells, which are notorious for their low incidence or absent primary cilia. Having said this, we did not detect any primary cilia in the TNBC cell lines, only in breast fibroblasts. The lower percentage of ciliated cells in untreated fibroblasts can be explained by their lack of synchronization, which is then modified under the presence of cytostatics.

That said, the finding that doxorubicin results in multiciliated cells is interesting and deserves additional discussion, perhaps referencing the example of multiciliated airway cells (even though these are motile cilia).

We appreciate the kind opinion of our reviewer and fully agree with him/her in this regard. Having said this, we cannot directly correlate the effect of the cytostatics on multiciliation in the treated cells of this study with naturally multiciliated airway cells, particularly because they serve different purposes, their function is linked with other signaling pathways, and are not structurally similar.

We thank once again to our reviewer and hope the made changes to our manuscript will be sufficient for its publication in your journal. We will happily oblige should further clarification be need on our part.

Best and kind regards,

Prof. Stanislav Filip, MD., Ph.D.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

**The toxic effect of cytostatics on primary cilia frequency and multiciliation.**

**Alžběta Filipová[1*], Daniel Diaz Garcia[2], Aleš Bezrouk[3], Dana Čížková[4], Josef Dvořák[5], Stanislav Filip[6*], Justin Sturge[7], Zuzana Šinkorová[1]**

[1]Department of Radiobiology, Faculty of Military Health Sciences in Hradec Králové, University of Defense, Hradec Králové, Czech Republic;
[2]University Hospital, Department of Clinical Biochemistry, Hradec Králové, Czech Republic.
[3]Charles University, Faculty of Medicine, Department of Medical Biophysics, Hradec Králové, Czech Republic;
[4]Charles University, Faculty of Medicine, Department of Histology and Embryology, Hradec Králové, Czech Republic;
[5]Charles University, Charles University and Thomayer Hospital, Department of Oncology, Prague, Czech Republic;
[6]Charles University, Faculty of Medicine, Department of Oncology and Radiotherapy, Hradec Králové, Czech Republic;
[7]University of Hull, School of Biological, Biomedical and Environmental Sciences, UK;

* Correspondence:

Alžběta Filipová, MSc, PhD.
Department of Radiobiology, Faculty of Military Health Sciences in Hradec Králové, University of Defense,
Třebešská 1575,
Hradec Kralove
500 01
Czech Republic
alzbeta.filipova@unob.cz

Prof. Stanislav Filip, MD, PhD, DSc.
Charles University, Medical Faculty and University Hospital, Department of Oncology and Radiotherapy
Sokolská 548
500 05 Hradec Králové
Czech Republic
stanislav.filip@fnhk.cz

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60


# Abstract

The primary cilium is considered as a key component of morphological cellular stability. However, cancer cells are notorious for lacking primary cilia in most cases, depending upon the tumour type. Previous reports have shown the effect of starvation and cytostatics on ciliogenesis in normal and cancer cells although with limited success, especially when concerning the latter. In this study we evaluated the presence and frequency of primary cilia in breast fibroblasts and in triple negative breast cancer cells after treatment with cytostatics finding that, in the case of breast fibroblasts, primary cilia were detected at their highest incidence 72 hours after treatment with 120 nM doxorubicin. Further, multiciliated cells were also detected after treatment with 80 nM doxorubicin. On the other hand, treatment with taxol increased the number of ciliated cells only at low concentrations (1.25 and 3.25 nM) and did not induce multiciliation. Interestingly, triple negative breast cancer cells did not present primary cilia after treatment with either doxorubicin or taxol. This is the first study reporting presence of multiple primary cilia in breast fibroblasts induced by Doxorubicin. However, the null effect of these cytostatics on primary cilia incidence in the evaluated TNBC cell lines requires further research.


**Key words:** Primary cilium, multiple cilia, cytostatics, toxicity, cell line .

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

# 1 Introduction

Primary cilium, an organelle found on nearly every cell in the human body, typically serves as a mechanical sensory tool; further, it is also involved in cell proliferation and embryonic development. This organelle is dynamically regulated during the cell cycle, appearing during the G0/G1 phases and resorbed prior to mitosis [1] . The exact solubilization moment of primary cilia is determined by cell type and the expression of genes affecting resorption, such as Aurora A, Plk1, TcTex-1 [2, 3] . Usually, a cell has only one primary cilium which, as previously mentioned, is involved in morphogenesis, cell proliferation and differentiation signalling [4, 5] . Should there be a multiplication of centrosomes, a higher number of primary cilia will appear on the surface of cells as well, often bearing the same length and construction design, and spawning from the same ciliary pocket; however, the presence of multiple cilia has been mostly recorded in solid tumours [6, 7] after exposure to ionizing irradiation [8, 9] or in ciliopathies [10] . Typically, primary cilia are always observed in myoepithelial cells and fibroblasts and with low incidence in luminal epithelium cells. Regarding cancer cells, a study of 26 breast cancer biopsy samples revealed the presence of primary cilia only on exceptional cases, especially in epithelial cells [11] .

Basal subtype B tumours, a classification of triple negative breast carcinomas (TNBC), is characterized by the absence of oestrogen, progesterone, and Her2/neu receptors [12], as well as by the rare presence of primary cilia. Among breast cancer tumours, TNBC has an estimated incidence of 10-20%, although from a histological point of view TNBCs are little differentiated and are often included in basal-like subgroups. From a clinical standpoint, these tumours are frequently resistant to treatment, have quick progression, low 5-year survival rate, increased local recurrence, and are highly metastatic. This kind of tumours can be observed at any age; however, they mostly occur accompanied by BRCA1 mutations in younger women (>40 years of age) [13]. Chemotherapy is the treatment of choice for triple negative breast cancer patients, of which Doxorubicin and Taxol are the standard chemotherapeutic agents used as anticancer therapy in combination with α-HER2/neu receptor targeted therapy. Doxorubicin belongs to the anthracyclines group, whereas Taxol is considered as a taxane. The former is an effective intercalating cytotoxic agent used in the treatment of various tumour types and commonly used in combination with the latter in adjuvant and neoadjuvant therapeutic strategies for breast cancer patients [14] .

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Previous research on the effect of cytostatics on primary cilia has focused in the effects of Taxol over the elongation and shortening of primary cilia. In a study by Sharma et al., Taxol was shown to block the emergence of primary cilia in mammalian cell cultures [15]. However, low concentrations result in an increased quantity of free tubulin subunits in the cytosol, leading to enlarged primary cilia [16, 17]. Ongoing research highlights two important questions in the cell biology of cancer and primary cilia: 1) the significance of having primary cilia in normal cells, and 2) the loss of primary cilia in cancer cells and its relation to drug resistance [18] . Therefore, the increased primary cilia frequency induced by cytostatics could be used in other studies trying to assess the toxicity of these drugs.

For Peer Review

# 2 Material and methods

## 2.1 Cell culture

Unless otherwise stated, all standard chemicals and antibodies were purchased from Sigma-Aldrich, Czech Republic. In this study, TNBC cell lines BT-549 (ATCC, USA) and MDA-MB-231 were used (kindly supplied by Mgr. Jaroslav Truksa, Ph.D., Laboratory of Tumor Resistance, Institute of Biotechnology CAS, Prague), as well as skin fibroblasts. BT-549 cells were cultured in DMEM 10% FBS (PAA, USA), 2% glutamine (Gibco, UK), 1% penicillin/streptomycin (Gibco, UK), 0.023 IU/ml insulin and incubated in a 5% $CO_2$ atmosphere at 37°C. MDA-MB-231 and fibroblasts were cultured in DMEM, 10% FBS (PAA, USA), 2% glutamine (Gibco, UK), 1% penicillin/streptomycin (Gibco, UK) and incubated in a 5% $CO_2$ atmosphere at 37°C. All cell lines were used until 10$^{th}$ to 12$^{th}$ passage and medium was replaced every two days for all experiments.

## 2.2 Skin fibroblasts

Human fibroblasts were isolated from skin biopsies obtained in accordance and approved by the Ethics Committee of the University Hospital Hradec Kralove, Czech Republic and the European Ethics committee under the directive approved on 10$^{th}$ July 2014 (Reference/license number: 201407 S12P). Donor patients signed an informed consent allowing us to work with the obtained samples. To isolate the fibroblasts, skin biopsies were kept in a basic solution (30 ml/2g of tissue) after surgery and transported to the laboratory, where the biopsies were washed in PBS and the subcutaneous tissue was removed. Each individual tissue sample was incubated in 5 ml of a 2U/ml dispase solution (Gibco, UK) for 18 hours at 4°C. After incubation, the

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

samples were washed 3 times in PBS at room temperature (22°C). The dermis was separated from the epidermis using tweezers, washed in PBS and cut into small sections (<5mm$^2$). Afterwards, the tissue was incubated in 10 ml of digestion solution (1g tissue/10 ml) in a rotating incubator at 37°C/6 *g*. After 4 hours, the samples were filtered through a 40 µm strainer into a new sterile 50 ml tube. The cell number was determined from this suspension and centrifuged for 10 min/150 *g*. The supernatant was decanted and the cell pellet was resuspended in culture media, plated in a T-75 flask (1x10$^5$ cells per flask) and incubated in a 5% $CO_2$ atmosphere at 37°C for 14 days.

## 2.3 Cytostatic drugs

Doxorubicin and Taxol were dissolved in 0.5% DMSO and kept in 1 mM stock solutions. Doxorubicin and Taxol were diluted in culture media before use at a ratio of 1:100 and 1:1000, respectively.

## 2.4 Cell treatment and Immunofluorescence

MDA-MB-231, BT-549, and fibroblasts cells were cultured in 6-well plates at a density of 3 x 10$^5$ cells per well and incubated at 37°C/5% $CO_2$ for 24h, each well contained a gelatine coated coverslip. After this period, the cells were treated with Doxorubicin (10, 20, 40, 80 and 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25, and 12.5 nM) for 72 hours. Control cells were kept under the same conditions in culture media with or without DMSO (0.5%). After treatment, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed three times with PBS. Immunostaining was performed as follows: cells were blocked with goat serum (Jackson Immunoresearch, USA) 1:20 for 30 minutes; anti-acetylated tubulin 1:800 for 1 hour; anti-gamma tubulin 1:500 for 1 hour; Cy3-conjugated donkey anti-mouse secondary antibody 1:300 for 45 minutes in the dark; Alexa 488 conjugated donkey anti-rabbit secondary antibody (JacksonImmunoresearch, USA) 1:300 for 45 min in the dark. Imaging analysis was performed using a Nikon Eclipse fluorescence microscope (Prague, Czech Republic) observed with an oil-immersion 60X objective. All experiments were performed in triplicate.

## 2.5 Cytotoxicity test

A WST-1 test (Roche; Basel, Switzerland) was used to determine cell viability after treatment with Doxorubicin (10, 20, 40, 80, 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25, 12.5 nM). All cell lines were plated and treated in a 96-well-plate (1 x 10$^3$ cells per well), and incubated at 37°C/5%$CO_2$ for 24h. After 72 h, 10µL of WST-1 reagent was added and incubated for 3 h at

For Peer Review

37°C/5% $CO_2$ and analysed in a Tecan SpectraFluor Plus spectrometer (Tecan Austria GmbH; Grödig, Austria) at a wavelength of 440 nm. All experiments were performed in triplicate.

### 2.6 Transmission electron microscopy (TEM)

Fibroblasts were fixed in 3% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.2) for 5 min. at 37°C and then for 3 h at room temperature, washed in cacodylate buffer (0.1 M, pH 7.2) and post-fixed in 1% OsO4 (in 0.1 M cacodylate buffer, pH 7.2) for 1 h at room temperature. After rinsing in cacodylate buffer (0.1 M, pH 7.2), the cells were dehydrated in graded alcohols (50%, 75%, 96%, 100%), cleared in propylene oxide and embedded in a mixture of Epon 812 and Durcupan (Sigma; polymerization for 3 days at 60°C). Semi-thin sections were stained with toluidine blue. Ultra-thin sections were cut on an Ultrotome Nova (LKB, Sweden), mounted into formvar carbon-coated copper grids, counterstained with uranyl acetate and lead citrate and examined in a JEOL JEM-1400Plus transmission electron microscope (at 120 kV JEOL, Japan). Images were captured with integrated 8Mpix CCD camera and software (JEOL, Japan).

### 2.7 Statistical analysis

Graphs were made using the GraphPad Prism 6 biostatistics software (GraphPad Software, USA). The statistical analysis between the evaluated groups was performed using one-way ANOVA followed by a post-hoc Tukey test ($p<0.05$).

## 3 Results

### 3.1 Proliferation and viability of BT-549, MDA-MB-231, and fibroblasts after treatment with Doxorubicin and Taxol

Cytostatics are known inhibitors of cell viability and proliferation, therefore we had to determine a suitable concentration in which the cells would stop proliferating without losing viability. To achieve this purpose, the MDA-MB-231, BT-549 and fibroblast cells were maintained with Doxorubicin or Taxol at various concentrations (10, 20, 40, 80 and 120 nM and 1.25, 3.25, 5.25, 6.25 and 12.5 nM, respectively), followed by a WST-1 test after 72 hours of treatment. The number of living cells was significantly lower after Doxorubicin (Figure 1A-C) and Taxol (Figure 1D-F) treatment across all cell lines.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

### 3.2 Primary cilia incidence and multi-ciliation induced by the cytostatics Doxorubicin and Taxol

Regarding Doxorubicin, the number of fibroblasts with primary cilia increased to ~70%, in comparison with control untreated cells, after 72 hours of treatment with various concentrations of this cytostatic (10, 20, 40, 80 and 120 nM), observing a higher incidence after treatment with 120 nM Doxorubicin. Overall, this effect could be observed evenly across the entire dose range used in this study (Figure 2A). After 72 hours of treatment, primary cilia were detected by immunostaining (Figure 2B – control cells; Figure 2C – 120nM Doxorubicin) and electron microscopy (Figure 2D – control; Figure 2E – 120 nM Doxorubicin). Interestingly, ~20-40% of fibroblasts treated with 20-120 nM Doxorubicin showed two or more cilia after 72 hours of treatment, observing a higher number of multi-ciliated cells at a dose of 80 nM Doxorubicin (~40%; Figure 3A). However, no multi-ciliated cells could be observed after treatment with 10 nM Doxorubicin or in untreated cells (Figure 3a). Multiple primary cilia were detected by immunostaining (Figure 3B) and electron microscopy (Figure 3C) after 72 hours of treatment.

Concerning Taxol, the treatment of fibroblasts with various concentrations (1.25, 3.25, 5.25, 6.25 and 12.5 nM) for 72 hours revealed a significantly higher incidence of primary cilia after treatment with 1.25 and 3.25 nM Taxol (~80%) (Figure 4A), as detected by immunostaining (Figure 4B) and electron microscopy (Figure 4C). However, this incidence rate was slightly decreased in the cells within the higher concentration range (5.25, 6.25 and 12.5 nM), although without change when compared to the untreated cells.

Interestingly, a complete absence of primary cilia was noted in MDA-MB-231 cells after treatment with either Doxorubicin (10, 20, 40, 80 and 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25 and 12.5 nM) for 72 hours, an effect that was repeated in BT-549 cells (Figure 5).

For Peer Review

# 4 Discussion

In the present work we sought to determine the effect that the cytostatics Doxorubicin and Taxol could have on primary cilia incidence in triple negative breast cancer cells. We chose these cytostatics because they are commonly used in the treatment of breast cancer. On one hand, Doxorubicin (DOX) acts by intercalating into the DNA strands inhibiting topoisomerase II activity and thus inducing strand breaks when DNA is being replicated; in addition, it also promotes the formation of reactive oxygen species (ROS), which are highly toxic. Taxol, on the other hand, inhibits microtubule depolymerization [19], resulting in shorter primary cilia

1
2
3 and affecting their frequency in the exposed cells [15] ; therefore, it was included as a negative
4
5 control drug in our experiments.
6
7 Solid tumours possess a characteristic absence or low incidence of primary cilia which,
8
9 when present, may also have a compromised structure and function. However, some tumour
10
11 types that are dependent upon the Hedgehog (Hh) signalling pathway often have an increased
12 frequency of primary cilia [20, 21]. Further, several types of tumours have been associated with
13
14 altered Hh, Wnt, NOTCH and Hippo signalling pathways, which are related to primary cilia;
15
16 therefore, compromised signal transduction could also be caused by defects in the formation,
17 structure or function of primary cilia [22] .
18
19 Primary cilia normally occur in approximately 70% of fibroblasts and from 7 to 19% of
20
21 epithelial cells from healthy breast tissue [11] . However, the study of 11 breast cancer cell lines
22
23 revealed that primary cilia were only present in 4 of these and at the very low frequency of
24 0.3% - 4%; curiously, these cell lines had the shared characteristic of being basal B subtypes,
25
26 which are analogous to triple negative breast cancer cells [11]. Regardless, primary cilia have
27
28 been found in some cases of TNBC, which hints at the existence of several TNBC subtypes in
29 which the reason for this exclusive presence of primary cilia is yet unclear. A possible
30
31 explanation could be that these tumour subtypes are dependent upon the Hh signalling pathway
32
33 and hence upon primary cilia, as it has been observed in most basal cell carcinomas and
34 medulloblastomas [22] .
35
36 In the present study, the chemosensitive triple negative breast cancer cell lines BT-549
37
38 and MDA-MB-231 were used, in addition to normal skin fibroblasts as a comparative control,
39
40 to test the effect that these cytostatics could have on primary cilia incidence. The cell lines were
41 treated with 10, 20, 40, 80 and 120 nM Doxorubicin and 1.25, 3.25, 5.25, 6.25 and 12.5 nM
42
43 Taxol for 72 hours to determine the dose in which the cells would become affected by the
44
45 cytostatic drug without compromising viability or inducing cell death. Accordingly, the number
46 of viable cells decreased with an increased concentration of cytostatics.
47
48 Regarding Taxol, the included cell lines showed consistently decreasing proliferation
49
50 and viability as the dosage of Taxol increased; therefore, low doses of 1.25, 3.25, 6.25 and 12.5
51
52 nM Taxol were chosen based on previous experiments [23] .
53
54 In our study, fibroblasts were treated with the mentioned dose range of Doxorubicin and
55 Taxol to establish their baseline effect on ciliogenesis. Concerning Doxorubicin, an even
56
57 increment in primary cilia frequency was observed across the full dose range (10, 20, 40, 80
58
59 and 120 nM) after 72h of treatment, reaching a maximum value of 70% after under a 120 nM
60



For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

dose. Interestingly, 20 to 40% of fibroblasts showed two or more cilia after treatment with doses of 20-120 nM, reaching the maximum value of 40% multiciliated cells after treatment with 80 nM. It must be mentioned that multiciliated cells also were in possession of multiple centrosomes, an effect that has also been observed in some tumour cells after exposure to cytostatics or ionizing radiation [7, 9]. The treatment with Taxol also resulted in increased primary cilia frequency, reaching a value of 80% after 72h of treatment with 1.25 and 3.25 nM; however, exposure to higher doses (5.25, 6.25 and 12.5 nM) resulted in a slightly decreased primary cilia frequency in the treated fibroblasts when compared to the lower dose range (1.25 and 3.25 nM) but not when compared against the untreated control. It must be highlighted that, unlike the treatment with Doxorubicin, the treatment with Taxol did not result in multiciliated cells.

For Peer Review

Once the baseline effect of Doxorubicin and Taxol was determined on healthy cells, we proceeded to test their effect on primary cilia frequency in the triple negative breast cancer cell lines MDA-MB-231 and BT-549. Unlike fibroblasts, however, the treatment of these cells with either Doxorubicin (10, 20, 40, 80, and 120 nM) or Taxol (1.25, 3.25, 5.25, 6.25, and 12.5 nM) did not increase the frequency of primary cilia, which was somewhat intriguing.

Based on previous results we were aware that primary cilia are absent or in very low percentage in the triple negative breast cancer cell lines BT-549 and MDA-MB-231 [11, 24, 25]. This absence of primary cilia has been associated with a loss of function mutation in the tumour suppressor gene p53 [26]. However, the effect that Doxorubicin could have on primary cilia frequency in these cell lines has been heretofore unreported.

A particular issue in this regard is that multiple primary cilia are associated with an abnormal number of centrosomes in the cell and, according to other unpublished results from our group, it appears that this is a relevant issue in understanding the relation between ciliogenesis and carcinogenesis. Elaborating in this regard, several tumours and some ciliopathies [6, 7, 10] possess a characteristic and aberrant number of centrosomes. Multiple centrosomes occur in tumour cells in which mutations of the p53, BRCA1 and BRCA2 genes are present [27–29]. Often, the surface of cells with multiple centrosomes display an increased number of primary cilia (2-6) with the same structure and similar length. Curiously, the total number of Smo receptors, serotonin type 6 receptors, fibrocystin protein, and Arl13b protein (ADP-ribosylation factor-like protein 13b) present on these multiple cilia remains the same as in single primary cilia. This suggests that since this number remains the same regardless of the number of cilia, the receptor and protein content along the total length of the primary cilia is

1
2
3 greater, meaning that the amount of these proteins per unit of length in the primary cilia is
4
5 smaller in multiciliated cases, thus resulting in a lower receptor density. Such dilution in
6
7 receptor density can lead to weaker signalling stimuli [30], which has been observed in the
8
9 alternative Wnt signalling pathways of cells with two or more primary cilia [31].
10 Primary cilia play an important role in cell growth and tissue homeostasis (Schneider et
11
12 al. 2005) and, in normal cells, the development of primary cilia is a dynamic process whose
13
14 formation can occur in either G0/G1 or, more commonly, during the S/G2 phases; however,
15
16 and almost without exception, the cilium is absorbed before entering mitosis and reappears once
17 again in post-cytokinetic phases of the cell cycle. This periodic cilium absorption is therefore
18
19 related to the cell cycle and affects cell sensitivity to external signals associated with cilia
20 receptors [32]. TNBC cells, however, are notorious for their lack of primary cilia, although they
21
22 can sometimes occur with extremely low frequency [33]. Regardless, this low primary cilium
23
24 frequency cannot be increased under serum starvation conditions in TNBC cells, which has
25
26 been a proven method in healthy cells [11].
27 Our study reports the null effect of Doxorubicin or Taxol on the incidence of primary
28
29 cilia in triple negative breast cancer cell lines BT-549 and MDA-MB-231. Previous reports had
30
31 only addressed the use of taxanes and their effect on ciliary length in breast cancer cell lines
32
33 but not of Doxorubicin, which has not been reported elsewhere. Further, we also report the
34
35 presence of multiple primary cilia in breast fibroblasts induced by Doxorubicin which, to the
36 best of our knowledge, is now reported for the first time. The null effect of these cytostatics on
37
38 primary cilia incidence in the evaluated TNBC cell lines, as opposed to their effect on healthy
39
40 cells, hints at some larger mechanism at play that could be involved with the inherent
41
42 characteristics of malignant cells; however, these considerations must be addressed further and
43 more in depth before an accurate conclusion can be reached.
44
45
46 **Acknowledgements and Funding**
47
48 This work was supported by the Ministry of Defense of the Czech Republic - long-term
49
50 organization development plan Medical Aspects of Weapons of Mass Destruction of the Faculty
51
52 of Military Health Sciences, University of Defense; the Ministry of Education, Youth and Sport,
53 Czech Republic (Specific Research Project No: SV/FVZ201703) and Progress, Q40/06,
54
55 Q40/09. Thanks also to Daniel Diaz, Ph.D. for his kind assistance in English language revision.
56
57
58 **Conflict of Interest**
59
60

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The authors declare no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

Alžběta Filipová and Daniel Diaz Garcia wrote the article. Alžběta Filipová performed experiments and analyzed the data. Daniel Diaz Garcia edited the article. Aleš Bezrouk and Dana Čížková performed experiments and analyzed the data from TEM. Stanislav Filip designed the research. Josef Dvořák, Justin Sturge and Zuzana Šinkorová assisted with the design of experiments. All authors read and approved the final manuscript.

**References**


[1] **Pugacheva EN, Jablonski SA, Hartman TR, et al.** HEF1-dependent Aurora A activation induces disassembly of the primary cilium. *Cell* 2007; 129; 1351–63.

[2] **Li A, Saito M, Chuang J-Z, et al.** Ciliary transition zone activation of phosphorylated Tctex-1 controls ciliary resorption, S-phase entry and fate of neural progenitors. *Nature Cell Biology* 2011; 13; 402–11.

[3] **Spalluto C, Wilson DI, Hearn T**. Evidence for reciliation of RPE1 cells in late G1 phase, and ciliary localisation of cyclin B1. *FEBS Open Bio* 2013; 3; 334–40.

[4] **Luesma MJ, Cantarero I, Castiella T, et al.** Enteric neurons show a primary cilium. *J. Cell. Mol. Med.* 2013; 17; 147–53.

[5] **Malone AMD, Anderson CT, Tummala P, et al.** Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104; 13325–30.

[6] **Nigg EA**. Origins and consequences of centrosome aberrations in human cancers. *Int. J. Cancer* 2006; 119; 2717–23.

[7] **Nigg EA, Čajánek L, Arquint C**. The centrosome duplication cycle in health and disease. *FEBS Lett.* 2014; 588; 2366–72.

[8] **Conroy PC, Saladino C, Dantas TJ, et al.** C-NAP1 and rootletin restrain DNA damage-induced centriole splitting and facilitate ciliogenesis. *Cell Cycle* 2012; 11; 3769–78.

[9] **Filipová A, Diaz-Garcia D, Bezrouk A, et al.** Ionizing radiation increases primary cilia incidence and induces multiciliation in C2C12 myoblasts: Induction of primary cilia by irradiation. *Cell Biology International* 2015; 39; 943–53.

[10] **Lee K, Battini L, Gusella GL**. Cilium, centrosome and cell cycle regulation in polycystic kidney disease. *Biochim. Biophys. Acta* 2011; 1812; 1263–71.

[11] **Yuan K, Frolova N, Xie Y, et al.** Primary cilia are decreased in breast cancer: analysis of a collection of human breast cancer cell lines and tissues. *J. Histochem. Cytochem.* 2010; 58; 857–70.

[12] **Habib JG, O'Shaughnessy JA**. The hedgehog pathway in triple-negative breast cancer. *Cancer Medicine* 2016; 5; 2989–3006.

[13] **Dubuc C, Savard M, Bovenzi V, et al.** Targeting intracellular B2 receptors using novel cell-penetrating antagonists to arrest growth and induce apoptosis in human triple-negative breast cancer. *Oncotarget* 2018; 9; 9885–906.

[14] **Hofman J, Skarka A, Havrankova J, et al.** Pharmacokinetic interactions of breast cancer chemotherapeutics with human doxorubicin reductases. *Biochemical Pharmacology* 2015; 96; 168–78.


For Peer Review

[15] **Sharma N, Kosan ZA, Stallworth JE, et al.** Soluble levels of cytosolic tubulin regulate ciliary length control. *Mol. Biol. Cell* 2011; 22; 806–16.
[16] **Bershteyn M, Atwood SX, Woo W-M, et al.** MIM and Cortactin Antagonism Regulates Ciliogenesis and Hedgehog Signaling. *Developmental Cell* 2010; 19; 270–83.
[17] **Kim J, Lee JE, Heynen-Genel S, et al.** Functional genomic screen for modulators of ciliogenesis and cilium length. *Nature* 2010; 464; 1048–51.
[18] **Goranci-Buzhala G, Gabriel E, Mariappan A, et al.** Losers of Primary Cilia Gain the Benefit of Survival. *Cancer Discov* 2017; 7; 1374–5.
[19] **Stinchcombe JC, Salio M, Cerundolo V, et al.** Centriole polarisation to the immunological synapse directs secretion from cytolytic cells of both the innate and adaptive immune systems. *BMC Biology* 2011; 9; 45.
[20] **Dvořák J, Sitorová V, Nikolov HD, et al.** Primární řasinky a jejich biologické funkce 2011; 5; 234–8.
[21] **Liu A, Wang B, Niswander LA**. Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development* 2005; 132; 3103–11.
[22] **Gerhardt C, Leu T, Lier JM, et al.** The cilia-regulated proteasome and its role in the development of ciliopathies and cancer. *Cilia* 2016; 5; 14.
[23] **Giannakakou P, Robey R, Fojo T, et al.** Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. *Oncogene* 2001; 20; 3806–13.
[24] **Menzl I, Lebeau L, Pandey R, et al.** Loss of primary cilia occurs early in breast cancer development. *Cilia* 2014; 3; 7.
[25] **Hassounah NB, Nunez M, Fordyce C, et al.** Inhibition of Ciliogenesis Promotes Hedgehog Signaling, Tumorigenesis, and Metastasis in Breast Cancer. *Mol. Cancer Res.* 2017; 15; 1421–30.
[26] **Alli E, Yang J-M, Hait WN**. Silencing of stathmin induces tumor-suppressor function in breast cancer cell lines harboring mutant p53. *Oncogene* 2007; 26; 1003–12.
[27] **Fukasawa K, Choi T, Kuriyama R, et al.** Abnormal Centrosome Amplification in the Absence of p53. *Science* 1996; 271; 1744–7.
[28] **Nigg EA**. Centrosome aberrations: cause or consequence of cancer progression? *Nat. Rev. Cancer* 2002; 2; 815–25.
[29] **Tutt A, Gabriel A, Bertwistle D, et al.** Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification. *Curr. Biol.* 1999; 9; 1107–10.
[30] **Mahjoub MR, Stearns T**. Supernumerary centrosomes nucleate extra cilia and compromise primary cilium signaling. *Curr. Biol.* 2012; 22; 1628–34.
[31] **Lancaster MA, Schroth J, Gleeson JG**. Subcellular spatial regulation of canonical Wnt signalling at the primary cilium. *Nat. Cell Biol.* 2011; 13; 700–7.
[32] **Plotnikova OV, Pugacheva EN, Golemis EA**. Primary cilia and the cell cycle. *Methods Cell Biol.* 2009; 94; 137–60.
[33] **Nobutani K, Shimono Y, Yoshida M, et al.** Absence of primary cilia in cell cycle-arrested human breast cancer cells. *Genes Cells* 2014; 19; 141–52.

**Figure and Legends**

**Figure 1.** WST-1 test after 72 hours of treatment with Doxorubicin (10, 20, 40, 80, and 120



For Peer Review

nM) in BT-549 (A), MDA-MB-231 (B), and fibroblasts (C). WST-1 test after 72 hours of treatment with Taxol (1.25, 3.25, 5.25, 6.25, and 12.5 nM) in BT-549 (D), MDA-MB-231 (e), and fibroblasts (f) (60x). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group.

**Figure 2.** Panel (A) shows the percentage ciliated fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Control cells with primary cilia were observed after 72 hours of culture. (C) Primary cilia were observed 72 hours after treatment with 120 nM Doxorubicin. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (d) Longitudinal section of a primary cilium (arrow) in fibroblast control cells. The cilium emerges from a basal body (open arrows). The proximal region of the cilium is situated within an invagination of the plasma membrane, a ciliary pocket (arrowheads). n – nucleus (Bar 500 nm). (e) Transverse section of a primary cilium in fibroblasts after treatment with 40 nM Doxorubicin, showing the structure of its axoneme (arrows): nine microtubule doublets arranged at the periphery, an undeveloped central microtubule doublet. Outer plasma membrane of the ciliary pocket surrounding the cilium shaft (arrowheads). n – nucleus (Bar 200 nm). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group (60x).

**Figure 3.** Panel (A) shows the percentage of multi-ciliated fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Multiple cilia were observed 72 hours after treatment with 80 nM Doxorubicin. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (C) Transverse section of multiple cilia in fibroblasts after treatment with 80 nM of Doxorubicin reveals the structure of its axoneme (arrows). The outer plasma membrane of the ciliary pocket surrounds the cilium shaft (arrowheads). n – nucleus (Bar 1.5 nm). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group. Magnification (60x).

**Figure 4.** Panel (A) shows the percentage of cells with primary cilia in fibroblasts after treatment with various concentrations of Taxol (1.25, 3.25, 5.25, 6.25 and 12.5 nM). (B) Primary cilia were observed after 72 hours of treatment with 1.25 nM Taxol. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme) and gamma-tubulin (green, basal body), nuclei (blue). (C) TEM image of fibroblasts after treatment with 1.25 nM Taxol shows the transverse section of a primary cilium (arrow), it contains six

For Peer Review

microtubule doublets arranged at the periphery and one centrally located microtubule doublet. The open arrows depict the outer plasma membrane of the ciliary pocket surrounding the cilium shaft (Bar 0.25 nm) (60x). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group.

**Figure 5.** Representative immunofluorescence images of BT-549 and MDA-MB-231 cells: acetylated tubulin (red), nuclei (blue). Primary cilia are absent after 72 hours of treatment with Doxorubicin and Taxol in BT-549 and MDA-MB-231 (60x).

**xxxxxxx**~~treatment with Taxol (1.25, 3.25, 5.25, 6.25, and 12.5 nM) in BT-549 (D), MDA-MB-231 (e), and fibroblasts (f) (60x). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group.~~

~~**Figure 2.** Panel (A) shows the percentage ciliated fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Control cells with primary cilia were observed after 72 hours of culture. (C) Primary cilia were observed 72 hours after treatment with 120 nM Doxorubicin. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (d) Longitudinal section of a primary cilium (arrow) in fibroblast control cells. The cilium emerges from a basal body (open arrows). The proximal region of the cilium is situated within an invagination of the plasma membrane, a ciliary pocket (arrowheads). n – nucleus (Bar 500 nm). (e) Transverse section of a primary cilium in fibroblasts after treatment with 40 nM Doxorubicin, showing the structure of its axoneme (arrows): nine microtubule doublets arranged at the periphery, an undeveloped central microtubule doublet. Outer plasma membrane of the ciliary pocket surrounding the cilium shaft (arrowheads). n – nucleus (Bar 200 nm). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group (60x).~~

~~**Figure 3.** Panel (A) shows the percentage of multi-ciliated fibroblasts after treatment with various concentrations of Doxorubicin (10, 20, 40, 80 and 120 nM). (B) Multiple cilia were observed 72 hours after treatment with 80 nM Doxorubicin. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme), gamma-tubulin (green, basal body), nuclei (blue). (C) Transverse section of multiple cilia in fibroblasts after treatment with 80 nM of Doxorubicin reveals the structure of its axoneme (arrows). The outer plasma membrane of the ciliary pocket surrounds the cilium shaft (arrowheads). n – nucleus (Bar 1.5 nm). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs.* control group. Magnification (60x).~~

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

~~**Figure 4.** Panel (A) shows the percentage of cells with primary cilia in fibroblasts after treatment with various concentrations of Taxol (1.25, 3.25, 5.25, 6.25 and 12.5 nM). (B) Primary cilia were observed after 72 hours of treatment with 1.25 nM Taxol. Representative immunofluorescence images of primary cilia: acetylated tubulin (red, axoneme) and gamma-tubulin (green, basal body), nuclei (blue). (C) TEM image of fibroblasts after treatment with 1.25 nM Taxol shows the transverse section of a primary cilium (arrow), it contains six microtubule doublets arranged at the periphery and one centrally located microtubule doublet. The open arrows depict the outer plasma membrane of the ciliary pocket surrounding the cilium shaft (Bar 0.25 nm) (60x). $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ *vs.* control group.~~

~~**Figure 5.** Representative immunofluorescence images of BT-549 and MDA-MB-231 cells: acetylated tubulin (red), nuclei (blue). Primary cilia are absent after 72 hours of treatment with Doxorubicin and Taxol in BT-549 and MDA-MB-231 (60x).~~

For Peer Review