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Environmental exposure to cadmium in breast cancer – association with the Warburg effect and sensitivity to tamoxifen

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ABSTRACT

The association between cadmium and breast cancer remains unexplained due to inconsistent epidemiological data and unknown underlying mechanisms. This study aimed to assess the relationship between environmental exposure to cadmium and the Warburg effect in breast cancer and, thus, its possible interference with breast cancer treatment. The observational study in two groups of breast cancer patients indicated a positive correlation between urinary cadmium concentration and tumor expression of *HIF1A* (a master regulator of the Warburg effect). Further explanatory research in MCF-7 cells showed no impact of cadmium exposure on molecular and biochemical markers of the Warburg effect. However, long-term exposure to a low and environmentally relevant concentration of cadmium led to the accumulation of the metal in MCF-7 cells and decreased their sensitivity to tamoxifen. To conclude, the association between cadmium and the Warburg effect was suggested in the observational study, although not confirmed *in vitro*. Nevertheless, cadmium seems to interfere with tamoxifen treatment which deserves further investigation in terms of its possible implication in intrinsic resistance to hormone therapy.

1. Introduction

The Warburg effect (aerobic glycolysis) is a phenomenon characterizing the energy metabolism of cancer cells, which is required for tumor growth [1]. Briefly, it is associated with increased glucose uptake and up-regulation of glycolysis, accompanied by high production of lactate [2,3]. Under physiological and aerobic conditions, the end product of glycolysis, pyruvate, enters the mitochondria and it is converted by the pyruvate dehydrogenase complex (PDC) to acetyl coenzyme A (acetyl-CoA). Subsequently, this conversion is followed by the Krebs cycle and oxidative phosphorylation, during which energy is generated in the form of adenosine triphosphate (ATP). Under anaerobic conditions, cells obtain ATP due to the reaction catalyzed by lactate dehydrogenase (LDH), which is a reduction of pyruvate to lactate (the process termed fermentation). It has been observed that in tumor cells, even in the presence of oxygen, metabolic activity is redirected towards glycolysis and energy generation by the reduction of pyruvate to lactate [2]. Although this specific metabolic phenotype of cancer cells was described by Otto Warburg 100 years ago, its function and direct causes remain unknown [3–5]. Initial Warburg's theory about mitochondrial

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dysfunctions, as the main root of cancer metabolic reprogramming, occurred to be confirmed but not in all cancers. The current research assumes that the Warburg effect is driven mainly by genetic instability, mutagenesis, aberrant gene expression, and altered signaling pathways [6].

Surprisingly, little research exists on the direct association between the known carcinogenic factors and the Warburg effect [7]. To investigate the above relationship, we focused on heavy metal cadmium - one of the most carcinogenic factors to humans, classified by the International Agency for Research on Cancer (IARC) as a carcinogen of group I [8]. Due to estrogenic properties, Cd raises a high scientific interest as an endocrine disruptor, implicated in several alterations of the endocrine system [9,10]. Epidemiological data suggest that Cd is involved in breast cancer development [11–13]. Notably, observational studies showed that Cd accumulates in breast cancer tissue [14–17], and experimental data indicated that chronic exposure to this metal leads to breast cancer progression [18]. Since Cd is a widely spread environmental pollutant [19], and at the same time it is a strong carcinogenic factor with accumulating properties, it is of interest to investigate its possible association with the Warburg effect in breast cancer. For this purpose, we conducted an observational study among two different groups of breast cancer women, from whom fragments of tumor tissue and peritumor tissue were collected, to compare Cd contents and molecular outcomes of the Warburg effect. In both types of the tissue we determined Cd content and mRNA expression of the key transcriptional regulator of the Warburg effect, that is hypoxia-inducible factor 1 alpha (HIF-1α), as well as other crucial proteins associated with the glycolytic phenotype, including glucose transporter 1 (GLUT1), glycolytic enzymes, LDH, pyruvate dehydrogenase kinase (PDK), pyruvate dehydrogenase (PDH), and the key kinases cascade regulating glycolysis, PI3K/AKT/mTOR. In addition, urinary Cd concentration (as a marker of environmental Cd exposure), and mRNA expression of estrogen receptor (ESR1) were analyzed. We observed that the expression of *HIF1A* in breast cancer tissues was associated with urinary Cd concentration in both study groups. To explore this association *in vitro*, we analyzed the effect of Cd exposure on molecular and biochemical markers of the Warburg effect in breast cancer cells. Considering the estrogenic potential of Cd, and at the same the recognized role of the Warburg effect and *HIF1A* in cancer drug resistance [20], joint effects of combined exposure to Cd and tamoxifen were analyzed in MCF-7 cells, to assess possible interference of this metal with the hormonal therapy of breast cancer. Tamoxifen (belonging to selective estrogen receptor modulators; SERMs) was selected as a model drug due to its common use in breast cancer treatment. Human breast cancer cells, MCF-7, were chosen as a model cell line due to their sensitivity to tamoxifen resulting from high expression of ESR1.

2. Methods

2.1. Breast cancer patients study

For this study, 166 female patients were recruited in the years (2006–2019) from two Polish oncology centers located in the north and the central part of Poland, including 65 women hospitalized at the Department of Surgical Oncology of Medical University of Gdansk (MUG) assigned to Group 1, and 101 women treated at the Department of Oncological Surgery of Provincial Multidisciplinary Centre of Oncology and Traumatology in Lodz, assigned to Group 2. In the case of patients from Group 1, the material came from the collection of the Department of Medical Laboratory Diagnostics - Fahrenheit Biobank BBMRI.pl of the MUG and included fragments of tissues secured immediately after the surgery at −70°C (tumor + peritumor tissue sets), as well as urine samples collected before the procedure. Group 1 included patients with histopathologically confirmed invasive breast cancer. In the case of patients from Group 2, the material included fragments of tissues collected intraoperatively and fixed in 4 % buffered formalin solution and embedded in paraffin for long-term storage (tumor + peritumor tissue sets), as well as urine samples collected before the surgery. Based on the results of the histopathological evaluation of the tissues, 24 patients were excluded from the study due to diagnosis other than invasive breast cancer (noninvasive breast cancer or other pathological changes in the breast). Altogether, 142 patients aged 28–91 years were included in further analysis, with ductal carcinomas accounting for more than half of the diagnoses. The detailed characteristics of the study group are presented in Table 1. Questionnaire data were collected from 91 (64 %) women with breast cancer (some patients did not agree to participate in the questionnaire study, and in the case of the archival collection, there was no possibility of obtaining complete questionnaire data). The study was conducted following the Declaration of Helsinki and approved by the Bioethical Committee at the Nofer Institute of Occupational Medicine in Lodz (Resolution No. 15/2017 of 08/09/2017) and by the Bioethical Committee at the Medical University of Lodz (Resolution No. RNN/361/18/KE of 13/11/2018). Patients gave written and informed consent to participate in the study.

2.2. MCF-7 cells study

Estrogen-dependent MCF-7 human breast cancer cells (ATCC, #HTB-22) were maintained in culture medium, i.e. Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, #D5671) supplemented with 5 % heat-inactivated Fetal Bovine Serum (Gibco, #10270–106), 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in an atmosphere of 6 % CO_2. For

Table 1
Study group characteristics.

Variable	Group 1	Group 2	All
N	65	77	142
Age (years)	62.7 ± 13.8 (28–91)	63.1 ± 12.9 (36–86)	62.3 ± 13.3 (28–91)
BMI (kg/m^2)	27.5 ± 4.9 (17.7–39.1)	26.8 ± 4.9 (20.5–37.1)	27.3 ± 4.9 (17.7–39.1)
Smoking			
Yes	10 (15.4 %)	3 (3.9 %)	13 (9.2 %)
No	52 (80.0 %)	22 (28.6 %)	74 (52.1 %)
No data	3 (4.6 %)	52 (67.5 %)	55 (38.7 %)
Menopausal status			
Pre	11 (16.9 %)	61 (79.2 %)	72 (50.7 %)
Post	52 (80.0 %)	16 (20.8 %)	68 (47.9 %)
No data	2 (3.1 %)	0 (0.0 %)	2 (1.4 %)
Breast cancer type			
Ductal	42 (64.6 %)	35 (45.4 %)	77 (54,2 %)
Lobular	9 (13.9 %)	37 (48.1 %)	46 (32,4 %)
Other	14 (21.5 %)	5 (6.5 %)	19 (13,4 %)
Tumor grading, n (%)			
G1	4 (6.2 %)	26 (33.8 %)	30 (21.1 %)
G2	37 (56.9 %)	34 (44.1 %)	71 (50.0 %)
G3	19 (29.2 %)	14 (18.2 %)	33 (23.3 %)
Not applicable	5 (7.7 %)	3 (3.9 %)	8 (5.6 %)
ER/PR status, n (%)			
ER+/PR+	41 (63.1 %)	41 (53.2 %)	82 (57.7 %)
ER+/PR-	6 (9.2 %)	11 (14.3 %)	17 (12.0 %)
ER-/PR-	14 (21.5 %)	14 (18.2 %)	28 (19.7 %)
No data	4 (6.2 %)	11 (14.3 %)	15 (10.6 %)
HER2 status, n (%)			
HER2+	20 (30.8 %)	9 (11.7 %)	29 (20.4 %)
HER2-	34 (52.3 %)	53 (68.8 %)	87 (61.3 %)
HER2 2+	6 (9.2 %)	4 (5.2 %)	10 (7.0 %)
No data	5 (7.7 %)	11 (14.3 %)	16 (11.3 %)
Urine samples, n (%)	18 (27.7 %)	33 (42.8 %)	51 (35.9 %)
Tissue sets for Cd analysis, n (%)			
-70°C	45 (69.2 %)	-	45 (31.7 %)
Formalin	-	29 (37.7 %)	29 (20.4 %)
Tissue sets for gene expression, n (%)			
-70°C	65 (100 %)	-	65 (45.8 %)
Formalin	-	77 (100 %)	77 (54.2 %)

testing, the cells were cultured/treated in E2-free medium, i.e. Dulbecco's modified Eagle's medium/F-12 without phenol red (DMEM/F-12) (Sigma-Aldrich, #D6434) supplemented with 5 % charcoal stripped Fetal Bovine Serum (Sigma-Aldrich, #F6765), 2.5 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) (37 °C, 6 % CO_2).

To confirm the lack of mycoplasma contamination of cell culture during experiments, we conducted testing against mycoplasma using MycoBlueTM Mycoplasma Detector (Vazyme Biotech, #D101–01, Nanjing City, China). Raw data from Mycoplasma testing are available upon request.

For cell line authentication we collected three cell pellets after completing the long-term experiment. A cell line authentication certificate confirming that the used cell line was MCF-7, is available upon request (obtained from Eurofins Genomics Europe Applied Genomics GmbH, Ebersberg, Germany).

2.3. Cell treatment

MCF-7 cells were seeded onto cell culture dishes, i.e. 96-well microplates (2×10^3 cells/well/100 μL) for cytotoxicity assays, and culture flasks (1.6×10^3 cells/T25/5 mL or 5×10^3 cells/T75/10 mL) for remaining assays, and left overnight in a culture medium for adherence. Then, following 48 h preincubation in an E2free medium, the cells were incubated for the next 72 h in the E2free medium in the absence or presence of $CdCl_2$ (Sigma-Aldrich, #202908). Two non-toxic concentrations of Cd were used in a short-term (72 h) experiment, including 1 μM and 10 μM, which were, respectively, 20 and 2 times lower than the IC30 value (IC30; the concentration evoking 30 % inhibition of growth/viability of cells) calculated for Cd after 72 h of exposure of MCF-7 to Cd (23.7 ± 1.7 μM). In a long-term experiment (6 months), cells were grown for 31–31 passages without and in the presence of Cd at a low concentration of 0.01 μM, being 2000 lower than IC30. The medium was changed twice a week to fresh, including once during the passage. Cells were collected from each culture variant (cells exposed to Cd 0.01 μM or Cd non-exposed cells) after 2, 4, and 6 months to determine the concentration of Cd. In both short and long-term experiments, cells preincubated with Cd were treated with tamoxifen metabolite, 4-OHT (Sigma-Aldrich, #H7904) at 15 μM (IC50; the concentration evoking 50 % inhibition of growth/viability of cells), for the last 24 h of experiment, and cells preincubated only with Cd for 72 h (including changing of medium with Cd to fresh after 48 h) were used as a control. IC30 for Cd calculated in the cells exposed to the mixture: Cd + 4-OHT (15 μM) was similar as in the case of cells not treated with 4-OHT (21.2 ± 1.9 μM). The IC30/IC50 values for each compound were calculated using GraphPad Prism ver. 7.04 (GraphPad Software, San Diego, CA, USA). Independent sets of cultures were run to perform three independent experiments in both short and long-term exposure. Control groups included non-treated cells and cells exposed in parallel to solvents used to prepare solutions of $CdCl_2$ (sterile-filtered water) and 4-OHT (ethanol, POCH, #396480111). The concentration of solvents was the same in control and all treated samples and was as follows: for ethanol: 0.3 % during the short or long treatment; for water: 0.1 % during the short-term treatment, and in the long-term treatment, the solvent was omitted due to very low concentration (<0.001 %). After the exposure, the MTT reduction test and the Sulforhodamine B assay were performed to assess cytotoxicity (as described below). Cell lysates were collected for gene expression analysis, Western Blot, and biochemical assays. Detailed cytotoxicity data for calculation of IC30 and IC50 are shown in Fig. S1.

2.4. MTT reduction test

The MTT assay is a colorimetric assay based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide; MTT) to purple insoluble formazan crystals by viable, metabolically active cells. After the exposure, the supernatants were removed, and the cells were incubated with the MTT solution (100 μL) in Hanks' Balanced Salt solution (HBSS; Sigma-Aldrich) at the concentration of 0.5 mg/mL for 3 h. After discarding MTT solution, DMSO (50 μL) was added to each well to solubilize formazan. The optical density (OD) of solubilized formazan product was determined using a MultiscanGO spectrophotometer (Thermo Fisher Scientific, Finland) with the 550 nm filter and the 620 nm filter as a reference. Results were expressed as the percent of cell survival (OD of exposed vs. OD of relevant control).

2.5. Sulforhodamine B (SRB) assay

The Sulforhodamine B assay is used for cytotoxicity assessment based on the measurement of cellular protein content. The cytotoxic effect is indicated by a decrease in the number of cells resulting in a concomitant decrease in the amount of SRB dye incorporated by the exposed cells. After the exposure, the cells were stained with SRB, as described previously [21].

2.6. Cadmium analysis

An ELAN® DRC-e ICP-MS (PerkinElmer SCIEX, USA), equipped with a Meinhard quartz nebulizer, quartz cyclonic spray chamber, and platinum sampler and skimmer cones, was used for cadmium determination in urine, tissues, and cells. Cadmium (^{114}Cd) was analyzed using the standard ICP-MS method and the Dynamic Cell Reaction (DRC-ICP-MS), which eliminates molybdenum oxide interferences. The DRC parameters were 1.0 mL/min methane (Linde Gas, Poland) flow rate and 0.85 RPq. Before samples collection, all polyethylene urine tubes were washed in 20 % nitric acid (24 h) and next rinsed with ultrapure water (Milli-Q Integral 3, Merck, Poland) to avoid contamination. Prior to the determination, urinary samples were centrifuged and supernatants (0.2 mL) were diluted with 1.8 mL of diluent (1 % nitric acid, 70 %, ULTREX™ II Reagent, J.T.Baker™, Witko, Poland). Prior to the analysis of tissues (20–50 mg) and cells (suspended in 0.5 mL of PBS - Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich, #D8537–6×500ML), samples were mineralized in 70 % nitric acid (ULTREX™ II Reagent, J.T.Baker™, Witko, Poland) in the microwave using wet digestion system (UltraWave, Milestone) and were next diluted with ultrapure water (Milli-Q Integral 3, Merck, Poland). External calibration ranges were 0.1–10 μg/L for cadmium (Multi-Element Calibration Standard, Perkin Elmer Pure Plus, Poland). Clinchek® urine (Recipe, Germany) was analyzed every ten samples as an internal quality control check. The laboratory participates in the external quality program for the Cd in urine determination organized by the Institute of Occupational Social and Environmental Medicine of the University of Erlangen, Nuremberg (G-EQUAS). Creatinine was determined using the colorimetric Jaffe method. Analysis was carried out at 520 nm on Cary 60 UV-Vis spectrometer Agilent Technologies (MS Spektrum, Poland).

2.7. Gene expression

Analysis of gene expression was performed in the tissues and MCF-7 cells. Fresh frozen tissues (~25 mg) were homogenized in Qiazol Lysis Reagent (Qiagen, Hilden, Germany, #79306), using ceramic beads (Lysing Matrix D, MP Biomedicals, #6913050) and FastPrep-24 5 G homogenizer (MP Biomedicals, Irvine, CA, USA). Total RNA was isolated with miRNeasy Mini Kit (Qiagen, Hilden, Germany, #217084) according to the manufacturer's instructions. In the case of paraffin-embedded tissues, total RNA was isolated with miRNeasy FFPE Kit (Qiagen, Hilden, Germany, #217504) following the manufacturer's instruction. Cells were directly lysed using Qiazol, and RNA was isolated with miRNeasy Mini Kit. All RNA samples' quality and quantity were determined spectrophotometrically by Multiskan GO multi-plate reader (ThermoFisher, Waltham, MA, USA). cDNA was synthesized from 100 ng of RNA,

using Transcriptor First Strand cDNA Synthesis Kit (#04897030001, Roche, Indianapolis, IN, USA). Primers and hydrolysis probes were obtained from Eurofins Genomics (Vienna, Austria). Oligonucleotide sequences for primers and probes are presented in Table S1. Target genes included 22 genes: *ESR1* (estrogen receptor alpha), *HIF1A* (hypoxia-inducible factor, alpha subunit), *GLUT1* (*SLC2A1*; glucose transporter), *HK1* (hexokinase 1), *HK2* (hexokinase 2), *PFKM* (muscle subunit of phosphofructokinase), *PFKL* (liver subunit of phosphofructokinase), *PFKP* (platelet subunit of phosphofructokinase), *PKLR* (pyruvate kinase; liver and RBC; isozyme), *PKM* (pyruvate kinase; muscle; isozyme), *LDHA* (lactate dehydrogenase A), *LDHB* (lactate dehydrogenase B), *LDHC* (lactate dehydrogenase C), *PDK1* (pyruvate dehydrogenase kinase 1), *PDHA1* (alpha subunit of pyruvate dehydrogenase), *PDHB* (beta subunit of pyruvate dehydrogenase), *AKT1* (serine-threonine protein kinase), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), *PIK3CB* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma beta, *PIK3CD* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta), *PIK3CG* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma), *MTOR* (mechanistic target of rapamycin). Three genes (*ACTB*, *RPLP0*, and *RPLP13A*) were selected as reference genes based on our previous study on breast cancer women [22]. Fast Essential DNA Probe Master (Roche, Indianapolis, IN, USA, #06924492001) was used for qPCR, and all gene expression experiments were run in duplicates, in the Light Cycler 96 Real-Time PCR System (Roche, Indianapolis, IN, USA). All procedures, including RNA isolation and the gene expression experiments, fulfilled MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiment).

2.8. Western blot

MCF-7 cells were lysed using RIPA lysis buffer (Abcam, UK #ab156034) with protease inhibitors (Abcam, UK, #ab201116). Proteins (20 μg) in 1xLaemmli Sample Buffer (Bio-Rad, USA #161–0747) and Precision Plus Protein Standard (Bio-Rad, USA #161–0375) were separated by the long-life TGX (Tris-Glycine eXtended) SDS-PAGE 4–20 % gels (Bio-Rad, USA #456–1093) and transferred to a PVDF mini membrane (Bio-Rad, USA #170–4156). The membranes were blocked by 3 % Blocker for 2 h, then incubated with: anti-Estrogen Receptor alpha antibody [6F11] (1:1000; Abcam, UK #ab93021) or anti-beta Actin antibody [AC-15] (1:10 000; Abcam, UK #ab6276) at 4 °C overnight. Next, the secondary antibody Goat Anti-Mouse IgG H&L (HRP) (1:20 000 for beta-Actin and 1:10 000 for receptor; Abcam, UK #ab205719) was incubated for 90 min at room temperature. The protein bands were visualized on the membrane with Amplified Opti-4CN Substrate Kit (Bio-Rad, USA #170–8238) in Gel Doc XR System (Bio-Rad, USA). Protein expression was quantified using ImageJ software (NIH, USA).

2.9. Biochemical markers

Colorimetric methods were used for biochemical analyses. The lactic acid concentration was measured in cell lysates and culture medium using the L-Lactate Assay kit (Abcam, #ab65331). Cells were lysed with a lysis buffer with protease inhibitors (Lactate Assay Buffer + 1 % Protease Inhibitor Cocktail II, Abcam, #ab201116). PK activity was determined in cell lysates with Pyruvate Kinase (PK) Assay Kit (Abcam, #ab83432).

2.10. Statistical analysis

Statistical analysis was performed using Statistica ver. 13.3 (TIBCO Statsoftware Inc.). Differences between groups were analyzed using Student t–test, U Mann-Whitney test or Wilcoxon test (for continuous variables), and Chi-square test (for categorical variables). For analysis of the correlation between two continuous variables, Spearman's rank correlation coefficient was calculated. Multiple comparisons were performed using one-way ANOVA followed by Tukey's or Holm-Sidak's post hoc test, or two–way ANOVA followed by Tukey's post hoc test. Data distribution was assessed with Shapiro-Wilk test. A p-value of 0.05 or lower was considered to be statistically significant.

3. Results

3.1. Observational study in breast cancer women

3.1.1. Study group characteristics

Clinicopathological data and basic demographic characteristics of the patients are presented in Table 1. Both groups were similar in terms of age ($p = 0.841$) and BMI ($p = 0.563$) but differed in menopausal status (Group 1 included mainly postmenopausal patients, whereas Group 2 was mainly premenopausal, $p < 0.001$). Most of the patients within Group 1 were non-smokers, and for Group 2, information on smoking was available only for one-third of the patients. The majority of the tumors were ductal, G2, and estrogen positive. In both groups, most patients were also HER negative.

3.1.2. Cadmium concentration in urinary samples and tissues

The mean concentration of Cd in urine was similar in both groups (0.99 μg/L and 0.89 μg/L, $p = 0.584$), with individual concentration values in all collected samples (a total of 51 women from two regions of Poland) ranging from 0.12 to 3.35 μg/L (0.34–3.45 μg/g creatinine) (Fig. 1a). A higher mean Cd content was observed in neoplastic tissue compared to peritumor tissue, both in Group 1 (0.086 vs. 0.056 μg/g) and Group 2 (0.052 vs. 0.020 μg/g), with a statistically significant difference shown only in Group 2 ($p < 0.0001$) (Fig. 1b). At the same time, Cd content was significantly and two times lower in tissue fragments fixed in formalin (Group 2) compared to fresh frozen tissue fragments (Group 1) (0.070 vs. 0.036 μg/g, $p = 0.0003$), indicating that Cd releases into the formalin solution during the material storage. We confirmed this observation by analyzing the metal concentration in the formalin solution from 7 randomly selected samples in which the mean value was 1.11 μg/L.

A significant positive correlation was found between the Cd content in the tumor and peritumor tissue, both in Group 1 (0.358, $p < 0.05$) and in Group 2 (0.451, $p < 0.05$). When analyzed both groups together, urinary Cd concentration was correlated with the Cd content in the tumor ($r = 0.25$, $p = 0.05$) and peritumor tissue ($r = 0.208$, $p = 0.09$).

In both groups, the associations between Cd markers and: age, BMI, smoking, menopausal status, hormone receptor status, and grading (G), were analyzed. In Group 1, urinary Cd concentration expressed as μg/g creatinine was significantly correlated with age ($r = 0.492$, $p = 0.038$) and associated with smoking ($p = 0.024$; higher values in smokers as compared to nonsmokers) and ER/PR receptor status ($p = 0.005$, with the highest levels observed in ER+PR+ samples). In Group 2, no significant correlations with the mentioned factors were observed, apart from a significant association between Cd content in the tumor and menopausal status ($p = 0.05$; higher values in premenopausal women).

3.1.3. Gene expression in tissues

Five samples from Group 1 and seven samples from Group 2 were excluded from further analysis due to poor RNA quality. Thus final analysis included 60 tissue sets (tumor + normal tissue) from Group 1 and 70 tissue sets from Group 2. Gene expression analysis was performed for 25 genes, including three reference genes (*ACTB*, *RPLP0*, *RPL13A*) and 22 genes related to the research hypothesis (*ESR1*, *HIF1A*, *PIK3CA*, *PIK3CB*, *PIK3CD*, *PIK3CG*, *AKT1*, *MTOR*, *GLUT1*, *HK1*, *HK2*, *PKM*, *PFKM*, *PFKP*, *PFKL*, *PKLR*, *LDHA*, *LDHB*, *LDHC*, *PDK1*, *PDHA1*, *PDHB*). After obtaining the experimental data, two genes (*PKLR* and *LDHC*) were excluded from further analysis due to the lack of expression (no reading or very high Ct values indicating poor expression). Ultimately, expression data were obtained for 20 target genes in 130

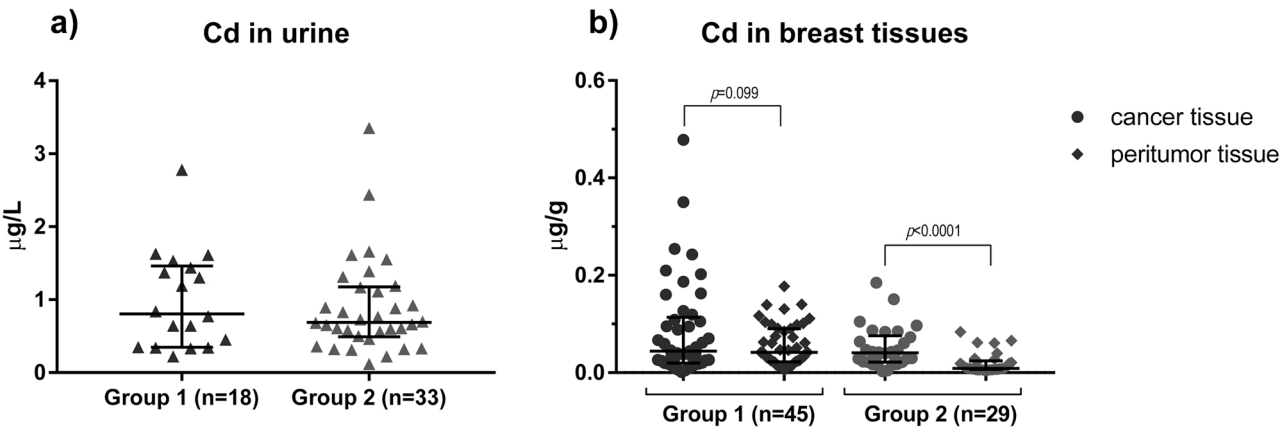

Fig. 1. Markers of environmental Cd exposure in the two groups of breast cancer patients. a) Urinary cadmium concentration, b) Cadmium content in cancer tissues and peritumor tissues. Group differences analyzed with the Wilcoxon test. Data shown as scatter dot-plots with median and interquartile range (IQR).

patients (Fig. 2). Taking into account the median values, in both study groups (1 and 2) analyzed separately, a significantly higher expression of 5 genes related to the Warburg effect was observed in the tumor tissues compared to the peritumor tissues, including *HIF1A*, *GLUT1*, *HK1*, *PKM*, and *PFKL*, with the highest levels observed for *GLUT1* and *PKM*. At the same time, the expression of 3 genes, including *HK2*, *PDHA1*, and *LDHB*, was shown in both study groups to be significantly lower in the tumor tissues compared to peritumor tissues. Additionally, in Group 1, significantly higher expression in the tumor tissues was observed for *PIK3CB* and *PDK1*, whereas in Group 2, significant changes in the tumor tissue were observed for *AKT1* and *PFKP* (higher expression) as well as *PIK3CA* and *PFKM* (lower expression). In the tumor tissues, *HIF1A* (master regulator of the Warburg effect) expression was significantly correlated with the expression of 9 target genes (*PIK3CG*, *AKT1*, *MTOR*, *HK2*, *PFKP*, *LDHA*, *LDHB*, *PDHA1*, and *PDHB*) in Group 1, and with the expression of 11 target genes (*PIK3CD*, *PIK3CG*, *AKT1*, *GLUT1*, *HK2*, *PFKP*, *LDHA*, *LDHB*, *PDK1*, *PDHA1* and *PDHB*) in the Group 2 (data not shown). Additionally, in the tumor tissues of patients from both Groups, *HIF1A* expression was negatively correlated with the expression of ESR1 ($r = -0.28$, $p = 0.032$ in Group 1 and $r = -0.21$, $p = 0.097$). As expected, based on histopathological examination, ESR1 expression in the tumor was significantly higher compared to peritumor tissues in ER+ patients in both 1 and 2 Groups ($p < 0.0001$, $p = 0.0004$, respectively; Fig. S2).

3.1.4. Tumor gene expression with respect to hormone receptor status and tumor grading

Following the observation of the negative correlation between *HIF1A* and *ESR1* expression in the tumor tissues, we analyzed *HIF1A* expression according to hormone receptor status. *HIF1A* expression was higher in tumors with negative expression of ER as compared to ER+ tumors, both in Group 1 ($p = 0.033$) and in Group 2 (border significance $p = 0.084$, Fig. S3). A significant association was also observed between *HIF1A* expression and PR receptor status, with significantly higher expression observed for PR-negative tumors as compared to PR+ , in both Group 1 and Group 2 ($p = 0.01$ and $p = 0.018$, respectively, Fig. S3). In the tumors from Group 1 and Group 2, ER/PR receptor status was also associated with the expression of *PFKP* ($p = 0.084$ and $p = 0.037$, respectively) and *PFKL* ($p = 0065$ and $p = 0.024$, respectively), though in the Group 1, the associations were of border significance. Additionally, there was a significant association between ER/PR status and tumor expression of *PDK1* ($p = 0.002$) in Group 2. HER2 status was significantly associated with the expression of *AKT1* ($p = 0.049$), *MTOR* ($p = 0.005$), *PKM* ($p = 0.035$), and *LDHA* ($p = 0.004$) in Group 1. Additional relationships included significant associations with tumor grading, for the expression of *LDHA* ($p = 0.025$) in Group 1, and for *PFKP* ($p = 0.007$) and *PDK1* ($p = 0.004$) in Group 2.

3.1.5. Correlation between markers of environmental exposure to cadmium and gene expression

Next, we analyzed the correlation between markers of environmental Cd exposure and tissue expression of dysregulated genes (Figures 3 and

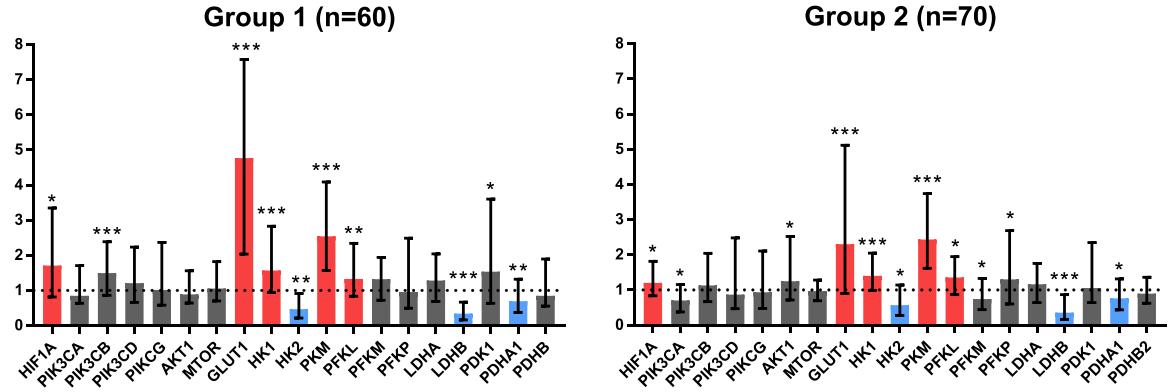

Fig. 2. Normalized relative mRNA expression of the Warburg effect related genes in the tumor tissue as compared to peritumor tissue, measured in the two groups of breast cancer women. a) Group 1 (n = 60 tissue sets) – RNA was isolated from fresh frozen tissues. b) Group 2 (n = 70 tissue sets) – RNA was isolated from FFPE tissue fragments. The color of the bars corresponds to upregulated (red) and downregulated (blue) genes in both study groups. *- $p < 0.05$, ** - $p < 0.001$, ***- $p < 0.0001$. Group differences analyzed with the Wilcoxon test. Data shown as median with interquartile range (IQR).

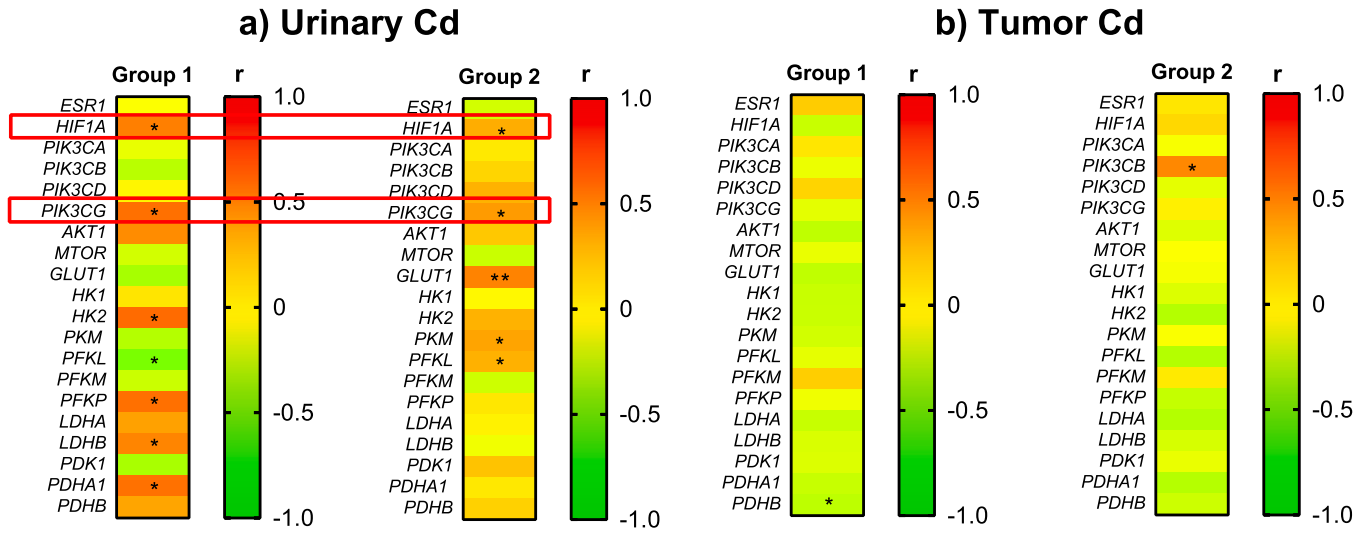

Fig. 3. Correlation between markers of Cd exposure and tumor expression of *ESR1* and the Warburg effect related genes in the two groups of breast cancer women. a) Urinary Cd concentration (expressed as μg/L); b) Cd content in the tumor (expressed as μg/g). Correlation coefficient (r) values correspond to the color in the scale bar and statistically significant correlations are indicated with * and * *, * - $p < 0.05$, * * - $p < 0.001$. Red rectangles indicate genes which expression was significantly and positively correlated with urinary Cd in both study groups (*HIF1A* and *PIK3CG*).

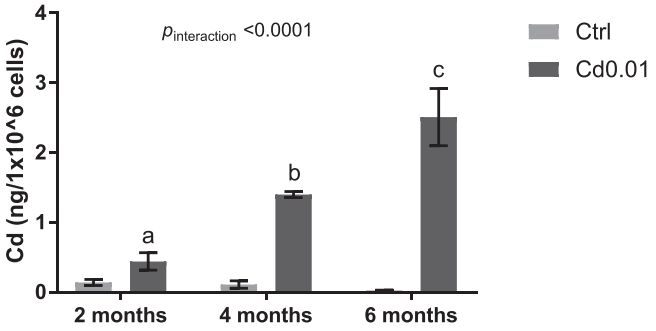

Fig. 4. Cd accumulation in MCF-7 cells during 6 months of exposure to Cd at a low concentration of 0.01 μM. Ctrl – Cd non-exposed cells. Different letters above the bars indicate a statistically significant difference between time points within cells exposed to Cd (two-way ANOVA with Tukey's multiple comparison test). Data shown as mean ± standard deviation (SD).

4, Tables S3 and S4). In Group 1, out of 8 upregulated genes, a significant positive correlation was observed between urinary Cd concentration and the expression of *HIF1A*, both in the tumor tissues (r = 0.502, $p = 0.035$) and in the peritumor tissues (r = 0.539, $p = 0.030$). Urinary Cd was also correlated with the expression of *PKM* in the peritumor tissues (r = 429, $p = 0.0088$). Expression of three other highly upregulated genes, *GLUT1*, *ESR1* and *PKM1* was significantly and positively correlated with tissue Cd content, though the correlation was observed only in the peritumor tissues (*GLUT1*: r = 0.437, $p = 0.002$; *ESR1*: r = 0.313, $p = 0.002$ l; *PKM*: r = 0.328, $p = 0.145$). In addition, in Group 1, urinary Cd was also positively associated with tumor expression of two non-dysregulated genes (*PIK3CG* and *PFKP*) and three downregulated genes (*PDHA1*, *HK2*, and *LDHB*).

In Group 2, out of 8 upregulated genes, a significant positive correlation was observed between urinary Cd concentration and tumor expression of four genes: *PKM* (r = 0.352, $p = 0.024$), *GLUT1* (r = 0.481, $p = 0.004$), *PFKL* (r = 0.301, $p = 0.050$) and *HIF1A* (r = 0.316, $p = 0.042$). Expression of *PFKL* was also positively correlated with tissue Cd content, but only in the peritumor tissue (r = 0.477, $p = 0.0051$). Within non dysregulated genes, there was a significant positive correlation between urinary Cd and tumor expression of *PIK3CG* (r = 0.387, $p = 0.0157$), significant positive correlation between Cd content in the tumor and tumor expression of *PIK3CB* (r = 0.459, $p = 0.0184$), and significant negative correlation between Cd content and the expression of *LDHA* (r = - 0.375, $p = 0.0245$) in the peritumor tissue.

3.2. Experimental study in MCF-7 cells - exposure to cadmium and 4-hydroxytamoxifen

3.2.1. Cadmium accumulation in MCF-7 cells during long-term exposure

In MCF-7 cells exposed for 6 months to 0.01 μM Cd, the mean concentration of the metal significantly increased over time, indicating its accumulation. Reported mean values, calculated per 1×10^6 cells, were 0.44 ± 0.13 ng, 1.40 ± 0.04 ng, and 2.51 ± 0.41 ng after 2, 4 and 6 months of exposure, respectively ($p < 0.0001$ for the effect of exposure; $p < 0.0001$ for the effect of time; $p < 0.0001$ for interaction, Fig. 4).

3.2.2. Effects of Cd exposure on the Warburg effect markers in MCF-7 cells

Five genes (*PIKCD*, *PIK3CG*, *PKLR*, *LDHC*, and *LDHB*) were excluded from gene expression analysis in MCF-7 cells due to lack of expression or very low Ct values. The expression profile of 17 genes subjected to further analyses is shown in Fig. S4 (72 h of exposure) and Fig. S5 (6 months of exposure). Short-term exposure (72 h) to Cd was associated with a dose-dependent decrease of *ESR1* expression (p for trend = 0.005), with a 5 % and 13 % lower expression observed upon Cd 1 μM and 10 μM, respectively. However, no changes were observed at the protein level for ESR1 (Fig. S6). No difference in the expression of the Warburg effect-related genes was observed upon 72 h of Cd exposure at 1 μM and 10 μM (Fig. S4).

In the long-term (6 months) exposure experiment, a significant decrease in *PKM* expression was observed in MCF-7 cells exposed to Cd at a concentration of 0.01 μM. No effect of Cd on the expression of other genes, including *ESR1*, was observed (Fig. S5). Protein expression of ESR1 also remained not changed upon long-term exposure to Cd (Fig. S6).

Biochemical markers (pyruvate kinase (PK) activity and lactate) are shown in Fig. S7. PK activity in cell lysates was not changed upon short-term (post hoc ANOVA $p = 0.9999$ and $p = 0.9999$ for 1 μM Cd and 10 μM Cd, respectively) and long-term exposure to Cd 0.01 μM (post hoc ANOVA $p = 0.9922$). Similarly, exposure to Cd at 1 μM, 10 μM (72 h), and 0.01 μM (6 months) did not induce changes in the concentration of lactate in cell lysates (post hoc ANOVA $p = 0.9992$, $p = 0.9997$, and

$p = 0.9912$, respectively) and lactate released into the medium, (post hoc ANOVA $p = 0.9314$, $p = 0.7838$, and $p = 0.2344$, respectively), though some non-significant trend toward increased extracellular lactate upon long-term exposure was observed (Fig. S7).

3.2.3. Effect of cadmium exposure on MCF-7 sensitivity to 4-hydroxytamoxifen

Treatment with 4-hydroxytamoxifen (4-OHT) (IC50) significantly decreased MCF-7 cells viability by over 50 % as assessed in the MTT test (46 %, $p < 0.0001$) and SRB test (41 %, $p < 0.0001$ for SRB). Short-term (72 h) exposure to Cd at 1 μM and 10 μM decreased MCF-7 sensitivity to 4-OHT in a dose-dependent manner (p for trend = 0.0394 in MTT test, and $p = 0.0164$ in SRB test, Fig. 5a). Significant change of the viability was observed upon treatment with Cd 10 μM, which decreased the cytotoxicity of 4-OHT by 17 % and 12 %, as observed both in the MTT test (46 % (4-OHT) vs. 63 % (Cd10 + 4-OHT), $p = 0.0372$) and in the SRB test (41 % (4-OHT) vs. 53 % (Cd10 + 4-OHT), $p = 0.0289$). A similar effect was observed for Cd 1 μM, but the difference was not significant (the viability for the combined exposure of Cd 1 μM + 4-OHT was 55 % in the MTT test ($p = 0.4018$) and 47 % in the SRB test ($p = 0.3752$).

Long-term (6 months) exposure to Cd at a very low concentration of 0.01 μM showed no toxic effects in MCF-7 cells. The viability of Cd-non-exposed cells treated with 4-OHT (IC50) on the last day of the long-term experiment was 27 % in the MTT test ($p = 0.0003$) and 46 % in the SRB test ($p < 0.0001$), and it was significantly lower as compared to cells exposed to Cd (0.01 μM) for 6 months and treated with 4-OHT on the last day, as shown both in the MTT test (27 % (4-OHT) vs. 57 % (Cd0.01 + 4-OHT), $p = 0.03$) and in the SRB test (46 % (4-OHT) vs. 68 % (Cd0.01 + 4-OHT), $p = 0.04$; Fig. 5b).

3.2.4. Cadmium interference with 4-hydroxytamoxifen at the level of gene expression and biochemical markers

4-OHT treatment led to significant changes in the expression profile of MCF-7 cells, both in short-term and long-term experiments (Fig. S4, Fig. S5). As expected, in a short-term experiment (Fig. S4), exposure to 4-OHT caused a significant 36 % decrease in *ESR1* expression ($p < 0.0001$ as compared to the control condition). At the same time, a

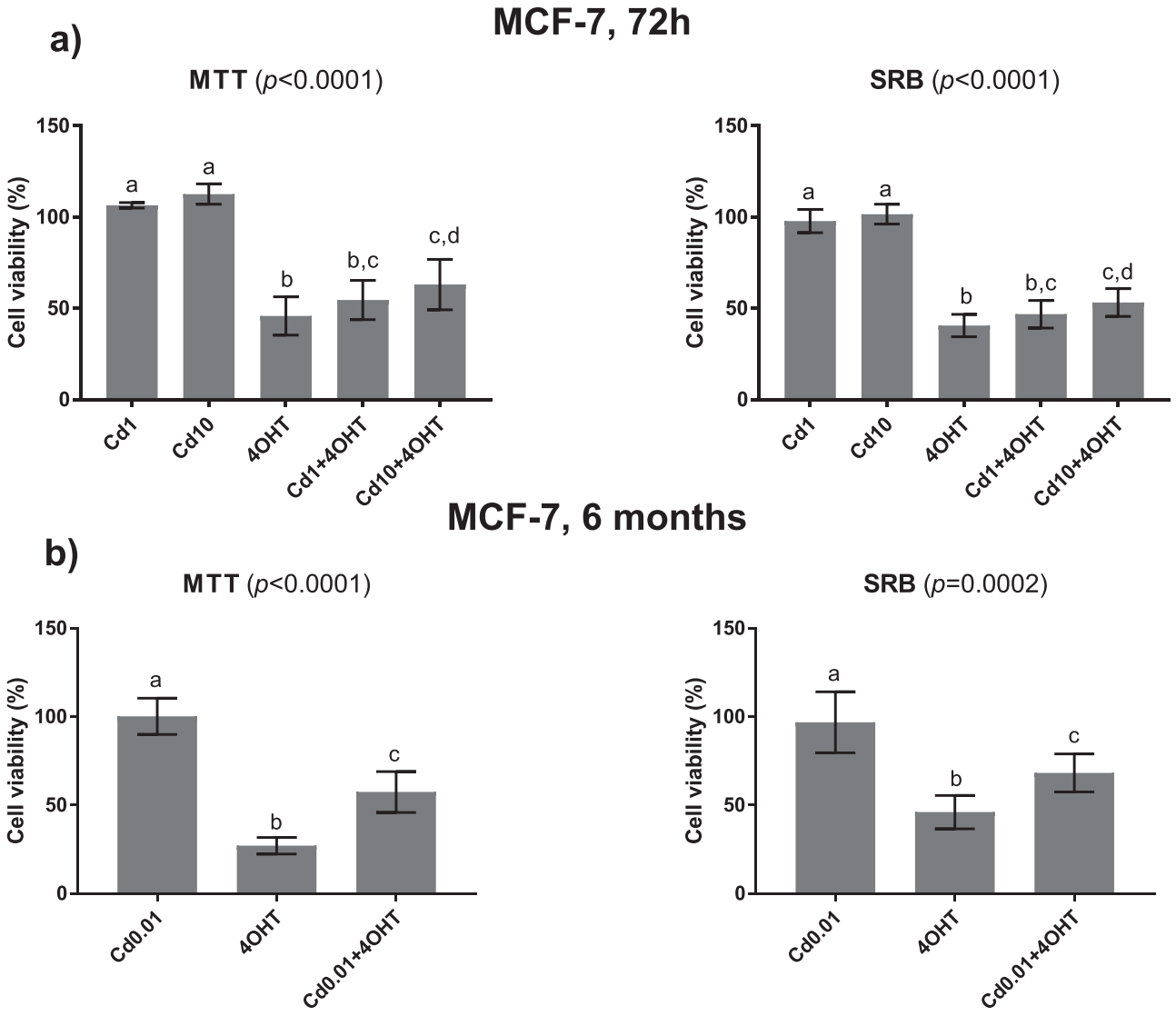

Fig. 5. Viability of MCF-7 cells exposed to Cd and 4-OHT assessed with MTT and SRB test. a) 72 h of exposure to 1 μM and 10 μM of Cd; b) 6 months of exposure to 0.01 μM Cd. Cells were co-exposed to 4-OHT (IC50; 15 μM) 24 h before cytotoxicity assays. Different letters above the bars indicate statistically significant difference (one-way ANOVA with Holm-Sidak's multiple comparison test).Viability for separate exposures (Cd or 4-OHT) was calculated with respect to the relevant control condition (medium, 0.1 % water or 0.3 % ethanol) and in the case of combined exposure (Cd+4-OHT), viability was calculated with respect to 0.3 % ethanol. Data shown as mean ± standard deviation (SD).

very strong increase in *PDK1* expression (by 268 %, $p < 0.0001$) and a strong increase in the expression of 7 other genes were observed, including *HIF1A* (50 %, $p < 0$, 0001), *HK2* (45 %, $p < 0.0001$), *PFKP* (33 %, $p < 0.0001$), *LDHA* (28 %, $p < 0.0001$), *PIK3CA* (27 %, $p = 0.044$), *GLUT1* (20 %, $p = 0.002$), and *PKM* (10 %, $p < 0.0001$). Preincubation of MCF-7 cells in Cd for 72 h (1 μM and 10 μM) did not change the expression profile of cells treated with 4-OHT, since similar changes were observed in all treatment groups, regardless of Cd exposure (Fig. S4).

In the long-term experiment (Fig. S5), exposure of MCF-7 cells to 4-OHT induced a significant, 55 % decrease in *ESR1* expression ($p = 0.0007$ as compared to the control condition). In general, changes in the expression of the Warburg effect-related genes upon 4-OHT were similar to those observed in the short-term experiment. The most highly upregulated gene upon 4-OHT treatment was *PDK1* (increase by 544 %, $p = 0.0001$). The other upregulated genes upon 4-OHT in the long-term experiment included *HK2* (103 %, $p = 0.0003$), *HIF1A* (45 %, $p = 0.002$), *PFKP* (41 %, $p = 0.0003$), *LDHA* (37 %, $p = 0.0008$), and *PKM* (13 %, $p < 0.0001$). The only downregulated gene upon 4-OHT in a long-term experiment was *AKT1* (decrease by 11 %, $p = 0.07$), and this change was not observed in the 72 h experiment. In the case of combined exposure (6 months of exposure to Cd 0.01 μM followed by treatment with 4-OHT on the last day), the demonstrated changes in mRNA expression were similar as compared to Cd-nonexposed cells, except for one gene - *PKM*, for which 4-OHT-induced overexpression was lower in cells preincubated with Cd as compared to Cd-nonexposed cells ($p = 0.03$, Fig. S5).

4-OHT treatment was not associated with changes in PK activity and cellular lactate concentration, regardless of Cd preincubation and time of Cd exposure (Fig. S7). However, a significant difference was observed in a long-term experiment in the concentration of lactate released into the medium, with significantly higher values observed in the case of combined exposure as compared to treatment with 4-OHT (923 vs 543 ng/μg, p for post hoc ANOVA = 0.01).

4. Discussion

In this study, we observed that Cd content measured in breast cancer tissues was higher than in peritumor tissues and correlated with urinary Cd concentration. Moreover, we have shown for the first time that long-term exposure to Cd at low concentration, led to the accumulation of the metal in breast cancer cells. Notably, Cd concentration used in the long-term experiment (0.01 μM; corresponding to the concentration of 1,12 μg/L) was within the range of Cd concentrations observed in the patients' urine in this study (0.12–3.35 μg/L) and only three times higher than Cd concentrations reported in human blood (geometric mean: 0.37 μg/L for men and 0.41 μg/L for women) [23]. Thus, the concentration investigated in this study in the long-term *in vitro* experiment may be regarded as environmentally relevant. Altogether, these data add some more biological plausibility for Cd implication in breast cancer development. This study confirms also other findings on high Cd content in breast cancer tissues, including our previous study conducted in a group of 42 Polish women and a recent case-control study on women from Serbia [15,17]. It should be noted that urinary Cd concentration (regarded as a good biomarker of environmental exposure to this metal [23] was relatively high in the studied individuals and confirms high exposure to Cd in Poland compared to other European countries [24].

Using data on Cd concentration in the urine and tissues of the patients, we aimed to investigate the relationship between environmental exposure to Cd and molecular markers of the Warburg effect in breast cancer. Interestingly, in both groups of breast cancer women, *HIF1A* was shown to be overexpressed in the tumor and significantly correlated with urinary Cd concentration. *HIF1A* encodes the alpha subunit of hypoxia-inducible factor 1, which is a key transcriptional regulator and the master player of the Warburg effect [25]. Activation of HIF-1α drives the Warburg effect mainly by inducing the expression of glucose transporters (GLUTs), glycolytic enzymes, LDH, and PDK1 [26]. Notably, urinary Cd was also correlated with another crucial gene related to the Warburg effect – *PIK3CG* (encoding phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma), though this gene was not overexpressed in the tumor tissues. Products of *PIK3* genes play an important role in the PI3K/AKT/mTOR axis and together with HIF-1, are central regulators of glycolysis, cancer metabolism, and cancer cell proliferation [27]. Intriguingly, tumor expression of *HIF1A* and *PIKCG* were strongly correlated with urinary Cd but not with the content of Cd in the tumor. Furthermore, exposure to Cd at different non-toxic concentrations had no impact on the expression of *HIF1A*, *PIKCG* and other genes involved in the Warburg effect in breast cancer MCF-7 cells. We hypothesize that the observed correlation between tumor expression of *HIF1A* (as well as *PIK3CG*) and urinary Cd could have been explained by an unknown confounder – for example, factor, which was directly correlated with *HIF1A/PIK3CG*, and independently correlated with urinary Cd as a result of joint exposure. The only significant effect of Cd observed in the experimental part of this study was limited to the increased extracellular concentration of lactate upon tamoxifen in cells exposed to Cd for 6 months. Thus, we conclude that, overall, this study does not give strong support for a causative relationship between Cd exposure and glycolytic phenotype. The origin of the Warburg effect and its direct role in cancer still lacks explanation and remains an issue of scientific debate [4]. So far, not much attention has been paid to the possible role of carcinogens in metabolic reprogramming, focusing mainly on arsenic (As) [7]. As, similar to Cd, is a carcinogenic heavy metal [8]. Experimental studies indicated that exposure to As in different cancer cell lines was associated with metabolic alterations, including increased glycolysis and lactate production [7], thus it could be a possible confounder in our study.

Independently of the main aim of the study, we confirmed that, similar to other cancers, breast cancer is associated with the Warburg effect [28]. Transcriptomic analysis of breast cancer tissues conducted separately in two different groups of breast cancer women enabled us to identify the most significant markers "universal" for breast cancer, regardless of clinicopathological features. We observed high consistency in the expression profile between two groups of patients, indicating tumor overexpression of crucial genes related to the Warburg effect, which, apart from the mentioned *HIF1A*, also included *GLUT1*, *HK1*, *PKM* and *PFKL*. *GLUT1* (also known as *SLC2A1*), which encodes the membrane protein responsible for cellular glucose transport, was the most overexpressed gene in the tumor. This observation confirms the unusual reliance of cancer cells on increased glucose uptake and supports data on the clinical importance of GLUT1 in breast cancer [29–31]. Importantly, *GLUT1* overexpression in breast cancer was generally associated with poor overall survival and disease-free survival, as shown by a recent meta-analysis [32]. The other three significantly upregulated genes, *HK1*, *PKM* and *PFKL*, encode specific isoforms of glycolysis rate-limiting enzymes: hexokinase (HK), pyruvate kinase (PK) and phosphofructokinase (PFK) [33]. All these three enzymes are largely implicated in cancer, though their role in breast cancer was less often investigated [34–36]. For example, a recent study conducted on breast cancer women from Pakistan showed increased mRNA expression of specific isoforms of HK, PK and PFK in the tumors characterized by late clinical stages, positive nodal involvement, and distant metastasis [34].

The most interesting observation in this study was related to the interference of Cd exposure with tamoxifen metabolite in breast cancer cells. Cd reduced the sensitivity of MCF-7 cells to 4-OHT in a dose-dependent manner in a short-term experiment, and similar effect was confirmed further in long-term experiment upon treatment to low and environmentally relevant concentration of the metal. It could be expected that Cd, acting as a metalloestrogen, interferes with the tamoxifen via activation of estrogen receptor, as was observed in the case of other xenoestrogens [37]. However, in our study, Cd did not affect mRNA and protein expression of ESR1 after 6 months of exposure, thus it may be speculated that the underlying mechanisms were not related to

the estrogenic activity of this metal. Furthermore, this effect was also not related to the changes in the expression profile of the Warburg effect-related genes as well as alterations of PK activity. However, a significantly higher concentration of extracellular lactate was observed upon combined treatment (Cd and 4-OHT) compared to treatment with 4-OHT only. Notably, lactate was suggested very recently to promote tamoxifen resistance in MCF-7 cells [38], so it is highly possible that, in our study, Cd reduced MCF-7 cells to 4-OHT via enhanced lactate production. Overall, these data may suggest the possible implication of Cd in intrinsic resistance to endocrine therapy in breast cancer. Further studies are required to explain, whether this effect is mediated directly by the estrogenic potential of the metal (via induction of estrogen-responsive genes which were not analyzed in this study), lactate overproduction, or other mechanisms. Considering the fact that the main non-occupational source of Cd exposure is tobacco smoking, it would be relevant to investigate the potential impact of smoking on treatment with tamoxifen in breast cancer patients.

Apart from the main results, this study yielded some other interesting data, including the effect of 4-OHT treatment on the expression of the Warburg effect-related genes. Exposure to tamoxifen metabolite led to a dramatic increase in several mRNA transcripts related to energy metabolism reprogramming. The highest upregulation was observed for *PDK1* which may confirm the suggested role of pyruvate dehydrogenase kinase in the development of tamoxifen resistance [39]. The other interesting observation was the negative correlation between *HIF1A* expression and ER status in the tumor tissues, which may reflect the regulatory relationship between these two proteins [40].

The major limitation of this study concerned the small sample size in the case of biological material collected from patients for Cd analysis (Cd content in the tissues was assessed in half of the patients, whereas urinary Cd was available for one-third of the subjects). Another limitation was that most of the correlations observed for urinary Cd concentration were significant when this marker was expressed as μg/L, whereas no significance was present in the case of values adjusted for creatinine. Nevertheless, all analyses were performed separately in two different groups of breast cancer patients, allowing for independent verification of the main observations. It should be also mentioned, that the experimental part of this study did not include an assessment of mitochondrial function upon Cd exposure. Originally, impairment of mitochondria was proposed by Otto Warburg as the primary cause of glycolytic switch, but this theory was undermined by further studies, showing normal mitochondrial activity in cancer cells [4]. Interestingly, some studies suggest that mitochondria represent the main target of Cd toxicity [41], which suggests that its role in the general energetic metabolism of cancer cells should be further explored.

5. Conclusions

This study failed to indicate a strong relationship between exposure to Cd and the Warburg effect in breast cancer. However, an interesting observation on Cd interference with tamoxifen in MCF-7 cells deserves further investigation because it may suggest that chronic exposure to this metalloestrogen, resulting in its accumulation in the tissues, may be implicated in intrinsic resistance to hormone therapy of breast cancer.

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Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Conceptualization, E.J. and J.R.; Methodology, E.J., E.R., J.R., B.J., M.L., K.T.; Validation, J.R., B.J.; Formal analysis, K.T., E.J., J.R., B.J., K. K., M.B.; Investigation, K.T., J.R., E.J., B.J., M.L., K.K., E.W., M.B., A.K-W., J.S.; Resources, E.R., L.K.; Data curation, E.J., A.K-W., J.S., M.B., L. K.; Writing – original draft preparation, K.T., E.J.; Writing – review & editing, J.R., B.J., M.L., E.R., E.W., M.B., A.K-W., K.K., J.S., L.K., Visualization, K.T., E.J.; Supervision, E.J.; Project administration, E.J.; Funding acquisition, E.J, E.R., L.K. All authors have read and agreed to the published version of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114435.

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