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Lucie Linhartova, Nathalie Costet, Farzad Pakdel, Tomas Cajthaml, Denis Habauzit. Key parameter optimization using multivariable linear model for the evaluation of the in vitro estrogenic activity assay in T47D cell lines (CXCL-test). Journal of Applied Toxicology, 2022, 42 (7), pp.1121-1136. 10.1002/jat.4280 . anses-03512680

HAL Id: anses-03512680

https://anses.hal.science/anses-03512680

Submitted on 26 Jan 2022

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Key parameter optimization using multivariable linear model for the evaluation of the *in vitro* estrogenic activity assay in T47D cell lines (CXCL-test)

Multivariable linear model use for estrogenic assay optimization

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Keywords
Estrogenic activity cellular assay, multivariate linear model (MLM), T47D, material variability, Cytokine CXCL12/SDF1

Abstract

In comparison to analytical tools, bioassays provide higher sensitivity and more complex evaluation of environmental samples and are indispensable tools for monitoring increasing in anthropogenic pollution. Nevertheless, the disadvantage in cellular assays stems from the material variability used within the assays, and an interlaboratory adaptation does not usually lead to satisfactory test sensitivities. The aim of this study was to evaluate the influence of material variability on CXCL12 secretion by T47D cells, the outcome of an estrogenic activity assay, the CXCL-test. For this purpose, the cell line sources, sera suppliers, experimental and seeding media, and the amount of cell/well were tested. The multivariable linear model (MLM), employed as an innovative approach in this field for parameter evaluation, identified that all the tested parameters had significant effects. Knowledge of the contributions of each parameter has permitted step-by-step optimization. The most beneficial approach was seeding 20,000 cells/well directly in treatment medium and using DMEM for the treatment. Great differences in both basal and maximal cytokine secretions among the three tested cell lines and different impacts of each serum were also observed. Altogether, both these biologically based and highly variable inputs were additionally assessed by MLM and a subsequent two-step evaluation, which revealed a lower variability and satisfactory reproducibility of the test. This analysis showed that not only parameter and procedure optimization but also the evaluation

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/JAT.4280

methodology must be considered from the perspective of interlaboratory method adaptation. This overall methodology could be applied to all bioanalytical methods for fast multiparameter and accurate analysis.

Short abstract
The CXCL-test is a sensitive *in vitro* assay developed in T47D cell line to identify estrogenic properties of anthropogenic chemicals and to monitor pollution caused by endocrine disruptors. As the test sensitivity can be modified by several factors, multivariable linear model was employed as an innovative statistical approach for fast evaluation of contributing factors. It revealed that cell lines, media, sera, cell number and cell density significantly affected CXCL12 secretion. This knowledge enables new interpretation of the test results.

1 Introduction

An environmental endocrine-disrupting chemical (EDC) was defined as an "exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al., 1996). To date, almost 800 chemicals have been identified as EDCs (Karthikeyan et al., 2021), and most of them are either currently being used and are released continuously into the environment as a result or persist in environmental matrices due to past contamination. The majority of EDCs are considered micropollutants, which cause widespread trace contamination, usually in concentrations below the detection limits of the available analytical methods. Nonetheless, even low EDC contamination in the range of ng/l poses an ecotoxicological and human health risk (Jackson et al., 2019; Wee & Aris, 2017), and the severity of EDC contamination has already been addressed in the European Union EDC screening program (Decision 2018/840/EU, 2018; Directive 2008/105/EC, 2008).

The development of sensitive and reliable methods is essential for EDC monitoring in the environment. While analytical methods assess the content of target chemicals, bioanalytical methods evaluate the biological effects of the whole mixture of pollutants in the sample. Moreover, chemical analyses provide lower sensitivity and only partially elucidate the biological effects evaluated by *in vitro* assays (Conley et al., 2017; Tousova et al., 2017; Valitalo et al., 2016).

The suitability and sensitivity of the current *in vitro* assays have been previously reviewed by several authors (Leusch et al., 2017; Li et al., 2020). However, even when the preconcentration of samples is taken into consideration, *in vitro* tests based on cell lines are among the most sensitive bioanalytical tools. The extensively used *in vitro* estrogenic activity assays employed in environmental sample evaluations are based on genetically modified estrogen receptor-positive cells transfected with the estrogen response element sequence with an appropriate detection marker (Adam et al., 2020; Legler et al., 1999; Leusch et al., 2017; Wilson et al., 2004). Stably transfected cells provide both sensitivity and technical simplicity. High sensitivity was also proven with several alternative methods, such as immuno- or electrochemical assays (Li et al., 2020). Nevertheless, all these methods are focused only on one estrogenic pathway or on the detection of one estrogenic compound. The most sensitive cellular assay that assesses complex cell response is the *in vitro* proliferation test, the E-screen (Leusch et al., 2017; Soto et al., 1995).

The CXCL-test, an *in vitro* assay also evaluating complex cell response to estrogenic compounds, was developed by Habauzit et al. (2010) as an alternative method to classical proliferation tests, and the sensitivity and time- and cost-effectiveness were improved later (Habauzit et al., 2017). The test employs the estrogen receptor-positive breast cancer cell line T47D derived by Keydar et al. (1979), which was previously employed in the E-screen (Soto

et al., 1995) or transfected cellular tests (Adam et al., 2020, Wilson et al., 2004) for the evaluation of estrogenic activity. The CXCL-test is based on the quantification of estrogen-stimulated secretion of cytokine stromal cell-derived factor 1 (CXCL12 previously referred to as SDF1) in T47D. As the regulation of *CXCL12* transcription is controlled by multiple estrogen-related pathways (Boudot et al., 2011), the test provides overall information about the cell response. The CXCL-test revealed the endocrine-disrupting properties of several widely used pharmaceuticals and personal care products (Ezechias et al., 2016; Habauzit et al., 2017; Michalikova et al., 2019). Moreover, the test was also used as a tool for the development of a predictive mixture toxicity model (Ezechias & Cajthaml, 2016), and its suitability for the evaluation of endocrine-disruptive activity of environmental samples was reviewed by Li et al. (2020).

The main disadvantage of *in vitro* cellular tests stems from the use of highly variable biological materials, which makes reproducibility and interlaboratory protocol transfer difficult and usually does not lead to satisfactory sensitivity; therefore, the assay is sometimes abandoned. The aim of this study was to evaluate the effects of crucial parameters on the CXCL-test, a sensitive and complex *in vitro* assay, by a multivariate statistical methodology. Moreover, suggestions for the evaluation of the test performance and management of the result assessment were also determined. The tested experimental parameters were selected based on our previous experiments. The cell line source, medium used for cell seeding, experimental medium, serum supplier and cell density before and during the experiment were tested in a step-by-step experimental optimization procedure, and the multivariate linear model (MLM) was employed as a suitable statistical approach that allowed simultaneous assessment of the effects of multiple parameters and their interactions on an outcome. Therefore, the MLM was applied within this study to evaluate the influence of all the tested parameters on the secretion of CXCL12. From this knowledge, a new method of data interpretation was determined, and its applicability was demonstrated on the estrogenic effect evaluation of five endocrine-disrupting compounds.

2 Materials and Methods

2.1 Chemicals

The compounds of interest 17β-Estradiol (E2, ≥98%), 17α-Ethinylestradiol (EE2, ≥98%), Genistein (≥98%), Genistin (≥97.5%), Daidzein (≥98%), and Daidzin (≥95%), were purchased from Merck (Merck, Darmstadt, Germany), and ethanol (99.9%) was obtained from VWR (VWR, Prague, Czech Republic). All media and supplements from Gibco, Life Technologies, and Invitrogen brands were purchased from Thermo Fischer Scientific (Thermo Fischer Scientific, Waltham, USA). Charcoal-stripped fetal bovine serum (chsFBS) was obtained directly in the estrogen-free (charcoal-stripped) form from two different suppliers, Merck and Biowest (Biowest, Nuaillé, France), and labelled in this study as chsFBS(Mer) and chsFBS(Bio).

2.2 Cell lines and cell maintenance

Three T47D breast carcinoma cell lines were used. T47D(ATCC) was directly purchased from American Type Culture Collection, ATCC® HTB-133™. T47D(IBT), kindly donated by Dr. Truksa (Laboratory of Tumor Resistance, Czech Academy of Sciences) were also originated from ATCC, as well as T47D(IRSET), which were kindly provided by Dr. Pakdel (Laboratory of Transcription, Environment and Cancer, Research Institute for Environmental and Occupational Health, France).

All cell lines were routinely maintained in RPMI 1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% non-essential amino acids solution (NEAA), 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (PS); for details, see Table 1. The cells were cultured at 37 °C and 5% CO_2 in a humidified incubator. The culture

medium was renewed every 3–4 days, and the cells were passaged by a trypsin (Gibco) treatment once per week.

2.3 Experimental design

Estrogenic treatments were performed in 96-well plates (Nunc™, Thermo Fisher Scientific) according to Habauzit et al. (2017). Several protocol modifications were applied to investigate their influence on CXCL12 secretion. All experiments were performed with three T47D cell lines, T47D(ATCC), T47D(IBT), and T47D(IRSET). Two treatment media were tested: phenol red-free RPMI 1640 (Gibco) supplemented with 1% GlutaMAX™ (Life Technologies) and PS and phenol red-free DMEM (Gibco) supplemented with 1% NEAA, PS, 1 mM sodium pyruvate, and 4 mM glutamine. Both phenol red-free treatment media were completed with 2.5% chsFBS(Bio) or chsFBS(Mer). For details see Table 1. Additionally, different amounts of cells/well and different cell densities in the flasks before the experiment were tested.
The schedule of all A-C experiments is given in Figure 1. The cells were seeded overnight (day 1). In Figure 1A, the cells were seeded at a total amount of 20,000 cells/well in phenol red culture medium. As shown in Figure 1B, the cells were seeded at 10,000 or 20,000 cells/well directly in treatment medium (phenol red-free RPMI or DMEM). Finally, the cell density in the flask before cell seeding was evaluated (Figure 1C). Twenty thousand cells per well were directly seeded in phenol red-free DMEM treatment medium. Cells were issued from low (40% confluent) and high (80% confluent) cell densities in the initial flask.
When the cells were initially seeded in culture medium (Figure 1A), they were double rinsing with Dulbecco's phosphate-buffered saline (DPBS). In all three setups, the medium was replaced by 200 µl of fresh treatment medium on day 2 and incubated for an additional 24 h. Thereafter, on day 3, the medium was removed and replaced by 150 µl of the treatment medium containing either E2 in the range of $2.5\ 10^{-12}$ – $1.0\ 10^{-8}$ M or pure vehicle (0.1% ethanol (v/v)). The cells were treated for 24 h, and the level of the secreted cytokine CXCL12 was quantified by enzyme-linked immunosorbent assay (ELISA) (day 4).

2.4 ELISA

The secreted cytokine CXCL12 was quantified by a human CXCL12/SDF-1 DuoSet® ELISA kit (R&D Systems, Minneapolis, USA). Quantification was performed according to the manufacturer's protocol, as previously described (Habauzit et al., 2010). The results of ELISA were assessed by measuring the absorbance at 450 and 570 nm with the Infinite M200 PRO microplate reader (Tecan, Switzerland).

2.5 Statistical and data analyses

Each experiment was analyzed separately using the MLM to test the respective effect of each experimental parameter and the E2 dose on the level of CXCL12 secretion. In experiment A, the effects of the cell line (reference = T47D(ATCC)), medium (reference = RPMI), serum (reference = chsFBS(Mer)) and E2 dose (reference = ethanol) were tested as the main fixed effects. Evaluated doses of E2 were $1.0\ 10^{-9}$ M, $1.5\ 10^{-10}$ M, $4.4\ 10^{-11}$ M, $2.8\ 10^{-11}$ M, $8.8\ 10^{-12}$ M, $2.5\ 10^{-12}$ M. To detect non-linearity in the E2 - CXCL12 secretion dose–response relationship and to estimate differential secretion at the specific E2 concentrations tested, the dose of E2 was considered a categorical variable. Moreover, the interactions among the cell line, dose of E2, medium, and serum were also evaluated in experiment A (data not shown).
In experiments B and C, biological replicates were performed and mixed effect models were implemented, in which the sample (experimental/biological) incurred a random effect, to consider the repeated measures for each sample. Moreover, the additional E2 dose of $2.5\ 10^{-12}$ M was also evaluated. In experiment B, the analysis included not only the same fixed factors as in experiment A but also the "number of cells per well" (20,000 vs 10,000 as a reference).

In experiment C, the "number of cells per well" was not included; the "cell density before experiment" was considered instead (low vs high as a reference), and the medium effect was not taken into account. Interactions were also tested between the cell line and dose of E2 or between the cell line and serum (data not shown).
Furthermore, to reveal potential differences in the effect of the E2 dose and experimental parameters on CXCL12 secretion among the tested cell lines, the same analyses were applied to each cell line separately. The results of the analyses are presented as the difference in mean level of CXCL12 secretion expected for each level of the different experimental parameter and E2 dose compared to the reference category, and all other parameters remain equal.

2.6 Methodology for optimal analysis

The ethanol controls reflect each tested parameter. To identify only the estrogenic effect under each experimental condition, the corresponding secretion level in the ethanol control was subtracted from the secretion level in each replicate of E2 treatment. Then, to compare the final estrogenic effect, a second correction was performed. Each individual biological replicate was expressed as a percentage of CXCL12 secretion treated with 1.0 10^{-9} M E2, which was considered as 100%.

2.7 Evaluation of endocrine-disrupting chemicals

The dose–effect relationship of EE2, Genistein, Genistin, Daidzein, and Daidzin was evaluated in the range of 1.0 10^{-12} M – 1.0 10^{-5} M (0.1% ethanol served as a control). After 24-h exposure, the cytokine secretion was quantified, and the results were adjusted by subtracting the respective ethanol control. Thereafter, each individual biological replicate was expressed as a percentage of CXCL12 secretion treated with 1.0 10^{-9} M E2, which was tested simultaneously and considered as 100%.

3 Results

3.1 Cell line source

T47D(ATCC) cells were obtained directly from the original supplier, ATCC. Although T47D(IBT) and T47D(IRSET) cells were kindly provided by two laboratories (Czech Republic, France), however the origin of the cell lines was ATCC as well. The cell lines showed slightly different dynamics of growth during maintenance (data not shown) and different resistances to buffer rinsing (see below). The cell comparison under a phase-contrast microscope showed identical cell shapes and very similar cell sizes (Figure S1).
As shown in Figures 2, 3, 4, and 5, the cell lines demonstrated different levels of basal (ethanol control) and maximal cytokine secretion. The lowest secreted level of CXCL12 was detected in T47D(ATCC) (basal 32–48 pg/ml, maximal 85–196 pg/ml); T47D(IBT) basally secreted 44–102 pg/ml of CXCL12 and maximally 210–332 pg/ml; and the highest secretion was detected in T47D(IRSET) (basal 103–217 pg/ml, maximal 280–412 pg/ml) when 20,000 cells/well were seeded in treatment medium (Figures 4 and 5). Moreover, involvement of the cell line origin in CXCL12 secretion was deciphered by MLM analysis (Table 2). When the T47D(ATCC) cell line was considered as a reference (in table marked as 0 (ref)), the secretion of CXCL12 was significantly higher in the T47D(IBT) cell line by 9 to 110 pg/ml and in the T47D(IRSET) cell line by 76 to 150 pg/ml according to the experiment. The difference in mean CXCL12 secretion between T47D(ATCC) and T47D(IBT) cells was not significant in experiment C when parameters, such as the medium, and the cell densities were fixed before the experiment. Nevertheless, T47D(IRSET) was the cell line with the significantly highest secretion regardless of the experimental design. Moreover, separate analyses for each cell line

were carried out to obtain deeper insight into CXCL12 secretion (Table 3). In general, technical parameters such as the medium, serum, amount of the cells/well, and cell density before the experiment mostly modified CXCL12 secretion in the same way, even though the magnitude (in pg/ml of the secreted CXCL12) and the statistical significance differed. For instance, the design in experiment C enabled a decrease in the detection limit and evidenced that the E2 treatment at 2.5 10^{-12} M induced a significant effect on CXCL12 secretion in T47D(ATCC) and T47D(IRSET) cells (Table 3).

3.2 Effects of the protocol and technical parameters on CXCL12 secretion

The MLM analyses confirmed that the protocol (medium used for the cell seeding or washing step) and technical parameters (cell line source, medium, serum, amount of the cells/well and cell density before the experiment) affected cytokine secretion. However, each cell line manifested their own levels of secretion and sensitivity to the parameters, and these were evaluated in two steps. First, the global effect of the experimental parameters was considered with all cell line sources together (Table 2). Second, the differences in cytokine secretion that were caused by the tested parameters in each cell line were examined (Table 3).

3.2.1 Cell seeding medium

In experiment A, the cells were seeded in culture medium, which contained estrogenic compounds (FBS hormones, phenol red). Therefore, a double buffer rinse was necessary before the medium was exchanged with the treatment medium. As previously mentioned, the microscope control revealed a different resistance to buffer washing in the tested cell lines. Indeed, during the washing step, the T47D(ATCC) and T47D(IBT) cells remained adhered to the surface, whereas the T47D(IRSET) cells were noticeably washed away. Subsequent tests of viability (see supplementary information) revealed up to approximately 50% loss of T47D(IRSET) cells when compared to those of T47D(ATCC) and T47D(IBT) cells (data not shown). Nonetheless, CXCL12 secretion by T47D(IRSET) cells was sufficient despite cell detachment (illustrative example Figure 2); hence, cell detachment was not observed until viability tests were performed. Since the cell lines manifested different adherence properties and stability in the DPBS washing step, the cells were seeded directly in the respective treatment medium, and the rinsing step was eliminated in the following optimization tests, where no differences in the adherence properties were detected by the viability tests. Seeding in culture medium was not conducted in any other experiment. Moreover, the tested E2 concentration was subsequently decreased to determine the method detection limit.

After adjusting for the effect of the other factors (medium, serum, replicate), it was apparent that T47D(ATCC) and T47D(IRSET) seemed to react in the same way. Unlike them, T47D(IBT) cells were more sensitive to the medium, DMEM increased the secretion by 165±30 pg/ml on average, and chsFBS(Bio) decreased the secretion (Table 3).

3.2.2 The amount of cells/well

In general, basal CXCL12 secretion significantly increased by 119±7 pg/ml on average when the number of cells/well was raised from 10,000 to 20,000 (Table 2, experiment B). When 10,000 cells/well was applied, both medium and serum were shown to affect the test sensitivity. As shown in Figure 3, T47D(ATCC) cells treated in chsFBS(Mer)- or chsFBS(Bio)-supplemented RPMI did not secrete sufficient amount of cytokine to manifest an E2 dose–response effect. Similarly, DMEM supplemented with chsFBS(Mer) did not affect the effect in T47D(IRSET) cells. In conclusion, the amount of 10,000 cells/well was applicable only for T47D(IBT) and in some conditions for the T47D(IRSET) cell lines.

When the cells were seeded at 20,000 cells/well, an E2 dose–response effect was detected in all the tested cell lines (Figure 4). As shown in Table 3 (experiment B), an increase by 39±4,

106±7, and 210±12 pg/ml of the secreted cytokine was manifested in T47D(ATCC), T47D(IBT), and T47D(IRSET), respectively.

3.2.3 Medium used for the cell treatment

DMEM induced a statistically significant increase in CXCL12 secretion compared to that of RPMI, regardless of the medium used for cell seeding. As shown in Table 2 (experiments A and B), increases of 75±15 and 39±7 pg/ml were detected in culture medium-seeded and treatment medium-seeded experiments, respectively. As previously detailed, the cell lines differed in sensitivity to the medium used for the treatment.

Nonetheless, in both experiments (A, B) where the media were compared, significantly different levels of CXCL12 secretion were detected. As shown in Table 3 (experiments A and B), DMEM increased cytokine secretion in all tested cell lines by 32±11, 165±30, and 34±16 pg/ml CXCL12 in culture medium-seeded T47D(ATCC), T47D(IBT) and T47D(IRSET) cells, respectively (experiment A). When the cells were seeded directly in treatment medium (experiment B), the secretion of CXCL12 increased in DMEM by 26±4 (T47D(ATCC)), 34±9 (T47D(IBT)), and 56±12 (T47D(IRSET)) pg/ml compared to RPMI. These effects on CXCL12 secretion were also clearly manifested in the ethanol control samples (Figure 4).

3.2.4 Treatment medium supplemented with charcoal-stripped serum

In fact, the serum, which was monitored in all the experiments, was shown to significantly affect cytokine secretion only in experiments B and C, where chsFBS(Bio) induced an increase in secretion (Table 2). Finally, the MLM analysis revealed a significant increase in cytokine secretion in all the tested cell lines in experiment C, where parameters such as the medium used for seeding and the treatment medium were fixed. As shown in Table 3 (experiment C), chsFBS(Bio) increased CXCL12 secretion by 36±10, 24±10, and 56±5 pg/ml in T47D(ATCC), T47D(IBT), and T47D(IRSET) cells, respectively.

3.2.5 Cell density before the experiment

The treatment was performed only in DMEM, which was previously shown to increase the level of secreted CXCL12 and in this manner to improve the test sensitivity. As shown in Table 3, the density of the cells before the experiment affected cytokine secretion; densely cultivated cells secreted significantly lower amounts of the cytokine in all the tested cell lines. When an approximately eighty percent confluence was formed in the cultivation flask prior to the experiment, T47D(ATCC), T47D(IBT), and T47D(IRSET) cells exhibited significantly decreased secretion by 30±10, 44±10, and 19±5 pg/ml, respectively (Table 3, experiment C and Figure 5).

3.3 General Analyses Methodology

The MLM methodology allows the additive effects of each parameter to be assessed on the global variance of CXCL12 secretions with different combinations of experimental conditions. As illustrated in Figure 6, the secretion observed in the ethanol controls reflects the mean secretion resulting from a particular combination of experimental conditions that generate different levels of secretion. For instance, the effect of serum was similar in T47D(ATCC) and T47D(IBT) cells, while T47D(IRSET) cells were more sensitive to the serum origin. The medium demonstrated a similar effect in T47D(IBT) and T47D(IRSET) cells, and the lowest response to the medium was observed in T47D(ATCC) cells. To identify only the estrogenic effect under each experimental condition, the corresponding secretion level in the ethanol control was subtracted from the secretion level in each replicate of E2 treatment.

Finally, all three cell lines and both sera were evaluated in the mean (and standard deviation) of all experiments performed in DMEM (Figure 7a). The results are expressed as the mean of 5 to 6 independent experiments. As shown in Figure 7a, the curves observed were quite

different from one another. The secretion of each E2 concentration was individually corrected by the secretion of the respective ethanol control (Figure 7b), and therefore CXCL12 secretion for control was set to 0 for all conditions tested. This first correction showed that the T47D(IRSET) and T47D(IBT) curves were quite similar in shape and magnitude. However, T47D(ATCC) showed quite different magnitudes, and this magnitude was still dependent upon the serum (Figure 7b). To compare the final estrogenic effect, the last correction was performed, and each individual biological replicate was expressed in percent of the secretion at $1.0\ 10^{-9}$ M E2, considered as 100% (Figure 7c). This two-step operation showed that regardless of the cell line considered or the serum used, the estrogenic effect on secretion was equivalent.

3.4 Evaluation of endocrine-disrupting chemicals

The applicability of the methodology was demonstrated on the evaluation of dose–response effects induced by five different molecules (one synthetic well-known estrogenic compound and four derived isoflavones). The methodology has permitted to evaluate the estrogenic effect of the tested molecules; thus, the method suitability for identifying endocrine disruptors was demonstrated (Figure 8). All the tested molecules induced CXCL12 secretion in a dose-dependent manner. Moreover, EE2 and isoflavones Genistein and Daidzein induced the maximal secretion of CXCL12 observed in the $1.0\ 10^{-9}$ M E2 treatment (Figure 8A, B and, E). The glycosylated forms of isoflavones Genistin and Daidzin did not change the sensitivity of the molecule stimulation in comparison to Genistein and Daidzein, respectively. Nevertheless, a slight increase in the estrogenic effect was observed for the derivatives (Figure 8 C and, E).

4 Discussion

Considering the wide diversity of material used among laboratories, interlaboratory protocol transfer requires an optimization procedure. Therefore, some of these parameters, such as cell line source, media used for cell seeding and treatment, serum supplier, amount of the cells and cell density before experiment, were evaluated. Moreover, the most appropriate protocol for endocrine disruption chemical evaluation, the CXCL-test, was proposed within this work. The involvement of each parameter in CXCL12 secretion was assessed during the three-step protocol optimization and subsequent MLM analyses, which were used for a global effect analysis and a stratified analysis according to the cell line.

The global MLM analysis revealed constant significant differences in CXCL12 secretion among the tested cell lines, even though the cell origin was initially the same; the original supplier (ATCC) and two laboratories (Czech Republic, France) purchased the cell line from ATCC (Habauzit et al., 2017; Tomkova et al., 2019). Identical cell shape did not indicate any diversity. However, the cell lines demonstrated immense differences in both basal and E2-stimulated CXCL12 secretion. Dissimilarities among the same cell lines have been discussed in recent decades (Resnicoff et al., 1987), especially in biomedical research. Our results are in agreement with the literature. The T47D cell line was shown to be genetically instable and undergoes ploidy changes spontaneously (Reddel et al., 1988). New populations of T47D were identified after extended weekly passages, and different characteristics of these sublines, such as steroid receptor levels and changes in ploidy, were demonstrated (Fernandez et al., 1994; Reddel et al., 1988). Increased copies of some chromosomes could be associated with an increased level of steroid receptors in T47D subpopulations (Graham et al., 1989). Moreover, vastly different responses to both estrogens and anti-estrogens were proven among the T47D sublines (Graham et al., 1989; Graham et al., 1990; Reddel et al., 1988). Advanced multi-omic analyses have discovered differences among the same commonly used cell lines (Kleensang et

al., 2016; Liu et al., 2019), and suppliers require upgraded quality declaration (Lorsch et al., 2014). Moreover, Kleensang et al. (2016) demonstrated genetic variability even in the same ATCC frozen batch of the estrogen receptor-positive breast cancer cell line MCF-7, which is frequently used for estrogenic activity testing. In addition, starkly different estrogenic responses were identified between the same-batch cells in Kleensang's study. Reassuringly, E2 treatment of all the tested T47D cells induced an increase in CXCL12 secretion in a dose-dependent manner, which disproved the cell line dependency of the test and confirmed their suitability for the CXCL-test. However, the experiments and subsequent MLM analyses affirmed that the cell line as well as other parameters played an important role in this treatment. In this work, each tested cell line required different optimal test conditions, and most of the tested parameters affected secretion differently according to the cell line. The global analysis did not allow us to observe the effect of parameters on the cell line individually. As formal testing of all the interactions between all the tested experimental conditions was not possible (there were too many parameters to estimate and interpret, inducing a lack of power), we focused on the interaction with the cell line and implemented a second MLM analysis stratified by cell line. This analysis allowed us to more precisely decipher the parameter impacts on the secretion in each cell line individually and the impact on new parameters studied on the secretion. The T47D(IRSET) cell line demonstrated suitability only with the treatment medium seeding design of the CXCL-test. The highest cytokine secretion (up to 412±12 pg/ml) was detected in T47D(IRSET) cells, and a positive effect for secretion was significantly identified when DMEM and chsFBS(Bio) were applied. Generally, cytokine secretion that is too high may also cause a problem with ELISA signal linearity, and overexpression of the cytokine in the assay should be resolved by additional supernatant dilution, as was previously described (Habauzit et al., 2010). In experiment A, T47D(IRSET) cells manifested equal or lower secretion than T47D(IBT) cells (e.g., DMEM, chsFBS(Mer)), which could be explained by the adherence properties of T47D(IRSET) cells. The lower secretion by T47D(IRSET) was not observed in any other experiment.

The T47D(IBT) cell line demonstrated sufficient E2-stimulated secretion, reaching 332±23 pg/ml (treatment medium-seeded experiments), in combination with quite low basal secretion in the controls. Compared to T47D(IRSET) and T47D(IBT), ATCC purchased cells (T47D(ATCC)) demonstrated the lowest CXCL12 secretion ability, which did not exceed 196±4 pg/ml in the treatment medium seeded experiments even in the favourable DMEM supplemented by chsFBS(Bio). However, low basal secretion enabled estrogenic treatment evaluation in the T47D(ATCC) cell line. In T47D(ATCC) cells, it was demonstrated that a decrease in basal secretion caused by unfavourable cell conditions could improve the test sensitivity in parameters such as fold of induction (FI). Nevertheless, the FI evaluation of the test sensitivity was considerably dependent on the test performance and on variability in the material. The material could increase basal secretion, but the maximal secretion was determined by the cell secretion ability. Therefore, the FI was not a convenient outcome to analyse the data obtained due to the variability of the ethanol controls in different conditions.

The number of cells per well (10,000 cells/well in a 96-well plate) was optimized in a preceding study by Habauzit et al. (2017) with T47D(IRSET) cells that secreted more CXCL12 than other tested cells evaluated within this work. In this study, the 10,000 cells/well did not provide a sufficient dose–response curve, especially in T47D(ATCC), where the amount of the secreted cytokine was in some cases also near the detection limit of the ELISA.

A marked effect of treatment medium was detected in all the experiments. DMEM significantly increased global CXCL12 secretion in all the tested cell lines compared to RPMI. Our results are in accordance with other studies where a medium impact on various cell outcomes was observed. Wu et al. (2009) showed that DMEM enhanced cell differentiation and induced the activity of important enzymes in periosteum-derived cells compared to that of RPMI medium.

The composition of DMEM and RPMI (available on the Thermo Fisher Scientific website) shows that DMEM contains higher amounts of almost all the components in common, which stimulate cell metabolism. The medium was also shown to play a key role in the up- or downregulation of almost 9,000 genes in MDA-MB-231 cells, the breast carcinoma cell line, when the most common media (RPMI, MEM, and DMEM) involvement was studied (Kim et al., 2015).

The test was affected by not only the medium used for the treatment but also the seeding medium. When the cells were seeded in the culture medium, where the buffer washing step was needed, highly reduced adhesiveness was observed in the T47D(IRSET) cells. Direct seeding into the treatment medium was therefore applied in further experiments. Moreover, the benefit of cell preparation in steroid-free medium prior to the experiment was shown earlier. For instance, Wilson et al. (2004) recommend cultivation in steroid-free medium even one week before the experiment.

The serum was also shown to be mostly involved in cytokine secretion. A significant stimulating effect of chsFBS(Bio) was found in the experiments, where the cells were seeded in the treatment medium. Different impacts of serum were expected, as serum is the most lot-to-lot variable component of medium with variable mitotic and toxic properties. The composition of FBS is dependent on, e.g., different animal genetics, location of origin, animal feed, etc. Zheng et al. (2006) showed that high-abundance proteins were detected in comparable relative amounts among the sera of different suppliers. However, even lot-to-lot variability was observed in the content of growth factors. Moreover, when serum is applied in an estrogenic activity evaluation assay, charcoal-dextran treatment of serum is necessary to eliminate serum estrogens. Nevertheless, this additional procedure increases the lot-to-lot variability. A great diversity and efficiency of several charcoal stripping procedures were demonstrated by Sikora et al. (2016). The final test efficiency and sensitivity could be strongly moderated by a small increase in basal cytokine secretion caused by a trace amount of hormone in chsFBS. This phenomenon is not rare. Experiences have shown us that an incomplete charcoal-dextran purification of serum even in the range of units of pg/ml E2 (declared in supplier's Certificate of Analysis) considerably increases basal secretion and markedly decreases test sensitivity.

The cell density in the initial flask before the experimental evaluation showed that a higher cell density significantly decreased cytokine secretion in all the tested cell lines. This result is concordant with the study by Kim et al. (2015), who proved there are diverse proteomic profiles of breast cancer cells at different cell densities. Moreover, decreased expression of CXCL12 in a growing tumor was proven earlier (Zhao et al., 2014). Nevertheless, the sensitivity of the CXCL-test was not modified because differences were observed in both control and E2-treated cells.

Two more parameters, technical and biological replicates, were included in the evaluation. While the technical replicates demonstrated equal cytokine secretion in the same treatment, the biological replicates provided statistically significant differences. This is not surprising, as for all optimization experiments, only two biological replicates were carried out. Nevertheless, the effects of the tested parameters were affirmed in both biological replicates. The optimization enabled us to show variability in the CXCL-test outcome when the original protocol was modified by interlaboratory material variability. Although some authors exhort strict protocol compliance (Kim et al., 2015), the current situation when great variability in cell lines is not fixed (Kleensang et al., 2016; Lorsch et al., 2014) and few methodology details are usually published does not allow us to follow their suggestion. The results show that some protocol modifications are required. However, the unavoidable variability in the biological material (chsFBS, cell line) allows only some general recommendations for estrogenic assay performance. The choice of serum without estrogen residues together with rich nutritive

properties supporting the secretion in treatment increases the test sensitivity. Furthermore, a preliminary test of every new chsFBS lot is advised. Seeding directly to the treatment medium was shown to be strongly favourable in all the tested cell lines. Conformed confluence affected the secretion, but a clear benefit was not detected in any tested variant. Additionally, the suitable number of cells/well was increased compared to the original study (Habauzit et al., 2017) to 20,000 cells/well, which suggests adjustment for this parameter if needed.

Finally, it was shown that the experimental conditions greatly affected the final results regarding the dose-response association of interest. The MLM statistical method offers real value for cell-based toxicological test evaluation. It provides a fast multiple parameter assessment, which simultaneously evaluates both fixed and random effects (when repeated experiments occur) and detects potential interactions among the monitored parameters. This statistical analytical methodology also permits the assessment of many parameters with a low number of replicates. A similar approach was used by deCastro & Neuberg (2007) in MCF-7 E-SCREEN assessment. A generalized linear mixed model was shown to represent an appropriate method to evaluate design variations such as plate-to-plate, well-to-well, and the interaction between plate-to-plate variation and the dose of E2. Nevertheless, only reproducibility in technical performance was considered in deCastro's study. To the best of our knowledge, this is the first paper evaluating the impact of a biological-based material variability such as different sera and different sources of cells using this statistical methodology.

The most common evaluation of toxicological tests based on a stimulated biological answer is the FI parameter, indicating how many times stronger the stimulated cell answer is in comparison to the appropriate control. However, this parameter could be considerably affected by differences in the basic protocol and the material variability, which was also proven within this work. Therefore, the test evaluation could be misinterpreted, and the test sensitivity could be undervalued. The most variable material based on biological origin (chsFBS and cell line) was finally evaluated in the general analysis, where all the experiments performed in DMEM were considered. First, the comparison of the means showed great differences among the six treatment variants (three cell lines, two sera). Nevertheless, among all possible interaction calculations, MLM did not permit evidence of any interaction among the parameters we tested. Because formal testing of all the interactions between all the tested experimental conditions was not possible (too many parameters to estimate and interpret, inducing a lack of power), we proposed a complementary analysis where only the test sensitivity (dose-response) was assessed after controlling for the effect of the combination of experimental parameters of each experiment. This normalization by the level of secretion in ethanol controls allows better control of the interactions between the experimental conditions. This first step of the data normalization was not sufficient for comparing all the parameters. Indeed, the T47D(ATCC) curves demonstrated a lower magnitude than the other cell lines. This fact could be due to external factors of the remaining interaction. However, when a second normalization step where all these values were subsequently expressed in percent of E2 at the concentration of $1.0\ 10^{-9}$ M was carried out, all the curves became identical. This analysis revealed much lower variability among the tested cell lines and satisfactory reproducibility of the test. Step-by-step protocol (CXCL-test) optimization and subsequent MLM and "general" analyses enabled us to propose several suggestions applicable for interlaboratory transfer procedures and evaluation of the results. This methodology was applied for the evaluation of estrogenic compounds, and it was shown that the estrogenic properties were more easily observable with this approach. In our examples we observed that glycosylation of the natural compounds did not substantially affect the estrogenic properties of the compounds.

In conclusion, the CXCL-test, one of only a few *in vitro* assays for estrogenic activity that are not based on a genetically modified organism, was optimized, and the impact of variable

material used in the test, the test procedure and the data interpretation were evaluated within this work to facilitate interlaboratory protocol transfer.

All the tested parameters demonstrated a significant impact on CXCL12 secretion, the outcome parameter of the test. The step-by-step optimization allowed us to formulate the most suitable protocol for the CXCL-test performance. The T47D(IBT) cell line was considered the most suitable cell line for further experiments. However, the variability of biological parameters, such as the cell line or serum, makes the definite recommendation of a particular serum or cell line inapplicable, further demonstrating the necessity for the optimization, for instance, of each new batch of serum. Nonetheless, the remaining parameter optimization clearly showed that the seeding of the 20,000 cell/well directly in the phenol red-free treatment medium and DMEM used as an exposure medium were the most suitable parameters for the CXCL-test.

Moreover, not only the impact of the material variability and the methodology procedure should be considered in the interlaboratory protocol adaptation but also the way data interpretation affects the final test results. For instance, the commonly used evaluation parameter FI was influenced by the material variability, and an innovative approach of the test assessment was determined. MLM analyses and two-step normalization decreased the variability and increased the reproducibility of the CXCL-test results. This statistical method provides an exploration of the factors involved in EDC evaluation and could be applied, for instance, in the assessment of the mixture of molecules (e.g., synergetic antagonism evaluation). These general requirements should permit us to compare all results obtained even from different laboratories and could be generalizable to other *in vitro* assays usually used for EDC evaluation.

Acknowledgements:

Authors are grateful to Dr. Truksa to have provided the T47D.

Funding

This research was financially supported by the Czech Science Foundation [Grant number 20-14318Y]

Conflicts of interests

The authors declare that they have no conflict of interest.

Authors' contribution statements

Conceptualization: [Denis Habauzit], [Tomas Cajthaml]; Methodology: [Denis Habauzit], [Lucie Linhartova]; Formal analysis and investigation: [Lucie Linhartova], [Denis Habauzit], [Nathalie Costet]; Writing - original draft preparation: [Lucie Linhartova], [Denis Habauzit]; Writing - review and editing: [Lucie Linhartova], [Denis Habauzit], [Nathalie Costet], [Farzad Pakdel], [Tomas Cajthaml]; Funding acquisition: [Tomas Cajthaml]; Resources: [Tomas Cajthaml], [Farzad Pakdel]; Supervision: [Denis Habauzit], [Tomas Cajthaml]

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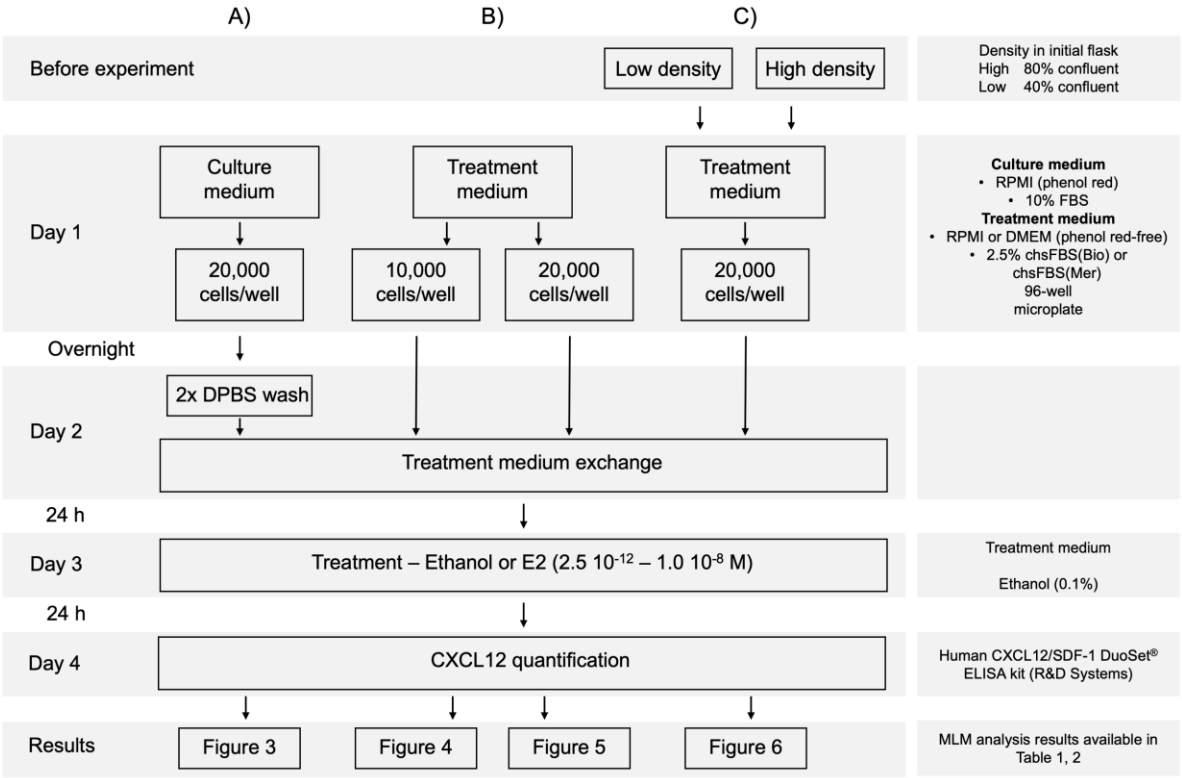


Figure 1. Experimental setup schedule (A, B, C) for the CXCL-test performed with T47D(ATCC), T47D(IBT), and T47D(IRSET) cells. (A) The cells were seeded overnight in culture medium at a total amount of 20,000 cells/well. The next day, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and a new portion of treatment medium (RPMI or DMEM supplemented with charcoal-stripped fetal bovine serum (chsFBS(Bio) or chsFBS(Mer)) was added and incubated for an additional 24 h. The cells were exposed to Estradiol (E2) or ethanol in the respective treatment medium. (B) A total of 10,000 or 20,000 cells/well were directly seeded in treatment medium (RPMI or DMEM) supplemented with chsFBS(Bio) or chsFBS(Mer). The next day, the medium was exchanged, and 24 h later, the cells were treated with ethanol or E2. (C) Twenty thousand cells/well originating from flasks of two different cell densities (low or high) were seeded in DMEM supplemented with chsFBS(Bio) or chsFBS(Mer). Then, the medium was renewed, and the cells were treated with ethanol or E2. In all the experiments, the treatment was terminated after 24 h of incubation. Secreted CXCL12 was immediately quantified by an enzyme-linked immunosorbent assay (ELISA), and the results were evaluated by a multivariate linear model (MLM).

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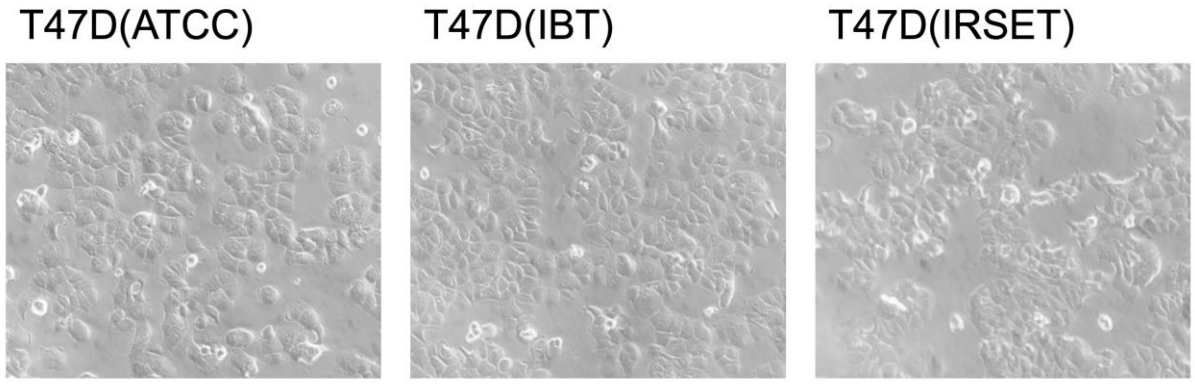


Figure 2. Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after a 24-h exposure to Estradiol (E2). Cells were seeded in cultivation medium and treated in two phenol red-free media (DMEM or RPMI) supplemented with two different charcoal-stripped sera (chsFBS(Bio) or chsFBS(Mer)).

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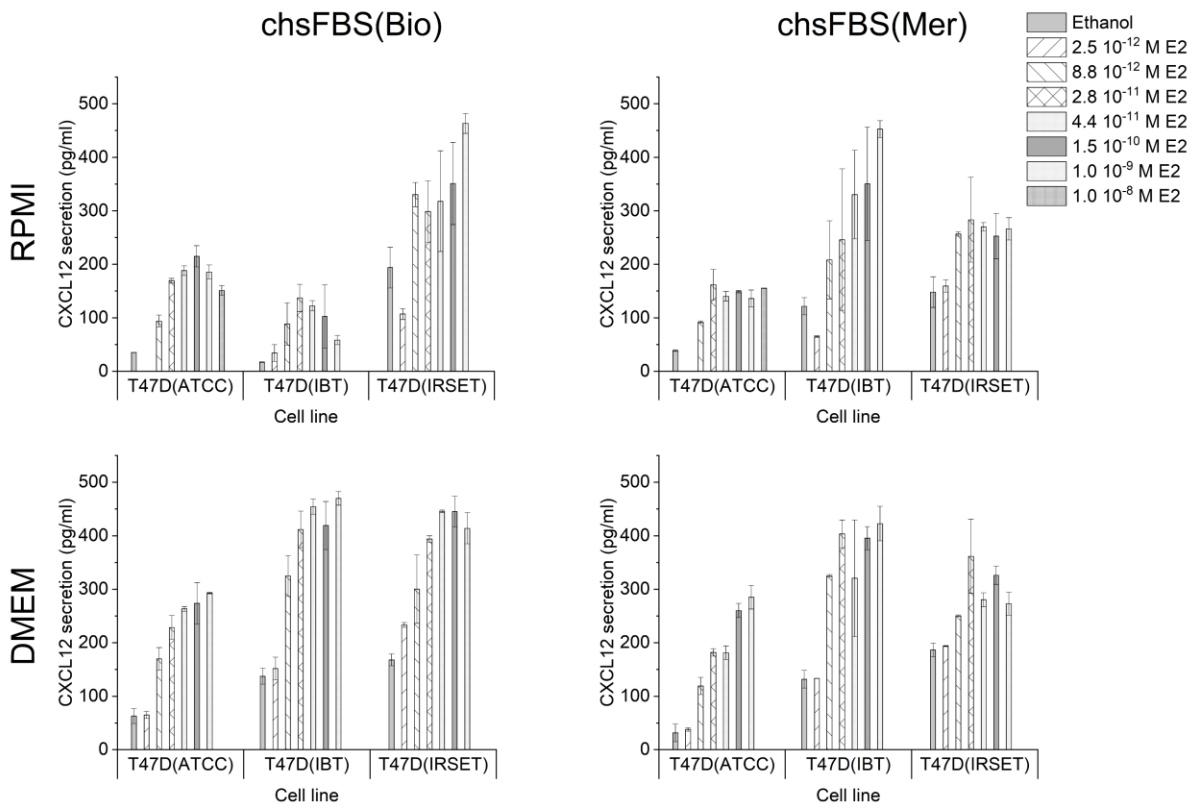


Figure 3. Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after 24-h exposure to Estradiol (E2). A total of 10,000 cells/well were seeded and treated in two phenol red-free media (RPMI or DMEM) supplemented with two different charcoal-stripped sera (chsFBS(Bio) or chsFBS(Mer)).

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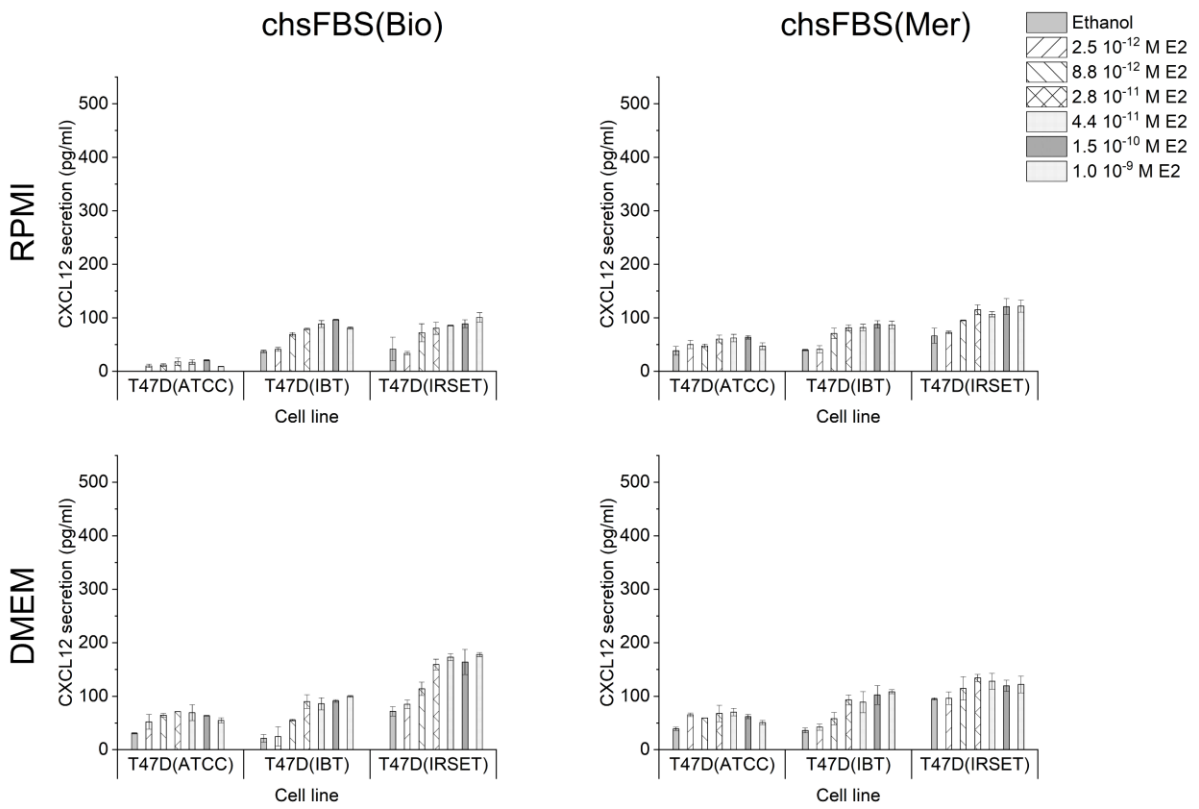


Figure 4. Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after 24-h exposure to Estradiol (E2). A total of 20,000 cells/well were seeded and treated in two phenol red-free media (RPMI or DMEM) supplemented with two different charcoal-stripped sera (chsFBS(Bio) or chsFBS(Mer)).

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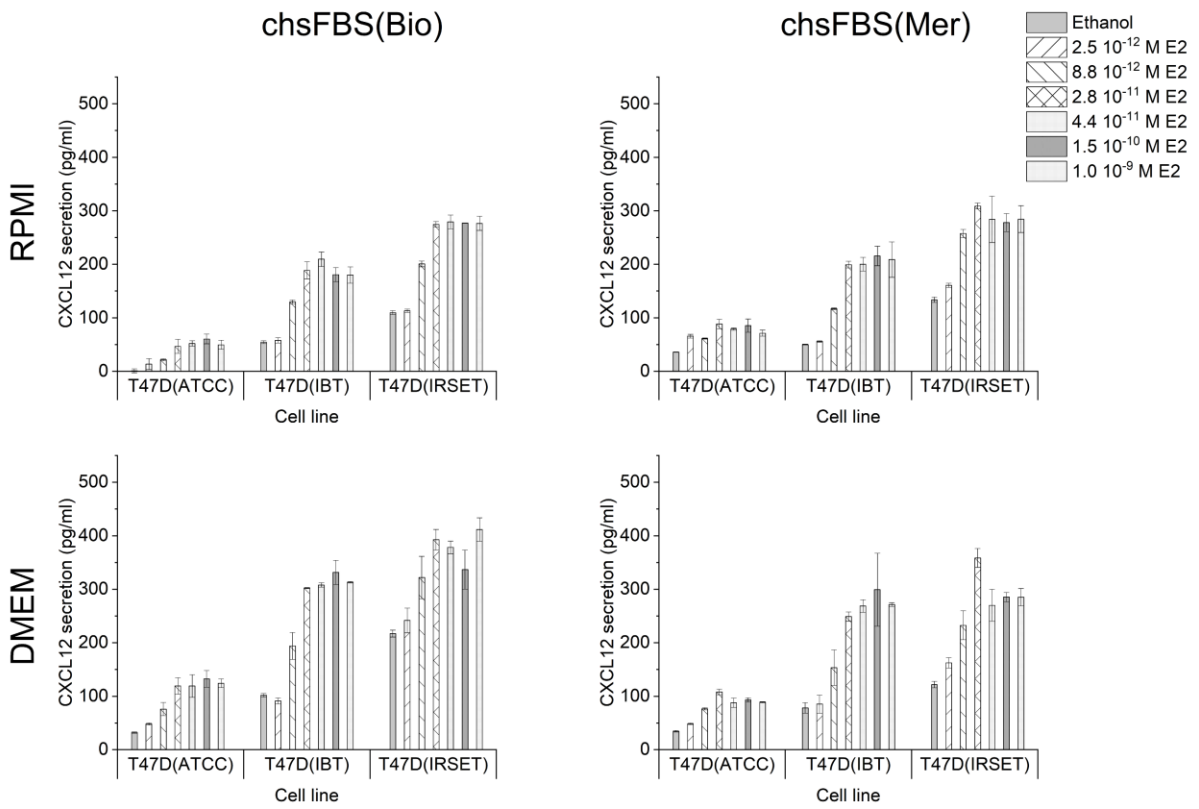


Figure 5. Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after 24-h exposure to Estradiol (E2). All the cell lines were exposed to DMEM supplemented with two different sera (chsFBS(Bio) or chsFBS(Mer)). Cells were seeded from two different cell culture densities; high and low density in the initial flask represent ca. 80 and 40% confluent, respectively.

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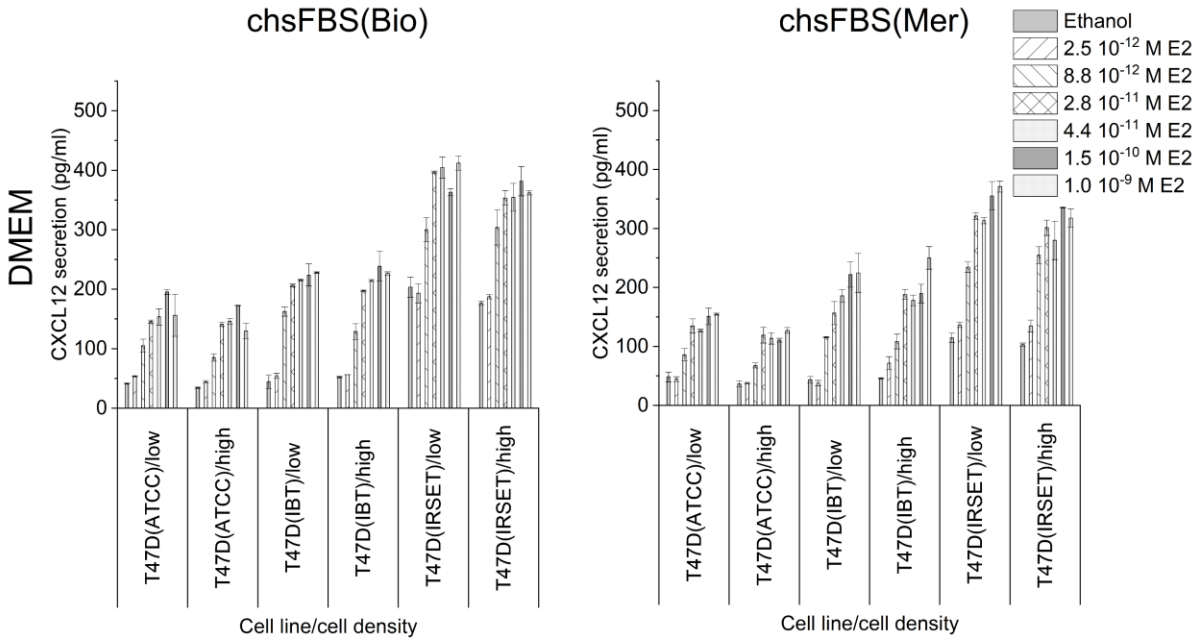


Figure 6. Illustration of the additional effect of the individual parameters on CXCL12 secretion in T47D(ATCC), T47D(IBT), and T47D(IRSET) cells. The amount of secreted CXCL12 was determined by a multivariate linear model for each parameter. The Merck-obtained charcoal-stripped fetal bovine serum (chsFBS(Mer)) and RPMI medium were considered references, whereas the effects of serum obtained from Biowest (chsFBS(Bio)) and DMEM were manifested in the columns with an effect of 1.0 10^{-9} M Estradiol (E2).

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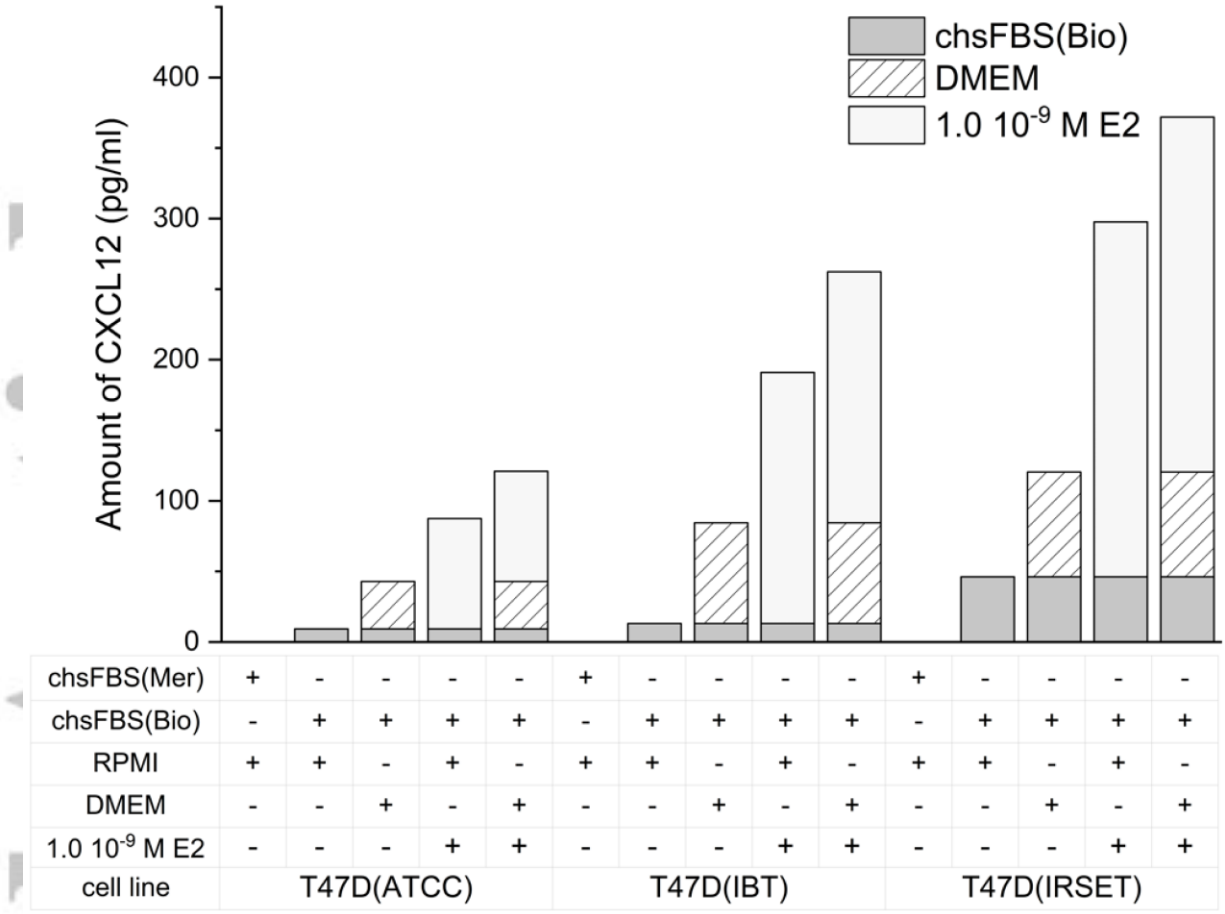
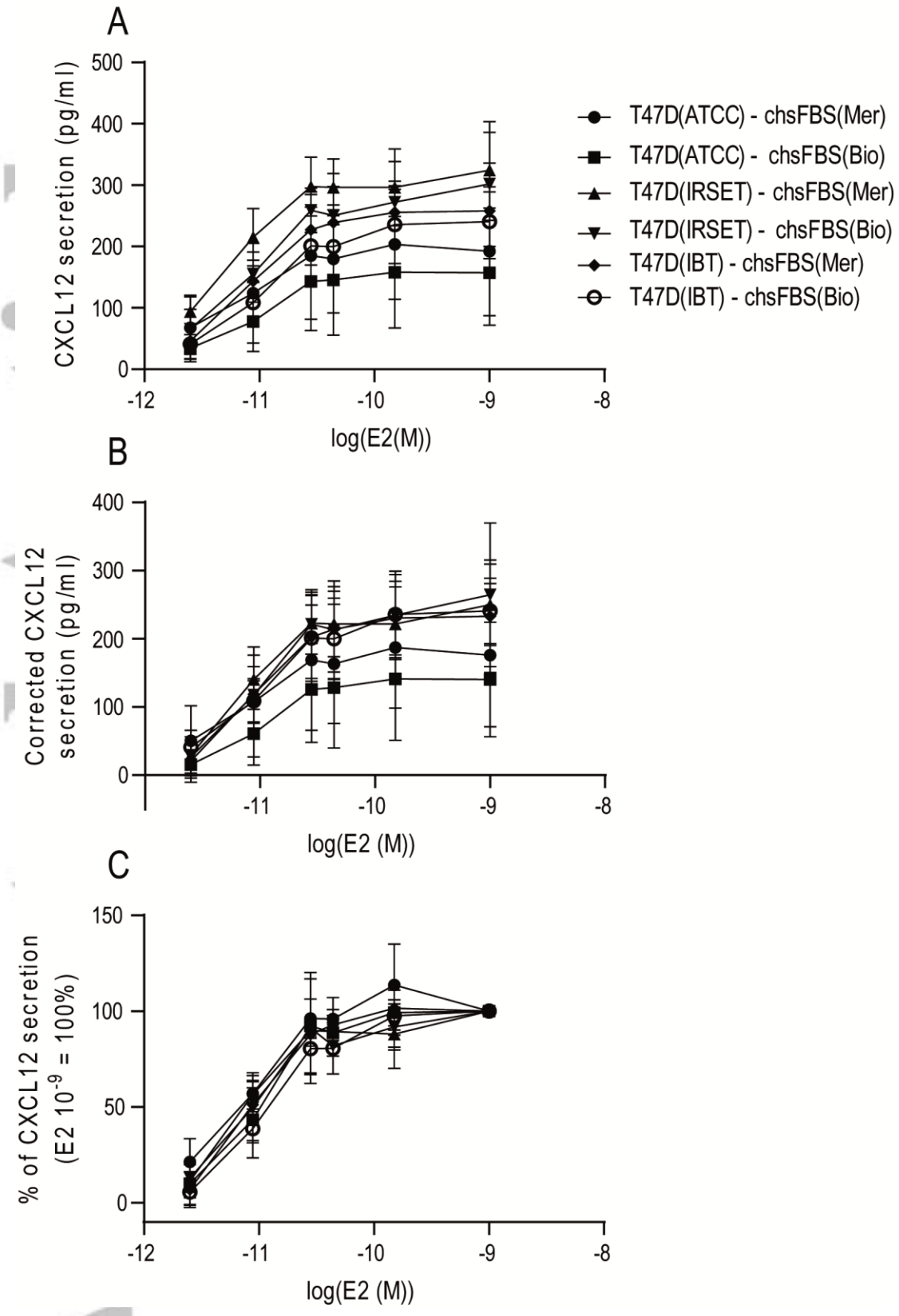


Figure 7. CXCL12 secretion considered from all DMEM seeded and treated experiments. The influence of the three T47D cell lines (ATCC, IBT, IRSET) and two charcoal-stripped fetal bovine sera (chsFBS(Mer) and chsFBS(Bio)) on CXCL12 secretion was evaluated after 24-h exposure to Estradiol (E2). The results are expressed as (A) the mean secretion, (B) the secretion of each E2 concentration corrected by the secretion of the respective ethanol control, and (C) the individual biological replicate in percent of cytokine secretion at 1.0 10^{-9} M E2 (considered as 100%).

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A
CXCL12 secretion (pg/ml)
500
400
300
200
100
0
-12
-11
-10
-9
-8
log(E2(M))
T47D(ATCC) - chsFBS(Mer)
T47D(ATCC) - chsFBS(Bio)
T47D(IRSET) - chsFBS(Mer)
T47D(IRSET) - chsFBS(Bio)
T47D(IBT) - chsFBS(Mer)
T47D(IBT) - chsFBS(Bio)
B
Corrected CXCL12 secretion (pg/ml)
400
300
200
100
0
-11
-10
-9
-8
log(E2 (M))
C
% of CXCL12 secretion (E2 10-9 = 100%)
150
100
50
0
-12
-11
-10
-9
-8
log(E2 (M))

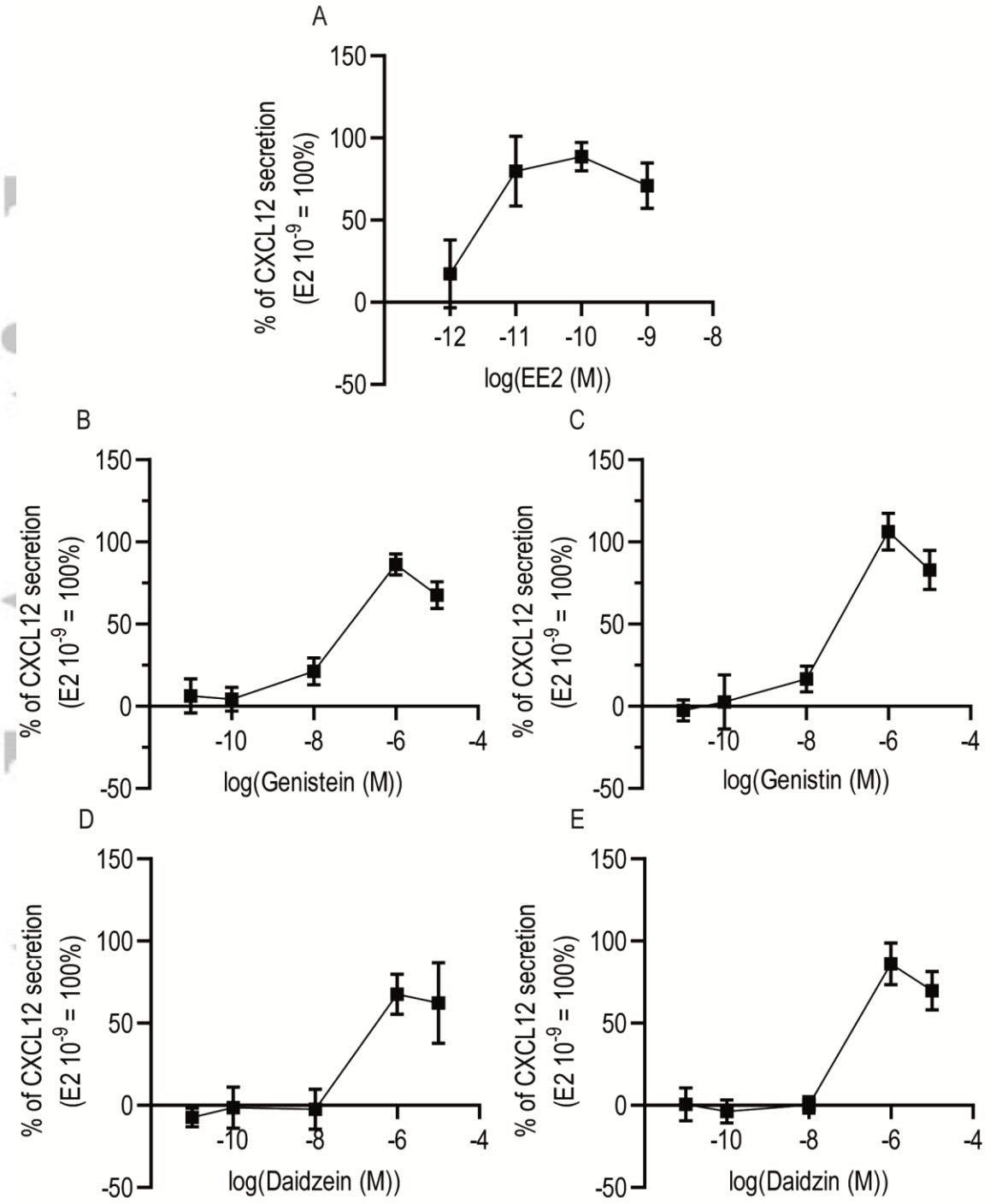


Figure 8. CXCL12 secretion after 24-h exposure to (A) Ethinylestradiol (EE2), (B) Genistein, (C) Genistin, (D) Daidzein, and (E) Daidzin. The cytokine secretion stimulated by the tested compounds was adjusted by subtracting the secretion of the respective ethanol control, and the results were expressed as a percentage of cytokine secretion at 1.0 10^{-9} M Estradiol (considered as 100%).

Table 1 Composition of the media.

	Medium Composition	Supplier, Catalogue number
Culture medium	RPMI 1640	Gibco, 21875
	10% FBS	Gibco, 10270
	1% NEAA	Gibco, 11140
	1% Sodium Pyruvate	Gibco, 11360
	1% PS	Gibco, 15140
Treatment medium (RPMI)	RPMI 1640	Gibco, 32404
	2.5% chsFBS	Merck, F6765 Biowest, S181F
	GlutaMAX™	Gibco, 35050
	1% PS	Gibco, 15140
Treatment medium (DMEM)	DMEM	Gibco, 31053
	2.5% chsFBS	Merck, F6765 Biowest, S181F
	1% NEAA	Gibco, 11140
	1% Sodium Pyruvate	Gibco, 11360
	1% Glutamine	Gibco, 25030
	1% PS	Gibco, 15140
Rinsing buffer	DPBS	Gibco, 14190

FBS = fetal bovine serum, NEAA = non-essential amino acids, PS = Penicillin-Streptomycin, chsFBS = charcoal-stripped FBS, HBSS = Hanks' balanced salt solution, AB = alamarBlue™ Cell viability reagent, CFDA = 5-carboxyfluorescein diacetate acetoxymethyl ester, DPBS = Dulbecco's phosphate-buffered saline

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Table 2 CXCL12 secretion changes in experiments A-C (Figure 1), where three T47D cell lines (ATCC, IBT, IRSET), two media (RPMI, DMEM), two charcoal-stripped fetal bovine sera (chsFBS, chsFBS(Bio) or chsFBS(Mer)), number of cells/well and different cell density before experiment were tested with or without Estradiol (E2) treatments. The data were analyzed by a multivariate linear model (MLM).

		MLM analysis		
		Experiment A	Experiment B	Experiment C
Tested parameter		Added value in pg/ml (*p*-value)		
Cell line	T47D(ATCC)	0 (ref)	0 (ref)	0 (ref)
	T47D(IBT)	110±24 (****)	83±11 (****)	9±10 (ns)
	T47D(IRSET)	145±24 (****)	150±8 (****)	76±10 (****)
Medium	RPMI	0 (ref)	0 (ref)	na
	DMEM	75±15 (****)	39±7 (****)	
Serum	chsFBS(Mer)	0 (ref)	0 (ref)	0 (ref)
	chsFBS(Bio)	22±14 (ns)	17±7 (*)	39±8 (****)
E2	Ethanol	0 (ref)	0 (ref)	0 (ref)
	1.0 10^{-9} M	195±25 (****)	108±13 (****)	221±16 (****)
	1.5 10^{-10} M	186±25 (****)	108±13 (****)	215±16 (****)
	4.4 10^{-11} M	174±25 (****)	97±13 (****)	194±16 (****)
	2.8 10^{-11} M	163±25 (****)	94±13 (****)	195±16 (****)
	8.8 10^{-12} M	100±25 (****)	47±13 (***)	115±16 (****)
	2.5 10^{-12} M	na	18±15 (ns)	28±16 (ns)
Cells/well	10,000	na	0 (ref)	na
	20,000		119±7 (****)	
Cell density	High	na	na	0 (ref)
	Low			31±8 (***)
Replicate	Experimental	9±6 (ns)	1±1 (ns)	2±2 (ns)
	Biological	11±24 (ns)	60±17 (***)	22±8 (**)

na = not analysed; ns = not significant; ref = reference; $p>0.05$ ns; $p\leq0.05$ *; $p\leq0.01$ **; $p\leq0.001$ ***; $p\leq0.0001$ ****

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Table 3 CXCL12 secretion changes in three T47D cell lines (ATCC, IBT, IRSET) in experiments A–C, where two media (RPMI, DMEM), two charcoal-stripped fetal bovine sera (chsFBS, chsFBS(Bio) or chsFBS(Mer)), number of cells/well and different cell density before experiment were tested in Estradiol (E2) treatment. The data were analyzed by a multivariate linear model (MLM).

					MLM analysis		
		Experiment A			Experiment B		
		T47D(ATCC)	T47D(IBT)	T47D(IRSET)	T47D(ATCC)	T47D(IBT)	T47D(IRSET)
Tested parameter						pg/ml (*p*-value)	
Medium	RPMI	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)
	DMEM	32±11 (**)	165±30 (****)	34±16 (*)	26± 4 (****)	34±9 (***)	56±12 (****)
Serum	chsFBS(Mer)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)
	chsFBS(Bio)	49±9 (****)	-80±30 (*)	81±16 (****)	2±4 (ns)	8±7 (ns)	39±12 (**)
E2	Ethanol	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)
	$1.0\ 10^{-9}$ M	170±17 (****)	249±53 (****)	180±27 (****)	47±8 (****)	120±13 (****)	160±23 (****)
	$1.5\ 10^{-10}$ M	178±17 (****)	215±53 (***)	170±27 (****)	55±8 (****)	118±13 (****)	154±23 (****)
	$4.4\ 10^{-11}$ M	166±17 (****)	205±53 (***)	155±27 (****)	43±8 (****)	109±13 (****)	141±23 (****)
	$2.8\ 10^{-11}$ M	142±17 (****)	198±53 (**)	160±27 (****)	44±8 (****)	103±13 (****)	136±22 (****)
	$8.8\ 10^{-12}$ M	70±17 (****)	135±53 (*)	110±27 (***)	19±8 (*)	54±13 (****)	67±22 (**)
	$2.5\ 10^{-12}$ M	na	na	na	20±10 (ns)	9±13 (ns)	38±29 (ns)
Cells/well	10,000	na	na	na	0 (ref)	0 (ref)	0 (ref)
	20,000				39±4 (****)	106±7 (****)	210±12 (****)
Cell density	High	na	na	na	na	na	na
	Low						
Replicate	Experimental	7±6 (ns)	29±14 (ns)	17±11 (ns)	2±1 (ns)	6±2 (**)	1±3 (ns)
	Biological	33±12 (**)	na	na	0±5 (ns)	42±9 (****)	34±13 (*)

na = not analysed; ns = not significant; ref = reference; $p>0.05$ ns; $p\leq0.05$ *; $p\leq0.01$ **; $p\leq0.001$ ***; $p\leq0.0001$ ****

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