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A toxicogenomic data space for system-level understanding and prediction of EDC-induced toxicity

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ABSTRACT

Endocrine disrupting compounds (EDCs) are a persistent threat to humans and wildlife due to their ability to interfere with endocrine signaling pathways. Inspired by previous work to improve chemical hazard identification through the use of toxicogenomics data, we developed a genomic-oriented data space for profiling the molecular activity of EDCs in an *in silico* manner, and for creating predictive models that identify and prioritize EDCs. Predictive models of EDCs, derived from gene expression data from rats (*in vivo* and *in vitro* primary hepatocytes) and humans (*in vitro* primary hepatocytes and HepG2), achieve testing accuracy greater than 90%. Negative test sets indicate that known safer chemicals are not predicted as EDCs. The rat *in vivo*-based classifiers achieve accuracy greater than 75% when tested for *in vitro* to *in vivo* extrapolation. This study reveals key metabolic pathways and genes affected by EDCs together with a set of predictive models that utilize these pathways to prioritize EDCs in dose/time dependent manner and to predict EDC evoked metabolic diseases.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a group of compounds which cause different adverse effects by perturbing with hormone systems (Foulds et al., 2017; Yang et al., 2018). High risk of exposure to these agents is possible due to their presence in different products including pesticides, cosmetics and pharmaceutical agents. Therefore, it is of great importance to label and prioritize these compounds in the environment and different areas of industry for hazard assessment (Karthikeyan et al., 2019). For this purpose, the Toxicity Forecaster (ToxCast™) program (Dix et al., 2007) has generated data using more than 800 *in vitro* high-throughput screening assays measuring activity of chemicals at endpoints, such as androgen (AR) and estrogen (ER) receptor activation, sensitive to endocrine disruption (Sipes et al., 2013; Becker et al., 2015; Silva et al., 2015). Many endpoints correspond to genes which are instrumental in identifying ED-related mechanisms of action and predict assay results for chemicals where little is known about their toxicity (Liu et al., 2015; Mansouri et al., 2016; Pham et al., 2019; La Merrill et al., 2020). However, the limited number of genes targeted in ToxCast hampers the implementation of accurate mechanistic analysis and toxicogenomics-based predictive tools (Vandenberg and Catanese, 2014). It is therefore necessary to study the ToxCast assay data in a broader biological context, by incorporating large scale toxicogenomics data which consider global gene expression after chemical exposure (Brockmeier et al., 2017; De Abrew et al., 2019).

Two major toxicogenomics data sources are the DrugMatrix (Ganter et al., 2005) dataset from the National Toxicology Program (NTP) and the TG-GATEs (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System) dataset from the Japanese National Institute of Biomedical Innovation (Uehara et al., 2010). TG-GATES includes time- and dose-course exposures across 160 chemicals, while DrugMatrix includes only time-course exposures across 130 chemicals. Both datasets include gene expression signatures derived from *in vitro* model of rat primary hepatocytes, and *in vivo* (rat) models exposed to hundreds of chemical compounds with varying hepatotoxicity, carcinogenicity and genotoxicity. TG-GATEs also provides toxicogenomic signatures derived from primary human hepatocytes. Furthermore, these two databases include the study of 26 known EDCs. Another example is the National Institute of Health Library of Integrated Network-Based Cellular

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Signatures (LINCS) 1000 project (Subramanian et al., 2017), which aims to model the gene expression response of compound exposures in cell lines. Toxicogenomics signatures retrieved from DrugMatrix, TG-GATEs and LINCS can be systematically analyzed to characterize mechanisms of action and potential toxic effects of compounds (Sutherland et al., 2018, 2019). In addition to the above databases, there are several manually-curated annotation databases, such as the Comparative Toxicogenomics Database (Davis et al., 2017, 2019), which provide information on compound-gene interactions, which correspond to molecular initiating events (MIEs) or early EDC-related key events (KEs), as well as compound-disease associations. CTD has been previously utilized to implement *in silico* methods for the identification of novel EDCs (Basili et al., 2018).

Mounting epidemiological and experimental evidence indicates that exposure to EDCs may predispose to several adverse health outcomes, such as certain cancers, neurodevelopmental disorders, immunological diseases and also various aspects of metabolic diseases, including obesity, fatty liver disease and disturbances of glucose and lipid metabolism (Heindel et al., 2017; Papalou et al., 2019). The main shortcomings of current knowledge are related to the lack of robust and predictive tests for metabolic outcomes, including challenges with interpretations based on rodent data, and the fact that much of the data is based on associations of single compounds with disease markers, while exposure usually entails complex mixtures of EDCs at low levels (Kassotis and Stapleton, 2019). Further, many EDCs are known to interact with several intracellular targets and may thus have impact on multiple biological pathways, potentially leading to adverse outcomes. For robust risk assessment, it would thus be of utmost importance to predict the overall biological phenotype emerging from EDC exposure and to identify suitable biomarkers for EDC exposure and effect.

On these premises, we decided to build a toxicogenomics data space for investigating and profiling the molecular activity of EDCs, with a specific focus on metabolic pathways. Compound-induced gene expression alterations in rat liver and rat primary hepatocytes, human primary hepatocytes and HepG2 cell line, were used to model mechanistic networks of compounds with a broad variety of potential toxicities, including compounds with known ED activity (Kohonen et al., 2017; Mulas et al., 2017; Li et al., 2019). Multiple toxicogenomic-driven gene networks are systematically built for in silico modelling of genes and pathways responsive to relevant ED-gene interactions (e.g. nuclear receptors) and adverse outcomes (e.g. metabolic diseases) (Heindel et al., 2017; Nadal et al., 2017; Papalou et al., 2019). Using the inferred genes and pathways as the basis for classification, we build (i) predictive models for identifying and prioritizing compounds with ED activity, and (ii) for inferring metabolic diseases EDCs may cause. Elastic-net-regularized classifiers were used to build the predictive models and also to reveal key genes and pathways in EDC-induced toxicity. The predictive models were then utilized to generate an overall EDC score for "untested" compounds starting from their known EDC-gene interaction information. Analyzing more than 10 000 chemicals annotated in CTD, this study provides the first massive toxicogenomics-driven in silico screening for endocrine disruptors. ToxCast, ToxPi and a set of known EDCs, which were excluded from the initial training, and negative test sets, were employed to extensively validate the trained predictive models. Drawing upon a system-level understanding of EDC-related toxicity, the results presented in this study provide insight into uncovered EDC–gene associations and pathway-level information to be used for the definition of novel adverse outcome pathways.

2. Materials and methods

2.1. Preparation of toxicogenomic-driven gene networks

Large-scale toxicogenomic datasets including LINCS, TG-GATEs and Drug Matrix were used to build toxicogenomic-driven gene networks. Pre-processed toxicogenomic signatures of TG-GATEs and Drug Matrix were downloaded from the diXa Data Warehouse (https://www.ebi.ac.uk/biostudies/diXa/studies). These datasets collect gene expression signatures of chemical exposures of rat hepatocytes *in vitro* and *in vivo* studies, and human hepatocytes *in vitro* and HepG2 cell lines. Log-fold changes between treatments and controls were considered for modelling the toxicogenomic signatures. Then, a different toxicogenomic-based gene network was built for the exposure scenarios that are studied in TG-GATEs and Drug Matrix (e.g., DrugMatrix-rat-*in vivo*-single-dose-1d, DrugMatrix-rat-*in vitro*-single-dose-1d, etc.). Co-expression networks were built from the selected toxicogenomics signatures via the R package wTO (Gysi et al., 2018). wTO compiles robust link weights between genes by using a bootstrapping approach. Eight gene networks were built from TG-GATEs. TG-GATEs includes a large variety of time- and dose-course exposures across 160 chemicals, 10 of which are classified as EDC. In order to avoid having a prediction system biased towards the toxicogenomic networks of TG-GATEs, we decided to focus on specific combinations of dose levels and time points of analysis. Six networks were built from gene expression signatures derived from *in vivo* models of rat liver. In more detail, three networks were defined from single-dose treatments, one day of exposure and three dose levels (high, low and middle). Then, other three networks were built from repeated-dose treatments and by using the available time points: 8, 15 and 29 days. The networks obtained from single-dose treatments are used to model dose-course exposures, while those obtained from repeated-dose treatments are utilized to model time-course exposures. Two more networks were defined from TG-GATEs by using *in vitro* exposure of human and rat primary hepatocytes, respectively. We considered only 24 h as time point of analysis when defining the *in vitro*-based networks of TG-GATEs. For the case of DrugMatrix, four gene co-expression networks specific to liver tissue were constructed based on compound-induced transcriptomics profiles of *rat primary hepatocytes* and rat liver for 1, 3 and 5 days of exposure. We decided to restrict our analysis to gene expression signatures that were profiled at time points greater or equal than 24 h. In general, we excluded short-term exposures lasting less than 24 h of duration, to study the ED-related effects of a given compound under a more realistic setting. All networks were built upon a set of genes that are known to be expressed in liver (Sutherland et al., 2016). The set of "liver-expressed genes" was used in order to increase the level of concordance of compound-induced transcriptional response in *rat liver* and cultured hepatocytes across DrugMatrix and TG-GATEs. A consensus co-expression network was also built for the rat *in vitro* networks in DrugMatrix and TG-GATEs. Expression signatures of human HepG2 cell lines exposed to compounds and drugs were selected from the LINCS database. In particular, a consensus network was implemented by integrating the gene network built for the compounds of the phase I with the network built for the compounds of the Phase II. A human protein–protein interaction (PPI) network derived from the StringDB database (Franceschini et al., 2013) was also included in this study in order to consider a gene interaction network without using toxicogenomics signatures. More detail on the defined toxicogenomic-driven gene networks can be found in Appendix Supplementary Methods and Results.

2.2. Selection of early key molecular events and adverse outcomes associated to EDs

Toxicogenomic-driven gene network were systematically used to find biological pathways that are activated by known compound-gene interactions or molecular initiating events. All gene-compound interactions were retrieved from the Comparative Toxicogenomics Database (CTD) (Davis et al., 2017, 2019). The genes related to reaction, binding, activity, expression and metabolic processing were considered as early key molecular events for the selected compounds. The chemicals with large numbers of interacting genes were considered as outliers and excluded based on an IQR filter. CTD provides a full list of gene interactions for each compound, regardless the experimental conditions in

which these putative MIEs are found. However, the list of gene interactions collected from CTD are systematically intersected with the genes of the networks. Since each network is modelled on a specific exposure scenario (e.g. 24 h after low-dose exposure), genes that are not dysregulated in a specific experimental condition are discarded. The CTD database is also used to determine known adverse outcomes (in our case diseases) that are known to be linked to EDCs.

2.3. Collection of core pathways

Gene sets annotated in KEGG (Kanehisa and Goto 2000; Kanehisa et al., 2017), REACTOME (Vastrik et al., 2007; Jassal et al., 2020), WikiPathways (Slenter et al., 2018) and Gene Ontology databases (The Gene Ontology Consortium, 2019) were retrieved by using the data source msigdb (Liberzon et al., 2015). Selected pathways from msigdb with annotation in adverse outcome pathways were retained and the other pathways were excluded from the list of the pathways. The pathways with gene size more than 200 were excluded from the pathways list, in order to exclude generic biological pathways. A detailed explanation for the preparation of the biological pathways is included in Appendix Supplementary Methods and Results.

2.4. Selection of known endocrine disrupting chemicals (EDCs) and negative controls

A set of chemicals with endocrine disruption activities based on evidence from both literature (DEDuCT) and *in vitro* studies related to nuclear receptors (ToxCast assay endpoints) were selected to define a robust set of known EDCs. The DEDuCT database provides a list of 686 endocrine disruptors, which are selected based on the review of more than 16,000 articles. The list of 686 EDCs was then crossed with the chemical inventory of ToxCast in order to rule out the agents for which only literature-based evidence data are available. In particular, ToxCast assays targeting nuclear receptors linked to endocrine disruptors were utilized to verify whether the 686 EDCs activate or inactivate relevant nuclear receptors (e.g. PPAR, PXR, ER, CAR, AR, etc.). Then, since the ToxCast™ nuclear receptor assays (Suppl. Table 1) are checked for down/up regulation, a test for proportion was performed to determine for each nuclear receptor which is the most common/frequent type of interaction with the selected EDCs (Suppl. Table 2). After selecting the most informative ToxCast assays (p-value less than 0.05) and discarding EDCs with hit calls largely inactive for these assays, the set of 304 EDCs was reduced to 287 agents. Finally, the CTD platform was used to define the final set of EDC-gene interactions, reducing the set of 287 EDCs to 197 agents. The CTD database was also used to select negative controls (or decoys), which correspond to chemicals that are not associated to early EDC-KEs of the 197 EDCs. The selection of decoys is a common practice in *in silico* screening methods (Réau et al., 2018). Appendix Supplementary Methods and Results provides detailed information on how the EDCs and related decoys were compiled.

2.5. The random walk with restart (RWR) and fast gene set enrichment analysis (FGSEA) procedure

The cascading effects of ED-gene interactions (or EDC-KEs) was modelled through network and pathway analysis. In particular, a network diffusion algorithm (random walk with restart, RWR) (Tong et al., 2006) was used in combination with Fast Gene Set Enrichment analysis (FGSEA) (Sergushichev, 2016) to generate compound-induced pathway activation scores. The RWR algorithm was needed to extend the initial sets of EDC-KEs, and to discover novel genes associated with EDs. The extended gene lists genes were then subjected to fast gene set enrichment analysis, or FGSEA (Sergushichev, 2016), in order to compile normalized enrichment scores (NESs) for pathway annotations. The R packages dnet (Fang and Gough, 2014) and FGSEA were used for the random walk-based analysis and the calculation of the NES scores, respectively. Detail on the approach RWR-FGSEA can be found in the Appendix Supplementary Methods and Results.

2.6. The RWR-based approach to identify putative EDC-KEs

The RWR-based approach estimated each gene's relevance (or steady probabilities) with regard to a given set of seed genes (ED-KEs or decoy-KEs) and a toxicogenomic gene network (e.g. 24 h after low-dose exposure). In order to identify novel EDC-associated markers, the odds ratio statistics (OR) was estimated from RWR steady probabilities to quantify the strength of marker gene associations between EDC and decoy within each network. Top 100 EDC-associated markers were selected by measuring, for each gene, the geometric mean of the best 60% OR estimates across the networks. Additionally, estimate percentiles were determined in each network for data visualization.

2.7. Machine learning based analysis

The matrix of pathway activation scores (Compounds × Pathways), resulting from the analysis of each toxicogenomic-driven network were utilized to train regularized and generalized linear models for two prediction tasks: i) identify chemicals with ED activity and ii) predict EDC-associated adverse. The employed ML approach is particularly useful for assessing the synergies and antagonisms (i.e. interactions) existing among pathway-based biomarkers for the identification and classification of EDCs. The classification models were trained and validated with repeated 10-fold-cross-validations, while the hyperparameter tuning was implemented with random search. Both steps were implemented by using the R package caret(Kuhn, 2008). Due to an imbalanced number of EDCs (~197) and decoys (or negative controls in data layers: ~1300), the F1 metric was applied as a measure of accuracy during cross validation. It considers both the precision and the recall of the test to compute the accuracy: an F1 score reaches its best value at 1 (perfect precision and recall) and worst at 0. ANOVA with post-hoc Tukey test was compiled to verify whether differences observed across the F1-scores were statistically significant. The Suppl. Table 3 lists the ANOVA with post-hoc Tukey test results, while Suppl. Tables S11-16 report information on the pathways and genes selected by the Elastic-net-approach, when addressing the classification between EDC and decoys, and the pathways selected when addressing the classification task between EDC-leading-to-adverse-outcomes (AOs) and EDC-not-leading-to-AOs. Univariate receiver operating characteristic (ROC) curve analysis (Sonego et al., 2008) was applied to verify which pathways among those selected for the classification tasks are informative based on univariate tests.

2.8. Validation of EDC class probabilities

The trained classifiers were tested on an independent set of positive (other chemicals which are known to be EDC) and negative chemicals (a list of safer chemicals). The set of positive chemicals correspond to compounds selected from the DEDuCT (https://cb.imsc.res.in/deduct/), which is a manually curated dataset of known Endocrine Disrupting Chemicals, and compounds selected by domain experts working within the EDCMET project. The Safer List of Chemicals Ingredient was downloaded from https://www.epa.gov/saferchoice/safer-ingredients. A third test was implemented in order to test the *in vivo* based EDC-classifiers with ED-KEs selected from *in vitro* assay data from ToxCast. For this test, the mesh IDs of EDCs and negative controls were translated to CAS ids and the active assay endpoints for each compound were identified from the ToxCast hit call matrix. For the comparison with ToxPi-based scores, we selected compounds, which have been characterized as endocrine disruptors by using the ToxPi scoring system (Filer et al., 2014). The comparative analysis was conducted on chemicals annotated with a CAS number the intersection. Detailed information about this analysis can be found in Appendix Supplementary Methods

and Results.

2.9. Classification of EDCs linked to adverse outcomes based on pathway activation scores

Three diseases including atherosclerosis, type 2 diabetes and metabolic syndrome were considered as AOs, and all compounds with an overall EDC score greater than 0.85 were selected. Then, by using the chemical-disease associations annotated in the CTD database, the predicted EDCs were divided in two groups: EDC → AO and EDC ⇏AO. All models were subjected to repeated k-fold-cross validation and the accuracy of the models for each data layer was evaluated with F1-scores in order to consider the class imbalance between EDC → AO and EDC ⇏AO. The ANOVA followed by Tukey's post hoc test was applied to identify statistically significant differences across the accuracy scores compiled from each data layer.

3. Results

3.1. A toxicogenomic data space to investigate the molecular activity of EDCs

A toxicogenomic-based computational framework was implemented to investigate the molecular activity of EDCs in an *in silico* manner. In this framework, toxicogenomic signatures, presented in a variety of forms such as gene networks and pathway activation scores (Hardt et al., 2016; Herwig et al., 2016; Liu et al., 2019), are systematically used to profile molecular activities of EDCs and to train models for EDC toxicity prediction (Kohonen et al., 2017; Alexander-Dann et al., 2018). Fig. 1 depicts the workflow of the proposed pipeline. Compound-induced expression profiles from *in vivo* and *in vitro* liver cell models were collected from large-scale toxogenomics datasets, including TG-GATEs, DrugMatrix and LINCS. The selected toxicogenomic signatures were then grouped based on similar exposure scenario (e.g. 24 h after low-dose exposure) in order to decipher the molecular mechanisms underlying the adverse effects of compounds tested in a given exposure scenario (ES). The pathway activation profiles estimated for each compound and toxicogenomic-driven network represent the bulk of the proposed toxicogenomic data space. They were used to model classification systems for prioritizing chemicals based on their ED potential, and for inferring associations between EDC exposures and metabolic-related diseases such as atherosclerosis, type 2 diabetes and metabolic syndrome. More details on the construction of the toxicogenomic-driven networks can be found in the section Materials and Methods and the Appendix Supplementary Methods and Results.

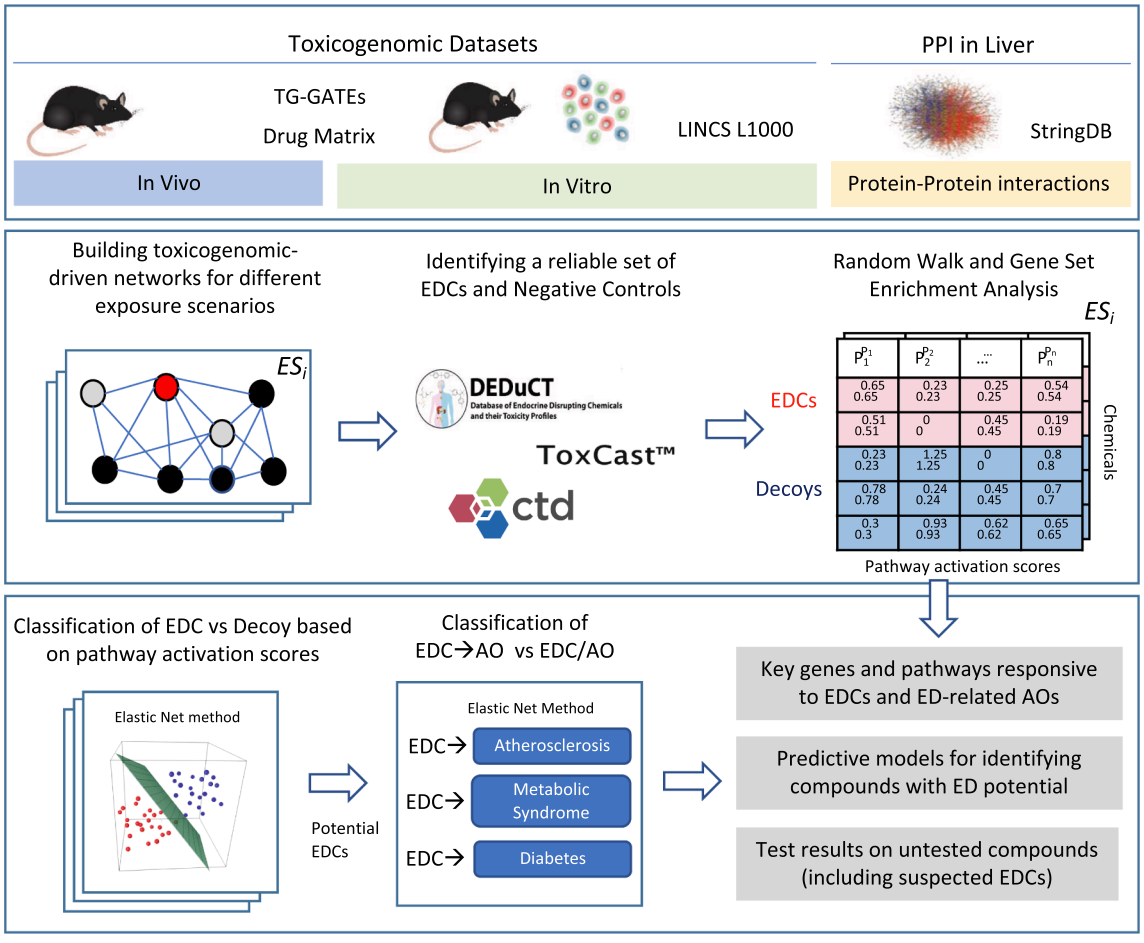

Fig. 1. Graphical illustration of the implemented computational platform for toxicogenomic-driven *in silico* analysis of EDCs. Modelling gene networks from gene expression profiles derived from *in vivo* (rat) and *in vitro* (primary rat hepatocytes, primary human hepatocytes and HepG2 cell line) exposure to different compounds; and a protein–protein interactions network (String DB). DeDUCT, ToxCast and CTD are utilized to select a reliable set of compounds with confirmed ED activity, and also to define a set of negative controls, namely decoys. Key molecular events of EDCs at gene level are transformed to pathway activation scores by random walk with restart on gene co-expression networks and gene set enrichment analysis. The pathway scores are used to train machine learning classifiers that identify and prioritize compounds with endocrine disrupting potential. Classification tasks aiming to link EDC-gene interactions to adverse outcomes (AOs) such as type 2 diabetes, atherosclerosis and metabolic syndrome are also addressed.

3.2. Selection of a robust set of endocrine disruptors

A set of 197 EDCs was selected by using the database DEDuCT (Karthikeyan et al., 2019), ToxCast (Dix et al., 2007), and the CTD (Davis et al., 2019). DEDuCT contains a large list of potential EDCs. Therefore, we conducted an extensive analysis in order to select chemical agents with ED potential based on evidence from ToxCast *in vitro* HTS assay data. Our analysis aimed to assess whether a chemical agent activate (or inactivate) nuclear receptors (e.g. PPAR, PXR, ER, CAR, AR, etc.), which are linked to endocrine-disrupting activity. Suppl. Table 1 lists ToxCast assays targeting nuclear receptors linked to endocrine disruptors, while Suppl. Table 2 indicates which are the most informative NR-based assays for the identification of known EDCs. After selecting known endocrine disruptors, a set of 1336 chemicals (referred to as negative controls or simply decoys) was identified by choosing compounds with a different set of EDC-gene interactions (or EDC-KEs). This selection does not guarantee that the negative controls are compounds with no endocrine disruption. However, the selection of decoys, i.e., assumed non-active molecules, in benchmarking datasets for *in silico* screening studies is also a common practice for *in silico* studies aiming to identify novel EDCs (McRobb et al., 2014). Detail on the selection of EDCs and decoys can be found in the section Materials and Methods and the Appendix Supplementary Methods and Results.

3.3. Classification models for in silico screening of compounds with ED potential

Toxicogenomic-driven networks were built to infer genes and pathways responsive to ED-related compounds. The full space of molecular responses caused by a set of known EDCs and decoys (or negative controls) was utilized to assess the classification accuracy on the task of distinguishing EDCs from decoys by using a machine learning approach. The Random Walk with Restart (RWR) algorithm was used to extend the set of known EDC-gene interactions extracted from CTD and the FGSEA method was applied to generate pathway activation scores (more details are provided in Materials and Methods section). Pathway activation scores for the selected EDCs and decoys were finally given input to regularized and generalized linear models (Zou and Hastie, 2005) in order (i) to build classifiers enabling the identification and prioritization of EDCs; and (ii) to discover biological pathways (or informative molecular features) that link EDC-KEs to EDC-associated adverse outcomes. Details of the implemented ML framework are included in the Materials and Methods and the Appendix Supplementary Methods and Results. Fig. 2A shows the accuracy achieved by EDC-based classifiers trained on different toxicogenomic data layer. Models based on pathway activation scores systematically achieved higher accuracies than those trained with known EDC-gene interactions. This result is in accordance with previous findings showing that pathway-based information are more informative for toxicity prediction or compound classification (Hardt et al., 2016).

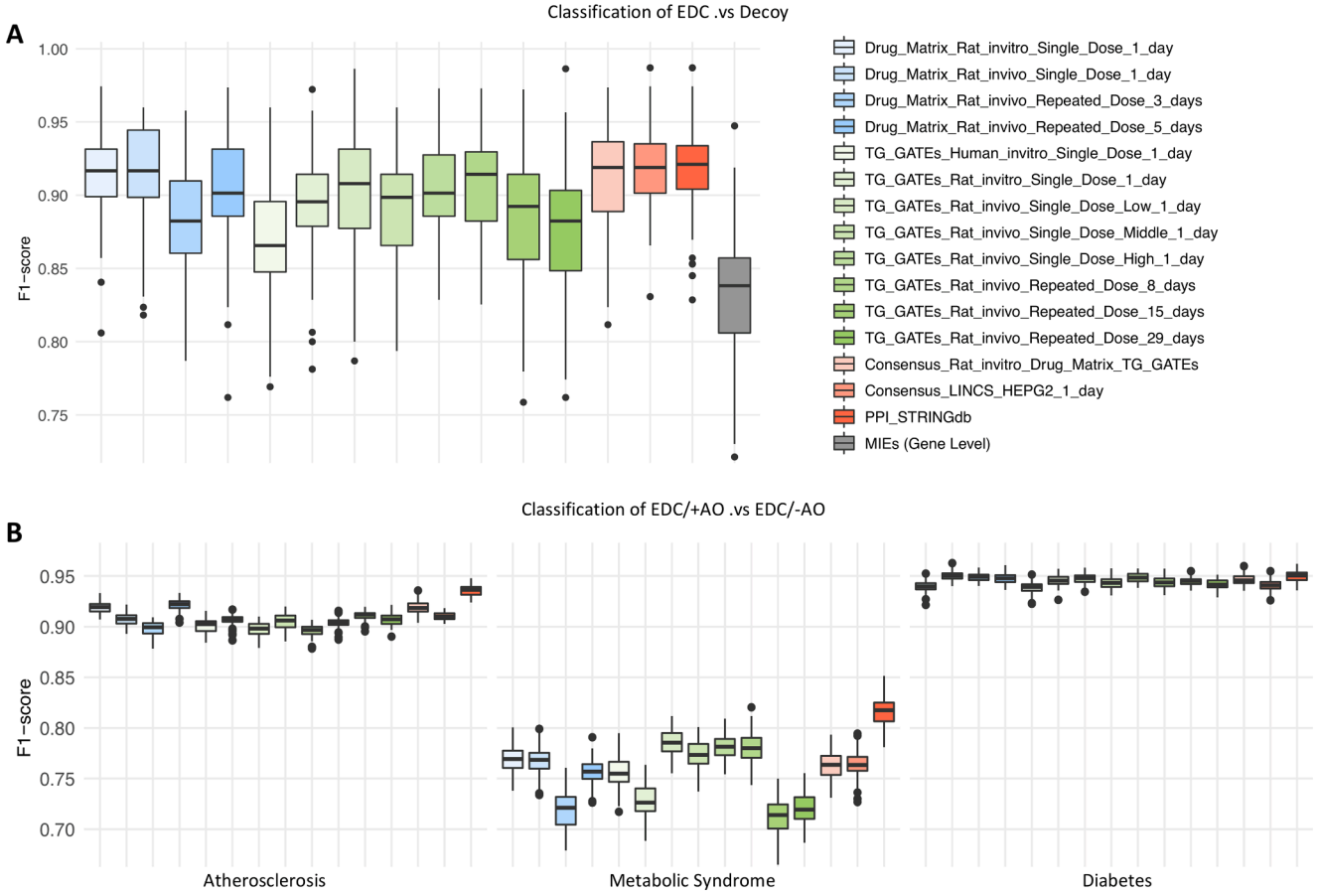

Fig. 2. Performance of classification models to identify EDCs and EDC-associated adverse outcome. (A) Classification performance of classifiers utilizing pathway activation scores to discriminate EDCs from decoys. The F1-score was used to estimate their accuracy with repeated 10-fold cross-validation. Each EDC-classifier refers to a specific toxicogenomic-data layer (e.g., 24 h after low-dose exposure). (B) Classification performance of classifiers trained to identify EDCs with the potential to lead to a given adverse outcome by using the most EDC-responsive genes and pathways (AO). Three metabolic-related diseases (atherosclerosis, metabolic syndrome and type 2 diabetes) were considered as adverse outcomes. Repeated 10-fold cross-validation and F1-score was used to estimate the classification accuracy.

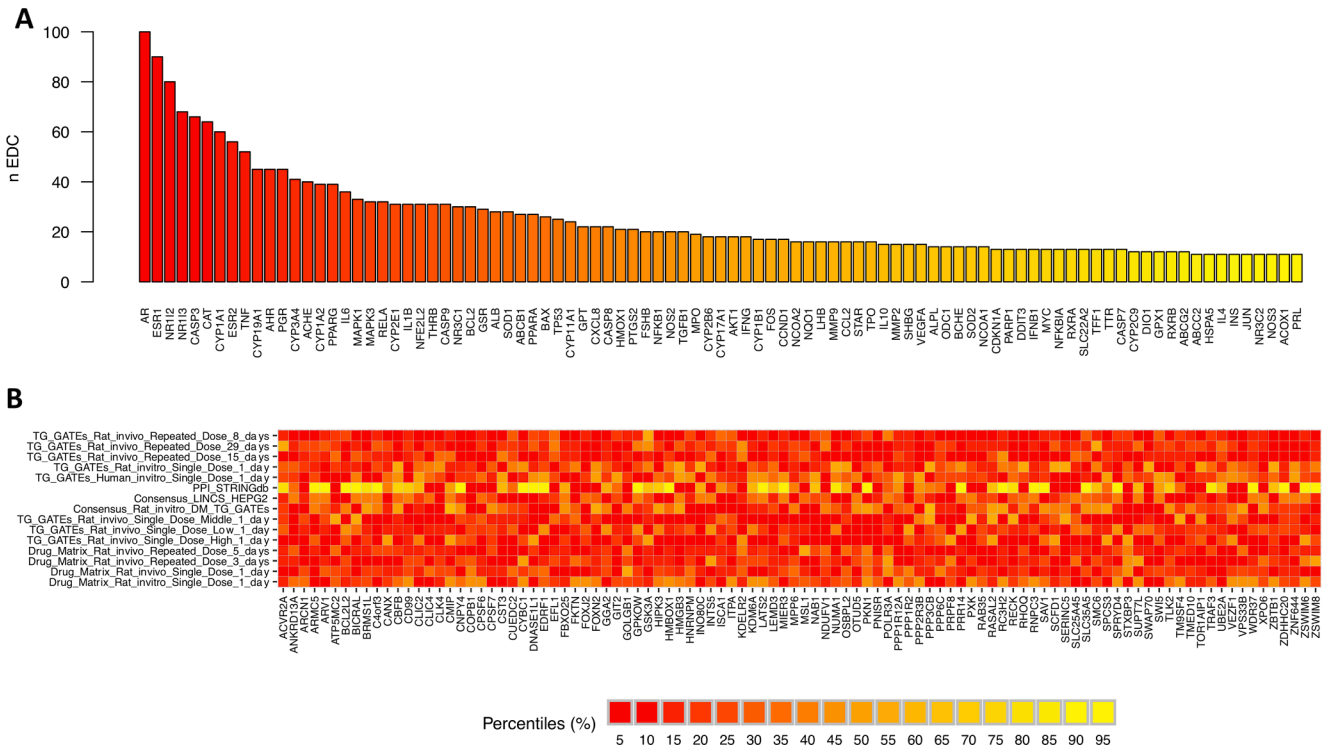

Fig. 3. Set of putative EDC-gene interactions. (A) A set of relevant associations between EDCs and genes characterized by using the computed toxicogenomic-driven networks. The associations are found by using the results obtained from the RWR procedure. The bar plot indicates how many times the same gene (or EDC-KEs) is found in the list of known EDCs. The y-axis indicates the number of genes, while the x-axis reports the most relevant EDC-responsive genes. (B) A set of novel and relevant EDC-gene associations which are identified by computing, for each gene, the odds ratio (OR) between the RWR-based steady probabilities from EDC-MIEs and decoy-MIEs in each toxicogenomics-driven network. The geometric mean of the OR estimates is calculated to select EDC-specific genes that are closely linked to known EDC-gene interactions across different toxicogenomics-driven gene networks. The included heat map shows network-related percentiles of descending OR estimates for the top 100 EDC-associated genes; lower percentiles correspond to higher OR values.

Analysis of variances (ANOVA) followed by a Tukey post hoc test was applied to compare accuracy (F1-scores) across all data layers (Suppl. Table 3). The top performing models are DrugMatrix rat *in vitro* single dose (after 24 h of exposures), DrugMatrix rat *in vivo* single dose (after 24 h of exposure), and models from consensus networks. The average accuracy of these models is greater than 90%. Overall, DrugMatrix-based classifiers slightly outperformed those relying on TG-GATEs. This may be due to the fact that DrugMatrix covers a set of 16 EDCs, while TG-GATEs includes 10 known EDCs. Remarkably, the consensus network generated from toxicogenomic signatures of rat *in vitro* hepatocytes in DrugMatrix and TG-GATEs achieved average classification accuracy of 92%, suggesting that the use in combination of pathway level information and ML methods reveal a level of concordance between DrugMatrix and TG-GATEs that has been observed before (Sutherland et al., 2016). Sensitivity and specificity measures of the trained classifiers are provided in Suppl. Table 4.

The EDC-classifiers were finally used to predict EDC probability scores for more than thousands of compounds annotated in CTD. The full list of ED probability scores is reported in Suppl. Table 5. Compounds exhibiting a high ED probability score across all networks can be considered as candidate substances. In particular, some non-endocrine targeting drugs, such as Amiodarone, Lithium, Propranolol, which are known to be linked to ED-related adverse outcomes (Sabir et al., 2019), were predicted as EDCs. This suggests that the proposed network-driven approach can discover and utilize alternative ED-responsive pathways for the identification of new ED candidates.

The information needed to calculate the EDC-score for new or untested compound is the list of genes (MIEs or KEs) associated with the selected compound. Genes interacting with untested compounds can be determined by using *in silico* systems (e.g., docking) or *in vitro* assay data (e.g., CTD, ToxCast, etc.).

Compounds classified as EDC were further analyzed to identify molecular pathways linking EDC exposures to metabolic diseases such as atherosclerosis, metabolic syndrome and type 2 diabetes. Manually curated association between EDCs and the selected metabolic adverse outcomes were retrieved from the CTD's chemical-disease database (Davis et al., 2018). Fig. 2B shows the test accuracy (F1-scores) obtained by classifiers aiming to distinguish EDCs leading to an adverse outcome (EDC/+AO) from EDCs not leading to that adverse outcome (EDC/-AO). Notably, the classifiers predicting the association between EDCs and type 2 diabetes (or atherosclerosis), achieve higher accuracy, while the classifier trained to predict EDCs leading to metabolic syndrome show a lower accuracy. This result could be explained by the fact that metabolic syndrome is characterized by a constellation of metabolically related abnormalities and cardiovascular risk factors (Magkos et al., 2009), while type 2 diabetes and atherosclerosis are more narrowly defined, specific diseases. Remarkably, all toxicogenomic data layers provide highly accurate EDC classifiers in T2D. Indeed, previously reported epidemiological data indicate that exposure to EDCs is linked to an increased risk of diabetes mellitus in adults (Alonso-Magdalena et al., 2011; Chevalier and Fénichel, 2016). Taken all together, these results demonstrate that the proposed toxicogenomic data space can be used to infer adverse outcomes from known EDC-gene interactions and pathways associated to ED activity. The trained EDC-AO-classifiers were finally used to infer EDC-disease associations for more than 10 K compounds annotated in CTD (Suppl. Tables 6–8).

3.4. Toxicogenomic-driven gene networks can reveal putative EDC-gene associations

An important novelty presented in this study is the modelling of a multi-layer, toxicogenomic gene network, which is then exploited to infer EDC-induced pathway activation scores. The large-scale public toxicogenomic resources, such as Open TG-GATEs, DrugMatrix and LINCS, provide the unique opportunity to characterize the mode of action of EDCs in terms of gene networks, and to discover genes underlying the molecular mechanisms leading to adverse metabolic effects. Therefore, starting from the pre-compiled toxicogenomic-driven gene networks and the RWR-based gene scores, a statistical analysis was conducted to quantify gene relevance with respect to known EDC-gene interactions. This analysis aims to detect genes that are closely related to known EDC-gene interactions (or EDC-KEs), but which are not reachable when using decoy-gene interactions. These genes are supposed to play a key role in recognizing novel EDCs. Technical details of the implemented analysis can be found in the Material and Methods section. Fig. 3A reports the top significant EDC-KEs; while the Fig. 3B indicates novel EDC-gene interactions, which are not currently annotated in CTD. These results confirm that only a small fraction of known EDC-KEs (e.g., AR, PXR, ER, etc.) is common to a large set of chemicals annotated as EDCs. However, many other EDC-KEs are common to specific small groups of known EDCs. Suppl. Table 9 includes the full list of detected EDC-gene interactions. Some of these genes have been found to be associated with metabolic pathways and adverse outcomes (Suppl. Table 10). Two of these genes (GSK3A and HIPK3) are also part of regulatory networks related to EDC-activated NRs (e.g., AR, ER and GR), while the others have no established connection to classical EDC targets or pathways. The genes associated with cholesterol homeostasis (ARV1, OSBPL2) are involved in the same regulatory networks as the farnesoid X receptor, also a target for EDC-mediated effects, but any direct associations between the function or regulation of these genes have not been discovered. Similarly, while the downstream effects of the discovered putative genes associated with EDC adverse effects indicate a role in the control of energy homeostasis and metabolic disruption, the regulatory processes affecting the expression or activity of these genes have not been elucidated. In most cases, these changes have been associated with mutations, transcript variants or observed in different disease states but we were unable to find evidence on changes in expression or activity caused by specific external stimuli. EDCs have been shown to affect signal transduction and epigenetic regulation by altering protein phosphorylation mostly in relation to carcinogenesis. However, earlier studies have linked these changes to obesity-associated dysfunctional adipose tissue, indicating various protein phosphatases (e.g. PP1 and PP4) or kinases (e.g. GSK3, HIPK3) affecting metabolic dysfunction. (Petrakis et al., 2017) Similarly, while the roles of different transporter proteins (e.g., SLC25A45 and SLC25A5) in the maintenance of energy homeostasis have not been fully elucidated, they have been strongly associated with emerging metabolic dysfunction (1 1 4). These findings open several new interesting avenues for further studies on the effects of EDCs on the regulation and function of these genes and pathways.

3.5. Molecular pathways for informing on potential endocrine disruptors and their metabolic effects

Systematic characterization of EDC-induced biological pathways was obtained by applying the feature selection system intrinsic to elastic-net classifiers. Hence, the elastic-nets were not only used to build EDC classifiers, but also to identify the most informative pathways in a multivariate fashion. The informativeness of pathway annotations was assessed by considering the following metrics: average of normalized enrichment scores for EDCs (EDC-NESs), regression coefficients and stability over repeated 10-folds cross validations (Suppl. Tables 11–14). The same approach was utilized to select the most informative genes from the gene-based classifier. The full list of genes that are relevant for the classification of EDCs are reported in Suppl. Table 15. Selected pathway annotations displayed a significant enrichment for metabolic pathways, organismal systems including immune and endocrine system pathways, and environmental information processing including membrane transport and signal transduction pathways (Fig. 4A). Remarkably, a large variety of metabolic-related pathways are affected across different models and conditions (e.g., doses and time points) suggesting their potential as biomarkers. A comparison of the most informative pathways for the classification of EDC in both *in vivo* and *in vitro* models revealed a large set of common pathways (Fig. 4B). These pathways can provide a bridge between the *in vivo* and *in vitro* data and improve the knowledge of metabolic-related pathways for EDC hazard identification. Selected pathways include many NR-related pathways (Sanderson, 2006; Vandenberg et al., 2012), arachidonic acid metabolism and the calcium metabolism (Heindel et al., 2017), the NFkappaB pathway (Zhu et al., 2015; Bansal et al., 2018) and ovulation cycle (Rattan et al., 2017). This result confirms that the defined predictive models rely on pathways (or features) that are involved in various endocrine and metabolic processes. Moreover, many gene ontology terms, which are linked to key events of known adverse outcome pathways, were found (Fig. 4A). The pathways selected with the ML approach were further analyzed by using univariate receiver operating characteristic (ROC) curve analysis. The ROC analysis was used to verify whether the individual pathways are able to distinguish EDCs from decoys. We identified very specific metabolism-related pathways when selecting AUC values greater than 0.7 (Suppl. Table 16). Many of these pathways exhibit very high differences of pathway activation scores between EDCs and decoys.

3.6. Validation of EDC probability scores with ToxCast in vitro assay data

Each EDC-classifier was first validated against the ToxCast *in vitro* assay data. The validation strategy aims to verify whether EDC scores correlate with hit calls in ToxCast assays that are designated to identify EDCs. ToxCast provides hit calls indicating negative (0) and active (1) assays for each compound. Therefore, the ROC curve analysis was used to measure how often compounds activating an ED-relevant ToxCast assay are ranked among tops in a given EDC classifier. Fig. 5A includes the AUC results for the most relevant associations between toxicogenomic data layers and ToxCast-endpoints, while Suppl. Table 15 contains the full list of results. The most informative EDC classifiers with respect to ED-related ToxCast assays are those derived from the PPI, HepG2 cell line, rat *in vitro* (in both TG-GATEs and DrugMatrix), and rat *in vivo* DrugMatrix (Fig. 5A). Notably, these classifiers are also among the most accurate models (Fig. 2D). Moreover, we observed that ToxCast assays showing higher correlations, such as those targeting ER, AR, PPARγ and progesterone receptor (PR), are often used for toxicity prediction of EDCs in literature (Sipes et al., 2013). Remarkably, the most informative EDC classifiers are those based on *in vitro* and *in vivo* toxicogenomics data. The set of EDC predictions obtained from the top 6 informative EDC classifiers were finally merged to build two ensemble scoring systems for *in silico* screening of chemicals with ED potential, by using the average and harmonic sum.

3.7. Validation of the EDC probability scores with positive and negative test sets

EDC probability scores were generated for more than 10 K compounds (Suppl. Table S5), and the scores compiled for each compound across the different EDC classifiers were summarized by using the average and the harmonic sum. The average of EDC scores is more stringent than the harmonic sum, leading to a few compounds with a high overall EDC score (Supp. Fig. 6). Then, positive and negative set of EDCs were identified to validate the proposed EDC scoring systems. The initial screening of EDCs from DEDuCT excluded hundreds of compounds due to the impossibility to map these compounds in ToxCast and the high frequency of inactive hit calls for ED-relevant nuclear receptors.

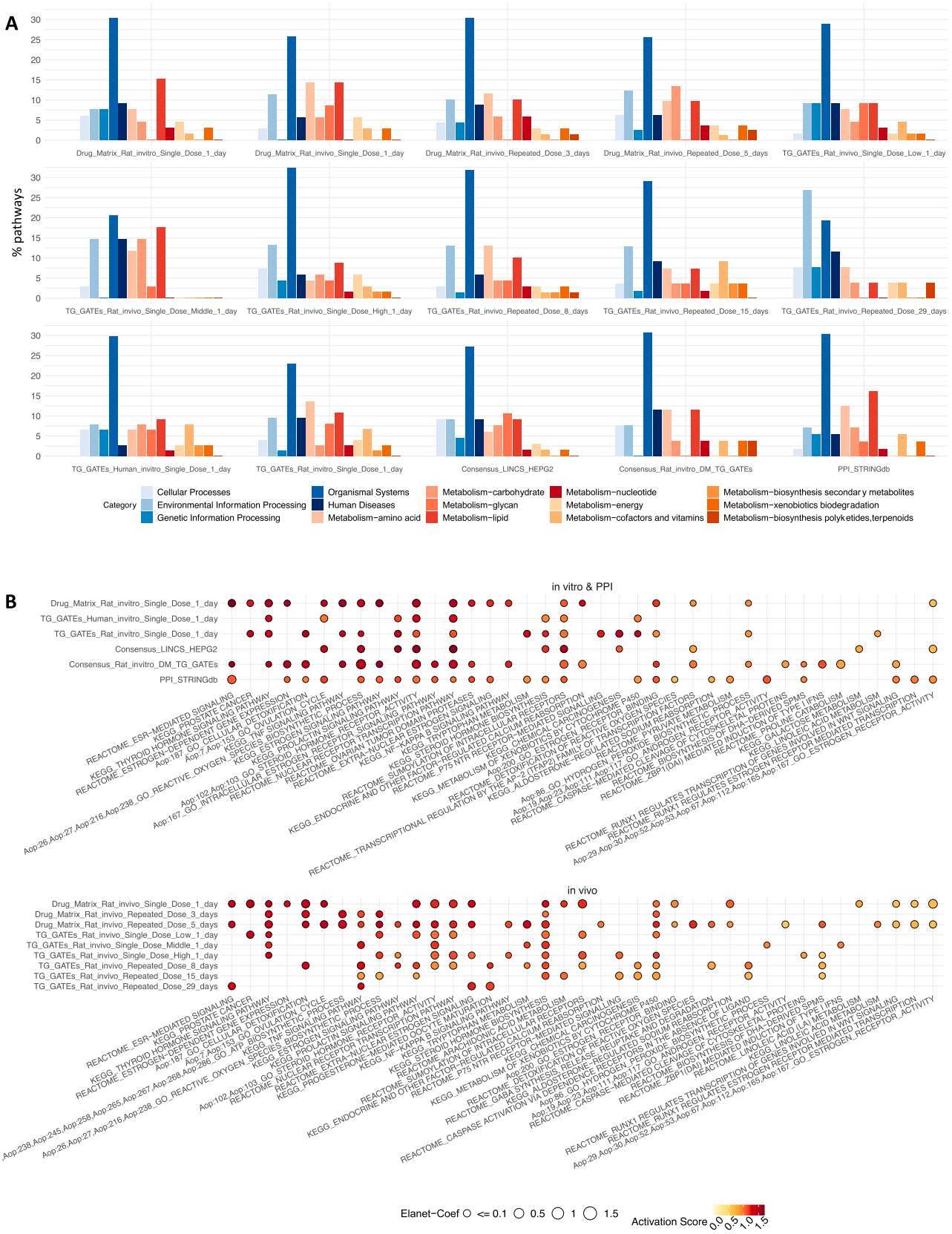

Fig. 4. Molecular pathways that are informative for the classification and prioritization of chemicals with ED potential. (A) Graphical illustration of the most enriched categories in KEGG pathways. Each bar graph displays the percentage of pathways activated for each category and network layer. Subcategories of pathways for metabolism were provided along with their percentages. (B) A set of common pathways selected based on the regression coefficient (>0.01) and the average of the normalized pathway activation scores (EDC-NES > 0.5). The size of the circles indicates the relevance of each pathway based on the regression coefficient, while the color indicates the average score of a pathway induced by EDC exposures in a given network layer.

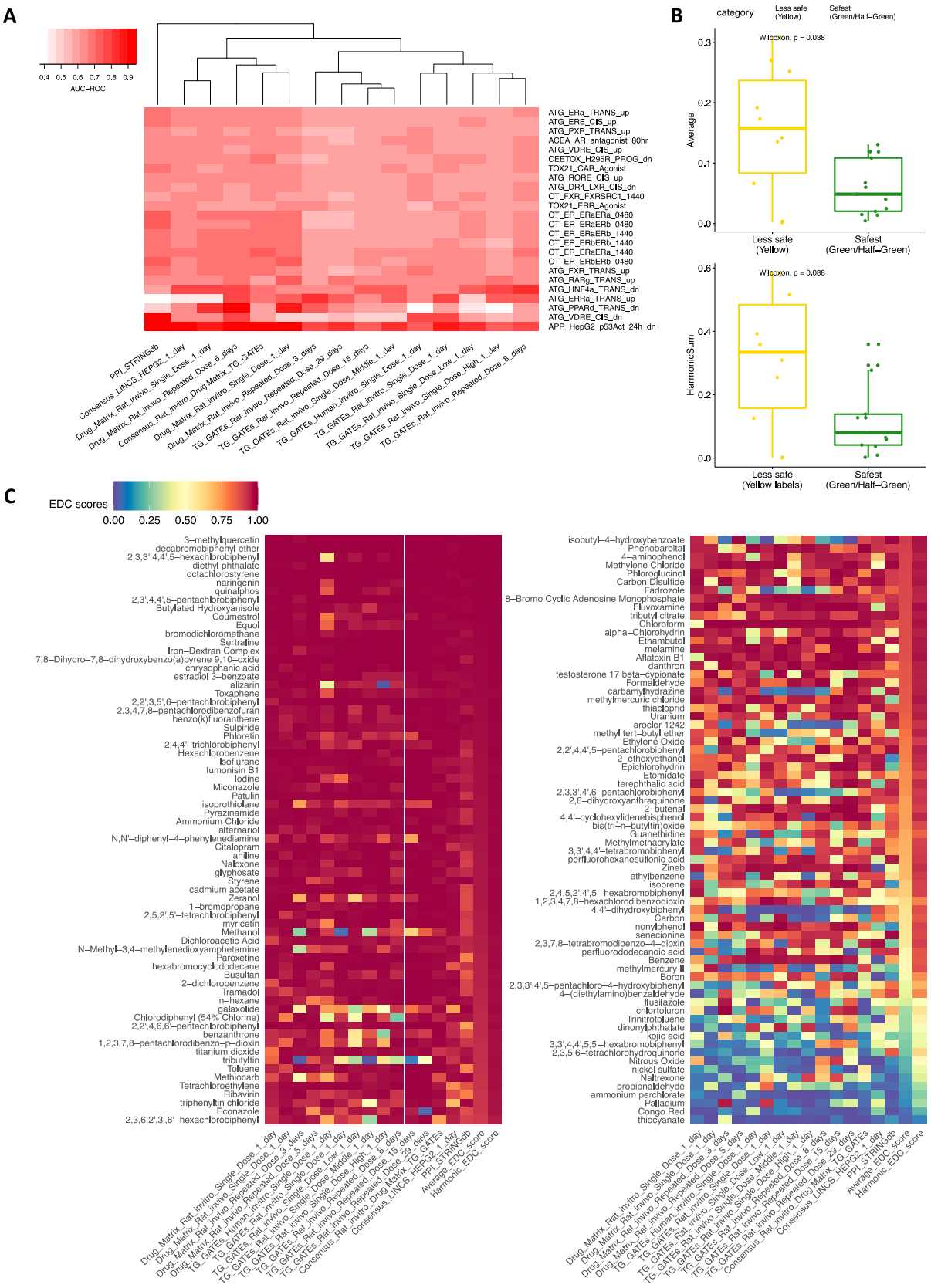

Fig. 5. Validation results of EDC probability scores. (A) Heatmap of area under the curve (AUC) values of ROC analysis between the predicted class probabilities of the compounds based on 15 data layers and hit call matrix of assay endpoints in ToxCast. (B) Boxplots showing the prediction results on the negative test set, which includes 23 chemicals selected from the EPA's Safer Chemical Ingredients List (C) Prediction results on a set of compounds with known ED potential. These compounds were not part of the training/testing sets and were selected from the DEDuCT database and literature-based information.

This set of known EDCs was merged with a further and independent test set of ED agents selected by domain experts of the EDCMET project (Küblbeck et al., 2020). Two further tests based on lists of compounds with suspected ED activity were conducted[2][3]. The full list of prediction results for the positive test sets is reported in Suppl. Tables 18–20. Finally, a negative test set was defined in order to evaluate the specificity of the trained EDC-classifiers. Validation results show that both the average and harmonic sum of EDC class probabilities provided accurate predictions of known EDCs (Fig. 5C). The overall accuracy, when having selecting compounds with EDC class probability greater than 0.6, is 89% for the harmonic sum and 81% for the average of EDC scores across all the networks. However, a limited set of EDCs was not correctly classified by our classification system, this may be due to the fact that, currently, our ensemble classification system utilizes only liver-based models. Nonetheless, the proposed toxicogenomics data space is extremely flexible. It could further be extended in order to contain gene-gene networks modelled from a new set of toxicogenomics signatures, involving different tissues (e.g., adipose tissue, skeletal muscle, pancreas, etc.), *in vitro* cell or rodent *in vivo* models (e.g., humanized mouse models) or conditions (e.g., different exposure times and doses). In order to evaluate the specificity of the trained classifiers, we implemented a negative test set. The negative test set aimed to verify whether the classifiers provide high EDC-scores for chemicals that met the Criteria for Safer Chemical Ingredients (https://www.epa.gov/saferchoice/how-list-chemical-safer-chemical-ingredients-list). The listed chemicals are safer alternatives, grouped by their functional-use class. The safer chemicals are marked with green (or half-green) circles and yellow triangles. The chemicals marked with complete, or half circles are to be considered with a safer status, while those marked in yellow may have some hazard profile issues. Both the average and harmonic sum of EDC class probabilities compiled for 23 safer chemicals are very low (Fig. 5B). Moreover, we can observe that both scores are sensitive to different levels of safety. Remarkably, chemicals that may have hazard profile issues tend to have slightly higher EDC scores.

3.8. A comparative analysis between the EDC scoring systems and ToxPi

To further validate the EDC scoring systems, we applied The Toxicological Priority index (ToxPi) on the training set of EDCs. An updated version of the ToxPi scoring schema for endocrine profiling was applied in order to take into account a larger set of endocrine disruption targets available in ToxCast (Filer et al., 2014). A comparison between the two integrated EDC scores and the ToxPi score for the compounds included in DEDuCT is graphically illustrated in Fig. 6 (Suppl. Table 21 includes the complete list of results). Although it is observed a partial agreement between the two scoring systems, methyl testosterone, mifepristone, zearalenone and spironolactone were found to be with both high ToxPi and harmonic sum EDC scores. On the other hand, many compounds such as carbaryl, colchicine and isoniazid exhibit low ToxPi scores, suggesting that our proposed toxicogenomics-based scoring system could help reduce the number of false negatives. Furthermore, there are many studies in literature showing that compounds like isoniazid are linked to endocrine disruptive activity (Dvorak et al., 2003; Karthikeyan et al., 2019) The observed discrepancy between our EDC scores and the ToxPi scores may be also due to the limited number of targeted genes in ToxCast assays. Indeed, our EDC scoring system, relying on large-scale toxicogenomics, enables the use of novel genes and pathways (or predictor) that are involved in hormone signaling pathway components downstream of receptor activation. Many of these genes (and related pathways) are not currently targeted in ToxCast.

[2] https://ec.europa.eu/environment/chemicals/endocrine/strategy/substances_en.htm#priority_listMany

[3] https://edlists.org/the-ed-lists

3.9. Prediction of in vivo EDC probability from in vitro MIEs

A validation test was conducted to test the trained classifiers with pathway scores derived from the extended list of EDC-gene interactions indicated by ToxCast *in vitro* assay data. In more detail, the *in vitro* ToxCast assay data were used to determine the MIEs (or KEs) of known EDCs. Then, EDC-classifiers trained on the basis of CTD-driven KEs, were tested on pathway activation scores of EDC-MIEs obtained from ToxCast *in vitro* assay data. Fig. 7 shows the EDC probability scores obtained by using the EDC classifiers derived from *in vivo* single dose rat exposures at one and five days. Suppl. Table 22 contains the full list of results, and the overall accuracy values (88% and 76%) obtained by the two selected classifiers. This result indicates a possibility to use the trained EDC classifiers for *in vitro* to *in vivo* extrapolation (IVIVE).

4. Discussion

Use of toxicogenomics data in order to prioritize chemicals for testing and management has expanded in the last decade, thanks to a wealth of publications, data-rich databases and analytical resources. However, inclusion of different genomic-based platforms, such as ToxCast, CTD, DrugMatrix, TG-GATEs and LINCS in developing novel tools for EDC toxicity prediction remains limited (Hardt et al., 2016; Herwig et al., 2016; Liu et al., 2019). In this study we present a toxicogenomics-driven computational framework that can effectively combine all these data sources to support the discovery of mechanistic information of EDCs and the development of predictive tools for the identification of substances with endocrine disrupting properties.

The first important contribution of this study is the definition of predictive models that can be used to confirm compounds suspected of acting as ED. Indeed, the prediction results obtained when testing our scoring method on the whole set of compounds annotated in CTD (see Suppl. Table S5), confirmed compounds suspected of acting as ED and highlighted new candidates. Prediction tests, with both positive and negative set of compounds, showed that the proposed approach can be used to aid the initial screening of potential EDs, avoiding the application of many *in vitro* (or *in vivo*) assays when screening large set of compounds. Furthermore, the trained classification models can be used for calculating EDC-scores from user's own data, to make prediction on new, untested compounds. The only information needed is the list of MIEs (or KEs) associated with the new compound. KEs can be retrieved from existing databases, such as CTD. Alternatively, MIEs (or KEs) could be derived from *in silico* molecular docking or *in vitro* assay data (e.g. ToxCast), and as last resort, by *in vivo* testing. The present study provides also classification models aiming at predicting adverse outcomes (AOs) from EDC-gene interactions. In particular, we focused on three metabolic diseases: atherosclerosis, type 2 diabetes and metabolic syndrome. An important novelty presented in this study is the use of a multi-layered network approach to characterize the mode of action of EDCs. As we demonstrated, the derived toxicogenomic-based gene networks can reveal putative EDC-gene interactions, or genes that are not currently annotated as MIEs or KEs, but which are included in relevant EDC-induced pathways. Furthermore, the compiled toxicogenomics networks, in combination with machine learning, contributed to the definition of an extensive catalogue of genes and pathways that are responsive to EDCs and their adverse outcomes (Suppl. Table S15 and Suppl. Table S16). The ED-related pathways could be used to define novel ED-related biomarkers. The implemented ML-based approach was also used to learn new molecular pathway linking EDC-gene interactions with adverse outcomes such as metabolic-related diseases. These pathways can inform on ED-related adverse outcome pathways. Moreover, the current study, which is limited to three metabolic disorders, can be further extended to study the connections between EDCs and other phenotypes and metabolic-related diseases. The implemented *in silico* methods also provides an important advantage over other traditional methods such as Quantitative structure–activity relationship (QSAR),

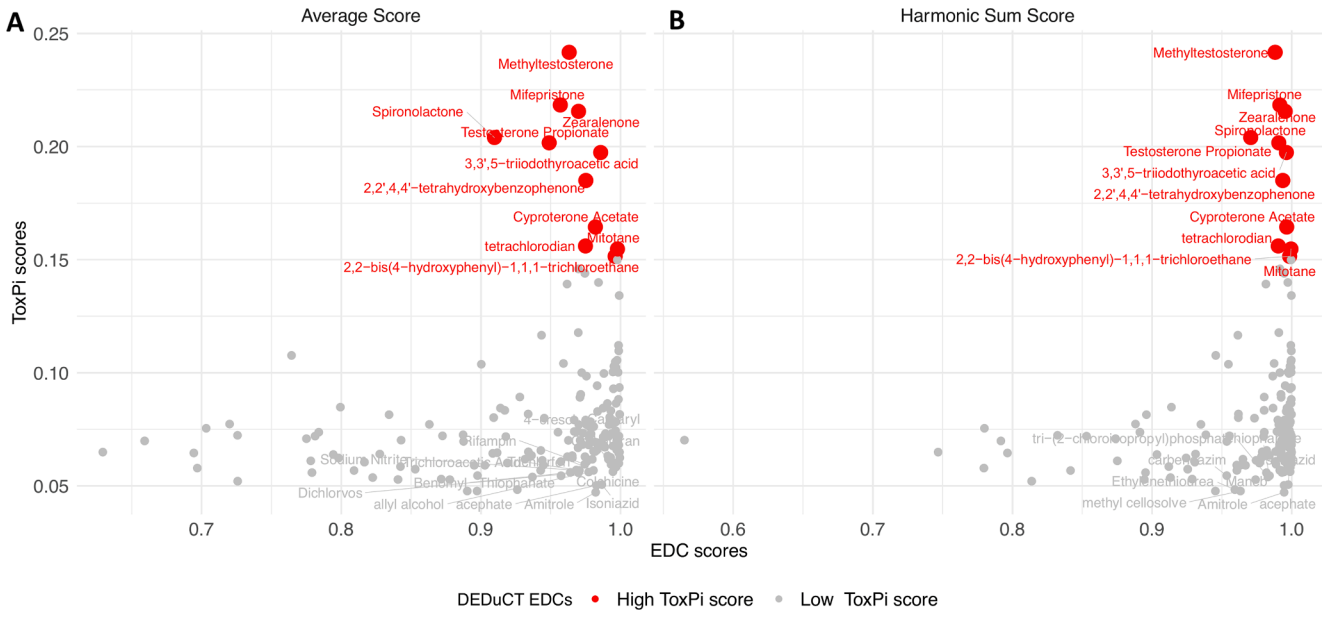

Fig. 6. Comparative results between EDC probability scores and ToxPi-based scores. (A) Comparison between the average of the EDC-class-probabilities and ToxPi scores compiled for the compounds included in DEDuCT. (B) Comparison between the EDC-harmonic-sum and ToxPi scores compiled for the compounds included in DEDuCT. The red color is used to indicate the compounds exhibiting concordant scores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

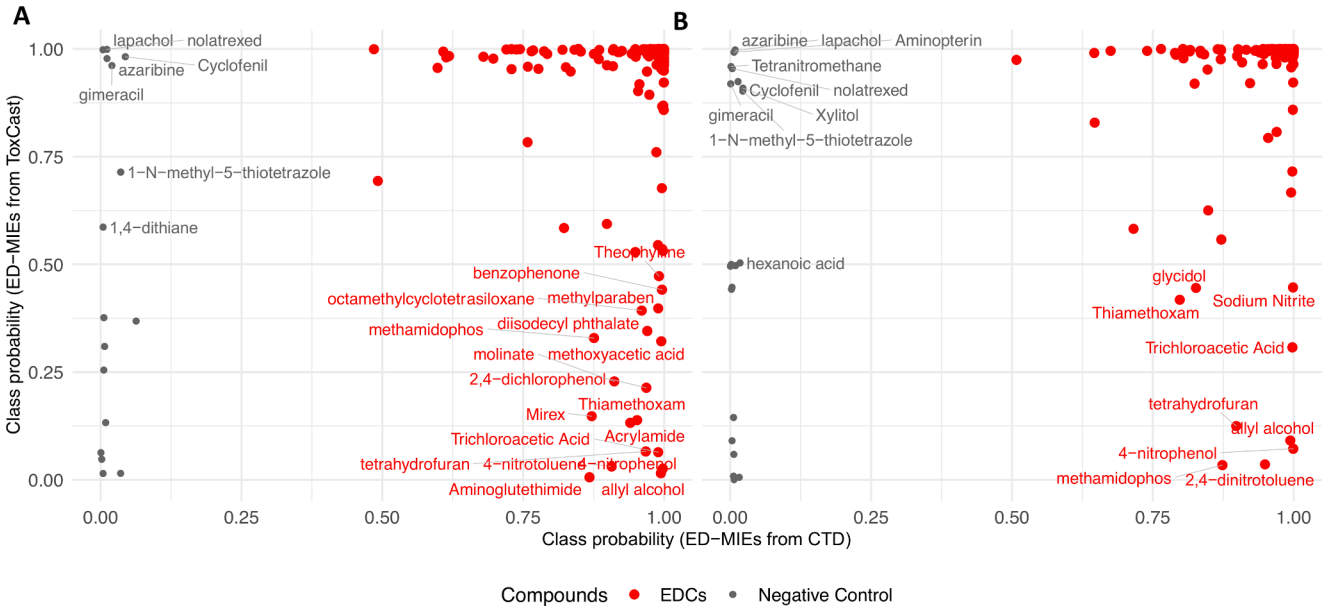

Fig. 7. Testing *in vivo* based EDC-classifiers with *in vitro* ED-MIEs. Two EDC-classifiers based on *in vivo* toxicogenomic networks were used to compiled EDC probability scores starting from ToxCast-driven EDC-gene interactions. (A) The EDC probability scores compiled from the classifier based on toxicogenomic signatures derived from *DrugMatrix-rat-in-vivo-single-dose-1-day*. (B) The EDC probability scores compiled from the classifier based on toxicogenomic signatures derived from *DrugMatrix-repeated-rat-in-vivo-doses-5-days*.

since it can be used to predict toxicity of mixtures as well as pure compounds. In this case, the EDC-KEs related to the mixture can be used as the input data to the pipeline. Finally, the proposed computational pipeline can be used to model new networks and predictive models based on upcoming toxicogenomic signatures characterizing ED-related exposures on different cell lines (e.g. HepaRG). Experimental validations were not conducted in this study. However, we strongly believe that the evaluation of the EDC predictions implemented on the evidence obtained by multiple data sources (e.g. DEDuCT, ToxCast, CTD and toxicogenomics datasets), provides substantial evidence that the proposed framework can indeed identify and prioritize EDCs.

To conclude, we strongly recommend using the proposed *in silico* method to (1) characterize of the Mode of Actions (MoAs) of compounds with suspected ED activity; (2) to identify new ED-gene associations; (3) to make predictions on new, untested compounds; (4) to predict whether ED-mediated effects of EDCs lead to metabolic diseases; (5) to inform on putative adverse outcome pathways linking ED-related key events to EDC-induced toxicity.

Data availability section

Freely available data were used in the project throughout. In particular, pre-processed TG-GATEs and DrugMatrix data were retrieved from the data warehouse hosted on https://www.ebi.ac.uk/biostudies/diXa/studies. Regarding LINCS, preprocessed level of (Level 5) gene expression data from L1000 landmark set. The PPI based network is based on the StringDB database. The pathway activation scores and the EDC class probability for more than 10 K chemicals are available in the Supplementary Excel Tables. Custom R code and methods implementing the presented computational framework are archived via GitHub repository (https://github.com/vittoriofortino84/EDTOX). Further information about the methods used in this work can be found in Appendix Supplementary Methods and Results.

CRediT authorship contribution statement

A. Sakhteman: Methodology, Software, Formal analysis, Data curation, Writing - review & editing, Investigation. **M. Failli:** Methodology, Software, Formal analysis, Data curation, Writing - review & editing. **J. Kublbeck:** Formal analysis, Writing - review & editing. **A.L. Levonen:** Writing - review & editing. **V. Fortino:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106751.

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