nature portfolio

Peer Review File

Multiscale Mapping of Transcriptomic Signatures for Cardiotoxic Drugs

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript by Hansen et al. describes a computational approach to identify transcriptomic signatures of drug-induced cardiotoxicity using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). The authors used bulk transcriptomic profiles and applied singular value decomposition to identify drug-selective patterns in cell lines obtained from multiple healthy human subjects. By integrating their findings with publicly available genomic, pathway, and single cell transcriptomic datasets, the authors provide a multiscale predictive algorithm to classify cardiotoxic and non-cardiotoxic drugs and to define potential downstream cellular pathways. Overall, the computational approach is interesting, particularly the techniques applied to differentiate cell line-specific effects (in normal healthy patients) versus drug-specific effects.

However, the major limitation of the study is the reliance on in vitro cell line models, which may not fully reflect the complexity of cardiotoxicity phenotypes in vivo. For TKIs in particular, the effects on VEGF signaling in endothelial cells (ECs) may be critical to the development of cardiotoxic phenotypes including cardiomyopathy. It is challenging to know how CM-specific transcriptional responses to TKIs may be modulated differently with or without signaling from other cell types. While the focus on CMs results in a high-throughput assay, a co-culture or organoid model would provide a more comprehensive picture that would likely phenocopy the cardiotoxicity seen in patients more accurately. Although this point is mentioned in the Discussion, it highlights the challenge in finding a use case where this platform could be leveraged in isolation to screen for cardiotoxic potential.

Other major comments are as follows:
1. Given that many of the drugs tested in this platform are life-saving when used to treat cancer, the concept of "permissive cardiotoxicity" is becoming increasingly popular - some drugs are known to be cardiotoxic, yet they are necessary for optimal cancer treatment. One example is ponatinib, which has a black box warning for cardiovascular events but is used for patients who either develop resistance to other TKIs or have a specific tumor mutation. How might the concept of permissive cardiotoxicity, or alternatively on-target cardiotoxicity (related to inhibition of the intended drug target) be incorporated into the proposed platform?

Since most of the TKIs tested have been reported to cause cardiotoxicity in some patients, including "non-cardiotoxic TKIs," it seems that a focus on each patient's individual risk may be more fruitful than a focus on drug-specific risk.

2. In patients, most TKIs result in a cardiomyopathy that is reversible upon cessation of the offending agent. TKI-induced cardiomyopathy is therefore considered to be mechanistically different than anthracycline cardiomyopathy, which results in CM apoptosis, ferroptosis, myofibrillar disarray, and other severe myopathic changes. Of the SCPs that overlapped with dilated or hypertrophic cardiomyopathy, have any of these been previously reported to be associated with TKI cardiotoxicity in vivo?

Minor Comment:
Supplemental Fig 3 - both cardiac-acting and non-cardiac-acting drugs are labeled in blue. Please use a different color scheme to differentiate these categories.

Reviewer #2 (Remarks to the Author):

In the present manuscript, authors applied sophisticated bioinformatics and computation tools to discriminate the TKI-induced cardiotoxicity observed by several anti-cancer TKI inhibitors (already approved by the FDA). The present computation study is mainly based on the two previous already published studies of the authors (Schaniel et al.: Stem Cell Reports 2021 and Van Hasselt et al.: Nat Commun 2020). The experimental part includes the generation and treatment of hiPSCs-cardiomyocytes (from 6 healthy individuals) with cardiotoxic and non-cardiotoxic TKI (and other additional compounds) as well as the transcriptome data are already published (Schaniel et al.: Stem Cell Reports 2021 and Van Hasselt et al.: Nat Commun 2020).
In the present computation study, authors reanalysed the huge transcriptome data in combination with genomic variations of t the DNA of the 6 individuals with the main aim to identify a specific cardiotoxicity signature for the TKI cardiotoxic compounds. The computation/bioinformatic tools

are indeed beyond the state of the art. In summary, the authors identified a specific gene signature/pathway that may specifically predict the cardiotoxicity of TKIs pending the genomic variations of an individual. The authors conclude that the specific TKI cardiotoxic signature might be useful for therapeutical applications to prevent drug-induced cardiotoxicity.
Certainly, this is an interesting computation study. However, the conclusions of the authors are over-enthusiastic. To my opinion, some weak points should be taken into consideration for improving the manuscript.
1. Although the computation and bioinformatics tools are very advanced and useful, the main wet lab work and the main transcriptome part have been already published.
2. Functional assays such as beating activity/electrophysiology of the cardiomyocytes are completely missing. Certainly, such studies can be performed with a few representative cardiotoxic and non-cardiotoxic TKI as well as doxorubicin as a control. Such studies are necessary to correlate the specific cardiotoxic SCPs observed by the cardiotoxic TKIs
3. The specific cardiotoxicity-associated SPCs for the TRIs are certainly also observed by treatment of the hiPSCs-cardiomyocytes with anthracyclines. There are a few publications already available but not cited by the authors. In this context, it was also published that ferroptosis is also a key process elicited in hiPSCs-cardiomyocytes by an anthracycline
4. However, it would be essential to demonstrate for at least a few cardiotoxic and non-cardiotoxic TRIs (as a control) whether the gene signatures/SCPs are reversible after removing the compounds for 48 h (or even longer). It might be very interesting to identify reversible and sustained gene signatures/SCPs. This will help us to develop predictive strategies by targeting sustained (non-reversible) gene signatures/SCPs.

Reviewer #3 (Remarks to the Author):

In the manuscript titled "Multiscale Mapping of Transcriptomic Signatures for Cardiotoxic Drugs," the authors discussed the hypothesis that drug-induced gene expression can serve as an indicator for drug toxicity. They tested a total of 54 FDA-approved drugs (including 27 antineoplastic tyrosine kinase inhibitors) on six healthy human iPSC-derived cardiomyocytes. Drug-selective transcriptomic signatures were obtained through singular value decomposition (SVD)-based analysis of the total transcriptomic responses. The authors also integrated other publicly available datasets to achieve precise predictions of cardiotoxicity for new drug development.

The overall design and workflow make this study a valuable method for evaluating cardiotoxicity during preclinical drug development.

Compared to other similar studies that use cardiomyocyte cell lines, this paper employs human-derived iPSCs and targets the induced differentiation into "ventricular cardiomyocytes," which better mimic the physiology and drug responses of human adult cardiomyocytes.

Major Points:
1. The differentially expressed genes (DEGs) identified in this study using HiPSC-derived cardiomyocytes can only account for part of the cardiotoxic effect. Cardiac fibroblasts, cardiomyocyte macrophages, or a co-culture system often play an equally important role in cardiotoxicity and if possible should be tested to evaluate this pipeline.
2. The unique cluster of DEGs may not represent or cover all potential mechanisms involved in cardiotoxicity. Defining these DEGs as the fingerprint of cardiotoxicity remains a challenge.
3. The manuscript currently lacks totally compelling evidence or a robust pipeline for identifying DEGs from drug-induced genes.
4. The authors have not integrated genome and transcriptome data effectively in the validation and prediction of cardiotoxicity. A similar paper published in Frontiers in Pharmacology in 2020, titled "Dual Transcriptomic and Molecular Machine Learning Predicts All Major Clinical Forms of Drug Cardiotoxicity," reported a model and the prediction using a large collected and curated dataset. It is better to comment on and compare the advantages of the method used in this study, if any.
5. Please functionally predict and validate the comprehensive and novel signature of drug-induced cardiotoxicity that has not been identified by traditional measurements, such as left ventricular ejection fraction (LVEF).

Minor Points:

1. Misuse of abbreviations: For example, the inconsistent use of DEGs and their full name in figure legends and results sections.
2. Please ensure uniform font and size in figures.

REVIEWER COMMENTS

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This manuscript by Hansen et al. describes a computational approach to identify transcriptomic signatures of drug-induced cardiotoxicity using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). The authors used bulk transcriptomic profiles and applied singular value decomposition to identify drug-selective patterns in cell lines obtained from multiple healthy human subjects. By integrating their findings with publicly available genomic, pathway, and single cell transcriptomic datasets, the authors provide a multiscale predictive algorithm to classify cardiotoxic and non-cardiotoxic drugs and to define potential downstream cellular pathways. Overall, the computational approach is interesting, particularly the techniques applied to differentiate cell line-specific effects (in normal healthy patients) versus drug-specific effects.

We thank the reviewer for the kind comments and appreciation of our work.

However, the major limitation of the study is the reliance on in vitro cell line models, which may not fully reflect the complexity of cardiotoxicity phenotypes in vivo. For TKIs in particular, the effects on VEGF signaling in endothelial cells (ECs) may be critical to the development of cardiotoxic phenotypes including cardiomyopathy. It is challenging to know how CM-specific transcriptional responses to TKIs may be modulated differently with or without signaling from other cell types. While the focus on CMs results in a high-throughput assay, a co-culture or organoid model would provide a more comprehensive picture that would likely phenocopy the cardiotoxicity seen in patients more accurately. Although this point is mentioned in the Discussion, it highlights the challenge in finding a use case where this platform could be leveraged in isolation to screen for cardiotoxic potential.

The reviewer raises a valid point about our data. The data we present by itself was not designed to fully explain the mechanisms of cardiotoxicity, rather it was designed to answer the question if cardiotoxic drugs have identifiable transcriptomic signatures in cardiomyocytes. In response to the referee's comments we decided to test if co-culturing cardiomyocytes with coronary endothelial cells modulated the transcriptomic response in cardiomyocytes to drugs. We tested in two of the six previously used iPSC-derived cardiomyocyte cell lines two TKI drugs (one with high cardiotoxicty (>10%) and one with lower cardiotoxicty (1-10%)) in absence or presence of human coronary arterial endothelial cells (HCAECs). Since the reviewer suggested that inhibition of VEGF signaling in endothelial cells may be critical for cardiotoxic responses, we selected the drug pazopanib, a multi-tyrosine kinase inhibitor that affects human umbilical vein endothelial cells by interfering with VEGF and bFGF signaling as well as murine angiogenesis [1]. Endothelial dysfunction caused by VEGF blockage is discussed as a potential mechanism involved in pazopanib-induced cardiomyopathy [2]. As a second selection, we choose dabrafenib - a TKI with less cardiotoxicty than pazopanib. The new data was processed using the same pipelines and the eight new lists of DEGs were added to the initial 266 lists of DEGs. The merged 274 lists of

DEGs were projected into the drug-selective subspaces, as identified from the initial 266 lists of DEGs. Obtained clustering results, F1 scores and predicted pathways showed only minor differences to the initial results. In particular, we do not observe any major differences between the data generated in presence or absence of the endothelial cocultures. We added a new paragraph to the results section of our manuscript. Since we did not see any major differences, we introduced the new paragraph with the headline “TKI transcriptomic signatures are similar in cardiomyoctes cocultured without and with human coronary artery endothelial cells” (page 9). The new paragraph describes the results shown in the additional supplemental figures 27-28. Coculturing with endothelial cells resulted in observable more coordinated beating of the cardiomyocytes suggesting communication between the two cell types. Drug treatment at the therapeutically equivalent concentrations as per our experimental design for the entire study did not produce any observable effects on endothelial cells and drug treated cells looked similarly healthy as control endothelial cells (Supp. Fig 27).

Other major comments are as follows:

1. Given that many of the drugs tested in this platform are life-saving when used to treat cancer, the concept of "permissive cardiotoxicity" is becoming increasingly popular - some drugs are known to be cardiotoxic, yet they are necessary for optimal cancer treatment. One example is ponatinib, which has a black box warning for cardiovascular events but is used for patients who either develop resistance to other TKIs or have a specific tumor mutation. How might the concept of permissive cardiotoxicity, or alternatively on-target cardiotoxicity (related to inhibition of the intended drug target) be incorporated into the proposed platform?

We thank the reviewer for pointing out the concept of permissive cardiotoxicity and his suggestion to link it to our approach. Associating cellular pathways to cardiotoxic side effects can form the basis for mitigating therapies, allowing continuation of anti-cancer treatment despite cardiotoxic side effects.
In one of our own studies that we cite in the discussion of our manuscript, we identified baclofen as a treatment for thoracic aortic aneurysm [3]. Our drug repurposing strategy specifically aimed at the reversal of expert-curated disease-relevant pathway activities and not on the reversal of all gene expression profiles. Similarly, identified cardiotoxic pathways that are changed by a cardiotoxic TKI and ideally are not involved in its antineoplastic effects, can be targeted using a similar approach. Perhaps cardiotoxic mitigating drugs such as mevacamten or SGLT2 inhibitors could be used in combination with ponatinib, especially since changes in cardiac contractility pathways are signatures for cardiotoxicity and downregulated by ponatinib with top ranks.
The opportunity for identification of mitigating therapies is summarized in figure 5. We have rewritten the discussion about mitigating therapies that could be predicted from our data and explicitly mentioned the concept of permissive cardiotoxicity (page 12, end of first paragraph of the discussion).

Since most of the TKIs tested have been reported to cause cardiotoxicity in some patients, including "non-cardiotoxic TKIs," it seems that a focus on each patient's individual risk may be more fruitful than a focus on drug-specific risk.

Based on observed frequencies in clinical studies that are either larger or smaller than 1%, we have separated the TKIs into a group of cardiotoxic and noncardiotoxic TKIs, respectively. We anticipated to identify TKI pathway activities that indicate a higher risk for cardiotoxic side effects over those with a lower risk. We aimed at identification of general mechanisms that can be used for preclinical toxicity screening, such as screening for HERG channel inhibition is used to predict potential arrhythmic side effects [4,5].

We agree with the reviewer that prediction of each patient's individual risk for side effect development is a very important task. Therefore, we have subjected our six genomic cell lines to whole genomic sequencing (WGS) and combined the WGS results with our transcriptomic signatures by mapping cell-line-selective variants to transcriptomic outlier responses and mechanisms involved in a TKI's pharmacokinetics or -dynamics. Fully recognizing that small numbers of subjects involved, we nevertheless tested if this approach could work. This strategy allowed reidentification of the variant *rs2229774* in the RARG gene that is casually linked to anthracycline-induced cardiotoxicity (AIC). It is one of three variants with the strongest evidence for AIC [6,7]. In addition, we hypothesize that knowledge of cardiotoxic pathway activities induced by TKIs can allow prioritization of genomic variants mapping to genes involved in those pathways over those that do not. To emphasize potential gains of our approach, we now stated in the results section that our data integration based strategy could allow "targeted genomic studies to identify risk determinants, even before a sufficient number of patients has developed side effects to meet minimum samples sizes required for traditional GWAS".

2. In patients, most TKIs result in a cardiomyopathy that is reversible upon cessation of the offending agent. TKI-induced cardiomyopathy is therefore considered to be mechanistically different than anthracycline cardiomyopathy, which results in CM apoptosis, ferroptosis, myofibrillar disarray, and other severe myopathic changes. Of the SCPs that overlapped with dilated or hypertrophic cardiomyopathy, have any of these been previously reported to be associated with TKI cardiotoxicity in vivo?

We searched the literature for both primary publications and review articles for in vivo demonstration of TKI cardiotoxic pathways. We were not able to find any papers that describe in vivo mechanisms. Most studies are cell based. A recent review [8] provides a nice summary. Our SCPs agree with many of the biological processes described in this review. The supplemental information of our manuscript contains a detailed comparison of our findings with those described in the literature.

Minor Comment:

Supplemental Fig 3 - both cardiac-acting and non-cardiac-acting drugs are labeled in blue. Please use a different color scheme to differentiate these categories.

We thank the reviewer for her/his suggestions and changed the colors for the cardiac-acting and non-cardiac-acting drugs in all our figures to dark blue and cyan. They now can be easily distinguished from each other.

Reviewer #2 (Remarks to the Author):

In the present manuscript, authors applied sophisticated bioinformatics and computation tools to discriminate the TKI-induced cardiotoxicity observed by several anti-cancer TKI inhibitors (already approved by the FDA). The present computation study is mainly based on the two previous already published studies of the authors (Schaniel et al.: Stem Cell Reports 2021 and Van Hasselt et al.: Nat Commun 2020). The experimental part includes the generation and treatment of hiPSCs-cardiomyocytes (from 6 healthy individuals) with cardiotoxic and non-cardiotoxic TKI (and other additional compounds) as well as the transcriptome data are already published (Schaniel et al.: Stem Cell Reports 2021 and Van Hasselt et al.: Nat Commun 2020).

In the present computation study, authors reanalysed the huge transcriptome data in combination with genomic variations of the DNA of the 6 individuals with the main aim to identify a specific cardiotoxicity signature for the TKI cardiotoxic compounds. The computation/bioinformatic tools are indeed beyond the state of the art. In summary, the authors identified a specific gene signature/pathway that may specifically predict the cardiotoxicity of TKIs pending the genomic variations of an individual. The authors conclude that the specific TKI cardiotoxic signature might be useful for therapeutical applications to prevent drug-induced cardiotoxicity.

Certainly, this is an interesting computation study.

We thank the reviewers for acknowledging our work and findings, in particular the computational and bioinformatics approaches.

We apologize for confusion regarding the misunderstanding about already published and unpublished data that was used in this manuscript. All data in the van Hasselt paper were on Promo cells, i.e. cells derived from the human adult heart. These do not beat and have mixed cardiomyocyte fibroblast properties. In this study we only use iPSC-derived cardiomyocytes obtained by use of a ventricular differentiation protocol that show rhythmic contraction.

Though the experiments to measure control (base line) and drug-induced gene expression in our six iPSC-derived cardiomyocyte cell lines were done in parallel, we have only published the control results in the Schaniel et al paper [9]. Gene expression measured after drug treatment is described in this manuscript for the first time.

To reduce confusion about published and unpublished data, we have rewritten the first paragraph of the results section and explicitly stated that “Untreated control data was generated at the same time and has been previously published as part of characterization of these cell lines [9].”

However, the conclusions of the authors are over-enthusiastic. To my opinion, some weak points should be taken into consideration for improving the manuscript.

We have added a concluding paragraph highlighting the limitations of the study (page 13, last paragraph).

1. Although the computation and bioinformatics tools are very advanced and useful, the main wet lab work and the main transcriptome part have been already published.

We again apologize for our confusing presentation about published and unpublished results. We have now clarified it.

2. Functional assays such as beating activity/electrophysiology of the cardiomyocytes are completely missing. Certainly, such studies can be performed with a few representative cardiotoxic and non-cardiotoxic TKI as well as doxorubicin as a control. Such studies are necessary to correlate the specific cardiotoxic SCPs observed by the cardiotoxic TKIs

While such studies are valuable they do not provide reliable measures of cardiotoxic especially at therapeutically relevant doses. In a previous study [10] we have compiled a list of functional and pathology studies that show very variable results between different studies for many TKIs (please see Figs 1d and e in van Hasselt et al. [10]).

3. The specific cardiotoxicity-associated SPCs for the TRIs are certainly also observed by treatment of the hiPSCs-cardiomyocytes with anthracyclines. There are a few publications already available but not cited by the authors. In this context, it was also published that ferroptosis is also a key process elicited in hiPSCs-cardiomyocytes by an anthracycline.

We apologize for these omissions. We have added a comparison of the up- and downregulated pathways between anthracyclines and very toxic TKIs (frequency of cardiotoxicity > 10%) (Suppl. Figures 25 and 26). We could identify multiple pathways mapping to the previously published mechanisms, as briefly discussed on page 9 (top paragraph).

4. However, it would be essential to demonstrate for at least a few cardiotoxic and non-cardiotoxic TRIs (as a control) whether the gene signatures/SCPs are reversible after removing the compounds for 48 h (or even longer). It might be very interesting to identify reversible and sustained gene signatures/SCPs. This will help us to develop predictive strategies by targeting sustained (non-reversible) gene signatures/SCPs.

We agree with the reviewer that the separation between permanent and reversible gene or SCP signatures is an interesting question for a follow-up study to emphasize which pathway activities are more urgent to be reversed. Similarly, reviewers 1 and 3 also suggested additional

experiments, i.e. the investigation of TKI-induced gene expression profiles in presence of an additional cell type of the human heart. Reviewers 1 and 3 correctly stated that our single cell culture condition might not represent the tissue environment in the human heart and omit cross cell type effects involved in TKI cardiotoxicity. Since we thought that both reviewers raise an important concern about our data, we decided to experimentally address their suggestions. This is in agreement with the editor's proposal "to validate our findings in another in vitro model, such as co-culturing". We stimulated two iPSC-derived cardiomyocyte cell lines with dabrafenib and pazopanib in presence or absence of endothelial cells. As described in a new results paragraph "TKI transcriptomic signatures are similar in cardiomyoctes cocultured with endothelial cells" (page 9) and presented in supplemental figures 27-28, we could not find evidence for different transcriptomic responses in cocultured cardiomyocytes, besides small variations that might be due to chance. We hope for understanding of this reviewer that we selected the experimental suggestions of the other reviewers.

Reviewer #3 (Remarks to the Author):

In the manuscript titled "Multiscale Mapping of Transcriptomic Signatures for Cardiotoxic Drugs," the authors discussed the hypothesis that drug-induced gene expression can serve as an indicator for drug toxicity. They tested a total of 54 FDA-approved drugs (including 27 antineoplastic tyrosine kinase inhibitors) on six healthy human iPSC-derived cardiomyocytes. Drug-selective transcriptomic signatures were obtained through singular value decomposition (SVD)-based analysis of the total transcriptomic responses. The authors also integrated other publicly available datasets to achieve precise predictions of cardiotoxicity for new drug development.

The overall design and workflow make this study a valuable method for evaluating cardiotoxicity during preclinical drug development.

Compared to other similar studies that use cardiomyocyte cell lines, this paper employs human-derived iPSCs and targets the induced differentiation into "ventricular cardiomyocytes," which better mimic the physiology and drug responses of human adult cardiomyocytes.

We thank the reviewer for his appreciation of our work.

Major Points:

1. The differentially expressed genes (DEGs) identified in this study using HiPSC-derived cardiomyocytes can only account for part of the cardiotoxic effect. Cardiac fibroblasts, cardiomyocyte macrophages, or a co-culture system often play an equally important role in cardiotoxicity and if possible should be tested to evaluate this pipeline.

We agree with the reviewer's concerns about the validity of the transcriptomic profiles that were generated in single cardiomyocyte cultures. Since reviewer 1 addressed the same idea, we repeated the stimulation of two cell lines with pazopanib and dabrafenib in presence or absence

of human coronary artery endothelial (HCAE) cells. As described in a new results paragraph “TKI transcriptomic signatures are similar in cardiomyoctes cocultured with endothelial cells“ (page 9) and presented in supplemental figures 27-28, we could not find evidence for different transcriptomic responses in cocultured cardiomyocytes, besides small variations that might be due to chance.

2. The unique cluster of DEGs may not represent or cover all potential mechanisms involved in cardiotoxicity. Defining these DEGs as the fingerprint of cardiotoxicity remains a challenge.

We agree with the reviewer that transcriptomic effect only cover one aspect indicative of cardiotoxic responses. For example, direct inhibition of important proteins is a common mechanism in many drug effects (e.g. channel inhibition) and should consequently contribute to cardiotoxicity development as well. We briefly mention this on page 10 in the fourth paragraph, when discussing how genomic potential variants could interfere with a drug’s cardiotoxicity by mapping to pathways targeted by TKIs, either directly or by changing levels of pathway gene expression.

To clearly state the limitation of our study that focusses only on transcriptomic responses, we added an additional paragraph at the end of our discussion (page 13, last paragraph) that suggests integration of more omic technologies for an advanced description of mechanisms involved in TKI-induced cardiotoxicty.

3. The manuscript currently lacks totally compelling evidence or a robust pipeline for identifying DEGs from drug-induced genes.

In agreement with the reviewer’s suggestion, we have uploaded our R- and C# code that we used to generate all figures and supplemental figures to https://github.com/DToxS/SVD-curated_transcriptomic_signatures_cardiotoxic_drugs. We apologize that we have not published our code within the first submission.

For the initial data analysis that is used as an input to our SVD pipeline, we have developed a pipeline that integrates multiple standard programs, such as STAR and edgeR. This pipeline has been described in our previous publications [10,11]

4. The authors have not integrated genome and transcriptome data effectively in the validation and prediction of cardiotoxicity. A similar paper published in Frontiers in Pharmacology in 2020, titled "Dual Transcriptomic and Molecular Machine Learning Predicts All Major Clinical Forms of Drug Cardiotoxicity," reported a model and the prediction using a large collected and curated dataset. It is better to comment on and compare the advantages of the method used in this study, if any.

We have now cited this paper. These authors used data from our group on the PromoCell cardiac tissue derived cells (published in the van Hasselt Nature Communications paper [10]) to validate their computational model. We appreciate the reviewer highlighting this paper.

5. Please functionally predict and validate the comprehensive and novel signature of drug-induced cardiotoxicity that has not been identified by traditional measurements, such as left ventricular ejection fraction (LVEF).

We appreciate the reviewer's interest in an experimental validation of our predicted pathway activities. Please note that many of the identified SCPs are part of mechanisms involved in drug-independent cardiomyopathy and are targeted by existing or suggested mitigation therapies, as we summarize in the supplemental information. Organ physiology experiments are beyond the scope of this study.

Minor Points:

1. Misuse of abbreviations: For example, the inconsistent use of DEGs and their full name in figure legends and results sections.

We apologize for the errors and have removed the described inconsistencies. The full term 'differentially expressed genes', for example, is now only used in headlines, at the beginning of the results section, first paragraph, and in the supplemental methods chapter 'Identification of differentially expressed genes'. In all other cases, we have replaced the term by 'DEGs'.

2. Please ensure uniform font and size in figures.

We appreciate the comment and have tried to comply as best as possible for figures generated by R that were combined in power point.

Additional comment:

We noticed that when applying a cutoff for the population-wide frequencies, we did not select 0.1, but 10 (i.e., 10%). Since the allele frequencies range from 0 to 1, this resulted in not applying a cutoff at all. We repeated our analysis using the correct cutoff (i.e., 0.1) and using the most recent versions of all databases and libraries used for the genomic analysis (e.g., DrugBank, transcription factor and kinase libraries). The new analysis affects figures 4E/G/H and Suppl. Figures 20, 21 and 32A/B/C/D, as well as the gene and variant counts in the results section (page 11, first, second and fourth paragraph). The updated DrugBank database also contained a link of doxorubicin to its target protein TOP2B (Figure 4C).

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have performed an additional experiment to assess cardiomyocyte transcriptional changes in the presence of coronary endothelial cells, and they have added several important points to the Discussion. The manuscript is significantly improved as a result.

Reviewer #2 (Remarks to the Author):

However, there remain a couple of principal concerns that warrant consideration. The authors have clarified that their current study utilizes iPSC-derived cardiomyocytes instead of the previous study, which used Promo cells. While this addresses one point adequately, the pre-existence of control data in the literature weakens the manuscript's novelty.
In my view, functional data holds immense significance in such studies. Transcriptome alterations induced by various drugs should manifest in functional consequences, which ought to be demonstrated by representative examples. The authors argue against the reliability of such functional studies in assessing cardiotoxicity, particularly at therapeutically relevant doses. However, this assertion lacks validity and could be used to undermine the present study. It seems not logical to identify gene signatures at therapeutic concentrations without demonstrating any adverse functional effects. Additionally, there exist numerous studies on anthracyclines and other compounds that have already been published, demonstrating adverse effects on cardiomyocyte function at therapeutic concentrations, which the authors appear to have overlooked.
I acknowledge the authors' efforts in conducting new experiments suggested by reviewers 1 and 3. However, I believe the manuscript could be enhanced if the authors could showcase the reversibility of effects induced by certain drugs. As previously mentioned, identifying reversible and sustained gene signatures/SCPs would be highly valuable. This would aid in the development of predictive strategies by targeting sustained (non-reversible) gene signatures/SCPs.

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The authors have performed an additional experiment to assess cardiomyocyte transcriptional changes in the presence of coronary endothelial cells, and they have added several important points to the Discussion. The manuscript is significantly improved as a result.

We thank the reviewer for their approval.

Reviewer #2 (Remarks to the Author):

However, there remain a couple of principal concerns that warrant consideration. The authors have clarified that their current study utilizes iPSC-derived cardiomyocytes instead of the previous study, which used Promo cells. While this addresses one point adequately, the pre-existence of control data in the literature weakens the manuscript's novelty.
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We agree with the reviewer's statement and have added the following sentence to the paragraph about the limitations of our study in the discussion (page 14, 2nd paragraph):

"Functional assays offer an additional opportunity to predict a drug's cardiotoxicity [73-80] and can be used to analyze if our transcriptomic findings translate into physiological effects that could be targeted by mitigating therapies."

I acknowledge the authors' efforts in conducting new experiments suggested by reviewers 1 and 3. However, I believe the manuscript could be enhanced if the authors could showcase the reversibility of effects induced by certain drugs. As previously

mentioned, identifying reversible and sustained gene signatures/SCPs would be highly valuable. This would aid in the development of predictive strategies by targeting sustained (non-reversible) gene signatures/SCPs.

We again want to emphasize that we agree with the reviewer, but due to limited funding we cannot perform these experiments at this stage. To acknowledge the reviewer's idea, we extended the last sentence in our paragraph about the limitations of our study in the discussion (page 14, 2nd paragraph): "Additionally, our study is at one time and one therapeutically relevant concentration and does not separate transient from permanent effects by analyzing reversal of transcriptomic changes after drug withdrawal."