Cardio quickPredict: A Targeted Metabolomics-Based Assay Using Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes to Identify Structural and Functional Cardiotoxicity Potential

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Associate Director of Toxicology
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Stemina Biomarker Discovery

• History
  – Established operations in 2007
  – State-of-the-art mass spectrometry and stem cell culture laboratories

• Expertise
  – Human stem cell models (hES and iPS cells) of toxicity and disease
  – Proprietary metabolomics platform
  – Biology of Autism Spectrum Disorders
  – Identification of biomarkers of toxicity and disease for diagnosis and more precise treatment, drug development, clinical study management, companion diagnostics
Stemina’s Approach

✓ Unique opportunity to evaluate chemical toxicity using a human endpoint
✓ Differentiated cells function similar to in vivo counterparts
✓ “Unlimited” supply
✓ Applicable to high-throughput systems
Stemina’s Approach

Metabolome: Measure of Function

- **Genomics**: DNA Structure & Function
- **Transcriptomics**: Gene Expression
- **Proteomics**: Protein ID

**LC-MS-based Metabolomics**

- Metabolites
- Functional Pathways
- Biochemical Phenotype

**Human Pluripotent Stem Cell Biology**

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Stemina’s Approach

- Understand compound risk profiles faster
- Multiple exposure levels
- Minimal compound required
- Cost Savings

Human Pluripotent Stem Cell Biology

Stemina’s In Vitro Toxicology Assays

LC-MS-based Metabolomics
Stemina’s *In Vitro* Toxicology Assays

*Exposure-based prediction of toxicity potential using targeted metabolomics and human pluripotent stem cells and differentiated cells*
A Targeted Metabolomics-Based Assay Using Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Identifies Structural and Functional Cardiotoxicity Potential

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Abstract

Implementing screening assays that identify functional and structural cardiotoxicity earlier in the drug development pipeline has the potential to improve safety and decrease the cost and time required to bring new drugs to market. In this study, a metabolic biomarker-based assay was developed that predicts the cardiotoxicity potential of a drug based on changes in the metabolism and viability of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM). Assay development and testing was conducted in 2 phases: (1) biomarker identification and (2) targeted assay development. In the first phase, metabolic data from hiPSC-CM spent media following exposure to 66 drugs were used to identify biomarkers that identified both functional and structural cardiotoxicants. Four metabolites that represent different metabolic pathways (arachidonic acid, lactic acid, 2′-deoxyribose, and thymidine) were identified as indicators of cardiotoxicity. In phase 2, a targeted, exposure-based biomarker assay was developed that measured these metabolites and hiPSC-CM viability across an 8-point concentration curve. Metabolite-specific predictive thresholds for identifying the cardiotoxicity potential of a drug were established and optimized for balanced accuracy or sensitivity. When predictive thresholds were optimized for balanced accuracy, the assay predicted the cardiotoxicity potential of 81 drugs with 86% balanced accuracy, 83% sensitivity, and 90% specificity. Alternatively, optimizing the thresholds for sensitivity yields a balanced accuracy of 86%, 90% sensitivity, and 79% specificity. This new hiPSC-CM-based assay provides a paradigm that can identify structural and functional cardiotoxic drugs that could be used in conjunction with other endpoints to provide a more comprehensive evaluation of a drug’s cardiotoxicity potential.

Key words: cardiotoxicity; drug discovery and development; in vitro; hiPSC-CM; metabolites.
Predicting Cardiotoxicity is a Challenge

- Cardiovascular (CV) toxicity is a major cause leading to compound attrition throughout the drug discovery and development process as well as withdrawal of FDA-approved drugs from the market.
- Broad range of drug-induced CV toxicity → Affects all parts and functions of the CV system.
- Current preclinical CV toxicity testing methods heavily focused on functional/electrophysiological assessment.
- Limitations of in vivo models due to species differences in ventricular repolarization and drug response.
- Ongoing initiatives aimed at addressing limitations of current methods for proarrhythmia (CiPA, CSAHi).
- Unmet need to test for structural CV toxicity early in drug development.
  - Limited number of in vitro assays available to determine potential for structural cardiotoxicity.

Functional (Electrophysiological)
- Main effect on electrical/mechanical function.

Structural
- Main effect on general cellular processes/Morphological damage to cardiomyocytes.
  - Cell viability
  - Metabolism
  - Mitochondrial function
  - Oxidative stress, etc.
Cardio\textsuperscript{qp} Development Overview

- Two phases of development: Biomarker Discovery & Targeted Assay Development
- Developed using iCell\textsuperscript{®} Cardiomyocytes\textsuperscript{2} (FUJIFILM Cellular Dynamics, Inc.)
- Used 81 well-characterized drugs with known cardiotoxicity outcomes → 52 cardiotoxic, 29 non-cardiotoxic
  - Cardiotoxic compounds classified by main effect type: (1) Structural; (2) Functional; (3) Structural & Functional (aka General)
  - Wide range of cardiovascular and non-cardiovascular drugs: channel blockers, antineoplastic, antiviral, COX-2 inhibitors, receptor agonists and antagonists, and tyrosine kinase inhibitors.

Phase 1a: Concentration-Response Metabolic Profiling Experiment
- 50 drugs evaluated with 8-point concentration-response curve
- Identify non-cytotoxic concentration that alters hiPSC-CM metabolism (used for single exposure experiment)

Phase 1b: Single Exposure Metabolic Profiling Experiment
- 66 drugs evaluated at a single, non-cytotoxic concentration
- Two independent replicates
- Identify most predictive mass features
- Evaluate biomarker reproducibility

Phase 2: UPLC-HRMS Method Development
- Develop rapid UPLC-HRMS method
- Confirm identity of most predictive mass features with UPLC-HRMS-MS

Phase 2: Metabolism-Based Assay for Predicting Cardiotoxicity
- 81 drugs evaluated with 8-point concentration-response curve
- Set predictive thresholds for each metabolite
- Determine most predictive, complementary metabolite combination
- Develop composite model for predicting cardiotoxicity potential
Cardio<sup>qP</sup> Development Overview: Phase 1

**Phase 1a: Concentration-Response Metabolic Profiling Experiment**
- 50 drugs evaluated with 8-point concentration-response curve
- Identify non-cytotoxic concentration that alters hiPSC-CM metabolism (used for single exposure experiment)

**Phase 1b: Single Exposure Metabolic Profiling Experiment**
- 66 drugs evaluated at a single, non-cytotoxic concentration
- Two independent replicates
- Identify most predictive mass features
- Evaluate biomarker reproducibility

**Goals:**
- Identify predictive secreted metabolites that discriminate cardiotoxictants from non-cardiotoxictants independent of changes in cell viability
- Confirm the reproducibility of the predictive response

- Two metabolic profiling experiments
- Measured changes in secretome using untargeted UPLC-HRMS methods
- 156 secretome “features”
- Selected 13 most predictive features (AUC >0.7, PPV >0.8, accuracy >20%)
- Cross-validation used to evaluate performance of feature combinations and select most predictive features for confirmation and use in Phase 2.
Cardio\textsuperscript{qp} Development Overview: Phase 2

- **Goal:**
  - Develop an exposure-based targeted metabolomics assay for rapid prediction of cardiotoxicity potential.
- Confirmed identity of 6 most predictive features
- Developed an optimized UPLC-HRMS method for predictive metabolites → 4-fold increase in throughput
- Four-fold cross-validation was used to assess the performance of metabolite combinations and select final model metabolites
- Final prediction model includes four metabolites: Lactic Acid, Arachidonic Acid, 2’-Deoxycytidine, Thymidine

**Phase 2: UPLC-HRMS Method Development**
- Develop rapid UPLC-HRMS method
- Confirm identity of most predictive mass features with UPLC-HRMS-MS

**Phase 2: Metabolism-Based Assay for Predicting Cardiotoxicity**
- 81 drugs evaluated with 8-point concentration-response curve
- Set predictive thresholds for each metabolite
- Determine most predictive, complementary metabolite combination
- Develop composite model for predicting cardiotoxicity potential

**Table 2. List of Predictive Metabolites Confirmed with UPLC-HRMS-MS.**

<table>
<thead>
<tr>
<th>Metabolite Name</th>
<th>Adduct</th>
<th>m/z</th>
<th>RT (Seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>[M-H]\textsuperscript{-}</td>
<td>303.2332</td>
<td>55</td>
</tr>
<tr>
<td>Thymidine</td>
<td>[M+Cl]\textsuperscript{-}</td>
<td>277.0618</td>
<td>132</td>
</tr>
<tr>
<td>2’-deoxycytidine</td>
<td>[M+Cl]\textsuperscript{-}</td>
<td>262.0615</td>
<td>296</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>[M-H]\textsuperscript{-}</td>
<td>89.0244</td>
<td>373</td>
</tr>
<tr>
<td>Alanine</td>
<td>[M-H]\textsuperscript{-}</td>
<td>88.0404</td>
<td>572</td>
</tr>
<tr>
<td>N-acetylaspartic acid</td>
<td>[M-H]\textsuperscript{-}</td>
<td>174.0410</td>
<td>695</td>
</tr>
</tbody>
</table>
Cardio\textsuperscript{qP} Process

Human iPSC-Cardiomyocytes

iCell Cardiomyocytes\textsuperscript{2} (CDI)

Control Treatments on Each Plate

Test Article Exposure

Cells

Cell Viability Analysis

[Graph showing cell viability analysis with various treatments and concentrations]

Spent Media

LC-MS Analysis

[Graphs showing LC-MS analysis of metabolites such as Lactic Acid, Acetohydroxamic Acid, Thymidine, and 2-Oxoglutathione]

Data Analysis

Test Article Exposure on Each Plate

LC-MS Analysis

[Graph showing Cardio\textsuperscript{qP} Model Metabolites and Cardio\textsuperscript{qP} Prediction Model]

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Metabolite-Based Prediction of Cardiotoxicity Potential

- Prediction model (PM) is a composite of 4 metabolites and cell viability.
  - Viability/Lactate
  - Viability/Arachidonic Acid
  - Thymidine
  - 2’-Deoxycytidine

- Each metabolite has its own prediction thresholds that were optimized based on the response at $10 \times C_{\text{max}}$ for all 81 drugs
  - Can be optimized for balanced accuracy or sensitivity
Cardio\textsuperscript{\textit{qp}} Prediction Model

- Prediction Distance (PD): measure of cardiotoxicity potential determined for each metabolite at each concentration based on difference between response and threshold.
Cardio\textsuperscript{qP} Prediction Model

- **Composite Prediction Distance (CPD):** maximum PD from response at each concentration (CPD)
  - Reflects the most sensitive ratio at a given concentration.
Cardio\textsuperscript{qP} Prediction Model

- Cardiotoxicity Potential Concentration (cTP): Point where CPD dose-response curve crosses the PM Cardiotoxicity Threshold (cTT)
Cardio\textsuperscript{qP} Prediction Model Performance

- Metabolite-specific prediction thresholds can be optimized to meet the needs of the end user.
  - Sensitivity-optimized thresholds $\rightarrow$ application during early drug discovery for hazard identification and elimination, when it is more important to accurately predict true positives.
  - Balanced accuracy-optimized thresholds $\rightarrow$ application after a candidate drug has been identified, when it is more important to have highly specific models.
- Response at $10\times C_{\text{max}}$ for each drug was used to score classification performance of the prediction models.
Cardio<sup>QP</sup> Accurately Predicts the Potential for Cardiotoxicity

### Balanced Accuracy Trained Model

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Balanced Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Functional*</th>
<th>Structural*</th>
<th>“General”*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic Acid</td>
<td>83%</td>
<td>65%</td>
<td>100%</td>
<td>100%</td>
<td>62%</td>
<td>80%</td>
<td>50%</td>
<td>67%</td>
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<tr>
<td>Arachidonic Acid</td>
<td>74%</td>
<td>56%</td>
<td>93%</td>
<td>94%</td>
<td>54%</td>
<td>47%</td>
<td>50%</td>
<td>67%</td>
</tr>
<tr>
<td>Thymidine</td>
<td>74%</td>
<td>52%</td>
<td>97%</td>
<td>96%</td>
<td>53%</td>
<td>33%</td>
<td>50%</td>
<td>67%</td>
</tr>
<tr>
<td>2’-Deoxyctydine</td>
<td>81%</td>
<td>65%</td>
<td>97%</td>
<td>97%</td>
<td>61%</td>
<td>67%</td>
<td>50%</td>
<td>76%</td>
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<tr>
<td>Composite Model</td>
<td>86%</td>
<td>83%</td>
<td>90%</td>
<td>93%</td>
<td>74%</td>
<td>93%</td>
<td>63%</td>
<td>90%</td>
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</table>

### Sensitivity Trained Model

<table>
<thead>
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<th>Metabolite</th>
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<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Functional*</th>
<th>Structural*</th>
<th>“General”*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic Acid</td>
<td>82%</td>
<td>75%</td>
<td>90%</td>
<td>93%</td>
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<td>87%</td>
<td>56%</td>
<td>81%</td>
</tr>
<tr>
<td>Arachidonic Acid</td>
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<td>69%</td>
<td>86%</td>
<td>90%</td>
<td>61%</td>
<td>53%</td>
<td>69%</td>
<td>81%</td>
</tr>
<tr>
<td>Thymidine</td>
<td>76%</td>
<td>62%</td>
<td>90%</td>
<td>91%</td>
<td>57%</td>
<td>40%</td>
<td>50%</td>
<td>86%</td>
</tr>
<tr>
<td>2’-Deoxyctydine</td>
<td>83%</td>
<td>73%</td>
<td>93%</td>
<td>95%</td>
<td>66%</td>
<td>87%</td>
<td>56%</td>
<td>76%</td>
</tr>
<tr>
<td>Composite Model</td>
<td>85%</td>
<td>90%</td>
<td>79%</td>
<td>89%</td>
<td>82%</td>
<td>100%</td>
<td>75%</td>
<td>95%</td>
</tr>
</tbody>
</table>

*Percent of Subclass Correctly Predicted at Cardiotoxic
Cardio$^{QP}$ Application for Unknown Compounds

- For compounds that do not have an established $C_{\text{max}}$ value, changes in hiPSC-CM metabolism can be used as a signal regarding the cardiotoxic potential of the compound.

- A concentration threshold can be applied to classify based on the predicted cTP concentration, e.g., 30 µM.
  - $\text{cTP} < 30 \ \mu\text{M} = \text{cardiotoxic}; \ \text{cTP} > 30 \ \mu\text{M} = \text{non-cardiotoxic}$

### Threshold Performance for 81 Drugs Used in Assay Development

<table>
<thead>
<tr>
<th></th>
<th>30 µM Threshold</th>
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<tbody>
<tr>
<td>Balanced Accuracy</td>
<td>85%</td>
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<tr>
<td>Sensitivity</td>
<td>94%</td>
</tr>
<tr>
<td>Specificity</td>
<td>76%</td>
</tr>
<tr>
<td>PPV</td>
<td>88%</td>
</tr>
<tr>
<td>NPV</td>
<td>88%</td>
</tr>
<tr>
<td>Functional*</td>
<td>100%</td>
</tr>
<tr>
<td>Structural*</td>
<td>81%</td>
</tr>
<tr>
<td>General*</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Percent of Subclass Correctly Predicted at Cardiotoxic
Metabolite Biomarkers Identified in this Study are Biologically Relevant for Cardiotoxicity

• Respond to cardiotoxicant exposure independent of changes in cell viability.
• Affected by a wide range of compound classes → Implies common metabolic pathways of toxicity shared by many cardiotoxicants.
• Have key roles in modulating oxidative stress and mitochondrial function and replication and are related to known mechanisms of cardiotoxicity.
• Broad range of response types observed for each biomarker indicate cardiotoxic drugs may effect different components in the pathways.
Lactic Acid: Mitochondrial Dysfunction

- Present in media but not utilized by cells under "normal" conditions → Secreted by cells into the media
- **Increase** in secretion most common effect; however, a few drugs **decreased** lactic acid relative to the controls → indicates increased uptake
- Provides insight into the state of hiPSC-CM mitochondrial energy metabolism
- Increased secretion reflects a shift from oxidative phosphorylation → anaerobic respiration and glycolysis – Hallmark of **mitochondrial dysfunction**
- Elevated levels have been shown to be associated with cardiotoxicity, especially during cardiac ischemia and heart failure.

---

**A.** Cell Viability

**B.** Lactic Acid

**C.** Response (Normalized to Controls)

Arachidonic Acid: Oxidative Stress Pathways

• Present in media but not utilized by cells under “normal” conditions → Secreted by cells into the media
• Increases (secretion) & Decreases (increased uptake) with toxicity → Indicative of multiple mechanisms
• Involved in lipid transport, lipid metabolism, and fatty acid metabolism.
• Activation of the arachidonic acid cascade due to redox state unbalance contributes to the pathogenesis of cardiovascular disease.
• Some cardiotoxic compounds known modulate CYP2J2-mediated arachidonic acid metabolism, leading to increased levels in plasma in vivo.
• Thiazolidinediones (e.g., rosiglitazone) increase arachidonic acid release from the cell membrane.
2’-Deoxycytidine & Thymidine: Altered Mitochondrial DNA Synthesis and Mitochondrial Replication

- Secreted by cells into the media
- Increase & Decrease with toxicity → Indicative of multiple mechanisms
  - Thymidine: Increase most common
  - 2’-Deoxycytidine: Decrease most common

2’-Deoxycytidine & Thymidine: Altered Mitochondrial DNA Synthesis and Mitochondrial Replication

- **Secreted** by cells into the media
- **Increase & Decrease** with toxicity → Indicative of multiple mechanisms
  - Thymidine: **Increase** most common
  - 2’-Deoxycytidine: **Decrease** most common

![Graphs showing cell viability and responses](image-url)
2’-Deoxycytidine & Thymidine: Altered Mitochondrial DNA Synthesis and Mitochondrial Replication

- Two of the principal nucleosides of DNA and components of the pyrimidine metabolism pathway.
- Changes reflect **inhibition of mitochondrial enzymes** necessary for mitochondrial DNA synthesis and mitochondrial replication.
- **Mitochondrial DNA damage** in cardiomyocytes → mechanism of doxorubicin-mediated cardiotoxicity.
- Many **antiviral and anticancer** agents known to cause cardiotoxicity are **nucleoside analogs** (e.g., zidovudine and fluorouracil).

Use Cardio$^{QP}$ to Determine Structural vs. Functional Liability

- Predict likelihood of functional vs structural effects based on metabolite response
  - Lactic acid $\rightarrow$ Functional
  - Arachidonic acid, thymidine, 2’-deoxycytidine $\rightarrow$ Structural
- Data can be used to understand differences in compounds within a compound class (i.e., TKI)
Use Of Cardio\textsuperscript{qP} To Assess Cardiotoxicity Of Tyrosine Kinase Inhibitors (TKIs)

**Challenge:**
Predict likelihood and type (functional vs. structural) of cardiotoxicity of TKIs based on metabolite response

**Cardio\textsuperscript{qP} Value-Add:**
- Prioritize which drug candidates should be undergo for additional safety pharmacology
  - Drugs that impact Via/Lac have higher potential to cause functional cardiotoxicity demonstrating higher risk for QT prolongation & torsade de pointes (TdP)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Via/Lac cTP (µM)</th>
<th>Via/AA cTP (µM)</th>
<th>Thy cTP (µM)</th>
<th>2dC cTP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axitinib</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Crizotinib</td>
<td>0.4</td>
<td>2.8</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>3.2</td>
<td>4.1</td>
<td>10.2</td>
<td>ND</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>56.8</td>
<td>57.4</td>
<td>9.5</td>
<td>ND</td>
</tr>
<tr>
<td>Imatinib</td>
<td>19.8</td>
<td>3.4</td>
<td>16.8</td>
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</tr>
<tr>
<td>Lapatinib</td>
<td>16.8</td>
<td>11.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>ND</td>
<td>61.9</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>10.5</td>
<td>2.0</td>
<td>9.1</td>
<td>ND</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>1.2</td>
<td>7.4</td>
<td>0.05</td>
<td>ND</td>
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<tr>
<td>Vandetanib</td>
<td>4.6</td>
<td>2.4</td>
<td>5.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

Cardio\textsuperscript{qP} Value-Add:
- Prioritize which drug candidates should be undergo for additional safety pharmacology
  - Drugs that impact Via/Lac have higher potential to cause functional cardiotoxicity demonstrating higher risk for QT prolongation & torsade de pointes (TdP)
Summary

• The combination of 4 metabolites predicted the cardiotoxicity potential of 81 compounds with ≥85% accuracy at therapeutically relevant concentrations.
• Each metabolite can identify multiple mechanisms of cardiotoxicity.
• PM can be used to determine concentration a compound shows the potential to cause cardiotoxicity.
• This method can be combined with other assays or endpoints (e.g., MEA or impedance) for a comprehensive understanding of a compound’s cardiotoxicity liability.
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- Beth Donley (CEO)

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