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PUBLISHABLE SUMMARY

In order to identify molecular response pathways and modules thereof partners have developed and implemented a pipeline for processing molecular omics data such as gene expression, proteomics and metabolomics data and to compute modules of interacting proteins and pathways applying graph theoretic approaches. The pipeline delivers key pathways that are predictive of drug toxicity.

Partners have conducted analysis of public data as well as project-related data in human and rat *in vivo* and *in vitro* material and identified molecular themes related to mitochondrial dysfunction as being predominantly affected by drug treatment. In this deliverable we highlight specific aspects and findings of the molecular analysis with respect to mitochondrial dysfunction. In particular, we have mined the literature for mitochondrial processes dysregulated according to cardiotoxicity and analysed multi-omics data with respect to anthracycline treatment in rat heart and human *in vitro* cardiac microtissues. We identified key molecules that are consistently regulated upon anthracycline treatment and that can be associated with molecular processes that build the basis for molecular modelling.

OBJECTIVES

Aim of the work is to analyze specific molecular aspects of toxicity, in particular mitochondrial dysfunction. Partners MPIMG and MD perform computational approaches for in-depth analysis of predictive potential, in particular in specific pathways related to mitochondrial dysfunction which will be used for model refinement in WP3 and WP4 and for the construction of adverse outcome pathways (AOPs) in WP11.

INTRODUCTION

Partners MD and MPIMG have reviewed public data, project data as well as literature in order to identify key mechanisms and proteins associated with mitochondrial function and related diseases. Using the computational pipeline built and described in Deliverable Report D2.3, partners identified molecular themes that are of primary interest for building models and Adverse Outcome Pathways (AOPs).

RESULTS

Mitochondrial dysfunction as a hallmark of toxicity

In accordance with ongoing experimental work in the project, modelling approaches are focusing on cardiotoxic drug effects of anthracyclines, esp. idarubicine and doxorubicine. Anthracyclines are important cancer therapeutics but they show strong side effects resulting in cardiotoxicity. Modelling of these toxic effects at the current stage is proceeding based on information extracted from literature and public data sources. The goal of our modelling approach is to represent key mechanisms on the regulatory and signalling level and to link them to the physiological models provided by the other partners of the project. As illustrated in Fig. 1 for doxorubicine different derivatives of anthracyclines are formed under physiologic conditions.

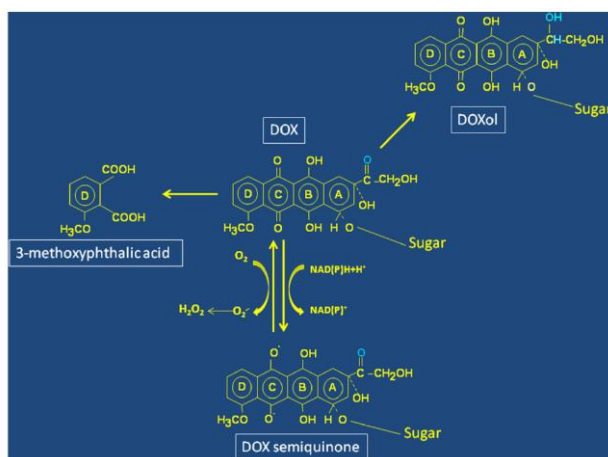


Fig 1: Structure and simplified scheme of molecular transformations of Doxorubicine (Gamella et al.). Redox cycling between the quinone and semiquinone forms (ring C) of Doxorubicine (DOX) leads to oxygen radicals' formation. The residue involved in DOXol formation following two-electron reduction of the carbonyl group in ring A is marked in blue. DOXol is directly linked to the pathway of iron homeostasis, dysregulation of which is one of the probable causes of mitochondrial dysfunction. Oxidative pathways involving a hydroquinone-derived semiquinone lead to formation of a diquinone (rings B and C), and eventual degradation of DOX with formation of 3-methoxyphthalic acid as remnant of ring D.

These derivatives interact via different pathways with the cardiac muscle cell resulting in different possible mechanisms for cardio-toxicity. As illustrated in Fig 2 these mechanisms include Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), iron homeostasis, calcium homeostasis, and mitochondrial dysfunction.

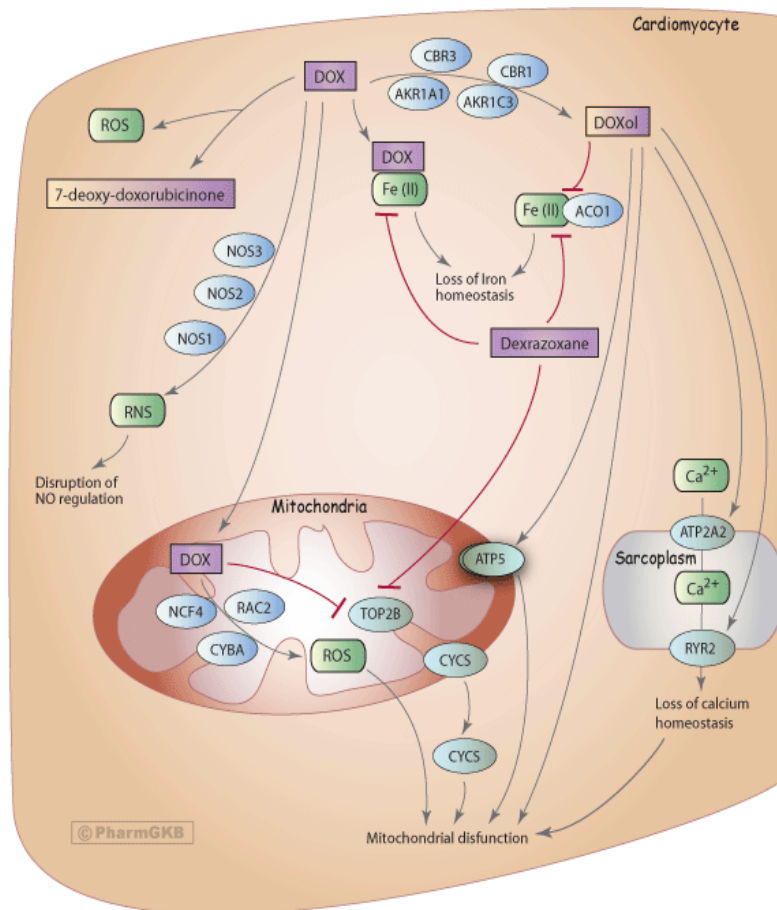


Fig 2: The major adverse effects of the antineoplastic drug doxorubicine (DOX) are acute and chronic cardiotoxicity/cardiomyopathy (Thorn et al.). DOX use is limited by cumulative, dose-related, progressive myocardial damage that may lead to congestive heart failure. The cardiotoxicity induced by DOX appears to be a multi-factorial process and many mechanisms have been proposed. The mechanisms of the therapeutic effects of DOX are thought to be different from those of the mechanisms of its cardiotoxicity.

Literature mining on modelling focus I: mitochondrial dysfunction

The text mining platform was used to identify genes related to idarubicine and doxorubicine. We compared genes showing a general compound association with genes related to both the compound and cardiotoxicity. We identified a list of 93 genes exclusively significant for cardiotoxicity of doxorubicine.

Since doxorubicine is used as chemotherapeutic agent, functional mapping of general compound related genes from literature mostly shows cancer related pathways (Fig 3). However, when using the genes identified for cardiotoxicity of doxorubicine, the functional enrichment strongly hints for the HIF-1 (Hypoxia-inducible factor 1)-pathway (p-value 10^{-11}). HIF-1 is a central transcription factor for the regulation of oxygen flux and has several targets that localize to the mitochondrion [Benita et al.; NAR 2009]. This pathway plays a key role in oxygen homeostasis but is at the same time cross linked with pathways for iron homeostasis (see below). Our modelling approach takes into particular regards the

cross linking effects of these two pathways via the regulatory elements like iron responsive element (IRE) controlling several key processes in iron homeostasis, synthesis of the heme group and hemoglobin synthesis.

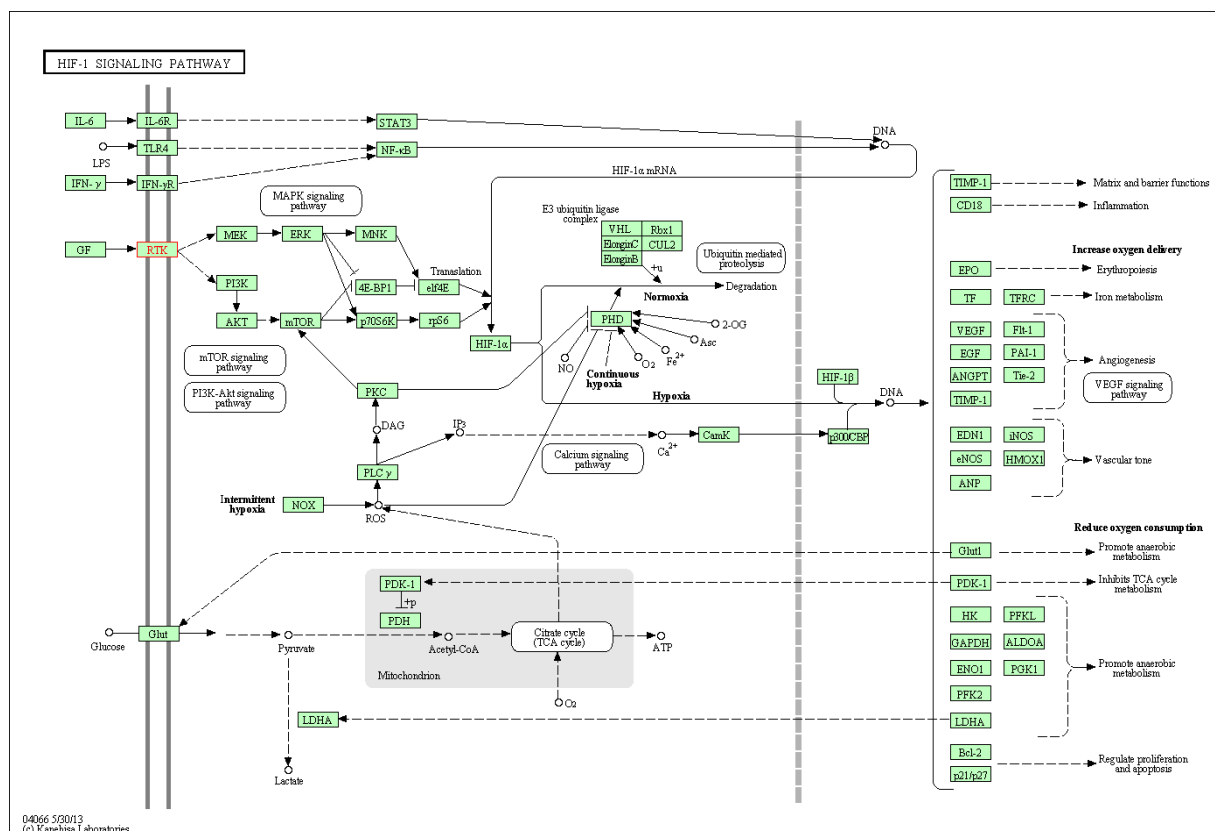


Fig 3: HIF-1 signaling pathway retrieved from KEGG. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that functions as a master regulator of oxygen homeostasis. It consists of two subunits: an inducibly-expressed HIF-1 α subunit and a constitutively-expressed HIF-1 β subunit. Under normoxia, HIF-1 α undergoes hydroxylation at specific prolyl residues which leads to an immediate ubiquitination and subsequent proteasomal degradation of the subunit. In contrast, under hypoxia, HIF-1 α subunit becomes stable and interacts with coactivators such as p300/CBP to modulate its transcriptional activity. Eventually, HIF-1 acts as a master regulator of numerous hypoxia-inducible genes under hypoxic conditions. The target genes of HIF-1 encode proteins that increase O₂ delivery and mediate adaptive responses to O₂ deprivation. Despite its name, HIF-1 is induced not only in response to reduced oxygen availability but also by other stimulants, such as nitric oxide, or various growth factors.

Microarray experiments for cardiotoxicity

Rat expression data for idarubicine and doxorubicine are obtained from DrugMatrix. Microarray data shows very strong down-regulation of different hemoglobin genes (>40 fold down-regulation) after five days for both idarubicine and doxorubicine. Furthermore we can observe a 5-fold up-regulation of myosin. The up-regulation of myosin may lead to a structural change in muscle phenotypes (Goldspink et al.; J. Anat. 1999).

Cross-omics identification of mitochondrial pathways and markers integrating public benchmark data and project data

MPIMG analysed publicly available microarray expression data from the DrugMatrix resource. Rat heart tissue was agglomerated for 72 different drugs and for each drug a set of most responsive pathways was identified using network analysis according to the pipeline set up in WP2 (cf. Deliverable Report D2.3). Strikingly, there were several pathways associated with mitochondrial function that occurred consistently in a large number of drug responses, such as “TCA cycle” (63 drugs out of 72), “electron transport” (63) and “oxidative phosphorylation” (61). Details of the analysis have been reported in Deliverable Report D12.1.

We were particularly interested in the cellular response to anthracyclines (such as doxorubicine, idarubicine, 4'-epidoxorubicine, daunorubicine) because they account for large numbers of cardiotoxicities in patients and are also of primary interest for HeCaToS. It has been shown by proteomics analysis that anthracyclines cause changes in proteins crucial for oxidative phosphorylation (Sterba et al. (2011) J Mol Cell Cardiol 50:849-862) which are in line with the observations that we made with the rat expression data.

There are 35 molecular pathways that were identified consistently in the network response modules of doxorubicine, idarubicine, 4'-epidoxorubicine and daunorubicine. Among these were predominantly pathways related to mitochondrial dysfunction as the ones described above. MPIMG has further investigated the expression time series of the genes that are associated with these pathways and identified 36 genes that are mostly reactive to the four compounds. The genes are listed in Table 1.

| Gene | Gene | Gene | Gene |
|--------|--------|--------|-------|
| MYL4 | RYR2 | NOS3 | RCOR1 |
| PCK1 | UCP2 | TKT | TPM4 |
| SNCA | ADIPOQ | PIK3R1 | DDIT3 |
| MYH7 | PDK4 | NR1H3 | PGAM2 |
| SLC2A4 | ALDOB | APRT | AK1 |
| NEB | APOE | GNAI3 | TPM2 |
| ATP1B4 | MYL1 | GAPDHS | |
| CYP2E1 | ATP1A2 | AKT1 | |
| IRS2 | PRKAG2 | BDNF | |
| BPGM | PDP2 | UCP3 | |

Table 1: Genes that have at least one fold-change >2 in the time series of the four anthracyclines under analysis in rat heart tissue. Red: gene predominantly up-regulated; green: gene predominantly down-regulated (median fold-change).

A question is which functions of the mitochondrion are targeted by the response genes. We used the ConsensusPathDB and performed over-representation analysis with the 36 genes. Pathways enriched are shown in Fig. 4.

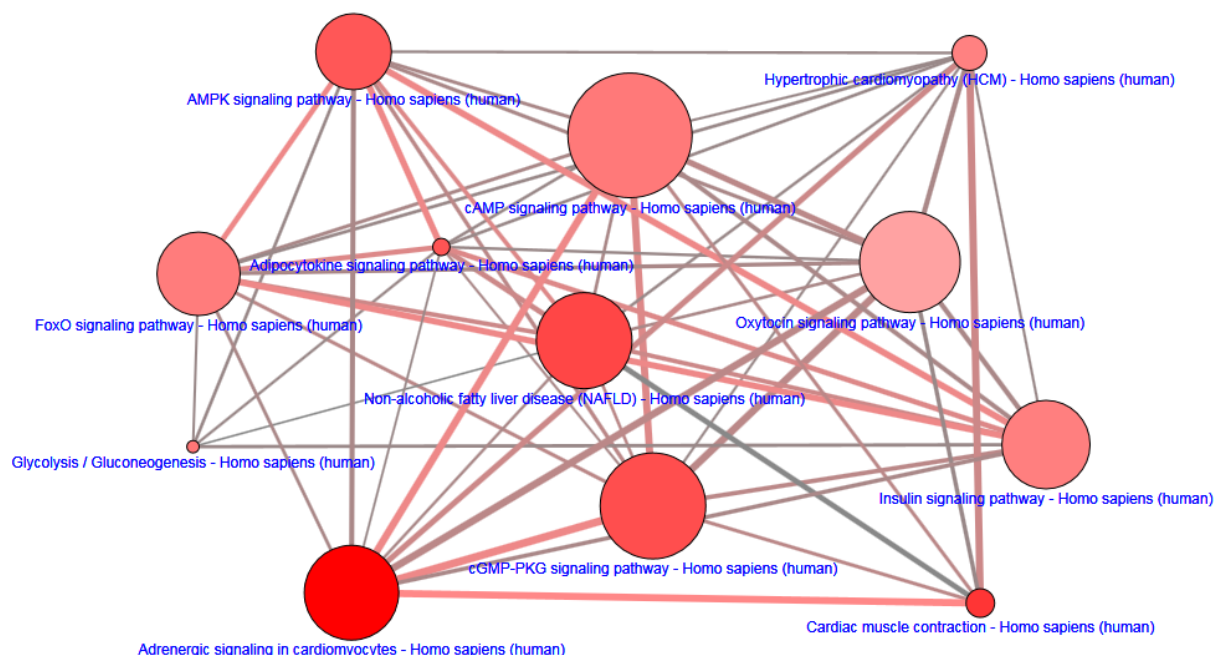


Figure 4: Pathway concepts enriched by the 36 genes. Thickness of the edges denote level of overlap between pathways; different node size reflects pathway size.

Key proteins that were identified in rat heart tissue expression (Table 1) were, for example MYL4 (myosin light chain 4) and TPM2 (tropomyosin 2 beta), PDK4 and RYR2:

- MYL4 encodes the protein Atrial Light Chain-1 (ALC-1), also known as Essential Light Chain. ALC-1 is expressed in fetal [cardiac ventricular](#) and fetal [skeletal muscle](#), as well as fetal and adult [cardiac atrial](#) tissue. ALC-1 expression is reactivated in human [ventricular myocardium](#) in various [cardiac muscle](#) diseases, including [hypertrophic cardiomyopathy](#), [dilated cardiomyopathy](#), [ischemic cardiomyopathy](#) and [congenital heart diseases](#).
- TPM2 is a protein coding gene. Diseases associated with TPM2 include [arthrogryposis multiplex congenita, distal, type 1](#) and [nemaline myopathy 4, autosomal dominant](#). Among its related pathways are [adrenergic signaling in cardiomyocytes](#) and [cardiac muscle contraction](#). GO annotations related to this gene include actin binding and structural constituent of muscle. An important paralog of this gene is [TPM1](#).
- Pyruvate dehydrogenase [lipoamide](#) kinase isozyme 4, mitochondrial (PDK4) is a member of the PDK/BCKDK protein kinase family and encodes a mitochondrial protein with a histidine kinase domain. This protein is located in the matrix of the mitochondria and inhibits the pyruvate dehydrogenase complex by phosphorylating one of its subunits, thereby contributing to the regulation of glucose metabolism. Expression of this gene is regulated by glucocorticoids, retinoic acid and insulin. Furthermore, it has been shown that PDK4 is activated by hypoxia.
- Ryanodine receptor 2 (RYR2) is a [protein](#) found primarily in [cardiac muscle](#). In humans, it is encoded by the RYR2 [gene](#). In the process of cardiac [calcium-induced calcium release](#), RYR2 is the major mediator for sarcoplasmic release of stored calcium ions. It has been suggested that binding of compounds, such as daunorubicin to RYR2 induce oxidation of RYR2 and contribute to anthracycline-induced cardiotoxicity during chemotherapy (Hanna et al., 2011). It is also involved in the loss of calcium homeostasis (cf. Fig. 2).

These genes show significant differential expression upon anthracycline treatment both at the gene expression and proteome level in human tissue and, thus, extrapolate from rat toxicity to human toxicity. Investigating RNA-seq data from cardiac microtissues upon idarubicine treatment reveals significant down-regulation of MYL4, TPM2 and RYR2 genes and up-regulation of PDK4 (consistent with rat *in vivo* data) at toxic doses. Additionally, genes show significant changes in protein expression at toxic doses.

Consistent with rat *in vivo* data (Fig. 4 above) the direct analysis of mRNA *in vitro* expression data generated in the HeCaToS project from cardiac microtissues (idarubicine data) identified the beta adrenergic signaling pathway as highly responsive. In figure 5 the corresponding KEGG pathway is displayed. Substantial part of this pathway is strongly down regulated under toxic conditions implying substantial changes in a number of membrane bound cellular transporters. In particular Ca-transport to the sarcoplasmic reticulum appears to be dysregulated. The sarcoplasmic reticulum is a compartment with essential function in control of muscle contraction.

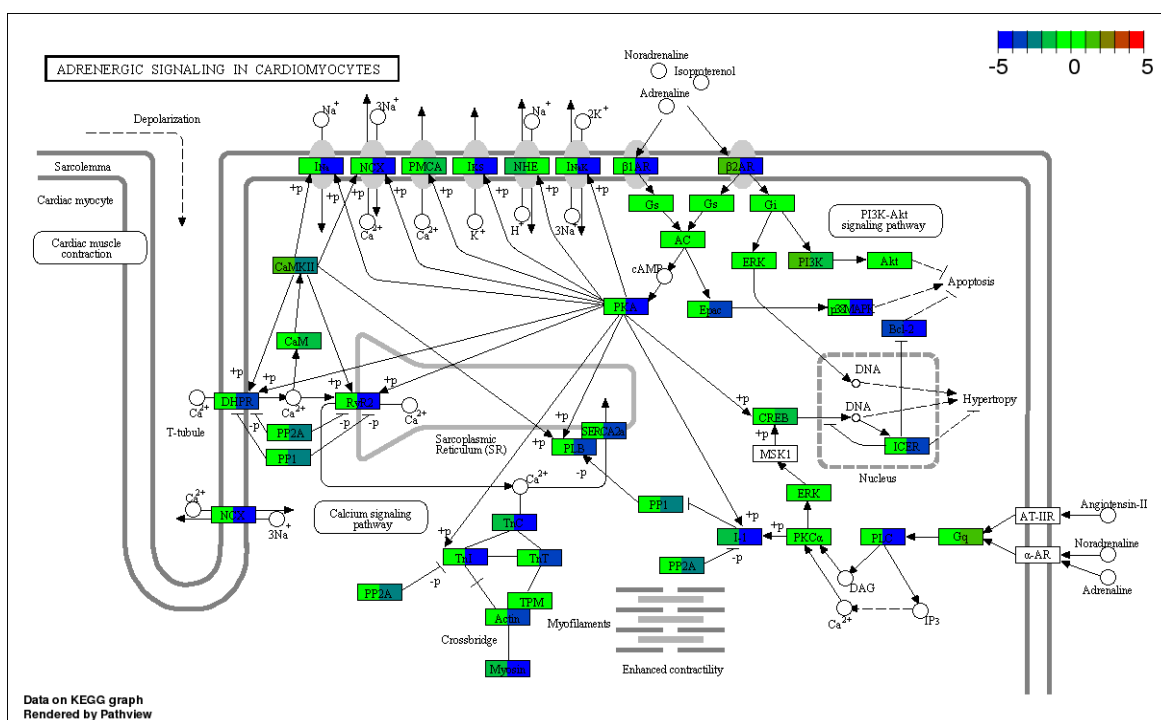


Fig 5: Idarubicine induced changes of mRNA levels comparing therapeutic and toxic doses. Colored fields encode the effect of therapeutic (left) and toxic doses of idarubicine. Colors correspond to log2 fold changes in expression from blue (-5, 32-fold suppression) to red (+5, 32-fold induction). Most genes in this pathway are essentially unaffected by the therapeutic dose (green) but strongly down-regulated under the effect of a toxic dose.

Constructing links between metabolic and regulatory models of mitochondrial function

One difficulty in approaching mitochondrial regulation is the fact that available models often completely focus on aspects of metabolic processing ignoring the important aspects of enzyme regulation on the level of mRNA and protein expression. Key events in mitochondrial energy metabolism are represented in the mitochondrial model by Smith and Robinson (see Fig 6). Since this model is a metabolic model it needs to be extended to include regulatory and signaling pathways. This is achieved by defining a relation between different entities such as metabolites and genes using information from knowledge bases. For the model of Smith and Robinson we obtained 193 compounds related to 415 genes using HMDB as reference. As a next step, we extend the model using differentially expressed genes from publicly available high-throughput data sets and text mining from literature. The link between genes and

enzymes enables prediction of potential effects of gene regulation (e.g. by a drug or a transcription factor) on the metabolic level. These effects in term can be incorporated to higher level physiological models as constructed by other partners in the HeCaToS project.

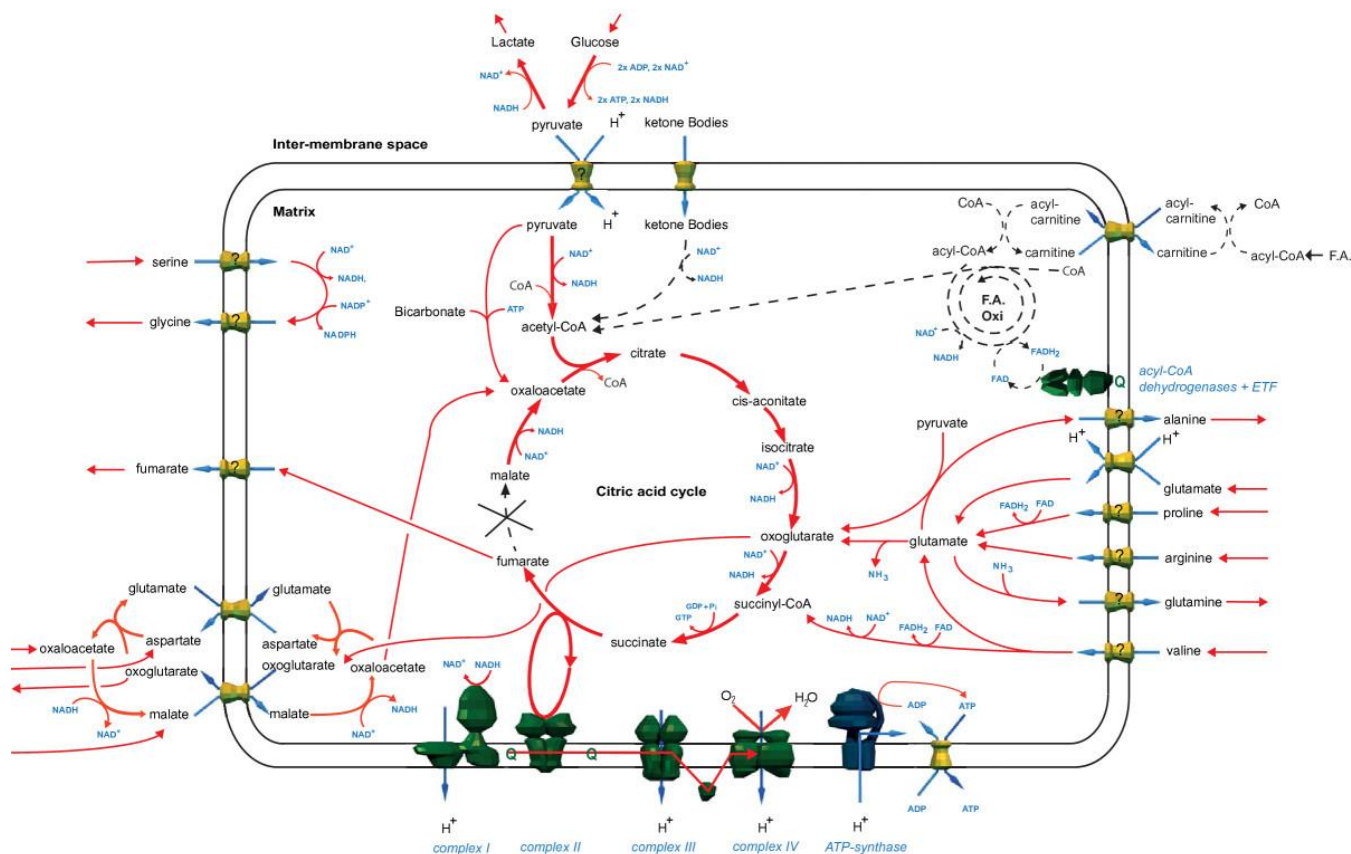


Fig 6: Key processes in mitochondrial metabolism related to energy and ion homeostasis (Smith and Robinson). Using database information a mapping from metabolites to related genes is achieved. Linking from genes to enzymes to metabolites and ions allows the prediction of changes in the metabolite profile which can be used to parameterize higher level physiological models.

Towards dynamic molecular models of mitochondrial toxicity

The above investigations based on public and proprietary data hint to a number of different but related mechanisms leading to anthracycline induced cardio-toxicity. One of the key processes is iron homeostasis linking drug effects with effects of reactive oxygen species and energy homeostasis. Starting point is modeling study (J. Chifman et al. JTB, 2012) for iron homeostasis. Key components of the model by Chifman et al. are iron responsive elements (IRE) regulating the concentrations of iron regulatory protein (IRP), iron transporter ferroportin and iron storage protein ferritin.

Based on information from the literature we extended the model to include the following effects:

- Transformation of IRP to Aconitase dependent on availability of iron from the labile iron pool (LIP);
- Degradation of Aconitase to the inactive IRP0 protein mediated by ROS;
- DOXol mediated reduction of active IRP.

With these extensions we arrive at an eight dimensional model for iron homeostasis. By adiabatic elimination under the assumption of relatively slow changes of LIP we arrive at a six dimensional model for iron homeostasis. Current efforts aim at the approximate determination of the resulting net parameters coupling DOXol with iron homeostasis.

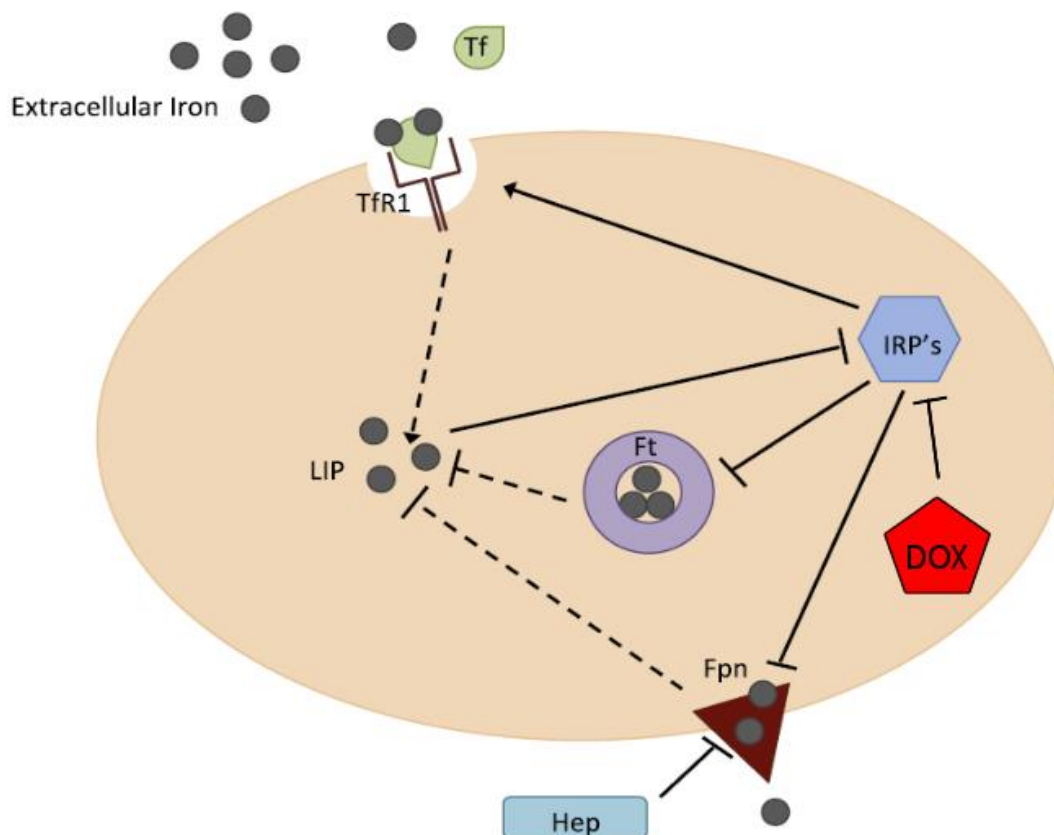


Fig 7: Model of iron homeostasis (Chifman et al.) extended by action of DOXol on the set of iron regulatory proteins. Catalysing the transition of iron regulatory protein (IRP) to an inactive null protein DOXol exerts an inhibitory net effect on IRP. This leads to a deregulation of iron homeostasis. Current investigations aim at a better understanding of the effect of DOXol on the dynamics and the attractor structure of the extended iron homeostasis model.

OUTLOOK

Combining all available data, our persistent goal is to extend existing mitochondrial models to represent mechanisms of cardio-toxicity of drugs like doxorubicine and idarubicine. The following key components are currently being investigated for their relevance to our model:

- HIF-1A pathway, oxygen homeostasis, regulation of HEME and globin synthesis;
- Iron homeostasis under action of DOXol;
- Sarcoplasmatic reticulum and calcium homeostasis;
- Structural changes related to MYL4 (myosin light chain 4), TPM2 (tropomyosin 2 beta), PDK4 and RYR2 and incorporation of these and other markers as potential model read-outs that are measurable with human in vitro omics data.

In the next reporting period partners will analyze these mechanisms with further project data and - together with WP11 - will explore modules in these pathways as potential key events in the construction of adverse outcome pathways (AOPs). The initial analysis performed here has already identified key mitochondrial markers that can be consistently measured with rat in vivo and human in vitro assays; thus, a key question that will be addressed in the next reporting period is whether these markers will also extrapolate to patient tissue. Such an extrapolation would allow including measurable molecular markers as model components and to use these components as read-outs that are predictive for human (cardio-)toxic phenotypes.

The long term goal is to combine these pathway level models with whole organ modeling of heart function by other project partners.

Formulating hybrid models combining elements of Bayesian modeling with classical ODE based modeling in order to represent mechanisms of which the current knowledge is limited as influence factors on the aspects modeled in more detail, e.g regulatory action of HIF1A and interaction of iron oxygen regulation.

DIFFICULTIES

Cardiac toxicity is mediated by many different pathways and mechanisms. This is true even for a single drug like doxorubicin. Few is known about interdependencies of these effects. While many molecular mechanisms are described in the literature few of them come along with quantitative information on interaction and rate constants. These parameters then need to be inferred by statistical methods leading to uncertainty about true time scales and relevance of effects observed in the model. The growing body of experimental data generated in the HeCaToS project will help to reduce these uncertainties.

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