Unbiased, network theoretic approaches to identify novel therapeutic targets in cardiovascular comorbidities

PhD thesis

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1 Introduction

1.1 Cardiovascular diseases and comorbidities

In the developed countries mortality and health care expenditures attributable to cardiovascular diseases and comorbidities far exceed the corresponding measures related to every other causes. Particularly, the overwhelming majority of cardiovascular mortality is due to ischemic heart disease, especially acute coronary syndrome. Although as a result of adequate revascularization therapy the early mortality of acute coronary syndrome is steadily decreasing, the occurrence of post-myocardial infarction heart failure showed a more modest reduction over the past decades. With a prevalence varying still between 14-36%, post-myocardial infarction heart failure is one of the main determinants of mortality after myocardial infarction.

A robust correlation between infarct size and risk of post-myocardial infarction heart failure is supported both by experimental and clinical evidences. It was previously demonstrated that significant reduction in the infarct size could be achieved by the intermittent occlusions of the affected coronary artery before or after the actual ischemic insult, called ischemic pre- or postconditioning, respectively. However, after successes with animal models of ischemia-reperfusion injury treated by drugs targeting mediators involved in the cardioprotective effect of ischemic pre- and postconditioning, studies to translate these drugs into the clinical practice were concluded with disappointing results uniformly.

Multiple recommendations were proposed to avoid further failures of the clinical translation including two central ideas, namely the consideration of the modifying effect of comorbidities and comedications and the application of unbiased target identification.

In the vast majority of the cases acute myocardial infarction develops in patients also affected by modifiable cardiovascular risk factors, such as metabolic disorders including diabetes mellitus, dyslipidemia and obesity. The sustained presence of these comorbid risk factors and agents administered to treat them lead to a remodeling process in both the vasculature and the myocardium, which could eventually impede the protective effect of the applied therapeutic interventions. Therefore, it is unavoidable to explore the effects of comorbidities and comedications on the cardiovascular system and to decipher molecular mechanisms responsible for these effects.

1.2 Unbiased, network theoretic target identification based on omics datasets

Hypothesis-driven target identification could have also contributed to the failure of clinical translation of candidate cardioprotective agents. Therefore, an unbiased workflow was recommended to be applied, which consists of high-throughput molecular biological measurements, bioinformatics evaluation of the resulting datasets, network theoretical target prediction and experimental validation of the selected targets.

High-throughput molecular biological methodologies together are called omics techniques. In case of complex, multifactorial diseases, in addition to static genomic data other omics techniques should be utilized to assess at least snapshots of the dynamical processes in the affected cells and tissues. Although epigenomics, proteomics and metabolomics measurements could provide us with truly valuable data on the functional state of the investigated cells, there is a great variety of technical difficulties that could still limit the utility of these methodologies. In contrast to these immature omics techniques, well-established, highly standardized and relatively cheap technologies like DNA microarray, NanoString nCounter and RNA sequencing (RNA-seq) are available for the global profiling of the transcriptome. Due to the limited number of microRNAs, which are unavoidable players in the post-transcriptional regulation of gene expression, the starting point of a parsimonious approach to uncover key players in the pathomechanism of various diseases can be the assessment of the microRNA fingerprint of the experimental model relying on the above transcriptomics measurement techniques. The analysis of the resulting huge omics datasets only possible by *in silico* approaches, which in turn requires in-depth understanding of the network theoretic bases of the available bioinformatics toolset and a detailed knowledge of the complex biology of the post-transcriptional regulation mediated by microRNAs.

A network consists of entities represented by nodes and relations between them symbolized by edges connecting the nodes. In case of a network with n nodes an $n \times n$ matrix A, called the adjacency matrix can be used to describe the presence or absence of links between pairs of nodes. The number of incoming edges of a node is called its degree, while the sum of the weight of the incoming edges is called the node strength. Those regions of the network where the degree of the nodes mostly counts for the connections within this region rather than the remaining parts of the network are called modules or communities.

Visualization of networks could facilitate the understanding of the functional relations between its constituents. However, in case of large, complex, real-world networks finding the best, meaningful layout is a challenging task, known as the "hairball" problem. This problem cannot be solved by the currently available network visualization algorithms, which also lack an objective measure to quantify the quality of the layout.

Information theoretic approach of relative entropy optimization offers a solution for both above problems. By assigning a two dimensional probability distributions to each node, the topology and the two dimensional visualization of the network can be represented by the adjacency matrix and the overlap matrix of these distributions, respectively. Kullback–Leibler divergence (information loss or relative entropy, *D*) can be calculated to express the difference between the information content of these two matrices. Therefore, minimization of the relative entropy function could result in achieving the optimal two dimensional layout of the network. Although this way the improvement of the visualization could be naturally and elegantly accompanied by a measure to quantify the layout quality, it remained an open question whether this algorithm can be implemented as a user-friendly application with reasonable time and space complexity, which could effectively avoid local minima of the state space.

MicroRNA-target interaction networks depict microRNAs and messenger RNA (mRNA) targets of microRNAs as nodes, and silencing interactions as edges. Relying on these networks either individual mediators or functional groups of mediators relevant in the pathogenesis of various diseases can be selected for experimental validation by network topological algorithms.

1.3 MicroRNA-target interaction networks

Besides RNA-binding proteins a central effector mechanism of the post-transcriptional regulatory network is RNA interference. RNA interference is an umbrella term for those small non-coding RNA mediated processes that lead to the reduction of the expression of a target gene at either the mRNA or the protein level. As one of the three classes of small non-coding RNAs that effectuate RNA interference, microRNAs (often abbreviated as miRNAs) with a mean length of approximately 22 nucleotides associate to and guide the members of the Argonaute protein family to the target mRNAs based on sequence complementarity of the 6-7 nucleotides long seed region. MicroRNA mediated gene silencing happens by mRNA destabilization, mRNA degradation or translational inhibition. The above

permissive pairing rule of microRNAs makes the regulatory network of microRNAs highly intricate by allowing for one microRNA to bind to multiple mRNAs and one mRNA to be targeted by multiple microRNAs. Therefore, it is obvious that the effect of alterations of microRNA expression profiles could only be predicted by taking into account the contribution of all microRNAs by network theoretic algorithms.

The primary prerequisite for inferring mRNA level transcriptomic changes from microRNA fingerprints is to identify the target genes of each microRNA showing expression changes between the studied phenotypes. Although, records in experimentally validated, manually curated databases, like miRecords, miRTarBase or DIANA-TarBase are relatively reliable, these datasets only cover a tiny portion of the possible microRNA-target interactions. Algorithms to predict microRNA targets, on the other hand, could provide full coverage of the possible interactions, they are still limited by a significant proportion of false positive findings.

An additional problem is the relative lack of software tools that can consider the effect of multiple microRNAs on the same target. Currently there are only a few algorithms that is capable of analyzing miroRNA-target interaction network as a whole. Here we propose that building and analyzing microRNA-target interaction networks by combining available experimental and predicted data could solve both above problems.

2 Objectives

We aimed to develop and validate two pieces of user-friendly software utilizing network theoretic algorithms to facilitate the identification of key pathways and functional modules in the interactome by the unbiased analysis of omics datasets.

Firstly, our goal was to implement a network layout algorithm that is capable to visually highlight functional modules in the layout of real-world networks and to objectively quantify the quality of the resulting network visualization.

The second purpose of this work was to develop a software that by evaluating transcriptomics profiles and by combining multiple publicly available microRNA-target interaction databases could select those genes that are most likely regulated by the set of differentially expressed microRNAs. We aimed to validate this latter software by analyzing the microRNA fingerprints of animal comorbidity models, to also contribute to the elucidation of the underlying pathomechanisms and to the identification of novel therapeutic targets.

3 Results

3.1 The EntOptLayout software

To address the “hairball” problem of network visualization and to facilitate the quantification of the layout quality the relative entropy optimization algorithm proposed by Kovács *et al*. was implemented as a user-friendly software called EntOptLayout and it was validated on several biological and synthetic networks. The source code is available at the following link: https://sourceforge.net/projects/entopt/

3.1.1 Software characteristics

The EntOptLayout software was implemented in Java programming language as a plugin (http://apps.cytoscape.org/apps/entoptlayout) for the cross-platform compatible Cytoscape network analysis and visualization framework, therefore its graphical user interface is available for every major operating systems including GNU/Linux, macOS and Microsoft Windows.

Adjacency matrices populated by edge weights of the network are stored using the compressed row storage (CRS) sparse matrix representation. CRS significantly reduces the memory space complexity of the plugin compared to the conventional dense, two dimensional array representation while it still enables efficient reading of the matrix entries. As elements of the overlap matrix are updated frequently and new values are read only a few times during the layout optimization, computation of the overlaps are performed on-the-fly, when they are needed without significant performance loss.

To reduce relative entropy and consequently improve the quality of the network visualization besides position standard deviation and normalization (height) of the above mentioned probability distributions can be also optimized in separate calculation tasks. These optimization steps were implemented as Newton-Raphson iterations on the first derivative of the functions describing the relation between relative entropy and the optimized parameter.

To highlight the structural characteristics of the network determined by mutual neighbors of the nodes, a special option to raise the adjacency matrix to the second power before starting calculations is also implemented in EntOptLayout.

3.1.2 Validation of the EntOptLayout software

The EntOptLayout algorithm was validated using both normal and diseased or perturbed versions of human and yeast interactomes and benchmark networks. Human validation datasets included a full (Interactome3D), an Alzheimer's diseases related and a human immunodeficiency virus (HIV) related protein-protein interaction network, a biological pathway network (Reactome) and a cancer signaling network. One yeast protein-protein interaction network was analyzed both in its normal and heat shocked state and also a Saccharomyces cerevisiae genetic interaction network was investigated.

It was observed that using EntOptLayout after preprocessing of the layout by Cystoscape's default Prefuse force-directed algorithm could result in better visualizations in terms of normalized information loss values compared to the case when EntOptLayout is applied alone.

On the Interactome3D protein-protein interaction network it was demonstrated that raising the adjacency matrix to the second power before the actual visualization could considerably reduce the normalized information loss both for layouts created by the so far best-performing Prefuse force-directed algorithm and those post-processed by EntOptLayout. In case of EntOptLayout the reduction was more substantial.

According to marked improvements in normalized information loss sequential position and width optimization of the studied biological networks using EntOptLayout consistently resulted in a better layout compared to the currently available network visualization algorithms. As shown in Figure 1 in case of the Interactome3D protein-protein interaction network the visualization by EntOptLayout after preprocessing by the Prefuse force-directed algorithm and using the second power of the adjacency matrix option yielded a much lower relative entropy value (D = 0.077) compared to the layouts prepared by either the spring-embedded (D = 0.294), the EClerize (D = 0.353) or the Prefuse force-directed algorithm alone (D = 0.299).

When visualizing synthetic graphs generated with predefined number and overlap of the modules, it was clearly demonstrated that EntOptLayout with the second power of adjacency matrix option could separate modules much better than the best currently available algorithm (Figure 2). This improvement was also reflected by the normalized information loss values.

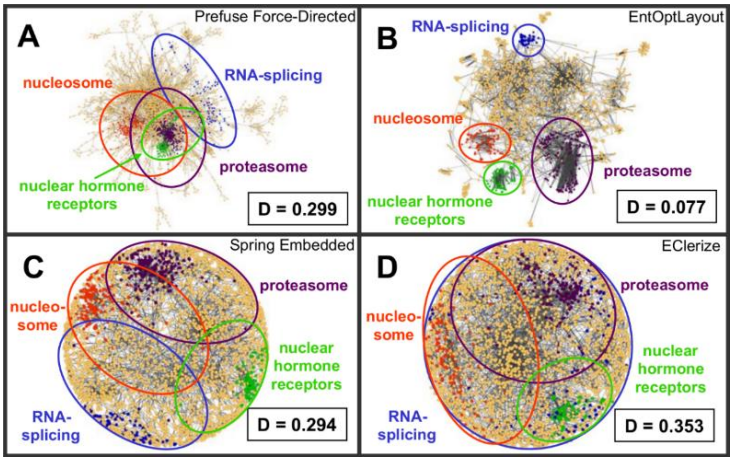

Figure 1 Visualization of the Interactome3D protein-protein interaction network by the Prefuse force-directed (A), the EntOptLayout (B), the spring-embedded (C) and the EClerize (D) algorithms. Normalized information loss (D value) was indicated for each layout.

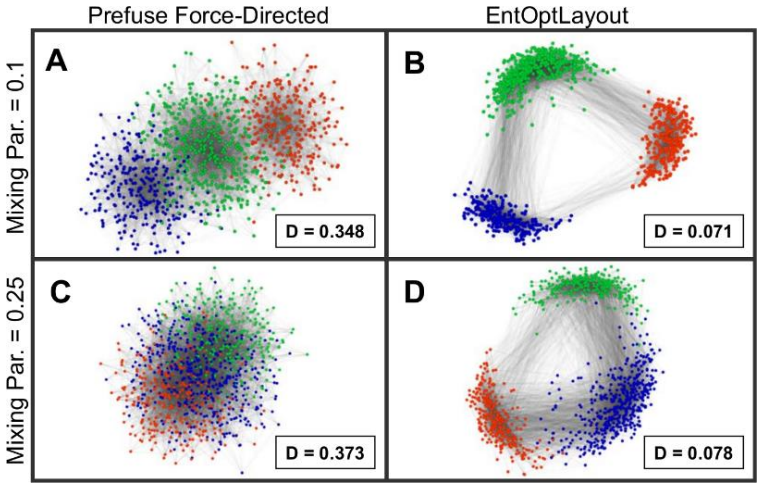

Figure 2 Generated networks with mixing parameter of 0.1 (A, B) and 0.25 (C, D) of the predefined modules (blue, green and red) visualized by the Prefuse force-directed (A, C) and the EntOptLayout (B, D) algorithms. Normalized information loss (D value) was indicated for each layout.

3.2 Software characteristics of the miRNAtarget software

A software with a user-friendly, web-based graphical user interface (https://mirnatarget.com) called miRNAtarget was implemented in C++ programming language for the prediction of the most relevant common targets of differentially expressed microRNAs. MiRNAtarget constructs a microRNA-target interaction network by combining data from experimentally validated, manually curated (miRTarBase) and predicted (microRNA.org, miRDB) microRNA-target interaction databases. In this generated microRNA-target interaction network target hubs (i.e. target nodes with the greatest degree or node strength) are identified as mediators with the highest probability to be regulated by differentially expressed microRNAs.

Target hubs predicted by the miRNAtarget software were experimentally validated in multiple projects including two studies to investigate the direct myocardial effect of hypercholesterolemia and sensory neuropathy detailed in the next two chapters.

3.3 Hypercholesterolemia-induced myocardial dysfunction

Cardiac microRNA fingerprint of normo- and hypercholesterolemic rats were analyzed by the miRNAtarget software and predicted targets were experimentally validated to elucidate the molecular pathomechanism behind the primary myocardial dysfunction associated to hypercholesterolemia.

3.3.1 Differentially expressed microRNAs

After demonstrating the presence of hypercholesterolemia and myocardial dysfunction in Wistar rats (*Rattus norvegicus*) fed by cholesterol-enriched diet, cardiac microRNA expression profiles of treated and control rats were assessed by DNA microarray technique in a previous study. Out of the 350 measured and 120 detectable microRNAs 10 and 47 microRNAs showed downregulation and upregulation, respectively (Figure 3).

3.3.2 Predicted microRNA targets

The microRNA-target interaction network constructed by miRNAtarget from differentially expressed microRNAs and their predicted targets is shown in Figure 3A. 11 targets were predicted to have a degree of at least 4 out of which 4 were selected for experimental validation based on review of the relevant literature.

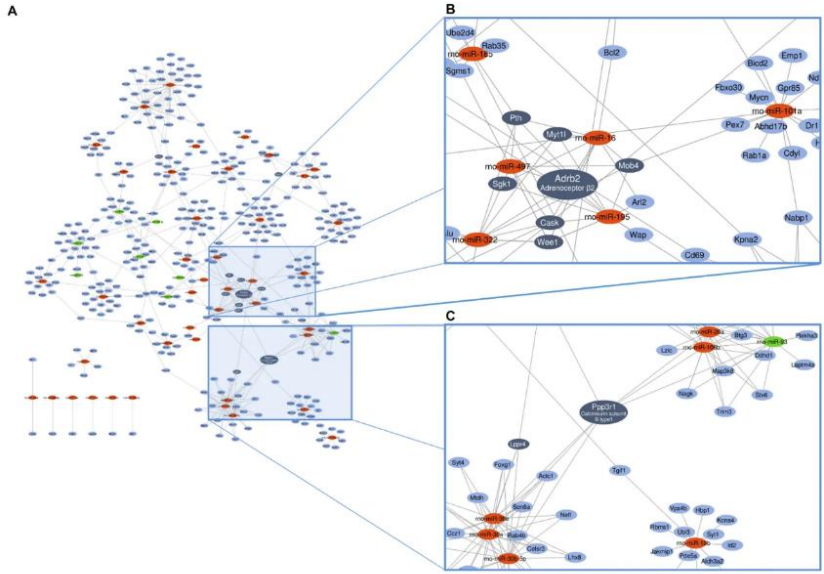

Figure 3 EntOptLayout visualization of the microRNA-target interaction network of the down- (green) and upregulated (red) microRNAs and their predicted targets (blue) in the rat model of the hypercholesterolemia-induced myocardial dysfunction. Target hubs are indicated by dark blue color (A). Four target hubs (Adrb2, Ppp3r1, Cask and Sgk1) selected for validation are highlighted in panel B and C.

The four candidate targets were beta-2 adrenergic receptor (Adrb2), calcineurin B type 1 (Ppp3r1), calcium/calmodulin-dependent serine protein kinase (Cask) and serum/glucocorticoid regulated kinase 1 (Sgk1).

3.3.3 Target validation

Predicted downregulation of Adrb2 in the hypercholesterolemic hearts compared to the control group was successfully validated both on the mRNA and the protein levels, by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot measurements, respectively. Although, the expected downregulation of calcineurin B type 1 could not be observed on the mRNA (Ppp3r1) level, the protein product (CNB1) of this gene was significantly downregulated. In case of Cask we could not validate our

predictions. Contrary to the data in the Human Protein Atlas on the cardiac expression of the human orthologue of Sgk1, its expression on the mRNA level was not detected in our rat heart samples. Direct interaction between Adrb2 and two selected microRNAs, namely rno-miR-195 and rno-miR-322, was also demonstrated by microRNA-luciferase reporter assay.

3.4 Sensory neuropathy-induced myocardial dysfunction

Similarly to the investigation of hypercholesterolemia-induced myocardial dysfunction an unbiased, microRNA omics based study was conducted to unravel the role of microRNAs in the development of diastolic dysfunction related to sensory neuropathy, and miRNAtarget was further validated on the resulting dataset.

3.4.1 Differentially expressed microRNAs

Sensory neuropathy and consequent diastolic dysfunction was successfully induced by systemic capsaicin treatment in male Wistar rats as a model for the neurological and related cardiac consequences of diabetes mellitus. By DNA microarray measurement performed on the rat hearts expression of 257 microRNAs was detected and in case of 8 microRNAs either significant differential expression or an absolute binary logarithm of fold change (log2FC) above 0.6 was demonstrated.

3.4.2 Predicted microRNA targets

With the use of miRNAtarget software 15 microRNA targets with a degree of at least 3 was identified in the predicted microRNA-target interaction network. Out of these 15 predicted targets insulin-like growth factor 1 (Igf1), solute carrier family 2 member 12 (Slc2a12), eukaryotic translation initiation factor 4E (Eif4e) and unc-51 like autophagy activating kinase 2 (Ulk2) were selected for experimental validation based on available literature data indicating their effect on myocardial function or their role in the pathogenesis of diabetes.

3.4.3 Target validation

In case of all four selected targets, namely Igf1, Slc2a12, Eif4e and Ulk2, the *in silico* predicted upregulation was observed even at the mRNA level by qRT-PCR.

4 Conclusions

We developed and successfully validated two user-friendly software tools, namely EntOptLayout and miRNAtarget for the unbiased, network theoretic analysis of omics datasets to identify potential molecular drug targets. We conclude that the present thesis is based on the following novel findings:

1. By utilizing the principle of relative entropy optimization and the novel approach of raising the adjacency matrix to the second power the EntOptLayout plugin performed markedly better in the spatial separation of functional modules of biological networks compared to the currently available best-performing software tools, this way facilitating the identification of key mediators even by visual inspection of the network layout.
2. Normalized information loss values calculated by EntOptLayout for the objective quantification of the quality of the network arrangements, also demonstrated that visualizations produced by EntOptLayout are superior than the ones assessed by traditionally best-performing algorithms.
3. The miRNAtarget software, which we made available also as a web based tool, for improved coverage and quality utilizes microRNA-target interaction data from multiple experimentally validated and predicted databases to construct and analyze a microRNA-target interaction network. MicroRNA-target hubs with the highest node degree, identified by miRNAtarget as the most relevant mediators, were successfully validated by multiple experimental approaches in two animal models of cardiovascular comorbidities, providing evidence for the utility of miRNAtarget.
4. In addition to the validation of miRNAtarget, by the unbiased, omics based investigation of the above two comorbidity models we first identified and validated potential new drug targets for the treatment of myocardial dysfunction induced by hypercholesterolemia (Adrb2, Ppp3r1) and sensory neuropathy (Igf1, Slc2a12, Eif4e, Ulk2).

5 List of own publications

5.1 Own publications involved in the current thesis

1. Ágg B, Baranyai T, Makkos A, Veto B, Faragó N, Zvara Á, Giricz Z, Veres D V., Csermely P, Arányi T, Puskás LG, Varga Z V., Ferdinandy P. (2018) MicroRNA interactome analysis predicts post-transcriptional regulation of ADRB2 and PPP3R1 in the hypercholesterolemic myocardium. Sci Rep, 8: 10134. [IF: 4.011]
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