In Silico Analysis of Differential Gene Expression in Three Common Rat Models of Diastolic Dysfunction

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Standard therapies for heart failure with preserved ejection fraction (HFpEF) have been unsuccessful, demonstrating that the contribution of the underlying diastolic dysfunction pathophysiology differs from that of systolic dysfunction in heart failure and currently is far from being understood. Complicating the investigation of HFpEF is the contribution of several comorbidities. Here, we selected three established rat models of diastolic dysfunction defined by three major risk factors associated with HFpEF and researched their commonalities and differences. The top differentially expressed genes in the left ventricle of Dahl salt sensitive (Dahl/SS), spontaneous hypertensive heart failure (SHHF), and diabetes 1 induced HFpEF models were derived from published data in Gene Expression Omnibus and used for a comprehensive interpretation of the underlying pathophysiological context of each model. The diversity of the underlying transcriptomic of the heart of each model is clearly observed by the different panel of top regulated genes: the diabetic model has 20 genes in common with the Dahl/SS and 15 with the SHHF models. Advanced analytics performed in Ingenuity Pathway Analysis (IPA®) revealed that Dahl/SS heart tissue transcripts triggered by upstream regulators lead to dilated cardiomyopathy, hypertrophy of heart, arrhythmia, and failure of heart. In the heart of SHHF, a total of 26 genes were closely linked to cardiovascular disease including cardiotoxicity, pericarditis, ST-elevated myocardial infarction, and dilated cardiomyopathy. IPA Upstream Regulator analyses revealed that protection of cardiomyocytes is hampered by inhibition of the ERBB2 plasma membrane-bound receptor tyrosine kinases. Cardioprotective markers such as natriuretic peptide A (NPPA), heat shock 27 kDa protein 1 (HSPB1), and angiogenin (ANG) were upregulated in the diabetes 1 induced model; however, the model showed a different underlying mechanism with a majority of the regulated genes involved in metabolic disorders. In conclusion, our findings suggest that multiple mechanisms may contribute to diastolic dysfunction and HFpEF, and thus drug therapies may need to be guided more by phenotypic characteristics of the cardiac remodeling events than by the underlying molecular processes.

Keywords: metabolic disease, endothelial and microvascular dysfunction, inflammation, heart failure, hypertension
INTRODUCTION

Comparatively isolated diastolic dysfunction or stiffness of the left ventricle is a chronic pathological condition that evolves into heart failure with preserved ejection fraction (HFrEF). An increasing body of evidence reports that HFrEF is frequent in women and the elderly (1–3). In fact, HFrEF accounts for about half the cases of heart failure (HF) and is the leading cause of hospital admission in patients over 65 years of age. Although individuals with HFrEF exhibit similar mortality rates as those that have heart failure with reduced ejection fraction (HFrEF) or impaired cardiac contractility, there are currently no proven effective medicines for this condition (1, 4–6). Thus, HFrEF is a major unmet medical need and there is an urgency for new therapeutic approaches and strategies that target mechanisms specific for HFrEF. Notably, HFrEF is associated with the co-presence of several comorbidities (7). Particularly among younger persons, HFrEF is associated with the interrelated cardiovascular risk factors of obesity and type II diabetes (8). Given the obesity epidemic that is attributed to the Western diet and lifestyle, and an aging population, it is not surprising then that HFrEF is predicted to be the primary cause of HF within a decade.

A clinical cardiac phenotype like HFrEF is difficult to study due to its complex nature that involves genetic, molecular, and environmental factors. Ultimately, increased cardiac stiffness in HFrEF is thought to arise from loss of nitric oxide signaling due to microvascular inflammation, increased cardiac fibrosis, and a concentric pattern of cardiac hypertrophy that also compromises ventricular chamber size. Several animal models are characterized by diastolic dysfunction and have been used to study HFrEF. However, it is unclear what these different models have in common, or their differences, as far as disease etiology and cardiac remodeling, which could reveal common or fundamental signaling pathways representing potential therapeutic targets. Given the rapid growth of experimental genetic data in repositories, such as Gene Expression Omnibus (GEO), data mining is a powerful approach to aid in deciphering genetic events that drive the detrimental cardiac remodeling in three rodent models with diastolic dysfunction, the Dahl Salt Sensitive rat (Dahl/SS), the spontaneously hypertensive rat (SHRF/N), and the type I diabetic rat.

Our in silico investigation aimed towards identifying novel pathological pathways in the heart that are relevant for the development of HFrEF. In our study, we make use of public datasets, to perform top-notch biostatistics and bioinformatics that aid in deciphering genetic events that drive the detrimental cardiac remodeling in three rodent models with diastolic dysfunction, the Dahl salt sensitive rat (Dahl/SS), the spontaneously hypertensive heart failure (SHHF) rat, and the type I diabetic rat.

METHODS

Geo Search

GEO search for microarray data from heart in three animal models with underlying diastolic dysfunction: the Dahl Salt Sensitive (GSE66617), the Spontaneous Hypertensive Heart Failure (GSE2876), and the Streptozotocin/Diabetes1 (GSE6880). The total number of samples of each dataset was n = 28, 10 and 6 for Dahl SS, SHHF, and diabetic, respectively.

Rat Models Selected

Dahl Salt Sensitive (SS) rats were fed either a 0.3% NaCl (normal salt, NS, control group) or with 8% NaCl (high salt, HS, heart failure group) diet until evidence of left ventricular dysfunction (9). The authors extracted total RNA from left ventricular samples at the stage of heart failure using mirVana (Applied Biosystems) according to the manufacturer’s instructions (9). Three samples from each group were pooled. cDNA and Amino Allyl aRNA was synthesized by Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion). CyeDye Coupling and fragmentation were performed according to the manufacturer’s protocol (TORAY Industries, Inc, Tokyo, Japan). Samples were then hybridized for 16 h at 37°C on a Rat Oligo chip 20 K (TORAY Industries, Inc, Tokyo, Japan). Scanning was performed on a 3D-Gene Scanner (TORAY Industries Inc., Tokyo, Japan).

Spontaneously Hypertensive and Heart Failure-prone (SHHF) rats were obtained by backcrossing a Kōtsky obese rat to a Spontaneously Hypertensive Rat (SHR/N) (10). Controls were 4 months old lean rats and heart failure rats were 10 months old obese rats (10). Total RNA was extracted with TRIzol (GIBCO) according to the manufacturer’s protocol and reverse transcribed with Superscript enzyme (Invitrogen) and labelling the cDNA with 33P. Purification of cDNA was performed using NucleoSpin Extract (Macherey-Nagel). The kit Array-Advantage GF (Ambion) was used to hybridize the samples into pan-genomic macroarray nylon membranes (RZPD, Berlin), which were exposed for 24 h and scanned with a Scan Typhoon 9400 (resolution at 50 μm per pixel).

Diabetic rats were induced with streptozotocin injection at 8 weeks, which led to a model of insulin-deficient type 1 diabetes (11). Rats were sacrificed at 12 weeks (so diabetic for 4 weeks) (11). RNA was extracted with TRIzol (GIBCO) and cleaned with RNaseq total RNA mini kit (Qiagen). Superscript II (Life Technologies) was used for first strand cDNA synthesis. Subsequent steps necessary to hybridize the samples on Affymetrix RAE 230A microarrays were performed according to the Affymetrix protocol. Arrays were scanned on an Hewlett-Packard Gene Array scanner.

Microarray Data Analysis

GEO2R has been used as a base for the analysis of the Dahl/SS dataset, whereas the Limma package was used to analyze the Diabetic and SHHF model. Distribution of value data for the Samples used was calculated (data not shown) and median-centered values indicated that the data were normalized and cross-comparable. Fold-changes per group were calculated using average Disease/Control. Statistical significance of differences in gene expression was assessed using the nonparametric Wilcoxon signed-rank test and the log2-transformed data.

The differentially expressed genes (DEGs) with fold-change (FC) >2 and < −2 and adjusted p-value < 0.05 have been selected for Dahl/SS and SHHF, whereas FC >1.5 and FC < −1.5 was chosen for Diabetic 1 due to the high level of confidence [false discovery rate (FDR) = 0, (Figure S1)] at p-values < 0.05.

The Benjamini and Hochberg false discovery rate method was selected by default because it is the most commonly used adjustment for microarray data and provides a good balance between...
discovery of statistically significant genes and limitation of false positives.

Gene Set Enrichment Analysis (GSEA) was performed for each of the three datasets using QIAGEN’s Ingenuity Pathway Core Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). The Ingenuity Knowledge Base was used as reference set and both direct and indirect experimentally confirmed relationships from humans and rodents. Z-scores of >2 or < −2 were considered significant. Details about the statistics used can be found at the Ingenuity website: http://www.ingenuity.com/products/ipa/#/tab-resources.

Genes have been mapped in IPA for further analysis (see Table 1).

### RESULTS

#### Animal Models of HFpEF

Our GEO search for published microarray data on hearts from animal models of diastolic dysfunction was narrowed down to the Dahl/SS, streptozotocin-induced diabetic, and SHHF rat models. Table 1 shows the overall number of DEGs found for each animal model. The top 250 DEGs (genes with the smallest *p*-value) calculated with GEO2R indicated that the diabetic model has 20 genes in common with the Dahl/SS and 15 with the SHHF models (Table 2). Given the high cumulative number of significant calls for the diabetic rat model (Figure S1) we included up to 1,139 genes with a local FDR (false discovery rate) of 0.

#### Cardiac Genes Assessed by GSEA

IPA generated annotations were derived for the DEGs in each of the datasets. Among the three animal models we selected, only the Dahl/SS and SHHF models showed that over-represented genes (*n* = 25 and *n* = 26, respectively) determined by DEG analysis are strongly associated with cardiovascular diseases. The cardiovascular disease phenotypes of these two models appear to have different gene datasets as none of the DEGs identified are in common (Table 3). SHHF with 17 genes is also associated with metabolic disease, whereas the diabetic rat is solely associated with metabolic disease with 175 genes (Table 4). These two datasets appear to have in common phosphoinositide-3-kinase interacting protein 1 (*PIK3IP1*), a negative regulator of PI3K and physiological, but not pathological cardiac hypertrophy (12).

#### Pathway Analysis and Pathophysiological Mechanisms

The contribution of every DEGs to the development of diastolic dysfunction was calculated in the context of molecular network and biological progresses using the algorithms described in the Materials and Methods section.

##### Dahl/SS

The biological activities occurring in the heart tissue were identified using the IPA Upstream Regulator analytic on the gene expression changes we observed in the dataset. The analysis inferred the likely activation states of 5 transcription regulators given by our DEG dataset. Hence, based on the comparison of the change of direction of the DEGs (i.e., expression in the Dahl/SS model to control) and what is known from the literature we found that the transcription regulators *TGFBI*, *RETNLB*, *TNF*, *INHBA*, and *IL17RA* (z-score 2.4, 2.2, 2.1, 2.0, 2.0, respectively) were predicted to be activated (Figure 1). A further examination of the underlying biological activities prompted by our dataset from the hearts of Dahl/SS rats revealed biological activities such as dilated cardiomyopathy, hypertrophy of heart, arrhythmia, and failure of heart (Figure 2).

##### SHHF

A total of 186 DEGs were observed. Of the 78 significantly upregulated genes, 43 fell into the rather nondescript category of “other”, followed by 10 in the second largest group transcription regulator. Of the 108 downregulated genes, 61 were characterized as “other”, 17 as enzyme, and 6 as transcription regulator. Differently from the Dahl/SS, the SHHF model did not show significant inferred activation of upstream transcription regulators, apart from a moderate activation (z-score 0.3) of *NUPR1*. In this model, the *ERBB2* upstream regulator was predicted to be inhibited (z-score −2.2). As shown in Figure 3, ERBB2 is a plasma membrane-bound receptor tyrosine kinases that is involved in the protection of cardiac myocytes, which is illustrated by the cardiotoxicity of ERBB2-targeted cancer therapies (13). Out of the 250 DEGs determined in the SHHF heart tissue, 26 genes were linked to cardiovascular disease including cardiotoxicity, pericarditis, ST-elevated myocardial infarction, and dilated cardiomyopathy. A list of these genes is provided in Table 3.

##### Diabetic 1

The diabetic rat model resulted in the highest number of DEGs (see Table 1) consistent with a FDR = 0. A total of 18 (9 activated and 9 decreased) DEGs were identified as in common (Table 3). SHHF with 17 genes is also associated with metabolic disease, whereas the diabetic rat is solely associated with metabolic disease with 175 genes (Table 4). These two datasets appear to have in common phosphoinositide-3-kinase interacting protein 1 (*PIK3IP1*), a negative regulator of PI3K and physiological, but not pathological cardiac hypertrophy (12).
inhibited) significant upstream transcriptional regulators that can explain the observed gene expression profile were identified. A list of these genes is displayed in Table 5. Not surprisingly, given the metabolic underpinnings of the model, most (110) of the 558 genes that were upregulated code for enzymes. Other groupings include 30 kinases, 20 G-protein-coupled receptors, 18 ion channels, and 6 growth factors. Upregulated as well is NPPA encoding for atrial natriuretic peptide (ANP), a biomarker for cardiac hypertrophy and heart failure (Figure 4). Two genes that code for recently proposed novel biomarkers for HFpEF were also upregulated: HSPB1, which codes for cardioprotective heat shock protein 27 (hsp27) (14, 15) and ANG which codes for angiogenin, a protein important for vascularization (16).

**DISCUSSION**

In our study, we selected three established rat models of diastolic dysfunction that are defined by three major risk factors associated with HFpEF. Remarkably, we found that the cardiac transcriptomics
of the models demonstrated little commonality, either in differentially regulated genes or specific biological functions. Overall, our findings highlight the case that divergent molecular processes may underpin a shared phenotype.

Diastolic dysfunction is primarily characterized by stiffening of the left ventricular (LV) tissue, which contributes to impaired relaxation and filling of the ventricle. The specific mechanisms leading to myocardial stiffening are undetermined, but broadly LV stiffness has active and passive components. Passive stiffness describes the inherent stretchiness of the myocardium and is governed by the pliability of both the extracellular matrix and the contractile units. The latter is set by the giant sarcomeric filament, titin, which also determines the elastic recoil of the myocardium, thereby contributing in a major way to LV filling via diastolic suction (17). Evidence indicates that abnormal high-energy phosphate metabolism, specifically increased free cytosolic ADP and enhanced AMP catabolism, may contribute to passive myocardial diastolic stiffness as well (18, 19).

Active stiffness, or reduced relaxation, of the LV tissue depends on Ca$^{2+}$ homeostasis (20). There are two interrelated aspects of intracellular Ca$^{2+}$ handling that determine diastolic function; the rate of cytosolic Ca$^{2+}$ decline following its release from the sarcoplasmic reticulum (SR), and the resting or end-diastolic Ca$^{2+}$ concentration (21). These parameters are set by the rate of Ca$^{2+}$ removal from the cell via the sodium calcium exchanger (NCX) and reuptake of Ca$^{2+}$ into stores by SERCA2, in addition to Ca$^{2+}$ leak from the SR. We did not observe any changes in the gene expression of calcium handling proteins (SERCA2, NCX, PLN, or CASQ2) in any of the three models of diastolic dysfunction. Of course, our approach does not address the possibility that in any of the models of diastolic dysfunction the activities of the Ca$^{2+}$ handling protein are negatively affected by posttranslational modification or change in their environment.

**Figure 1** displays the targets of TGFB1 that are differentially expressed in the Dahl/SS dataset. TGFB1 plays a central role in hypertension-induced cardiac hypertrophy and fibrosis (22,
### Table 4: DEGs associated with metabolic disease

| Expr Log Ratio | Symbol   | Location       | Type(s)                      | Expr Log Ratio | Symbol   | Location       | Type(s)                      |
|----------------|----------|----------------|------------------------------|----------------|----------|----------------|------------------------------|
| -0.99          | AGER     | Plasma Membrane | Transmembrane receptor      | 1.136          | ABAT     | Cytoplasm      | Enzyme                      |
| -1.454         | ARSA     | Cytoplasm      | Enzyme                      | -0.602         | ABCD8    | Plasma Membrane | Transporter                  |
| 1.534          | ARSB     | Cytoplasm      | Enzyme                      | 0.92           | ABOG8    | Plasma Membrane | Transporter                  |
| -1.764         | C1S      | Extracellular Space | Peptidase                  | 0.629           | ACAA2    | Cytoplasm      | Enzyme                      |
| -1.194         | CXCL16   | Extracellular Space | Cytokine                    | -0.776         | ACAI2    | Cytoplasm      | Enzyme                      |
| -1.621         | DCX      | Cytoplasm      | Other                        | -0.697         | ADAM10   | Plasma Membrane | Peptidase                    |
| -1.697         | LIG1     | Nucleus        | Enzyme                      | -1.219         | ADRA1B   | Plasma Membrane | G-protein coupled receptor   |
| -1.204         | NAT8B    | Cytoplasm      | Enzyme                      | -3.176         | ADRA1D   | Plasma Membrane | G-protein coupled receptor   |
| -1.234         | PDK1     | Cytoplasm      | Kinase                       | -0.691         | AKT1     | Cytoplasm      | Kinase                       |
| 1.444          | PK2IP1   | Cytoplasm      | Other                        | -1.784         | ALDH1A1  | Cytoplasm      | Enzyme                      |
| -1.668         | POLB     | Nucleus        | Enzyme                      | -1.459         | ALDH1L1  | Cytoplasm      | Enzyme                      |
| -1.381         | PRNP     | Plasma Membrane | Other                       | 2.492          | ALOX15   | Cytoplasm      | Enzyme                      |
| -1.427         | PSEN1    | Plasma Membrane | Peptidase                   | 2.875          | AMY2B    | Extracellular Space | Enzyme                  |
| 1.257          | PTGS1    | Cytoplasm      | Enzyme                      | -1.363         | APLN     | Extracellular Space | Other                  |
| -1.237         | SNRNP70  | Nucleus        | Other                        | -1.284         | APOBEC1  | Cytoplasm      | Enzyme                      |
| 1.267          | SV2A     | Cytoplasm      | Transporter                  | -2.127         | APB1     | Cytoplasm      | Enzyme                      |
| -2.219         | TUBA1C   | Cytoplasm      | Other                        | -0.711         | ATP1A2   | Plasma Membrane | Transporter                  |
| -1.378         | UQCC2    | Cytoplasm      | Other                        | 2.899          | ATP1A4   | Plasma Membrane | Transporter                  |
|                |          |                |                              | -1.124         | ATP7A    | Plasma Membrane | Transporter                  |
|                |          |                |                              | -2.395         | AVPR2    | Plasma Membrane | G-protein coupled receptor   |
| 1.312          | BCL2L1   | Cytoplasm      | Other                        | 0.848          | BMP2     | Extracellular Space | Growth factor                  |
| 1.046          | BRCA1    | Nucleus        | Transcription regulator      | -0.927         | CA4      | Plasma Membrane | Enzyme                      |
| 1.386          | CACNA1B  | Plasma Membrane | Ion channel                 | 1.014          | CACNA1S  | Plasma Membrane | Ion channel                 |
| -2.783         | CASP8    | Nucleus        | Peptidase                    | 0.704          | CAT      | Cytoplasm      | Enzyme                      |
|                |          |                |                              | -0.717         | CAV1     | Plasma Membrane | Transmembrane receptor       |
| 0.795          | CBLB     | Nucleus        | Enzyme                      | 0.951          | Ccl2     | Extracellular Space | Cytokine                  |
| -0.861         | CCNB1    | Cytoplasm      | Kinase                       | -1.161         | CCND1    | Nucleus        | Transcription regulator      |
| -1.261         | CCND2    | Nucleus        | Other                        | -1.099         | CD44     | Plasma Membrane | Other                      |
| -0.861         | CCNB1    | Cytoplasm      | Kinase                       | -1.914         | CDKN2B   | Nucleus        | Transcription regulator      |

Continued
### Table 4 (Continued)

| Expr Log Ratio | Symbol | Location | Type(s)          |
|---------------|--------|----------|------------------|
| **SHHF**      |        |          |                  |
| 0.879         | CEBPD  | Nucleus  | Transcription regulator |
| -1.19         | CEL    | Extracellular Space | Enzyme |
| -1.443        | CHRNA5 | Plasma Membrane | Transmembrane receptor |
| 2.334         | CHRNA8 | Plasma Membrane | Transmembrane receptor |
| 3.049         | CNR1   | Plasma Membrane | G-protein coupled receptor |
| -1.606        | COL18A1| Extracellular Space | Other |
| -1.411        | COL1A1 | Extracellular Space | Other |
| -1.683        | COL1A2 | Extracellular Space | Other |
| -1.979        | COL2A1 | Extracellular Space | Other |
| -0.757        | COL4A1 | Extracellular Space | Other |
| -0.879        | COL4A2 | Extracellular Space | Other |
| -1.037        | COL5A1 | Extracellular Space | Other |
| -0.883        | COL5A2 | Extracellular Space | Other |
| -1.291        | COL5A3 | Extracellular Space | Other |
| -0.693        | COL6A1 | Extracellular Space | Other |
| -1.026        | COL6A3 | Extracellular Space | Other |
| -1.023        | COL8A1 | Extracellular Space | Other |
| 0.586         | CPT1A  | Cytoplasm | Enzyme |
| 1.683         | CTRP   | Extracellular Space | Other |
| 0.823         | CST3   | Extracellular Space | Other |
| -2.373        | CTSK   | Cytoplasm | Peptidase |
| -1.26         | CXCL12 | Extracellular Space | Cytokine |
| -0.965        | CYP11A1| Cytoplasm | Enzyme |
| 2.541         | CYP2E1 | Cytoplasm | Enzyme |
| -1.687        | CYP7B1 | Cytoplasm | Enzyme |
| -0.808        | Dclk1  | Cytoplasm | Kinase |
| 1.204         | DECR1  | Cytoplasm | Kinase |
| 0.926         | DKK3   | Cytoplasm | Enzyme |
| -1.807        | DPP4   | Extracellular Space | Cytokine |
| 1.335         | DRD4   | Extracellular Space | Cytokine |
| -0.918        | EIF2AK2| Extracellular Space | Peptidase |
| 1.828         | EIF4EBP1| Extracellular Space | Peptidase |
| -0.766        | EPHA4  | Plasma Membrane | G-protein coupled receptor |
| 1.681         | FABP1  | Plasma Membrane | G-protein coupled receptor |
| -0.902        | FABP5  | Plasma Membrane | G-protein coupled receptor |
| -1.589        | FBN1   | Extracellular Space | Other |
| -1.834        | FBN2   | Extracellular Space | Other |
| 0.68          | FDFT1  | Cytoplasm | Enzyme |

| **Diabetes 1** |        |          |                  |
|---------------|--------|----------|------------------|
| 0.879         | CEBPD  | Nucleus  | Transcription regulator |
| -1.19         | CEL    | Extracellular Space | Enzyme |
| -1.443        | CHRNA5 | Plasma Membrane | Transmembrane receptor |
| 2.334         | CHRNA8 | Plasma Membrane | Transmembrane receptor |
| 3.049         | CNR1   | Plasma Membrane | G-protein coupled receptor |
| -1.606        | COL18A1| Extracellular Space | Other |
| -1.411        | COL1A1 | Extracellular Space | Other |
| -1.683        | COL1A2 | Extracellular Space | Other |
| -1.979        | COL2A1 | Extracellular Space | Other |
| -0.757        | COL4A1 | Extracellular Space | Other |
| -0.879        | COL4A2 | Extracellular Space | Other |
| -1.037        | COL5A1 | Extracellular Space | Other |
| -0.883        | COL5A2 | Extracellular Space | Other |
| -1.291        | COL5A3 | Extracellular Space | Other |
| -0.693        | COL6A1 | Extracellular Space | Other |
| -1.026        | COL6A3 | Extracellular Space | Other |
| -1.023        | COL8A1 | Extracellular Space | Other |
| 0.586         | CPT1A  | Cytoplasm | Enzyme |
| 1.683         | CTRP   | Extracellular Space | Other |
| 0.823         | CST3   | Extracellular Space | Other |
| -2.373        | CTSK   | Cytoplasm | Peptidase |
| -1.26         | CXCL12 | Extracellular Space | Cytokine |
| -0.965        | CYP11A1| Cytoplasm | Enzyme |
| 2.541         | CYP2E1 | Cytoplasm | Enzyme |
| -1.687        | CYP7B1 | Cytoplasm | Enzyme |
| -0.808        | Dclk1  | Cytoplasm | Kinase |
| 1.204         | DECR1  | Cytoplasm | Kinase |
| 0.926         | DKK3   | Cytoplasm | Enzyme |
| -1.807        | DPP4   | Extracellular Space | Cytokine |
| 1.335         | DRD4   | Extracellular Space | Cytokine |
| -0.918        | EIF2AK2| Extracellular Space | Peptidase |
| 1.828         | EIF4EBP1| Extracellular Space | Peptidase |
| -0.766        | EPHA4  | Plasma Membrane | G-protein coupled receptor |
| 1.681         | FABP1  | Plasma Membrane | G-protein coupled receptor |
| -0.902        | FABP5  | Plasma Membrane | G-protein coupled receptor |
| -1.589        | FBN1   | Extracellular Space | Other |
| -1.834        | FBN2   | Extracellular Space | Other |
| 0.68          | FDFT1  | Cytoplasm | Enzyme |

Continued
| Table 4 | Continued |
|-----------------|-----------------|
| **SHHF** | **Diabetes 1** |
| Expr Log Ratio | Symbol | Location | Type(s) | Expr Log Ratio | Symbol | Location | Type(s) |
| -0.97 | FLNA | Cytoplasm | Other | -0.801 | FYN | Plasma Membrane | Kinase |
| 3.723 | GAL | Extracellular Space | Other | -1.286 | GAP43 | Plasma Membrane | Other |
| 1.627 | G0KR | Nucleus | Other | 1.926 | GDF15 | Extracellular Space | Growth factor |
| -0.697 | GFM1 | Cytoplasm | Translation regulator | 1.799 | GLRB | Plasma Membrane | Ion channel |
| -1.371 | GDP2 | Cytoplasm | Enzyme | 1.401 | GPNMB | Plasma Membrane | Enzyme |
| 0.725 | GUCY1A3 | Cytoplasm | Enzyme | 0.885 | HADHA | Cytoplasm | Enzyme |
| 0.593 | HADHB | Cytoplasm | Enzyme | 1.203 | HBG2 | Cytoplasm | Other |
| -1.033 | HK2 | Cytoplasm | Kinase | 0.846 | HMGCL | Cytoplasm | Enzyme |
| -0.703 | HMGCR | Cytoplasm | Enzyme | 3.441 | HMGCS2 | Cytoplasm | Enzyme |
| -1.702 | HOMER1 | Plasma Membrane | Other | 1.727 | HP | Extracellular Space | Peptidase |
| -1.439 | HRH1 | Plasma Membrane | G-protein coupled receptor | 3.249 | HSPA1L | Cytoplasm | Other |
| -0.714 | HSPB1 | Cytoplasm | Other | 1.882 | HTR1D | Plasma Membrane | G-protein coupled receptor |
| 0.894 | ID1 | Nucleus | Transcription regulator | -0.657 | IGF1 | Extracellular Space | Growth factor |
| 0.675 | IGF1R | Plasma Membrane | Transmembrane receptor | 1.624 | IGFBP3 | Extracellular Space | Other |
| -2.072 | IGFBP5 | Extracellular Space | Other | 0.664 | IL1R1 | Plasma Membrane | Transmembrane receptor |
| 1.38 | IN6K1 | Cytoplasm | Other | 0.95 | INSR | Plasma Membrane | Kinase |
| -1.237 | KCNA2B3 | Plasma Membrane | Ion channel | -0.611 | KCNJ11 | Plasma Membrane | Ion channel |
| 0.764 | KCNK3 | Plasma Membrane | Ion channel | 1.819 | KCTD16 | Plasma Membrane | Other |
| 1.063 | KLF10 | Nucleus | Transcription regulator | -0.613 | KRAS | Cytoplasm | Enzyme |
| Continued | | | |
| SHHF | Diabetes 1 |
|---------------------------------|---------------------------------|
| **Expr Log Ratio** | **Symbol** | **Location** | **Type(s)** | **Expr Log Ratio** | **Symbol** | **Location** | **Type(s)** |
| 2.749 | LEPR | Plasma Membrane | Transmembrane receptor |
| -0.69 | LIEM1 | Plasma Membrane | Other |
| 1.188 | LIPC | Extracellular Space | Enzyme |
| 0.887 | LRP8 | Plasma Membrane | Transmembrane receptor |
| -1.079 | MAGI3 | Cytoplasm | Kinase |
| -1.167 | MAOB | Cytoplasm | Enzyme |
| -0.87 | MED2 | Nucleus | Transcription regulator |
| -0.634 | MGP | Extracellular Space | Other |
| 1.685 | MSA9A | Other | Other |
| -1.838 | MSTN | Extracellular Space | Growth factor |
| 2.404 | NEFH | Cytoplasm | Other |
| -2.808 | NOS2 | Cytoplasm | Enzyme |
| 0.816 | NOTCH4 | Plasma Membrane | Transcription regulator |
| -0.977 | NRAS | Plasma Membrane | Enzyme |
| 1.836 | NTRK1 | Plasma Membrane | Kinase |
| 2.42 | OLR1 | Plasma Membrane | Transmembrane receptor |
| -1.059 | OXCT1 | Cytoplasm | G-protein coupled receptor |
| -1.268 | OPCR1 | Cytoplasm | Enzyme |
| 2.329 | PAK1 | Cytoplasm | Kinase |
| 0.7 | PECAM1 | Plasma Membrane | Other |
| 1.068 | PIK3P1 | Cytoplasm | Other |
| 2.175 | PKL1 | Cytoplasm | Kinase |
| -2.188 | PLPP3 | Plasma Membrane | Phosphatase |
| 0.697 | POR | Cytoplasm | Enzyme |
| -0.754 | PPT2 | Cytoplasm | Enzyme |
| -0.61 | PRKCA | Cytoplasm | Kinase |
| -0.713 | PRKCB | Cytoplasm | Kinase |
| 0.894 | PPM6B | Cytoplasm | Other |
| -1.882 | PTGIS | Cytoplasm | Enzyme |
| -1.791 | QPT | Cytoplasm | Enzyme |
| -1.546 | RAMP3 | Plasma Membrane | Transporter |
| -3.845 | RET | Plasma Membrane | Kinase |
| -0.767 | S100A10 | Cytoplasm | Other |
| 1.12 | S100B | Cytoplasm | Other |
| -0.842 | SERPINE2 | Extracellular Space | Other |
| -0.833 | SERPINF1 | Extracellular Space | Other |
| -0.586 | SER | Nucleus | Phosphatase |
| 2.599 | SLC12A1 | Plasma Membrane | Transporter |
| 2.338 | SLC12A3 | Plasma Membrane | Transporter |

*Continued*
| Expr Log Ratio | Symbol    | Location  | Type(s)          |
|---------------|-----------|-----------|------------------|
| -1.043        | SLC2A4    | Plasma Membrane | Transporter |
| 1.018         | SLC9A1    | Plasma Membrane | Ion channel    |
| -0.882        | SLC2A2A1  | Plasma Membrane | Transporter    |
| 0.834         | SORL1     | Cytoplasm  | Transporter      |
| 1.193         | SCK2      | Nucleus    | Transcription regulator |
| 0.936         | SQSTM1    | Cytoplasm  | Transcription regulator |
| 2.28          | SSTFR2    | Plasma Membrane | G-protein coupled receptor |
| 0.737         | SSTFR3    | Plasma Membrane | G-protein coupled receptor |
| 2.067         | STAR      | Cytoplasm  | Transporter      |
| 0.641         | TANGO2    | Cytoplasm  | Other            |
| 1.49          | TGFBR3    | Plasma Membrane | Kinase         |
| -2.148        | TGM1      | Plasma Membrane | Enzyme         |
| -0.6          | THBD      | Plasma Membrane | Transmembrane receptor |
| 0.734         | THR2      | Nucleus    | Ligand-dependent nuclear receptor |
| 0.828         | TIMP3     | Extracellular Space | Other       |
| -3.057        | TLR7      | Plasma Membrane | Transmembrane receptor |
| -0.685        | TMEM116   | Other     | Other            |
| 0.736         | TNNT2     | Cytoplasm  | Other            |
| -2.696        | TOP2A     | Nucleus    | Enzyme           |
| -0.705        | TPS3      | Nucleus    | Transcription regulator |
| 2.123         | TSPAN5    | Plasma Membrane | Other         |
| -0.83         | TUBA8     | Cytoplasm  | Other            |
| 0.823         | XDH       | Cytoplasm  | Enzyme           |

Green indicates the downregulated gene; red indicates the upregulated gene.
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and consistent with that fact, genes involved in fibrosis/extracellular matrix (ECM) accumulation (TGFB1, POSTN, CTGF, TIMP1) (24) and hypertension-related pathological hypertrophy (NPPA and MYH7) (25–28) are upregulated with hypertension. Interestingly, the Dahl/SS model of heart failure is associated with upregulation of the Wnt signaling inhibitor secreted frizzled-related protein-1 (SFRP1), which protects against ventricular dilation and hypertrophy (29), as well as increased expression of the transporter of large neutral amino acids (SLC7A5). Downstream targets of TGFB1 upregulated in Dahl/SS rats include EGR2 and NUPR1. Based on computational analysis of the transcriptomes at five time points in male murine hearts subjected to transverse aorta banding, the transcription factor EGR2 was recently identified as a new candidate key regulator of cardiovascular-associated genes (30). Nupr1 is a nuclear basic helix-loop-helix protein and transcriptional coregulator that is induced in response to stress in the heart (31).

Recent evidence indicates that Nupr1 activation contributes to cardiac fibrosis, while protecting against autophagy and apoptosis that lead to heart failure (32). Elevated circulating levels of both interleukin (IL)-6 and TNF are observed in HFP EF (33), and in the Dahl/SS model TNF shares several downstream targets with TGFB1, including TIMP1, INHBA, and SFRP1 (Figure 1). The role of INHBA or RETNLB in cardiac remodeling is unreported. The predicted downstream target of RETNLB, THBS4, has been shown to attenuate pressure-overload cardiac fibrosis (34). TNF is also linked to S100A9 and S100A8 expression (both significantly increased in our DEG list), as is IL17RA (Interleukin 17 receptor A). IL17RA has been linked to myocardial collagen metabolism in hypertension-induced diastolic dysfunction and heart failure (35, 36). The "damage-associated molecular patterns" (DAMPs), proteins S100A8/A9 contribute to cardiac fibrosis by activating pro-inflammatory NF-κB signaling in cardiac fibroblasts via the receptor for advanced glycation end products (RAGE) and inducing the expression of multiple chemokines and cytokines (37). NF-κB is predicted to be activated in the Dahl/SS model (z-score of 1.92), as evidenced by the marked upregulation of CTGF, CXCL11, SLC7A5, TIMP1, and CDC25B. TNF is also linked to upregulation of the chemokine CXCL11 and the hormone Apelin, which appear to have conflicting actions on cardiac inflammation (38) and hypertrophy (39), respectively. Interestingly, TNF is linked to upregulation of Purkinje cell protein-4 (PCP4), a putative regulator of calmodulin and Ca²⁺/calmodulin-dependent kinase II (CaMII) signaling, within the His-Purkinje network and contributor to cardiac arrhythmias (40). The role if any played...
by DNER, REG3A, and IRX1 to diastolic dysfunction and cardiac hypertrophy is not reported, although genetic variants of the IRX1 gene were recently reported to contribute to the pathogenesis of congenital heart disease (41). Finally, expression levels of CXCL11 were increased in hearts of Dahl/SS rats fed a high salt diet, which is consistent with the concept that inflammation plays a key role in the pathogenesis of HFpEF. The closely related CXCL9, CXCL10, and CXCL11 are chemoattractants for monocytes and lymphocytes. Accumulating evidence has implicated these chemokines in several cardiovascular diseases, including atherosclerosis, hypertension, cardiac hypertrophy, and heart failure, as well as in transplant coronary artery disease and heart transplant rejection (42). Of note, circulating levels of these chemokines were found to provide additional diagnostic utility beyond NT-pro BNP levels for detecting LV diastolic dysfunction in hypertensive patients (43). In addition, we observed that serum levels of CXCL10 are increased in patients with symptomatic heart failure (44). Although CXCL9, -10, and -11 all bind to CXCR3, there is some evidence that these agonists activate opposing responses due to biased signaling that is a fixture of G protein-coupled receptors (44). Whereas CXCL9/CXCL10/CXCR3 interactions drive effector Th1 polarization, CXCL11/CXCR3 binding seems to induce an immunotolerizing state characterized by polarization into regulatory Tr1 lymphocytes that produce anti-inflammatory IL-10.

For the SHHF dataset, none of the DEGs stood out mechanistically in the context of heart failure. Expression of the gene for frizzled class receptor 5 (FZD5), which plays a role in endothelial cells in angiogenesis (45), was increased, as was the gene for the repressor of the protective, anti-oxidant transcription factor Nrf2, KEAP1 (46).

Unexpectedly, the genes for 9 different collagen types were downregulated in diabetic 1 hearts, although a number of genes involved in ECM remodeling and fibrosis were upregulated, including MMP14 (24), as well as SDC4 and LOX, which together have been linked to myocardial stiffness and diastolic dysfunction (47). PDGFD, which codes for platelet-derived growth factor D, a potent pro-fibrotic cytokine (48), was upregulated. Two signaling components of the pro-fibrotic cytokine TGF-β were upregulated, SMAD3 (49) and TGFBR3 (TGF-β receptor 3), although TGFBR1 (TGF-β receptor 1) was downregulated and the inhibitory SMAD7 was upregulated. However, CAV1 was downregulated. CAV1 encodes for caveolin 1, which may inhibit both TGF-β-dependent and –independent cardiac fibrosis (50). Other relevant pro-fibrotic genes that were upregulated, include: the chemokines CCL2 and CXCL3, linked to fibrosis and inflammation (51–53); pro-inflammatory cytokine IL-3 (54) and the innate immune cytokine IFNB1; and the K+ channel KCNN4, which is linked to angiotensin II-induced cardiac fibroblast proliferation and collagen production (55). The gene for the tyrosine kinase receptor EGFR was also upregulated. EGFR is implicated in cardiac fibrosis and hypertrophy (56–58), as well as heart protection (59). In contrast, the gene for TIMP3, which has anti-fibrotic actions (60), was upregulated.
Several pro-fibrotic genes were downregulated, including: VCAN (Versican) (61); LUM (Lumican), an ECM-localized proteoglycan that binds collagen and is important for fibrosis, is associated with inflammation, and is increased in experimental and clinical heart failure (62); SERPINE2, which is increased in pressure overload- and angiotensin II-induced cardiac remodeling downstream of ERK1/2 signaling and increases collagen deposition (63); and the integrin ITGA6, associated with collagen deposition (64).

Notable genes linked to metabolism were increased in diabetic hearts, including FABP1 and GDF15. The later encodes a heart-derived hormone, which regulates body growth (65). PFKFB3 encodes a pro-glycolytic enzyme that is upregulated in cardiac progenitor cells by diabetes (66). The gene for the leptin receptor (LEPR), which is implicated to both cardiac hypertrophy and cardioprotection in obesity (67), was upregulated. However, a number of genes associated with cardiac hypertrophy and heart failure due to pressure-overload or angiotensin II infusion were downregulated. CCND2 (cyclin D2), which was implicated in cardiac hypertrophy, was downregulated (68), but so was the cyclin-dependent kinase inhibitor CDKN2B. TP53, a major player in the development of systolic heart failure (69, 70), was downregulated, as was PRKCA. Activation of PKCa is implicated in cardiac hypertrophy and heart failure (71–73).

Oxidative stress is a feature of the diabetic heart (74) and several genes associated with inflammation and oxidative stress were upregulated. CAT, encoding the key antioxidant enzyme catalase was upregulated. On the other hand, TXNIP, which inhibits the anti-oxidative function of thioredoxin, was also upregulated. TXNIP is linked to increased oxidative stress, along with fibrosis and arrhythmias in diabetes (75), as well as diabetic cardiomyopathy (76). The thioredoxin system 2 is a major buffer against H2O2 emission from mitochondria (77), and the present

**FIGURE 3** | Significant inhibition of ERBB2 kinase in SHHF. Asterisks (*) indicate that multiple identifiers in the dataset file map to a single gene in the Global Molecular Network. Figure has been generated using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Inc., Cambridge, MA, USA).
Evidence showing that TXNIP is strongly upregulated in the diabetic heart may account for the overall oxidative environment found in the diabetic heart. TSPAN5, encoding a cell surface protein, was upregulated. Evidence was recently reported that endothelial Tspan5- and Tspan17-ADAM10 complexes contribute to inflammation by maintaining VE-cadherin expression and promoting T lymphocyte transmigration (78). Another injury-related gene that was upregulated was BNIP3, which is associated with mitochondrial autophagy and apoptosis and is increased in the heart by pressure overload and various stresses (79, 80).

Notably, a number of protective genes were upregulated in the type 1 diabetic heart. The gene for the Smad corepressor, transforming growth interacting factor (TGIF), was increased, as was BMP2. BMP2 may antagonize BMP4-induced cardiomyocyte hypertrophy and apoptosis (81) and TGFβ-induced fibrosis (82). Three genes linked to inhibition of cardiac hypertrophy were upregulated, viz., DKK3 (Dickkopf-3) (83), FBXO32 (encoding E3 ligase, Fbxo32) (84), and GADD45B, which blocks MKK7-induced JNK activation (85). The anti-apoptotic gene BNIP3, which is associated with mitochondrial autophagy and apoptosis and is increased in the heart after acute MI (91, 92).

Interestingly, we found that there were no genes similarly affected among all three models of diastolic dysfunction, nor between the Dahl SS and SHHF models. However, the type 1 diabetes model shared 20 regulated genes with the Dahl SS model and 15 with the SHHF model. Human diseases traditionally have been differentiated and categorized on the basis of which organ system they primarily affect. An alternate view is now emerging, which emphasizes that different diseases typically have common underlying mechanisms and intermediate pathophenotypes or endo(pheno)types. According to this construct, expression of a specific disease reflects the interplay between relevant endophenotypes and the local organ-based environment. Important examples of such endophenotypes are inflammation, fibrosis, and metabolic dysfunction, which play essential roles in many developing diseases. In this study, we identified the endophenotype networks of three models of diastolic dysfunction and explored their relation to cardiovascular diseases in particular. We identified the subnetworks of the top regulated microarray genes that are playing a role in inflammation, fibrosis, and metabolism. Although our three models of diastolic dysfunction exhibit little overlap in regulated genes, each is still significantly enriched with disease-associated genes. Moreover, they are enriched also with differentially expressed genes linked to cardiovascular disease (risk).

Our findings illustrate as well the observation that HFP EF is a syndrome with multiple extracardiac comorbidities, such as aging, hypertension, and obesity, rather than a disease of clearly defined etiology (93). Pharmacological targeting of HFP EF may need to be informed more by phenotypic characteristics of the cardiac remodeling events that occur than by the underlying molecular processes alone. These phenotypic events may include concentric hypertrophy, myocardial stiffness, fibrosis, endothelial...

### Table 5: Significant upstream transcriptional regulators in the diabetic rat model

| Upstream Regulator | Molecule Type       | Predicted Activation State | Activation z-score | Target molecules in dataset |
|--------------------|---------------------|----------------------------|--------------------|---------------------------|
| RA8L6              | Other               | Inhibited                  | −2.010             | ABAT, AUFK8, BIK, CCNB1, DAPK1, H2AFX, MCM5, MCM7, RFC3, SERPINH1, TGF2, TP53 |
| MAX                | Transcription regulator | Inhibited                | −2.000             | BMP2, CCNB1, CYP1B1, GDF15 |
| HLX                | Transcription regulator | Inhibited                | −2.000             | E2F2AK2, IF35, MX1, E2F6L, USP18 |
| IFNL1              | Cytokine            | Inhibited                  | −2.186             | FBXO32, GADD45B, KLF10, TXNIP |
| MNT                | Transcription regulator | Inhibited                | −2.000             | ALOX15, ASS1, BCL2L1, CASPR8, CAT, Cdx2, CCL22, CYP2E1, DDR2, DPP4, E2F2AK2, ICOBLG, LOC102723996, IFIT1, BLOX, MX1, NOS2, NPY2R, SDC4, SERPINB9, SLC15A3, STAR, TGF1, TXNIP, UBD, USP18 |
| IFNG               | Transcription regulator | Inhibited                | −2.668             | E2F2AK2, IF35, IFNB1, MX1, TPS3, UBE2L6, USP18 |
| IFNA2              | Cytokine            | Inhibited                  | −2.236             | CCND1, CCND3, CC44A, GJ1A, RET |
| PCDH11Y            | Other               | Inhibited                  | −2.236             | CCND1, CCND3, CC44A, GJ1A, RET |
| SPDEF              | Transcription regulator | Activated                | 2.498              | CDH1, COL1A1, COL4A1, COL4A2, COL5A1, COL5A2, COL6A1, COL6A3, DKK3, EGRF, JTG6, LAMC1, PRKCA, SMAD3 |
| KIAA1524           | Other               | Activated                  | 2.359              | C4BPA, CDKN2B, COL6A1, E3N3, GPNMB, LUM, RHOC, SERPIN2, TUBA4A |
| miR-29b-3p         | Mature microRNA     | Activated                  | 2.449              | COL1A2, COL5A2, GAST, LAMC1, MYBL2, TUBB2A |
| CST5               | Other               | Activated                  | 2.584              | ACAT2, AKAP12, AP1M2, CAV1, CD44, DECR1, DRAF1, ELK3, EMPI, EPN3, FER, FHL1, LAMC1, NR2F1, NRP1, P1L3, PRSS8, S100A11, TAF1, TGF1, TSPAN5, VCAN |
| PTGS2              | Enzyme              | Activated                  | 2.425              | BCL2L1, CD2, IFNB1, ITGA6, MCL1, MMP14, PPA1, TP53 |
| SIRTI              | Transcription regulator | Activated                | 2.049              | ATXN10, BNP3, CCNB2, CPT2, PTA1, CPA1, CPA1, HMGCR, U9F1, TP53 |
| KITLG              | Growth factor       | Activated                  | 2.000              | BCL2L1, IL1R1L1, PRKCA, PRKGB |
| FOXO3              | Transcription regulator | Activated                | 2.197              | BNP3, CAT, CCNB2, CDKN2B, EGR4, FBXO32, GADD45B, MX1, TXNIP |
| NEDD9              | Other               | Activated                  | 2.646              | BNP2, BNP3, ELF3, FABP1, GDF15, MMP14, TXNIP |
| POU2F2             | Transcription regulator | Activated                | 2.236              | ALDOLC, KCCN4, MCM7, PFKFB3, SSTR2, VIP |

Orange/blue indicate the predicted activation state, namely activated/inhibited, of the transcriptional regulator.
dysfunction, capillary rarefaction, and abnormal ventricular-arterial coupling, as well as changes in the shape and stiffness of the left atrium.

There are some limitations and caveats to our approach. Changes in gene expression may not necessarily be reflective of changes in protein levels, given the contribution of additional levels of post-transcriptional regulation, including microRNA mediated regulation. Alterations in protein stability and function may occur as well. In addition, in any disease context, changes in gene expression have a temporal and dynamic component, with alterations in expression of any particular gene and its protein levels at a given time point being impacted upon by changes in other network or system components. Nonetheless, global changes in gene expression are reflective of basic underlying pathological processes and offer insight into stress-related responsiveness to the disease process. In addition, there are several other models of HFpEF, such as abdominal aortic constriction and partial nephrectomy. Certainly, it would be of interest to extend our analysis in the future to include these other models. It should be mentioned as well that streptozotocin affects other organ systems, including vascular function; however, HFpEF in many patients is increasingly recognized as a systemic disorder involving vascular inflammation and dysfunction (94).

**Figure 4** | Pathological processes in the diabetic rat model. Asterisks (*) indicate that multiple identifiers in the dataset file map to a single gene in the Global Molecular Network. Figure has been generated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc., Cambridge, MA, USA).
In conclusion, our *in silico* analysis of differential gene expression in three common rat models of diastolic dysfunction highlights the diversity in causality and molecular basis for the impaired cardiac function. A better understanding of the phenotype changes that accompany diastolic dysfunction, such as concentric hypertrophy, and their impact on genetic changes, is required to gain better insight into the disease process. An effective pharmacological approach for diastolic dysfunction and HFpEF may arise from a global strategy based on a better understanding of altered cardiac ultrastructure.

**AUTHOR CONTRIBUTIONS**

RA conceived the study, analyzed the data, designed the figures and tables, and helped write the manuscript. FZ and RB assisted in the analysis of the data and writing of the manuscript. SB and AC helped write the manuscript. GB assisted in the analysis and interpretation of the results, and contributed to the writing and editing of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcvm.2018.00011/full#supplementary-material
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