The gene expression profile of patients with new-onset heart failure reveals important gender-specific differences†

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Aims

We sought to test the hypothesis that inherent biological factors contribute to gender differences in disease pathophysiology of new-onset heart failure (HF), which can be detected from the transcriptome of a single endomyocardial biopsy (EMB).

Methods and results

We analysed samples from male (n = 29) and female patients (n = 14) with idiopathic dilated cardiomyopathy (IDCM) and new-onset HF with U133 Plus 2.0 microarrays (Affymetrix) and significance analysis of microarrays (SAM). There were 35 overexpressed and 16 downregulated transcripts in men vs. women [q < 5%, fold change (FC) > 1.2]. In addition to overexpression of Y-chromosome-related transcripts (n = 18), such as USP9Y (FC > 13.1), DDX3Y (FC > 11.3), RPS4Y1 (FC > 9.9), and EIF1AY (FC > 11.8) in males, there was overexpression of CD24 (FC > 5.6) and KCNK1 (FC > 1.5). In females, XIST was highly overexpressed (FC > 28.9), together with X-linked zinc finger proteins (FC > 1.9) and autosomal genes GATAD1 (FC > 1.6), SLC2A12 (FC > 2.9), and PDE6B (FC > 1.5). Analysis of a public data set of end-stage IDCM (n = 15) resulted in ~85% overlap with our findings.

Conclusion

This is the first study that identified gender-specific transcriptomic differences in new-onset HF. Our findings may offer novel insights into fundamental biological differences in the pathophysiology of HF between sexes and provide a platform for personalized medicine.

Keywords

Heart failure • Transcriptomics • Cardiomyopathy • Gene expression • Gender

Introduction

Gender differences of cardiovascular pathophysiology and disease presentation are a well-known phenomenon described both in animal models1 as well as clinical trials.2–8 Although this phenomenology is well accepted, the underlying biological basis for those differences remains controversial. One suggested explanation for adverse outcomes in females after acute myocardial infarction was the so called Yentl syndrome,9 describing gender-biased treatment response by the physician, which is influenced by different presentation of symptoms in women vs. men.9 While this phenomenon may explain observations in part, many additional conditions and comorbidities warrant consideration.

There is strong evidence for a hormonal component that protects the cardiovascular system from atherosclerotic changes in pre-menopausal women,10 even though female vasculature appears to have stronger predisposition for stress-induced changes of vessel walls.11,12 Further female-specific conditions, such as gestational diabetes and hypothalamic hypoestrogenemia, carry increased risk for ischaemic cardiomyopathy.13
High throughput technology, using gene expression microarrays or genome-wide association studies, has offered molecular insights into gender-specific differences.\(^{14,15}\) In this regard, investigators discovered that even in the normal heart, the female transcriptome is distinct from the male transcriptome,\(^1\) with the majority of differentially expressed genes located on sex chromosomes.\(^1\) Furthermore, analysis of samples from patients with end-stage heart failure (HF) at the time of left-ventricular assist device (LVAD) placement revealed important gender-biased differences in the transcriptome.\(^16\) In the current study, we sought to test the hypothesis that in patients with new-onset HF and idiopathic dilated cardiomyopathy (IDCM), there are gender-specific differences in gene expression that are only found at new-onset HF and that those discrepancies can be divided into a subset that is specifically related to sex and another subset that is related to heart function and therefore may be causative for higher ejection fraction (EF) in women. The reported findings suggest fundamental molecular differences in new-onset HF, and therefore support the notion of a true biological basis as opposed to purely environmental issues. As such, these findings offer insights into future gender-based individualized drug development.

**Methods**

**Patients**

Endomyocardial biopsies (EMBs) were collected from patients referred to the Johns Hopkins Hospital between 1997 and 2006 for evaluation of cardiomyopathy and HF (n = 350).\(^{17,18}\) In addition to a comprehensive history and physical examination, patients underwent right-heart cardiac catheterization and echocardiography.\(^17\) Patients with a history suggestive for ischaemic heart disease or at least two standard risk factors for atherosclerosis were further evaluated with coronary angiography.\(^17\) Blood tests were performed to measure cardiac enzymes, thyroid-function, and antinuclear antibodies.\(^17\)

Four to six biopsy specimens were obtained from each patient for standard histologic staining, Congo red for the identification of amyloidosis, and Prussian blue if haemochromatosis was suspected.\(^17\) If clinical evaluation resulted in more than one potential cause of cardiomyopathy, the single most likely cause was chosen.\(^17\) After this extensive evaluation, IDCM was a diagnosis of exclusion.\(^17\)

In addition to diagnostic biopsies, one EMB was flash frozen and stored in liquid nitrogen for later microarray analysis.\(^{17,18}\) Participants gave written informed consent. Endomyocardial biopsies were obtained from the right-ventricular septum via the right internal jugular vein using an Argon disposable endomyocardial biopsy forceps (Jawz).\(^{17,18}\)

**Selection of patients**

We used microarray data from previously processed samples of the above-described biorepository (IDCM; males: n = 29, females: n = 14).\(^{17,18}\) In the present study, we re-analysed data for gender-specific differences in gene expression at new-onset HF. Then we compared our findings with samples from end-stage HF (n = 15) at the time of heart transplantation (http://cardiogenomics.org).

To evaluate to which extent the primary data set (n = 43) reflects the actual relationship of baseline conditions in men vs. women of a broad population, we analysed clinical data of all patients with IDCM (n = 182) within our biorepository. Finally, to identify the most robust set of gender-specific genes, which is unlikely to be related to heart function, we analysed a subset of samples (males: n = 20, females: n = 12) from the original data set in a case–control fashion, matched for similar EF and left-ventricular internal diastolic diameter (LVIDD).

**Microarray analysis**

EMBs were analysed with the Human Genome U133 Plus 2.0 Array from Affymetrix as presented previously.\(^{18}\) Importantly, no additional step of amplification was performed before hybridization. After normalization with robust multiaarray average (RMA),\(^{15,18}\) microarray data were analysed with significance analysis of microarrays (SAM)\(^19\) to identify gender-specific differences of gene expression. Heatmaps were created with an unsupervised clustering approach based on Euclidean distance in R. GeneGo Inc. identified gender-related genes targeted by pharmacotherapy.

Statistical analysis of baseline parameters was performed with Student’s t-test for numerical and Fisher exact test for categorical variables. Statistical analysis was performed using SigmaStat 3.5.\(^{16}\) Before each parametric test, a normality test (Kolmogorov–Smirnov) was performed to check for data distribution. Significance level was based on two-sided tests, and a P-value < 0.05 was considered as statistically significant.

**Validation with realtime RT–PCR**

Transcriptomic data was validated in a randomly selected subset of samples from our original cohort (males: n = 7, females: n = 7). In order to obtain sufficient starting material for efficient cDNA synthesis, total RNA was amplified with the MessageAmp II aRNA Amplification Kit (Applied Biosystems Inc., CA, USA). First-strand cDNA was synthesized from 100 ng total RNA, using High-Capacity cDNA Reverse-Transcription Kit (Applied Biosystems Inc., CA, USA). TaqMan primers (FAM-labelled) designed for CD2A, KCNK1, and ribosomal 18S RNA as housekeeping gene, were used for quantification with realtime RT–PCR according to manufacturer’s protocol. Data were analysed by threshold cycle (Ct) relative-quantification method.

**Western blot analysis**

Human heart samples were analysed with an anti-GLUT12 primary antibody (Abcam) and a polyclonal anti-GAPDH antibody (Santa Cruz Biotechnologies Inc.).

**Results**

**Clinical data of female vs. male patients with new-onset heart failure and idiopathic dilated cardiomyopathy**

The purpose of this study was to compare male vs. female patients with new-onset HF and IDCM in order to identify gender-related cardiac transcriptomic differences. Table 1 contains baseline conditions of both cohorts. There was a non-statistical trend towards older age in female patients relative to males. Although, New York Heart Association (NYHA) classification was similar in both groups, left-ventricular ejection fraction (LVEF) was 30 ± 4% in females vs. 20 ± 2% in males (P = 0.009).\(^{20}\) Left-ventricular internal diastolic diameter was 5.2 ± 1 in females vs. 6.5 ± 1 in male patients (P < 0.01).

Haemodynamic and structural parameters, such as left-ventricular internal systolic dimension, right-ventricular systolic pressure, right-ventricular diastolic pressure, systolic and diastolic pressure, and wall thickness were also significantly different in both groups (Table 1).
Transcriptomic differences in new-onset heart failure

We used transcriptomic analysis of EMBs to identify gender-specific differences in gene expression in new-onset HF. There were 35 overexpressed (Figures 1 and 2, see Supplementary material online, Table S1) and 16 downregulated (Figures 1 and 2, Supplementary material online, Table S1) transcripts in male vs. female patients [q-value < 5%, fold change (FC) > 1.2]. While most overexpressed transcripts in males were sex chromosome related, such as USP9Y (FC > 13.1), DDX3Y (FC > 11.3), RPS4Y1 (FC > 9.9), and EIF1AY (FC > 11.8), there was overexpression of KCNK1 (FC > 1.5) and PLEKHAB (FC > 1.8), located on chromosome 1 and 12, respectively (Figure 3A and B). Furthermore, CD24 located on the Y-chromosome, chromosome 6 and 15 (Figure 3A) was overexpressed in males (FC > 5.6). The X-chromosome inactivator (XIST) was highly overexpressed (FC > 28.9) in females, together with X-linked zinc finger proteins (FC > 1.9), GATAD1 zinc finger domain (FC > 1.6) located on chromosome 7, SLC2A12 located on chromosome 6 (FC > 1.9), and PDE6B (FC > 1.5) located on chromosome 4 (Figure 3B). Validation by realtime RT–PCR and western blot confirmed our results from microarray analysis, with overexpression of CD24 and KCNK1 in males (Figure 4), and 2.9-fold overexpression of SLC2A12 in females (Figure 5), respectively.

Transcriptomic changes during disease progression

Finally, we sought to evaluate the preservation of gender-specific patterns in gene expression during disease progression and analysed samples that were obtained from patients with end-stage HF at the time of heart transplant. To establish that new-onset and end-stage samples were representative for different stages of HF, expression level of canonical HF-specific genes were analysed. As expected, we observed expression level of CTGF, MYH7, NPPA, and NPPB were significantly increased in end-stage vs. new-onset patients (P < 0.001 for all genes, two-way ANOVA), whereas no difference was detected between male and female patients (Figure 6). There were only 24 differentially expressed genes in male vs. female patients, of which 85% were already detected in new-onset HF. Genes that overlapped between both analyses included: USP9Y, DDX3Y, RPS4Y1, EIF1AY, CD24, and XIST. However, from a total of 22 overexpressed genes in males with new-onset HF, 13 (59%) were not detected any longer in end-stage HF (see Supplementary material online, Table S1). Moreover, of 11 downregulated genes, 9 (82%) were not present in advanced HF (see Supplementary material online, Table S1).

Table 1 Baseline conditions

<table>
<thead>
<tr>
<th></th>
<th>Male IDCM (n = 29)</th>
<th>Female IDCM (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>44 ± 3</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>NYHA, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0 (0)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>II</td>
<td>11 (38)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>III</td>
<td>13 (45)</td>
<td>9 (64)</td>
</tr>
<tr>
<td>IV</td>
<td>5 (17)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>20 ± 2</td>
<td>30 ± 4*</td>
</tr>
<tr>
<td>LVIDD, cm</td>
<td>6.5 ± 1</td>
<td>5.2 ± 1†</td>
</tr>
<tr>
<td>LVIDS, cm</td>
<td>5.4 ± 1</td>
<td>4.0 ± 1</td>
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<tr>
<td>RVSP, mmHg</td>
<td>34.2 ± 2</td>
<td>44.2 ± 5</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>7.9 ± 1</td>
<td>9.1 ± 2</td>
</tr>
<tr>
<td>DBP</td>
<td>122.8 ± 4</td>
<td>133.7 ± 6</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>74.5 ± 2</td>
<td>78.1 ± 3</td>
</tr>
<tr>
<td>Systolic</td>
<td>21 ± 2</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Diastolic</td>
<td>17 ± 1</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>Pulmonary capillary wedge pressure, mmHg</td>
<td>15 ± 1</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td></td>
<td></td>
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<tr>
<td>β-Antagonist</td>
<td>21 (72)</td>
<td>9 (64)</td>
</tr>
<tr>
<td>ACE-inhibitor</td>
<td>18 (62)</td>
<td>11 (79)</td>
</tr>
<tr>
<td>Aldosterone antagonist</td>
<td>3 (10)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Diuretic</td>
<td>6 (21)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Intravenous inotropic therapy</td>
<td>8 (28)</td>
<td>7 (50)</td>
</tr>
</tbody>
</table>

NYHA, New York Heart Association classification; LVEF, left-ventricular ejection fraction; LVIDD, left-ventricular internal diameter in diastole; LVIDS, left-ventricular internal diameter in systole; RVSP, right-ventricular systolic pressure; RVDP, right-ventricular diastolic pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; PAP, pulmonary artery pressure; ACE-inhibitor, angiotensin-converting enzyme inhibitor.

Table S1

Validation by realtime RT–PCR and western blot confirmed our results from microarray analysis, with overexpression of CD24 and KCNK1 in males (Figure 4), and 2.9-fold overexpression of SLC2A12 in females (Figure 5), respectively.

Fundamental sex-specific differences in gene expression that are less likely related to functional status of the heart

Analysis of a sub-cohort of patients with IDCM and new-onset HF (n = 32), matched for two main functional parameters of the heart—LVEF and LVIDD—revealed 48 differentially expressed transcripts, of which 33 were overexpressed and 15 were downregulated in male vs. female patients (FC > 1.2, q < 5%). Among overexpressed transcripts, there was 85% overlap with the original cohort of our study (n = 43). Notably, genes that were not differentially expressed in the case–control setting were KCNK1, PLEKHAB 8, 9, SLC2A12, and PRKX. The overlap among downregulated genes was 80%, while GATAD1 and PDE6B were only

blood pressure, pulmonary artery pressure, and pulmonary capillary wedge pressure did not differ between groups. All patients received recommended standard therapy for HF, and intravenous inotropic therapy tended to be higher in females (50% of patients).

Analysis of all patients with IDCM within the biorepository (males: n = 112, females: n = 70) reproduced results of the cohort used for transcriptomic profiling. Left-ventricular ejection fraction was higher in women vs. men,20 27 ± 1 vs. 23 ± 1% (P = 0.028), and LVIDD was lower in women vs. men, 5.6 ± 0.1 vs. 6.4 ± 0.1. This finding confirmed that the data set of IDCM and new-onset HF (n = 43) that was used for transcriptomic analysis is representative of the entire sample (n = 180).

Transcriptomic differences in new-onset heart failure

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present in the original data set \( (n = 43) \), but not in the case–control study.

**Discussion**

The major new findings of this study are that gender-specific transcriptomic changes occur in new-onset HF. Many but not all of these are related to sex chromosomes, a biologically plausible observation. In addition, our analysis identified key pathways that have potential therapeutic implications for individualized medicine. Together our findings highlight the value of a comprehensive transcriptomic approach to addressing potentially important pathophysiologic insights in differing demographic groups with a similar disease phenotype.

It is increasingly appreciated that gender differences impact disease presentation and clinical trajectory of patients with cardiovascular disorders.\(^{2,7,8,20,21}\) While hormonal factors,\(^{10}\) structural differences in the cardiovascular system,\(^{11,12}\) and possible differences in treatment response\(^{9}\) are described contributing factors, comprehensive evaluation of the transcriptomic profile provides a substantial opportunity to advance understanding of gender-specific cardiovascular disease pathophysiology and could lead to insights about sex-related individualized therapy.\(^{14,22,23}\)

Transcriptomic differences have been studied in the normal heart,\(^{1}\) as well as end-stage HF,\(^{16,24}\) but previous studies have not addressed differences in the male vs. female transcriptome at the onset of HF. While we showed an overlap between our findings and those from Haddad et al.,\(^{24}\) as well as Fermin et al.,\(^{16}\) substantial differences were identified. We believe that these differences may be important and this study gave us the unique opportunity to examine HF pathophysiology at disease onset and to reveal plausible therapeutic targets.

Isensee et al.\(^{1}\) described gender-specific differences in gene expression in healthy human heart and found 13 genes to be overexpressed and 4 genes to be downregulated in men vs. women. In agreement with our results, most of the differentially expressed genes were located on sex chromosomes. Among four downregulated genes in healthy heart of men vs. women, one gene (XIST) overlapped with our study of new-onset HF, while there was higher overlap of overexpressed gender-related genes with our data (77%). Most notably, all of the overlapping genes were located on sex chromosomes, suggesting that these are the most fundamental transcripts that are entirely independent of heart function or disease stage.

A study about gender-related transcriptomic changes in patients with advanced HF was published by Haddad et al.,\(^{24}\) who analysed pooled normal heart tissue vs. heart from patients with IDCM and end-stage HF at the time of transplant, and then compared which alterations in gene expression in diseased vs. normal heart were detectable in both gender. This study found 55 genes to be differentially expressed in female patients with IDCM vs. normal heart, of which 35% overlapped with results in men. There was no overlap between these findings and our results in new-onset HF. However, this was not surprising, since this study design was entirely different from Isensee et al. and ours. Haddad et al. pre-selected a separate subset of genes for each gender, which was
affected during transition from normal to diseased heart and then analysed the overlap between the resulting gene lists of each sex. Consequently, most sex chromosome related genes will not be revealed in the final analysis.

Gender-specific patterns in gene expression in patients with new-onset heart failure and idiopathic dilated cardiomyopathy

Sex-linked changes
Expression profiling revealed a total of 49 differentially expressed genes between male and females, of which the majority were sex-chromosome related. Among 33 overexpressed transcripts in males, 73% were located on the Y-chromosome (Figure 3A and B). The CD24 antigen, overexpressed in males and located on the Y-chromosome, chromosome 6 and 15, is expressed in numerous cell lineages, where it functions as glycosylphosphatidylinositol (GPI)-anchored molecule preserving homeostasis between immune defense and autoimmunity. This finding is of potential importance because of the well-described lower prevalence of autoimmune diseases in males vs. females. Moreover, autoimmunity is a key pathophysiologic mechanism implicated as contributor to DCM. An attempt to target this antigen with local cell or molecular therapy in autoimmune disorders with cardiac involvement, such as sarcoidosis, systemic lupus erythematosus, or giant cell myocarditis, should be evaluated as potential novel therapeutic approach.

Another pathway of interest is RPS4Y1, overexpressed in males and found to encode ribosomal protein 54, which has a functionally interchangeable counterpart on the X-chromosome, RPS4X. Interestingly, the human RPS4X gene escapes X inactivation, which would suggest its overexpression in any tissue in females vs. males, due to resultant higher genetic load in women. Surprisingly, this was neither the case in our data nor in findings from Isensee et al. 1

Autosomal changes
Overexpressed autosomal genes in male vs. female patients with new-onset HF are KCNK1, coding for a double-pore potassium channel, and PLEKHA8, involved in glycolipid transport.
The product of KCNK1 is not a functional channel by itself, but may get activated by additional non-pore forming proteins and plays a critical role in renal potassium homeostasis. PLEKHA8, also known as FAPP2, is crucial for molecular transport between trans-Golgi network and plasma membrane.

Figure 3 (A) Chromosomal distribution of genes that were overexpressed in men (n = 29) vs. women (n = 14) with idiopathic dilated cardiomyopathy and new-onset HF: The majority of genes were located on the Y-chromosome. (B) Chromosomal distribution of genes that were downregulated in male (n = 29) vs. female (n = 14) patients with idiopathic dilated cardiomyopathy and new-onset HF: The majority of genes were located on the X-chromosome.
Among downregulated genes in men vs. women, we identified three autosomal genes, GATAD1, SLC2A12, and PDE6B. Expression alteration of these genes may contribute to explain differences in heart function between male and female patients, inhibition of phosphodiesterases such as PDE6B is known to modulate inotropic responses and cardio-protective effects, SLC2A12 codes a glucose transporter (GLUT12) expressed in the heart during fetal development, and activated in response to insulin stimulation. GATAD1 binds both the GTPase rab6 and its activating protein rab6-GAP which are important regulators of adrenergic and angiotensin receptor intracellular trafficking. Cyclic nucleotide metabolism, glucose transport and energetics, and neurohumoral stimulation are strongly implicated in HF pathology; thus alterations in the expression of these genes represent potential mechanisms to explain gender differences in new-onset HF.

Differentially expressed genes targeted by pharmacotherapy

One of the important insights of this study is the notion of gender-specific therapeutic targets in cardiomyopathy. We observed overexpression of the potassium channel KCNK1 in males, which is directly inhibited by Ibutilide, and the type la antiarrhythmics Quinine and Quinidine through interaction with the extracellular region of the channel protein. Furthermore, we noted PDE6B downregulation in males, an enzyme that is inhibited by direct binding of Sildenafil and Tadalafil. Sildenafil has been shown to have cardioprotective function, which in part may be caused by further inhibition of PDE6B. Clearly more work is necessary to explore these provocative hypotheses.

Gender-specific differences during disease progression

A large proportion of differentially expressed genes in male vs. females observed at disease presentation were no longer evident in end-stage HF, suggesting reduction of gender-related differences in gene expression during disease progression. However, the data set consisted of a smaller number of samples (n = 15), possibly leading to stronger impact of individual variance of gene expression levels and therefore detection of only highly robust genes.
Comparison of our findings with a second data set of female vs. male patients with late-stage HF by Fermin et al. revealed significant overlap. Among genes that our analysis identified to be overexpressed in females both in new-onset HF as well as endstage, 63% (DDX3Y, RPS4Y1, EIF1AY, USP9Y, and JARID1D) correlated with data from Fermin et al. Among downregulated genes, we detected only two transcripts both in early as well as late-stage HF (XIST and UTX) in females vs. males, of which XIST overlapped with results by Fermin et al. Additionally, a subgroup of gender-related transcripts (CD99, CYorf14, CYorf15B, and PRKX) that we detected solely in patients with new-onset HF, but not in the public data of advanced stage (n = 15, http://cardiogenomics.org), were also presented in Fermin et al. As speculated earlier, this may be caused by a lower number of samples with end-stage HF in the published data set (n = 15) vs. data from Fermin et al. (n = 102), leading to detection of only highly robust genes in our first overlap analysis.

Transcriptomic differences related to heart function

Clinical differences between men and women of this study warrant mention. Female patients tended to have higher EF and lower LVDD. As a result, the difference in heart function between males and females may have affected gene expression. On the other hand, it is equally possible that the observed distinct patterns in gene expression in men vs. women were major contributing factors for better function of the female heart. To test if functional differences in our subset (n = 43) reflect the nature of cardiovascular disease in men vs. women, and if this discrepancy is representative for a broad population, we analysed clinical data from all patients with IDC (n = 182) and successfully reproduced our results. Similar observations were made by other groups.

Subsequently, we performed a case–control study, to identify if genes related to heart function. A total of 11 samples from the original data were excluded, in order to achieve matched baseline conditions. Particularly autosomal genes from the original analysis (e.g. KCNK1, PLEKHA9, GATAD1, and PDE6), were not detectable in patients matched for EF and LVDD, suggesting that autosomal genes, such as KCNK1 and PDE6, may be major contributing factors for better heart function in women. This may be of importance, considering that those genes are targets of commonly used drugs in medicine.

In summary, we identified fundamental transcriptomic differences of male vs. female patients with IDC and new-onset HF. While the majority of genes were located on sex chromosomes, an additional small set of autosomal gender-specific genes was detected, of which some are directly affected by commonly used drugs in medicine. Interestingly, the amount of differentially expressed genes decreased during disease progression, suggesting an alignment of the male and female transcriptome during disease progression.

Supplementary material

Supplementary material is available at European Heart Journal online.

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