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DISSERTATION

Sex-specific gene expression in human cardiomyocytes subjected to left ventricular pressure overload

Geschlechtsspezifische Genexpression in durch linksventrikuläre Drucküberbelastung beeinflussten humanen Kardiomyozyten

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Vorwort

Im Rahmen dieser Dissertation könnte ein Teil der in der vorliegenden Arbeit vorhandenen Ergebnisse, Tabellen und Abbildungen, am 15.01.2020 von der Arbeitsgruppe Gaignebet L, Kańduła MM, Lehmann D, Knosalla C, Kreil DP, Kararigas G. unter dem Titel „Sex-Specific Human Cardiomyocyte Gene Regulation in Left Ventricular Pressure Overload“ in Mayo Clinic Proceedings veröffentlicht worden sein (Copyright 2019 Mayo Foundation for Medical Education and Research) [1]. Wie durch das “JOURNAL PUBLISHING AGREEMENT“ und das „Authors rights“ des Herausgebers „Elsevier“ festgelegt, ist eine Wiederbenutzung dieser Inhalte in einer Dissertation durch den Autor ohne Erlaubnis möglich, vorausgesetzt, dass der Artikel zitiert wird.

Inhaltsverzeichnis

Abbildungsverzeichnis

Tabellenverzeichnis

Abkürzungsverzeichnis

ACE:	angiotensin converting enzyme
AS:	aortic stenosis
AT1:	angiotensin 1
AV:	aortic valve
AVR:	aortic valve replacement
CHD:	coronary heart disease
CCN:	cellular communication network
CMR:	cardiac magnetic resonance
CV:	cardiovascular
ES:	enzyme solution
HF:	heart failure
HPRT1:	hypoxanthine phosphoribosyltransferase 1
LV:	left ventricular
LVEF:	left ventricular ejection fraction
LVH:	left ventricular hypertrophy
LVMI:	left ventricular mass index
MHC-β:	myosin heavy chain beta
NF-κB:	nuclear factor kappa B subunit 1
QPCR:	quantitative real-time PCR
RPLP0:	ribosomal protein large P0
PO:	pressure overload
RWT:	relative wall thickness
TGF:	transforming growth factor
TAC:	transverse aortic constriction
TF:	transcription factor

Abstract (Deutsch)

Eine der aktuell bedeutendsten Herausforderungen der klinischen Kardiologie stellt die Herzinsuffizienz dar, die in sehr unterschiedlichen Erscheinungsformen auftritt und zu einer Reduktion der Lebensqualität, wiederholten Krankenhausaufenthalten und erhöhter Mortalität führt. Häufig beobachtete Vorstufen dieses progredienten Krankheitsbildes sind kardiales Remodeling und ventrikuläre Hypertrophie. Bei erhöhter Druckbelastung, verursacht z.B. durch arterielle Hypertonie oder eine Aortenklappenstenose, kommt es zu molekularen und zellulären Veränderungen des Myokards. In zahlreichen Studien wurden diese Veränderungen bereits untersucht und wiederholt erhebliche Geschlechterunterschiede beschrieben. Unter anderem wurde festgestellt, dass Männer häufiger maladaptives Remodeling aufweisen, gekennzeichnet durch überschießende Hypertrophie, Dilatation sowie entzündliche und fibrotische Veränderungen.

Um diese molekularen Mechanismen genauer zu entschlüsseln, wurden in der dargestellten Arbeit Kardiomyozyten von Patienten*innen mit Aortenklappenstenose, die sich einem Aortenklappenersatz unterzogen und eine linksventrikuläre Hypertrophie aufwiesen, isoliert und deren Genexpression untersucht. Die Haupthypothese dieser Arbeit ist, dass männliche Patienten eine höhere Expression von Genen aufweisen, die mit maladaptivem Remodeling, Hypertrophie und Entzündung assoziiert sind. Zudem wurde hypothetisiert, dass die Expression von entzündungsbezogenen Genen bei Männern mit ihrer linksventrikulären Auswurffraktion zusammenhängt.

Vom 20. März 2016 bis zum 24. Mai 2017 wurden am Deutschen Herzzentrum in Berlin interventrikuläre Septumproben von 34 Patienten*innen (17 Männer, 17 Frauen) gesammelt und Kardiomyozyten für die Genexpressionsanalyse isoliert. Zusätzlich wurden von jedem*r Patienten*in präoperativ klinische und echokardiographische Daten erfasst, die zeigten, dass Alter, Body-Mass-Index, Komorbiditäten und Medikation bei den 17 männlichen und 17 weiblichen Patienten*innen vergleichbar waren. Männliche Patienten wiesen einen höheren mittleren linksventrikulären enddiastolischen Durchmesser (52 ± 9 vs. 45 ± 4 mm; P=0,007) und eine höhere Hinterwanddicke (14,2 ± 2,5 vs. 12,1 ± 1,6 mm; P=0,03), aber eine niedrigere linksventrikuläre Auswurffraktion (49% ± 14% vs. 59% ± 5%; P=0,01) auf.

Wie hypothetisiert, war die Genexpression der Mehrheit der ausgewählten Gene bei den männlichen im Vergleich zu den weiblichen Patienten*innen höher. Darüber hinaus wurde mit der verwendeten Modellierungsanalyse eine signifikante männerspezifische negative Assoziation zweier entzündungsbezogener Gene (*CCN2* und *NFKB1*) zur Ejektionsfraktion (P=0,03 bzw. P=0,02) beobachtet, was auf eine Beziehung zwischen Entzündung und Herzfunktion bei männlichen Patienten hinweist.

Dies sind neuartige Befunde in humanen drucküberlasteten Kardiomyozyten, die helfen, zuvor erwähnte molekulare und zelluläre Veränderungen zu verdeutlichen sowie deren Einfluss auf diverse klinische und funktionelle Erscheinungsformen zu verstehen. Weitere Studien, die diese Erkenntnisse einbeziehen, würden helfen, unterschiedliche zeitliche Abläufe der Herzinsuffizienz besser zu verstehen. Vor allem könnten geschlechtsspezifische Therapieansätze in frühen Stadien des Remodelings entwickelt werden, die die Prognose verbessern.

Abstract (Englisch)

A major issue currently faced in clinical cardiology is heart failure, which comes in many clinical presentations and leads to reduced life quality, repeated hospitalizations and high mortality. Being a time-evolving pathology, a common precursor of heart failure is ventricular remodeling and hypertrophy. While undergoing pressure overload caused for example by hypertension and aortic stenosis, the myocardium undergoes molecular and cellular changes. These changes have been explored and major differences have been described between the sexes. Among other findings, men were more likely to exhibit maladaptive remodeling, characterized by excessive hypertrophy and dilatation as well as inflammatory and fibrotic changes.

In the present work, to further investigate those molecular mechanisms, gene expression was assessed in cardiomyocytes isolated from patients with aortic stenosis undergoing aortic valve replacement and showing left ventricular hypertrophy. The main hypothesis of this work was that male cardiomyocytes display higher expression of genes linked with maladaptive remodeling, hypertrophy and inflammation. It was further hypothesized that expression of inflammation-related genes in males would be linked with myocardial function as described by left ventricular ejection fraction.

From 20th March 2016 through 24th May 2017, at the German Heart Centre in Berlin, interventricular septum samples from 34 patients were collected and isolated cardiomyocytes were used for gene expression analysis. Additionally, clinical and echocardiographical data from each patient were collected pre-operationally, showing that age, body mass index, systolic and diastolic blood pressure, comorbidities, and medication were similar between the 17 male and 17 female patients. Male patients showed higher mean ± SD left ventricular end-diastolic diameter (52 ± 9 vs 45 ± 4 mm; *P*=0.007) and posterior wall thickness (14.2 ± 2.5 vs 12.1 ± 1.6 mm; *P*=0.03) but lower ejection fraction (49%±14% vs 59%±=5%; *P*=0.01).

As hypothesized, the expression of the majority of the selected genes was higher in the cardiomyocytes of male vs. female patients. Furthermore, a significant male-specific negative association of two inflammation-related genes *(CCN2* and *NFKB1*) to ejection fraction (*P*=0.03 and *P*=0.02, respectively) was observed, which indicates an association between inflammatory factors and cardiac function in male patients.

These are innovative findings in human cardiomyocytes subjected to pressure overload, which help illustrate and explain the sex differences in forementioned molecular and cellular changes. Further research considering these molecular mechanisms could help understand different clinical presentations of heart failure and foremost, help develop sex-specific therapeutic approaches in early stages of remodeling.

Manteltext

1. Introduction

1.1 Cardiovascular diseases

Cardiovascular (CV) diseases are the number one cause of death globally representing 31.5% of all deaths (approx. 17.3 million deaths annually) [2]. In Europe, CV diseases are responsible for the mortality of an even larger percentage of the population, i.e. 46%, which represents over 4 million deaths per year [3]. CV diseases have been the leading cause of death for many years also in Germany and accounted for around 44% of deaths in women and 36% in men in the year 2008 [4]. As a cause of premature death (before the age of 65), CV diseases lead to a substantial loss of potential life years and disease-free life years [5]. Moreover, CV diseases are the disease group with the highest costs compared with all other disease groups. For example, in 2015, 14% of direct medical costs - around 46 000 billion euros - were caused by CV diseases [6], as well as 14% of all hospitalization admissions [7]. Among CV diseases, leading causes of hospitalization and mortality are coronary heart disease (CHD), myocardial infarction, stroke, and heart failure.

1.2 Heart failure

Heart failure (HF) affects 1-2% of the population and the prevalence in the population older than 70 years reaches up to 10%. Though the incidence is supposed to be stable, in developed countries like Germany faced with a global ageing population, the prevalence of HF is increasing. About 141 000 persons in Germany aged 80 and over were affected by HF in 2009, and it is expected that by 2050 the affected individuals will be over 350 000. [8]. The current societal burden is remarkably high considering heart failure. In 2016, it represented the first cause for hospitalization in Germany (after healthy birth cases) and the second cause of death among women (5,5 % vs 3,3 in men) [9]. Worldwide, the estimated prevalence of HF is growing rapidly, thereby making HF a major public health issue on a global scale.[10].

HF leads to reduced quality of life and loss of autonomy. Furthermore, it is currently one of the most complex issues in cardiology. The definition of HF in the current guidelines shows already the difficulties underlying its diagnosis and therapy. Described as a clinical syndrome caused by cardiac structural or functional abnormality, it is understandable that the diversity of causes leading to HF leads to a challenge of timing therapeutic, symptomatic, or preventive treatment. In 1993, the Framingham Heart Study linked congestive HF with an extremely poor prognosis. The median survival time was 1.7 years in men and 3.2 years in women, with a 5-year survival rate of 25% in men and 38% in women [11]. More recent studies show an improvement in 5-

year-survival after the time of diagnosis up to 56.7%, which is reduced to 49.5% for patients aged over 75 years. However, this difference is not explained alone by the improvement in therapies, as other parameters like earlier time of diagnosis play a role as well [12,13]. For this reason, it is now globally recognized that HF precursors have to be detected and addressed before the onset of HF to ensure the best prognosis [14].

1.3 Heart failure precursors

Heart failure, in its chronic form, is often preceded by structural changes and functional dysfunction of the ventricles. This dysfunction can be systolic or diastolic and the aetiology leading to this condition is diverse and multi-causal. Furthermore, the pathologies mostly leading to HF are common in the population.

Considering a time-evolving pathophysiology, there are now known precursors of heart failure, one of the most frequent one is myocardial hypertrophy. It is also the leading symptom in a number of heart diseases (such as cardiomyopathies) and is a negative prognostic sign by itself [15–21]. Individual characteristics, particularly biological sex, influence the progression of cardiac remodeling, hypertrophy, and outcome. Mechanisms leading to myocardial hypertrophy are induced by different stimuli, such as pressure overload (PO) as in aortic valve stenosis disease or hypertension, ageing, diabetes, or myocardial ischemia [22].

1.4 Pressure overload as risk factor for HF

Pressure overload due to aortic stenosis (AS) or hypertension is one of the most common risk factors for heart failure. In patients affected by PO, the left ventricle hypertrophies in order to maintain functional cardiac output and maintain normal wall stress, and up to 50% of those patients will develop HF [23]. The prevalence of aortic valve stenosis ranges about 2-7% in the adult population and is the third more common CV disease after CHD and hypertension [24,25]. AS severity is clinically classified according to narrowing of the aortic valve and its progression controlled through echocardiographic examination. It is also used as a hallmark of indication for aortic valve replacement (AVR) operation. However, it has been widely shown that symptom onset and global outcome are also determined by the myocardial response, as measured by left ventricular (LV) ejection fraction (LVEF), left ventricular hypertrophy (LVH), and myocardial fibrosis [15–21,26].

The development of myocardial hypertrophy caused by PO is associated with changes in cardiac geometry (size and shape) that are described as ventricular remodeling. In early stages, remodeling consists of an adaptive response to stress, where the heart walls thicken to compensate for increased stress and maintain function and high output. There are different mechanisms implicated in myocardial hypertrophy, concerning both cardiomyocytes and the

extracellular matrix. Extracellular matrix remodeling, as demonstrated by interstitial fibrosis, is a tissue state, where increased production, turnover or deposition of extracellular matrix components occur. Cardiomyocytes under PO conditions undergo several cellular and molecular changes, such as cell size increase, enhanced or modified protein synthesis, and reorganization of cellular architecture [27]. On the cellular level, this increase in the number of contractile elements leads to an improvement of the contractile force. On the molecular level, humoral and mechanical stimulus leads to reactivation of the fetal gene program. These molecular and cellular mechanisms lead to the reorganization of the entire ventricular architecture on the organ level and myocardial wall thickening. This helps reduce ventricular stress through Laplace's law and preserve systolic function despite high ventricular pressure. Overall, cardiac output is then maintained. This state has been defined as “adaptive hypertrophy”. If PO is not removed, this compensatory mechanism usually leads to pathologic hypertrophy, either “concentric”, characterized by loss of relaxation, an increase in filling pressures and ventricular decompensation, or “eccentric”, defined by ventricular dilation and impaired contractile function. In the long term, the myocardial tissues undergo fibrosis, contributing to onset of HF [22]. It can be noted that eccentric hypertrophy also occurs typically in the case of volume overload (i.e. mitral regurgitation). Overall, the modifications described above do not take place in a homogenous chronological or local order in different individuals, and many factors influence these structural, cellular and molecular modifications, one of the most considerable one being sex.

1.5 Sex differences in HF

Sex differences are present across the entire spectrum of CV diseases. The influence of menopause and other hormonal parameters, as well as differences in epidemiology, pathology and outcome have been described thoroughly. Menopause for example has been considered for a long time to be a major risk factor concerning CV diseases risk in women. The more pronounced age-related cardiovascular risk seen in postmenopausal compared with premenopausal women has been commonly associated with withdrawal of endogenous sexual hormones and early menopause clearly heightens HF risk in women [28–30]. However, experimental and population data suggest that hormonal changes alone are unlikely to completely explain the observed sex differences in cardiovascular presentations and outcomes. Even if the relationship between endogenous estrogen and CV diseases has been described in multiple settings, [31–35], it is still difficult to investigate in which extent menopause is a direct cause of higher cardiovascular risk, considering concurrent timing as well as interrelation between hormonal changes and “classic” cardiovascular risk factors like diabetes, age, obesity, hypertension etc. [36]. Recent meta analyses indicate that more than menopause itself, early age at menopause or other parameters like total reproductive time

show more influence on cardiovascular risk [37,38]. However, those population-based studies explore “higher cardiovascular risk” (higher CV mortality / incidence of any CV adverse events), which is more extensively defined than HF onset and specific data about HF are lacking. What has been thoroughly described in animal models is the cardioprotective influence of endogenous estrogens in HF and fibrosis, as discussed later.

In male individuals, earlier onset of CV diseases, including HF, has been reported [39].The EuroHeart Failure Survey demonstrated that men present more frequently with systolic dysfunction, while women have more frequently diastolic dysfunction [40]. For years, in clinical settings, HF has been mainly defined as LVEF reduction. Nowadays, HF is mainly classified in two different categories: HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF). This clinical presentation, linked with diastolic dysfunction and loss of relaxation/ increase of stiffness, is more often displayed in women (around 60% of cases) [32,40,41]. Moreover, even if prognosis and outcome of HFpEF has been described similar to the ones of HFrEF, there is limited clinical research performed on the pharmacological treatment or other therapeutic interventions.[42]. Few clinical trials exploring β-Blocker therapy, one of the most important pharmacological interventions for reduction of mortality in HFrEF, showed growing evidence that β-Blocker therapy does not benefit patients with HFpEF [43,44]. Addressing the right pathophysiological pathway in patients with HF seems to be a major priority, as treating HF syndrome as one entity has shown to be counterproductive before.

This could be a consequence of sex-specific physiological and pathophysiological adaption and remodeling discussed as follows.

1.6 Sex differences in pressure overload and remodeling

Numerous studies have described the influence of sex on LV geometry and LV function in PO [45–48]. In particular, the sex-dependent structural adaptation of LV geometry can be described through smaller, thicker walls and less dilation in female than male hearts [46]. Furthermore, some studies reported higher transvalvular pressure gradient [46,48], and high left ventricular mass index (LVMI) in women when corrected for stenosis severity, age, LV function, and concomitant bypass surgery [49,50]. Other studies have shown that men are more likely to have higher LVMI, lower LVEF, and increased diastolic myocardial stiffness. Women, on the other hand, showed higher relative wall thickness (RWT) [51,52]. These parameters indicate the development of a more compact, stiffer heart undergoing concentric remodeling in women.

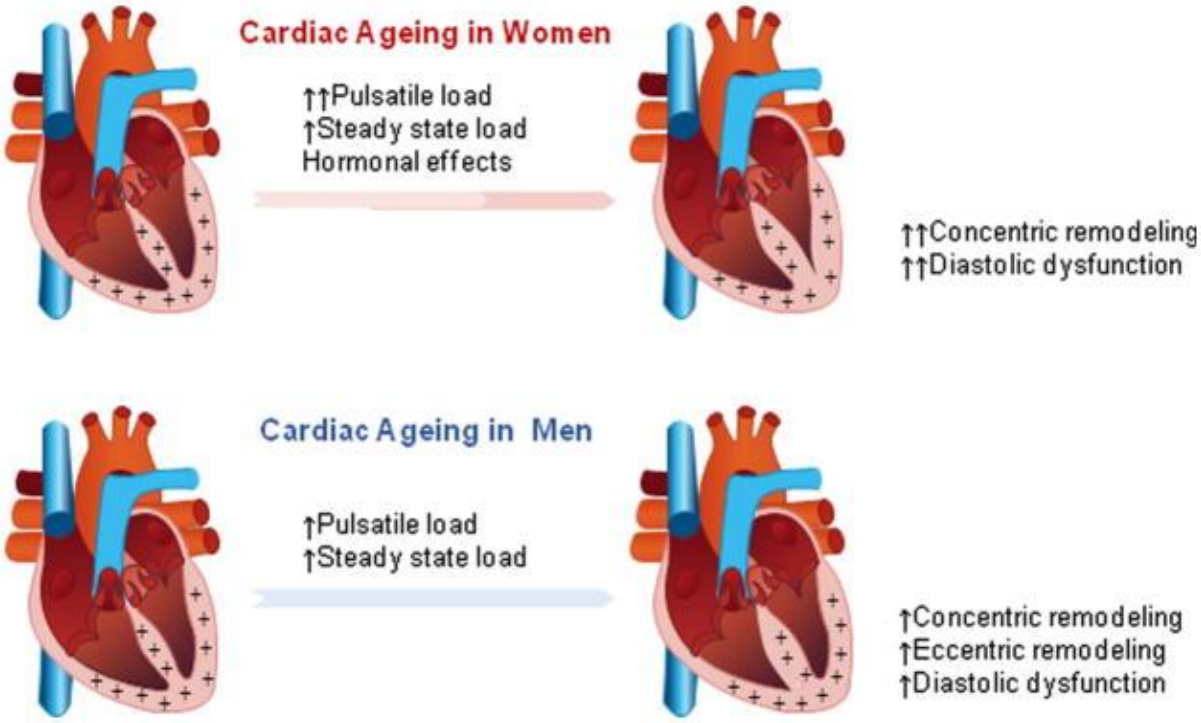


Fig. 1: Patterns of cardiac ageing in men and women. Reprinted from "Sex differences in cardiovascular ageing," by Merz AA and Cheng S, 2016, Heart Br Card Soc; 102(11):825–31. Copyright 2016 by BMJ Publishing Group Limited. [53] Reprinted with permission.

In another study, however, LVMI was similar between the sexes and when LVH of AS patients was analysed in a large population-based cohort, LVH was more prevalent in women than men [54]. This was also shown in another, more recent study with patients undergoing AVR, where similar percentages of women and men had increased LV diameters, but women more frequently exhibited LVH than men when compared with data from a healthy cohort [55]. These findings have been repeatedly confirmed, lately with even more precise investigations and use of cardiac magnetic resonance (CMR) measurement [52,56]. Women, independently of LV size, more frequently preserve better ventricular function. This is shown by the presence of higher fractional shortening and preserved ejection fraction. Overall, myocardial contractility indicated by mid-wall shortening (MWS) is better in women than men during AS progression [57]. It has been shown that this functional difference is linked with LV geometry and typical female remodeling [48].

These differences in geometry and function may influence outcome. It has been shown that LVEF and MWS were better preserved during the progression of AS in women than men [48,57]. This could mean that women develop initially a form of remodeling that is more adaptive to PO in terms of function. The progression of the stenosis itself was not significantly different between the sexes, but women showed 31% lower all-cause mortality independently

of treatment, age or blood pressure [57]. Overall, presentation of inappropriately high LVM has been associated with a strong impairment of survival [16]. In a similar manner, diffuse fibrosis and remodeling as detected in CMR imaging has been linked to increased mortality [26]. A study with AS patients undergoing AVR surgery demonstrated that increased LV diameters persisted 1 week after surgery in 34% of men but only in 12% of women [55]. LVH reversed more frequently in women than in men and showed a better reversibility after unloading the ventricle by aortic valve replacement in women than in men [55]. In a study considering prognosis of patients after transcatheter AVR, women showed better long time outcome and survival [58]. Furthermore, prognosis of heart failure is better in women than men, even if diagnosis and therapy are more problematic, as shown in the CHARM study [59]. Further studies showed better or faster reversibility of LV dysfunction (described as reverse remodeling) in women when receiving heart failure treatment [60,61]. In the β-blocker Evaluation of Survival Trial (BEST) study, the effect of female sex was as powerful as the effect of β-blocker therapy alone [62]. This suggests a therapeutic effect of interest for sex-based therapies.

1.7 Animal models of heart failure and pressure overload

In order to investigate the role of biological sex in CVD, numerous studies have been performed using experimental models, such as mice and rats [63]. To mimic PO and induce HF in these models, transverse aortic constriction (TAC) has been used among others [64] [65]. These models, as well as others, have shown pronounced sex differences and are consistent with clinical studies. Males consistently develop more severe disease symptoms following PO in a variety of experimental settings. As presented before in men and women with AS, males from studies with mice and rats develop more eccentric cardiac hypertrophy, as well as increased fibrosis, compared with females [66]. On the molecular level, female hearts showed lower expression of fetal genes, such as atrial natriuretic peptide (ANP, *NPPA*), myosin heavy chain beta (MHC-β, *MYH7*) and matrix metalloprotease (MMP) was lower in females vs. males [67]. Even when showing a similar degree of hypertrophy or remodeling, female rats were more likely to preserve a better cardiac function than males [68]. Not only gene expression differs between sexes, but proteomic response as well [69]. Estrogen-mediated cardioprotection has been suggested as a mechanism, since 17-β-oestradiol supplementation or treatment in different animal disease models in *vivo* or *in vitro* repeatedly ameliorated cardiac dysfunction and hypertrophic development. The results of those animal model studies are summarised in Table 1.

Table 1. Summarized results from relevant animal studies

Study	Model	Material analysed	Reported regulation, incl. gene expression/pathways
Douglas 1998 [68]	TAC in female and male rats, compared with sham females and males	Echocardio-graphic data at 6 and 20 weeks after TAC and perfusion-fixed tissues	Males: more chamber dilatation, more collagen content
Witt 2008 [70]	TAC in female and male mice	Left ventricle, 2 weeks after TAC	Female: higher expression of mitochondrial-related genes (PGC-1, cytochrome oxidase, carnitine palmitoyl transferase, acyl-CoA dehydrogenase, pyruvate dehydrogenase kinase) Male: higher expression of ribosomal proteins and genes associated with extracellular matrix remodeling (collagen 3, matrix metalloproteinase 2, TIMP2, and TGF-β2)
Fliegner 2010 [66]	TAC, WT vs. ERβKO and male vs. female mice	Left ventricle, 9 weeks after TAC	Female: conservation of mitochondrial function (oxidative phosphorylation) Male: Upregulation of matrix remodeling and cell proliferation pathways
Westphal 2012 [71]	TAC in ovariectomized female mice, treated with E2, raloxifene, 16α-LE2	Left ventricle, 9 weeks after TAC	E2- & 16α-LE2-treated: less TGF-β and CTGF expression, slower development of LVH & systolic dysfunction progression
Prévilon 2014 [72]	TAC in male vs female vs sham mices	Left ventricle, 4 weeks after TAC	Sex differences in CaMPK-MEF pathway and calcineurin-NFAT pathway Male: higher αSK mRNA level
Montalvo 2012 [73]	TAC,in male vs female vs orchidectomized males mice (1-year old)	Left ventricle, 2 weeks after TAC	Orchidectomized males: diminished up-regulation of TGF-β after TAC
Donaldson 2009 [74]	TAC in ovariectomized mice, E2-treated vs Placebo	Left ventricle, 2 weeks after TAC	E2 treated females: mitigated increase of LV mass, ANP, and β-MHC gene expression

Skavdahl 2005 [75]	TAC in male vs female mice	Cardiac tissue (not specified), 2 weeks after TAC	Male: higher TNF receptor 12b, matrix metalloproteinase 14 (MMP14) and transforming growth factor-β3 (TGF-β3)
Loyer 2007 [76]	TAC in male vs female Wistar rats	Left ventricle, 2, 14 and 20 weeks after TAC	Female: delayed NOS1 expression and activity
Weinberg 1999 [67]	TAC in male vs female rats	RNA isolated from LV tissue and isolated LV cardiomyocytes, 6 weeks after TAC	Multicellular LV tissue: higher RNA overexpression of ANF, β-MHC, less SERCA in males Cardiomyocytes: expression of ER-receptor
Pedram 2005 [77]	In vitro angiotensin II (ANG II) or endothelin 1 (ET-1) treatment, in presence or absence of E2	Isolated rat neonatal cardiomyocytes (1-3 days)	E2-treated cells: 17beta-oestradiol prevents AngII-ET-1-induced new protein synthesis and skeletal muscle actin expression. Induces high MCIP1 levels, thereby enhancing calcineurin inhibition.
Wang 2017 [78]	G-Protein Estrogen Receptor (GPER) KO mice	Isolated mouse cardiomyocytes from adult mice (18–20 weeks)	GPER KO females: mitochondrial genes are upregulated GPER KO males: inflammatory response genes are upregulated

1.8 Mechanisms contributing to the observed sex differences

It has been shown that gene expression differs significantly between the sexes in healthy and failing LV cardiac tissues [79,80]. The genomic correlates and corresponding molecular pathways responsible for HF are influenced by sex, as well as age and other factors. A study with human myocardial tissues of AS patients showed the importance of TGF-β1 gene expression levels and could detect a sex-dependent regulation of the downstream SMAD pathway [81]. The levels of collagen and fibronectin, as well as the correlating high TGF-β1 levels, were more important in men with worse LVEF and more dilatation than men with normal LVEF. In women, a proportionally higher expression of myosin light chain 2 (*MYL2*) and a correlation with the hypertrophic response were reported, as well as the expression of genes encoding sarcomeric proteins and TGF-β1. In another study of LV tissue samples from AS patients, men had significantly higher collagen I (*COL1A1*) and III (*COL3A1*) and matrix metalloproteinase 2 (*MMP2*) gene expression than women. The expression of these genes in women with PO was not significantly higher than the controls. These genes are characteristic

for matrix synthesis and turnover, and these results show the difference in regulation between women and men, with a higher increase of fibrotic gene expression in males [55]. Another more recent study highlighted the sex-related role of TGF-β1 and the SMAD signalling in regulation of fibrotic genes programmes: they were more expressed in men with higher cardiac fibrosis, but not in women [82]. Furthermore, periostin, a downstream target of TGF-β1 and formerly described as modulator of PO-induced hypertrophy, was significantly higher in AS males. Lately, a genome wide analysis of LV tissues from AS patients corroborated these findings and identified entire pathways, where sex differences could be observed. In male tissues, fibrosis-related genes/pathways were induced, while extracellular matrix-related and inflammatory genes/pathways were repressed in female tissues [83]. Of course, activation of inflammatory factors is necessary to react to stress or injury and initiate tissue recovery, cell multiplication and functional adaptation. Inflammation is a timely regulated process and growing evidence tends to point out that unbalanced, persistent inflammation contributes to disease progression. On the single gene level, relevant differences between sexes could be seen in the expression of TIMP metallopeptidase inhibitor 2 (*TIMP2*), chemokine (C-X3-C motif) receptor 1 (*CX3CR1*), interleukin 33 (*IL33*), and Toll-like receptor 7 (*TLR7*), more increased in males only, and the expression of TGF-β1 (*TGFB1*), decreased in females only, corresponding to the pathways regulation detected in the genome wide assay [83].

1.9 Further relevant actors of remodeling

CCN2, which encodes cellular communication network factor 2, also known as connective tissue growth factor (CTGF), is a member of the CCN family of cytokine. It has been shown to be induced by TGF-β1 and to be a cofactor for and downstream mediator of TGF-β1, and therefore another candidate of sex-specific regulation of inflammation [84]. It is also a good example of interaction between inflammation and fibrosis, as it showed a crucial role in promoting fibrotic processes und ventricular hypertrophy [85]. High levels of *CCN2* have been reported previously before in human and animal failing cardiac tissues [86,87].

NFKB, which is a well-known transcription factor (TF) playing a central role in immune and inflammatory responses, has been described as sustaining chronic inflammation through the induction of inflammatory factors und thus being a hallmark of pathological hypertrophy [27] [88].

Sex differences are often noticed but not explained and the importance of linking gene expression with clinical, *in vivo* measurements is highlighted. The evidence for estrogen-mediated cardioprotection in human remains debatable because the mechanisms contributing to sex differences in cardiac hypertrophy and HF and their relevance in each sex are not fully understood.

2. Aim and hypothesis

The aim of the present work was to assess sex differences in the expression of selected genes in isolated human cardiomyocytes (hCMs). The hypothesis was that the levels of maladaptive remodeling genes in hCMs from AS patients are higher in male vs. female patients. Furthermore, it was hypothesized that there is a male-specific association between ejection fraction and the expression of inflammation-related genes.

3. Methods

3.1 Study population

Septum biopsy was collected from 34 patients (50% men) with AS undergoing AVR. They were recruited between March 20, 2016, through May 24, 2017 at the German Heart Institute Berlin. Inclusion criteria were informed consent and availability of septum biopsy. Exclusion criteria were emergency surgery, reoperation, transapical or transarterial AVR, severe aortic leakage or other significant (stage II) concomitant mitral or tricuspid valve disease, and infectious or suspected infectious diseases, such as endocarditis, myocarditis, hepatitis. The characteristics of the patients are shown in Table 5. The study was approved by the local ethics committee, followed the principles outlined in the Declaration of Helsinki and written consent was obtained from all patients.

3.2 Echocardiography

Patients received two-dimensionally guided M-mode transthoracic echocardiography during the week before and after operation. The M-mode tracings were digitally recorded and analysed. LVEF was calculated using the Quiñones formula and LV mass using the Devereux formula [89,90]. LV mass was indexed on body surface area to calculate LVMI and RWT was calculated as 2xLV posterior wall thickness (PWT) at end diastole/LV end-diastolic diameter (LVEDD). Aortic valve area was calculated with the continuity equation und indexed on body surface to obtain the aortic valve area index. Pressure gradients were calculated with the Bernoulli equation.

3.3 Myocardial sampling

During surgery, myocardial biopsies were taken from the lateral LV wall. Right after sampling, the biopsy was put in in 25ml Falcon tube filled with ice cold modified Tyrode solution (S1), oxygenated for at least 15 min beforehand and supplemented with 2,3-Butanedione monoxime with end concentration of 1 mg/ml. The falcon tube was then put on ice in a polystyrene box and transported within 20 min or less to the laboratory for further processing.

Table 2: S1 Ca2+ free Tyrode solution

Material	MW	Final [ ] mM	g/1L ddH_2O	Company	Reference
Sucrose	342.30	88	30.1224	Sigma	S1888-1KG
NaCl	58.44	88	5.1427	Sigma	S7653-1KG
KCl	74.55	5.4	0.4026	Sigma	P9333-500G
$NaHCO_3$	84.01	4	0.3360	Sigma	S5761-500G
NaH_2PO_4	119.98	0.3	0.036	Sigma	S5011-100G
$MgCl_2.6H_2O$	203.30	1.1	0.2236	Sigma	M2670-100G
HEPES	238.30	10	2.383	Sigma	H4034-500G
Taurine	125.15	20	2.503	Sigma	T0625-500G
D-(+)-Glucose	180.16	10	1,802	Sigma	G7528-250G
Sodium pyruvate	110.04	5	0.55	Sigma	P2256-25G

3.4 Cardiomyocyte isolation

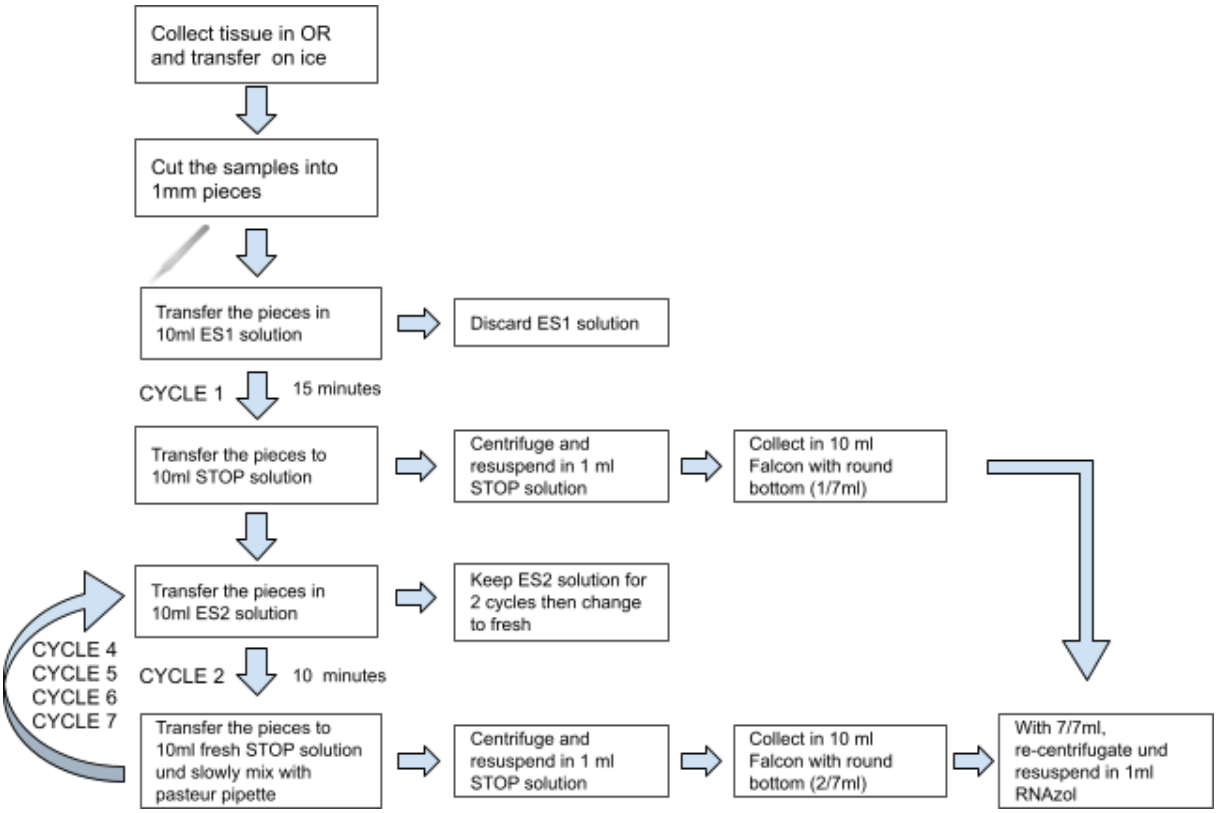


Fig. 2: Overview of the cardiomyocyte isolation protocol. *Source*: Own representation.

After tissue collection, the samples would be transferred to a petri dish on ice. Any visible fat was removed, and samples were cut in 1 mm pieces. The pieces were put in an Erlenmeyer flask containing 10ml enzymatic solution (ES1) for digestion containing 0.115 % collagenase and 0,02 % protease and put in a 35°C bath for 15min. During all digestion steps, the solution was continuously oxygenated. After this first step, another 10min digestion step in 0.062 % collagenase enzyme solution (ES2) followed. After this, pieces of biopsy would be delicately transferred to a cold solution (STOP solution) and mixed slowly with Pasteur pipette to facilitate separation. This last step (digestion + pipetting) would be repeated until complete digestion of the tissue. The cell solution would undergo each time centrifugation (RT, 50g, for 3min) in bovine serum albumin (BSA) solution, isolated cells were frozen in 1ml RNAzol and stored at -80°C until further processing.

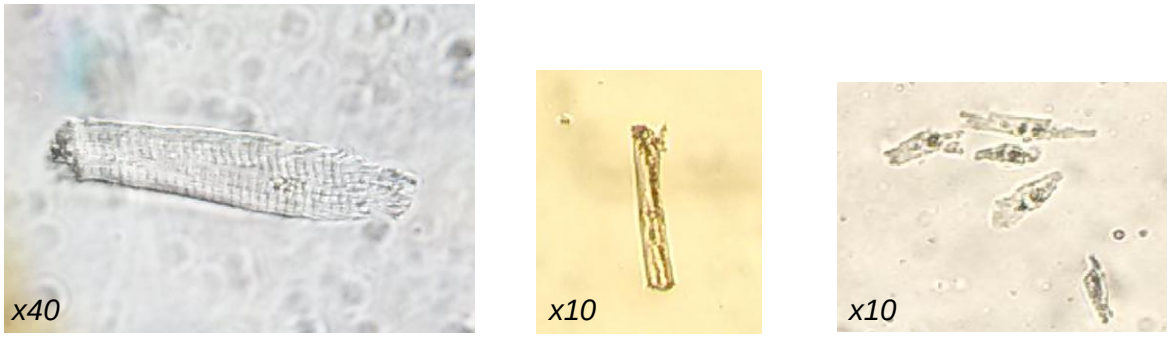


Fig. 3: Cardiomyocytes in isolation solution, light microscopy. *Source*: Own pictures.

Table 3: Enzyme solutions

Solution	Company
Base of Ca free Tyrode (S1)	
+ Blebbistatin with end concentration of 2µM	Sigma; B0560-5MG
ES1: 10ml Blebbistatin-enriched S1	
+ 6.5 mg Collagenase type 2	Worthington; LOT: 40N12244; 298 u/mg
+ 2.5 mg Proteinase	Sigma; P8038-250MG
+ 20.5 mg BSA (2 mg/ml)	Sigma; A6003-25G
ES2: 30ml Blebbistatin-enriched S1	
+ 18.5 mg Collagenase type 2	Worthington; LOT: 40N12244; 298 u/mg
+ 60.5 mg BSA (2 mg/ml)	Sigma; A6003-25G

3.5 RNA isolation

RNA isolation was performed on all available samples. Frozen 1ml RNAZol-cells samples were mixed with 0,2ml Chloroform and then centrifugated for 10 min (4°C, 14000 U/min). The upper, water-based layers of each sample were isolated and mixed 1:1 with isopropanol to induce precipitation of nucleic acids. After overnight precipitation at -20°C, a second 30 min centrifugation step (4°C, 14000 U/min) led to collection of an RNA-pellet. Surfactant was then discarded, and the pellet was washed und centrifuged repeatedly with 80% ethanol and left to air dry. After removing all alcohol, total RNA was eluted in RNase-free water. RNA quality and quantity were controlled by measurement of total RNA Yield with a nanodrop. The quality of the RNA was assessed by the appropriate 240/260 ratios of the samples. RNA samples were measured and diluted in the right amount of water for transcription.

3.6. Reverse transcription

For each tissue, 0.5 g of total RNA was reverse transcribed into complementary DNA (cDNA) using the Applied Biosystems™High-Capacity cDNA Reverse Transcription Kit, which uses the random primer scheme for initiating cDNA synthesis, according to the manufacturer's instructions.

3.7. Quantitative real-time PCR (QPCR)

QPCR was conducted on all samples. QPCR amplification mixtures were performed in 25 µl final volume containing 3 ng template cDNA, 2x SYBR Green PCR Master Mix and the corresponding pair of primers as seen in Table 4. The cycling conditions comprised 10 minutes polymerase activation at 95oC and 40 cycles at 95oC for 15 seconds and the annealing temperature of 60oC for 60 seconds. To normalize cDNA concentration in different samples, the expression of hypoxanthine phosphoribosyltransferase 1 *(HPRT1*) and ribosomal protein large P0 (*RPLP0*) and used as a normalization factor: their expression levels was calculated, by which the quantity of the target gene was divided.

Table 4: Primer data

Gene	Forward primer	Reverse primer
ACTC1	5'-CATCCAGGCAGTGCTATCCC-3'	5'-ACCATCCCCAGAGTCCAGAAC-3'
CCN2	5'-TACCAATGACAACGCCTCCT-3'	5'-TGGGAGTACCGATGCACTTT-3'
GATA4	5'-GGAAGCCCAAGAACCTGAAT-3'	5'-GGGAGGAAGGCTCTCACTG-3'
GJA1	5'-GCCTGAACTTGCCTTTTCAT-3'	5'-CTCCAGTCACCCATGTTGC-3'
HPRT	5'-TGTTGTAGGATATGCCCTTGACT-3'	5'-GGCTTTGTATTTTGCTTTTCCA-3'
NPPA	5'-ACCGTGAGCTTCCTCCTTTT-3'	5'-TTCCAAATGGTCCAGCAAA-3'
NPPB	5'- CACCGCAAAATGGTCCTCTA-3'	5'-TTAATGCCGCCTCAGCAC-3'
MYH7	5'-AGAGGGTGGCAAAGTCACTG-3'	5'GCCATGTCCTCGATTTTGTC-3'
MYH6	5'-TGCGCATTGAGTTCAAGAAG-3'	5'-CTTCATCCAGGGCCAATTCT-3'
MYL2	5'-GCAGGCGGAGAGGTTTT-3'	5'-AGTTGCCAGTCACGTCAGG-3'
MYL3	5'-GGCAGAAGTGCTCCGTGT-3'	5'-AGTCCATCATCTTGGTATTGAGC-3'
MYL4	5'-GAACCCTACCAATGCCGAGG-3'	5'-GAAATGTGCTGCAGGATGGG-3'
NFKB1	5'-CCAGATTCGATTTTATGAAGAGG-3'	5'-GAGTTTTGAAGACAATGGCAAAT-3'
RPLP0	5'-ACGGGTACAAACGAGTCCTG-3'	5'-AGCCACAAAGGCAGATGGAT-3'

3.8 Statistical analysis

The statistical analysis of clinical data and gene expression was performed using the PRISM 7 software (GraphPad Software Inc). Every data set was tested for normality with the Shapiro-Wilk test and for outliers with the Grubbs Test. Depending on data distribution, either the unpaired *t* test or the Mann-Whitney *U* test was used for comparisons between two groups. Comparisons for categorical variables were made with the χ^2 test or Fisher's exact test. A value of $P \leq .05$ was considered statistically significant.

To analyse the relation between LVEF and gene expression, linear regression models were used based on iterative inverse variance weighted least squares regression with an M-estimator with Huber ψ and median absolute deviation scale estimation as implemented by the *rlm* function of the *MASS* R library. [91,92]. Logit transform was applied to all ratios in order to meaningfully use linear regression [93,94]. The regression models were first run based only on gene expression for every gene, age, sex, or body mass index alone. To test the validity of the models, leave-one-out robustness analysis of the model structure was performed. For example, each gene-based regression model would be tested for significance when excluding one different patient every time. By always removing one patient out of the equation and recording significance of the regression model, it excludes the possibility that this patient is responsible alone for the performance of the model. When leaving out once each patient where

LVEF data was available (24), if a model is significant every time, then it is robust 24 out of 24 times (24/24). By doing so, it suggests that possible results are not affected by outliers. Robustness is then reported under two thresholds: 5%, i.e. $P<.05$, and 10%, i.e. $P<.1$.

4. Results

4.1 Patient population

In the present study, 17 male and 17 female patients with AS undergoing aortic valve replacement were recruited. The characteristics of the patients are shown in Table 2. There were no significant differences in age, body-mass-index (BMI), or heart rate. The systolic and diastolic blood pressure are also similar between groups and the proportion of patients with arterial hypertension is not significantly different. In the same way, the distribution of CHD between men and women was not significantly different, nor concomitant hyperlipidaemia. Among further relevant comorbidities, the only difference between the cohorts was a significantly higher proportion of male patients with type 2 diabetes mellitus.

Concerning medication, the number of statin-treated patients was higher in the male cohort. Another medication, calcium antagonists, was found in proportionally more women than men. The occurrence of other relevant medications like angiotensin converting enzyme (ACE) inhibitors and angiotensin 1 (AT1) receptor antagonists showed no significant differences between sexes in this cohort.

LVEF was significantly higher in women than men, even if the aortic valve (AV) area index was similar in both sexes. Hypertrophy parameters, such as LVEDD, LVMI and PWT, were significantly higher in men than women. The parameters related to pressure, i.e. peak or mean AV gradient pressure, were not significantly different between men and women.

Table 5**:** Baseline and echocardiographic characteristics of the study population

	Total (*n* = 34)	Men (*n* = 17)	Women (*n* = 17)	*P* value
Age, y	68 ± 9	68 ± 10	69 ± 8	.49
BMI, kg/m²	28 ± 4.9	28 ± 3	28 ± 5.2	.93
Systolic blood pressure, mmHg	133 ± 24	133 ± 19	133 ± 31	.98
Diastolic blood pressure, mmHg	72 ± 11	74 ± 10	70 ± 12	.32
Diabetes Mellitus, %	38	47	24	.15
Hyperlipidemia, %	41	41	41	1.0
Hypertension, %	73	76	71	.69
CHD, %	47	52	41	.40
ACE inhibitors, %	42	47	40	.68
AT1 receptor antagonists, %	28	29	27	.86
Beta-blockers, %	54	71	40	.08
Diuretics, %	36	41	33	.65
Calcium antagonists, %	21	6	40	**.02**
Statins, %	57	77	40	**.03**
LVEDD, mm	48 ± 7	52 ± 9	45 ± 4	**.007**
Interventricular septum, mm	14.4 ± 2.8	15.3 ± 3.2	13.6 ± 2.2	.07
Posterior wall thickness, mm	13.2 ± 2.3	14.2 ± 2.5	12.1 ± 1.6	**.03**
LVMI, g/m²	140 ± 41	158 ± 45	123 ± 29	**.04**
RWT	0.54 ± 0.1	0.6 ± 0.12	0.5 ± 0.08	.87
Aortic valve area index, cm²/m²	0.4 ± 0.16	0.4 ± 0.19	0.5 ± 0.15	.96
Mean pressure gradient, mmHg	42 ± 13	39 ± 12	45 ± 14	.17
Maximal pressure gradient, mmHg	64 ± 18	57 ± 15	69 ± 19	.07
LVEF, %	54 ± 11	49 ± 14	59 ± 5	**.01**

Values are shown as mean ± SD. *Note:* From “Sex-Specific Human Cardiomyocyte Gene Regulation in Left Ventricular Pressure Overload” by Gaignebet L, Kańduła MM, Lehmann D, Knosalla C, Kreil DP, Kararigas G, Mayo Clin Proc. 2020 Apr;95(4):688-697 [1]

4.2 Gene Regulation

Samples were collected from the interventricular septum during AVR and used these samples to isolate cardiomyocytes to assess cardiomyocyte-specific gene expression between male and female patients. The targeted genes were selected because of their implication in synthesis of structural proteins encoding actin cytoskeleton and cardiac muscle structural and contractile proteins, as well as inflammatory factors: *ACTC1*, *CCN2*, *GATA4*, *GJA1*, *MYH6*, *MYH7*, *MYL2*, *MYL3*, *MYL4*, *NFKB1*, *NPPA*, and *NPPB*. Most of these genes showed higher expression levels in male cardiomyocytes. Only the levels of *MYH7*, *MYL2*, and *MYL3* were similar between male and female cardiomyocytes.

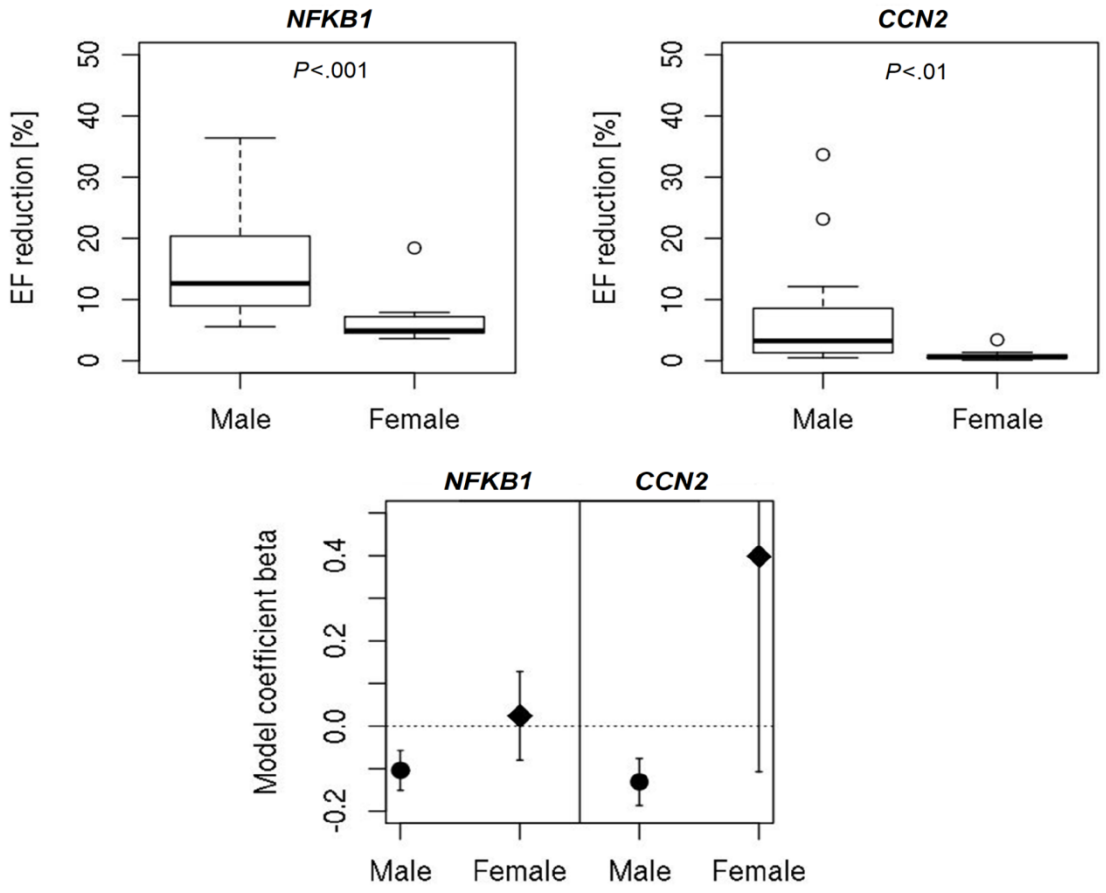
4.3 Relationship Between Gene Expression and LVEF

To explore these significant sex differences in gene expression and test the hypothesis that they are linked to cardiac function, as assessed by LVEF, further systematic statistical analysis was performed to identify relations between the two. A systematic statistical regression analysis has been used, completed by leave-one-out robustness testing for validation, which revealed a significant relationship between LVEF and *NFKB1* (*P*=0.01) as well as *CCN2* (*P*=0.02) genes (Table 2). A higher expression level of *NFKB1* or *CCN2* was negatively related to LVEF (respectively *P*=0.01, robust at <5% in 24 of 24 leave-one-out validation cohorts and *P*=0.02, robust at <5% in 22 of 24 and <10% in 24 of 24 validation cohorts), and LVEF would decrease on average by 11% and 4% for every 2-fold increase of *NFKB1* or *CCN2* expression. This effect was not pronounced equally when considering males and females separately (*P*<0.001 for *NFKB1* and *P*=0.005 for *CCN2*). LVEF reduction related to increased gene expression was more distinct in male (17%) as female patients (7%) for *NFKB1*, as well as *CCN2*, (8% in males vs 1% in male and female patients).

In order to investigate the influence of sex on the relation between gene expression and LVEF for these 2 genes, regression models for gene-sex interaction were analysed and robust sex-specific effects could be identified: for *NFKB1*, *P*=0.02, robust at less than 5% in 22 of 24 and less than 10% in 24 of 24 leave-one-out validation cohorts; for *CCN2*, *P*=0.02, robust at less than 10% in 24 of 24 leave-one-out validation cohorts. Even given the small numbers of patients, an higher gene expression of those genes was related to a reduced LVEF in male patients (*P*=0.03 for *NFKB1* and *P*=0.02 for *CCN2*), while there was no significant effect in female patients (*P*=0.81 for *NFKB1* and *P*=0.41 for *CCN2*). Specifically, a 2-fold increase of gene expression of *NFKB*1 corresponded to a reduction of LVEF by 15% on average (*P*=0.04, robust at <5% in 22 of 24 and <10% in 24 of 24 leave-one-out validation cohorts). The significant male-specific effect for gene expression in CCN2 yielded an average reduction in

LVEF of over 7% for every 2-fold increase. (*P*=0.02, robust at <5% in 22 of 24 and <10% in 24 of 24 leave-one-out validation cohorts).

Fig. 4: Relation between gene expression and LVEF



Note: From “Sex-Specific Human Cardiomyocyte Gene Regulation in Left Ventricular Pressure Overload” by Gaignebet L, Kańduła MM, Lehmann D, Knosalla C, Kreil DP, Kararigas G, Mayo Clin Proc. 2020 Apr;95(4):688-697, [1]

5. Discussion

5.1. Overall insights in cardiac remodeling at early stage

The main und novel finding of this work is that gene expression in hCMs isolated from AS patients is significantly different between the sexes. In particular, the expression of numerous genes associated with maladaptive remodeling was higher in hCMs of male patients compared with female patients. Additionally, a novel relation between the expression of two inflammatory genes, i.e. *CCN2* and *NFKB1*, and LVEF was identified, showing a negative relation, in a sex-specific manner. Among the numerous genes related with maladaptive remodeling, structural

genes have been previously described as involved in the development of PO-induced LVH and LV remodeling. [95]. The expression of *ACTC1*, *GJA1*, *MYL4*, *NPPA* and *NPPB* was higher in male vs female hCMs. In the same manner, the levels of the important transcription factor *GATA4*, involved in the development of LVH and HF [96], were higher in male vs female hCMs, as well as the levels of its downstream targets *MYH6* and *NPPA.* The overall higher induction of these hypertrophic factors in male hCMs correspond to the hypothesis that male patients show more maladaptive remodeling and have therefore a worse outcome.

In a similar way to the aforementioned structural genes, the expression of genes related to inflammation, i.e. *CCN2* and *NFKB1*, was higher in male cardiomyocytes compared with female cardiomyocytes. Notably, their expression is negatively related to LVEF in male patients. This is the first work studying *CCN2* expression at an early phase of remodeling in hCMs. Therefore, this suggest that it exerts a pro-inflammatory action and that it may contribute to increased vulnerability to cardiac dysfunction and worse prognosis in men.

NFKB1 codes for the nuclear factor kappa B subunit 1 (NF-κB), whose activation has been repeatedly linked to the hypertrophic response of the heart in rodents and has been described in tissue from HF patients [88]. It has not been much studied at such an early phase of remodeling. We put forward that *CCN2* has a pro-inflammatory role together with NF-κB in male hCMs.

The modelling approach used revealed that increasing the expression of *CCN2* and *NFKB1* two-fold yielded a 7% and 15% average reduction in LVEF of male patients, respectively. This suggests that male-specific activation of pro-inflammatory factors may contribute to LV remodeling and cardiac dysfunction, or that they are at least mechanisms present in early steps of the failing myocardium. These findings, confirmed in bigger cohorts, could have many therapeutic implications. Beyond offering insight into new therapeutic targets or mechanisms, this work reflects the diversity of pathological mechanisms implicated in the development of HF. It illustrates the necessity of doing more research in this field, as the definition of HF as a uniform phenotype and pathophysiology has been strongly refuted. Different pathological mechanisms could be better targeted in different individuals, and this could even help to understand the efficacy of current therapies, to improve cardioprotective effects and reduce their Number Needed to Treat. Doing so would lead to a more personalized medicine and a better management of this very frequent syndrome with poor outcome.

5.2 Further research

The present findings open new research avenues. In order to better understand remodeling, a follow up study where clinical data of patients is assessed at a later time point could be

established. Information about long term development of heart function could help to identify genes and pathways which are linked to reversible, adaptive remodeling or permanent malfunction. This could help to identify patients at increased risk. As the present study has been based on a targeted approach, it would be interesting to perform a genome-wide analysis of gene expression in hCMs. This could highlight entire pathways as it has been done previously in the mouse adult heart or in whole heart tissue of humans [69,70]. Another point that requires further research is the investigation of the underlying mechanisms that contribute to the observed sex-specific gene expression. Among others, epigenomic approaches and studies of microRNA have been developed lately [97].

Another possibility would be a pharmacological interventional approach. An experimental setting with isolated hCMs submitted to pharmacological interventions would offer information about the influence of physiological mechanisms (for example estrogen-related cardioprotection) or pharmacological mechanisms on gene expression. Clinical studies of larger cohorts of patients with different pharmacological treatment or interventional studies could help to identify the influence of treatment on undergoing molecular remodeling.

Given the broad spectrum of remodeling and leading paths to HF, it is not a surprise that HF therapy has been focussed mostly to volume load control and heart frequency management. Remodeling mechanisms and ways of failing are multiple and there is growing evidence that the female cardiovascular system presents more often less explored pathomechanisms like pathological tissue stiffness, loss of distensibility and diastolic dysfunction. On the other hand, fibrotic progression, inflammation, and cell death are more likely to be found in men.

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Eidesstattliche Versicherung und Anteilserklärung

„Ich, Léa Gaignebet, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Sex-specific gene expression in human cardiomyocytes subjected to left ventricular pressure overload / Geschlechtsspezifische Genexpression in durch linksventrikuläre Drucküberbelastung beeinflussten humanen Kardiomyozyten“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Léa Gaignebet hatte folgenden Anteil an der folgenden Publikation:

Sex-Specific Human Cardiomyocyte Gene Regulation in Left Ventricular Pressure Overload.
Gaignebet L, Kańduła MM, Lehmann D, Knosalla C, Kreil DP, Kararigas G. Mayo Clin Proc. 2020 Apr;95(4):688-697. doi: 10.1016/j.mayocp.2019.11.026. Epub 2020 Jan 15. PMID: 31954524.

Beitrag im Einzelnen:
Idee: Kararigas G
Datensuche: Gaignebet L, Kararigas G
Patientenrekrutierung und -aufklärung: Gaignebet L, Knosalla C
Probenasservierung und -verarbeitung, Datengenerierung – und analyse: Gaignebet L, Lehmann D
Statistische Aufarbeitung: Gaignebet L (Table 1, Fig. 1), Kańduła MM (Table 2 and 3, Fig. 2)
Text: (Abstract, Einführung, Methode außer “Statistical analysis”, Ergebnisse außer “Relationship Between Gene Expression and EF”): Gaignebet L, Kararigas G
Text: (Methoden: Statistische Aufarbeitung, Ergebnisse: “Relationship Between Gene Expression and Diskussion): Kańduła MM
Supervision, Corrections: Kreil DP, Kararigas G

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Unterschrift der Doktorandin

Auszug aus der “Journal Summary List"

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCISelected Categories: **“MEDICINE, GENERAL and INTERNAL”**
Selected Category Scheme: WoS
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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NEW ENGLAND JOURNAL OF MEDICINE	344,581	70.670	0.686700
2	LANCET	247,292	59.102	0.427870
3	JAMA-JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION	156,350	51.273	0.300810
4	Nature Reviews Disease Primers	4,339	32.274	0.019740
5	BMJ-British Medical Journal	112,901	27.604	0.152760
6	JAMA Internal Medicine	15,215	20.768	0.095580
7	ANNALS OF INTERNAL MEDICINE	57,057	19.315	0.096020
8	PLOS MEDICINE	30,689	11.048	0.071200
9	Journal of Cachexia Sarcopenia and Muscle	2,799	10.754	0.005870
10	BMC Medicine	13,630	8.285	0.045220
11	Cochrane Database of Systematic Reviews	67,607	7.755	0.158690
12	**MAYO CLINIC PROCEEDINGS**	**14,695**	**7.091**	**0.025750**
13	CANADIAN MEDICAL ASSOCIATION JOURNAL	15,351	6.938	0.016500
14	JOURNAL OF INTERNAL MEDICINE	10,547	6.051	0.015700
15	Journal of Clinical Medicine	2,315	5.688	0.007210
16	MEDICAL JOURNAL OF AUSTRALIA	11,134	5.332	0.012600
17	PALLIATIVE MEDICINE	5,682	4.956	0.009860

MAYO CLINIC

ORIGINAL ARTICLE

Sex-Specific Human Cardiomyocyte Gene Regulation in Left Ventricular Pressure Overload

Lea Gaignebet, MB; Maciej M. Kańduła, PhD; Daniel Lehmann, MS; Christoph Knosalla, MD; David P. Kreil, PhD; and Georgios Kararigas, PhD

Abstract

Objective: To assess gene expression in cardiomyocytes isolated from patients with aortic stenosis, hypothesizing that maladaptive remodeling and inflammation-related genes are higher in male vs female patients.

Patients and Methods: In this study, 34 patients with aortic stenosis undergoing aortic valve replacement from March 20, 2016, through May 24, 2017, at the German Heart Centre in Berlin, Germany, were included. Isolated cardiomyocytes from interventricular septum samples were used for gene expression analysis. Clinical and echocardiographic data were collected preoperatively.

Results: Age, body mass index, systolic and diastolic blood pressure, comorbidities, and medication were similar between the 17 male and 17 female patients. The mean ± SD left ventricular end-diastolic diameter (52±9 vs 45±4 mm; P=.007) and posterior wall thickness (14.2±2.5 vs 12.1±1.6 mm; P=.03) were higher in male vs female patients, while ejection fraction was lower in male patients (49%±14% vs 59%±5%; P=.01). Focusing on structural genes involved in the development of cardiac hypertrophy and remodeling, we found that most were expressed higher in male vs female patients. Our modeling analysis revealed that 2 inflammation-related genes, *CCN2* and *NFKB1*, were negatively related to ejection fraction, with this effect being male specific (P=.03 and P=.02, respectively).

Conclusion: These findings provide novel insight into cardiomyocyte-specific molecular changes related to sex differences in pressure overload and a significant male-specific association between cardiac function and inflammation-related genes. Considering these sex differences may contribute toward a more accurate design of research and the development of more appropriate therapeutic approaches for both male and female patients.

 ■ Mayo Clin Proc. 2019;■■(■):1-10

The pressure overload (PO) that develops in aortic stenosis (AS) or hypertension leads to left ventricular (LV) hypertrophy (LVH). The initial compensatory mechanism is expected to reduce wall stress and to maintain systolic function of the heart. In later stages, the myocardium undergoes pathologic molecular, cellular, and tissue changes. This maladaptive LV remodeling includes reactivation of the fetal gene program, induction of fibrosis, and dilation, ultimately contributing to the development of heart failure (HF).

The development of PO-induced LVH differs significantly between men and women.[1] In particular, sex-specific LV remodeling leads to a more concentric form of LVH in women. This structural adaptation of LV geometry can be described by less LV dilation and wall thinning in female than in male hearts.[2] However, the molecular mechanisms contributing to these sex differences are incompletely understood. So far, analysis of cardiac biopsy specimens from patients with AS has revealed a higher level of fibrosis-related genes in male hearts compared with female hearts.[3,4] Studies in

From the Charité — Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Germany (L.G., D.L., G.K.); Department of Biotechnology, BOKU University, Vienna, Austria (M.M.K., D.P.K.); DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Germany (D.L., C.K., G.K.); and German Heart Centre, Berlin, Germany (C.K.).

TABLE 1. Baseline and Echocardiographic Characteristics of the Study Patients[a,b]

Variable	Total (N=34)	Male (n=17)	Female (n=17)	*P* value
Age (y)	68±9	68±10	69±8	.49
Body mass index (kg/m^2)	28±4.9	28±3.0	28±5.2	.93
Systolic blood pressure (mm Hg)	133±24	133±19	133±31	.98
Diastolic blood pressure (mm Hg)	72±11	74±10	70±12	.32
Diabetes mellitus	12 (38)	8 (47)	4 (24)	.15
Hyperlipidemia	14 (41)	7 (41)	7 (41)	>.99
Hypertension	25 (73)	13 (76)	12 (71)	.69
Coronary artery disease	16 (47)	9 (52)	7 (41)	.40
ACE inhibitors	15 (42)	8 (47)	7 (41)	.69
AT1 receptor antagonists	10 (29)	5 (29)	5 (29)	.78
β-Blockers	19 (55)	12 (71)	7 (41)	.08
Diuretics	12 (35)	7 (41)	6 (35)	.68
Calcium antagonists	7 (21)	1 (6)	6 (35)	.03
Statins	20 (59)	13 (77)	7 (41)	.04
LVEDD (mm)	48±7	52±9	45±4	.007
Interventricular septum (mm)	14.4±2.8	15.3±3.2	13.6±2.2	.07
Posterior wall thickness (mm)	13.2±2.3	14.2±2.5	12.1±1.6	.03
Left ventricular mass index (g/m^2)	140±41	158±45	123±29	.04
Relative wall thickness	0.55±0.10	0.60±0.12	0.50±0.08	.87
Aortic valve area index (cm^2/m^2)	0.40±0.16	0.40±0.19	0.40±0.14	.96
Mean pressure gradient (mm Hg)	42±13	39±12	45±14	.17
Maximal pressure gradient (mm Hg)	64±18	57±15	69±19	.07
Left ventricular ejection fraction (%)	54±11	49±14	59±5	.01

[a]ACE = angiotensin-converting enzyme; AT1 = angiotensin II type 1 receptor; LVEDD = left ventricular end-diastolic diameter.
[b]Data are presented as mean ± SD or No. (percentage) of patients.

animals have also identified higher levels of fibrotic and hypertrophic mediators, as well as lower levels of mitochondrial factors, in male vs female LV tissues under PO.[5-9]

However, the investigation of these sex differences has been based on multicellular LV tissue samples. Consequently, the use of whole tissues leads to the loss of information on cell-specific gene regulation.[10] The heart consists of a number of different cell types that coordinately regulate cardiac physiology and the response to injury.[11] Given the fundamental role of cardiomyocytes in myocardial function, our goal was to assess gene expression in isolated human cardiomyocytes. We focused on genes relevant for cardiomyocyte function and adaptation, including hypertrophic markers and genes involved in LV remodeling. We hypothesized that the cardiomyocyte levels of maladaptive remodeling genes are higher in male vs female patients and that there is a male-specific association between ejection fraction (EF) and the expression of inflammation-related genes.

PATIENTS AND METHODS

Study Population

For this exploratory study, 34 patients (17 [50.0%] male) with AS undergoing aortic valve replacement (AVR) from March 20, 2016, through May 24, 2017, at the German Heart Centre in Berlin, Germany, were recruited. Inclusion criteria were informed consent and availability of septum biopsy specimens. Exclusion criteria were emergency operation, reoperation, transcatheter AVR, more than mild aortic regurgitation, or other severe concomitant mitral or tricuspid valve disease, as well as infectious diseases such as endocarditis, myocarditis, or hepatitis. The baseline characteristics of the patients are presented in Table 1. The study was approved by the local ethics committee and conforms with the principles outlined in the Declaration of Helsinki. Written consent was obtained from all patients.

Echocardiographic Analysis

Patients underwent 2-dimensional M-mode transthoracic echocardiography during the week before AVR. The M-mode tracings were digitally recorded and analyzed. Ejection fraction was calculated using the Quiñones formula and LV mass using the Devereux formula.[12,13] Left ventricular mass was indexed on body surface area to calculate the LV mass index, and relative wall thickness was calculated as 2 × LV posterior wall thickness at end-diastole/LV end-diastolic diameter. Aortic valve area was calculated with the continuity equation and indexed on body surface to obtain the aortic valve area index. Pressure gradients were calculated with the Bernoulli equation.

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Cardiomyocyte Isolation

During AVR, myocardial biopsy specimens were obtained from the interventricular septum and quickly transported from the operating room to the laboratory in a Tyrode solution, oxygenated and supplemented with 2,3-butanedione monoxime.[14] Cardiomyocytes were isolated as previously described.[15] Briefly, following tissue collection, any fat was removed and samples were cut in 1-mm pieces. The pieces were put in an enzymatic solution containing 0.5 mg/mL collagenase and 0.5 mg/mL proteinase for digestion, followed by another digestion step in 0.4 mg/mL collagenase. Following centrifugation in a bovine serum albumin solution, isolated cells were frozen in RNAzol (Sigma-Aldrich) and stored at −80°C until further processing.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Reverse transcription and quantitative real-time polymerase chain reaction were performed as described previously.[16,17] Total RNA was reverse transcribed and amplified using One-Step RT qPCR MasterMix (Eurogentec). Reactions in which RNA or reverse transcriptase had been omitted were used as negative controls. Polymerase chain reaction products were obtained using gene-specific, intron-spanning primers and SYBR Green (Applied Biosystems) in an ABI PRISM 7000 instrument (Applied Biosystems). The levels of all candidate genes were normalized to *HPRT1* (for expansion of gene symbols, use search tool at www.genenames.org) housekeeping messenger RNA levels. Primer sequences are presented in the Supplemental Table (available online at http://www.mayoclinicproceedings.org).

Statistical Analyses

Clinical and gene expression data were analyzed statistically using Prism 7 (GraphPad Software, Inc). Normality of the data was tested with the Shapiro-Wilk test and outliers with the Grubbs test. Depending on data distribution, either the unpaired *t* test or the Mann-Whitney *U* test was used for comparisons between 2 groups. Comparisons for categorical variables were made with the χ^2 test or Fisher exact test. A value of $P \leq .05$ was considered statistically significant.

Before fitting regression models, logit transformation was applied to all ratios to make them amenable to meaningful linear regression.[18,19] We used iterative inverse variance weighted least squares regression using an M-estimator with Huber ψ and median absolute deviation scale estimation as implemented by the rlm function of the MASS R library.[20,21] For an unbiased analysis, we considered all potential factors in regression models. In order to avoid overfitting in view of the available patient numbers, we first focused on the most simple models, each only including 1 of the 12 genes or 1 of the clinical features of age, sex, or body mass index. For all of these tests, we report a measure of evidence strength (Akaike information criterion) and significance (*P* value), characterizing the different models in Table 2. For each model, we then additionally performed leave-one-out robustness

TABLE 2. Models With One Regressor[a,b]

Variable	β	SE	*P* value	AIC	Robust <5%	Robust <10%
ACTC1	−.03	0.02	.08	32.46	1/24	8/24
CCN2	**−.15**	**0.06**	**.02**	**31.86**	**22/24**	**24/24**
GATA4	−.02	0.01	.07	35.08	3/24	15/24
GJA1	−.04	0.02	.04	35.22	9/24	21/24
MYH6	−.04	0.02	.04	35.36	7/24	20/24
MYH7	−.06	0.02	.01	33.80	23/24	23/24
MYL2	−.04	0.02	.01	34.92	21/24	23/24
MYL3	−.02	0.02	.25	36.98	0/24	2/24
MYL4	−.01	0.01	.12	35.83	0/24	6/24
NFKBI	**−.12**	**0.05**	**.01**	**29.83**	**24/24**	**24/24**
NPPA	−.01	0.01	.09	33.36	3/24	9/24
NPPB	−.01	0.01	.55	35.57	0/24	0/24
Age	.00	0.01	.68	39.87	0/24	0/24
Sex	**−.35**	**0.16**	**.04**	**35.50**	**14/24**	**24/24**
BMI	.01	0.02	.39	38.39	0/24	0/24

[a]AIC = Akaike information criterion; BMI = body mass index. For expansion of gene symbols, use search tool at www.genenames.org.

[b]β = model coefficient; SE = standard error of the β coefficient; *P* value = statistical significance of the model; AIC = assessed goodness of the model fit (lower values are better); Robust <5% or <10% = the number of leave-one-out robustness tests significant under the selected *P* value threshold. Bold entries are significant and robust and are the focus of our further analysis.

analysis. Specifically, for every variable considered, including every individual gene and clinical feature, we confirmed the relevance of the model variable by testing the significance of the model trained on a validation cohort that excludes a patient. This means that we split the 24 patients with measured EF into a group of 23 patients to train our regression model and 1 patient to be left out from this training. We did this 24 times, always removing a different patient and recording the significance of the regression model. If a model was significant for all 24 different subgroups of 23 patients, we report that the model is robust 24 of 24 times. This indicates that no single patient is alone responsible for the performance of the model, ensuring that results are not affected by outliers. We report robustness under 2 thresholds: 5% (ie, $P<.05$) and 10% (ie, $P<.1$). Examining the gene expression for the 2 robustly significant gene factors suggested that they may be sex specific. We expressly tested this hypothesis in a model of the sex-specific effects, assessing evidence strength, significance, and robustness as before (Table 3).

RESULTS

Patient Characteristics

In this exploratory study, 34 patients (17 [50%] male) with AS undergoing AVR were included (Table 1). Age and body mass index were comparable between male and female patients. Systolic and diastolic blood pressures were also similar between male and female patients. There was no statistically significant difference between male and female patients in the proportion of patients with diabetes ($P=.15$), arterial hypertension ($P=.69$), concomitant hyperlipidemia ($P>.99$), or coronary heart disease ($P=.40$). The number of patients treated with calcium antagonists was higher in female than in male patients. In contrast, there were more male patients treated with statins than female patients. No significant differences were observed between male and female patients in other relevant medications, such as use of angiotensin-converting enzyme inhibitors ($P=.69$), angiotensin II type 1 receptor antagonists ($P=.78$), β-blockers ($P=.08$), and diuretics ($P=.68$).

Echocardiographic Characterization

Echocardiographic assessment of the patients revealed significant sex differences (Table 1). The LV end-diastolic diameter was significantly higher in male than in female patients (52±9 vs 45±4 mm; $P=.007$). Similarly, the posterior wall thickness (14.2±2.5 vs 12.1±1.6 mm; $P=.03$) and LV mass index (158±45 vs 123±28 g/m^2; $P=.04$) were higher in male patients. The EF was significantly lower in male than in female patients (49%±14% vs 59%±5%; $P=.01$). The aortic valve area index and transvalvular pressure gradients were similar between male and female patients.

Gene Regulation in Isolated Cardiomyocytes of Male and Female Patients

In order to assess cardiomyocyte-specific gene expression between male and female patients, we collected samples from the interventricular septum during AVR and used these specimens to isolate cardiomyocytes. We focused on genes that lead to increased synthesis of structural proteins encoding actin cytoskeleton and cardiac muscle structural and contractile proteins, as well as inflammatory factors. These genes were *ACTC1*, *CCN2*, *GATA4*, *GJA1*, *MYH6*,

TABLE 3. Models of the Interaction Between the Robustly Significant Genes and Sex[a,b]

Variable	β	SE	*P* value	AIC	Robust <5%	Robust <10%
***CCN2*:sex**			**.02**	**33.02**	**22/24**	**24/24**
CCN2:male	−.13	0.06	**.02**			
CCN2:female	.40	0.51	.41			
***NFKBI*:sex**			**.02**	**30.62**	**22/24**	**24/24**
NFKBI:male	−.10	0.05	**.03**			
NFKBI:female	.02	0.10	.81			

[a]AIC = Akaike information criterion. For expansion of gene symbols, use search tool at www.genenames.org.

[b]β = model coefficient; SE = standard error of the β coefficient; *P* value = statistical significance of the model; AIC = assessed goodness of the model fit (lower values are better); Robust <5% or <10% = the number of leave-one-out robustness tests significant under the selected *P* value threshold. Bold entries are significant and robust and are the focus of our further analysis.

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MYH7, *MYL2*, *MYL3*, *MYL4*, *NFKB1*, *NPPA*, and *NPPB*. Our analysis revealed that the levels of most of these genes were higher in male cardiomyocytes than in female cardiomyocytes (Figure 1). Only the levels of *MYH7*, *MYL2*, and *MYL3* were similar between male and female cardiomyocytes.

Relationship Between Gene Expression and EF

Given the significant sex differences in cardiac function, as assessed by the EF, and in gene expression, we performed a systematic analysis of dependencies between the two. In order to identify relevant links between the EF and specific genes, we combined systematic statistical regression analysis with leave-one-out robustness testing for validation. We found statistically significant and highly robust gene-based models for the *NFKB1* ($P=.01$) and *CCN2* ($P=.02$) genes (Table 2). Our analysis revealed that a higher expression level of *NFKB1* was negatively related to EF ($P=.01$, robust at <5% in 24 of 24 leave-one-out validation cohorts). Similarly, a higher expression level of *CCN2* was negatively related to EF ($P=.02$, robust at <5% in 22 of 24 and <10% in 24 of 24 validation cohorts). Notably, a 2-fold increase of *NFKB1* or *CCN2* expression was related to an average reduction of the EF by 11% and 4%, respectively. Considering males and females separately, we identified that the change of EF was significantly different between the sexes ($P<.001$ for *NFKB1* and $P=.005$ for *CCN2*). In particular, a 2-fold increase of *NFKB1* expression was related to an average reduction of the EF by 17% in male patients and 7% in female patients (Figure 2A). In the case of *CCN2*, the average reduction of the EF was 8% and 1% in male and female patients, respectively (Figure 2B).

For each of these 2 robustly significant genes, we next investigated how the effect of their expression on the EF is influenced by sex. Even in this pilot cohort, it was possible to identify highly significant sex-specific effects (Table 3) that were strong enough to robustly yield significant models for the 2 genes: *NFKB1*—$P=.02$, robust at less than 5% in 22 of 24 and less than 10% in 24 of 24 leave-one-out validation cohorts; *CCN2*—$P=.02$, robust at less than 10% in 24 of 24 leave-one-out validation cohorts. For both genes, regression for an explicit gene-sex interaction term revealed that higher expression was related to a reduced EF in male patients ($P=.03$ for *NFKB1* and $P=.02$ for *CCN2*), while there was no significant effect in female patients ($P=.81$ for *NFKB1* and $P=.41$ for *CCN2*; Figure 2C and Table 3). In particular, for *NFKB1*, a significant male-specific effect was detected ($P=.04$, robust at <5% in 22 of 24 and <10% in 24 of 24 leave-one-out validation cohorts), with a 2-fold increase yielding an average EF reduction of 15%. For *CCN2*, a significant male-specific effect was detected ($P=.02$, robust at <5% in 22 of 24 and <10% in 24 of 24 leave-one-out validation cohorts), with a 2-fold increase being related to an average reduction in EF of over 7%.

DISCUSSION

The present study is the first to show that in cardiomyocytes isolated from patients with AS, gene expression differs significantly between the sexes. In particular, the expression of a number of genes associated with maladaptive remodeling was higher in cardiomyocytes of male patients compared with female patients. An additional novel and important finding of this study is that the expression of 2 inflammatory genes, *CCN2* and *NFKB1*, was negatively related to EF and in a sex-specific manner.

The development of PO-induced LVH differs significantly between men and women.[2,22,23] To this extent, we found sex-specific remodeling of the heart. In particular, at a similar degree of LV outflow obstruction, male hearts had significantly increased LV end-diastolic diameter ($P=.007$) and decreased EF ($P=.01$) compared with female hearts. These sex differences in geometry and function may influence outcome. It has been reported that EF and mid-wall stress were better preserved during the progression of AS in women than in men.[24,25] This difference indicates that women develop initially a form of

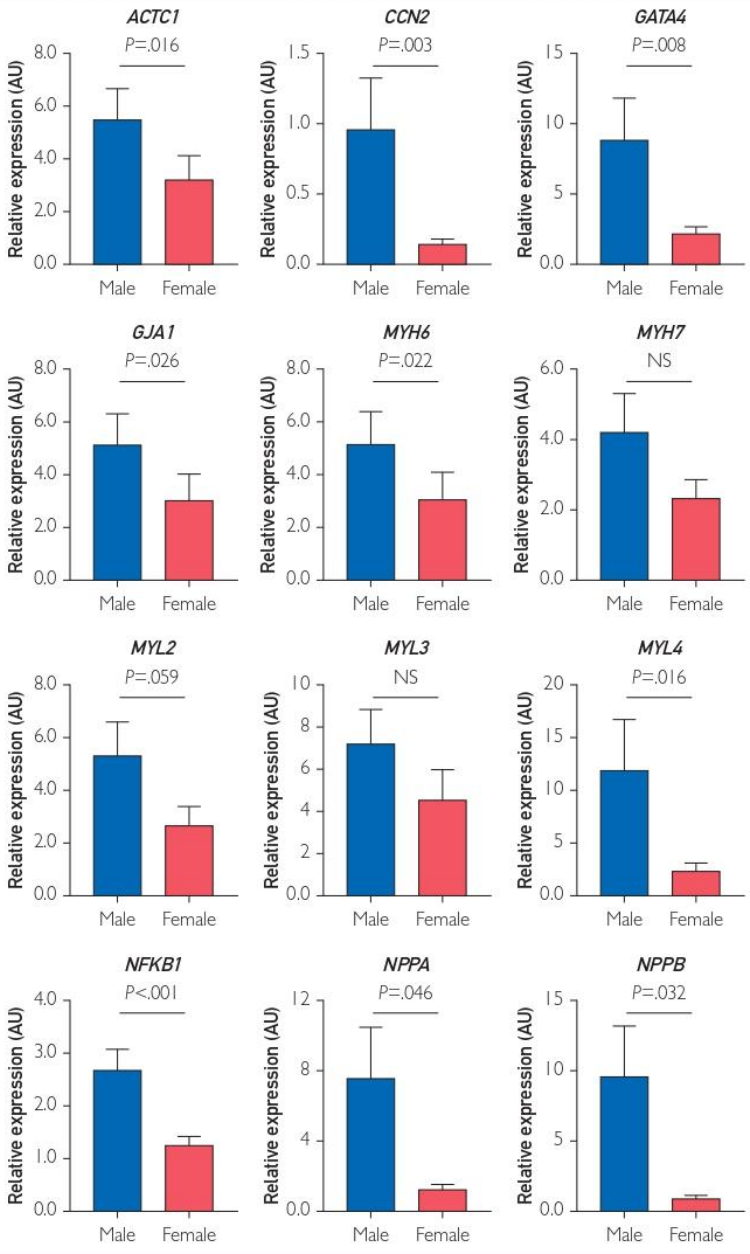

FIGURE 1. Gene expression analysis in isolated human cardiomyocytes. Data present mean ± SEM in groups of 12 to 13 patients. AU = arbitrary unit; NS = not significant.

remodeling that is more adaptive to PO in terms of function. Of note, although the progression of the stenosis itself was not significantly different between the sexes, women had 31% lower all-cause mortality independent of treatment, age, or blood pressure.[25]

Previous studies investigating samples from patients and animals have provided

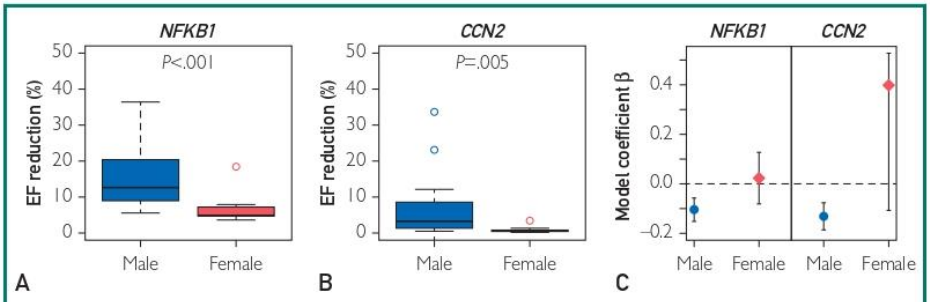

FIGURE 2. Sex-specific factors. Box plot showing the sex-specific reduction in the ejection fraction (EF) on a 2-fold increase in expression of the *NFKB1* (A) and *CCN2* (B) genes. In each box, the median is presented by the horizontal bold lines and the corresponding quartiles (whiskers) are shown. C, Plot showing the β coefficients of the gene:sex interaction terms with bars indicating standard errors for male (blue circles) and female (red diamonds) sex. Notably, the coefficients for male-specific effects have tight error bars, clearly excluding 0. Correspondingly, the *NFKB1* and *CCN2* effects for males are significantly different from 0 (P=.03 and P=.02, respectively). In contrast, the female-specific interaction terms are not statistically significant (P=.81 and P=.41, respectively), reflected by them not being distinguishable from 0, with 0 falling within the range of the error bars.

insight into potential mechanisms underlying sex differences in PO.[3-9] However, they were based on multicellular LV tissue samples. Consequently, for this study our goal was to assess gene expression in cardiomyocytes isolated from patients with AS. As a pilot study, we took a targeted approach focusing on structural genes, because they are involved in the development of PO-induced LVH and LV remodeling. We found that the expression of *ACTC1*, *GJA1*, *MYL4*, *NPPA*, and *NPPB*, which are associated with LVH and maladaptive LV remodeling,[26] was higher in male cardiomyocytes than in female cardiomyocytes. In addition, the levels of *GATA4*, which is an important transcription factor involved in the development of LVH and HF,[27] as well as the levels of its downstream targets *MYH6* and *NPPA*, were higher in male cardiomyocytes than in female cardiomyocytes. Although several factors may influence disease progression and outcome,[28,29] the overall induction of these hypertrophic factors in male cardiomyocytes indicates maladaptive remodeling at the molecular level occurring in male patients and supports the view that they may play a key role in the worse outcome observed in men with PO. Notably, the novel finding of increased gene expression of maladaptive remodeling factors in cardiomyocytes of male patients compared with female patients suggests that the male and female hearts remodel differently through divergent mechanisms. This difference raises the question to what extent current knowledge, which mostly stems from research on males, is relevant for females, ultimately calling for more research in females.

We further identified that the expression of 2 inflammation-related genes, *CCN2* and *NFKB1*, was higher in male cardiomyocytes compared with female cardiomyocytes, suggesting a male-specific activation of inflammatory factors. Of note, assessment of the relationship between these 2 genes and cardiac function revealed that their levels were negatively related to EF in male patients, pointing to the involvement of inflammatory factors with heart function and failure in a sex-specific manner. Inflammation is a necessary process that follows stress and injury, such as myocardial infarction and hypertrophy. Consequently, initial activation of inflammatory factors is crucial for the heart to maintain its integrity and function. However, persistent inflammation contributes to disease progression and may lead to HF.

CCN2 encodes cellular communication network factor 2, also known as connective tissue growth factor, which is a member of the CCN family of cytokines. Nevertheless, CCN2 does not act as a traditional growth factor or cytokine but rather as a matricellular protein, induced by transforming growth factor β, as well as a cofactor for and downstream mediator of transforming growth factor β,[30,31] thereby contributing to cardiac fibrosis.[32] The levels of CCN2 have been reported previously to be induced in cardiac tissues of humans and animals with HF[30,33] but have not been studied previously at an earlier phase of LV remodeling, ie, compensated LVH, or in isolated human cardiomyocytes. In fact, the role of cardiomyocyte-derived CCN2 is not clear, whereas fibroblast-derived CCN2 appears to be crucial for the development of cardiac fibrosis in mice.[34] We postulate that a proinflammatory role might be more relevant for cardiomyocyte-derived CCN2 (discussed below). Collectively, we propose that the increased expression of *CCN2* in male cardiomyocytes under PO may contribute to increased vulnerability to cardiac dysfunction and worse prognosis in men.

NFKB1 codes for the nuclear factor κB subunit 1 (NF-κB), which belongs to a family of transcription factors playing a central role in immune and inflammatory responses. Rodent studies have linked NF-κB to the hypertrophic response of the heart. Its prolonged activation is detrimental, triggering chronic inflammation through the induction of inflammatory factors, ultimately leading to HF.[35] Along this line, activation of NF-κB has been reported in cardiac tissues of patients with HF,[36,37] but as with CCN2, any regulation of NF-κB at an earlier phase of LV remodeling or in isolated human cardiomyocytes is poorly understood. Interestingly, CCN2 has been found to activate NF-κB in the mouse aorta and kidney, thereby promoting proinflammatory factors and inflammatory cell infiltration.[38,39] This finding suggests a proinflammatory role of CCN2 together with NF-κB in male cardiomyocytes. Their persistent induction might render male patients more prone to the development of HF with depressed systolic function.

Our modeling approach revealed that increasing the expression of *CCN2* and *NFKB1* 2-fold yielded a 7% and 15% average reduction in EF of male patients, respectively. This finding suggests that male-specific activation of proinflammatory factors may contribute to LV remodeling and cardiac dysfunction. On the basis of this finding, we propose that male patients with compensated LVH might benefit from therapeutic interventions targeting CCN2 and NF-κB. This could address the unmet need for effective anti-inflammatory therapies, because, for example, antagonism of the proinflammatory cytokine tumor necrosis factor in HF has been discouraging.[40,41] It must be noted, however, that such anti-inflammatory strategies were used in chronic HF. Our data indicate that activation of inflammatory factors already occurs during compensated LVH. Therefore, we posit that onset of treatment against these targets at an earlier phase of cardiac remodeling may prove useful for the prevention of the progression from compensated LVH to HF. Novel anti-inflammatory strategies targeting the CCN2 and NF-κB pathways merit further research to identify selective inhibitors reaching therapeutic efficacy and minimizing systemic toxicity.

These findings also provide insight into the diversity of pathologic mechanisms implicated in the development of HF. Over the past few years, the clinical definition of HF as a uniform phenotype and pathophysiology has been strongly refuted. Although EF has been used for decades as a clinical quantification of HF, current research has highlighted the high prevalence of HF with preserved EF and its strong association with the female sex.[42,43] The present findings support the hypothesis that different molecular mechanisms would initiate diverse disease trajectories and highlight the need for more research in order to understand the background and individuality of every presentation of clinical symptoms of HF, starting with sex-specific differences. Doing so would lead to more personalized

medicine and better management of this very frequent syndrome with poor outcome.

The sample size in our study might be considered relatively small. However, this cohort is not unusually small, given that cardiac samples were used from patients who have to continue their lives with a functioning heart following biopsy sampling. Therefore, it is not realistic or relevant to expect cohort sizes similar to those using, for example, blood samples that can be more readily available in larger numbers. Notably, this exploratory sample was adequate for identifying significant sex differences in cardiomyocyte gene expression. In fact, this unique pilot data set could already illustrate the remarkable strength and robustness of both the overall gene expression effect and the interaction effect with sex, reflected by the solid results obtained. Surely, with a larger sample size, more features could be added to statistical models without losing interpretability or leading to model overfitting. This factor underlines the need to confirm the present findings in follow-up studies with bigger cohorts. Moreover, in light of the recent advances in single-cell sequencing of the mouse adult heart,[10] it would be interesting to perform a genome-wide analysis of gene expression in isolated human cardiomyocytes. Further research is warranted.

CONCLUSION

The present findings illustrate pronounced sex differences in gene expression in isolated human cardiomyocytes and a significant male-specific association between cardiac function and inflammation-related genes. Together, these factors might contribute to the transition of compensated LVH to HF in the male pressure-overloaded heart, thereby determining postoperative recovery and the development of symptomatic HF with depressed systolic function. Taking these sex differences into account may contribute toward a more accurate design of research and the development of more appropriate therapeutic approaches for both men and women.

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Drs Gaignebet and Kańduła contributed equally to this work.

SUPPLEMENTAL ONLINE MATERIAL

Supplemental material can be found online at http://www.mayoclinicproceedings.org. Supplemental material attached to journal articles has not been edited, and the authors take responsibility for the accuracy of all data.

Abbreviations and Acronyms: **AS** = aortic stenosis; **AVR** = aortic valve replacement; **EF** = ejection fraction; **HF** = heart failure; **LV** = left ventricular; **LVH** = LV hypertrophy; **NF-κB** = nuclear factor κB subunit 1; **PO** = pressure overload

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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