MEDIA: Executive Summary

- Diastolic Heart Failure (DHF) currently accounts for more than 50% of all Heart Failure (HF) cases and its importance relative to Systolic Heart Failure (SHF) continues to rise at an alarming rate of 1% per year.
- In contrast to SHF, no pharmacological treatment for DHF has consistently proven to ameliorate prognosis or symptoms. This failure has mainly been ascribed to a lack of understanding of the mechanisms operative in DHF.
- The MEDIA project hypothesized that DHF originates from deranged metabolism related to obesity, metabolic syndrome and type 2 diabetes, all of which give rise to a systemic inflammatory state that promotes interstitial collagen deposition and affects signaling from myocardial microvascular endothelial cells to adjacent cardiomyocytes, which therefore become hypertrophied and stiff.
- The MEDIA project largely succeeded in turning this hypothesis into an established paradigm by achieving the following results and foregrounds:
    - DHF could be induced in metabolically compromised small rodent or large pig animal models;
    - Myocardial microvascular inflammation and high cardiomyocyte stiffness were demonstrated in biopsy material of DHF patients;
    - Extent of epicardial fat deposition was shown to relate to diastolic LV dysfunction in patients;
    - Cardiomyocyte stiffness was impaired because of modified elastic properties of the giant cytoskeletal protein titin as a result of hypophosphorylation of specific sites, oxidative damage, stretch-induced damage and epigenetic effects;
    - Myocardial collagen deposition was attributed to dysregulation of the axis consisting of procollagen I carboxy terminal peptidase (PCP), procollagen I carboxy terminal peptidase enhancer (PCPE) and lysine oxidase (LOX)(i.e. the PCP-PCPE/LOX axis) in myocardial fibroblasts. Similar dysregulation was also observed for several matricellular proteins such as osteopontin;
    - Comprehensive RNAomics and miRNAomics in DHF myocardium showed differentially expressed mRNAs or miRNAs to be mainly involved in metabolism and inflammation;
    - Inflammatory biomarkers, especially endothelial cell adhesion molecules, have high predictive value for development of DHF in metabolic risk patients and for prognosis in DHF patients.
- Apart from these results, the MEDIA project also developed a stringent protocol for exercise stress echocardiography in DHF and paved the road for phenotype specific treatment of DHF patients by demonstrating efficacy of a cyclicGMP enhancing therapeutic strategy in DHF patients with combined pre- and postcapillary pulmonary hypertension (CpcPH phenotype).
- Because of these achievements, MEDIA is having a profound societal and socio-economic impact. In terms of societal impact, MEDIA altered the worldwide understanding of DHF which is now more and more appreciated as a syndrome where HF meets internal medicine and systemic inflammation in particular. The socio-economic impact of MEDIA is evident from the current exploitation of its results:
    - animal models for the pharmaceutical industry,
    - patents for effects of alfaB-crystallin on cardiomyocyte stiffness,
    - screening platforms for antifibrotic therapy,
    - protocols for exercise echocardiography,
    - DHF therapies for specific phenotypes.
- In conclusion: MEDIA showed DHF to be a manifestation of systemic inflammation induced by coexistent metabolic comorbidities. This finding has a profound impact on diagnosis and treatment of DHF.

MEDIA: Project Context and Main Objectives

Diastolic Heart Failure (DHF) or Heart Failure with preserved Ejection Fraction (HFpEF) currently accounts for more than 50% of all Heart Failure (HF) cases and its importance relative to Systolic Heart Failure (SHF) or Heart Failure with reduced Ejection Fraction (HFrEF) continues to rise at an alarming rate of 1% per year. The prognosis of DHF is equally grim compared to SHF with reported annual mortality rates averaging 10% per year. In contrast to SHF, no pharmacological treatment for DHF has consistently been proven to ameliorate prognosis or symptoms. This failure has been ascribed to a lack of understanding of the pathophysiology of DHF, to inadequate design of trials and to poor DHF patient recruitment into trials. The rising prevalence of DHF in Western societies is mirrored by the rising prevalence of obesity and type 2 diabetes mellitus. The MEDIA project therefore hypothesized that DHF originates form deranged metabolism related to obesity, metabolic syndrome, insulin resistance and type 2 diabetes mellitus. All the aforementioned conditions give rise to a systemic inflammatory state that affects signaling from myocardial microvascular endothelial cells to adjacent cardiomyocytes, which therefore become hypertrophied and stiff.

As lack of understanding of the underlying pathophysiology of DHF greatly contributes to the lack of appropriate treatment for DHF, providing proof of principle for the hypothesis underlying the MEDIA project was of paramount importance. Thanks to the 5 year research effort of MEDIA, the MEDIA hypothesis evolved into an established paradigm which gained widespread acceptance in the cardiological community as evident from the high number of citations (n=436) of the original MEDIA publication describing the novel paradigm for DHF (J Am Coll Cardiol 2013;62:263). The Figure summarizing the metabolic risk induced origin of myocardial dysfunction and remodeling in DHF is shown below (Figure 1) and has been reproduced in numerous articles and prestigious textbooks (e.g. Braunwalds textbook on Cardiology, partim Heart Failure). The large interest for the DHF paradigm proposed by MEDIA led several editors to invite the coordinator of MEDIA to write respectively an “In-depth State of the Art” review (Circulation 2016;134:73) and a “Perspective”review (Journal of Cardiac Failure 2016; December Issue) on DHF discussing the conceptual, diagnostic and therapeutic implications of the new DHF paradigm developed by the MEDIA project. It is fair to conclude that the MEDIA project has profoundly altered the worldwide appreciation of DHF which thanks to the MEDIA efforts is now considered to be a myocardial manifestation of systemic inflammation mainly related to coexistent metabolic comorbidities.

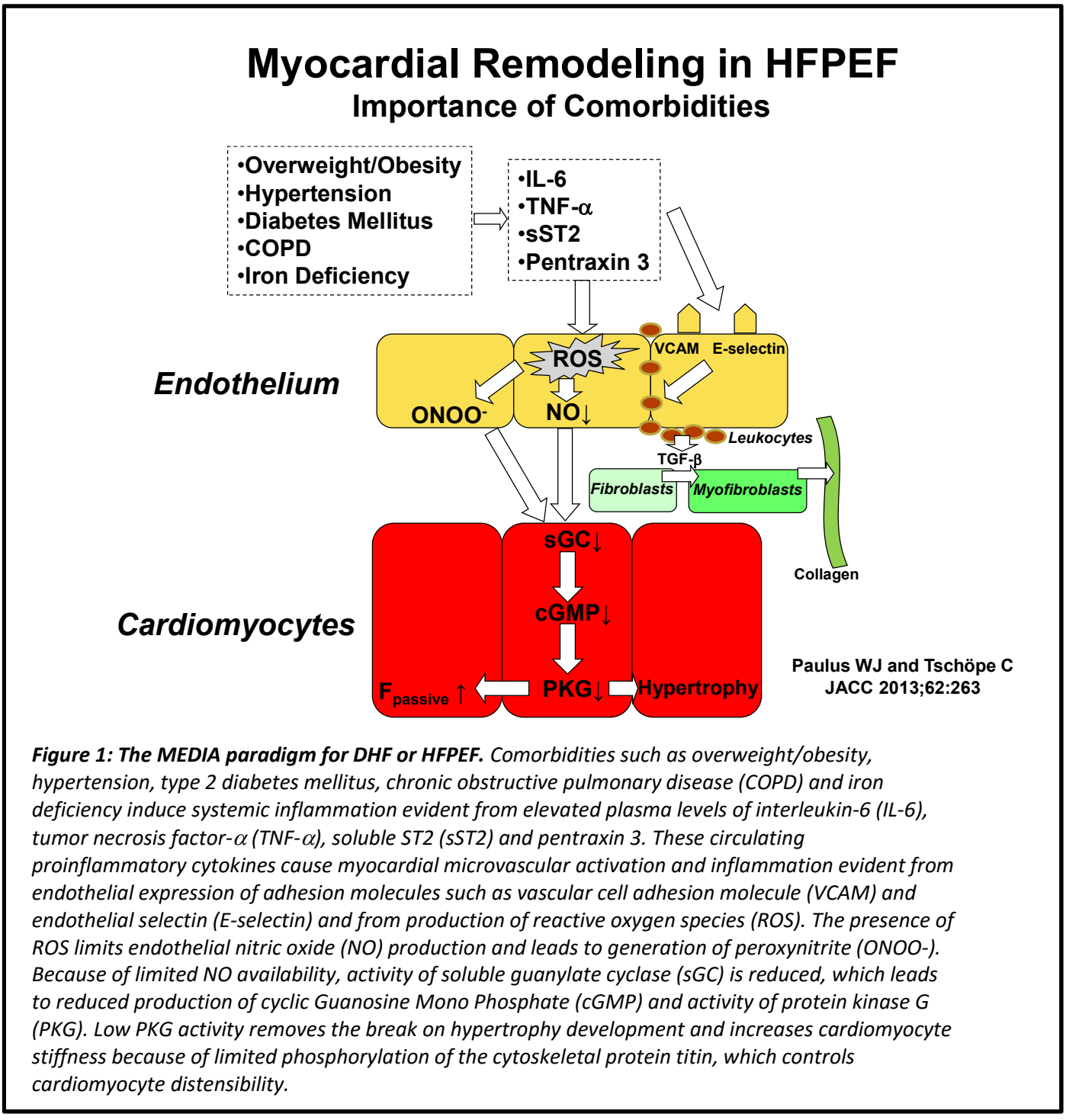


Figure 1: The MEDIA paradigm for DHF or HFPEF. *Comorbidities such as overweight/obesity, hypertension, type 2 diabetes mellitus, chronic obstructive pulmonary disease (COPD) and iron deficiency induce systemic inflammation evident from elevated plasma levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), soluble ST2 (sST2) and pentraxin 3. These circulating proinflammatory cytokines cause myocardial microvascular activation and inflammation evident from endothelial expression of adhesion molecules such as vascular cell adhesion molecule (VCAM) and endothelial selectin (E-selectin) and from production of reactive oxygen species (ROS). The presence of ROS limits endothelial nitric oxide (NO) production and leads to generation of peroxynitrite (ONOO-). Because of limited NO availability, activity of soluble guanylate cyclase (sGC) is reduced, which leads to reduced production of cyclic Guanosine Mono Phosphate (cGMP) and activity of protein kinase G (PKG). Low PKG activity removes the break on hypertrophy development and increases cardiomyocyte stiffness because of limited phosphorylation of the cytoskeletal protein titin, which controls cardiomyocyte distensibility.*

The main objectives of MEDIA were fourfold:

1. Development of animal models of DHF by subjecting rodents or pigs to metabolic risk.
2. Elucidation of mechanisms responsible for high myocardial stiffness involving both the cardiomyocytes and the interstitium.
3. Development of new diagnostic algorithms for DHF using plasma biomarkers or advanced imaging techniques.
4. Providing new therapeutic inroads for DHF.

1.Development of animal models of DHF by subjecting rodents or pigs to metabolic risk.

With respect to development of animal models of DHF, the MEDIA project succeeded to develop both a small rodent and large pig animal model. The rodent model consisted of obese ZSF1 rats which in contrast to their lean counterparts developed DHF after 20 weeks of unlimited access to food. The ZSF1 rats resulted from crossbreeding obese Zucker rats with spontaneously hypertensive rats. The model was extensively described by the MEDIA investigators (Circ Heart Fail 2013;6:1239) and currently used by multiple laboratories, both academic and pharmaceutical (Amgen, Ironwood..), as a valid model of DHF. The addition of a metabolic component to the arterial hypertension resulted in persistent concentric left ventricular (LV) remodeling in contrast to pure hypertensive rat models in which concentric LV remodeling always preceded eccentric LV remodeling. Apart from a rodent rat model, the MEDIA consortium also created a large animal DHF model consisting of pigs treated with a limited dose of streptozotocin and subjected to ¾ nephrectomy. Of interest in this model was the necessity to subject the animals to exercise for the elevation of LV filling pressures to become manifest. The use of exercise for the diagnosis of DHF was also clinically explored by the MEDIA investigators.

2.Elucidation of mechanisms responsible for high myocardial stiffness involving both the cardiomyocytes and the interstitium.

Hitherto, high myocardial diastolic LV stiffness was mainly attributed to interstitial deposition of collagen. The MEDIA project also intensively studied involvement of cardiomyocytes, which can also contribute to myocardial stiffness. Cardiomyocyte stiffness is determined by the giant cytoskeletal protein titin which modulates stiffness through isoform switches, posttranslational modifications, oxidative or stretch-induced damage. All these mechanisms were investigated by the MEDIA project. Studies on interstitial collagen deposition focused on procollagen I C terminal peptidase (PICP), procollagen I C terminal peptidase enhancer (PCPE), lysine oxidase (LOX) and the matricellular protein osteopontin. Animal and clinical investigations of MEDIA resulted in a clinical, biomarker based method for assessment of myocardial collagen cross-linking consisting of the ratio of collagen I terminal peptide/ matrix metalloproteinase-1 (CITP/MMP-1) (J Am Coll Cardiol 2016;67:251-260) and in the elucidation of comprehensive interactions between cystatin C, the matricellular protein osteopontin, LOX and collagen crosslinking. Furthermore, using a systems biology approach, the MEDIA project also evaluated the relative contributions of cardiomyocytes and interstitial collagen to overall myocardial stiffness in DHF. The relative contributions appeared to be highly variable with cardiomyocytes being the main contributor in the obese ZSF1 rat, collagen the main contributor in the pig DHF model and both cardiomyocytes and collagen being involved in DHF patients.

3.Development of new diagnostic algorithms for DHF using plasma biomarkers or advanced imaging techniques.

With respect to improvement of diagnostic algorithms, the MEDIA project assessed the usefulness of novel biomarkers and of advanced imaging techniques such as exercise-stress-echocardiography and cardiac late gadolinium enhancement (LGE) magnetic resonance imaging. The MEDIA project established two cohorts: the retrospective METR-DHF cohort (n=912) and the newly recruited MEDIA-DHF cohort (n=626). The METR-DHF cohort were patients with a metabolic risk factor. In the METR-DHF cohort, linkage of diastolic LV dysfunction to metabolic risk profile was present only when diastolic LV dysfunction was defined by the 2007 ESC consensus algorithm and not by the 2009 EAE/ASE recommendations. Development of diastolic LV dysfunction was clinically related to low glomerular filtration rate (eGFR) and use of loop diuretics or betablockers. Patients which developed diastolic LV dysfunction had significant elevations of galectin 3, procollagen III n-terminal peptide (PIIINP), procollagen I c-terminal peptide (PICP), C-reactive protein (CRP), N-terminal-pro brain natriuretic peptide (NT-proBNP), platelet-selectin (P-selectin), intercellular

adhesion molecule 3 (ICAM3) and endothelial-selectin (E-selectin). Addition of 4 biomarkers (PICP, NT-proBNP, E-selectin, ICAM3) raised the AUC of the ROC curve for diastolic LV dysfunction to 92.5%. In the newly recruited MEDIA-DHF cohort, the biomarker profile was fully supportive of the novel DHF paradigm in so far that prognosis (a composite of all cause death and cardiovascular hospitalization) was significantly related to low eGFR and elevated ICAM3 but not to NT-proBNP. The strong predictive value for unfavourable outcome of ICAM3 in contrast to NT-proBNP is an important finding as it again demonstrates the importance of systemic inflammation for DHF.

4.Providing new therapeutic inroads for DHF.

Three therapeutic inroads were investigated: 1) the effects of renal artery denervation on diastolic LV dysfunction and on LV hypertrophy were investigated using sequential MRI. In line with a similar study (Eur J Heart Fail 2016; doi:10.1002/ejhf.502), the outcome was neutral. The neutral outcome of a therapeutic manoeuver focusing on arterial hypertension again supports the hypothesis that DHF develops because of metabolic risk induced systemic inflammation and not because of myocardial overload; 2) the cGMP enhancing therapeutic strategy (cGETS) study enhanced myocardial cGMP content through simultaneous administration of statins, angiotensin converting enzyme inhibitors (ACEIs) and phosphodiesterase 5 inhibitors (PDE5I). The study mainly observed improved right ventricular performance because of pulmonary vasodilation; 3) the DROP-PIP DHF study, which looked at the antifibrotic effects of the diuretic torasemide was terminated on June 30th 2016 and its outcome is currently being assessed.

MEDIA: Main S & T Results/Foregrounds

1) Production of a novel rat DHF model.

- During the two initial years of MEDIA (from 1/1/2011 to 31/12/2012) a novel rat metabolic risk induced DHF animal model was developed within the MEDIA consortium. The model consisted of obese ZSF1 rats fed either a regular (D1.1) or a Western diet (D1.2). The ZSF1 rat model is the result of crossbreeding between spontaneously hypertensive rats and obese Zucker rats with leptin deficient receptors.
- The ZSF1 obese rat model of DHF was produced at the University of Porto (Portugal) and at the University of Antwerp (Belgium). It was published in 2013 in a joint publication of several MEDIA partners (UniPorto, VUMC, Pamplona, Charite, Antwerp) (Hamdani N et al. Circ Heart Fail 2013;6:1239). The uniqueness of the MEDIA model was highlighted in an accompanying editorial comment (Lewinter MM and Meyer M. Circ Heart Fail 2013;6:1112).
- The model displayed all structural features of concentric LV remodeling, myocardial hypertrophy and lung congestion as present in patients with DHF (Figure 2).

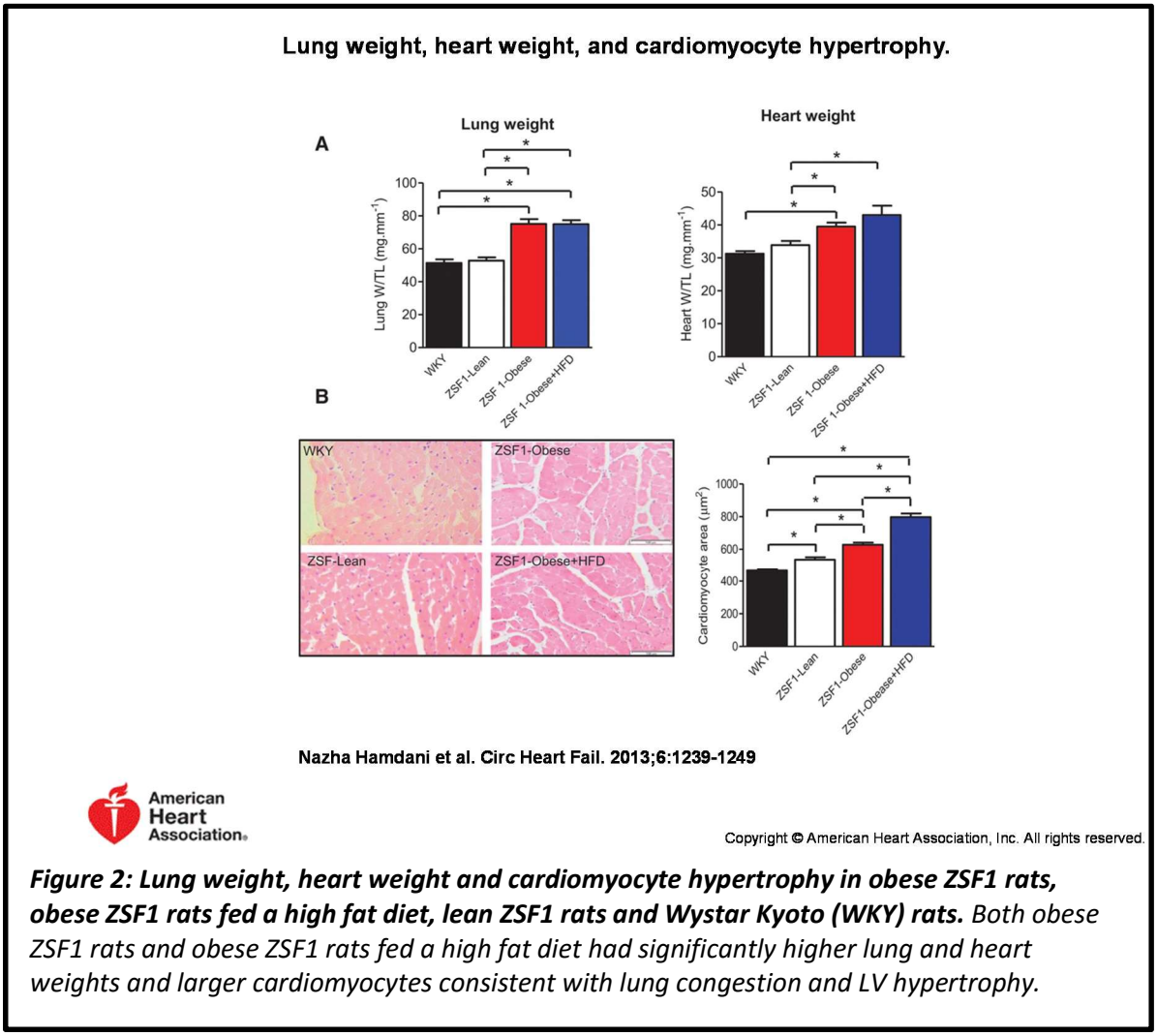


Figure 2: Lung weight, heart weight and cardiomyocyte hypertrophy in obese ZSF1 rats, obese ZSF1 rats fed a high fat diet, lean ZSF1 rats and Wystar Kyoto (WKY) rats. *Both obese ZSF1 rats and obese ZSF1 rats fed a high fat diet had significantly higher lung and heart weights and larger cardiomyocytes consistent with lung congestion and LV hypertrophy.*

- In the initial years, ZSF1 rats were sacrificed at a single time point (20 weeks of age). In 2013, additional time points of sacrifice (15 and 25 weeks) were also investigated. Apart from additional time points of sacrifice, ZSF1 rats were also treated for a period of 6 weeks (from week 14 to 20) with sildenafil (100mg/kg). At 20 weeks of age, sildenafil treated rats had less

diastolic dysfunction, improved myocardial energetics and better exercise tolerance than untreated animals.

- The ZSF1 rat model was also used in 2015 to explore coronary microvascular inflammation induced by metabolic comorbidities. The findings were published in JACC-HF (DOI: j.jchf.2015.10.007) and again accompanied by a favourable editorial comment written by Gomberg-Maitland M, Shah SJ and Guazzi M (Inflammation in heart failure with preserved ejection fraction: Time to put out the fire; JACC-HF 2016;4:325).
- The model is currently used by multiple laboratories, both academic and pharmaceutical (Amgen, Ironwood..) and is rapidly becoming the gold standard model of DHF. The addition of a metabolic component (i.e. insulin resistance) to arterial hypertension resulted in persistent concentric left ventricular (LV) remodeling in contrast to pure hypertensive rat models in which concentric LV remodeling is temporary as it always preceded eccentric LV remodeling.

2) Production of a novel pig DHF model.

- The MEDIA project succeeded in reproducing DHF in a large animal model consisting of diabetic pigs with renal insufficiency. In total, 25 pigs were treated and instrumented. The publication describing the studies in pigs is currently under revision for publication.
- As titin isoform composition differs between rodents (99% N2B; 1% N2BA) and man (70% N2B; 30% N2BA), a large animal model with a titin isoform composition resembling human titin was considered desirable (MEDIA DOW 5/46). This large animal model consisted of Yorkshire x Landrace pigs. The elaboration of this model was performed at Erasmus Medical Center, Rotterdam and proceeded in a stepwise fashion.
- Initially, 9 pigs were injected with streptozotocin (50 mg/kg/day) for 3 consecutive days and then fed an atherogenic high fat diet for 6 months without food restriction. At sacrifice, magnetic resonance imaging, invasive hemodynamics, blood samples and cardiac biopsies were obtained. Obese animals had clearcut evidence of metabolic syndrome as evident from elevated total cholesterol-triglycerides-glucose and low HDL cholesterol. Proinflammatory cytokines (IL-6, TNFα) were elevated consistent with the novel paradigm for DHF proposed by MEDIA. Responses of the microcirculation were determined with a reduced response to bradykinin, which was however not ascribed to lack of NO but to lack of EDHF (Endothelium Derived Hyperpolarizing Factor). Cardiomyocytes (30 cells per pig) were isolated from the biopsies. Cardiomyocyte resting tension was comparable but cardiomyocyte developed tension lower in the obese pigs. Collagen content was significantly higher in the obese animals. Magnetic resonance imaging revealed lower end-diastolic volume index and lower stroke volume index in the obese pigs which combined with unaltered LV end-diastolic pressure implied lower LV diastolic compliance.
- In 2014, the large animal pig model (>100 kg) was further elaborated. The major drawback of the pig model in relation to the clinical profile of DHF patients was the absence of arterial hypertension. In order to add arterial hypertension to the risk factor profile of the pigs, pigs underwent microcirculatory occlusion of the right kidney and of part of the left kidney using infusion of cospheric polyethylene spheres (3/4 nephrectomy). In these pigs mean arterial blood pressure rose from 75 to 115 mmHg 14 weeks following the renal microcirculatory occlusion procedure. Ten animals were treated. After 6 months the animals were sacrificed. Before sacrifice, MRI imaging and cardiac catheterization were performed. Blood samples and tissue samples were procured. The pigs had a severely deranged metabolic status with elevated glucose, cholesterol and triglycerides. Important steatosis of the kidneys was observed. Plasma levels of proinflammatory cytokines were elevated similar to DHF patients. Microvessels had blunted vasodilator responses. Oxidative stress was enhanced with eNOS uncoupling and enhanced NADPH stimulated superoxide production. In contrast to pigs

which were only made diabetic, cardiomyocyte resting tension was increased. Collagen content was enhanced.

- Finally, in 2015 some animals also underwent exercise testing which resulted in a whopping increase in LV filling pressures similarly to DHF patients subjected to exercise stress testing.

3) Acquisition of myocardial tissue of DHF patients.

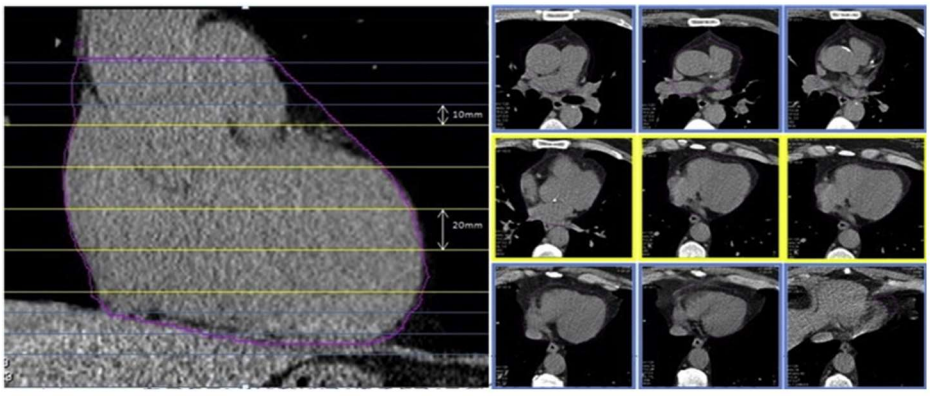
- Acquistion of myocardial tissue of DHF patients provided the MEDIA consortium with the unique possibility of directly exploring pathophysiological mechanisms in human myocardium. Because of the availability of myocardium of DHF patients, the MEDIA consortium collaborated with John Hopkins Hospital Baltimore for determination of phosphodiesterase 9 expression which appeared to be upregulated in human DHF myocardium (Nature 2015;519:472-476).
- Transvascular LV endomyocardial biopsies were procured from patients admitted to hospital because of worsening DHF, diagnosed in accordance to the HFA-ESC guidelines (Eur Heart J 2007;28:2539) and suspected of having restrictive cardiomyopathy (VUMC, CHARITE). In accordance to the AHA-ESC guidelines for biopsy procurement, suspicion of restrictive cardiomyopathy is a class IIa indication for biopsy procurement . Biopsies free of myocardial inflammation or infiltration were withheld for further investigations.
- Peroperative biopsies were procured in patients with aortic stenosis, who had evidence of diastolic LV dysfunction in accordance to the HFA-ESC guidelines (Eur Heart J 2007;28:2539) and who underwent aortic valve replacement (UNI-Porto). These biopsies consisted of tissue extracted from myectomy routinely performed to enlarge concomitant outflow tract narrowing (Morrow procedure).
- Normal human LV samples were obtained from rejected explanted donor hearts (VUMC; RUB). Use of rejected explanted donor hearts provided larger amounts of normal LV myocardial tissue for the MEDIA participants.
- The total number of DHF/HFPEF biopsies equalled n=244. The total number of AS biopsies equalled n=238.
- An overview of the number of biopsies procured and investigated by the consortium is provided in the Table with reference to the corresponding number in the publication list of MEDIA and to the DOI of the publication:

Number Publication Listing	Number DHF or HFPEF	Number SHF or HFREF	Number AS	Number Controls	DOI of the publication
1			62		10.1161/CIRCULATIONAHA.111.025270
16	67	43	67		10.1161/CIRCULATIONAHA.111.076075
31	21			10	10.1093/cvr/cvt100
36			34	18	10.1042/CS20120612
45	12		8	10	10.1038/nature14332
64				4	10.1016/j.freeradbiomed.2015.02.036
82	31			7	10.1161/HYPERTENSIONAHA.113.02654
83	39			7	10.1002/ejhf.246
86	38				10.1016/j.jacc.2015.10.063
93	36	43	67	4	10.1016/j.jchf.2015.10.007

4) Relation between epicardial fat and DHF

Obesity has been associated with subclinical left ventricular (LV) diastolic dysfunction and increased risk of heart failure. Few data are available on the relative contribution of adiposity distribution and changes in myocardial structure and function. We evaluated the influence of visceral versus

subcutaneous abdominal adipose tissue and epicardial fat on LV diastolic function after acute myocardial infarction. One month after acute myocardial infarction, 225 consecutive patients were prospectively enrolled and underwent anthropometric evaluation, bioimpedance analysis, detailed echocardiography, and multidetector 64-slice computed tomography scan for quantification of epicardial fat volume (EFV) (Figure 3) and of total, subcutaneous and visceral abdominal fat areas. A significant association was found between LV diastolic dysfunction parameters and body mass index, fat-mass percentage, and waist-to-height ratio. E' velocity and E/E' ratio were correlated with total and visceral abdominal fat (r=0.27, p<0.001 and r=0.21, p<0.01 respectively), but not with subcutaneous fat. After multivariate analysis, increasing EFV was associated with decreased E'velocity (adjusted β -0.11, 95% confidence interval -0.19 to 0.03, p<0.01) and increased E/E'ratio (adjusted β 0.19, 95% confidence interval 0.07 to 0.31, p<0.01). Patients with diastolic dysfunction showed higher EFV (116.7±67.9 ml vs. 93.0±52.3 ml, p=0.01) and there was a progressive increase in EFV according to diastolic dysfunction grades (p=0.001). None of the adiposity parameters correlated with ejection fraction or S' velocities. In conclusion, in patients after myocardial infarction, impaired LV diastolic function was associated with increased adiposity, especially with visceral and central fat parameters. Increasing EFV was independently associated with worse LV diastolic function (Am J Cardiol 2014;114:1663-1669).



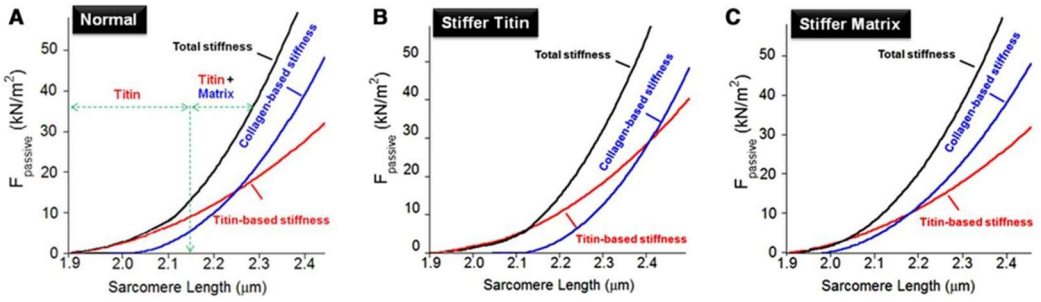
The American Journal of Cardiology, Volume 114, Issue 11, 2014, 1663–1669

Figure 3: Measurement of EFV by computer tomography. *The level of axial slices is shown in a coronal projection. Epicardial adipose tissue was identified within the limits of the pericardial sac. Pericardial contour was traced for every 10 mm, starting from the lower visible level of pulmonary artery bifurcation until the top level of the pulmonary valve, for every 20 mm from there until the first slice where the diaphragm becomes visible and for every 10 mm from this point until the last slice where pericardium is still visible. Final epicardial adipose tissue volume (EFV) was measured as the sum of all slices fat values.*

5) Titin Pathophysiology in DHF.

- The MEDIA consortium emphasized the importance of both cardiomyocyte stiffness and interstitial collagen deposition for total myocardial diastolic stiffness (Figure 4).

Titin- and matrix-based components of the myocardial passive-length tension relation.

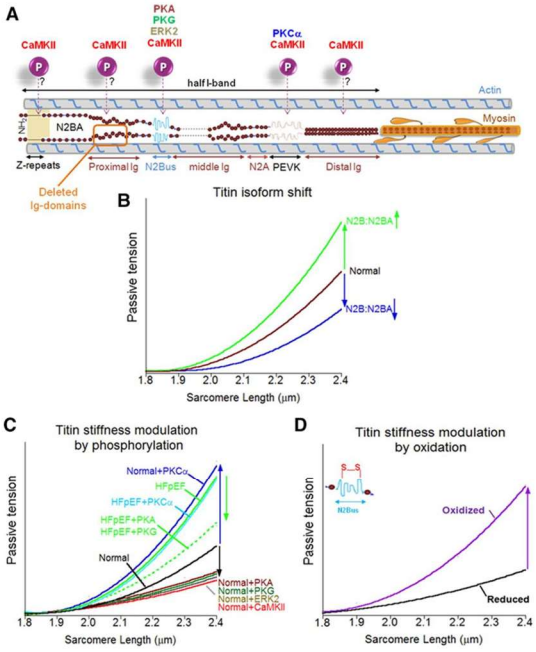


Nazha Hamdani, and Walter J. Paulus Circulation. 2013;128:5-8

Figure 4: Titin- and matrix-based components of the myocardial passive-length tension relation. *Myocardial passive-length tension relation and its components in normal myocardium (A), in the presence of a stiffer titin (B), and in the presence of a stiffer matrix (C). $F_{pressure}$ indicates the resting tension of isolated cardiomyocytes.*

- Cardiomyocyte stiffness is determined by the elastic properties of the giant cytoskeletal protein titin. These elastic spring properties can be modified by isoform shifts (Figure 5), posttranslational modifications such as phosphorylation and oxidation (Figure 5) and stretch-induced damage .

Adjustable spring properties of titin.



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American Heart Association.

Figure 5: *Adjustable spring properties of titin. A,Structural components and phosphorylation sites of N2BA titin; B through D, Modulation of cardiomyocyte stiffness (passive-length tension relation) by titin isoform shift (B), by titin phosphorylation (C), and by oxidation (D). CaMKII indicates calcium calmodulin dependent kinase II; ERK2, extracellular signal-regulated kinase-2; N2-Bus, unique sequence; P, phosphorylation site; PKA, protein kinase A; PKCα, protein kinase Cα; PKG, protein kinase G; and S, sulfide.*

a) Absence of Titin Isoform Shifts in DHF

- Titin isoform expression was characterized in the newly established obese ZSF1 rats. Titin isoform expression was determined at VUMC, Amsterdam and RUB in 25 myocardial samples of Wystar-Kyoto, Lean ZSF-1, Obese ZSF-1 and Obese ZSF-1 rats on Western Diet. Cardiomyocyte stiffness of isolated cardiomyocytes was determined in parallel to the titin proteomics (overall > 500 cardiomyocytes) at VUMC. Apart from titin isoform analysis, titin proteomics also included determination of titin phosphorylation. This was again determined in 25 samples from each group at VUMC and RUB. In the MEDIA ZSF1 model, no significant titin isoform shifts were observed compared to control Wystar Kyoto and lean ZSF1 rats. The increased cardiomyocyte stiffness observed in the obese ZSF1 rats resulted solely from titin hypophosphorylation. These results were reported in Circ Heart Fail 2013;6:1239.
- In 14 human DHF samples, no myocardial titin isoform shifts were observed in DHF patients and in DHF patients with diabetes-induced diastolic LV dysfunction. Again, titin hypophosphorylation was responsible for the increased cardiomyocyte stiffness. The data were extensively described in Circulation 2011;124:1151-1159. This manuscript resulted from a collaborative effort of VUMC, University of Porto and University of Debrecen.

b) Presence of Titin Hypophosphorylation in DHF

- In 2011, MEDIA investigators demonstrated diastolic LV dysfunction in elderly dogs with experimental hypertension to result from myocardial titin hypophosphorylation and to be corrected by short-term cGMP enhancing therapy with sildenafil and BNP (Circulation 2011;124:2882-91). MEDIA investigators also analysed phosphorylation of titin in a human model of DHF, namely aortic stenosis patients with diabetes mellitus. Cardiomyocytes of these patients were stiffer and the stiffness change could again be attributed to hypophosphorylation of titin (Circulation 2011; 124:1151)
- In 2012, PKG activity was compared in myocardial samples of patients with DHF, systolic heart failure (SHF) and aortic stenosis. This comparative analysis of myocardium of patients with aortic stenosis, DHF and SHF revealed high cardiomyocyte stiffness in DHF patients as a result of titin hypophosphorylation because of low PKG activity. The latter could be related to low NO bioavailability. These results were published (Circulation. 2012;126:830-9) and accompanied by a favourable editorial comment (Circulation 2012;126:797-9).
- In 2012, myocardial tissue of the ZSF1 rats (MEDIA-DHF model) became available. Apart from titin isoform analysis, titin proteomics also included determination of titin phosphorylation. Phosphorylation of the titin molecule was not only assessed as overall phosphorylation but also as site specific phosphorylation using site-specfic antibodies and some of the hypophosphorylated amino acids were actually identified (e.g. serine 3991). These results were published in Circ Heart Fail 2013;6:1239.
- Furthermore, the MEDIA consortium also investigated involvement of other kinases than PKG in the phosphorylation of titin. In this respect, the MEDIA consortium achieved a breakthrough in titin physiology by showing calcium/calmodulin dependent protein kinase II to be able to phosphorylate titin. This finding establishes a unique link between slow early diastolic calcium reuptake, calcium/calmodulin kinase activation and late diastolic LV distensibility (Hamdani N. Circ Res 2013;112:664).

c) Oxidative Stress Related Effects on Titin

- The MEDIA project also focused on oxidative changes of the myofilamentary protein titin. In the metabolic risk-induced DHF model developed by the MEDIA investigators, disulfide bounds formed by oxidation of sulfhydryl groups did not account for the observed rise in cardiomyocyte resting tension, which was ascribed to carbonylation of titin. There was a gradual increase in resting tension of cardiomyocytes from Wystar Kyoto, lean ZSF1, obese ZSF1 and obese ZSF1 with western diet. This increase was paralleled by increased carbonylation of titin. Exposure to Fenton greatly increased the extent of carbonylation.
- MEDIA also focused on carbonylation of other myofilamentary proteins in the ZSF1 DHF model. The investigators found no significant carbonylation of myosin or of actin using specific antibodies against carbonylated epitopes. Similarly, they observed no disulfide bounds using specific antibodies against sulfhydryl groups and looking at actin and myosin binding protein C. Furthermore, the carbonylation of titin was shown to depress active tension and to raise resting tension of isolated human cardiomyocytes. The antioxidant DTT was unable to correct either the decreased active tension or the raised resting tension. The data were extensively described in “Exp and Clin Cardiol 2013;12:202”.
- As an extension of this work, carbonylation of myofilaments was also investigated in a mouse myocardial infarction model. In contrast to the ZSF1 DHF rat model, infarcted myocardium displayed extensive carbonylation of actin and myosin heavy chain in both remote and infarcted myocardium (Cardiovasc Res 2014;101:108). These findings again convincingly demonstrate the site of oxidative stress to differ between DHF and SHF induced by unfavourable post myocardial infarction remodeling. In DHF, the oxidative stress within the cardiomyocyte compartment is limited to the giant myofilamentary protein titin, which accounts for diastolic distensibility whereas in SHF, it also involves actin and myosin, which are responsible for systolic contractile performance. These findings support the DHF

paradigm proposed by the MEDIA investigators which suggests DHF to result mainly from oxidative stress in the endothelial cell compartment and SHF from oxidative stress in the cardiomyocyte compartment (J Am Coll Cardiol 2013;62:263).

- Effects of oxidative stress on the unfolding and refolding characteristics of the IgG segments of the giant cytoskeletal protein titin were also investigated. The MEDIA consortium developed a unique system of atomic force microscopy (AFM), which allowed force of individual titin molecules to be measured at different molecule lengths. In the presence of oxidative stress unfolding and refolding of IgG segments was profoundly altered with loss of stepwise unfolding and absence of appropriate refolding. The effects were caused by glutathionylation of cryptic cysteines within the IgG domain of titin. These observations were published in the prestigious journal Cell and credit the FP7 MEDIA programme (Cell 2014;156:1235).

d) The Small Heat Shock Protein α-B Crystallin Restores Stretch-Induced Damage of Titin

- Cardiomyocytes (CM) with a less distensible titin and interstitial collagen contribute to the high diastolic stiffness of failing myocardium. Their relative contributions and mechanisms underlying loss of titin distensibility were assessed in failing human hearts.
- Left ventricular tissue was procured in patients with aortic stenosis (AS, N=9) and in patients with dilated cardiomyopathy (DCM, N=6). Explanted donor hearts (N=8) served as controls (Ctrl).
- Stretches were performed in myocardial strips and an extraction protocol was applied to differentiate between passive tension ($F_{passive}$) attributable to cardiomyocytes or to collagen. $F_{passive}$-Cardiomyocytes was higher in AS and DCM especially at shorter muscle lengths whereas $F_{passive}$-Collagen was higher in AS and DCM especially at longer muscle lengths.
- Cardiomyocytes were stretched to investigate titin distensibility. Cardiomyocytes were incubated with alkaline phosphatase (AP), subsequently reassessed after a period of prestretch and finally treated with the small heat shock protein α-B crystallin. AP shifted the $F_{passive}$-sarcomere length (SL) relation upward only in Ctrl. Prestretch shifted the $F_{passive}$-SL relation further upward in Ctrl and upward in AS and DCM. Alfa-B crystallin shifted the $F_{passive}$-SL relation downward to baseline in Ctrl and to lower than baseline in AS and DCM (Figure 6). Only in failing myocardium did confocal lasermicroscopy reveal α-B crystallin in subsarcolemmal aggresomes.
- High CM stiffness contributes to stiffness of failing human myocardium and relates to reduced titin distensibility. The latter was not improved by in-vivo phosphorylation status, resulted from damage incurred by prior in-vivo stretch and was corrected by α-B crystallin. These findings open the perspective on treatment of diastolic LV dysfunction through induction or administration of small heat shock proteins such as α-B crystalline.

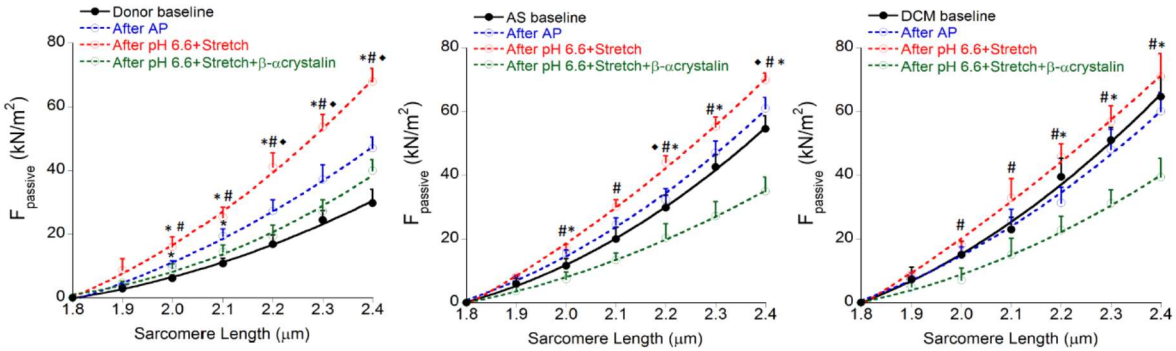


Figure 6: Effects of Stretch and α-B crystalline on $F_{passive}$ in single cardiomyocytes. *A: In donor cardiomyocytes, $F_{passive}$ significantly increased after administration of alkaline phosphatase and increased further after performing a prestretch in an acidic environment. After in vitro administration of α-B crystallin, $F_{passive}$ fell to a position slightly lower than after AP. B: In AS cardiomyocytes, no significant change in $F_{passive}$ was observed after incubation with AP. After the prestretch in pH 6.6, $F_{passive}$ increased significantly compared to baseline, but in vitro treatment with α-B crystallin lowered $F_{passive}$ to a level significantly lower than baseline. C: In DCM cardiomyocytes, incubation with AP had no effect on $F_{passive}$, but performing a prestretch in an acidic environment significantly increased passive stiffness. After in vitro treatment with α-B crystallin, $F_{passive}$ fell to a level significantly below baseline.*
** P<0.05 AP vs Baseline; # P<0.05 pH 6.6 + prestretch vs AP; ǂ P<0.05 α-B crystallin vs pH 6.6 + prestretch; § P<0.05 α-B crystallin vs baseline.*

6) Epigenetic effects of metabolic risk on DHF myocardium.

The pivotal role of CaMKII activation for epigenetic control, titin phosphorylation and maladaptive remodeling became firmly established by the activities of the MEDIA consortium. This involved demonstration of *a) Effects of CaMKII on titin*; *b) Effects of CaMKII on HDACs*, which exert epigenetic control of gene expression; *c) Effects of CaMKII on calcineurin/NFAT signaling*; *d) Effects of Smyd proteins on titin and on epigenetic control*.

a) Effects of CaMKII on titin.

- Expression of SERCA in the myocardium of ZSF1 rats, the MEDIA DHF model, was low.
- Low expression of SERCA increased diastolic Ca2+ in the cytosol of cardiomyocytes and triggers CaMKII activation.
- CaMKII was shown to be able to phosphorylate titin and to alter cardiomyocyte resting tension.
- The precise phosphorylation sites of CaMKII on the titin molecule were identified.
- In failing human myocardium increased CaMKII dependent phosphorylation of titin was demonstrated.

b) Effects of CaMKII on HDAC, which exerts epigenetic control of gene expression.

- CaMKII and PKA exert antagonistic roles on gene expression via proteolysis of HDACs which exert epigenetic control of gene expression.
- PKA was shown to exert its protective effect on HDAC activity by altering lipolysis. This was demonstrated in a ABHD5 knock-out mice. ABHD5 is a protein associated with lipid droplets.

HDAC knock-out mice were created. These mice had transient diastolic dysfunction during exercise stress and altered metabolism evident from increased aldolase activity during exercise and increased perilipin. From these experiments it appears that altered epigenetic control by HDACs affects expression of genes involved in metabolic risk.

- In line with these findings, effects of CaMKII knock-out on insulin signaling and on cardiac performance were also demonstrated.

c) Effects of CaMKII on calcineurin/NFAT signaling.

- The MEDIA consortium also explored the role of CaMKII in a double knock-out mouse lacking the 2 cardiac CaMK genes δ and γ. By the use of this model, it was shown that CaMKII induces maladaptive cardiac remodeling while it inhibits calcineurin_dependent hypertrophy.

d) Effects of Smyd proteins on titin and on epigenetic gene control.

- Protein lysine methylation controls gene expression and repair of deoxyribonucleic acid in the nucleus and also occurs in the cytoplasm, where the role of this posttranslational modification is less understood. Members of the Smyd protein family of lysine methyltransferases are particularly abundant in the cytoplasm, with Smyd1 and Smyd2 being most highly expressed in the heart and in skeletal muscles.
- Smyd2 methylates histones and non-histone proteins, such as the tumor suppressors, p53 and retinoblastoma protein, RB. Smyd2 was shown to have an intriguing function in the cytoplasm of myocytes, where it methylates the chaperone Hsp90, thus promoting the interaction of a Smyd2–methyl-Hsp90 complex with the N2A-domain of titin. This complex protects the sarcomeric I-band region and myocyte organization.
- MEDIA provided experimental evidence that Smyd2 is also important for cardiac function. In the cytoplasm of cardiomyocytes, Smyd2 was found to associate with the sarcomeric I-band region at the titin N2A-domain.
- Binding to N2A occurred in vitro and in yeast via N-terminal and extreme C-terminal regions of Smyd2.
- Smyd2-knockdown in zebrafish using an antisense oligonucleotide morpholino approach strongly impaired cardiac performance.
- MEDIA therefore concluded that Smyd2 and presumably several other Smyd family members are lysine methyltransferases which have, next to their epigenetic effects on histone methylation, specific regulatory functions via association with Hsp90 and titin N2A domain.

7) Dysregulation of the PCP-PCPE/LOX axis and Myocardial Collagen Deposition in DHF.

- Clinical data supported a role for increased activity of procollagen I carboxy terminal peptidase (PCP), procollagen I carboxy terminal peptidase enhancer (PCPE) and lysine oxidase (LOX) (i.e. the PCP-PCPE/LOX axis) in the development of myocardial fibrosis in patients with heart failure and reduced LV EF (HFrEF). In these patients, torasemide treatment lowered PCP-PCPE/LOX axis activity, reduced myocardial fibrosis and improved diastolic LV function or clinical outcome.
- The MEDIA consortium therefore investigated myocardial PCP-PCPE/LOX axis activity in patients with DHF related to hypertension, diabetes, obesity or combinations thereof.
- Assessment of PCP-PCPE/LOX activity was performed *in ZSF1 rats* and *in DHF patients* and related to invasive and non-invasive diastolic LV function data. The latter allowed for a comprehensive assessment of myocardial PCP/PCPE/LOX axis dysregulation, myocardial fibrosis and diastolic LV dysfunction.

ZSF1 rats

- As shown in figure 7 below, myocardial collagen volume fraction and collagen crosslinking were unaltered in the ZSF1 rat model. Similarly, no changes were observed in expression of PCP and PCPE.

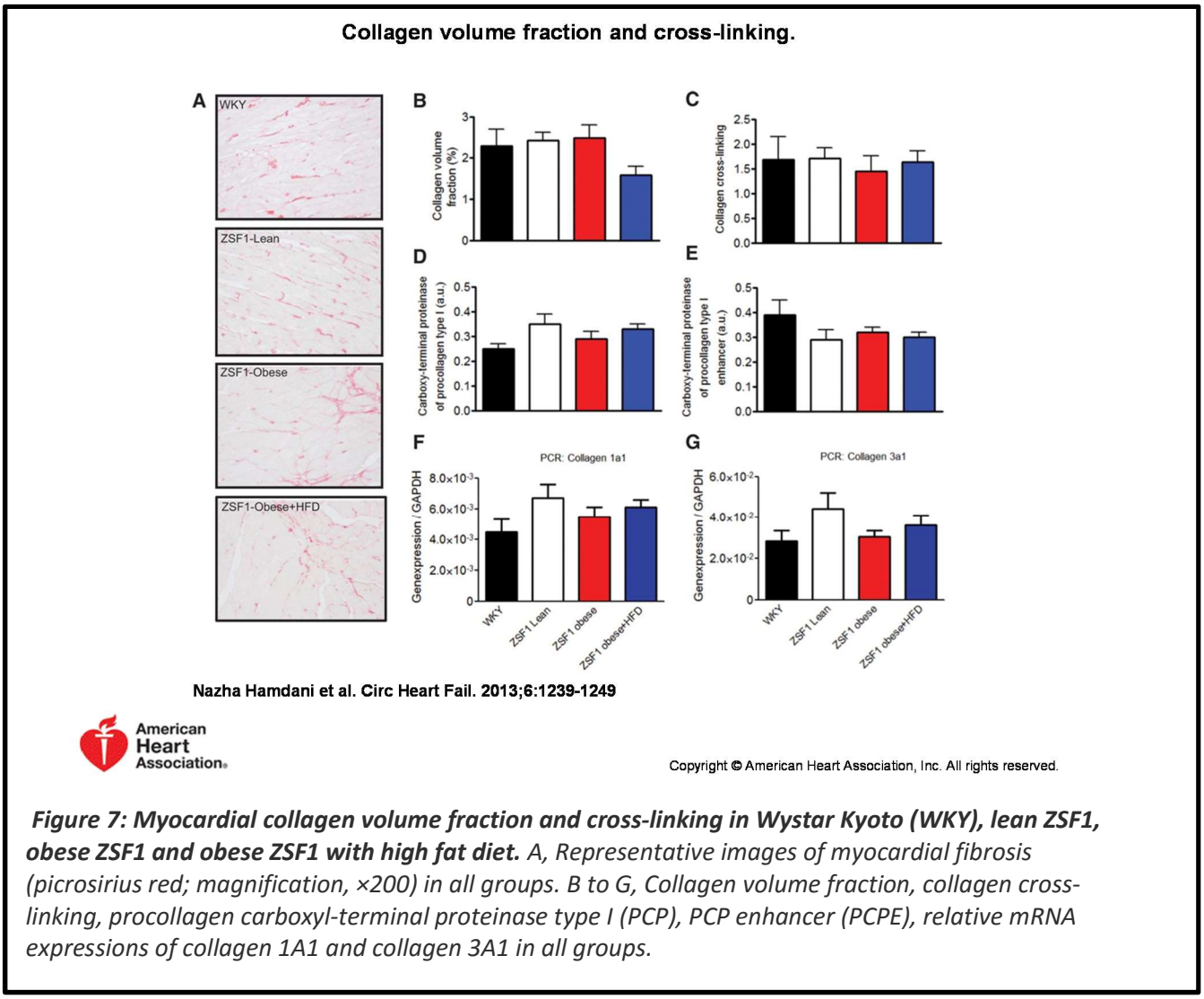
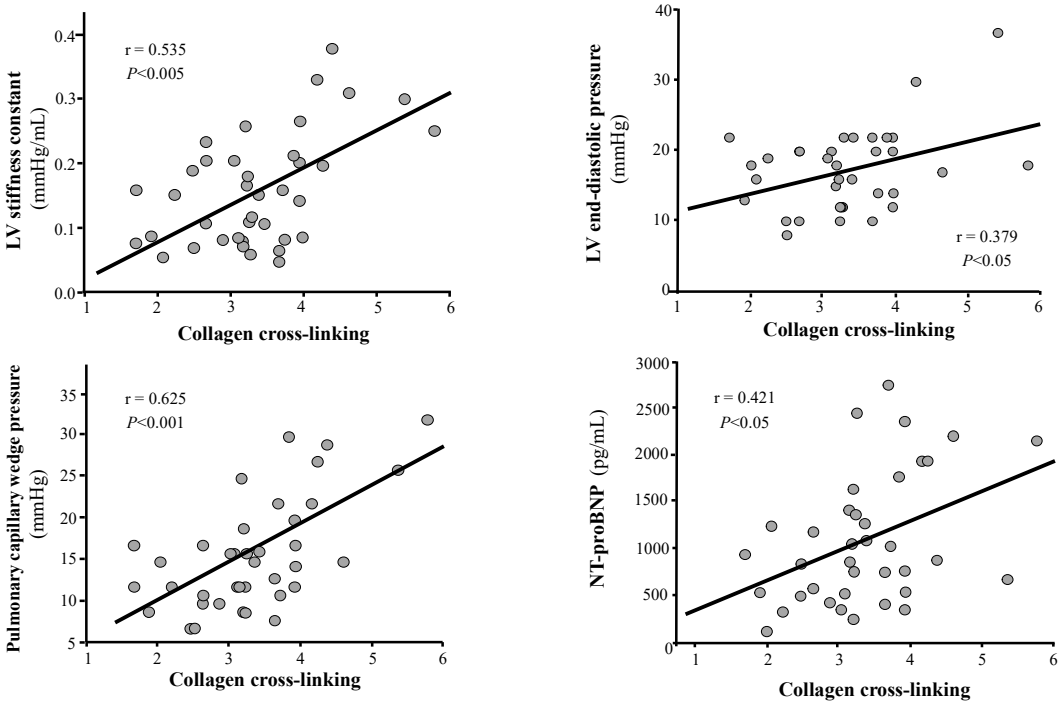


Figure 7: Myocardial collagen volume fraction and cross-linking in Wystar Kyoto (WKY), lean ZSF1, obese ZSF1 and obese ZSF1 with high fat diet. *A, Representative images of myocardial fibrosis (picrosirius red; magnification, ×200) in all groups. B to G, Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP), PCP enhancer (PCPE), relative mRNA expressions of collagen 1A1 and collagen 3A1 in all groups.*

DHF patients

- In a series of publications from the MEDIA investigators, the following observations were made concerning myocardial collagen crosslinking, LOX activity and effects of osteopontin on LOX activity in DHF/HFPEF patients:
  - Collagen crosslinking is related to diastolic LV dysfunction in HFPEF (Figure 8);
  - Collagen crosslinking can be assessed noninvasively by a ratio of biomarkers namely CITP/MMP1 (Figure 9) (J Am Coll Cardiol 2016;67:251);
  - The matricellular protein osteopontin stimulates LOX and cystatin C facilitates the extracellular accumulation of osteopontin (Figures 10-11). The latter is especially relevant to renal insufficiency with elevated cystatin C levels.

Association of collagen cross-linking with LV mechanics and hemodynamics in patients with HF of hypertensive etiology *

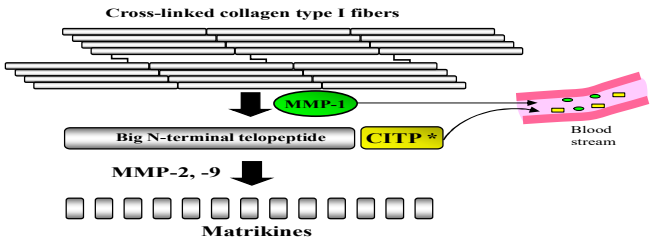


* Framingham+ESC criteria NYHA II-IV. Age range: 50-75 y. Men 70%.
All with HHD. No DM, IHD.
LV stiffness constant was calculated from the T_{DEC} (*AJP* 2001;280:H554-H61)
Pressures were measured invasively. NT-proBNP was measured by ELISA

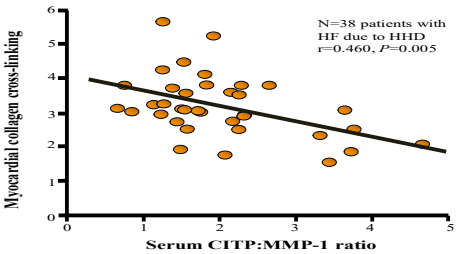
(Data obtained after review of López B et al, *Hypertension* 2009;53:236-242; *Hypertension* 2012;60:677-683; *Cardiovasc Res* 2013;99: 111-120; *J Am Coll Cardiol* 2015 [in press])

Figure 8: Collagen crosslinking is related to diastolic LV dysfunction in HFPEF. *Collagen crosslinking is significantly related to diastolic LV stiffness, LV end-diastolic pressure, pulmonary capillary wedge pressure and NT-proBNP in DHF patients with arterial hypertension.*

Histologic validation of a circulating biomarker of collagen type I cross-linking in patients with HF of hypertensive etiology



Rationale
The higher is the degree of collagen cross-linking the higher will be the resistance of the collagen type I fiber to degradation by MMP-1 and, thus, the lower will be the generation of CITP.
This can be captured by a low serum CITP:MMP-1 ratio

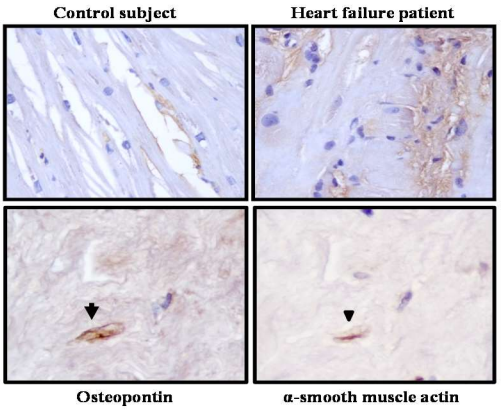


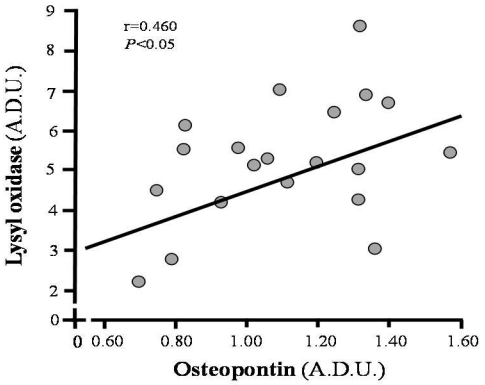
* CITP, C-terminal telopeptide of collagen type I

(López B et al, *J Am Coll Cardiol* 2015 [in press])

Figure 9: Noninvasive evaluation of myocardial crosslinking by the ratio of collagen I telopeptide/ matrix metalloproteinase 1 (CITP/MMP1).

Myocardial osteopontin in patients with HF of hypertensive origin *





* Framingham+ESC criteria
NYHA II-IV.
All with HHD. No IHD or DM
(64.4 ys, [CI 58.89-68.97])

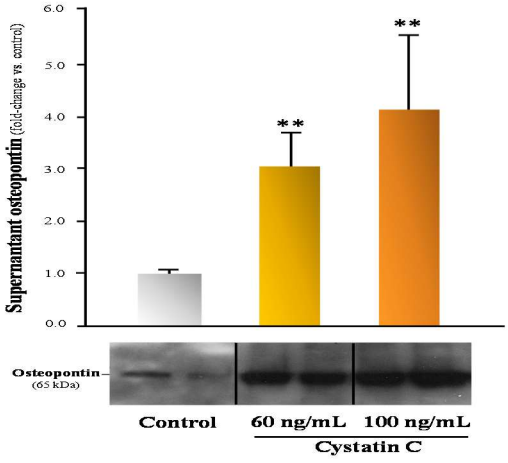
(López B et al, *Cardiovasc Res* 2013;99:111-120)

Figure 10: Relation between Lysyl Oxidase (LOX) and Osteopontin in myocardium of DHF patients.

Cystatin C facilitates extracellular accumulation of osteopontin in cultured primary human cardiac fibroblasts

	Control	Cystatin C		*P*
		60 ng/mL	100 ng/mL	
Intracellular osteopontin				
mRNA (A.D.U.)	1.01±0.03	0.93±0.06	1.13±0.08	NS
Protein (A.U.)	1.01±0.06	1.02±0.11	1.24±0.23	NS

Values are expressed as M±SEM (N≥10)



(Huerta A et al, *J Hypertens* 2016;34:130-138)

Figure 11: Accumulation of Osteopontin in relation to Cystatin C concentration.

8) Dysregulation of Matricellular Proteins in DHF Myocardium.

- Because of their role as an interface between matrix and cardiomyocytes, dysregulation of matricellular proteins is an important mechanism for diastolic LV dysfunction. This dysregulation was studied by the MEDIA consortium in experimental models and in patients with DHF.
- MEDIA investigated expression of matricellular proteins in myocardium of obese ZSF1 rats (MEDIA DHF model). mRNA expression and protein expression of thrombospondin 2, thrombospondin 4, osteoglycin, periostin and SPARC were determined. The matricellular proteins thrombospondin-2 and SPARC were not upregulated. Periostin, osteoglycin and thrombospondin-4 were upregulated in the obese ZSF1 rats. Osteoglycin was 1.3 times higher. The expression of periostin was 2.7 times higher.
- From 2013 onwards, the MEDIA group further focused on the role of osteoglycin and osteopontin. In osteoglycin knock-out mice, infarct healing was impaired and collagen crosslinking reduced. At 1 year of age, osteoglycin knock-out mice therefore have larger LV diastolic and systolic volumes. These investigations on the role of osteoglycin resulted in a publication that acknowledges MEDIA (Circ Res 2015;116:425).
- Osteoglycin interacts with the Toll-like 4 receptor. Administration of osteoglycin thereby limits inflammation and knock-out of osteoglycin increases inflammation. These effects were investigated in osteoglycin knock-out mice at different ages and following chronic infusion of subhypertensive doses of angiotensin II. As a result of diminished inflammation, osteoglycin also reduces fibrosis and diastolic LV dysfunction. These effects were again more outspoken following chronic infusion of angiotensin II in subhypertensive doses.
- Clinical investigations on matricellular proteins were also pursued by MEDIA. An excess of osteopontin was shown to be associated with increased LOX and insoluble collagen, as well as with LV stiffness in patients with HFPEF. In addition, osteopontin was shown to up-regulate LOX in human fibroblasts. It was therefore suggested that the osteopontin–LOX axis might facilitate the formation of insoluble collagen (i.e. stiff and resistant to degradation) and the subsequent alteration in LV diastolic properties and function in patients with HFPEF (López B et al., *Cardiovasc Res* 2013;99:111-120).

9) RNAomics and miRNAomics in DHF

- The myocardium of control Wystar Kyoto, lean ZSF1 rats, obese ZSF1 rats and obese ZSF1 rats exposed to Western diet was analysed by RNA Agilent array for mRNAs analysis and by microRNA array for microRNAs analysis. The array analyses were implemented by validation qPCR, which recapitulated the array data (R^2=0.75) (see Figure 12).

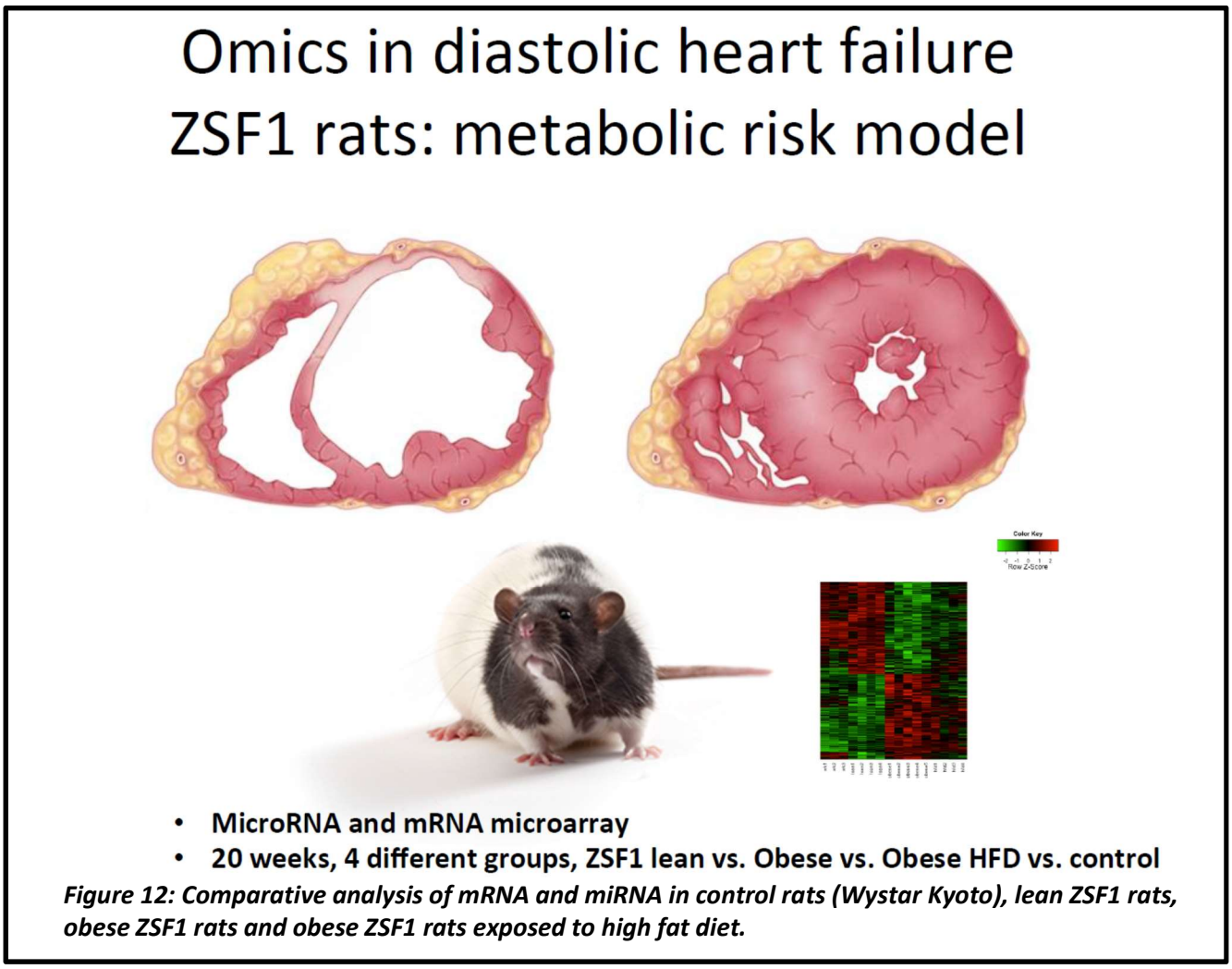


Figure 12: Comparative analysis of mRNA and miRNA in control rats (Wystar Kyoto), lean ZSF1 rats, obese ZSF1 rats and obese ZSF1 rats exposed to high fat diet.

- The differential regulation of mRNAs is shown in the Table below.

Differential Regulation mRNA

- Time point: 20 weeks
- Overall 13,391 genes expressed > 1 counts/million reads
- Significant if:
  - adjusted p-value (FDR) < 0.05

	Obese vs Lean	Obese HFD vs Lean	Obese HFD vs Obese
Total	**1572**	2022	193
Upregulated	**748**	977	86
Downregulated	**824**	1045	107

- At 20 weeks, expression of 13391 genes was analysed. Significant differential expression was observed for 1572 genes when comparing obese vs. lean animals. Differential upregulation was observed for 748 genes and differential downregulation for 824 genes. Significant differential expression was observed for 2022 genes when comparing obese animals exposed to Western diet to lean animals with upregulation of 977 genes and downregulation of 1045 genes. Significant differential expression was observed in 193 genes when comparing obese animals exposed to Western diet to obese animals given regular rat chow with upregulation of 86 genes and down regulation of 107 genes.
- The differential regulation of microRNAs is shown in the Table below.

Differential Regulation miRNA

- Time point: 20 weeks
- Overall 308 miRNAs expressed > 10 reads in min 3 samples
- Significant if:
  - p-value < 0.05

	Obese vs Lean	Obese HFD vs Lean	Obese HFD vs Obese
Total	**32**	21	17
Upregulated	**14**	13	12
Downregulated	**18**	8	5

- At 20 weeks, expression of 308 miRNAs was analysed. Significant differential expression was observed for 32 genes when comparing obese vs. lean animals. Differential upregulation was observed for 14 genes and downregulation for 18 genes. Significant differential expression was observed for 21 genes when comparing obese animals exposed to Western diet to lean animals with upregulation in 14 genes and downregulation in 18 genes. Significant differential expression was observed in 17 genes when comparing obese animals exposed to Western diet to obese animals given regular chow with upregulation of 12 genes and downregulation of 5 genes.
- Using RNA-sequencing technique (RNA-seq) and principal component analysis (PCA), clustering of gene expression was observed for mRNAs but not for microRNAs (see Figure 13).

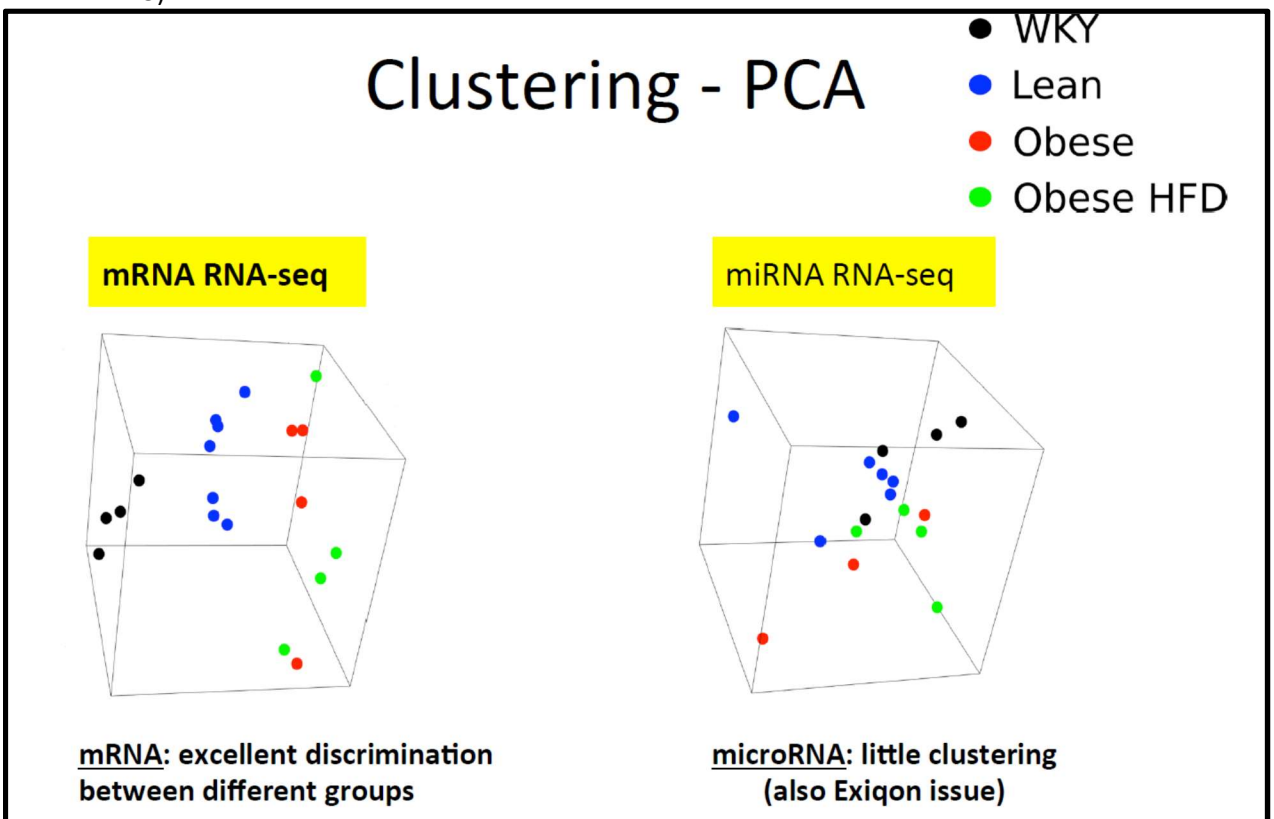


Figure 13: Clustering of differentially expressed mRNAs and miRNAs in myocardium of Wystar Kyoto rats (WKY), lean ZSF1 rats, obese ZSF1 rats and obese ZSF1 rats exposed to high fat diet.

- The differentially expressed mRNAs were mainly involved in inflammation and metabolism (fatty acid metabolism, glucose metabolism and insulin signaling). Genes involved in fibrosis were notoriously absent. Similarly, among the differentially expressed miRNAs, miRNA involved in glucose metabolism were also present such as miR-103 and miR-107. MiR-103 and miR-107 differ only by 1 nucleotide outside the functionally important seed sequence and have been described as metabolic miRs regulating insulin and glucose homeostasis in the liver. Their role in the heart is currently unknown. We hypothesize that cardiac miR-103/-107 regulate the metabolic switch during (diastolic) heart failure. The metabolic switch occurring in chronic HF was mimicked by exposing isolated cardiomyocytes to normoxia or hypoxia in the absence or presence or miRNA mimics. Increasing the levels of miR-103/-107 in cardiomyocytes resulted in a marked downregulation of several metabolic candidate genes. Next, signaling pathways that may be involved in the differential regulation of metabolic gene expression were investigated, and we found evidence for increased AMP-kinase activity in cardiomyocytes transfected with miR-103/107 mimics. When cardiomyocytes transfected with miR-103/-107 mimics were exposed to 24 h hypoxia, a 20% increase in the effect of insulin on glucose uptake was detected. The collective findings suggest that miR-103/-107 are involved in cardiomyocyte glucose uptake and utilization.
- In conclusion, miRNA and RNA omics were performed in the MEDIA model of DHF, the obese ZSF1 rat. Differential expression of numerous miRNAs and RNAs was established most of them involved in inflammation and metabolism consistent with the prominent role of microvascular inflammation and metabolic comorbidities in DHF.

10) Importance of Inflammatory Plasma Biomarkers for Development and Prognosis of DHF

- The MEDIA project established two cohorts: the retrospective METR-DHF cohort (n=912) and the newly recruited MEDIA-DHF cohort (n=626). The METR-DHF cohort were patients with a metabolic risk factor.
- After extensive deliberations a list of biomarkers was established that was determined in the plasma samples of the METR-DHF cohort. The biomarkers that were selected corresponded to the new paradigm for DHF proposed by the MEDIA investigators, which relates LV remodeling and dysfunction in DHF to extracardiac comorbidities via coronary microvascular inflammation (J Am Coll Cardiol 2013;62:263). Biomarkers analysed are listed in the Table below.

Final selection of biomarkers (METR-DHF)

Neurohormonal activation
- Aldosterone
- ADMA

Inflammation
- hCRP
- IL 6, 8
- TNF-α
- MCP-1
- ST2 receptor
- Pentraxin-3

Cardiomyocyte injury
- Troponin T (cTnI)

Endothelial dysfunction
- Soluble E-selectin
- ICAM-3
- P-Selectin
- Thrombomodulin

Fibrosis
- PICP, PIIINP, CITP
- MMP2, MMP9
- Galectin -3
- NGAL

Cardiomiocyte growth
- GDF - 15
- Cardiotrophin-1
- Nt-proBNP

- Data analysis yielded the following results:
  - Significant elevations were observed for: Galectin 3, PIIINP, PICP, CRP, NT-proBNP, P-selectin, soluble ICAM3. Significant reductions were observed for: E-selectin.

- Based on clinical profiling, the AUC of the ROC curve was 91% for development of diastolic LV dysfunction. Addition of 4 biomarkers (PICP, NT-proBNP, E-selectin, ICAM3) raised the AUC value to 92.5%.

- An array of biomarkers was also determined in the newly recruited DHF cohort (n=625). The biomarkers were the same as the biomarkers determined in the METR-DHF cohort. Biomarkers were evaluated for their prognostic value (all cause death and cardiovascular hospitalization). Clinical predictors for all cause death and cardiovascular hospitalization were diastolic blood pressure (p=0.018) and glomerular filtration rate (p<0.0002). Only ICAM-3>150 ng/ml significantly improved the predictive value of the clinical model in the overall population as indicated in the Table below.

Table 8: C-index, increase of c-index, IDI at 1 year and cNRI at 1 year for biomarkers in overall population

	Events/ patients	C-index	Δ C-index		IDI at 1 year		cNRI at 1 year	
			Indice (CI 95 %)	p-value	Indice (CI 95 %)	p-value	Indice (CI 95 %)	p-value
Clinical model	116/536	0.608	-	-	-	-	-	-
Clinical + E-Selectin > 20 ng/ml	111/524	0.624	0.023 (-0.009 to 0.054)	0.17	1.1 % (-0.1 to 3.6 %)	0.080	10.5 % (-0.1 to 20.6 %)	0.056
Clinical + ICAM-3 > 150 ng/ml	111/524	0.640	0.038 (0.014 to 0.062)	0.002	-0.2 % (-0.6 to 1.4 %)	0.72	5.2 % (-3.4 to 15.9 %)	0.24

Clinical model contained baseline clinical settings.
IDI: Integrated Discrimination Improvement; cNRI: continuous Net reclassification Improvement; CI: confidence interval.

11) Protocol for Exercise Stress Echocardiography in DHF

- A common exercise protocol using a two stage ramped cycling exercise with echocardiographic imaging intermission has been finetuned by the MEDIA investigators and is shown in Figure 14.

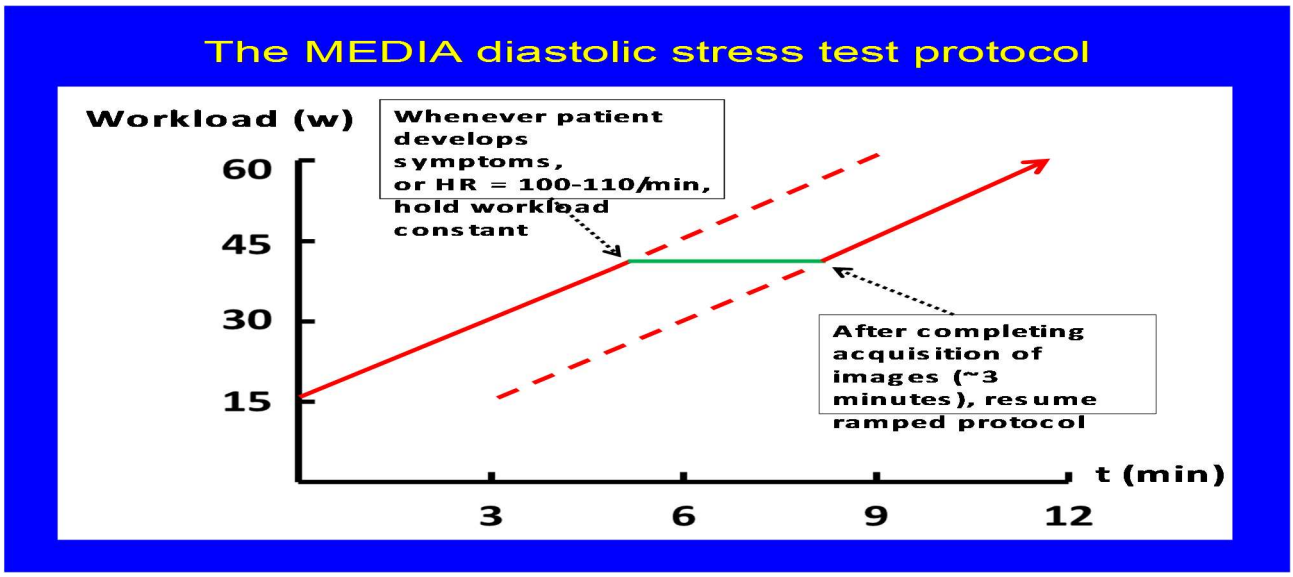


Figure 14: Echocardiographic exercise stress test protocol suitable for diagnosis of DHF.

- Using this protocol, reduced functional reserve during exercise in DHF was investigated. Furthermore, MEDIA investigated which Dopplerechocardiographic indices best identified an abnormal response to exercise in DHF.

- To establish an abnormal response to exercise in DHF, DHF patients diagnosed in accordance to the 2007 ESC consensus document were compared to breathless controls and to healthy controls. The baseline echocardiographic characteristics were comparable between the breathless and healthy controls. DHF patients had higher E/e', (Ard-Ad) and NTproBNP. The investigators observed an important finding namely that a simple index such as the increase in e' (Δe'avg) provided the best discrimination between DHF and healthy controls in contrast to the more frequently used ΔE/e'avg. A similar discriminatory effect was also observed for long-axis shortening velocity (Δs'). This finding has important implications for the use of exercise stress testing as diagnostic tool in DHF.
- These investigations were published (Eur J Heart Failure 2014;16:1345). This publication provides important guidelines for the execution of exercise stress echocardiography for the cardiological community at large and is therefore also of high societal relevance.

12) Treatment of DHF patients with CpcPH (Combined pre- and postcapillary Pulmonary Hypertension).

- MEDIA tested in a small phase II clinical trial a cGMP enhancing therapeutic strategy (cGETS) which consisted of upstream upregulation of myocardial cGMP production by combined administration of ACEIs and statins and downstream inhibition of cGMP breakdown by administration of the PDE5 inhibitor sildenafil.
- The flow chart of cGETS is shown in Figure 15.

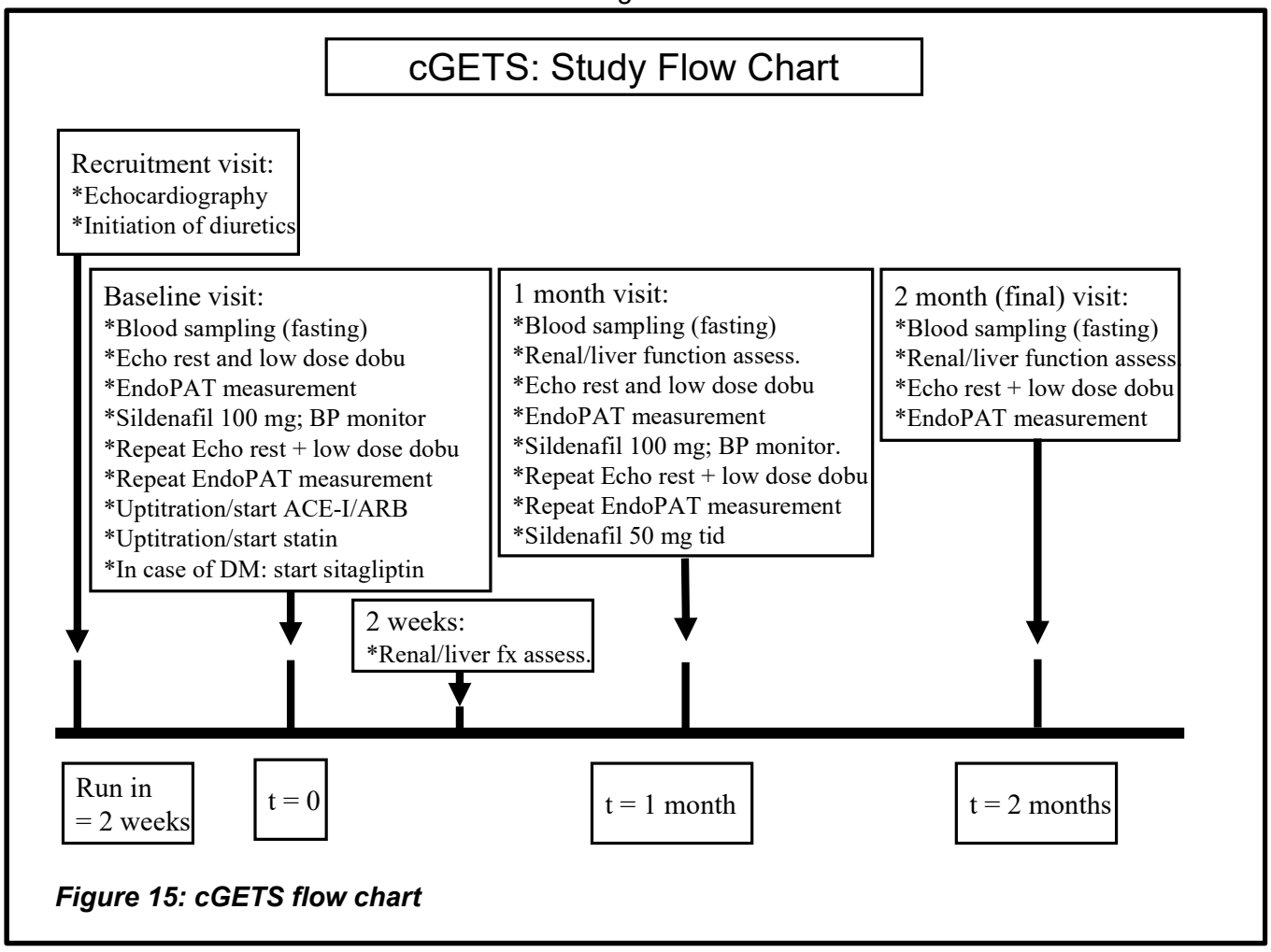


Figure 15: cGETS flow chart

- DHF patients suitable for cGETS were recruited and followed for 2 months. Patient characteristics are shown in the Table below:

Baseline characteristics

	cGETS patients (n=10), NYHA II-III
Age (years)	64.3 ± 8.7
Female gender (%)	100
Hypertension (%)	100
Diabetes mellitus (%)	25
Body mass index (kg/m2)	34.0 ± 6.1
eGFR (ml/min)	71.0 ± 17
Diuretic (%)	38
Angiotensin converting enzyme inhibitor (ACE-I;%)	25
Angiotensin receptor blocker (ARB;%)	25
ACE-I/ARB (%)	50
Mineralocorticoid receptor antagonist (%)	13
Betablocker (%)	75
Statin (%)	63
Calciumantagonist (%)	13
Nt-proBNP (pg/ml)	235±101
Number of patients with ntproBNP>125 pg/ml (%)	50

- The results on are shown in Figure 16.

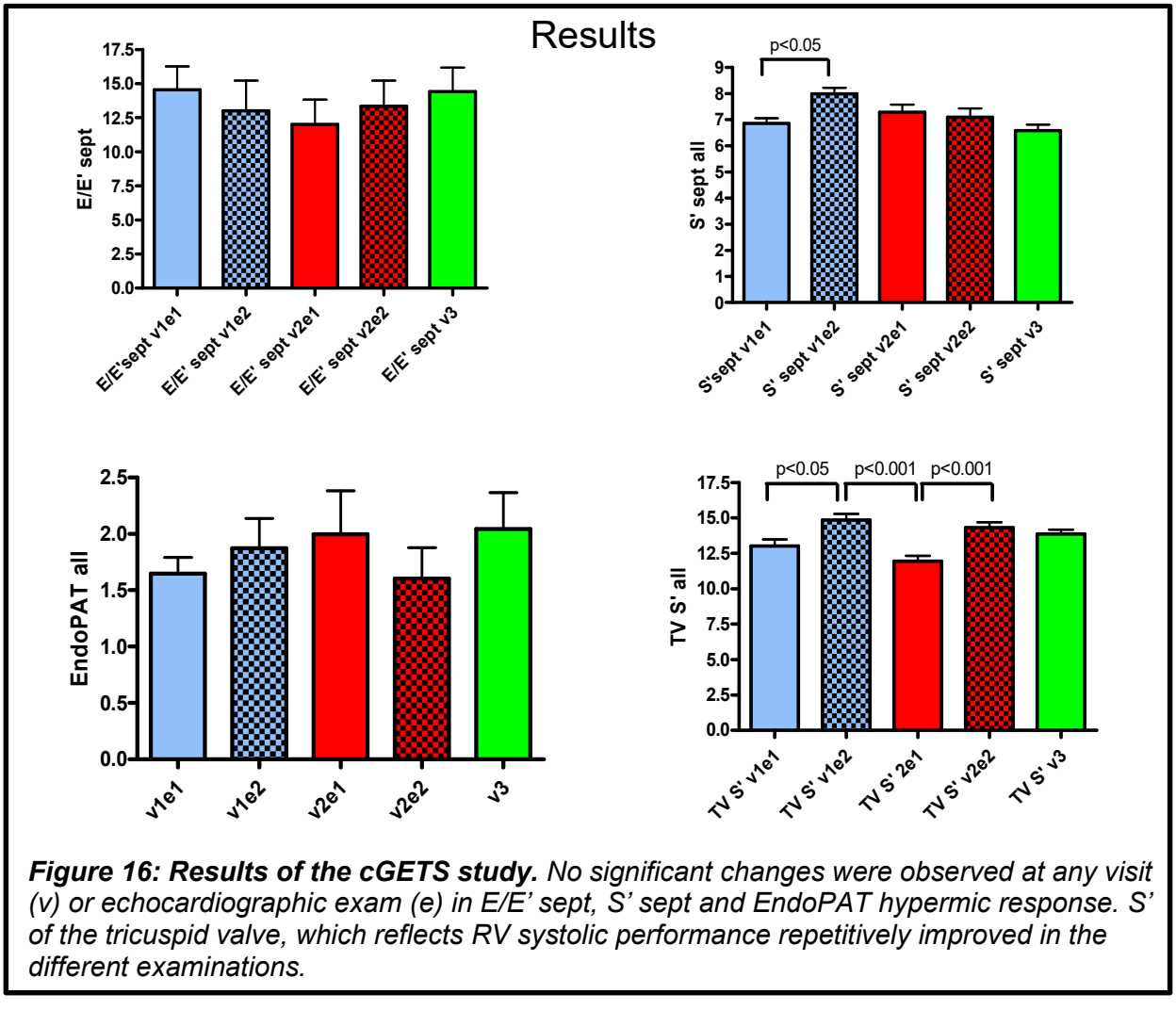


Figure 16: Results of the cGETS study. *No significant changes were observed at any visit (v) or echocardiographic exam (e) in E/E' sept, S' sept and EndoPAT hypermic response. S' of the tricuspid valve, which reflects RV systolic performance repetitively improved in the different examinations.*

- The most important result of cGETS is a repetitive increase in right ventricular S' consistent with pulmonary vasodilation induced by sildenafil. During chronic sildenafil administration, this pulmonary vasodilatory effect is only partially lost probably as a result of some tachyphylaxis to sildenafil.
- This response is especially relevant to treatment of DHF patients with exaggerated pulmonary arterial hypertension also labelled patients with CpcPH (Combined pre- and postcapillary pulmonary hypertension).

MEDIA: Potential Impact

Socio Economic Impact

The MEDIA project has reached all of its objectives and is having a profound socio-economic impact on the worldwide understanding, diagnosis and treatment of DHF. The main objectives of MEDIA were: 1) Development of animal models; 2) Elucidation of mechanisms responsible for high myocardial stiffness; 3) Improved algorithms for diagnosis and prognostication and 4) Exploration of novel therapeutic inroads.

1) Development of animal models

The MEDIA project succeeded to produce two animal models of DHF: a small rodent animal model (the obese ZSF1 rat) and a large animal model (the metabolically compromised and partially nefrectomized pig). The obese ZSF1 rat has achieved widespread acceptance as a valid model for DHF (Circ Heart Fail 2013;6:1239) and is currently used by several pharma companies (Amgen, Ironwoodpharma…) in the pursuit of novel therapies for DHF.

2) Elucidation of mechanisms responsible for high myocardial stiffness

- The MEDIA project was especially successful in unraveling mechanisms responsible for increased myocardial stiffness in DHF. Among its numerous contributions are: 1) demonstration of phosphorylation deficits in titin, the giant cytoskeletal molecule responsible for cardiomyocyte stiffness; 2) presence of oxidatively damaged titin with altered biomechanical characteristics; 3) potential of small heat shock proteins such as alfaB-crystallin to restore elasticity of oxidatively damaged titin; 4) presence of epigenetic mechanisms such as proteolysis of HDAC4 in DHF; 5) preponderance of metabolic disturbances in gene ontology or pathway analysis of DHF myocardium; 6) involvement of the Procollagen Peptidase-Procollagen Peptidase Enhancer (PCP-PCPE) / Lysine Oxidase (LOX) axis in the myocardial collagen accumulation in DHF; 6) involvement of matricellular proteins in myocardial fibrosis in DHF; 7) evidence of microvascular inflammation in DHF myocardium…
- These new insights acquired by the MEDIA project enabled the MEDIA investigators to propose a novel overarching paradigm for development of DHF (J Am Coll Cardiol 2013;62:263), whereby comorbidities induce a systemic inflammatory state with microvascular inflammation. This leads to deranged myocardial signaling from endothelial cells to cardiomyocytes and results in cardiomyocyte hypertrophy and stiffening. This paradigm gained widespread worldwide acceptance as evident from the number of citations (n=450) since its publication medio 2013.
- Some of these contributions such as the use of alfaB-crystallin to restore elasticity of DHF cardiomyocytes have meanwhile been patented (P110088EP00) or are being considered for patent application (e.g. use of PDE9I for phosphorylation of titin in DHF cardiomyocytes).
- Other contributions such as involvement of the Procollagen Peptidase-Procollagen Peptidase Enhancer (PCP-PCPE) / Lysine Oxidase (LOX) axis in the myocardial collagen accumulation in DHF have led to design and development of a specific myocardial antifibrotic therapy. The MEDIA consortium developed a screening bioassay using the collagen production of adult myocardial fibroblasts following TGF administration. Dose response curves were first made for several small peptides. EBP5 was identified as the most promising compound as it was able to reduce in a dose-dependent manner TGF-induced collagen production without affecting baseline collagen production. The molecule was however found to have a very short half-life and therefore not suitable for further pharmacological use. Because of the limited stability of EBP5, use of small peptides to block PCPE-1 was abandoned and a screen of small molecules for inhibitory activity on PCPE-1 was executed. The screening procedure is illustrated in the Figure 17. The screening revealed 40 compounds with satisfactory behaviour, of which 15 were subsequently selected and withheld for further studies. Among these small molecules, three compounds showed inhibitory activity comparable to the EBP5

small protein and will therefore undergo further in-vivo testing for pharmacological suitability.

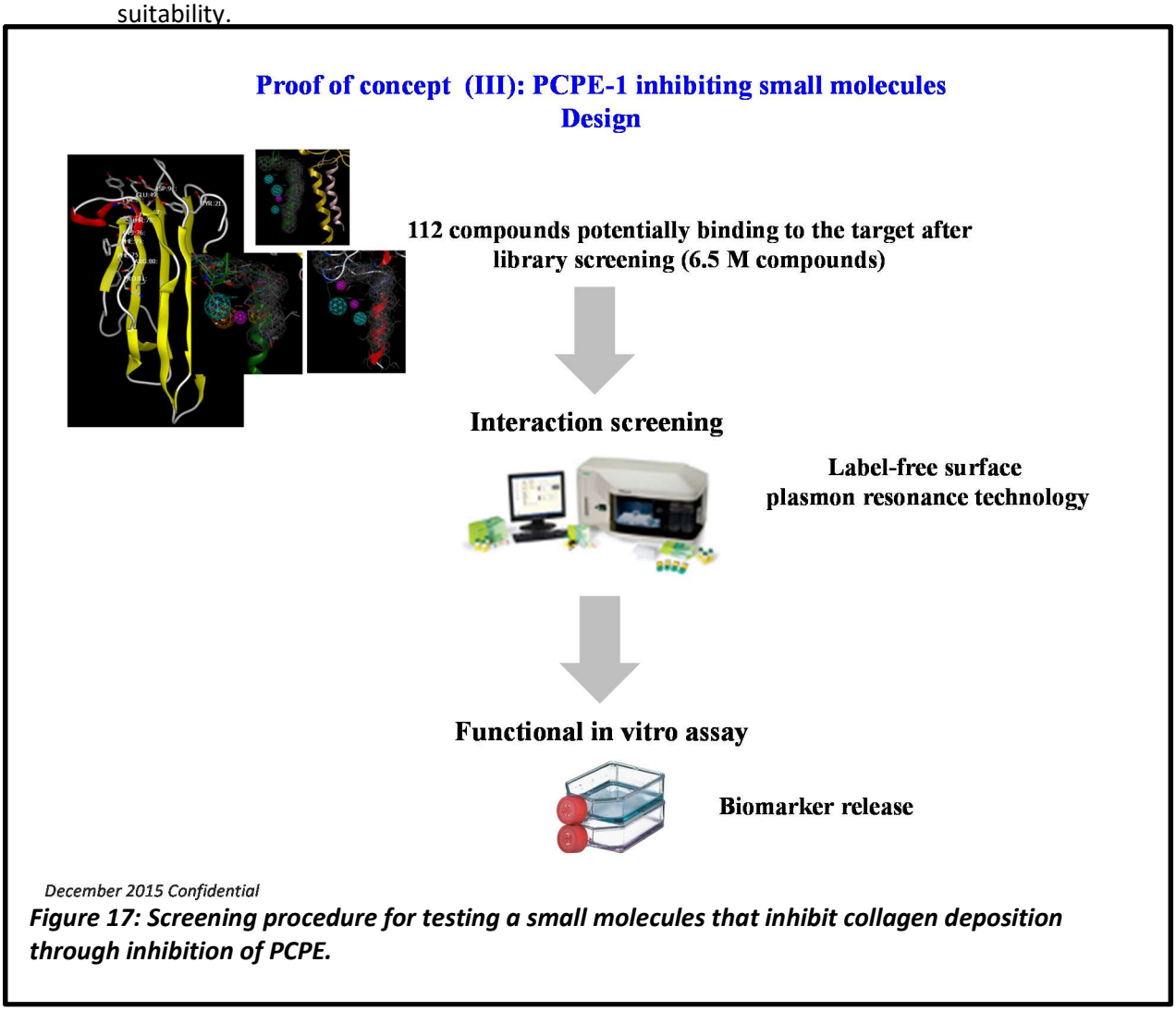


Figure 17: Screening procedure for testing a small molecules that inhibit collagen deposition through inhibition of PCPE.

3) Improved algorithms for diagnosis and prognostication

The MEDIA project contributed to improved diagnosis and prognostication of DHF patients. A key element in the diagnosis of DHF is the demonstration of elevated left ventricular filling pressures. The latter is however frequently obscured by prior administration of diuretics and only becomes evident following volume loading or exercise testing. In this respect the MEDIA project provided a stringent echocardiographic exercise stress testing protocol (Eur J Heart Fail 2014;16:1345). MEDIA also provided novel insights into prognostication of DHF. In patients with metabolic risk factors, development of diastolic left ventricular dysfunction could be accurately predicted (AUC 0.92) by a panel of clinical features combined with a panel of 4 biomarkers consisting of ICAM, E-selectin, NT-proBNP and PINP. Similarly, one year events (death, hospitalization) were predicted by renal insufficiency and ICAM. The usefulness of adhesion molecules as prognosticators is consistent with the novel paradigm for DHF proposed by the MEDIA project and opens the prospect of simple risk stratification of DHF patients.

4) Exploration of novel therapeutic inroads

The MEDIA project also explored new therapeutic inroads for DHF. Use of renal artery denervation failed to live up to the expectations in line with recent evidence provided by other investigators. In contrast, the cGETS study demonstrated improvement of right ventricular function in DHF patients.

The therapeutic approach proposed by cGETS is therefore especially useful for DHF patients with CpcPH (Combined pre and post capillary pulmonary hypertension). As such, the MEDIA project paved the road for a phenotype-specific treatment strategy for DHF (Circulation 2016;134:73), which is shown in Figure 18.

Phenotype-specific HFpEF treatment strategy using a matrix of predisposition phenotypes and clinical presentation phenotypes.

HFpEF Predisposition Phenotypes	HFpEF Clinical Presentation Phenotypes: Lung Congestion	+Chronotropic Incompetence	+Pulmonary Hypertension (CpcPH)	+Skeletal muscle weakness	+Atrial Fibrillation
Overweight/obesity/ metabolic syndrome/ type 2 DM	• **Diuretics (loop diuretic in DM)** • **Caloric restriction** • Statins • Inorganic nitrite/nitrate • Sacubitril • Spironolactone	+Rate adaptive atrial pacing	+Pulmonary vasodilators (e.g. PDE5I)	**+Exercise training program**	+Cardioversion +Rate Control **+Anticoagulation**
+Arterial hypertension	+ACEI/ARB	+ACEI/ARB +Rate adaptive atrial pacing	+ACEI/ARB +Pulmonary vasodilators (e.g. PDE5I)	+ACEI/ARB **+Exercise training program**	+ACEI/ARB +Cardioversion +Rate Control **+Anticoagulation**
+Renal dysfunction	+Ultrafiltration if needed	+Ultrafiltration if needed +Rate adaptive atrial pacing	+Ultrafiltration if needed +Pulmonary vasodilators (e.g. PDE5I)	+Ultrafiltration if needed **+Exercise training program**	+Ultrafiltration if needed +Cardioversion +Rate Control **+Anticoagulation**
+CAD	+ACEI +Revascularization	+ACEI +Revascularization +Rate adaptive atrial pacing	+ACEI +Revascularization +Pulmonary vasodilators (e.g. PDE5I)	+ACEI +Revascularization **+Exercise training program**	+ACEI +Revascularization +Cardioversion +Rate Control **+Anticoagulation**

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Figure 18: Phenotype-specific HFpEF treatment strategy using a matrix of predisposition phenotypes and clinical presentation phenotypes. *A stepwise approach is proposed that begins in the left hand upper corner of the matrix with general treatment recommendations, presumed to be beneficial to the vast majority of HFpEF patients as they address the presentation phenotype of lung congestion and the predisposition phenotype of overweight/obesity present in >80% of HFpEF patients. Subsequently, supplementary (+) recommendations are suggested for additional predisposition-related phenotypic features when moving downward in the matrix and for additional presentation-related phenotypic features when moving rightward in the matrix. Arterial hypertension, renal dysfunction, and coronary artery disease are proposed as additional predisposition phenotypes. Additional clinical presentation phenotypes, in which specific therapeutic interventions could be meaningful, include chronotropic incompetence, pulmonary hypertension (especially combined precapillary and postcapillary pulmonary hypertension [CpcPH]), skeletal muscle weakness, and atrial fibrillation. Only therapeutic measures indicated in bold are currently established. All other therapeutic measures require further testing in specific phenotypes. ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blockers; CAD, coronary artery disease; DM, diabetes mellitus; HFpEF, heart failure with preserved ejection fraction; and PDE5I, phosphodiesterase 5 inhibitor.*

Wider Societal Impact

The MEDIA project had a profound impact on the worldwide understanding of DHF as a syndrome where HF meets internal medicine and systemic inflammation in particular. This worldwide appreciation was strikingly evident from a recently published "In Depth – State of the Art" study produced by a collaborative effort of the MEDIA investigators, John Hopkins Medical Center and MAYO clinic (Circulation 2016;134:73). The concept of DHF being an interface between HF and multiple other organs in the human body is shown in Figure 19.

Systemic and myocardial signaling in HFPEF. Comorbidities induce systemic inflammation, evident from elevated plasma levels of inflammatory biomarkers such as soluble interleukin 1 receptor-like 1 (IL1RL1), C-reactive protein (CRP), and growth differentiation factor 15 (GDF15).

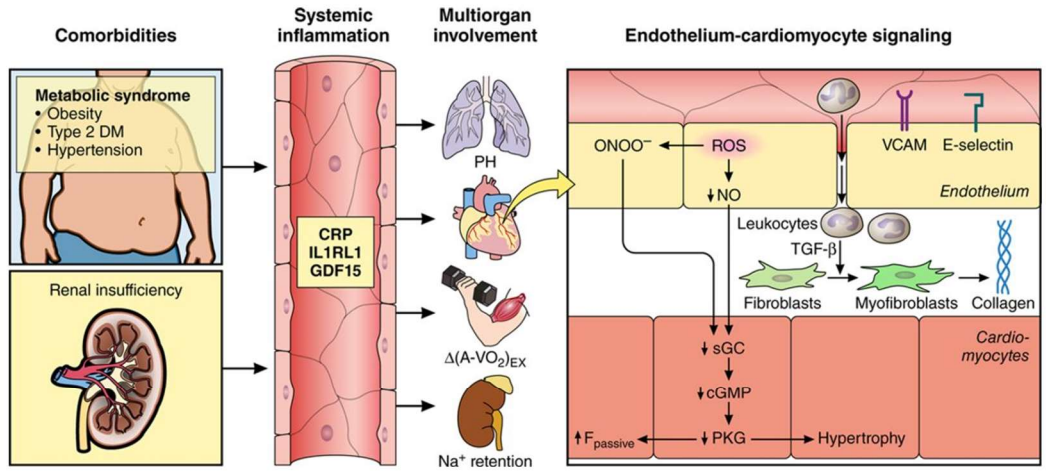

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Figure 19: Systemic and myocardial signaling in HFPEF. *Comorbidities induce systemic inflammation, evident from elevated plasma levels of inflammatory biomarkers such as soluble interleukin 1 receptor-like 1 (IL1RL1), C-reactive protein (CRP), and growth differentiation factor 15 (GDF15). Chronic inflammation affects the lungs, myocardium, skeletal muscle, and kidneys leading to diverse HFpEF phenotypes with variable involvement of pulmonary hypertension (PH), myocardial remodeling, deficient skeletal muscle oxygen extraction ($\Delta A\text{-}Vo_2$) during exercise (Ex), and renal Na^+ retention. Myocardial remodeling and dysfunction begins with coronary endothelial microvascular inflammation manifest from endothelial expression of adhesion molecules such as vascular cell adhesion molecule (VCAM) and E-Selectin. Expression of adhesion molecules attracts infiltrating leukocytes secreting transforming growth factor β (TGF-β), which converts fibroblasts to myofibroblasts with enhanced interstitial collagen deposition. Endothelial inflammation also results in the presence of reactive oxygen species (ROS), reduced nitric oxide (NO) bioavailability, and production of peroxynitrite ($ONOO^-$). This reduces soluble guanylate cyclase (sGC) activity, cyclic guanosine monophosphate (cGMP) content, and the favorable effects of protein kinase G (PKG) on cardiomyocyte stiffness and hypertrophy. HFpEF indicates heart failure with preserved ejection fraction.*

Since the introduction by MEDIA of this concept, numerous studies reported on presence of diastolic LV dysfunction or of diastolic heart failure in other systemic inflammatory conditions such rheumatoid arthritis, psoriatic arthritis, colitis ulcerosa or Crohn's disease.

Main Dissemination Activities

Publications: The DOI of publications acknowledging MEDIA are listed on the EU FP7 reporting website. The MEDIA consortium succeeded to have several publications in prestigious journals such as Nature, Cell, Circulation… So far the MEDIA consortium accredited the FP7 program of the EU in 95 peer reviewed publications.
Symposia: MEDIA consortium meetings have been organized in Amsterdam (17-18/1/11), in Leiden (23-24/1/12), in Leiden (14-15/1/13), in Leiden (3-4/2/14), in Leiden (26-27/1/15) and in Amsterdam (6-7/12/15). The final scientific conference took place in Amsterdam on 6-7/12/2015. The MEDIA consortium also contributed to the HFA-ESC meeting on HFPEF in Budapest (23-24/9/11), to the HFPEF meeting in Bergamo (15-16/6/12) and to the HFPEF-Biomarker meeting in Graz (13-15/10/12).
PhD Theses: PhD students were employed by the MEDIA project. This resulted in 9 PhD theses realized thanks to MEDIA.

Exploitation of Results

- The MEDIA project produced two animal models of DHF: a small rodent animal model (the obese ZSF1 rat) and a large animal model (the metabolically compromised and partially nefrectomized pig). The obese ZSF1 rat has achieved widespread acceptance as a valid model for DHF (Circ Heart Fail 2013;6:1239) and is currently used by several pharma companies (Amgen, Ironwoodpharma…) in the pursuit of novel therapies for DHF.
- The use of alfaB-crystallin to restore elasticity of DHF cardiomyocytes has been patented (P110088EP00).
- The MEDIA consortium developed a screening bioassay using the collagen production of adult myocardial fibroblasts following TGFβ administration for testing of small peptides or small molecules as antifibrotic therapy.
- The MEDIA project provided a stringent echocardiographic exercise stress testing protocol (Eur J Heart Fail 2014;16:1345) for diagnosis of DHF in patients with normal resting LV diastolic function.
- Vascular adhesion molecules were shown to be prognosticators in DHF. This opens the prospect of simple risk stratification of DHF patients using ICAM3.
- A cyclicGMP enhancing therapeutic strategy (cGETS) was shown to be beneficial in DHF patients with CpcPH (Combined pre and post capillary pulmonary hypertension). This finding paved the road for a phenotype specific treatment strategy in DHF (Circulation 2016;134:73).