Features of (reverse) Remodeling in Heart Failure

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Features of (reverse) Remodeling in Heart Failure

Histopathology and molecular biology of (reverse) remodeling during
Left Ventricle Assist Device (LVAD) Support in Heart Failure Patients

Histopathologische en moleculair biologische aspecten van het 'reverse-remodeling'
proces tijdens LVAD ondersteuning bij patiënten met hartfalen

(met een samenvatting in het Nederlands)

Proefschrift

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ter nagedachtenis aan mijn vader / in remembrance of my father

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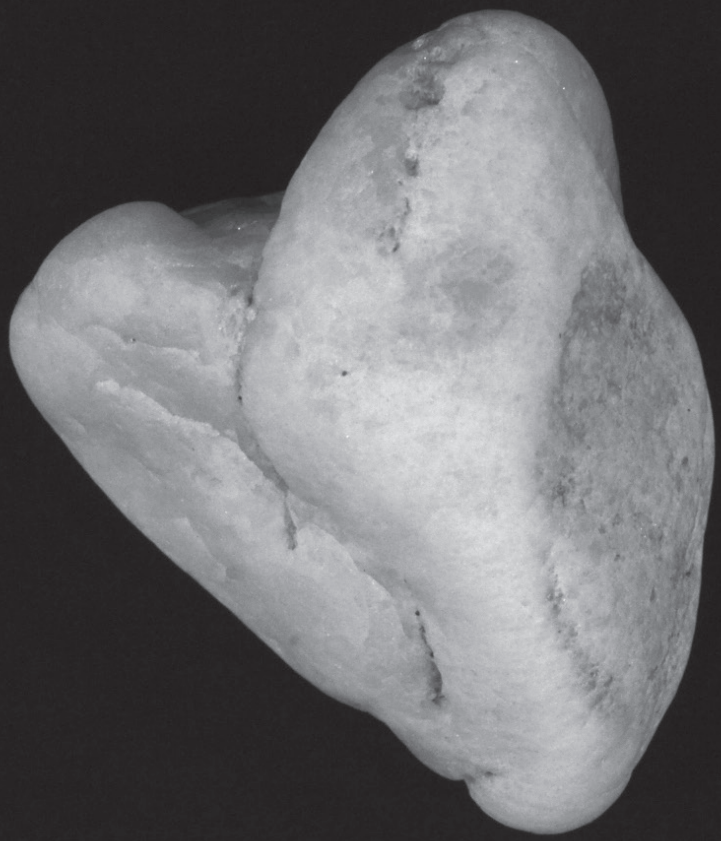
1 Introduction



Heart Failure

The clinical syndrome of heart failure results from a complex disorder of the heart muscle, caused by any structural or functional defect, resulting in a relative weakness of the heart muscle, which hinders the ventricles to fill or to eject blood (1;2). Approximately in two/ thirds of patients presenting with left ventricular output dysfunction coronary artery disease, resulting in poor oxygen supply and cardiac ischaemia is known as the underlying cause, called ischaemic heart disease (IHD) (3;4). Other main causes of heart failure are valvular disorders, systemic hypertension and cardiomyopathies of known and unknown etiologies, divided in dilating cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM) and restrictive cardiomyopathy (5-7). In the end all patients with the clinical signs of heart failure display the same structural abnormalities in the heart muscle, regardless of the cause of the disease.

Clinical Features

The general clinical features of heart failure are dyspnea and fatigue, leading to limited exercise tolerance and fluid retention, followed by chronic pulmonary congestion and peripheral edema in the lower extremities. Patients can remain asymptomatic or have only mild symptoms for a long time after the first signs of heart muscle damage.

In the classification of heart failure patients four stages are used by the New York Heart Association (NYHA I ª IV) (1;2). The classification of heart failure by the American College of Cardiology and the American Heart Association defines the following groups: Stage A patients are at high risk for developing heart failure, but without structural disorders. Stage B patients have structural disorders, but without clinical symptoms. Stage C patients have symptoms of heart failure caused by any structural disease of the heart muscle. Stage D defines the patient population with end-stage heart failure, which may be treated with maximal inotropic medication, mechanical support or heart transplantation (1).

Epidemiology

The increase of the heart failure patient population nowadays (8;9) is an emerging problem in the health care system and a challenge for the organization of cardiovascular medicine. The lifetime risk to develop heart failure is one in five (8). This is due to the world-wide aging population in general, caused by the better treatment and subsequent longer survival

of patients in all kind of diseases (cancer treatment, infectious diseases). But the improved survival of patients after acute myocardial infarction and the far better pharmacological treatment of heart failure patients (10), irrespective the cause of the disease, is another important reason. The epidemic increase in heart failure patients and hospitalizations during 1970-1990 (9;10) seem now to stabilize, but survival time is still increasing due to the improvements in treatment and treatment availabilities.

Therapeutic options

Despite these advances in treatment and irrespective of the cause of the disease, the general prognosis of heart failure is poor. Progressive pump failure and ventricular arrhythmias are the most common causes of death in this patient group (11). Maximal medical therapy with ACE inhibitors, angiotensin receptor blockers, aldosterone antagonists and beta-blockers has proven to be successful in heart failure, improving prognosis in general. In the case of valvular or coronary artery disease, invasive therapies like valvular surgery, percutaneous coronary intervention (PCI) or coronary bypass surgery (CABG) has to be considered. Implantation of a cardioverter defibrillator or a pacemaker could be necessary to prevent sudden cardiac death by ventricular arrhythmias or fibrillation. To improve the overall condition of the patient general measures, like regular exercise, cessation of smoking, alcohol intake and weight reduction may influence survival substantially (12).

Diagnostic procedures

Early diagnosis of the underlying disease in heart failure could be important (8;13). Several clinical and laboratory parameters and radiologic diagnostic procedures, like ECG, echo, CT and MRI are available to show evidence of dimensions and structural abnormalities in the heart. Sometimes histopathological evaluation of endomyocardial biopsies (EMB) is necessary to evaluate microscopical abnormalities, eventually followed by microbiological techniques to demonstrate an infectious agent (13;14). This can result in specific and individualized treatment possibilities in an earlier phase of the disease in order to prevent transition to end stage pump failure.

Circulatory support

Artificial support of the circulation in critical ill patients with progressive pump failure and during major cardiac surgery has been used from the middle of the last century. In the slipstream a wide range of ventricular assist devices have been and are still developed and used around the world (15). Their main function is supporting blood circulation, organ perfusion and unloading the failing heart. Both short-term and long-term extracorporal and intracorporal systems are now available, including a Total Artificial Heart (TAH) (16).

Left ventricle assist devices

The first implantable devices for end-stage heart failure patients were the pulsatile volume displacement pumps like Heartmate XVE (Thoratec, Pleasanton, CA, USA) and the Novacor (WorldHeart, Ottawa, Canada) with a large metal case around the pump and a large external driveline. In the following years an enormous technical development has resulted in several examples of assist devices based on different technologies, like the elegant continuous flow axial pumps (Cf-LVAD or Heartmate II) with a small tubular case and an impeller suspended inside (17).

Unfortunately the use of mechanical support for patients with end stage heart failure is limited in time and in principle only used as a bridge to transplantation (BTT) for patients on the waiting list for heart transplantation to improve the general condition and organ function (18;19). The second indication to use an assist device is as a bridge to recovery (BTR). In some patients during mechanical support their own cardiac function improves sufficiently to allow, after a period of weaning, the removal of the assist device. These specific groups of patients are mostly recovering from a fulminant type myocarditis, treated with corticosteroids and immunosuppressive therapy, sometimes combined with anti-viral medication.

But also in other groups of patients with an assist device a “reverse remodeling” process has been described in the heart (20) and will be further explained below. Considering the shortage of donor hearts (21;22) and the improvements in the design and the durability of the devices, the use of mechanical support as destination therapy (DT) could be the third application form in the near future as an alternative for heart transplantation.

Complications

Like any device in the cardiovascular system, the risk of thrombosis and thrombo-embolism is substantial. This is mainly caused by the absence of an intact endothelial lining on the inner surface of the device. Anticoagulation prevents thrombosis, but increases the risks of blood loss, with sometimes a fatal cerebral or gastrointestinal bleeding. Another risk of morbidity and sometimes mortality is infection and abcess formation around the device because of the contact with the outside world (the driveline) or excessive giant cell reaction with calcification of scar tissue around the device, caused by the presence of foreign body material.

Remodeling process

During progression of heart failure a complex process of architectural abnormalities take place in the myocardium, in general called the remodeling process (20;23). Clinically the heart changes in size, shape and function, essentially displaying an increase in cardiac mass and dimensions, as well as the shape of the ventricles (24). Irrespective the etiology of the disease, the common mechanisms of this process are adaptations of the heart to maintain normal cardiac function after acute or gradual damage of the cardiomyocytes. This process is strongly influenced by the haemodynamic load, neurohormonal activation and local acting cytokines (25;26). Remodeling is initiated by individual cardiomyocyte degeneration, necrosis as well as programmed cell death, called apoptosis, followed by compensatory hypertrophy of the remaining cardiomyocytes (27). But the close interplay between cardiomyocytes and the extracellular matrix during oxidative and mechanical stress, inducing fibroblast proliferation and progressive interstitial fibrosis, play also an important role in remodeling of the supportive frame work of the heart (28). Influencing this remodeling process by medication or mechanical support, unloading the left ventricle, possibly could prevent progression of heart failure and even serve as potential therapy (25).

Reverse remodeling

During mechanical support, as such unloading of the left ventricle by LVAD support, this results in a reversion of the remodeling process, normalizing cell size and shape of cardiomyocytes, increasing both sarcomeric and non sarcomeric cytoskeletal proteins, resulting in a stronger cell contraction (27;29) and regression of cellular hypertrophy (30). This is accompanied by reduction of interstitial fibrosis and functional improvement of the

left ventricle (31;32). Unravelling the underlying mechanical and molecular mechanisms controlling this reverse remodeling process on tissue level can possibly lead to the explant of the device in a subgroup of patients. But based on this knowledge, also novel therapeutic interventions could be considered during LVAD support, inducing myocardial recovery and subsequently treatment of heart failure itself (29).

Selection of patients

In the clinical setting all end stage heart failure patients suffer all of an otherwise not specified 'cardiomyopathy'. These cardiomyopathies are a heterogenous group of diseases, due to a variety of, partly genetic, causes (33). The many classification systems to standardize discussion about diagnostics and treatment, based on their phenotypical appearance, including morphological and physiological characteristics, are not sufficient anymore. Reclassifying cardiomyopathies, partly according to a causative genetic defect and if possible combined with the morphological phenotype in the myocardium, will provide a more useful method to classify patients with progressive heart failure (34). The selection of patients for different types of therapy should be based on this new classification.

Histopathology

In 1975 Human Pathology reports about cardiomyopathy (CMP) in autopsy reports of patients, who died due to congestive heart failure (35). They considered 2 types of cardiomyopathy: hypertrophic and dilating cardiomyopathy (36;37). The remaining group of autopsy findings in cardiac deaths were classified as ischaemic heart disease, caused by coronary artery obstruction. Up to the 80-ties of the last century CMP was a rare condition, mostly affecting young people. Several theories were suggested with the limitations of only the morphologic description and special stains for fibrosis. Yet the underlying etiologic or pathogenetic pathways of cardiomyopathy remained obscure (38). Histopathology of end-stage heart failure patients became available at autopsy and after heart transplantation. The morphological substrate of heart failure was all the same in these patients, irrespective the cause or the duration of the disease. Different groups were made, based on known (Ischaemic Heart Disease, IHD) or unknown (Dilating Cardio-Myopathy, DCM) etiology, to compare clinical, pathophysiological and morphological parameters in research protocols. These hearts showed extensive interstitial fibrosis, hypertrophic cardiomyocytes, sometimes completely without evident intercellular connections or intercalated discs and extremely

large irregular nuclei (36). So in end stage heart failure it is in most cases not possible to classify the cardiomyopathy based upon histopathological features alone (37-39).

Genetic defects

With the progression of molecular and genetic knowledge, the phenotypic presentation of the different entities, in casu the traditional histopathological description does not reflect accurately the complex cluster of myocardial diseases (38-40). The identification of many genetic defects in different genes appeared to be responsible for up to 30% of idiopathic dilated cardiomyopathies. They display many forms and different entities (either congenital, familial or spontaneous mutations with different penetration) with a large phenotypic and genetic heterogeneity (40;41). So genotyping cardiomyopathies is becoming increasingly important in the clinical practice of cardiomyopathies for disease diagnosis, genetic advice in family planning and prognosis (42).

Role of the pathologist

The expertise of the pathologist, specialized in cardiovascular pathology, is necessary (41) in transplant monitoring, myocarditis diagnosis and in the detection of underlying causes of cardiomyopathy, like storage diseases, cardiac involvement in systemic diseases like amyloidosis or tumor diagnosis (13;43). The recent developments in bacterial and viral molecular biology also need the expertise of the pathologists to select tissue specimen, both formalin fixed or snap frozen, for analysis (44-46).

The combination of extensive histopathological evaluation of complete hearts after heart transplantation or autopsy in, for example, sudden cardiac death (47) will provide more detailed information and better interpretation of cardiac imaging, like MRI and CT (48;49).

The advances in genetic techniques (genotyping) in combination with the histopathological substrate (phenotyping) will provide new insights in the etiology and pathogenesis of cardiomyopathy (44;45;49;50) in general.

New classification

In 2008 a new classification system has been proposed for use in clinical practice (46). This includes subdividing patients in specific morphological and functional phenotypes: hypertrophic cardiomyopathy, restrictive cardiomyopathy, dilated cardiomyopathy,

arrhythmogenic right ventricular cardiomyopathy and cardiomyopathy unclassified, subdivided in familial and non-familial subgroups. As the diagnosis of a cardiomyopathy starts with the clinical features and not with genetic testing, it is important to use a classification frame work, which provides in diagnostic procedures, possible treatment options and predictions about the clinical course (34;40).

Aim of this thesis

The first two research projects in the department of Pathology with pre- and post-LVAD tissue specimen were focussed on the histopathological and immunohistochemical features of cardiomyocytes (27) and extracellular matrix (32). Their conclusions were that a complete recovery of cardiomyocytes (23;51), important for stronger cell contraction, or restoration of the extracellular matrix including basal membrane connections, essential for mechanotransduction (28), were not observed.
Based on these findings the main starting-points of this study were the search for markers on molecular level (messengerRNA, gene profiling, proteomics and microRNA), involved in reverse remodeling during pulsatile mechanical support, in order to unravel the basic regulatory mechanisms of this process. The research data of the patients with a continuous flow pump are published elsewhere (52-54).

The questions to be answered in our study were:
1. Which molecular biological parameters give information about the remodeling and reverse remodeling status of the diseased myocardium in end stage heart failure patients?
2. Should the combination of these molecular findings with the pathogenesis of the underlying disease, give new insights about influencing the reverse remodeling process successfully? As such this could ultimately lead to new and more personalized treatment strategies.

The results of this study are described in the following chapters: in Chapter 2 the altered messengerRNA and gene expression profiles in IHD- and DCM patients during LVAD support are explained. Chapter 3 discusses the results and the consequences of proteomic profiling in the two patient groups, IHD and DCM. In Chapter 4 and 5 specific extracellular matrix proteins (osteopontin and integrins), involved in the complex interplay between the cardiac interstitium and the cardiomyocytes, are studied. In Chapter 6 the attention is focussed on the changes in regulatory microRNA expression during the reverse remodeling process.

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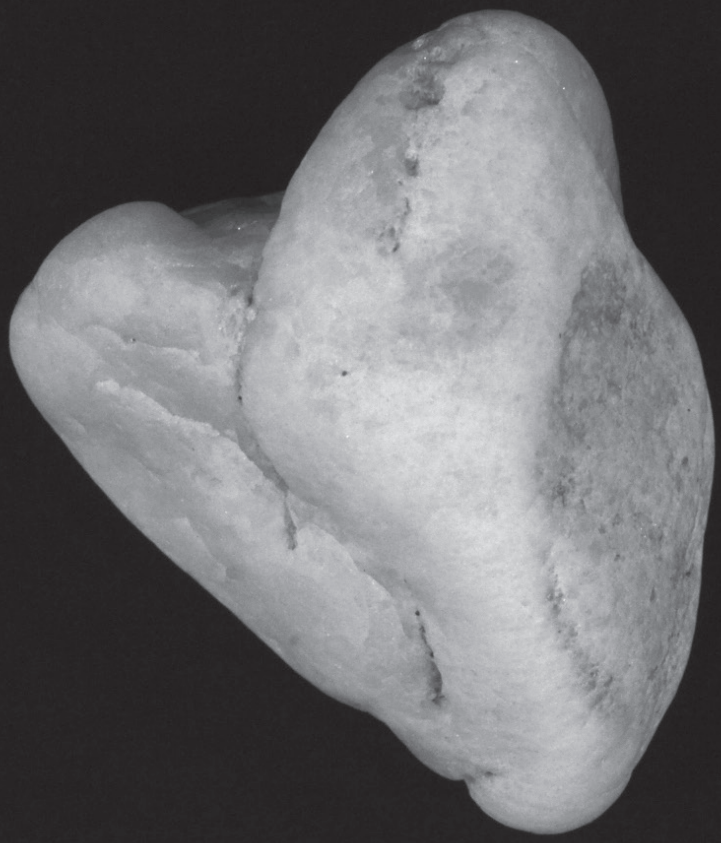
Altered expression of mRNA and miRNA during mechanical support of the failing human heart



Introduction

Remodeling during heart failure is characterized by structural rearrangement of the cardiac ventricular wall architecture. It involves hypertrophy of cardiomyocytes, fibroblast proliferation, and increased deposition of extra cellular matrix (ECM) proteins (Brower et al., 2006). Support of the left ventricle with a Left Ventricular Assist Device (LVAD) in patients with end-stage heart failure results in less neurohormonal activation (Estrada-Quintero et al., 1995; Frazier and Myers, 1999; Bruggink et al., 2006a), improvement of the patient's general condition (De Jonge et al., 2001; Grady et al ., 2003), reduction in ventricular diameter (reverse remodeling), and limited recovery of contractile elements in cardiomyocytes (Muller et al., 1997; De Jonge et al., 2002). Furthermore, reduction of ECM volume (Milting et al., 2008; Goldsmith and Borg , 2002; Bruggink et al., 2006b), diminished production of tumor necrosis factor (Thohan et al., 2005; Bruggink et al., 2008)), and reduction in brain natriuretic protein serum levels (Bruggink et al., 2006a; Kemperman et al., 2004) have been described during LVAD support. The changes in ECM during this process of reverse remodeling resulted not only in a time dependent change of type I and type III collagen protein (Goldsmith and Borg, 2002; Stamenkovic, 2003), but also in considerable changes in composition of the basal membrane. These included amongst others reduced collagen type IV content in the cardiomyocyte basal membrane, as a result of increased matrix metalloproteinase activity (Bruggink et al., 2007; Spinale, 2002; Li et al., 2001; Klotz et al., 2005). So, during LVAD support changes occur at the level of both the cardiomyocytes and the ECM.

The mechanics of the heart require a close interplay between cardiomyocytes and the ECM (Parker and Ingber, 2007) and therefore, one may anticipate a coordinated change in the molecules responsible for this interaction. These changes may not be the same in all heart failure patients supported by a mechanical support device. Some patients' heart may improve and may be eligible for removal of the support device without a heart transplantation (bridge to recovery and weaning from the device), whereas other hearts do not improve on support or may even deteriorate and these patients remain on the device (destination therapy) or will ultimately receive a heart transplant (bridge to transplantation). To make the proper choice of the type of therapy for each patient a good set of (bio)markers is required (De Weger and De Jonge, 2009).

In this chapter, we describe whether mRNA expression patterns could be indicative for the state of heart functionality supported by a LVAD (Heart-Mate I, Thoratec, Pleasanton, CA, USA). The changes in mRNA profiles that are detectable in myocardial biopsies taken from patients with end-stage heart failure due to dilated cardiomyopathy (DCM) or ischaemic heart disease (IHD) before and after LVAD support were analyzed, and compared with biopsies taken from control hearts as a reference (Table 1). Furthermore, the expression of 109 genes is described, which are involved in the process of mechanotransduction in the heart. Their expression was studied by Quantatative(Q)-PCR. The cohort comprised selected genes encoding for ECM filaments (such as collagens), transmembrane proteins (molecules that connect cells and matrix components like integrins and sarcoglycans), adhesion molecules, intracellular molecules related to mechanotransduction and signal transduction, ion-channel molecules and factors involved in pro- and anti-fibrotic processes.

Table 1. Patient characteristics.
DCM: dilating cardiomyopathy, IHD: ischemic heart disease, LVAD: left ventricular assist device.

Nr	*Age*	*Diagnosis*	*Gender*	*Days on LVAD*	*Medication during LVAD-support*
1	56	IHD	Male	138	None
2	57	IHD	Male	225	None
3	45	IHD	Male	259	2,5 mg Ramipril
4	57	IHD	Male	263	None
5	36	IHD	Male	325	2x 4 mg Perindopril
6	26	IHD	Male	357	None
7	39	IHD	Male	548	3x 6,25 mg Capoten
8	34	DCM	Female	55	3x 6,25 mg Capoten
9	17	DCM	Male	111	None
10	47	DCM	Male	190	None
11	35	DCM	Male	196	None
12	32	DCM	Female	219	3 mg Captopril
13	25	DCM	Male	263	1x 25 mg Losartan
14	32	DCM	Male	286	4 mg Perindopril
15	25	DCM	Male	330	2x 10 mg Fosinopril
16	46	DCM	Male	484	3x 50 mg Capoten

The expression of mRNA is however not always directly related to the protein production, due to post-transcriptional regulation. Recently, it is has been shown that intracellular gene expression is regulated in part by small RNA molecules: microRNA's (miR's). These miR's are highly expressed in heart tissue (Ji et al., 2007; Cheng et al., 2007) and have also been related to heart diseases (Chen, 2007; Van Rooij et al., 2006). The list of regulatory microRNA's involved in heart disease is constantly increasing (Coutinho et al., 2007; Markham and Hill, 2010). Each miR can regulate various mRNA expressions and which mRNA is regulated by which miR is not determined with certainty for most genes.

An additional goal of this study was therefore to analyse the changes in expression of 4 miR's, that are known to be expressed in the myocardium (Chen, 2007; Ikeda et al., 2007): miR-1, miR-133a, miR-133b and miR-208 These miR have been related to heart failure, as well. The expression of these miRs was measured in the same group of patients as the group of patients used to analyse the mRNA expression after LVAD support. This was done to study whether the LVAD induced remodelling of the heart was accompanied by changes in the expression of miR's, that could influence the protein expression of the mRNA studied, and could make the expression of some mRNAs less suitable as biomarker for the state of the supported heart. Whereas, the expression of miR's may serve as better markers either in the myocardium or the serum.

Tissue distribution of mRNA and miR in the myocardium

Tissue samples taken at various location of a cross section of the heart, showed that the expression of both mRNA and miR in the right and left ventricular wall did not show significant variation. Only at the site of the infarcted areas the expression of mRNA and miR was low to absent.

Hierarchical clustering of gene expression in myocardial tissue of IHD and DCM patients

The gene profiles in DCM and IHD heart tissue, detected by Q-PCR, were compared using TIGR software (www.tm4.org). Figure 1 shows the whole data set for all pre-LVAD samples versus the median of control samples. Hierarchical clustering was performed on all 92 detectable genes. The genes that were not detectable (n=14) and house keeping genes (n=3) were excluded; Table 2. The clustering segregated two groups: one group consists of 6

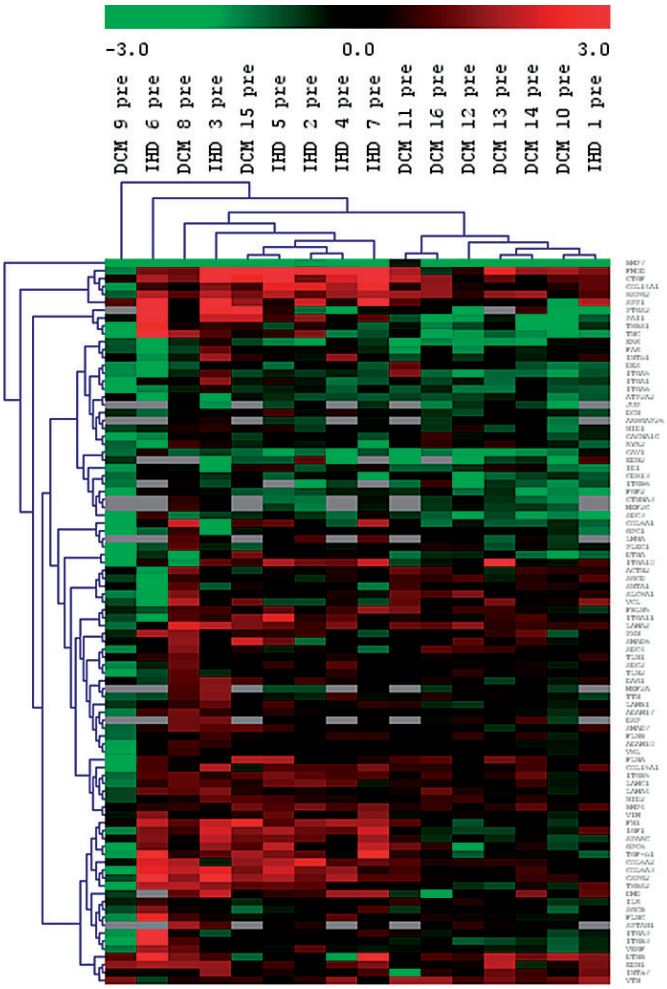


Figure 1. Unsupervised hierachical clustering of gene expression profiles of IHD and DCM pre-LVAD. Clustering was performed on all 92 detectable genes. Unsupervised hierarchical clustering was performed on normalized data using the multi-experiment viewer (MeV, version 4.3) of the TIGR software (www.tm4.org). The RQ of each sample per gene was normalized:

Normalized signal of sample x = Log2 (RQ sample x / median RQ).

To compare DCM and IHD the median was taken from the RQ of control hearts. To compare pre- and post-LVAD in DCM and IHD samples the median of all DCM or all IHD samples were taken, respectively. Clustering was performed on the whole dataset, and distance metric selection (Euclideon distance) and linkage metric selection (Complete linkage clustering) were used (www.tm4.org). This segregated two groups; one group consisting of 6 DCM patients with 1 IHD patient, and the other of 5 IHD patients with 2 DCM patients. Two patients (one DCM and one IHD) clustered outside these groups. Red: mRNA expression is higher than the median of control hearts. Green: mRNA expression is lower than the median of control hearts. Grey: not done.

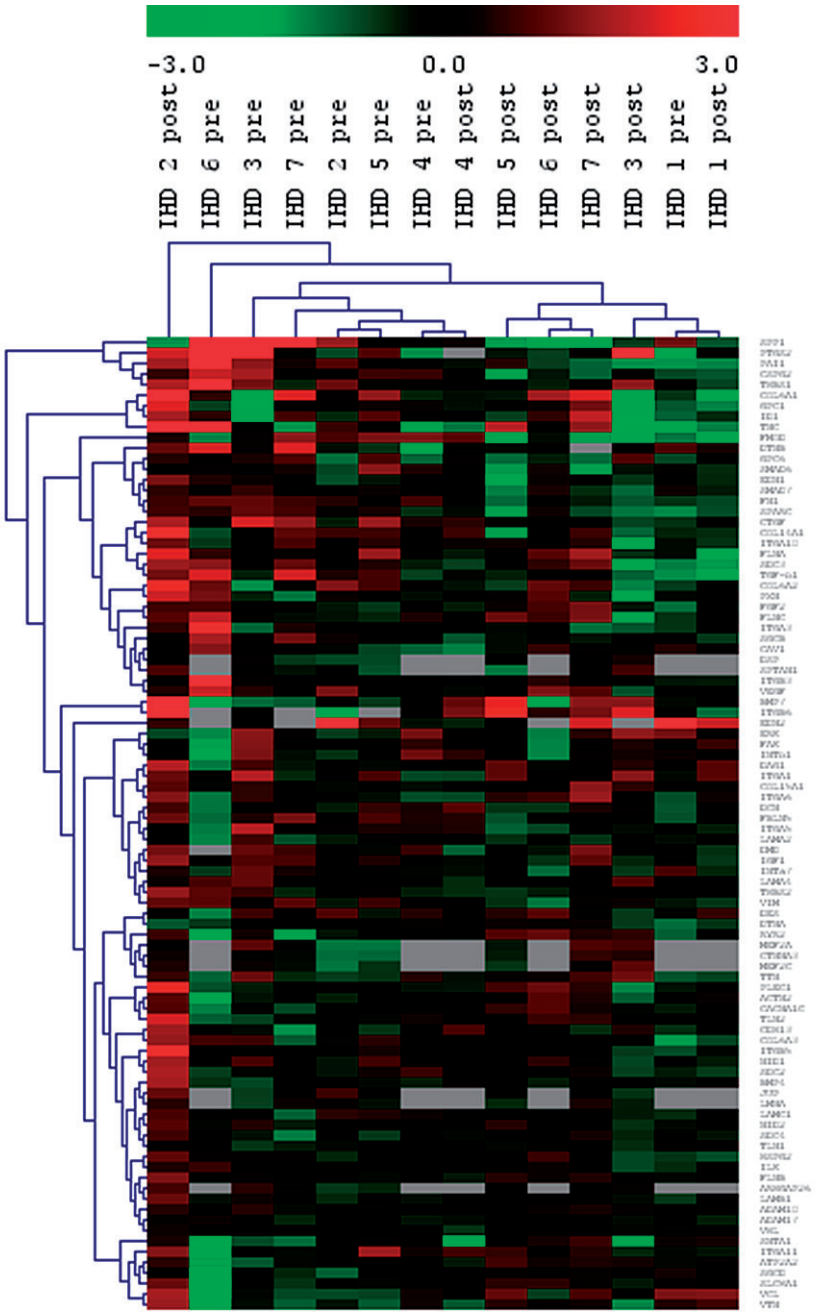


Figure 2. Unsupervised hierarchical clustering of gene expression profiles pre- and post-LVAD in IHD patients.

Clustering was performed on all 92 detectable genes and it segregated the patient group into a pre- and post-LVAD group. See for explanation Figure 1. Red: mRNA expression is higher than the median of all IHD samples. Green: mRNA expression is lower than the median of all IHD samples. Grey: not done.

Table 2. Statistical analysis of gene expression profiles in DCM and IHD patients.

				DCM					IHD				
Category	Gene name	Gene code AB	Assay code AB	p-value pre vs post	fold change pre vs post	p-value pre vs control		p-value post vs control	p-value pre vs post	fold change pre vs post	p-value pre vs control		p-value post vs control	
ECM	collagen, type XIV, alpha 1	COL14A1	Hs00385388_m1	0.033	1.75	0.576		0.055	0.542		0.014	>	0.197	
	collagen, type XV, alpha 1	COL15A1	Hs00266332_m1	0.003	1.76	0.353		0.131	0.079		0.645		0.161	
	collagen, type VI, alpha 1	COL6A1	Hs00242448_m1	0.104		0.716		0.181	0.374		0.903		0.451	
	collagen, type VI, alpha 2	COL6A2	Hs00242484_m1	0.154		0.129		0.121	0.488		0.061		0.195	
	collagen, type VI, alpha 3	COL6A3	Hs00365098_m1	0.042	1.53	0.842		0.048	> 0.703		0.075		0.081	
	chondroitin sulfate proteoglycan 2 (versican)	CSPG2	Hs00171642_m1	0.990		0.470		0.547	0.078		0.065		0.472	
	decorin	DCN	Hs00370385_m1	0.049	0.56	0.051		0.903	0.273		0.382		0.964	
	fibulin 5	FBLN5	Hs00197064_m1	0.343		0.490		0.662	0.721		0.481		0.660	
	fibromodulin	FMOD	Hs00157619_m1	0.250		0.097		0.196	0.062		0.009	>	0.134	
	fibronectin 1	FN1	Hs00415006_m1	0.445		0.587		0.885	0.024	-0.76	0.023	>	0.589	
	heparan sulfate proteoglycan 2 (perlecan)	HSPG2	Hs00194179_m1	0.024	0.96	0.491		0.067	0.805		0.052		0.122	
	osteonectin	SPARC	Hs00277762_m1	0.592		0.260		0.236	0.190		0.018	>	0.195	
AFF	bone morphogenetic protein 4	BMP4	Hs00370078_m1	0.029	0.93	0.215		0.023	> 0.120		0.017	>	0.064	
	bone morphogenetic protein 7	BMP7	Hs00233476_m1	0.034	2.14	0.006	<	0.732	0.072		0.018	<	0.693	
	inhibitor of DNA binding 1	ID1	Hs00357821_g1	0.031	0.87	0.099		0.288	0.323		0.166		0.540	
	prostaglandin-endoperoxide synthase 2/COX2	PTGS2	Hs00153133_m1	0.464		0.911		0.358	0.752		0.445		0.490	
	mothers against DPP homolog 7	SMAD7	Hs00178696_m1	0.300		0.522		0.876	0.295		0.065		0.681	
PFF	connective tissue growth factor	CTGF	Hs00170014_m1	0.258		0.318		0.082	0.398		0.048	>	0.112	
	endothelin 1	EDN1	Hs00174961_m1	0.710		0.332		0.442	0.913		0.040	>	0.184	
	endothelin 2	EDN2	Hs00266516_m1	0.431		0.084		0.185	0.419		0.267		0.150	
	fibroblast growth factor 2	FGF2	Hs00266645_m1	0.306		0.022	<	0.369	0.143		0.113		0.849	
	insulin-like growth factor 1	IGF1	Hs00153126_m1	0.084		0.938		0.114	0.903		0.003	>	0.076	
	mothers against DPP homolog 6	SMAD6	Hs00178579_m1	0.089		0.193		0.881	0.125		0.571		0.030	<
	transforming growth factor beta 1	TGF-β1	Hs00171257_m1	0.565		0.728		0.965	0.223		0.318		0.855	
	vascular endothelial growth factor	VEGF	Hs00900054_m1	0.586		0.352		0.134	0.254		0.880		0.538	
BM	glypican 1	GPC1	Hs00157805_m1	0.546		0.738		0.916	0.285		0.330		0.749	
	glypican 6	GPC6	Hs00170677_m1	0.200		1.000		0.373	0.839		0.095		0.104	
	laminin, alpha 2	LAMA2	Hs00166308_m1	0.505		0.072		0.111	0.798		0.078		0.003	>
	laminin, alpha 4	LAMA4	Hs00158588_m1	0.965		0.933		0.949	0.234		0.610		0.935	
	laminin, beta 1	LAMB1	Hs00158620_m1	0.001	0.82	0.338		0.255	0.268		0.696		0.201	
	laminin, gamma 1 (formerly LAMB2)	LAMC1	Hs00267056_m1	0.058		0.273		0.024	> 0.938		0.026	>	0.019	>
	lamin A/C	LMNA	Hs00153462_m1	0.261		0.287		0.717	0.938		0.055		0.112	
	nidogen 1	NID1	Hs00159600_m1	0.264		0.424		0.973	0.763		0.491		0.953	
	nidogen 2 (osteonidogen)	NID2	Hs00201233_m1	0.871		0.950		0.957	0.395		0.718		0.332	
	plasminogen activator inhibitor-2	PAI1	Hs00167155_m1	0.491		0.321		0.476	0.336		0.433		0.657	
	plectin 1	PLEC1	Hs00356977_m1	0.145		0.963		0.138	0.161		0.624		0.294	
	syndecan 4	SDC4	Hs00161617_m1	0.445		0.516		0.903	0.242		0.484		0.905	
	osteopontin	SPP1	Hs00167093_m1	0.001	-1.37	0.805		0.042	< 0.039	-1.82	0.064		0.155	
	thrombospondin 1	THBS1	Hs00170236_m1	0.943		0.094		0.118	0.452		0.931		0.322	
	thrombospondin 2	THBS2	Hs00170248_m1	0.656		0.785		0.523	0.764		0.040	>	0.264	
	tenascin C	TNC	Hs00233648_m1	0.654		0.389		0.538	0.890		0.538		0.364	
	vitronectin	VTN	Hs00169863_m1	0.464		0.010	>	0.061	0.171		0.366		0.071	
TAM	ADAM metallopeptidase domain 10	ADAM10	Hs00153853_m1	0.960		0.697		0.614	0.378		0.654		0.211	
	ADAM metallopeptidase domain 17	ADAM17	Hs00234224_m1	0.461		0.832		0.294	0.596		0.051		0.095	
	ATPase, Ca++ transporting, cardiac muscle	ATP2A2	Hs00155939_m1	0.416		0.096		0.109	0.008	1.02	0.002	<	0.297	
	calcium channel, alpha 1C subunit	CACNA1C	Hs00167681_m1	0.018	0.92	0.296		0.319	0.041	1.04	0.125		0.439	
	caveolin 1, caveolae protein, 22kDa	CAV1	Hs00184697_m1	0.267		0.011	<	0.027	< 0.442		0.291		0.126	
	cadherin 13, H-cadherin (heart)	CDH13	Hs00169908_m1	0.002	0.89	0.118		0.356	0.057		0.168		0.414	
	dystroglycan 1	DAG1	Hs00189308_m1	0.324		0.599		0.126	0.089		0.244		0.054	
	integrin, alpha 1	ITGA1	Hs00235030_m1	0.014	0.98	0.064		0.500	0.337		0.998		0.511	
	integrin, alpha 10	ITGA10	Hs00174623_m1	0.023	2.47	0.651		0.060	0.832		0.017	>	0.072	
	integrin, alpha 11	ITGA11	Hs00201927_m1	0.150		0.264		0.030	> 0.581		0.258		0.049	>
	integrin, alpha 3	ITGA3	Hs00233722_m1	0.309		0.856		0.525	0.364		0.398		0.818	
	integrin, alpha 5	ITGA5	Hs00233732_m1	0.370		0.206		0.945	0.039	-0.41	0.810		0.367	
	integrin, alpha 6	ITGA6	Hs00173952_m1	0.007	1.40	0.035	<	0.457	0.046	1.23	0.249		0.303	
	integrin, alpha 7	ITGA7	Hs00174397_m1	0.062		0.102		0.518	0.401		0.237		0.366	
	integrin, beta 1	ITGB1	Hs00559595_m1	0.231		0.313		0.648	0.377		0.747		0.840	
	integrin, beta 3	ITGB3	Hs00173978_m1	0.908		0.643		0.676	0.325		0.406		0.769	
	integrin, beta 5	ITGB5	Hs00609896_m1	0.510		0.252		0.132	0.410		0.012	>	0.288	
	integrin, beta 6	ITGB6	Hs00168458_m1	0.163		0.138		0.602	0.026	9.34	0.070		0.766	
	syndecan 3	SDC3	Hs00206320_m1	0.059		0.066		0.163	0.825		0.345		0.325	
	sarcoglycan, beta	SGCB	Hs00165095_m1	0.107		0.090		0.045	< 0.047	-0.76	0.333		0.083	
	sarcoglycan, delta	SGCD	Hs00165726_m1	0.538		0.152		0.051	0.056		0.589		0.002	>
	solute carrier family 8 ,member 1	SLC8A1	Hs00253432_m1	0.587		0.287		0.285	0.110		0.590		0.188	
IF	actinin, alpha 2	ACTN2	Hs00153809_m1	0.685		0.212		0.569	0.171		0.610		0.073	
	Rho GTPase activating protein 26	ARHGAP26	Hs00209395_m1	0.294		0.350		0.876	0.349		0.869		0.445	
	desmin	DES	Hs00157258_m1	0.416		0.617		0.156	0.829		0.109		0.136	
	dystrophin	DMD	Hs00244243_m1	0.196		0.202		0.203	0.882		0.036	>	0.044	>
	desmoplakin	DSP	Hs00189422_m1	0.559		0.869		0.367	0.015	0.59	0.918		0.120	
	dystrobrevin, alpha	DTNA	Hs00263201_m1	0.428		0.375		0.673	0.890		0.981		0.885	
	dystrobrevin, beta	DTNB	Hs00222463_m1	0.458		0.041	>	0.074	0.313		0.143		0.263	
	filamin A, alpha	FLNA	Hs00155065_m1	0.227		0.131		0.095	0.575		0.155		0.214	
	filamin B, beta	FLNB	Hs00181698_m1	0.003	0.92	0.895		0.034	> 0.177		0.161		0.068	
	filamin C, gamma	FLNC	Hs00155124_m1	0.118		0.248		0.564	0.705		0.677		0.804	
	junction plakoglobin	JUP	Hs00158408_m1	0.505		0.050	<	0.076	0.947		0.052		0.093	
	paxillin	PXN	Hs00236064_m1	0.077		0.514		0.121	0.393		0.494		0.319	
	syntrophin, alpha 1	SNTA1	Hs00162045_m1	0.312		0.112		0.399	0.454		0.372		0.964	
	spectrin alpha	SPTAN1	Hs00162203_m1	0.948		0.361		0.353	0.065		0.848		0.232	
	talin 1	TLN1	Hs00196775_m1	0.653		0.210		0.127	0.370		0.113		0.037	>
	talin 2	TLN2	Hs00322257_m1	0.005	1.13	0.936		0.022	> 0.135		0.428		0.286	
	titin	TTN	Hs00399225_m1	0.120		0.803		0.401	0.126		0.951		0.195	
	vinculin	VCL	Hs00247826_m1	0.093		0.154		0.503	0.078		0.934		0.108	
	vimentin	VIM	Hs00185584_m1	0.304		0.833		0.513	0.551		0.160		0.474	
STF	catenin (cadherin-associated protein), alpha 3	CTNNA3	Hs00379052_m1	0.377		0.446		0.900	0.002	1.09	0.163		0.492	
	Integrin-initiated extracellular signal-regulated kinase	ERK	Hs00177066_m1	0.102		0.054		0.848	0.433		0.435		0.838	
	focal adhesion kinase	FAK	Hs00178587_m1	0.016	0.57	0.312		0.496	0.235		0.907		0.471	
	integrin-linked kinase	ILK	Hs00177914_m1	0.526		0.527		0.292	0.947		0.692		0.720	
	myocyte enhancer factor 2A	MEF2A	Hs00271535_m1	0.164		0.239		0.864	0.030	0.94	0.367		0.865	
	myocyte enhancer factor 2C	MEF2C	Hs00231149_m1	0.491		0.564		0.280	0.023	0.69	0.302		0.956	
	ryanodine receptor 2 (cardiac)	RYR2	Hs00181461_m1	0.045	0.70	0.476		0.007	> 0.023	1.73	0.364		0.046	>
	syndecan 1	SDC1	Hs00174579_m1	0.348		0.838		0.495	1.000		0.890		0.920	
	von Hippel-Lindau tumor suppressor	VHL	Hs00184451_m1	0.234		0.762		0.699	0.979		0.762		0.774	

Significant changes are indicated in yellow. The genes are grouped by function/location. Abbreviations: extracellular matrix proteins (ECM), pro- and anti-fibrotic factors (P/AFF), basal membrane proteins (BM), transmembrane and adhesion molecules (TAM), intracellular filaments (IF), and signal transduction factors (STF). Applied Biosystems (AB). > and <gene expression significantly higher or lower compared to control.
The expression of 14 genes was below level of detection and are therefore not included in this table: ADAM 12 (ADAM metallopeptidase domain 12), ADAM 15 (ADAM metallopeptidase domain 15), AGC1 (aggrecan 1), ANK1 (Ankyrin 1), DSPG3 (dermatan sulfate proteoglycan 3), EDN3 (endothelin 3), LAMC3 (laminin, gamma 3), MMP-7 (matrix metallopeptidase 7), MUC16 (mucin 16), NOS1 (nitric oxide synthase 1), SCN1A (sodium channel, voltage-gated, type I, alpha), SCN2A2 (sodium channel, voltage-gated, type II, alpha 2), SDC1 (syndecan 1), TNXB (tenascin XB).

DCM and 1 IHD patients and the other group consists of 5 IHD patients with 2 DCM patients. One DCM and one IHD patient were clustered outside both groups. So, there is a strong tendency of segregation between IHD and DCM. Therefore, the DCM and IHD patient groups were analyzed separately in the rest of this study.

Hierarchical clustering of gene expression in myocardial tissue pre- and post-LVAD support

Hierarchical clustering of the IHD samples only, showed a clear segregation into a pre- and a post-LVAD group (Figure 2). In DCM patients a similar segregation into a pre- and a post-LVAD group was not evident (data not shown).

Differential expression of genes in myocardial tissue pre- and post LVAD

Changes in gene expression were tested individually using the paired t-test in DCM and IHD separately. Furthermore, these gene profiles were compared with gene profiles of controls, to test whether gene profiles normalized or showed a tendency to deviate more from normal after LVAD therapy using the unpaired t-test. Table 2 shows all genes, grouped by function/ location: extracellular matrix proteins (ECM), basal membrane proteins (BM), transmembrane and adhesion molecules (TAM), intracellular filaments (IF), signal transduction factors (STF) and pro- and anti-fibrotic factors (P/AFF) with the p-values and fold changes.

In Table 3 only the genes that show significant changes are indicated. Only a minority of genes showed a significant difference between pre- and post-LVAD: DCM 19/92 genes (21 %) and IHD 12/92 genes (13 %). Most of these genes showed an up regulation post-LVAD (DCM 18/19 genes and IHD 8/12 genes). In DCM pre-LVAD 6 genes and post-LVAD 9 genes were up regulated compared to control. Only one gene, encoding caveolin, showed a decreased expression in both pre- and post-LVAD compared to control. In IHD pre-LVAD 12 genes were up regulated and 2 downregulated compared to control. Post-LVAD 6 were up regulated and 2 down regulated. Among these, two genes (dystrophin and laminin gamma 1) showed an increased expression compared to control in both pre- and post-LVAD samples.

Table 3. Summary of significant alterations in gene expression.

DCM

Gene name	pre vs post	pre vs control	post vs control
osteopontin	▼	=	>
bone morphogenetic protein 4	▲	=	>
collagen, type VI, alpha 3	▲	=	>
filamin B, beta	▲	=	>
laminin, gamma 1	▲	=	>
ryanodine receptor 2 (cardiac)	▲	=	>
talin 2	▲	=	>
cadherin 13, H-cadherin (heart)	▲	=	=
calcium channel, alpha 1C subunit	▲	=	=
collagen, type XIV, alpha 1	▲	=	=
collagen, type XV, alpha 1	▲	=	=
decorin	▲	=	=
focal adhesion kinase	▲	=	=
heparan sulfate proteoglycan 2 (perlecan)	▲	=	=
inhibitor of DNA binding 1	▲	=	=
integrin, alpha 1	▲	=	=
integrin, alpha 10	▲	=	=
laminin, beta 1	▲	=	=
bone morphogenetic protein 7	▲	<	=
integrin, alpha 6	▲	<	=
dystrobrevin beta	=	>	=
vitronectin	=	>	=
integrin, alpha 11	=	=	>
sarcoglycan, beta	=	=	<
fibroblast growth factor 2	=	<	=
junction plakoglobin	=	<	=
caveolin 1	=	<	<

▼: decreased or ▲: increased gene expression after LVAD support, =: no change, >:higher or <:lower expression pre- or post-LVAD compared to control. The shaded (green) genes are significantly altered in both DCM and IHD.

Table 3 (continued)

IHD

Gene name	pre vs post	pre vs control	post vs control
fibronectin 1	▼	>	=
integrin, alpha 5	▼	=	=
osteopontin	▼	=	=
sarcoglycan, beta	▼	=	=
ryanodine receptor 2 (cardiac)	▲	=	>
cadherin 13, H-cadherin (heart)	▲	=	=
calcium channel, alpha 1C subunit	▲	=	=
catenin (cadherin-associated protein), alpha 3	▲	=	=
desmoplakin	▲	=	=
integrin, alpha 6	▲	=	=
integrin, beta 6	▲	=	=
myocyte enhancer factor 2A	▲	=	=
myocyte enhancer factor 2C	▲	=	=
spectrin alpha	▲	=	=
ATPase, Ca++ transporting, cardiac muscle	▲	<	=
dystrophin	=	>	>
lamin, gamma 1	=	>	>
bone morphogenetic protein 4	=	>	=
collagen, type XIV, alpha 1	=	>	=
connective tissue growth factor	=	>	=
fibromodulin	=	>	=
insulin-like growth factor 1	=	>	=
integrin, beta 5	=	>	=
integrin, alpha 10	=	>	=
integrin, alpha 6	=	>	=
osteonectin	=	>	=
thrombospondin 2	=	>	=
integrin, alpha 11	=	=	>
laminin, alpha 2	=	=	>
sarcoglycan, delta	=	=	>
talin 1	=	=	>
mothers against DPP homolog 6	=	=	<
bone morphogenetic protein 7	=	<	=

Genes encoding extracellular matrix proteins:

In DCM, 5 genes encoding ECM proteins were up-regulated post-LVAD. However, except for collagen type VI alpha3, these genes did not differ significantly (either pre- or post LVAD) from control. This indicates that the increased expression induced by the LVAD support of these 5 genes is significant but as a group are not different from the control group (Fig. 3). In IHD most differences between pre-LVAD and control were observed in genes encoding ECM proteins. This difference from control is not observed in post-LVAD samples, suggesting a high expression of ECM gene activity pre-LVAD.

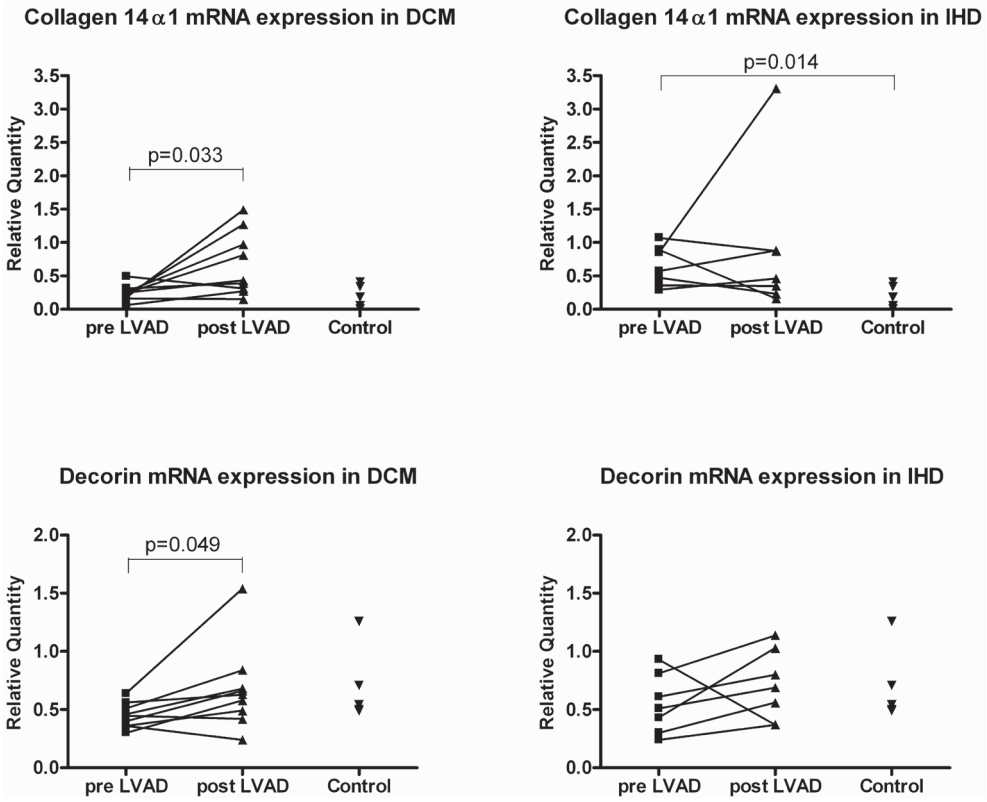


Figure 3. Relative mRNA expression of genes encoding ECM proteins.

Relative mRNA expression was determined pre- and post-LVAD of DCM and IHD and tested in paired t-test. Increase of collagen 14α1 mRNA expression is significant in DCM but not in IHD. Compared to the control, only the mRNA expression pre-LVAD of IHD patients is significantly higher (unpaired t-test). Decorin is significantly increased post-LVAD in DCM. Compared to the control none of the pre- and post-LVAD samples differed significantly.

Genes involved in the fibrotic pathway:

In the fibrotic pathway a remarkable difference between DCM and IHD was observed. In DCM patients the expression of genes encoding pro-fibrotic factors (TGFβ1, FGF, IGF, endothelin and CTGF) remained the same, in contrast, those encoding anti-fibrotic proteins (BMP-4, BMP-7, decorin and ID1) increased after LVAD support. Pre-LVAD the expression of the pro-fibrotic factor FGF2 and the anti-fibrotic factor BMP-7 was low compared to control. Post LVAD the expression of the anti-fibrotic factor BMP-4 was increased compared to control. In IHD patients however, there was no change in gene expression during LVAD support but

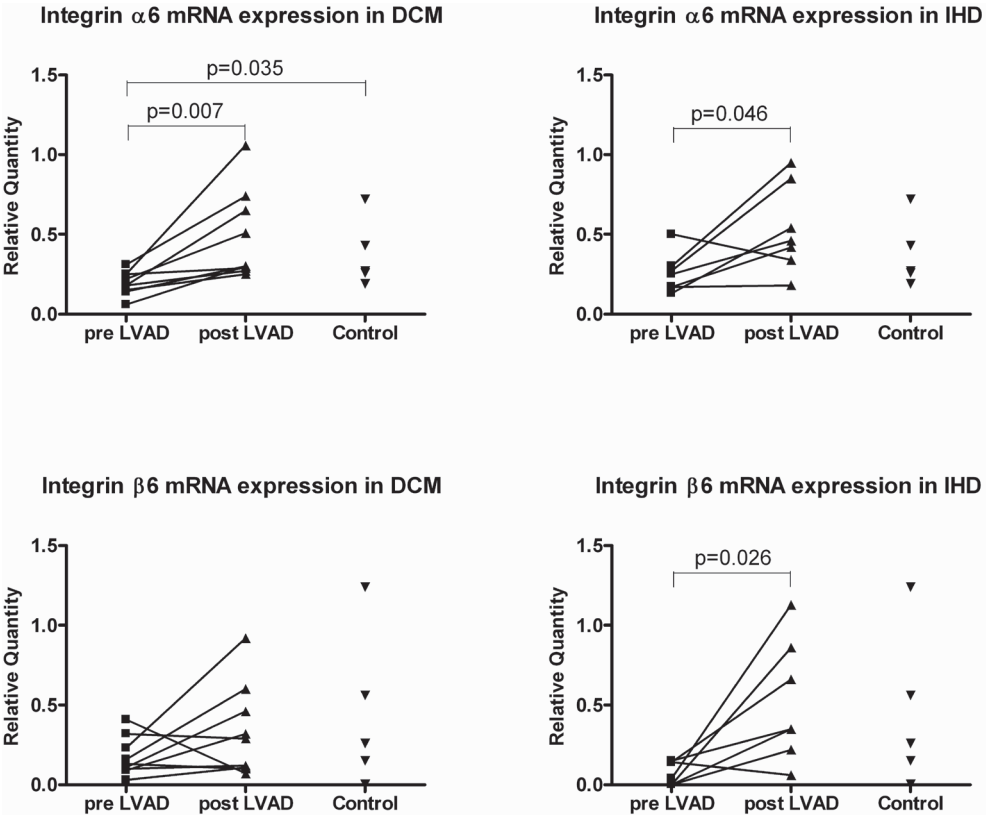


Figure 4. Relative mRNA expression of genes encoding different integrins.
Relative mRNA expression was determined pre- and post-LVAD of DCM and IHD and tested with the paired t-test. Increase of integrin α6 mRNA expression was significant in both DCM and IHD during LVAD support. Compared to the control, only the mRNA expression pre-LVAD of DCM patients is significantly lower (unpaired t-test). Integrin β6 is only significantly increased post-LVAD in the IHD group. Compared to the control none of the pre- and post-LVAD samples differed significantly.

in pre-LVAD samples the pro-fibrotic genes are expressed stronger than in control, whereas the expression of the anti-fibrotic gene BMP-7 is lower than in control. The post-LVAD expression pattern is comparable to that of control.

Genes encoding basal membrane proteins:

The gene encoding osteopontin is the most remarkable member of the BM group. In both DCM and IHD a significant reduction in its expression is observed after LVAD support. Other

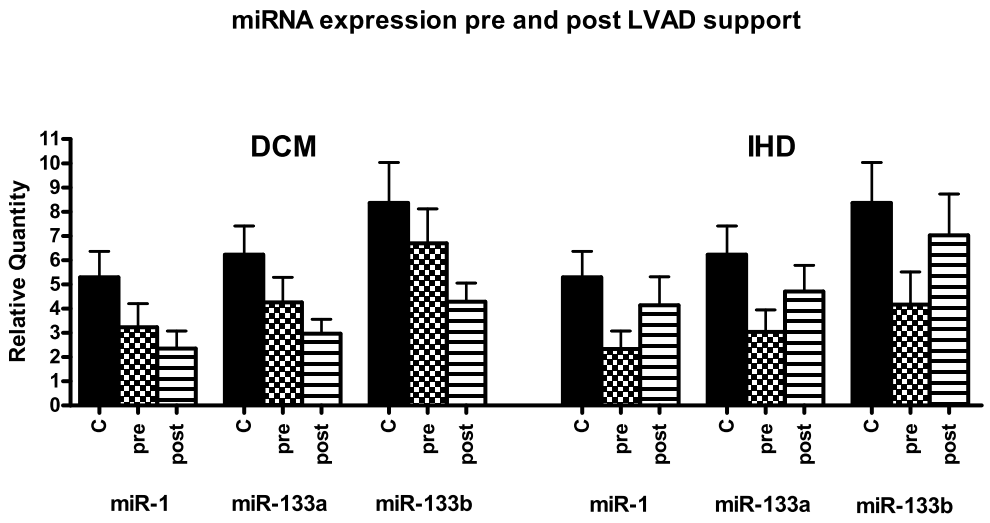


Figure 5. Changes in miR expression after LVAD support

The relative quantities of miR-1, miR-133a and miR-133b measured in heart tissue obtained form patients suffering from IHD (n= 8) or DCM (n=9) or C: controls (n=5; Pre = pre LVAD support ; Post= post LVAD support.

Table 4. Statistical analysis of miR expression changes after LVAD support

miR	DCM-patients			IHD-patients		
	1	2	3	1	2	3
miR-1	0.20	0.24	0.06	0.08	0.05	0.44
miR-133a	0.73	0.19	0.04	0.11	0.07	0.35
miR-133b	0.02	0.44	0.04	0.11	0.09	0.44

The p-values for the various differences in relative quantitative expression of the miRs in the myocardium obtained from DCM and IHD patients, respectively, before and after LVAD support
1: pre-LVAD versus post LVAD; 2: pre-LVAD versus control; 3: post LVAD versus control

BM proteins showed hardly any change, apart from laminin, vitronectin and thrombospondin (anchoring proteins).

Several integrins showed differential expression (mostly up-regulation) in both DCM and IHD. In particular, integrin β6 gene expression showed a strong increase pre- to post-LVAD

in the IHD group (Fig 4). Other membrane molecules, like caveolin, sarcoglycan and ATPase calcium transporting molecule, showed a low expression compared to control either pre- or post-LVAD.

Genes encoding intracellular proteins:

Expression of some intracellular filament genes changed significantly after LVAD support in DCM (2/19: filamin, talin) and in IHD (1/19: desmoplakin), suggesting only a minor intracellular filament involvement. In this group it was remarkable that in IHD the gene encoding dystrophin was upregulated both pre- and post-LVAD.

In the expression of signal transduction factors, relatively more changes were observed during LVAD support both in DCM (2/8: Focal Adhesion Kinase and ryanodine receptor 2) and in IHD (4/8: catenin, myocyte enhancer factor 2A and 2C, and ryanodine receptor 2).

Changes in miR expression during LVAD support

Total RNA was isolated from heart tissue of heart failure patients pre- and post-LVAD. The relative quantity of miRNA1, miRNA133a, miRNA133b and miRNA-208 was established with the Taqman® MicroRNA assay (Applied Biosystems, Foster City, CA, USA). In Figure 5 the expression of miR-1, miR-133a and of miR133b is shown for DCM and IHD patients on LVAD support. Compared to control levels the miR expression in both heart failure groups is low for all miR tested. This decrease was more significant in IHD than in DCM. After LVAD support the levels did not change significantly, although in IHD there is a tendency that the miR expression levels return to normal. In patients with DCM this was not the case. On the contrary there was a tendency of further decrease. The expression of miR-208 showed similar changes (data not shown) as did the other three. However, the expression was too low to make a reliable statistical analysis.

The results of the statistical analyses are given in Table 4. These data confirm that in DCM, LVAD support did not reverse the decrease in miR-expression, as in DCM post-LVAD the miR expression was significantly lower than in the controls. In patients with IHD there were no significant differences in expression of the miRs in the controls compared to the values after LVAD support, indicating that a considerable restoration of the expression did occur.

Discussion

During unloading, the myocardium of the failing heart shows various changes, both macroscopically and microscopically. Major changes include reduction of cardiomyocyte size, and changes in the volumes of ECM and BM components (Goldsmith and Borg, 202; Bruggink et al., 2006; Parker and Ingber, 2007). In many studies analyzing the effect of mechanical support on heart failure, only marginal differences have been observed between IHD and DCM (Bruggink et al., 2006a; De Jonge et al., 2001, 2002; Grady et al., 2003). However, in the present study hierarchical clustering of all expressed genes in end stage heart failure showed that DCM and IHD segregated and could be identified as separated entities (Figure 1). For this reason both groups were analyzed separately. In the IHD group pre- and post-LVAD samples did segregate by hierarchical clustering (Figure 2). In the DCM group no such separation of pre- and post-LVAD samples was observed. The reason for this difference between both groups is unknown. DCM may have a genetic background that leads primarily to hypertrophy and fibrosis. This attribute of DCM would then cause a gene expression that differs from controls in several aspects, but is not completely reversed by LVAD support. By contrast, in IHD, tissue damage and repair are involved by gene expression alterations that are induced by infarction, and that are partly normalized by the unloading of the heart. The differences in mRNA expression between IHD and DCM may give an important clue in finding targets that are informative for the state of the (un)supported hearts.

Compared to control, in DCM pre-LVAD samples, only 7 genes (2 up- and 5 down-regulated) were differentially expressed which increased to 10 genes post-LVAD (7 up- and 3 down-regulated). In IHD pre-LVAD samples, the expression of 15 genes (13 up- and 2 down-regulated) differed from control, which decreased to 8 genes post-LVAD (7 up- and only 1 down-regulated). In both groups most genes that were differentially expressed pre-LVAD normalized to control levels after LVAD support. On the other hand, LVAD support can also induce a down- or up-regulation of genes that pre-LVAD did not differ from control level (Table 2). Eleven genes showed significant changes in both DCM and IHD. However, in only 3 cases these changes were the same (calcium channel alpha 1C subunit, integrin alpha11 and rhyanodine receptor 2). In DCM, the expression of caveolin remained low (both pre and post LVAD) compared to control. This is in contrast to the described up-regulation of caveolin protein after LVAD support (Uray et a., 2003). In IHD the expression of dystrophin and laminin (gamma 1) remained high after LVAD support. Changes in expression of both

genes have been described by others (Vatta et al., 2004; Birks et al., 2005; Refaat et al., 2008).

LVAD-induced changes in ECM and cardiomyocytes have been described (Milting et al., 2008; Bruggink et al 2006b; Thohan et al., 2005). In this respect, the total number of genes coding for various structural elements, that were differentially expressed pre- and post-LVAD was surprisingly low. Morphological changes during LVAD support were paralleled by changes in collagen turn over and expression of genes encoding for structural collagens (Type I and III; 11). So, the minor changes observed in expression of ECM genes in the present study may imply that most ECM changes are induced post-transcriptionally, either by micro-RNA regulation (Schipper et al., 2008) or in the matrix itself (e.g. by MMP). The latter is supported by significant changes in mRNA expression of MMP during LVAD support (Li et al., 2001; Klotz et al., 2005). Interestingly the anchoring and connecting collagens (Types VI, XIV and XV) and molecules involved in ECM assembly like fibulin, fibronectin, osteonectin and proteoglycans (fibromodulin, heparan sulfate and decorin; Pollard et al., 2008) did change although not similar in DCM and IHD. The changes in these molecules, also observed by others (Jahanyar et al., 2007; Gabrielsen et al., 2007), may contribute to the increased rigidity of the heart after LVAD support, in addition to the differences in tissue concentrations of structural collagens (Klotz et al., 2005).

Previously, we have shown that during unloading the immunohistochemical expression of collagen IV in the BM decreased (Bruggink et al., 2007). In contrast, immunoreactivity of laminin did not show substantial changes. Of the 17 tested genes that encode BM proteins only few showed expression changes during LVAD support, indicating a disbalance between mRNA expression and protein expression. The few genes that did show changes, either in DCM or IHD, are involved in cell-adhesion (laminin β1 and γ1, osteopontin). Together with the changes observed in the gene expression of the integrin, cadherin and sarcoglycan family members, this underlines the importance of these specific anchoring or connecting proteins in the structural changes observed (Birks et al.,2005; Gabrielsen et al., 2007; Latif et al., 2007; Kim et al.,1999).

Only minor changes were observed in the expression of genes encoding intracellular proteins. In DCM, alterations in cytoskeletal filaments (dystrobrevin, filamin, junction plakoglobin, and talin) are more pronounced than in IHD (desmoplakin, dystrophin and talin; Gabrielsen et al., 2007). This could indicate that this class of genes is more affected in DCM than in IHD, which may be explained by the different onset of the tissue damage in both diseases.

In the fibrotic pathway a remarkable difference between DCM and IHD is observed. In DCM the expression of pro-fibrotic factors (TGFβ1, FGF, IGF, endothelin and CTGF) did not change, but the expression of anti-fibrotic genes (BMP-4, BMP-7, decorin, and Id1) increased after LVAD support. This is paralleled by the observed reduction in fibrosis in DCM (Bruggink et al., 2006b). In patients with IHD the expression of both anti- and pro-fibrotic factors remain unchanged. However, in IHD pre-LVAD the pro-fibrotic response genes are expressed stronger than in control whereas the expression of the anti-fibrotic gene BMP-7 is lower than in control. This will favour fibrosis in the hearts of patients with IHD. In these patients, the post-LVAD situation may be associated with a return of gene expression to control values, leading to a reduction of fibrosis. So, pro- and anti-fibrotic gene expression is in agreement with previously described reduction of fibrosis after LVAD support (Goldsmith and Borg, 2002; Gabrielsen et al., 2007), although the mechanism between these two entities is different.

In view of the changes in mRNA expression that did not seem to be paralleled by the linked protein expression special emphasis was given to miR expression during LVAD support. These miR are important in the post-transcriptional regulation of mRNAs, also in the heart (Chen, 2007; Couzin, 2008). Remarkable was the relative low expression in the myocardium of heart failure patients of the miR tested (miR-1, miR-133a and miR133b), compared to controls. In IHD the level of miR expression tended to return to control levels. In DCM however, the levels tended to decrease even further, which suggest that genes under the control of these miR could be expressed even stronger. Chen et al. (2006) have described that miR-1 and miR-133 promote myogenesis and myoblast proliferation respectively of skeletal muscles. Similar data have been produced by Liu et al (2007) and Ikeda et al (2008) for heart failure. The relative low expression of the miR in heart failure compared to control, may be related to the observed myocardial hypertrophy (De Jonge et al., 2002), as over expression of both miR-1 and miR-133 leads to cardiac hypertrophy (Care et al., 2007).
This difference in miR expression between DCM and IHD patients after LVAD support may be explained by the fact that there is no need for cell proliferation in DCM but it is in IHD. Remodeling of DCM involves mainly a reduction of hypertrophy of cardiomyocytes, whereas IHD involves tissue repair including cell proliferation. This may indicate that the studied miR's are more involved in regulation of the proliferative processes than in reduction of the hypertrophy. As already mentioned, the reduction in miRNA expression in IHD patients is not

restored completely to control levels during LVAD support. Neither in patients supported for a short period of time nor in patients supported over more than one year.
The miR data do show that miR change during heart failure (Busk and Cirera, 2010) and LVAD support, and in that respect it is interesting to note that there are initial indications that miR released in the serum could act as important biomarkers for screening of heart conditions. (Adachi et al., 2010) and be targets for therapy (Seok and Wang, 2010).

In conclusion, the set of genes coding for proteins involved in mechanotransduction, selected for the analysis of changes in mRNA expression pre- and post-LVAD, resulted in an identification of IHD and DCM as separate entities. The morphologic and structural changes observed in the failing human heart after LVAD support are only partly reflected in changes of mRNA expression of genes encoding proteins involved in mechanotransduction. This suggests that most changes in ECM and intracellular filaments are not regulated at the mRNA level. However, expression of genes encoding membrane bound proteins such as cadherin and integrins, and anchoring proteins such as collagen type VI and proteoglycans, are clearly affected by LVAD support and contribute to adaptation to decreased loading conditions. Also the genes involved in fibrosis showed adaptation to the LVAD support, and their expression runs parallel to the observed morphological changes. These genes may prove to be important targets in the development of protocols which decide whether LVAD supported patients should undergo heart transplantation or could rather continue their LVAD therapy for a longer period of time. The role of miR in this decision making, but also as biomarker or therapeutic targets is promising as well.

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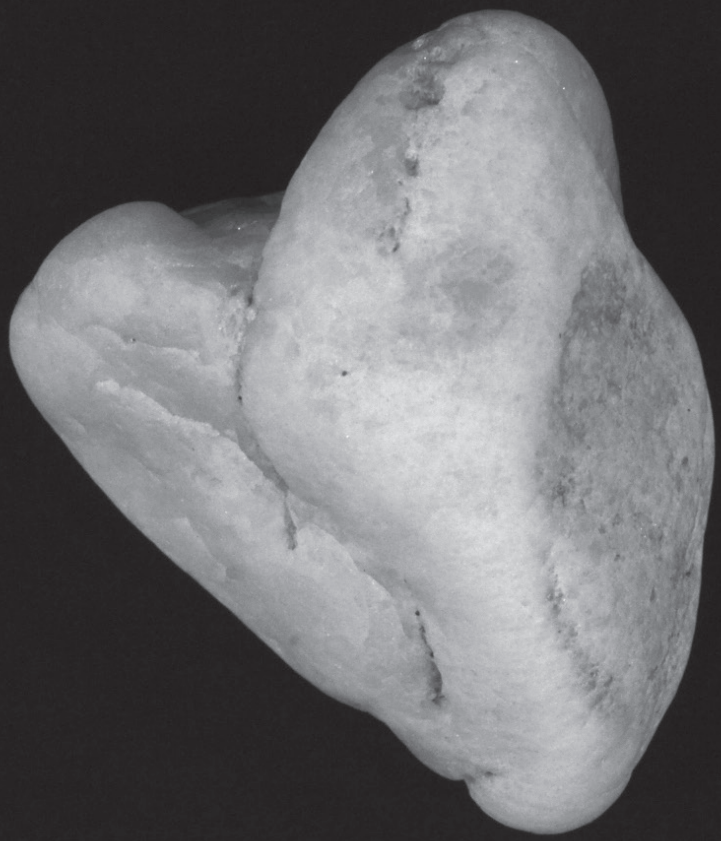
Proteomic profiling of the human failing heart after Left Ventricular Assist Device (LVAD) support



Abstract

Background- Left ventricular-assist device (LVAD) support is commonly used in patients with heart failure as a bridge to heart transplantation (HTx). Whereas myocardial gene expression profile changes have been well established following LVAD support, the consequences on the protein level largely remain unclear.

Methods and Results- Changes in protein expression in the human heart during LVAD support were studied, using fluorescent 2-dimensional difference gel-electrophoresis (2D-DIGE). Myocardial tissue from patients diagnosed with dilating cardiomyopathy (DCM) and ischemic heart disease (IHD) was obtained before LVAD-implantation (pre-LVAD) and at the time of HTx (post-LVAD). Proteins from pre- and post-LVAD tissues were analyzed by 2D-DIGE and differentially expressed proteins were identified by mass-spectrometry. In the DCM group, we detected 16 proteins that showed statistically significant downregulation from pre- to post-LVAD tissue. In IHD patients, 50 proteomic changes were found, including both up- (n=12) and downregulated (n=38) proteins. The identified proteins in both groups partially overlap and include proteins from the cytoskeleton and mitochondrial energy metabolism. The latter changes are paralleled by severe alterations in mitochondrial morphology, as shown by electron microscopy. Post-LVAD proteomes of both DCM and IHD patients largely mimic the protein profiles of non-failing hearts.

Conclusions- We conclude that LVAD-induced cardiac remodeling in both DCM and IHD patients is associated with specific atrophic changes in proteins expression profiles, predominantly involved in cytoskeleton integrity and mitochondrial energy metabolism. These data may facilitate prognostic prediction of the individual response to LVAD support and determine the liability of patients for LVAD weening or HTx.

Introduction

Left ventricular assist devices (LVAD) are commonly used in patients with heart failure as a bridge to heart transplantation (HTx). In most cases, LVAD support extends the patient's life span and improves the quality of life.[1,2] The pressure and volume unloading of the left ventricle can reverse left ventricular dilatation and lead to regression of the left ventricular hypertrophy and neurohormonal changes.[3-6] The understanding of this process, referred to as 'reverse remodeling'[7], is important for a better insight in both myocardial events during LVAD support and the processes leading to heart failure. Recently, LVAD's are more often used as destination therapy and also the number of institutions that perform LVAD explantations without the need for HTx (weaning) increases.[4,8-11] Both therapies require a more profound knowledge of the process of reverse remodeling to learn for diagnostic purposes, which changes are beneficial or detrimental.

In cardiac unloading experiments, we and others have previously demonstrated partial recovery of the contractile myofilaments in the cardiomyocytes[12-16], changes in the extra cellular matrix (ECM) components[13,17], changes in cytoskeletal proteins[18] and metabolic genes[19], and decreased natriuretic peptide levels both in the plasma of the patients, as well as in the heart.[5,6] Most of these changes were detected at the mRNA level by quantitative PCR (Q-PCR) and some were confirmed at the protein level by immunohistochemistry and Western blotting. The changes in expression of mRNA did not always parallel the change in corresponding proteins. Whereas myocardial gene expression profile changes have been well established following LVAD support, the consequences on the protein level largely remain unclear.

To obtain a better understanding of the major changes in proteins that occur in the failing human heart after LVAD support, we analyzed in the present study the changes in the total protein fraction of heart tissues obtained pre- and post-LVAD by using a proteomics approach. Fluorescent 2-dimensional difference gel electrophoresis (2D-DIGE) was used to profile differences in protein expression. Differentially expressed proteins were excised and identified by tandem mass spectrometry. Major protein changes that occur during LVAD support are observed in intra- and extra-cellular filaments and at the level of the energy metabolism. The latter changes are paralleled by severe alterations in mitochondrial morphology, as shown by electron microscopy.

Methods

Patients and tissue collection

Subjects of this study were patients with refractory end-stage heart failure. Patients characteristics are summarized in Table 1. Six patients were diagnosed with ischemic heart disease (IHD) and five patients suffered from dilated cardiomyopathy (DCM). They were all treated with a pneumatic or electric LVAD (Heart-mate, Thoratec, Pleasanton, California) as a bridge to heart transplantation (HTx). At the time of LVAD implantation, all patients were in NYHA functional class IV, and in NYHA functional class I while on LVAD support. All patients were on ACE inhibitor treatment in the stable phase of heart failure. Before LVAD implantation these were stopped and all patients received intravenous inotropics because of hemodynamic deterioration. Cardiac medication was discontinued initially in all patients after LVAD implantation. However, in 30-60% of patients at some stage hypertension was treated by ACE inhibitors (Table 1). Informed consent to participate in this study was obtained from all patients. The myocardial biopsy at the time of LVAD implantation consisted of the LV apical core, which was removed during LVAD implantation. These biopsies (pre-LVAD) were compared with LV tissue specimens of the explanted heart after HTx (post-LVAD),

Table 1. Clinical characteristics of study subjects.

	Pre-LVAD Cardiomyopathy	
	DCM* *(n=6)*	IHD* *(n=5)*
Age (y)	36 (32-45)	50 (47-61)
Male (%)	66	100
NYHA classification	IV	IV
Duration of LVAD support (days)	207 (132-257)	266 (264-361)
Left ventricular ejection fraction (%)	17 (15-20)	24 (18.5-24.5)
Left ventricular end-diastolic diameter (cm)	7.5 (7.3-7.7)	7.2 (7.1-7.2)
Pulmonary artery pressure (mm Hg)		
Systolic	41 (40-46)	50 (45-53)
Diastolic	26 (23-26)	25 (24-27)
Pulmonary capillary wedge pressure (mm Hg)	23 (21-26)	24 (23-26)
Cardiac index ($L.min^{-1}.m^{-2}$)	2.5 (2.2-2.8)	2.0 (1.5-2.3)
Medications while on LVAD (%)		
β-Antagonists	17	20
ACE inhibitors	33	60
Diuretics	33	40
Spironolacton	0	20

*Values are median (25^{th}-75^{th} percentiles)

taken from the apical half of the LV, outside the suture area of the inflow canula. All biopsies were directly frozen after LVAD implantation and after HTx. A pool of heart tissues from two rejected donor-hearts and three non-failing hearts obtained at autopsy served as reference tissue [Age (y) = 65 (52-69) and Male (%) = 66, values are median (25^{th}-75^{th} percentiles)].

Fluoresence 2-dimensional difference gel electrophoresis

Tissue samples in 50 mM Tris (pH 7.4), 150 mM NaCl, and 1% NP-40 were mixed and incubated for 30 min at 4°C. Lysates were cleared by centrifugation at 14,000 rpm for 10 min. Cell free extracts (75 μg) were precipitated using the Plus One 2D Clean-up kit as recommended by the manufacturer (GE Healthcare; Uppsala, Sweden) and solubilized in 8 M urea, 2 M thiourea, and 4% Chaps (75 μl). Pre- and post-LVAD samples (50 μl of a concentration of 1 μg/μl) were labeled with 400 pmol of either 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide N-hydroxysuccinimidyl ester (Cy3) or 1-(5-carboxypentyl)-1'-methylindodicarbocyanine halide N-hydroxysuccinimidyl ester (Cy5). Mixtures (1:1) of both samples were labeled with 3-[(4-carboxymethyl)phenylmethyl]-3'-ethyloxacarbocyanine halide N-hydroxysuccinimidyl ester (Cy2), which functions as internal control. The labeling reactions were stopped by adding 0.2 mM Lysine, diluted with rehydration buffer (8 M urea, 2 M thiourea, 4% Chaps, 150 mM DTT, 1% biolyte pH 3-10, and 0.002% bromophenol blue), and combined according to the experimental design. Samples (150 μg) were re-hydrated passively into immobilized pH gradient strips (24 cm; pH 3-10, non-linear) for 15 h at room temperature (RT) prior to iso-electric focusing in the IPGphor system (GE Healthcare, Uppsala, Sweden) for 64 kVh. IPG strips were reduced for 60 min in 1% (w/v) DTT, 6 M Urea, 2% (w/v) SDS, 30% (v/v) glycerol, 75 mM Tris, pH 8.8, and alkylated for 30 min in the same buffer containing 2.5% (w/v) iodocetamide instead of DTT. The strips were overlayed on a 12% SDS/PAGE gel (20 × 24 cm), immobilized to a low-fluorescent glass plate and electrophorezed for 18 h at 1 W per gel. The Cy2, Cy3, and Cy5-labeled images were acquired on a Typhoon 9400 scanner (GE Healthcare, Uppsala, Sweden) at the following excitation/emission values: 488/520, 532/580, and 633/670 nm, respectively. Dye swaps were included to exclude preferentially labeled proteins from the analysis. Relative quantification of matched gel features was performed by using Decyder DIA and BVA software (GE Healthcare, Uppsala, Sweden). For inter-gel analyses, the internal standard method was used.[20] Statistical analysis of gel spot volume quantification was performed by Student t-test. $P<0.05$ was regarded as statistically significant. Differentially expressed proteins (i) differed at least 1.5-fold in fluorescence

intensity, (ii) differed statistically significant (P<0.05), and (iii) could be detected in at least 3 patients. Proteomic comparison of post-LVAD samples (IHD, n=3 or DCM, n=3) with one pool of tissues from 5 non-failing hearts was performed as described above. Decyder EDA software (GE Healthcare, Uppsala, Sweden) was used for hierarchical clustering and a heat map that used Euclidean distance with complete linkage. The Amigo Gene Ontology system was used for functional classification of the proteins (www.amigo.geneontology.org).

Tandem mass spectrometry

Selected spots were excised robotically (Ettan Dalt Spot Cutter, GE Healthcare, Uppsala, Sweden). In-gel digestion and mass spectrometry (MS) analysis was outsourced to ServiceXS (Leiden, The Netherlands). Excised gel plugs were washed twice with water, twice with 50 mM ammoniumbicarbonate in 50% acetonitrile and dehydrated using 100% acetonitrile (Merck). The proteins were reduced with 10 mM DTT (Sigma, St. Louis, USA) and subsequently alkylated with 55 mM iodoacetamide (Sigma). Following washing with 50 mM ammonium bicarbonate, 50 mM ammonium bicarbonate/50% acetonitrile and 100% acetonitrile, the gel plugs were rehydrated using 10 µl 50 mM ammonium bicarbonate containing 50 ng of trypsin and incubated on ice for 30 minutes. If necessary, 50 mM ammonium bicarbonate was added to completely cover the gel plugs. Protein digestion by trypsin was allowed to proceed overnight at 37°C. The supernatant was acidified using TFA to a final concentration of 0.1%, desalted over a Zip-Tip (Millipore) and spotted directly on a MALDI target plate using 1 µl α-cyano-4-hydroxycinnamic acid (HCCA, 0.3 mg/ml in ethanol:acetone 2:1) (Bruker Daltonics, Bremen, Germany) as the Zip-Tip eluent. MS and tandem MS (MS//MS) spectra were acquired on an Ultraflex™ II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). The MS and MS/MS spectra were searched against the NCBI database using the MASCOT search algorithm (version 2.1) using mass tolerances of 0.15 Da for MS and 0.5 Da for MS/MS. Carbamidomethylcysteine was taken as a fixed modification and oxidation of methionines as a variable modification. The search parameters allowed for 1 missed cleavage.

Electron microscopy

Left ventricular (LV) biopsies of the apex (pre-LVAD) and LV wall (post-LVAD) were used for transmission electron microscopy (TEM). Heart tissues were fixed in Karnovsky's fixative, followed by 4% OsO_4. After dehydration in alcohol, they were embedded in epon, following

routine procedures. Ultrathin sections were stained with 5% uranyl acetate and 2.5% lead citrate, randomly analyzed and photographically recorded under a TEM (Jeol 1200 EX-2).

Results

To determine proteomic changes that occur following left ventricular unloading in the LVAD-associated cardiac reverse remodeling process, we analyzed differential protein expression in paired heart samples from patients with end-stage cardiomyopathy (DCM and IHD) obtained at the time of LVAD implantation (pre-LVAD) and at the time of HTx (post-LVAD). All end-stage patients exhibited a severely reduced ejection fraction, left ventricular dilation, elevated pulmonary arterial and wedge pressures, and reduced cardiac index (Table 1). We used fluorescence two-dimensional difference gel electrophoreses (2D-DIGE) in combination with tandem mass-spectrometry (MS) to compare the proteomes of the human heart following LVAD support. Pre- and post-LVAD samples were labeled with a red fluorescent-dye (Cy5) and a green fluorescent-dye (Cy3), respectively. Both samples were combined and separated on the same 2D-gel. A pool of all samples labeled with Cy2 was included as internal standard, allowing adequate comparison between gels. Gels were sequentially scanned at excitation and emission wavelengths specific for each fluorescent label, and images were overlaid digitally. Figure 1 shows representative examples of a DCM (Figure 1A) and an IHD (Figure 1B) patient. Red and green spots represent down- and upregulated proteins following LVAD support, respectively. For DCM, a total of ~1,400 proteins were resolved of which 16 proteins were downregulated (~1.1%; log peak volume change >1.5 fold, in at least 3 patients, $P<0.05$) following LVAD support. No upregulated proteins were detected. For IHD, a total of ~1,700 proteins were resolved of which 38 proteins were downregulated (~2.2%; log peak volume change >1.5 fold, in at least 3 patients, $P<0.05$) and 12 proteins were upregulated (~0.7%; log peak volume change >1.5 fold, in at least 3 patients, $P<0.05$) following LVAD support. Proteomic protein profiles following LVAD support in DCM and IHD patients only partially overlap. The majority of the detected cardiac protein changes from pre- to post-LVAD equalled the protein expression levels of non-failing reference subjects (Table 2 and 3). In a hierarchical clustering algorithm with changed protein spots, 5 out of 6 DCM samples and 5 out of 5 IHD samples formed distinct clusters by pre- and post-LVAD status (Figure 2). Taken together, these data indicate that 2D-DIGE is a powerful method to

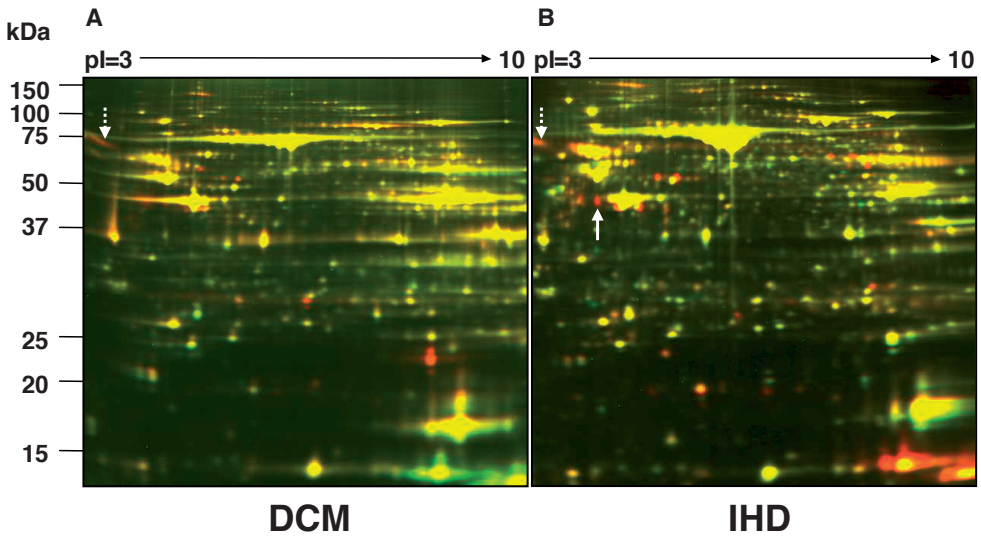


Figure 1. Proteomic profiling of the human failing heart following LVAD support. Pre- and post-LVAD biopsy samples (100 µg) of DCM (A) or IHD (B) patients were labeled with green or red fluorescent dyes, respectively. Samples were combined and separated by 2D gel electrophoresis. Protein spots shared between the two samples appear yellow and represent the unaffected proteome. Protein spots that are reduced in abundance following LVAD support are red, while upregulated spots appear red. These experiments were performed with at least 5 patients, allowing adequate statistical analysis. Protein spot intensity was regarded changed when peak volume ratios were >1.5-fold and $P<0.05$. The white arrow points to the identified structural protein desmin that is further characterized in figure 3.

profile proteomic changes in the human heart and to distinguish between etiologies and individual LVAD statuses.

LVAD support affects mitochondrial energy metabolism and morphology.

Employing 2D-DIGE, the differential protein expression in paired heart samples from patients with end-stage cardiomyopathy (DCM and IHD) obtained pre- and post-LVAD was analyzed. Changed spots were excised from 2D gels and the majority was identified by tandem MS. Several spots that consistently changed following LVAD support treatment could not be identified, most likely because protein levels were too low. We were able to detect both known and novel protein changes. It has been well established that expression of the structural protein desmin decreases in cardiomyocytes of the failing heart following LVAD support.[21] In agreement with this, we identified desmin to be downregulated in both DCM

Table 2. Overview of cardiac protein changes in DCM patients following LVAD support**.** Changed cardiac protein identities from DCM patients were determined by 2D-DIGE and MS. Details are provided from pre- to post-LVAD and from post-LVAD to reference tissue. NCBI accession numbers and the change of spot intensity (average ratio) are indicated. Theoretical molecular weight (Mw) and isoelectric point (pI) details are from the Swiss-Prot database.

Dilating cardiomyopathy (DCM)	*Pre → Post LVAD*							*Post LVAD → Reference*		
Protein identity	*Change*	*Average ratio*	*P-value*	*Number in Hierarchical Clustering (Fig. 3)*	*NCBI accession number*	*Theoretical*		*Change*	*Average ratio*	*P-value*
						Mw (kDa)	*pI*			
Alpha-1-antichymotrypsin	↓	-3.39	0.016	5	gi|177809	48.6	5.8	↑	1.85	0.0074
Alpha-1-antichymotrypsin	↓	-2.95	0.003	4	gi|177809	48.6	5.8	↑	2.25	0.00050
Fibrinogen gamma	↓	-2.56	0.00023	10	gi|119625326	47.3	5.5	=		
Ceruloplasmin	↓	-2.31	0.0061	1	gi|1620909	115.4	5.3	=		
Ubiquinol-cytochrome c reductase core protein 1	↓	-2.03	0.0033	9	gi|515634	52.6	5.9	=		
Alpha-1-antichymotrypsin	↓	-2.01	0.014	3	gi|77809	48.6	5.8	↑	2.21	0.016
Alpha-1-antichymotrypsin	↓	-1.89	0.015	2	gi|77809	48.6	5.8	↑	2.29	0.000049
Heat shock protein 27	↓	-1.69	0.020	14	gi|662841	22.3	9.1	=		
ATP synthase subunit beta	↓	-1.68	0.014	8	gi|89574029	48.1	4.8	=		
Heat shock protein 27	↓	-1.66	0.018	13	gi|662841	22.3	9.1	=		
Cardiac muscle alpha actin 1	↓	-1.65	0.021	12	gi|885049	42.0	5.1	↑	1.63	0.029
Mutant desmin	↓	-1.57	0.037	11	gi|1907570	53.5	5.0	↑	2.03	0.0063
ATP synthase subunit alpha	↓	-1.53	0.018	6	gi|158259937	54.5	8.3	↑	1.70	0.0036

Table 3. Overview of cardiac protein changes in IHD patients following LVAD support. Changed cardiac protein identities from IHD patients were determined by 2D-DIGE and MS. Details are provided from pre- to post-LVAD and from post-LVAD to reference tissue. NCBI accession numbers and the change of spot intensity (average ratio) are indicated. Theoretical molecular weight (Mw) and isoelectric point (pI) details are from the Swiss-Prot database.

Ischaemic heart disease (IHD)	*Pre → Post LVAD*							*Post LVAD → Reference*		
Protein identity	*Change*	*Average ratio*	*P-value*	*Number in Hierarchical Clustering (Fig. 3)*	*NCBI accession number*	*Theoretical*		*Change*	*Average ratio*	*P-value*
						Mw (kDa)	*pI*			
Desmin	↓	-5.92	0.000011	1	gil55749932	53.5	5.1	=		
Desmin	↓	-4.88	0.0019	2	gil1408188	53.4	5.1	↑	2.76	0.0059
Cardiac muscle alpha actin 1	↓	-4.78	0.000016	3	gil4885049	42.0	5.1	↑	2.35	0.0097
GDP dissociation inhibitor 2	↓	-3.78	0.037	11	gil119606836	48.3	8.5	=		
Cardiac muscle alpha actin 1	↓	-2.71	0.00023	12	gil4885049	42.0	5.1	=		
Vinculin	↓	-1.93	0.050	20	gil7669550	123.7	5.4	=		
Pro-apolipoprotein A-I	↓	-1.87	0.0017	50	gil178777	30.7	5.5	=		
Mutant desmin	↓	-1.85	0.047	18	gil71011081	53.6	5.1	=		
Vinculin	↓	-1.85	0.043	23	gil7669550	123.7	5.4	=		
Alpha-actin	↓	-1.83	0.0070	21	gil178027	42.1	5.1	=		
Ubiquinol-cytochrome c reductase core protein 1	↓	-1.82	0.016	25	gil515634	52.6	5.9	=		
Fibrinogen beta	↓	-1.78	0.010	27	gil182436	55.9	8.5	=		
Enolase 1	↓	-1.74	0.013	24	gil62896593	47.1	7.7	=		
Vinculin	↓	-1.72	0.046	17	gil7669550	123.7	5.4	=		
Cardiac muscle alpha actin 1	↓	-1.67	0.0017	30	gil4885049	42.0	5.1	=		
RNH1 protein	↓	-1.60	0.022	28	gil15029922	48.3	4.7	↑	2.71	0.00047
Apolipoprotein A-I	↓	-1.60	0.0062	49	gil90108664	28.1	5.1	↓	-2.19	0.00078
Aminopeptidase	↓	-1.53	0.049	48	gil119615217	92.8	5.1	=		
Malate dehydrogenase	↓	-1.51	0.0082	47	gil89574129	31.9	9.2	=		
Phosphoglycerate mutase	↑	1.91	0.027	37	gil50593010	28.7	9.6	=		
Fructose biphosphate aldolase	↑	1.89	0.0058	38	gil4885063	39.4	6.5	=		
NADH dehydrogenase [ubiquinone] FeS protein 2	↑	1.87	0.011	33	gil3540239	52.5	7.9	=		
Creatine kinase, muscle	↑	1.84	0.046	36	gil119577741	45.7	6.5	↑	1.81	0.011
ATP synthase subunit alpha	↑	1.68	0.0084	39	gil158259937	54.5	8.3	=		
Fructose-bisphosphate aldolase A	↑	1.62	0.037	40	gil 28614	39.3	9.2	=		
Glyceraldehyde 3-phosphate dehydrogenase	↑	1.55	0.019	43	gil134254708	17.3	9.8	=		
Pyruvate dehydrogenase alpha 1	↑	1.55	0.022	44	gil7688679	40.7	6.5	↑	1.67	0.0078
ATP synthase subunit beta	↑	1.53	0.012	45	gil89574029	48.1	4.8	=		
Mitochondrial aldehyde dehydrogenase	↑	1.52	0.00025	46	gil6137677	53.9	5.6	=		

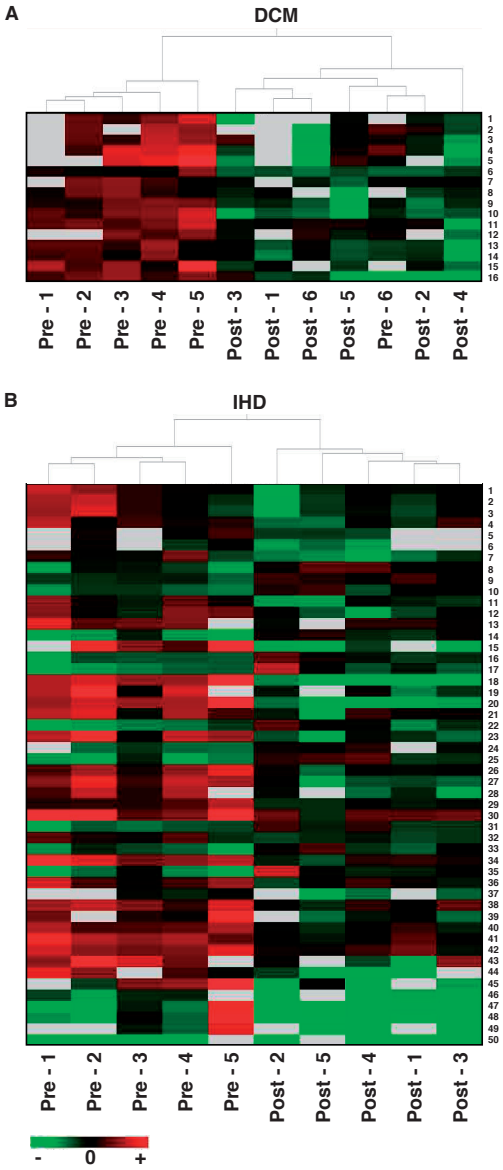


Figure 2. Hierarchical clustering and tree view of differentially expressed proteins in DCM and IHD patients following LVAD support. Each row represents 1 protein and each column represents 1 patient sample biopsy. Protein numbers correspond to protein identities as shown in Table 2 and 3. Protein expression levels greater than the mean expression level in the entire set of samples is shaded red and those below the mean are shaded green. For hierarchical clustering Euclidean distance with complete linkage was used. (A) Hierarchical clustering, heat map, and tree view of differentially expressed proteins (log peak volume change >1.5 fold, $P<0.05$) in DCM patients pre- and post-LVAD support. (B) Hierarchical clustering, heat map, and tree view of differentially expressed proteins (log peak volume change >1.5 fold, $P<0.05$) in IHD patients pre- and post-LVAD support.

and IHD patients. Figure 3 shows detailed gel images with corresponding 3D landscapes of desmin expression, pre- and post-LVAD support that occurred in all six IHD patients.

We identified 13 downregulated proteins from DCM patients (Table 2). These downregulated proteins include components of the cytoskeleton (n=2) and oxidative phosphorylation (n=3), and proteins involved in various other processes (Figure 4). From biopsies of IHD patients (Figure 1B), 19 downregulated and 10 upregulated proteins could be identified (Table 3). Following LVAD support, downregulated proteins include components of the cytoskeleton (n=5) and proteins involved in various other processes, whereas the majority of proteins that were upregulated following LVAD support were identified as metabolic proteins involved in oxidative phosphorylation, glycolysis, and energy metabolism (n=9) (Figure 4). In this cluster of metabolic proteins, only 3 were downregulated. These changes likely affect the metabolic energy producing pathways of mitochondria. To investigate whether the observed changes in metabolic protein expression following LVAD support affect mitochondrial integrity and morphology, transmission electron microscopy was performed (Figure 5). LVAD-induced cardiac remodeling in IHD patients indeed coincided with severe alterations in mitochondrial morphology. Although changes in proteins in DCM patients were less conspicuous, similar mitochondrial changes were observed pre- and post-LVAD (Figure 5). Mitochondria

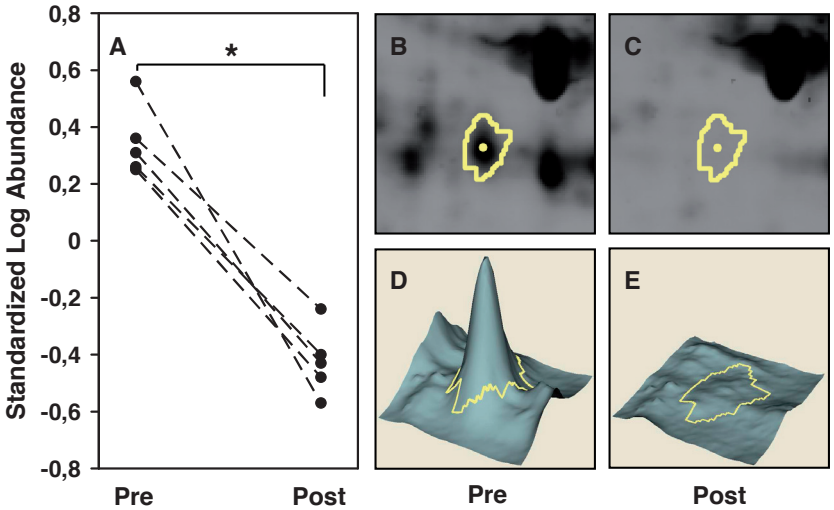


Figure 3. Desmin is downregulated following LVAD support. (A) Desmin protein levels are downregulated in the heart tissue of all six IHD patients. (Desmin protein is indicated by the white arrow in figure 1B). (B-E) Representative gel images of desmin in IHD patients pre- (D) and pots-LVAD (E) and corresponding 3D representations are depicted (F-G).

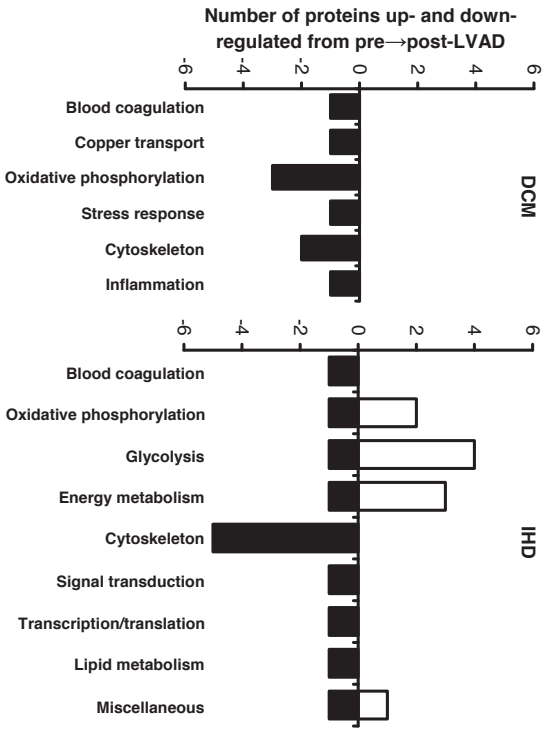


Figure 4. Number and functional classification of changed proteins in DCM and IHD heart biopsies following LVAD support. Proteins were functionally categorized according to the Amigo Gene Ontology classification (www.amigo.genetontology.org).

appeared larger and were less electron-dense in post-LVAD biopsies as compared with pre-LVAD samples. Mitochondria in normal heart resemble the mitochondria in the pre-LVAD samples (data not shown).

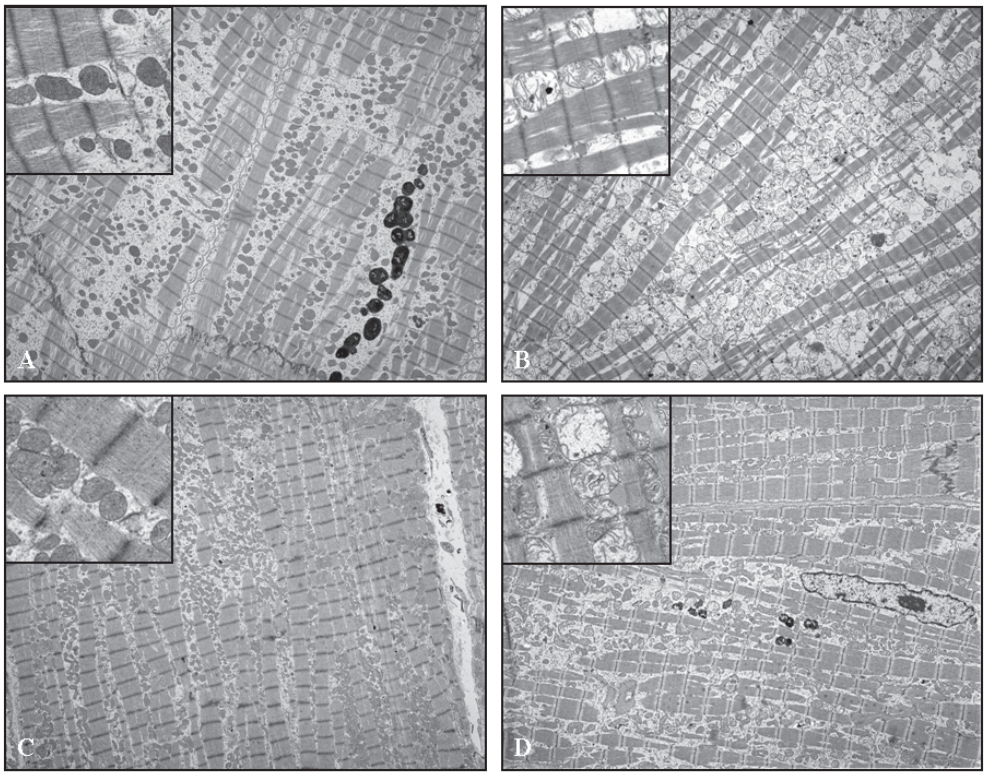


Figure 5. Ultrastructure of cardiomyocytes of DCM and IHD patients biopsies pre- and post-LVAD support. (A-B) Representative slides of a DCM patient pre-LVAD (A) and post-LVAD (B). (C-D) Representative slides of an IHD patient pre-LVAD (C) and post-LVAD (D). Magnifications of all overview pictures are x 2,000 and all inserts are x 4,000. Note the change in the mitochondrial morphology, which is similar in DCM and IHD.

Discussion

During LVAD support of a failing heart many changes occur in the myocardium. Most remarkable changes are reductions in the size of myocardial cells and ECM volume with concomitant altered production of various soluble factors, like BNP and TNFα.[5,6,12,13,17-19] In general, there is a tendency that the heart returns from an enlarged and non-rigid morphology to a more normal and compact size. In this reverse remodeling process, however, reshaping of the failing heart to a more normal heart morphology does not occur in all aspects, such as partial repair of intracellular filaments and loss of collagen IV in the basal

membrane.[12,17] The latter suggest that next to a partial return to normal, the heart shows signs of atrophy.[22] In the present study, we have employed a proteomic strategy to analyze how these morphological aspects reflect on the protein level in cardiac tissue samples of both DCM and IHD patients. We found that LVAD-induced cardiac reverse remodeling is associated with specific atrophic changes in protein expression profiles, predominantly affecting cytoskeleton integrity and mitochondrial energy metabolism.

In DCM cardiac patient samples, all changed proteins (n=16) were downregulated during LVAD support and 13 proteins thereof could be identified by MS (Table 2). We confirmed the previously well established downregulation of desmin by LVAD support and high HSP27 expression in heart failure.[21,23,24] Novel proteins that were downregulated following LVAD support in DCM patients include 4 isoforms of α-1-antichymotrypsin, which may represent 4 different glycosylation variants (Table 2). Increased plasma levels of this acute-phase protein in ischemic patients and concomitant differences in α-1-antichymotrypsin glycosylation profiles have been reported in heart failure.[25,26] LVAD support could reduce this inflammatory response. Also two ATPase synthetase subunits (α and β) were downregulated following LVAD support in DCM patients. In remarkable contrast, both α- and β-ATPase synthetase subunits were upregulated in IHD patients (Table 3), strongly suggesting diametrically opposed energy metabolism pathways in DCM and IHD patients following LVAD support. Further research is required to address this intriguing controversy. Like IHD, however, DCM cardiac tissue samples showed downregulation of several structural proteins after LVAD support (Table 2 and 3). This is consistent with a reduction in ECM during LVAD support in the failing heart as described in various previous studies.[13,14,18]

In IHD cardiac patient samples, 50 proteins showed a significant change during LVAD support and 29 could be identified by MS (Table 3). Among the 38 downregulated proteins, we confirmed the previously well established downregulation of the cytoskeleton components desmin and vinculin.[18,21] We detected at least 3 isoforms of desmin, vinculin, and actin that are consistently downregulated following LVAD support (Table 3). Twelve proteins were upregulated of which 10 could be identified by MS. Intriguingly, all detected upregulated proteins were involved in glycolysis, energy, and oxidative phosphorylation pathways (Figure 4), strongly suggesting an increased mitochondrial energy metabolism in LVAD-supported hearts in IHD patients. This is in agreement with our observation that upregulation of these proteins coincided with changes in mitochondrial morphology that resemble mitochondria in atrophic muscles (Figure 5). This suggests that LVAD support of the heart

muscle allows cardiomyocytes to relax and show signs of atrophy, a response that might be compensated for by an increase in mitochondrial energy metabolism.[22] Interestingly, similar morphological changes in mitochondria were observed in DCM patients (Figure 5), which is however not associated with a dramatic increase in proteins involved in energy metabolism as observed in IHD patients (Table 2 and 3). Oxidative phosphorylation components in DCM patients were even downregulated following LVAD support (Figure 3, Table 2). The reason for this discrepancy remains unclear. One possibility might be that proteins enhancing mitochondrial energy metabolism pathways are upregulated more subtle (≤1.5 fold and/or P≥0.05) in DCM patients as compared with IHD patients and were therefore omitted from our analysis. Alternatively, it should be mentioned that although 2D-gel electrophoresis is potentially capable of resolving several thousand individual protein spots on a single gel, not all proteins could be visualized because of extremes of molecular weight or charge, low-abundance, or because of overlapping gel features in some areas. Finally, other yet to be defined mechanisms, independent of energy metabolism, that result in the apparent atrophic mitochondrial morphology (Figure 5) might play a role.

LVAD's are commonly used in patients with heart failure until a suitable donor organ becomes available. To date, LVAD are more often used as destination therapy and also the number of institutions that perform LVAD explantations without the need for HTx increases.[4,8-11] However, it remains unclear which patients are appropriate for LVAD explantation. In the present study, we have demonstrated the feasibility of 2D-DIGE as a powerful method to profile proteomic changes in the human heart and to distinguish between individual LVAD statuses. The total number of proteins that change from pre- to post-LVAD is relatively small (~2 % of the total number of proteins) (Figure 1). Nevertheless, hierarchical clustering with proteins that were differentially expressed in pre- and post-LVAD samples showed a clear demarcation of pre- versus post-LVAD samples in both DCM and IHD patients (Figure 2). Interestingly, the number and type of proteins that differ in pre- and post-LVAD in DCM and IHD patients are distinct (Figure 4; Table 2 and 3). This is in contrast to most other studies in which differences in pre- and post-LVAD samples were difficult to find between DCM and IHD.[1,10,12,13] Our data indicate for the first time that both types of heart failure indeed are clear different entities, showing a characteristic and different reverse remodeling-associated protein profile in DCM and IHD myocardia. Thus, hierarchical clustering analysis on certain protein profiles could not only determine the status and etiology of the myocardium of the human heart, but could also facilitate prognostic prediction of the individual response

to LVAD support. In the future, more dedicated tailor-made protein arrays, preferably performed on patients' plasma, could provide promising diagnostic tools to determine a priori which patients would most likely respond favorably to long-term LVAD, weaning from LVAD, or HTx.

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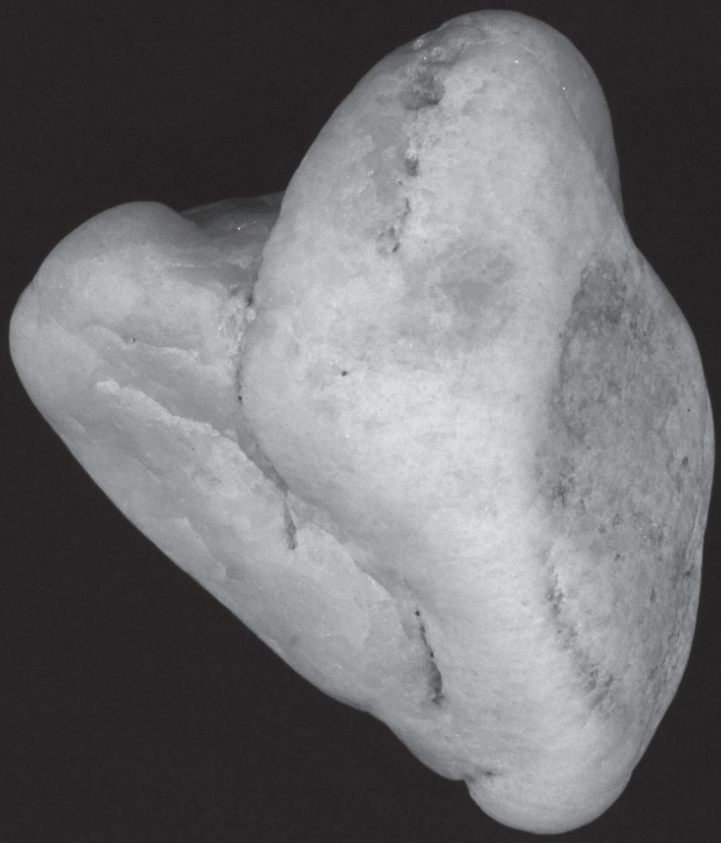
Integrin expression during Reverse Remodeling in the Myocardium of Heart Failure patients



Abstract

Background: The main anchoring proteins of myocardial cells with each other and with the extracellular matrix are integrins present in the membranes of myocardial cells. These integrins are important for maintaining the architecture of the myocardial tissue and the mechanotranduction in the heart. Heart failure leads to various alterations in the myocardium, such as changes in morphology and in expression of mRNA's, miRNA's and proteins. Left ventricular assist device (LVAD) support in heart failure patients has been described to induce reverse remodelling of the myocardium and thus to some degree of reversal of the afore mentioned alterations. In this study, we evaluated whether changes in expression of integrins alpha-1, -3, -5, -6, -7, -10, -11 and beta1, -3, -5 and -6 play a role during reverse remodelling.

Methods: Three step immunoperoxidase staining procedures were applied on frozen heart tissue sections to locate the various integrins tested. Integrin mRNA's were established by standard Q-PCR procedures.

Results: It was shown that mRNA expression of several integrins changes significantly during LVAD support. However,without subsequent changes in immonuhistochemical detectable quantities. Various integrins showed different locations within the myocardium.

Summary: Changes in integrin expression during reversed remodelling in the failing heart after LVAD support were studied. mRNA expression of several integrins did change without concomitant changes in immunohistochemically detectable amounts. Various integrins were found at different locations in the myocardium.

Introduction

Transmembrane receptors such as integrins are important for the dynamic interaction between intracellular processes and the extracellular environment [1,2]. Integrins are expressed in all cellular compartments of the myocardium. They are critical to its form and function and are essential in regulating cellular processes [1-3] . Anchoring cardiomyocytes to the extracellular matrix (ECM) is mainly mediated by integrins and in this respect very important for maintaining the proper architecture of the total myocardium[4]. Structural remodelling during the development of heart failure is characterized by rearrangement of the architecture of the cardiac ventricular wall. It involves among others hypertrophy of the myocytes, fibroblast proliferation, increased deposition of ECM proteins, and altered expression of miRNA's[5-7].

Left ventricular assist devices (LVAD) are mostly used as bridge to heart transplantation (HTx) in patients suffering from end-stage heart failure and induces partial recovery of ventricular functions [8], improved condition of the patients[9], reduction in cardiomyocyte size[10], changes in contractile fibres [11,12] and, depending on the type of heart failure (ischaemic heart disease (IHD) or dilated cardiomyopathy (DCM)), to partial recovery of miRNA expression [7]. Furthermore structural and volume changes of ECM and basal membrane components have been described [13].

The goal of this study was to analyse the changes in mRNA expression by quantitative PCR of several integrins (a1, -3, -5, -6, 7,- 10, -11 and b1, -3, -5 and -6) in the myocardium of heart failure patients before and after LVAD support. To establish the location of integrin-a5, -a6, -a7, -b1 and β6 immunohistochemical techniques have been used.

Methods

Patients and tissue samples

Sixteen patients (age: 38 ± 12 years; 14 men and 2 women) with refractory end-stage heart failure diagnosed with IHD (n=7) or with DCM (n=9) were selected for this study (Table 1). Because of the different etiologies of DCM and IHD both groups were analyzed separately. All patients were treated with a pulsatile LVAD (Heart-Mate I, Thoratec, Pleasanton, CA,

USA) as a bridge to HTx, between 2000 and 2005. At the time of LVAD implantation all patients were in NYHA functional class IV, and in NYHA functional class I while on LVAD support. Prior to LVAD implantation, all patients received intravenous inotropics because of hemodynamic deterioration. Cardiac medication was discontinued initially in all patients after LVAD implantation (except for aspirin), but resumed if necessary (table 1). Informed consent to participate in this study was obtained from all patients before LVAD implantation. The pre-LVAD biopsy (LV apical core) was obtained at the time of LVAD implantation. These biopsies were compared with LV tissue specimens of the explanted heart after HTx (post-LVAD), taken from the apical part of the LV. All biopsies were directly frozen. Normal myocardial tissue was obtained from vital organ donors from which the heart could not be used because of non-cardiac reasons (n=2) and from autopsy on patients with no pathology of the heart (n=3). These biopsies served as a control.

Table 1. Patient characteristics.

Nr	*Age*	*Diagnosis*	*Gender*	*Days on LVAD*	*Medication during LVAD-support*
1	56	IHD	Male	138	None
2	57	IHD	Male	225	None
3	45	IHD	Male	259	2,5 mg Ramipril
4	57	IHD	Male	263	None
5	36	IHD	Male	325	2x 4 mg Perindopril
6	26	IHD	Male	357	None
7	39	IHD	Male	548	3x 6,25 mg Capoten
8	34	DCM	Female	55	3x 6,25 mg Capoten
9	17	DCM	Male	111	None
10	47	DCM	Male	190	None
11	35	DCM	Male	196	None
12	32	DCM	Female	219	3 mg Captopril
13	25	DCM	Male	263	1x 25 mg Losartan
14	32	DCM	Male	286	4 mg Perindopril
15	25	DCM	Male	330	2x 10 mg Fosinopril
16	46	DCM	Male	484	3x 50 mg Capoten

DCM: dilated cardiomyopathy, IHD: ischemic heart disease, LVAD: left ventricular assist device.

Immunohistochemistry

Three-step immunoperoxidase staining to detect the localization of various integrins was performed on sections prepared from frozen heart tissue samples obtained pre- and post LVAD. Eight micrometer thick sections were mounted on silan coated glass slides. Frozen sections were air dried at room temperature, fixed in acetone (10 minutes), washed in PBS/Tween-20 for 10 min and incubated with the primary antibodies (goat anti Integrin alpha-5; -anti Integrin alpha-6, and Integrin alpha-7, mouse anti Integrin beta 1D and rabbit anti Integrin beta-6 respectively) for 1 hr at room temperature.

Next, sections were washed in PBS/Tween-20 (10 minutes) and fixed in formalin (4%) to cross link the antibody to the tissue. Endogenous peroxidase was blocked by incubation in a blocking buffer (20 min) followed by washing in PBS/Tween-20 (30 min) and the sections were incubated with appropriate PO-labeled secondary antibodies for 30 min at room temperature. All secondary antibodies had been absorbed before use with 10 % normal human serum to avoid cross reaction to human IgG. After another washing step in PBS/Tween-20 (30 min) the sections were incubated with Rabbit HRP Powervision (Immunologic, KliniPath, The Netherlands) for 30 min at room temperature. Finally the slides were washed again in PBS/Tween-20 for 30 min and incubated in a 3.3.di-aminobenzidineterachloric acid (DAB) solution for 10 min (room temperature), washed with aqua dest (10 min) and counterstained with Mayer's Haematoxylin. Slides were dehydrated and mounted in Pertex. The intensity of the immunohistochemical staining was scored (in a blinded fashion by two observers) on a semi quantitative scale ranging from negative (score = 0), till intermittent/mild staining (score= 1), moderate/diffuse staining (score=3) and strong/continuous staining (score=5). At least 5 adjacent high power microscopic fields (magnification x 40) per tissue section were scored. Per patient at least 6 sections were studied. In case of IHD only vital cardiomyocytes were examined. Necrotic or fibrotic areas were excluded from examination.

Quantitative PCR

The mRNA was isolated from 20 frozen tissue sections (thickness 10 µm) of myocardial biopsies using Dynabeads® Oligo$(dT)_{25}$ (Invitrogen Dynal AS, Oslo, Norway). cDNA synthesis was performed using oligo-dT, random primers and superscript-III. The primer/probe combinations used for Q-PCR were from Applied Biosystems (Foster City, CA) either in a low density array (LDA) or as single tests (TGEA). The LDAs were used according the manufacturer's instructions and for TGEAs per reaction 12.5 ml Taqman universal master

mix (Applied Biosystems) 1.25 ml primer/probe, 6.25 ml milliQ was used and 5 ml cDNA sample was added. The Q-PCR reactions were carried out by the 7900 sequence detection system of Applied Biosystems. Thermal cycling comprised a denaturation step at 95^0 C for 10 min followed by 45 cycles of 95^0 C for 15 sec and 60^0 C for 60 sec. All the experiments were performed in duplicate. For standardization the expression of 3 endogenous control genes was tested in parallel. These 3 genes showed differences in expression level but the relative expression remained the same. The mean quantification cycle threshold (Cq) value of all samples was 29.31 ± 0.91 for HMBS, 19.35 ± 1.05 for GAPDH, and 25.06 ± 1.24 for PGK-1. We decided to use GAPDH for quantification as its expression level lies within the most reliable Ct-range. To quantify the data, the comparative Cq method was used, resulting in relative mRNA quantities (RQ)[14].

Statistical analysis

The Q-PCR data ware analyzed using the paired and unpaired t-test when appropriate (based on normal distribution tested by the Kolmogorov Smirnov test). All data were calculated with the statistical package of Prism 4.0 for Windows. A p-value < 0.05 was considered statistically significant. The fold change between pre and post-LVAD gene expression for the differentially expressed genes was determined by calculating the RQ post/RQ pre ratio for each patient. Furthermore the average ratio of all patients was determined. Fold change was Log2 (average ratio).

Results

Immunohistochemistry

To evaluate the location and expression of the different integrins in the cardiovascular system pre and post LVAD support, frozen tissue sections were stained by a conventional three-step immunoperoxidase staining and scored.

The location of integrin-α5, -α6, -α7, –β1 and –β6 was established. The results, as summarized in Table 3, show that integrin-α5 is found in the cytoplasm of the cardiomyocytes, in stromal tissue as well as in the 'larger' blood vessels. Integrin-α6 is restricted to the capillaries and integrin-β1 to the membrane of the cardiomyocytes. Integrin-α7 occurs in the cardiomyocyte

Table 2. Overview of antibodies used for immunohistochemistry.

Primary Antibody against	Obtained from	Dilution	Secondary Antibody / Dilution: 1/250	Tertiary Antibody
Integrin-a5	Tebu,Heerhugowaard, The Netherlands	1/50	Rb-anti-Go PO	Rabbit-powervision
Integrin-a6	Bioconnect, Frankfurt am Main, Germany	1/250	Rb-anti-Go PO	Rabbit-powervision
Integrin-a7	Tebu,Heerhugowaard, The Netherlands	1/250	Rb-anti-Go PO	Rabbit-powervision
Integrin-b1D	Abcam,Cambridge, United Kingdom	1/20	Rb-anti-Mo PO	Rabbit-powervision
Integrin-β6	Santa Cruz Antibody, Heidelberg, Germany	1/10	Rb-anti-Ra PO	Rabbit-powervision
Perlecan	Bioconnect, Frankfurt am Main, Germany	1/250	Rb-anti0Mo PO	Rabbit-powervision

Table 3. Location of Integrins in the cardiovascular system. Integrins were detected by three-step immunoperoxidase staining

Integrin	Cardiomyocyte membrane	Intercalated discs	Cardiomyocyte cytoplasm	capillaries	Stromal tissue	Larger vessels
a5	negative	negative	***positive***	negative	***positive***	***positive***
a6	negative	negative	negative	***positive***	negative	negative
a7	***positive***	***positive***	***positive***	negative	negative	negative
b1	***positive***	negative	negative	negative	negative	negative
β6	negative	***positive***	negative	negative	negative	***positive***

* Integrins were detected by three-step immunoperoxidase staining

membrane, the intercalated discs and in the cytoplasm of the cardiomyocytes. Integrin-β6 was expressed in vascular smooth muscle cells and intercalated discs.

There were no statistically significant differences in the degree of positive staining of the various integrins examined in the cardiovascular system before and after LVAD support. The results obtained were similar in tissues obtained from IHD patients and from DCM patients.

mRNA expression

Messenger RNA expression of integrin-a1, -a3, -a5, -a6, -a7, -a10, -a11, -b1, -b3, -b5 and -b6 were tested by Q-PCR. Statistically significant changes in mRNA expression due to LVAD support were only observed in 5 out of 11 integrins tested (Figure 1). However, these 5 integrins did not show significant differences with the healthy controls (both pre- and post-LVAD) except for integrin-a6 in DCM patients. In this case the pre-LVAD level is significantly lower than control level (Fig 1).

Expression of Integrin-a1, -a6 and -a10 mRNA significantly increased after LVAD support in DCM patients. The increases observed were 0.98 fold ($p=0.014$), 1.40 fold ($p=0.007$) and 2.47 fold ($p=0.023$) respectively.

In IHD patients significant increases in mRNA expression were seen after LVAD support for integrin-a6 and -b6; 23 fold ($p=0.046$) and 9.34 fold ($p=0.026$) respectively. Whereas, a decrease (0.41 fold, $p=0.039$) was found for integrin-a5.

Discussion

Integrins mediate interactions between cells and the extracellular matrix (ECM), that are essential for several cellular processes. Intact integrin function has been related to anti-apoptotic signalling and cell survival [15], induction of post-infarct cell migration and myocardial repair [16], activation and regeneration involving epithelial-mesenchymal transition [17], as well as to a normal progression of cardiomyocytes through the cell cycle [18].

Structural remodelling of the ventricular wall in patients with heart failure also involves changes in the ECM [5,6,19]. Since integrins form the contact between cells and their surrounding matrix, it might be expected that if changes in the ECM occur this will be reflected in differences of the integrin expression in the myocardial components. Furthermore, it has been described that LVAD support in heart failure patients leads to at least partial normalization of the heart condition [8,9] among others reflected in changes in regulatory miRNA expression [7].

In this study, it has been shown that LVAD support also induces significant differences in the mRNA expression of integrins. The alterations observed were different in DCM and

Figure 1. Immunohistochemical staining of myocardial tissue pre- (A,C and E) and post- (B, D and F) LVAD support. Integrin alpha-6 (A,B) was mainly found in the smaller capillaries; integrin alpha-7 showed intermittent membrane staining (score 1; C) or continuous membrane staining (score 3; D); integrin beta-1 showed continuous diffuse membrane staining (score 3; E) and continuous non-diffuse membrane staining (score 3; F).

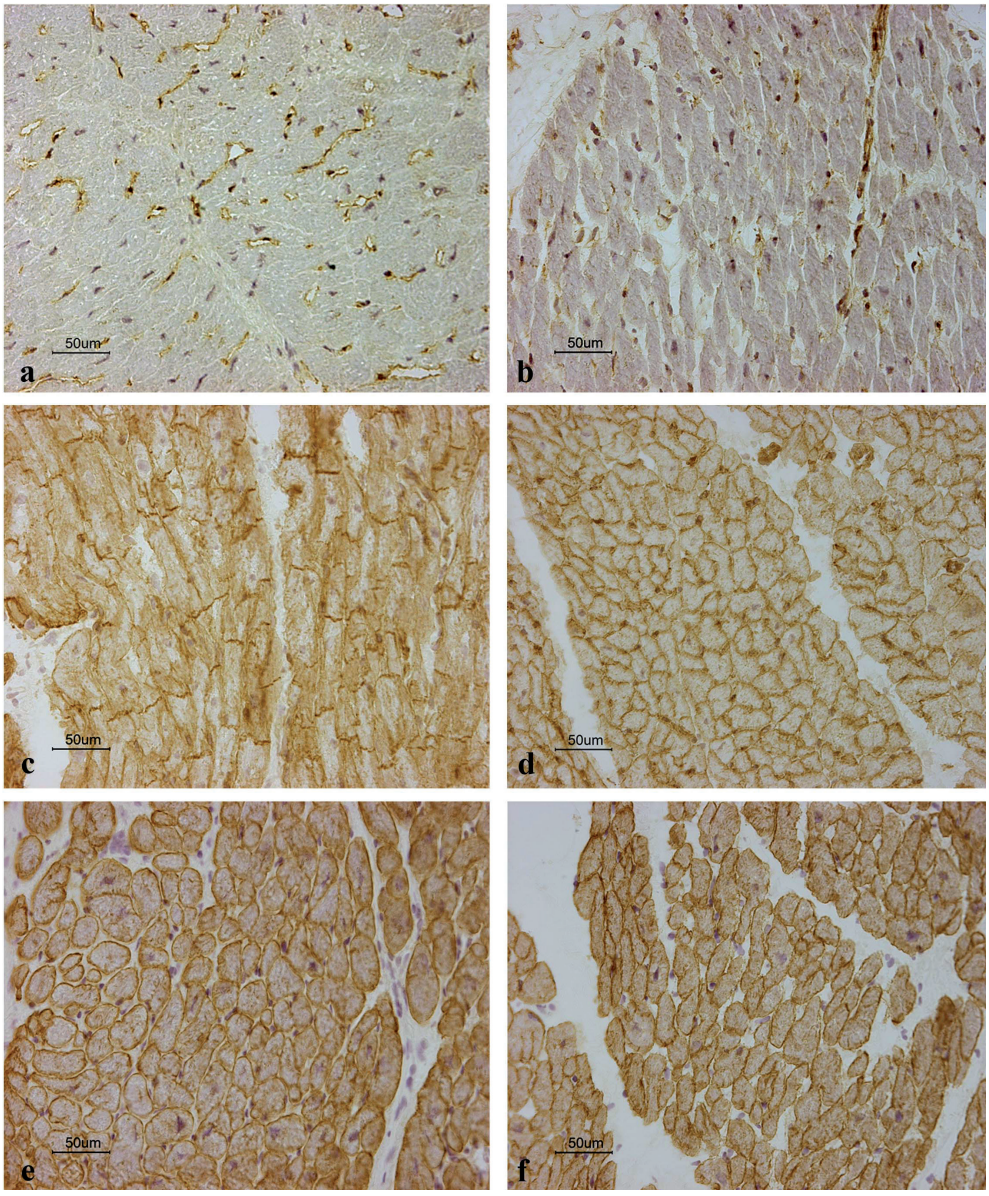


Figure 2. Immunohistochemical detection of Perlecan protein. Perlecan was found to be widely expressed on the membrane of cardiomyocytes: in control tissue (C) as well as in cardiomyocytes from HF patients pre- (A) and post- (B) LVAD support.

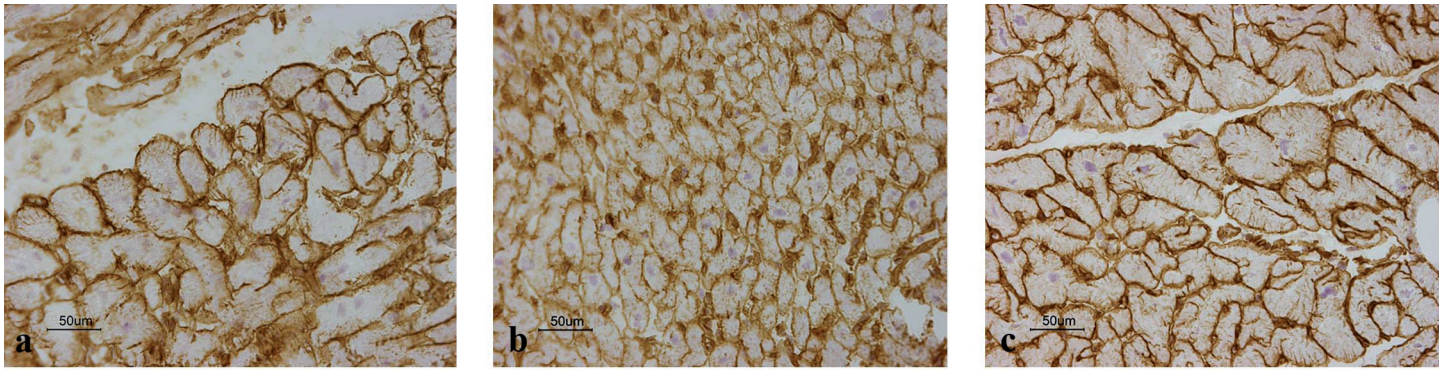
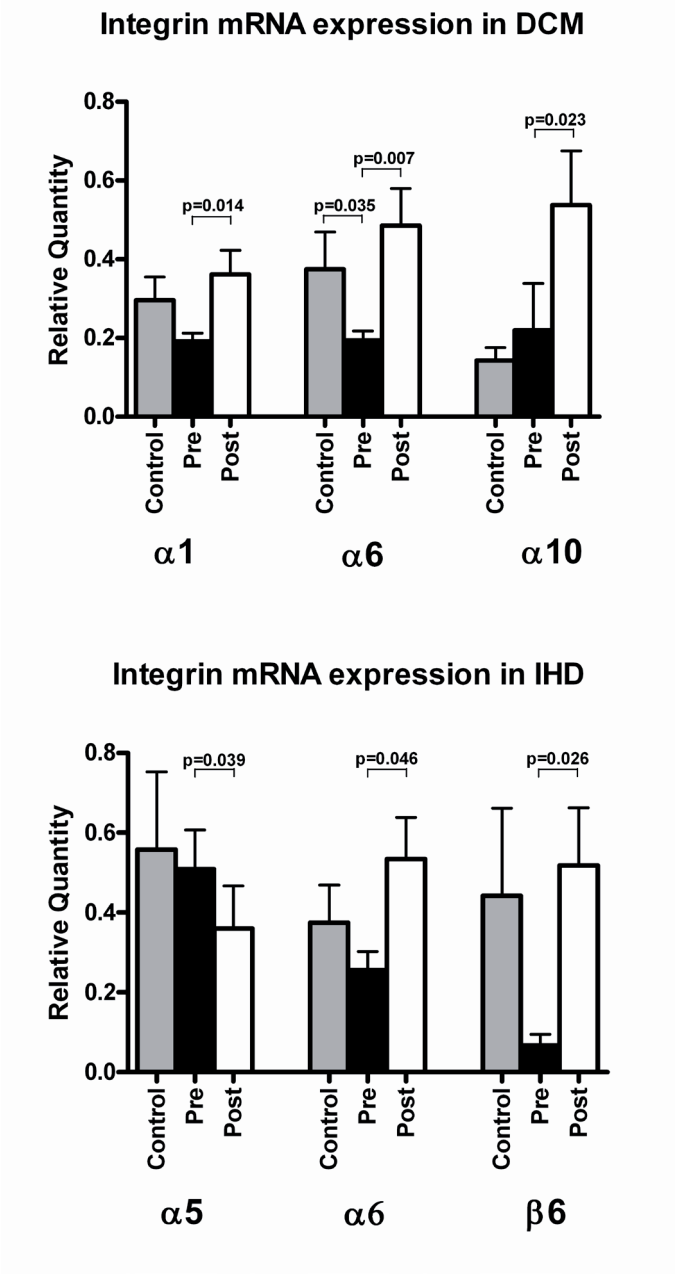


Figure 3. Significant mRNA changes during LVAD support of different integrins in patients with DCM (dilating cardiomyopathy) or suffering from ischaemic heart disease (IHD).



IHD patients (Figure 2). In DCM patients LVAD support caused a significant increase in the expression of integrin-a1, and -a10. Whereas in IHD patients a significant decrease in the expression of integrin-a5 and an increase in the expression of integrin-b6 was observed. This is interesting as integrin-a5 is the only known ligand of integrin-b6, but the mRNA expression of both follow a different pattern. The only similarity between the two patient groups was the increase in the expression of integrin-a6 mRNA.

Despite the differences observed in the mRNA expression, we did not detect differences in quantities of integrin expression by immunohistochemical staining. Whether this is due to a high turnover of the integrin proteins or is a consequence of integrin shedding [3] needs further study.

As a result of the immunohistochemical analyses we did find differences in the location of the integrins studied (Table 3).

Integrin-b6 mRNA was strongly upregulated after unloading in IHD patients (Fig. 1). This integrin is known to be upregulated during tissue remodelling and wound healing [20] and similar processes may be involved in reverse remodelling. It is likewise remarkable, that the only integrin whose mRNA expression was increased after LVAD support in both patients groups (integrin-a6) was located especially in the wall of the capillaries in the myocardium and not in the cardiomyoctes. It has been described that integrin-a6 is important for regeneration and repair processes [16,17] and so, it might stimulate the regeneration processes indirectly by inducing the development of more capillaries (resulting in a better blood supply) during the remodelling of the myocardium. This hypothesis is supported by the fact that the presence of integrin-a6 attracts mesenchymal stem cells [21] that might help to accomplish repair processes in the affected myocardium. As has been reported injection of mesenchymal stem cells into the myocardium of heart failure patients has often only limited beneficial effects and only in a limited number of patients. So, it might be worthwhile to study whether heart failure patients do have a high or low expression of integrin-a6 in their myocardial complex before they are treated with mesencymal stem cells [22]. High expression of integrin-a6 might facilitate the homing and promote the settlement of the stem cells in the myocardial environment leading to improved beneficial effects for these patients. Finally, a plea suggesting a positive role for alpha-6 expression is that in healthy controls the expression of this integrin is higher than in DCM and/or IHD (pre-

LVAD) myocardia. So, finding ways to stimulate the expression of alpha-6 might ultimately contribute to better treatment (i.e. LVAD support) results of these patients.

Acknowledgement

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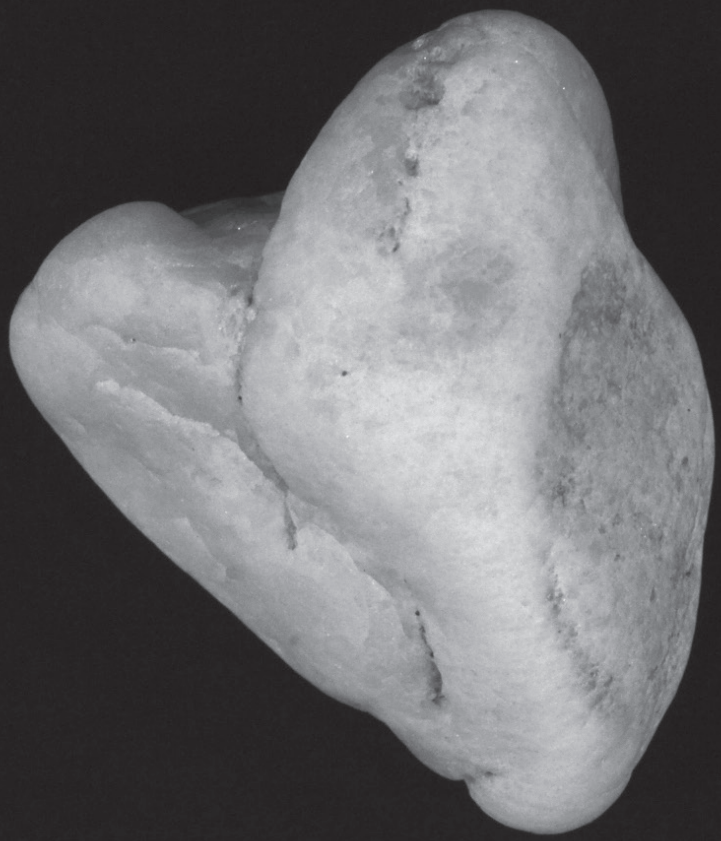
5 Osteopontin: a Potential Biomarker for Heart Failure and Reverse Remodeling after Left Ventricular Assist Device support



Abstract

Background: Left Ventricular Assist Device (LVAD) support in end-stage heart failure leads to recovery of the patient's condition, size reduction of cardiomyocytes, volume reduction and change in composition of the extracellular matrix (ECM).

Objectives: Myocardial expression of ECM osteopontin (OPN) protein increases with severity of HF. It was analyzed whether OPN mRNA expression in heart tissue and/or OPN protein in plasma are associated with reverse remodeling during LVAD support.

Methods: Plasma and heart tissue specimens of 25 end-stage HF patients before and after LVAD implantation and subsequent heart transplantation (HTx) were used to determine the concentrations of OPN protein (EIA) and OPN mRNA (Quantitative PCR). To locate OPN protein and mRNA, immunohistochemistry (IHC) and *in situ* hybridization (ISH) were performed.

Results: The high OPN protein levels in plasma of HF patients did not differ significantly before and after LVAD support. Some individual patients showed a decrease in OPN plasma levels. After HTx the OPN plasma levels decreased to control levels. In contrast, expression of OPN mRNA in heart biopsies decreased significantly after LVAD support. ISH and IHC revealed that OPN was present in cardiomyocytes and in the ECM.

Conclusions: In HF patients OPN mRNA in the myocardium showed a significant decrease after LVAD support, whereas OPN protein expression did not. Plasma OPN protein did reduce after HTx, but not after LVAD implantation. Changes observed at individual level suggest that changes in OPN plasma levels during LVAD support may be used as biomarker for heart condition.

Abbreviations

BNP = Brain natriuretic protein

DCM = Dilating cardiomyopathy

ECM = Extracellular matrix

EIA = Enzyme immunometric assay

HF = Heart failure

HRP = Horse radish peroxidase

HTx = Heart transplantation

IHC = Immunohistochemistry

IHD = Ischemic heart disease

ISH = *In Situ* hybridization

LMD = Laser microdissection

LVAD = Left ventricular assist device

NYHA = New York Heart Association

OPN = Osteopontin

TNF = Tumor necrosis factor

WB = Western blotting

Introduction

Heart failure (HF) is a progressive and disabling disease, caused by ischemic damage of the heart muscle, by myocarditis or by more obscure congenital syndromes. HF is characterized by ongoing structural rearrangement in the architecture of the ventricular myocardium. This involves loss of cardiomyocytes followed by compensatory hypertrophy, proliferation of fibroblasts and inflammatory cells, fibrotic scarring and other changes in the extracellular matrix (ECM)(1). This ongoing process of remodeling finally results in clinical and pathophysiological end-stage HF.

As a bridge to heart transplantation (HTx) patients with end-stage HF can be supported by a Left Ventricular Assist Device (LVAD). This mechanical unloading of the diseased heart results in an improved general condition of HF patients (2-5), a reduction in cardiomyocyte size, in left ventricular diameter and in a limited recovery of contractile elements in the cardiomyocytes (6-8). Furthermore, LVAD support leads to a reduction in ECM volume (9-11), as well as to a diminished production of tumor necrosis factor (TNF)(12) and brain natriuretic protein (BNP)(4,13). Other important adaptations as a consequence of LVAD support are alterations in the ratio of type I and type III collagen proteins (10) and a reduction in type IV collagen of the basal membrane of the cardiomyocytes due to MMP-2 activity (14-16). These data strongly suggest that changes occur in both the cardiomyocytes and the ECM influencing their interaction (17). A coordinated change on the molecular level, responsible for this interaction, is essential. The search for biomarkers produced during this reverse remodeling and indicative for the severity of the structural changes and / or recovery of the heart muscle during mechanical unloading, focused our attention on the possible importance of osteopontin (OPN).

Several groups (18-20) have reported that especially the expression/production of the non-collagenous ECM protein OPN increased with the severity of HF. Both OPN plasma levels as well as OPN myocardial protein expression increased in HF. OPN protein is a highly glycosylated and phosphorylated protein (21, 22). Full length OPN is 66 kDa and highly cleaved by thrombin and MMPs in various active fragments (22, 23). OPN has an important function as anchoring protein, immunoregulator, chemotactic agent, and regulates cell-adhesion and apoptosis (21).

The aim of the present study is to investigate the role of OPN in the pathophysiology of heart failure and reverse remodeling during LVAD support. Therefore, we determined

the concentrations of OPN protein in plasma and the myocardium, as well as the mRNA expression in the myocardium by quantitative PCR (Q-PCR) in various HF patients before and after LVAD support, and in the same HF patients after HTx.

Methods

Patients and tissues

In this study, 25 patients with end-stage HF were included, 14 diagnosed with ischemic heart disease (IHD) and 11 with dilating cardiomyopathy (DCM). Informed consent to participate in this study was obtained from all patients before LVAD implantation and/or HTx and approval of the local institutional review board was acquired. The patients were in NYHA class IV at time of LVAD (Heart-Mate I, Thoratec, CA, USA) implantation. During LVAD support all patients returned to NYHA class I. Cardiac medication was discontinued in all patients after LVAD implantation. However, in 30% of patients at some stage hypertension was treated with ACE inhibitors. Pre-LVAD heart tissue of the left ventricular apex was obtained during LVAD implantation. Post-LVAD tissue of the left ventricle of the explanted heart was collected after HTx. For control tissue, hearts were used not suitable for transplantation (n=2) or autopsy hearts (n=3). The latter were obtained within 4-6 hours after death. Another source of control myocardial tissue were heart biopsies taken after heart transplantation. All biopsies were directly frozen in liquid nitrogen and stored at -80°C until use, or were fixed in buffered formalin and embedded in paraffin.

OPN plasma concentration

EDTA blood samples (plasma) from LVAD-patients (n=22) were collected just before LVAD implantation, 3 and 6 months after LVAD implantation. When the LVAD-patients were transplanted, plasma samples were obtained just before HTx and at 3 and 6 months after transplantation. Control plasma samples were obtained from healthy individuals. The samples were centrifuged 10 min at 3,000 rpm and plasma was stored at -20°C until use. OPN concentrations were determined using the TiterZyme EIA (Enzyme Immunometric Assay) kit for human OPN (Assay Designs, MI, USA). The test was used according the manufacturer's instructions.

Quantitative PCR

Messenger-RNA was isolated from 20 frozen sections (thickness 10 µm) using Dynabeads® Oligo(dT)$_{25}$ (Promega, Madison, WI). All isolated mRNA was converted into cDNA using superscript III (Invitrogen, Oslo, Norway) and both oligo-dT and random primers (Promega). The primer/probe combinations (Taqman® Gene Expression Assays; TGEA) used for Q-PCR were from Applied Biosystems (Foster City, CA) as single tests. The TGEAs were used according the manufacturer's instructions: 12.5 µl master mix, 1.25 µl primer/probe, and 6.25 µl milliQ was used and 5 µl cDNA sample was added. The expression of the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was tested in parallel. Placental cDNA was used as a calibrator. The Q-PCR reactions were carried out by the 7900 sequence detection system of Applied Biosystems. Thermal cycling comprised a 10 min denaturation step at 95°C, followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C.

To quantify the data, the comparative Cq (quantification cycle) method was used. Relative quantity was defined as $2^{-\Delta\Delta Cq}$, in which ΔCq = Cq (target) – Cq (endogenous control), ΔΔCq = ΔCq (sample) – ΔCq (calibrator). Cq values above 35 were defined negative.

In situ hybridization (ISH)

To determine the location of OPN mRNA expression, RNA ISH was performed as recently described (3). Briefly, the OPN mRNA sequence (23) was used to create a specific 479 bp probe by PCR, using a forward primer: 5' ACACATGGAAAGCGA-GGAG 3' and a reverse primer: 5' CAACCAATAAACTGAGAAAG-AAGC (Eurogentec S.A., Seraing, Belgium). This PCR product was labeled with digoxigenin-11-dUTP (Roche diagnostics, Mannheim, Germany) by re-PCR using the same forward and reverse primers. Cycling was performed at a ramping rate of 50%. The labeled probe was used for ISH. Frozen tissue sections of 4 µm were fixed with 4% formaldehyde, subsequently the endogenous peroxidase was blocked. The slides were incubated with proteinase K, incubated with Triton X100, dehydrated and dried. The ISH mixture was applied to the slides and incubated for 10 min at 74°C and hybridized overnight at 37°C. Afterwards, the probe/ target-hybrid was detected for the presence of the digoxigenin label in the tissue and visualized immunohistochemically, using a monoclonal mouse anti-digoxigenin (Roche diagnostics), PowerVision poly HRP-Anti-Mouse IgG (Immunologic, Duiven, the Netherlands) and 3-3'-diaminobenzidintetrahydrochlorid (DAB; Sigma-ALDRICH, Deisenhofen, Germany). Negative controls were obtained by leaving out the

probe from the hybridization mixture. The intensity of the staining of the cardiomyocytes, the cells in the ECM and the vascular smooth muscle cells was analyzed.

Laser micro-dissection (LMD)

To determine the main source of OPN in cardiac tissue, microdissection was performed. Frozen tissue sections of 6 µm were placed on 1.0 PEN-membrane slides (Carl Zeiss MicroImaging, Munich, Germany). The slides were shortly dehydrated and stained with RNase free haematoxylin before use. Specific areas consisting mainly of cardiomyocytes or ECM were micro-dissected. RNA was isolated using the PicoPure™ RNA isolation kit (Arcturus Bioscience Mt., CA, USA), according to the manufacturer's instructions. OPN mRNA expression was analyzed by Q-PCR using TGEAs from Applied Biosystems, as described above.

Immunohistochemistry (IHC)

Four µm paraffin sections were cut and stretched for 30 min on a 70°C plate and overnight at 57°C. The slides were deparaffinized in xylene and rehydrated. Subsequently, the endogenous peroxidase was blocked. The slides were treated with pepsin (Sigma-ALDRICH Chemie). To detect OPN, both a mouse monoclonal antibody (1:50 diluted in PBS/1%BSA; Emelca Bioscience, Breda, The Netherlands) and a rabbit polyclonal antibody (1:200 diluted in PBS/1%BSA; Abcam, Cambridge, UK) were used. PowerVision poly HRP=Anti-Mouse IgG ready to use and PowerVision poly-Anti-Rabbit IgG ready to use (Immunologic) were used as a secondary antibody. The HRP was developed with a DAB solution of the DakoREAL Envision Detection System (Dako, Glostrup, Dennmark) and nuclei were counterstained with Mayer's haematoxilin.

Western Blotting (WB)

Proteins were isolated from frozen myocardial biopsies. Twenty frozen sections (thickness 20 µm) were dissolved in 1x RIPA lysis buffer (Upstate cell signaling solutions, CA, USA), containing protease and phosphatase inhibitors (Roche diagnostics). After centrifugation, the protein concentration of the samples was measured with a BCA protein assay kit (Pierce, Illinois, USA). Equal amounts of proteins were separated on 10% agarose gel (Pierce) and transferred to a PVDF membrane. Western blot analysis was performed using a rabbit

polyclonal (Abcam) antibody (1:1,000 diluted in PBS/0.05%Tween-20/5%BSA) to detect OPN. The membranes were washed with PBS/0.25%Tween-20. After incubation with a horseradish peroxidase-conjugated (HRP) secondary goat anti-rabbit antibody (1:10,000 diluted in PBS/0.05%Tween-20/5%BSA), the blot was developed using Amersham ECL system (GE Healthcare Limited, Buckinghamshire, UK).

Statistical analysis

All data were analyzed using the statistical package of Prism 4.02. The Grubbs test was used to eliminate statistical outliers and Kolmogorov Smirnov was used to determine normality. A paired t-test or Wilcoxon signed rank test was used to compare pre- and post-LVAD samples and HTx samples. To compare the patient samples with control, a non-paired t-test or Mann Whitney test was used. A p-value < 0.05 was considered statistically significant.

Results

Changes in OPN protein levels in plasma after LVAD support and HTx

Plasma samples were collected before LVAD support, as well as 3 months and 6 months after LVAD support. Plasma was also sampled just before HTx and 3 and 6 months after HTx. OPN levels were measured with an EIA (Fig 1A and 1B). The OPN plasma levels in both heart failure groups were significantly higher than in healthy controls. After LVAD support no reduction in OPN levels were noted. In contrast, after HTx the plasma concentration of OPN dropped significantly to normal levels within 3 months.

The OPN plasma levels of each individual patient before LVAD implantation and at the time of HTx (post-LVAD) are presented in Figure 2A and 2B. In both patients groups, but especially in the DCM group, 2 subgroups seemed to emerge. One set of patients showed an increase in OPN levels, whereas the other set showed a decrease.

Changes in OPN mRNA in heart tissue after LVAD support

The OPN mRNA levels obtained from heart tissue biopsies were quantified by Q-PCR. A significant decrease of OPN mRNA was detected after LVAD support. The OPN mRNA levels were especially high in IHD patients (Fig. 3), and the decrease in mRNA expression in this

Figure 1. Osteopontin (OPN) plasma concentrations during LVAD support and after HTx are shown for (A) DCM (n=11) and (B) IHD (n=11) patients. After LVAD support (only after 6 months is shown), no change in plasma OPN concentration was detected. The plasma OPN level is high in both groups, compared with the control group and is higher in IHD patients than in the DCM patient group. After HTx the OPN concentration drops to control levels. *$p < 0.05$ compared with pre-LVAD/HF. Error bars show the standard deviation. (DCM: Dilating Cardiomyopathy; HTx: Heart Transplantation; IHD: Ischemic Heart Disease; LVAD: Left Ventricular Assist Device; OPN: Osteopontin).

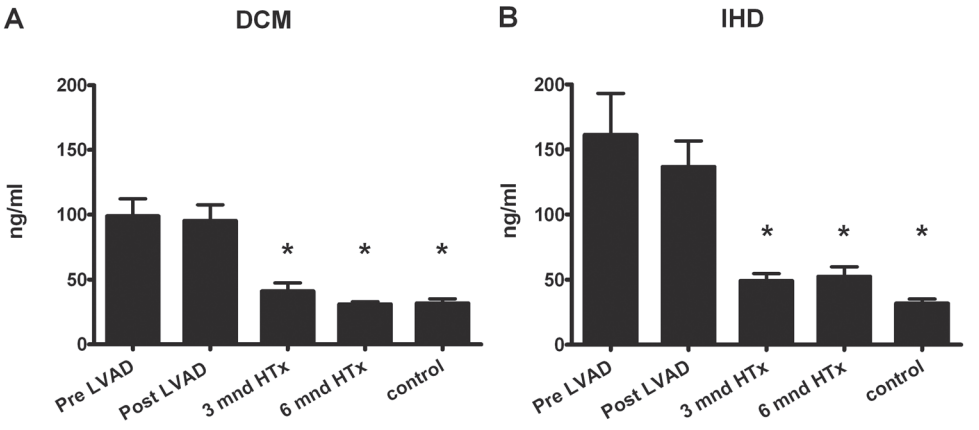


Figure 2. Individual osteopontin (OPN) protein plasma concentrations for (A) 11 patients with DCM and (B) 11 patients with IHD were determined at LVAD implantation and at various times (between 100 and 500 days) after LVAD support at time of HTx. Especially in the DCM group a number of patients showed a decrease and another group clearly showed an increase of OPN plasma levels after LVAD support, but this separation is less evident in the IHD group.

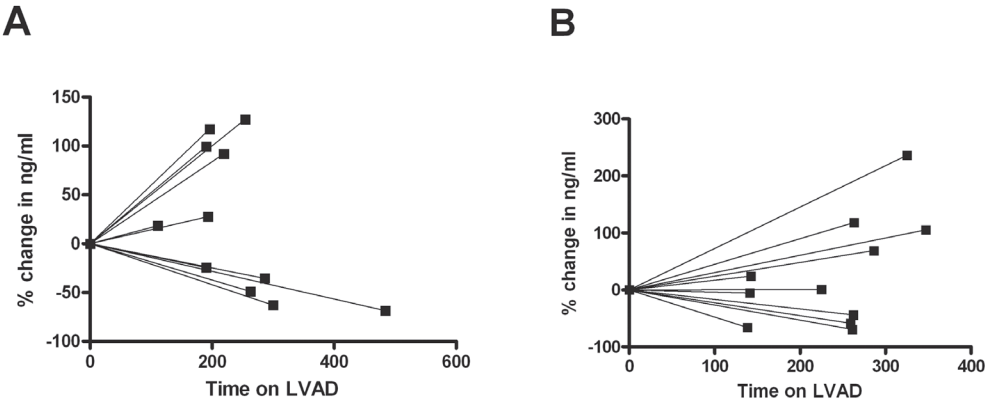


Figure 3. After LVAD support Osteopontin (OPN) messengerRNA levels decreased significantly in the DCM patient group and in the patients with IHD. The OPN expression in the IHD patient group starts at a higher level compared with the DCM group. After LVAD support both groups are at a control level (set at 100). *$p < 0.005$ pre-LVAD vs post-LVAD. Error bars show the standard deviation.

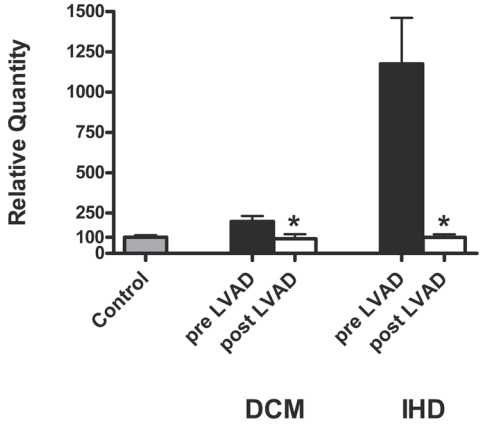
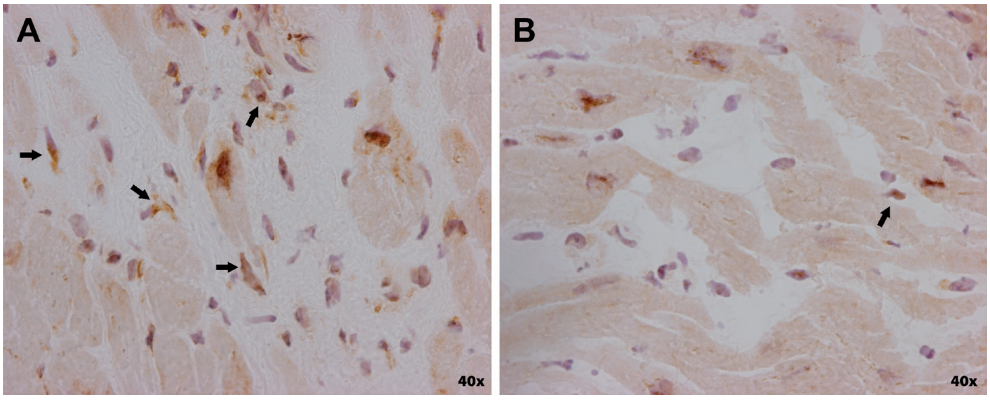


Figure 4. Photomicrographs show Osteopontin (OPN) messengerRNA (mRNA) in situ hybridization. (A) In the tissue of patients before LVAD support, cells in the extracellular matrix (ECM) stained positive for OPN mRNA (arrows), as did the nuclei of the cardiomyocytes. (B) In the tissue specimen after LVAD support, the cells of the ECM are almost all negative, whereas the nuclei of the cardiomyocytes are still slightly positive (original magnification 40x).



patient group after LVAD support was stronger compared to the DCM group. OPN mRNA levels pre-LVAD tend to be higher than in controls. OPN mRNA levels after LVAD support were equal to OPN mRNA levels in control heart tissue and in heart biopsies after HTx (data not shown separately, included in the controls). The decrease in OPN mRNA did not show a correlation with the duration of LVAD support (data not shown)

Location of OPN mRNA before and after LVAD support

Frozen heart tissue sections were incubated with a labeled OPN probe to determine the location of OPN mRNA (Fig. 4A and 4B). The staining in DCM and IHD tissues was similar, both the cardiomyocytes and cells in the ECM stained positive, especially pre-LVAD. The nuclei of the cardiomyocytes stained positive, in a dot-like pattern. In some patients positive staining was observed in the vascular smooth muscle cells. After LVAD support, cells in the ECM showed a striking decrease in OPN expression compared to pre-LVAD. A minor decrease was observed in the cardiomyocytes.

OPN mRNA location as detected by Q-PCR of mRNA isolated from laser micro-dissected tissue

To confirm the location of the OPN mRNA expression, laser microdissection (LMD) was performed on frozen tissue sections. Either the cardiomyocytes or the ECM were micro-dissected. Subsequently, mRNA was isolated and Q-PCR was performed. The results (Figure 5) show that the cardiomyocytes do express some OPN mRNA, however, the cells in the ECM are the main source of OPN mRNA in these end-stage HF patients.

OPN protein location determined by immunohistochemistry (IHC)

For the IHC of OPN both a monoclonal mouse (Fig 6A) and a polyclonal rabbit (Fig 6B) antibody were used. The two antibodies gave a different expression pattern. The monoclonal antibody stained predominantly the cells in the ECM, for example mast cells, and blood vessel endothelium. The polyclonal antibody stained the ECM, and also the basal membrane of the cardiomyocytes. Both antibodies stained positive in the cardiomyocytes and did not show any difference in the staining pattern of the myocardium pre- and post-LVAD (not shown).

Figure 5. Osteopontin (OPN) messenger (mRNA) expression was measured in cardiomyocytes and extracellular matrix (ECM) in laser microdissected tissue specimen. Quantitative polymerase chain reaction (Q-PCR) of the isolated mRNA showed that OPN mRNA expression was highest in the ECM. The cardiomyocytes expressed OPN mRNA at a lower level. Error bars show the standard deviation. .

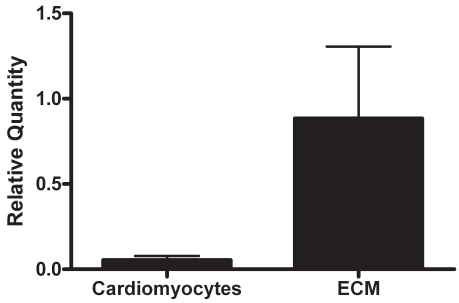
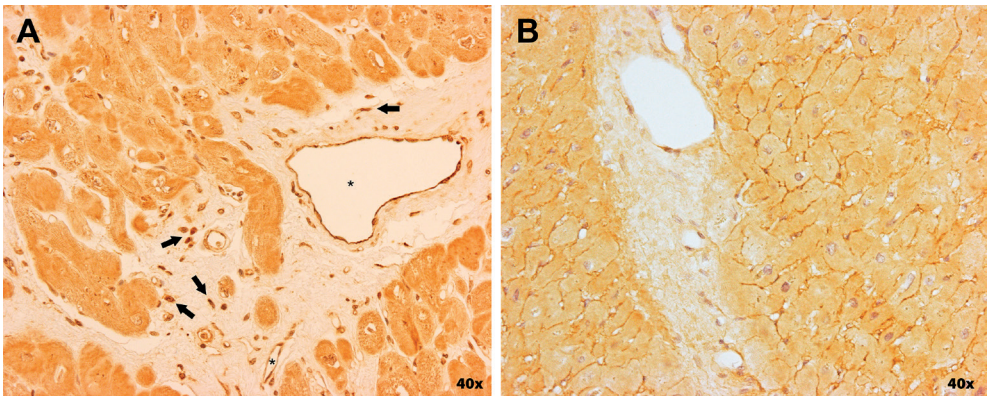


Figure 6. In the immunohistochemical analysis of Osteopontin (OPN) location in the myocardium, the (A) mouse monoclonal antibody stained predominantly the cells of the extracellular matrix (ECM: arrows), whereas the (B) rabbit polyclonal antibody stained the ECM itself and also the ECM between the cardiomyocytes. Both antibodies showed the same results in IHD and in DCM patients and no clear effect of the LVAD support was observed (original magnification 40x).



Quantification of OPN proteins isolated from pre- and post-LVAD heart tissue

To determine the OPN protein expression in the myocardium, proteins from heart tissue specimen pre- and post-LVAD were isolated and analyzed by WB. The mouse monoclonal antibody was not suitable for application in WB. According the manufacturer's information the rabbit polyclonal antibody detects only the full length OPN of 66 kDa, and the cleavage fragments of 32 kDa and 15 kDa. However, the blots showed various additional bands, that could represent other OPN fragments, but this was not verified (data not shown). The staining patterns did not differ pre- and post-LVAD but due to the a-specific bands the data were not considered reliable.

Discussion

In this study it is shown that OPN protein levels in plasma of heart failure patients do not decrease during LVAD support. After heart transplantation the OPN levels drop to control levels. In agreement with this observation, also the protein level in the myocardium after LVAD support did not change. In contrast to the OPN protein levels, the mRNA expression in the myocardium did significantly reduce after LVAD support, both in DCM and IHD patients.

OPN is a well known cross-linking protein in the ECM and plays an important role in remodeling processes (20). OPN is expressed by osteoblasts, osteoclasts, macrophages, vascular smooth muscle cells, fibroblasts, myocardial cells, and hematopoietic cells (18-19). OPN is known to be secreted in the blood plasma. These plasma levels (20-22) and OPN myocardial expression (22) increase with the severity of HF, suggesting OPN is produced in the diseased myocardium.

We investigated the role of OPN in the pathophysiology of heart failure and revere remodeling during LVAD support. The present study confirms the previous published data, that OPN protein plasma levels are significant higher in patients with end-stage HF compared to normal individuals (20-22), suggesting that OPN as such is produced in the failing heart. The control group of healthy individuals had overall significant lower OPN plasma levels. After LVAD support the OPN protein plasma levels, as well as the OPN protein levels in

the tissue specimens, did not differ significantly. However, after HTx the OPN plasma levels reduced immediately, again indicating that the main source of OPN was indeed from the failing heart. In this respect OPN showed remarkable different kinetics compared to brain natriuretic protein (BNP). The high plasma levels of BNP in heart failure patients reduced rapidly after LVAD support and remained low after HTx (3).

So, BNP and OPN plasma levels are indicative for conditions of heart failure and in that respect BNP and OPN may be used as important additional biomarkers. A raise in BNP levels is indicative for increased myocardial wall tension, and therefore deceases after unloading. OPN could be more related to tissue damage and repair, which continues after LVAD implantation. It is interesting to note that OPN plasma levels investigated at an individual level (at time of HTx) showed different patterns. In some patients a decrease and in others an increase was observed after LVAD support. This suggets that on the basis of OPN plasma levels a distinction can be made between patients that improve in heart condition (reduced OPN levels) and patients that remain at the same condition or even deteriorate (increased OPN levels) during LVAD support. The first group could then be considered for weaning and the other group must be considered for destination therapy or HTx. This is subject for further evaluation.

The lack of reduction in OPN protein plasma and tissue levels was in striking contrast with the tissue OPN mRNA levels. These showed a significant decrease in the myocardium after LVAD support towards control level or even below control level. This could indicate that OPN protein turnover in the myocardium is slow and the reduction of mRNA is only partially reflected in OPN protein reduction. The high OPN plasma levels after LVAD support suggest that despite the absence or low expression of mRNA, OPN protein is still released from the diseased heart, as this high OPN plasma level rapidly disappears after HTx. IHC analysis of pre- and post-LVAD tissues did not indicate a reduced OPN protein level in the myocardium after LVAD support. The *in situ* hybridization analysis showed that the mRNA reduction was particular strong in the cells in the ECM and less pronounced in the cardiomyocytes. Singh et al (20) suggested previously that the OPN protein splice variants may differ between OPN produced by cardiomyocytes or cells in the ECM. If this is the case, the reduction of tissue mRNA is mainly due to cells in ECM. Also laser microdissection analyses showed that this was the main source of mRNA expression. The sustained high OPN protein plasma level may be due to cardiomyocyte produced OPN. The exact cause of this discrepancy is, however, still unclear.

Based on our IHC analysis of pre- and post-LVAD heart tissue and on the mRNA expression studies on microdissected tissue specimens, the main source of OPN mRNA was located in the ECM. But in spite of our efforts to characterize the OPN producing cell these specific stromal cells could not be identified with certainty (data not shown). The increase and decrease of fibrous tissue (10, 14-16, 25) before and after LVAD support, and the general role of OPN in fibrotic processes, suggests an important role for fibroblasts in OPN production (20). These cells are difficult to identify by IHC as most reagents are not specific enough. This could also explain the relative high OPN plasma levels in IHD patients compared to DCM patients, as in the first group fibrosis in the infarcted area is much more evident, than the dispersed fibrosis in DCM (20).

Conclusions

Because of a shortage of donor hearts, LVAD support is at present not only used as a bridge to transplantation, but for an increasing number of patients suitable as destination therapy or bridge to recovery. In accordance with the worldwide search for biomarkers (26,27), indicative for the severity of myocardial changes in HF patients and recovery after LVAD support, plasma level changes of OPN could be a valuable future candidate to determine the state of recovery. If the OPN plasma level is indeed related to the extent of fibrosis (20), a reduced OPN plasma level in patients may be indicative for minor fibrosis, and therefore these patients may be prone for weaning from LVAD support (28). Combination of OPN and BNP plasma levels could be even more informative in this respect, as these markers can have an important additional value in the decision making between HTx, weaning from mechanical support, or considering LVAD support as destination therapy.

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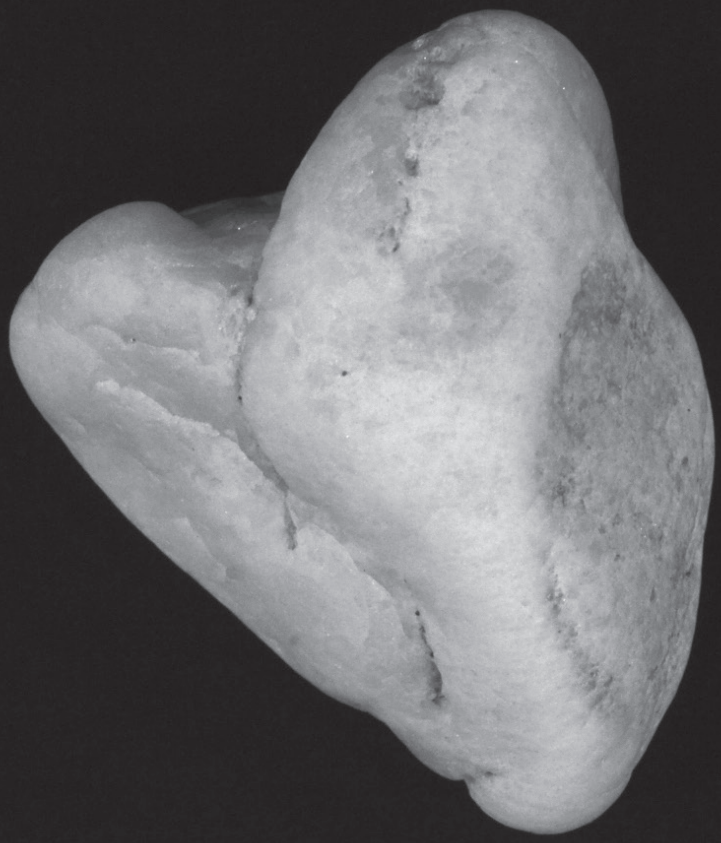
6 Changes in Regulatory MicroRNA Expression in Myocardium of Heart Failure patients on Left Ventricular Assist Device support



Abstract

Left ventricular assist device support in heart failure patients leads to various changes in the morphology of the myocardium and of mRNA and protein expression. As microRNA's are identified as important regulators of various cellular processes, changes in their expression were tested. In patients suffering from DCM , LVAD support leads to a decrease of the expression of miR-1, miR-133a and miR-133b. In contrast patients suffering from IHD showed an increase in the expression of these miR's during LVAD support. Both phenomena were independent from the duration of LVAD support. The expression of miR's pre- and post-LVAD in heart failure patients was low compared to the level of miR's in control myocardial tissue.

Introduction

Structural remodelling in heart failure is characterized by rearrangement of the architecture of the cardiac ventricular wall. It involves hypertrophy of the myocytes, fibroblast proliferation, and increased deposition of extra cellular matrix (ECM) proteins[1,2].

Left ventricular assist devices (LVAD) are used as bridge to heart transplantation (HTx) in patients suffering from congestive heart failure (CHF). As described previously, LVAD support can induce reverse left ventricular remodelling leading to partial recovery of ventricular functions [3], improved condition of the patients[4], reduction in cardiomyocyte size[5] and changes in contractile fibres [6,7]. Furthermore structural and volume changes of ECM and basal membrane components have been described [8]. These structural changes are paralleled by changes in various physiological and biochemical changes, such as reduction in Brain Natriuretic Peptide (BNP), increase in Matrix MetalloProteases (MMP) activity [9] and collagen degradation [8]. Although not for all parameters studied in heart failure patients on LVAD support, in general there is a tendency that LVAD support normalizes the heart condition compared to heart failure patients.

Recently it has been shown that intracellular regulation of gene expression is regulated at least in part by small RNA molecules: microRNA's (miR's). These miR's are highly expressed in heart tissue [10,11]. They do play a role in heart disease [12,13] although their exact function has not been elucidated yet the list of regulatory microRNA's involved in heart disease is constantly increasing [14].

The goal of this study was to analyse the changes in expression of four miR's, that are known to be expressed in the myocardium [15]: miR-1, miR-133a, miR-133b and miR-208. The expression of these miRs was measured in heart disease patients suffering from either Ischaemic Heart Disease (IHD) or Dilated CardioMyopathy (DCM) before and after LVAD support. This was done to study whether the LVAD induced remodelling of the heart was accompanied by changes in the expression of miR's.

It was shown that, compared to the controls (patients not suffering from any heart disease), the expression of all four miR's tested was decreased in both IHD and DCM patients. LVAD support led to a substantial increase of the expression of miR-1, miR-133a and miR-133b in patients suffering from IHD. However, in patients suffering from DCM this effect was not found. On the contrary, LVAD support in these patients caused a further decrease of miR expression.

Methods

Patients and materials

Seventeen patients (mean age: 40 ±13 years; 15 men and 2 women) with refractory end-stage heart failure were included in this study. All were treated with a LVAD (Heart-mate, Thoratec, Pleasanton, California) as a bridge to HTx. Eight patients suffered from IHD and 9 patients suffered from DCM. All patients underwent a successfull heart transplantation. The mean duration of Left Ventricular (LV) unloading was 262 ± 129 days (range 57-557 days). All patients were in NYHA class IV at the time of LVAD implantation, and in NYHA class I while on LVAD support. Informed consent to participate in this study was obtained from all patients. Myocardial tissue was taken at time of LVAD implantation from the LV apex. These tissue samples (pre-LVAD) were compared with LV tissue specimens of the explanted hearts after HTx (post-LVAD). All specimens were obtained from the mid half of the LV, just 2-3 cm above the apex outside the suture area of the inflow canula. Control tissue was taken either from the left ventricle of a donor heart not approved for transplantation (n=1) or from hearts dissected during autopsy (n=5) from patients that did not die due to a heart disease. Autopsies were performed within 2 hrs after death of the patients.
All biopsies were directly frozen and only included if no scar tissue was present.

Quantitative RT-PCR for miRNA

Total RNA was isolated from frozen heart tissue sections (before and after LVAD support) using RNeasy Kit (Qiagen, Benelux BV, Venlo, The Netherlands). Five ng of total RNA was applied for cDNA synthesis using the Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For the Taqman® MicroRNA assay (Applied Biosystems) 1.3 µl cDNA per reaction was used (following the manufactures instructions) for respectively hsa-miR-1; hsa miR-133a; hsa miR-133b; hsa miR-208. The small nuclear RNA U6B was used as an internal control (RNU6B [16]).
The ABI Prism 7900HT sequence detection system (Applied Biosystems) was used as a read out system. The relative miR quantity was determined using the comparative Ct method [17].

Statistical analysis

Q-PCR data were analysed using the paired Wilcoxon signed rank test or the Mann Whitney test. All data were calculated with the statistical package of Prism 4. A p value < 0.05 was considered statistically significant.

Results

Total RNA was isolated from heart tissue of heart failure patients before and after LVAD support (pre- and post-LVAD). The relative quantity of miRNA1, miRNA133a, miRNA133b and miRNA-208 was established. As shown in Figure 1A-C the expression of miR-1, miR-133a and of miR133b was decreased in both types of heart failure compared to normal hearts. This decrease was more significant in IHD than in DCM. Remarkably LVAD support did give rise to partial restoration of the expression in patients suffering from IHD whereas in patients with DCM it did not. On the contrary there was a tendency of further decrease.
The expression of miR-208 showed similar changes (data not shown) as did the other three. However, the expression was too low to make a reliable statistical analysis.
The results of the statistical analyses are incorporated in Figure 1.. These statistical data confirm that in cases of DCM , LVAD support did not reverse the decrease in miR-expression, as in the DCM patients post-LVAD the miR expression was significantly lower than in the controls (Fig 1). In patients with IHD there are no significant differences in expression of the miRs in the controls compared to the values after LVAD support, indicating that a considerable restoration of the expression did occur.
Similar levels of miR-expression were found in (control) tissue obtained either from a donor heart after 2 hrs of warm ischaemia or from post mortal ones.

Discussion

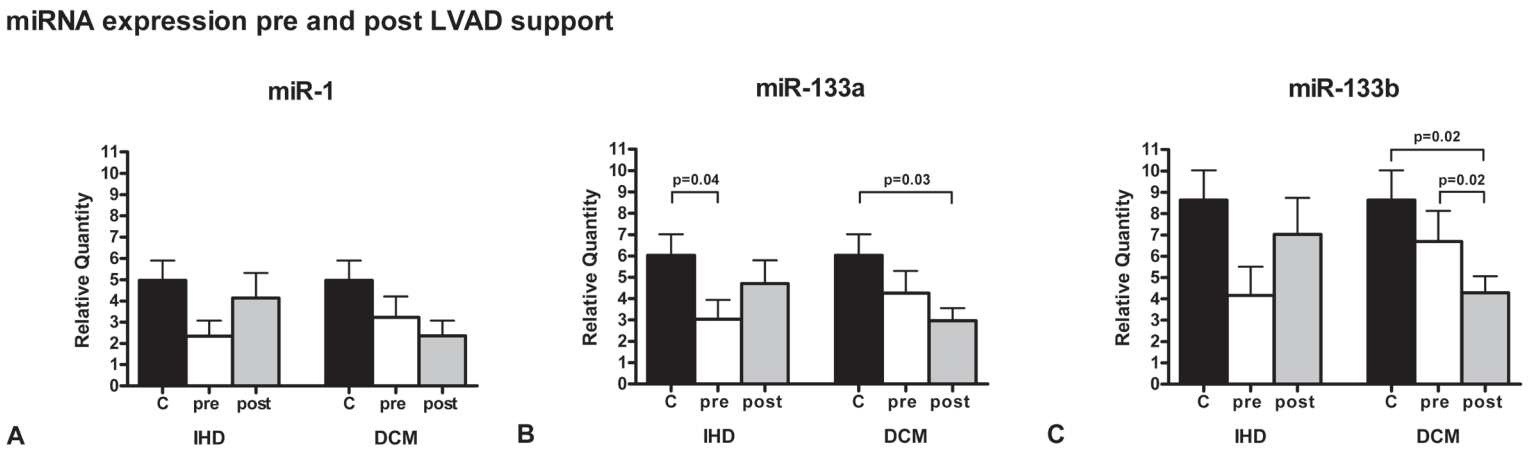
In heart failure significant changes occur in the myocardium, both structurally and physiologically. These changes tend to reverse at least in part after LVAD support [3-10]. Various cellular processes are controlled at the level of mRNA transcription by miRNA's. Also in the heart the role of these miRNA has been described in various processes such as cardiomyocyte hypertrophy (miR-208; miR-133), cardiomyocyte hyperplasia (miR-1 and 2) and ventricular arrhythmias(miR-1) [12,15] .

In this paper it is shown that during heart failure the expression level of miR-1, miR-133a and miR133b did undergo severe changes. The expression of all three miRs was reduced in patients with heart failure compared to the controls as has recently been shown by Ikeda et al[15]. LVAD support leads to a considerable restoration of the expression. However, this is only seen in patients suffering from IHD. Chen et al.[18] have described that miR-1 and miR-133 promote myogenesis and myoblast proliferation respectively of skeletal muscles . Similar data have been produced by Liu et al. [19] and Ikeda et al.[15] for heart faillure. This is the first paper showing changes in miR's during LVAD support. Besides the well known scarring/fibrosis that occurs in tissue damaged by ischaemia, both myogenesis and myoblast proliferation might also occur in IHD-hearts during LVAD support. The possible side effects of overexpression of both miR-1 and miR-133 may even be favorable for the restoration process as this leads to suppression of cardiac hypertrophy [20].

In contrast to IHD patients, in DCM patients no significant restoration of the expression of the miRs occurred after LVAD support. The main reason for this is the less dramatic reduction of miR's in DCM compared to IHD heart failure patients. So, during LVAD support restoration in DCM is more difficult to obtain than in IHD. This difference between DCM and IHD patients after LVAD support may be explained by the fact that there is no need for cell proliferation in DCM as it is in IHD. DCM involves mainly a reduction of hypertrophy of cardiomyocytes whereas IHD involves tissue repair including cell proliferation. This may indicate that the studied miR's are more involved in regulation of proliferative processes than in reduction of the hypertrophy. As already mentioned, the reduction in miRNA expression in IHD patients is not restored completely to control levels during LVAD support. Neither in patients supported for a short period of time nor in patients supported over more than one

Figure 1. The relative quantities of miR-1 (A), miR-133a (B) and miR-133b (C) measured in heart tissue obtained form patients suffering from IHD (n= 8) or DCM (n=9).

C: controls (n=6; black bars); Pre: pre LVAD support (white bars); Post: post LVAD support (grey bars).



year. In how far other factors regulating inflammatory and fibrotic processes hamper this, is yet unknown.

In conclusion, the results of this study indicate that in agreement with the findings of Ikeda et al.[15] changes occur at the level of regulatory miRNA expression in the myocardium obtained from patients suffering from heart failure. In contrast to many other heart parameters, LVAD support did not lead to a complete restoration of the expression level of the miRNA tested. A partial restoration was seen in IHD patients but not in DCM patients. On the contrary, the decrease was not stopped by LVAD support. These results indicate that the basic alterations occurring at the level of microRNAs are most likely not only fundamental to constitute aberrations in developing hearts [21,22] but might also play a role in repair processes occurring after IHD.

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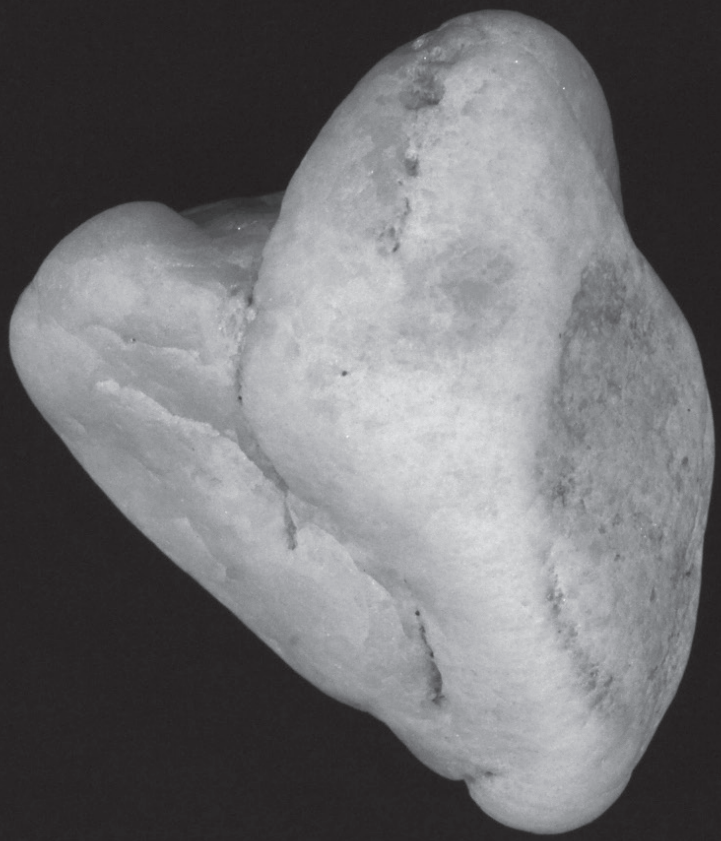
7 General Discussion



Introduction

The clinical syndrome of heart failure is the end point of many different diseases affecting the heart (1). The functional disorder is characterized by poor filling and contracting ventricles, resulting in a low cardiac output fraction. The underlying causes of heart failure are highly variable. Two/third of patients suffer from abnormalities in the heart itself, like coronary artery insufficiency or valvular disease. In one/third of patients, presenting with progressive heart failure the cause is completely unknown. Yet the origin of the disease is not always important for therapeutic decision making. Despite the proven effectiveness of new medicamentous therapies (1;2), the treatment options for end-stage heart failure are limited. Considering the few amount of hearts, suitable for transplantation and the limitations of LVAD support, other treatment strategies have to be developed.

The major architectural alterations, taking place in the heart during progression of heart failure and called remodeling process, are difficult to monitor. Exact clinical, serological and morphological parameters giving information about the status of the diseased myocardium are lacking. But also data about the reverse remodeling of the myocardium, induced by mechanical support could be important in order to know which patient should show recovery of the normal pump function after weaning of the device. Moreover during this reverse remodeling process improvement of the size and function of the cardiomyocytes including the contractile elements is described (3).

To select patients for weaning of the support device (Bridge To Recovery, BTR), LVAD destination therapy (DT) or heart transplantation (Bridge To Transplantation, BTT), it is necessary to be informed in detail about this reverse remodeling precess in the myocardium during the support period. For this reason the search for valuable serological and tissue based biomarkers (messengerRNA, gene expression, proteomic profiling and microRNA) has been the startingpoint of this study. The possibility of influencing successfully the reverse remodeling with new therapies was an additional goal.

Two distinct groups of heart failure patients were made: patients with ischaemic heart disease (IHD) caused by any form of cardiac damage following poor oxygen supply. The second group of patients with a dilating cardiomyopathy (DCM) of unknown origin, are a heterogenous group of diseases with a different etio-pathogenetic background, including post-myocarditis, toxic damage and all genetic abnormalities leading to a cardiomyopathy.

The aim for information concerning the cardiac reverse remodeling process, expressed by +several biomarkers, using pre- and post LVAD serum and tissue specimen, was the main hallmark of this thesis. The ultimate goal was to predict which patient should show recovery of the myocardium, which patient had to remain on the device as destination therapy and subsequently which patient was eligible for heart transplantation.

Chapter 2. Altered expression of mRNA and miRNA

The close interplay between cardiomyocytes and extracellular matrix is responsible for the normal pump function of the heart. Changes in the size of cardiomyocytes, the amount of extracellular matrix and basal membrane components are accompanied by up- and down regulation of protein production and different gene expression profiles.

We studied the mRNA expression patterns, monitoring the reverse remodeling process and the expression of 109 genes, studied by Q-PCR, involved in the process of mechanotransduction was analyzed. The hierarchical clustering of all expressed genes showed that DCM and IHD indeed are different entities, because the pre- and post-LVAD samples of DCM patients did not show segregation and displayed a different gene expression profile compared with controls. The (partly) genetic background in the cause of DCM could be responsible for this aspect. In IHD patients the gene expression profiles, induced by ischaemic necrosis, normalized partly to control levels during LVAD support. These differences in mRNA expression of genes encoding proteins could give an important clue (1) for the status of the failing heart; (2) the future targeted therapy to support and possibly heal the myocardium; and (3) the selection of patients for transplantation.

Special attention was given to the post-transcriptional regulation of mRNA by microRNA's. Because the mRNA expression is not always directly related to protein production, those proteins could be less suitable as a biomarker for the quality and the functional status of the supported myocardium compared to the miR's.

Chapter 3. Proteomic profiling of the human failing heart

Unloading the heart by LVAD support can decrease dilatation and lead to regression of myocyte hypertrophy (4). This reverse remodeling process during pulsatile LVAD support is expressed by up- and down regulation of different proteins, known to play a role in cytoskeleton integrity and mitochondrial energy metabolism (5;6).In this study the regulation of protein synthesis in pre-LVAD and post-LVAD tissue specimens of end stage heart failure patients

was analyzed by fluorescent 2-dimensional difference gel electrophoresis. The expressed proteins were identified by mass spectrometry. In DCM patients 1400 proteins have been analysed, and only 16 were downregulated during LVAD support. Upregulation of protein production was not detected. For IHD patients 1700 proteins have been tested. Thirty eight were downregulated and 12 were upregulated during LVAD support. The protein profiles in DCM and IHD patients were only partially identical, indicating that the pathogenesis of both types of heart disease are partly different entities, showing a characteristic different reverse remodeling-associated protein profile. Moreover the post-LVAD protein profiles proved to be identical with the protein profiles of non-failing hearts, irrespective the cause or the treatment of the pre-existent heart disease. The downregulation concerned proteins like alfa-1-chymotrypsin and other proteins, important for maintenance of the cytoskeleton integrity. The upregulated proteins were essential for metabolic processes, like mitochondrial energy metabolism, glycolysis and oxidative phosphorylation. Transmission electron microscopy was performed to show mitochondrial morphology and integrity. It was found that in post-LVAD heart tissue specimen larger and less electron-dense mitochondria, resembling the mitochondria in normal myocytes, are present.Alpha-1-antichymotrypsin showed the most striking and consistent changes in DCM and IHD patients after LVAD support. This protein was downregulated in almost all DCM patients with a mean of 1.8 fold decrease and also in nearly all IHD patients with a mean of 3.1 fold decrease. This may point to the future role of this protein to select patients for weaning, long term LVAD support or heart transplantation (7). In general the hierarchic clustering analysis on protein profiles gives an indication of the individual status and response of the patient in the reverse remodeling response during LVAD support. More dedicated tailor-made plasma protein arrays could also provide, like the miR's mentioned later, elegant diagnostic tools to select patients for short- and long term LVAD support, weaning as a bridge to recovery or heart transplantation.

Chapter 4. Integrin expression during reverse remodelling

The role of integrins in the myocardial reverse remodeling process has been studied in detail. Integrins are extracellular matrix proteins, anchoring cardiomyocytes and maintaining the cellular frame work of the myocardium. In this way these proteins play an important role in mechanotransduction and contraction (8). Furthermore integrins are essential for cell survival, differentiation, apoptose and repair mechanisms. The extracellular matrix undergoes massive structural changes during the progression of heart failure (9).

Subsequently this process should be accompanied by substantial changes in integrin- , but also by changes in miRNA expression.

Within the scope of this thesis we studied changes in expression of integrins (alpha 1, 3, 5, 6, 7, 10, 11 and beta 3, 5 6) during the reverse remodeling process. IHC and Q-PCR techniques were applied on frozen heart tissue specimens taken before and after LVAD support. With IHC there were no significant differences in the staining patterns of the various integrins in the pre- and post LVAD tissue specimen and the results were similar for IHD and DCM patients. But the Q-PCR results showed changes in mRNA expression for several integrins on different locations in the myocardium during LVAD support. These data suggest an adaptation mechanism to unloading on mRNA level and not in protein synthesis.

These changes are significantly different for patients with DCM and IHD, suggesting that the etiology of the underlying disease could play an essential role in the reverse remodeling and recovery process. Unfortunately it was not possible on IHC level to locate reliably the different levels of integrins in tissue sections. So we were not able to unravel the morphological substrate of the underlying regeneration and repair process. In literature (8;10) the presence of integrin-alpha-6 is shown to facilitate a better homing and settlement of mesenchymal stem cells by the induction of new capillaries and a subsequently better blood supply. This integrin as such can contribute to the healing process of the myocardium after acute damage and necrosis by ischemia or myocarditis. But the slowly deteriorating myocardium in DCM patients with mostly unknown structural myocyte abnormalities is accompanied by different interstitial fibrotic patterns, expressed by different integrin mRNA levels in the reverse remodeling process during LVAD support.

Chapter 5. Osteopontin: A Potential biomarker

In the search for a solid serum biomarker to indicate the severity of heart failure, related to the morphological abnormalities in the cardiac interstitium we investigated the role of osteopontin protein. This highly glycosylated and phosphorylated protein is known to be produced by the deteriorating myocardium of patients with end-stage heart failure (11).

We correlated the OPN plasma and tissue levels, measured with an Enzyme Immuno Assay, with the osteopontin messengerRNA expression using Q-PCR, in heart tissue specimen of patients during LVAD support, in order to have an indication of the stage of the remodeling process. The remarkable finding of this study was the striking decrease in OPN mRNA in all heart failure patients after LVAD support. This was not associated with a decrease in

OPN protein plasma levels in this group of LVAD-patients. Surprisingly OPN serum levels decreased to normal in all patients after heart transplantation. We concluded that the diseased myocardium continues to produce OPN protein during LVAD support. Only removing the whole organ diminishes the serum OPN immediately.

In all tissue sections and subsequently proven with 3 different morphological techniques (Lightmicroscopy, Immunohistochemistry and in situ Hybridisation) the OPN mRNA and protein was clearly present in the extracellular matrix and less pronounced in cardiomyocytes. The role of fibroblasts and the progressive fibrosis in the production of OPN, was difficult to prove, because of the lack of specific immunohistochemical markers for fibroblasts. Only the amount of fibrotic and fibroblastic tissue in between the cardiomyocytes could partially indicate the level of OPN synthesis, as IHD patients show more fibrous tissue compared with the tissue specimen of the DCM patients. The immediate decrease of OPN after HTx was in contrast with another biomarker, the in the clinical setting frequently used BNP (brain natriuretic protein). Our findings suggest that the diseased myocardium is the main source of the serum OPN, because it did not decrease after LVAD support. On the contrary the BNP serum levels decrease rapidly during LVAD support and remain also low after HTx (12). These differences between OPN and BNP plasma levels must have their background in the ongoing reverse remodeling process during LVAD support. It is known that BNP levels increases with left ventricular wall tension and decreases after unloading the heart. In contrast OPN plays a role in tissue damage and repair and shows different levels in different patients, possibly associated with the amount, the type and the localization of fibrosis. This could explain the relative high OPN plasma levels in IHD patients. However because the underlying cause of the disease in the DCM patients was unknown, it was not possible to take into account the striking differences between the fibrotic patterns in our patient groups.

End-stage diseased cardiomyocytes are not able to recover their intrinsic functions. Moreover they can not influence anymore the amount and type of surrounding interstial fibrosis during the myocardial supportive therapy. Consequently, as such in these patients the reverse remodeling process during LVAD support, will never result in improvement of heart function, since fibrosis plays an important role in the progression of heart failure. But like in liver diseases fibrosis is a partly reversible process. Influencing the fibrotic process in an early phase can decrease the amount and the composition of the fibrotic interstitium (13-15).

If OPN plasma levels could be correlated to the amount and especially the type of interstitial fibrosis, a serum biomarker is found to select patients for weaning, LVAD destination therapy and HTx.

Chapter 6. Changes in Regulatory micro-RNA's

In this chapter the effect of regulatory micro-RNA's on the reverse remodeling process is given. The intracellular regulation of gene expression and mRNA transcription for tissue repair and reverse remodeling is shown to be done by small RNA molecules: the micro-RNA's (miR's). These molecules are highly expressed in heart tissue, suggesting that they play a role in a substantial amount of heart diseases, associated with myocyte proliferation, hyperplasia and hypertrophy (16;17). The list of miR's involved in the heart and in any other disease process is increasing every month. This study of changes in miR's, normally expressed in the myocardium like miR-1, miR-133a, miR-133b and miR-208, was performed in LVAD patients in order to assess the expression and the possible role of these miR's in regulating protein production during reverse remodeling. Both literature data (18) and the results in our study show a decrease in expression of those 4 miR's, compared to controls in all patients with heart failure. This is independent of the underlying disease and more pronounced in IHD patients. But after LVAD support an increase in miR expression was seen in all IHD patients, even giving in some patients a partial normalization. This was in contrast to the DCM patients after LVAD support. They showed a further and overall decrease of miR expression, resulting in a significantly lower miR expression compared to the controls. These different findings between the 2 groups suggest an important role for the underlying etio-pathogenetic process, originally leading to the failing myocardium. Damage and repair, driven by inflammatory processes and fibro-neogenesis, followed by compensatory cardiomyocyte hypertrophy are important during the recovery and remodeling after ischaemic infarction. But there is no relation with structural abnormalities in the cardiomyocytes themselves. As mentioned before, the tested miR's play a role in myocyte functions like proliferation, hypertrophy and hyperplasia. The cardiac degeneration proces, induced by genetic abnormalities, represent various other and mostly unknown etio-pathogenetic mechanisms, affecting the intrinsic myocyte function. The same should be true for patients with end stage heart failure due to myocarditis. Subdividing the DCM group in patients with and without structural (genetic) myocyte abnormalities could give more and better information about the regulatory mechanisms of miR's to control and promote protein production in the

recovery of the myocardium after severe damage. Furthermore, as shown in this study, the regulatory functions of miR's in the reverse remodeling process during LVAD support are better understood. More substantial information about the function of different miR's in the myocardium and the myocyte can possibly lead to therapeutic strategies to influence miR expression in repair processes (19). In this respect unraveling the role of miR's in reverse remodeling could be promising for future decision making.

Conclusions

Basic research in end stage heart failure patients has considerable limitations. The heterogeneous group of patients with many different, partly unknown underlying diseases and the small patient groups, available for research purposes, substantially reduce the adequate interpretation of the results.

For heart failure patients the search for biomarkers is difficult, but challenging. It was the main subject of this study to obtain information about the reverse remodeling process in the heart during mechanical support (20). Some biomarkers are promising, like Osteopontin (11;21), but not yet available for clinical testing. Different proteins (like alpha-1-antichymotrypsine) provide data about the cytoskeleton integrity of the myocardium and could possibly play a diagnostic role in testing patients for weaning (7). The results of our study especially indicate for the first time the importance of the miR's in the reverse remodeling process. Influencing the regulatory functions and expression of miR's can be seen as novel therapeutic targets (22). The development of anti-miR's therapies will provide a wide range of future applications and are subject of different research projects in cardiovascular diseases.

Other consistent findings in our study are the differences between the patients with IHD and DCM, indicating basically different mechanisms in the progression and the quality of the (reverse) remodeling process. In the heterogenous group of DCM patients better information about the pathogenesis of the underlying disease should give more consistent data about the possible repair mechanisms during mechanical support. The abnormalities in post-infectious myocarditis patients and patients with toxic damage of the myocardium are not associated with structural defects in the cardiomyocytes or the myofibrils themselves and they show another fibrotic pattern. As early fibrosis is associated with substantial regression in adequately treated liver diseases, influencing the fibrotic progression in heart failure

patients can be a promising possibility. Besides changing the reverse remodelling process with new pharmacological therapies, avoiding end stage fibrosis with early LVAD support should be considered as another goal in heart failure treatment. In this respect more personalized therapeutic options become available, especially for the DCM patient group.

Classification

Many new classification systems of heart failure and cardiomyopathies are proposed the last years, partly based on the morphological and functional phenotype, partly on genetics or familial background, but not quite suitable for the general medical practice (23). Nevertheless the rising number of heart failure patients and the development of new therapeutic options made it necessary to know more about the basic etio-pathogenesis of the different heart diseases. This knowledge should give a better interpretation of the abnormalities in the diseased myocardium, especially in relation with the (reverse) remodeling process in heart failure patients and could subsequently provide the opportunity to design more personalized therapy strategies for each patient (24). The recent developments in molecular biology to detect genetic defects as the basis of cardiomyopathies, force clinicians, geneticists and pathologists to look in an innovative way to heart failure patients and their tissue abnormalities.

Data base

In this respect it is important to give attention to the development of, an eventually by PALGA (Pathologisch Anatomisch Landelijk Geautomatiseerd Archief) supported, nation wide (European/International) data base, to collect systematically all relevant characteristics of patients (clinical parameters, pharmacological treatment, radiologic and histopathologic abnormalities, genetic testing etc) with progressive heart failure in any stage of the disease and with any known or unknown etio-pathogenesis (25). This framework should provide clinicians adequate information about their patients to make a personalized diagnostic and prognostic plan. The selection for different types of therapy should be based on these data. The researchers can compare their results and interprete better the abnormalities in the diseased myocardium between patient groups with different etiologies of heart failure, using the information in this data base.

Future role of the pathologist

Although in some types of targeted cancer therapy the (molecular) pathologist has the leading role in diagnostics and prognostication (26), for the treatment of heart failure patients, this is not an option. The role of the cardiovascular pathologist in heart failure diagnostics is modest, but essential (27). A maximal effort to unravel the basic pathogenetic mechanisms of heart failure, to document in detail the cardiac histopathologic abnormalities before and after LVAD support and the ongoing research for serum and tissue biomarkers, indicating the status and the regulation of the (reverse) remodeling process are their main tasks.

The combination of these findings with all other clinical and diagnostic information, provided by the data base (23) and discussed in clinical meetings, will result in better decision making, as well as in practising new and personalized treatment strategies for heart failure patients in future.

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Samenvatting in het Nederlands

Hoofdstuk 1. Inleiding

Hartfalen is een klinisch syndroom dat veroorzaakt wordt door een complex samenspel van afwijkingen in het myocard, resulterend in een zwakte van de hartspier. Daardoor zijn de ventrikels van het hart niet meer in staat om zich voldoende te vullen en het bloed rond te pompen. Bij bijna twee-derde van de patienten met hartfalen wordt dit veroorzaakt door ziekten in de coronair-arterien (arteriosclerose), waardoor er zuurstoftekort (ischaemie) van de hartspier ontstaat (Ischaemic Heart Disease/IHD). Andere oorzaken van hartfalen zijn bijvoorbeeld hartklepafwijkingen en hoge bloeddruk. Maar er is ook een heterogene groep van ziekten in de hartspier, waarvan de oorzaken veelal nog onbekend zijn. Omdat bij deze patienten de ventrikels in de loop van hun ziekte steeds wijder worden en verslappen, wordt deze ziekte een Dilaterende Cardiomyopathie (DCM) genoemd. Echter alle patienten met hartfalen, onafhankelijk van de oorzaak, hebben in het eindstadium van hun ziekte uiteindelijk dezelfde structurele afwijkingen in het hart.

De toename van het aantal patienten met hartfalen is een groot probleem in de gezondheidszorg. Dit wordt enerzijds veroorzaakt door de wereldwijd steeds ouder wordende bevolking, maar ook omdat er meer behandelingsmogelijkheden zijn voor patienten met hartfalen, waardoor zij langer overleven. Verbetering van de algemene conditie van de patient door meer te bewegen en te stoppen met roken, maximale medicamenteuze therapie en soms invasieve procedures zoals hartklep vervanging of bypass chirurgie, kunnen de prognose gunstig beinvloeden. Desondanks is de uiteindelijke prognose van hartfalen slecht. In het eindstadium is alleen harttransplantatie een optie.

Door de enorme ontwikkelingen in de hartchirurgie sinds het midden van de vorige eeuw, kwamen er voor patienten met hartfalen ook andere mogelijkheden ter beschikking. Er werden diverse soorten (externe) mechanische pompen (Left Ventricular Assist Device/ LVAD) ontworpen om de pompfunctie van de ventrikels te ondersteunen. Aanvankelijk werden er bij patienten met hartfalen pompjes met een pulsatile flow (Heart Mate XVE en Novacor) geimplanteerd, later werden de veel kleinere, continue flow pompen (Heartmate II) gebruikt. Maar helaas is het gebruik van deze pompen beperkt en worden deze in principe alleen toegepast bij patienten die in aanmerking komen voor harttransplantatie (Bridge To Transplantation/BTT). Door het gebrek aan donor-harten en de innovatieve ontwerpen voor langer bruikbare pompen, komt de toepassing van mechanische ondersteuning als

Destination Therapy (DT) echter steeds dichterbij. Er blijken echter ook patienten te zijn, waarbij gedurende LVAD ondersteuning de functie van het eigen hart dermate verbetert, dat na een periode van 'ontwenning', de pomp weer verwijderd kan worden. Deze indicatie wordt Bridge To Recovery (BTR) genoemd.

(Reverse) remodeling

Tijdens de progressie van het hartfalen vinden er in het myocard (de hartspier) een groot aantal veranderingen plaats, die samen het 'remodeling' proces worden genoemd. De adaptatie mechanismen waarmee het hart probeert de normale pompfunctie op peil te houden, ondanks het toenemende verlies aan hartspiercellen, veranderen de vorm en grootte van het hart. Dit komt omdat de overblijvende hartspiercellen (cardiomyocyten) groter worden (hypertrofie) en de hoeveelheid extracellulaire matrix (het steunweefsel van de cardiomyocyten) toeneemt. Hier wordt ook steeds meer littekenweefsel gevormd, waardoor de bloedvoorziening verder verslechtert en de functie van de overblijvende cardiomyocyten steeds verder achteruitgaat. Uit eerder onderzoek is gebleken dat gedurende mechanische ondersteuning, waarbij de ventrikels niet meer hun uiterste best hoeven te doen om het bloed rond te pompen, dit remodeling proces stopt en zelfs omkeert (reverse remodeling). De hypertrofie van de cardiomycyten verdwijnt, de cytoskelet eiwitten, de structurele eiwitten waarmee de spiercontracties worden uitgevoerd, herstellen zich en de verbindweefseling van het steunweefsel wordt minder. Dit alles resulteert ook in een verbetering van de functie van de ventrikels. Om te weten te komen wat zich op weefselnivo afspeelt bij dit reverse remodeling proces en hoe dit op DNA nivo wordt aangestuurd, werd deze studie uitgevoerd. Daarnaast was het belangrijk om te kijken of er ook biomarkers zijn, die tijdens de LVAD ondersteuning, een indicatie kunnen geven of het hart zich inderdaad aan het herstellen is. Een bijkomend doel was om patienten, die in aanmerking komen voor LVAD ondersteuning misschien wel nieuwe therapeutische mogelijkheden te bieden om het reverse remodeling proces positief te beinvloeden.

Hoofdstuk 2. Expressie van messengerRNA en microRNA tijdens LVAD ondersteuning

In dit hoofdstuk wordt uiteengezet of de messengerRNA (mRNA) en de microRNA (miRNA/miR) expressie-profielen een indicatie zouden kunnen zijn voor de conditie van het hart

tijdens LVAD ondersteuning, of die expressie-profielen iets zeggen over de (rest) functie van het hart en of op basis daarvan de beslissing genomen kan worden om de LVAD ondersteuning te beeindigen. De veranderingen in mRNA expressie profielen werden gemeten in het hartweefsel van patiënten met een Dilaterende Cardiomyopathie (DCM) en met een Ischaemische Hartziekte (IHD) voor en na p-LVAD ondersteuning en dit werd vergeleken met hartweefsel van normale controles. De expressie van 109 genen, die van belang zijn voor het proces van mechanotransductie in het hart, met name genen die coderen voor extracellulaire matrix (ECM) filamenten (collageen), transmembraan eiwitten (integrines en sarcoglycanen), intracellulaire en adhesie moleculen, die iets te maken hebben met de signaal transductie, ionen-kanaal moleculen en factoren die invloed uitoefenen op het fibroserings proces, werden met behulp van Q-PCR bekeken. In de eerste plaats bleek dat, ten gevolge van de post-transcriptionele regulatie, die waarschijnlijk grotendeels plaatsvindt door microRNA's, de expressie van mRNA niet direct gerelateerd is aan de eiwit productie. Welke miR's verantwoordelijk zijn voor de expressie van welke mRNA's of genen is nog niet bekend, maar het is evident dat de lijst met miR's die een rol spelen bij hartziekten steeds langer wordt. Deze studie naar mRNA expressie werd dan ook meteen gebruikt om te kijken naar de veranderingen in expressie van de 4 miR's, die een rol spelen bij hartfalen. Het doel was niet alleen om te kijken naar mRNA expressie bij de remodeling van het hart, maar ook welke invloed deze miR's daarbij uitoefenen en of deze miRs een betere biomarker (in het serum of in het weefsel) zouden kunnen zijn voor de functionele status van het hart op dat moment.

De resultaten waren in zoverre opmerkelijk dat er voor de IHD patiënten een duidelijke hiërarchische clustering pre- en post LVAD ondersteuning werd gezien in mRNA expressie voor eiwitten, die te maken hebben met de samenstelling van de extracellulaire matrix, fibrosering, basaalmembraan, adhesie moleculen, intracellulaire filamenten en signaal transductie. Bij de DCM patiënten was deze hiërarchische clustering niet aanwezig. Bij het testen van de veranderingen in 3 verschillende hart-miR's, pre- en post LVAD, viel op dat de hoeveelheden in zowel de DCM als de IHD groep lager waren dan normaal, maar dat post LVAD de miR's in de IHD groep stegen, terwijl die in de DCM groep verder afnamen. Uit deze hiërarchische clustering kan worden geconcludeerd dat het inderdaad om 2 verschillende groepen patiënten gaat met een geheel verschillende etiologie wat betreft de oorzaak van het hartfalen. De pathogenese van de IHD groep ligt grotendeels vast en is direct gerelateerd aan progressieve vascularisatie stoornissen van het hart. De pathogenese van het hartfalen

in de DCM groep heeft een zeer heterogene achtergrond en is ook grotendeels onbekend. Dat deze achterliggende oorzaak direct van invloed kan zijn op het proces en het patroon van remodeling in het falende hart en dus gepaard gaat met een heel andere gen-expressie profiel, wat niet herstelt na LVAD ondersteuning, zal duidelijk zijn. Ook het feit dat de gen-expressie van intracellulaire eiwitten in de DCM groep duidelijker naar voren kwam, dan in de IHD groep, kan er op wijzen dat deze genen meer zijn aangedaan ten gevolge van de preexistente (vaak ook genetische) afwijkingen, die ten grondslag liggen aan het hartfalen bij een deel van de DCM patiënten. Dat bij de IHD patiënten tijdens LVAD ondersteuning het gen-expressie profiel de neiging heeft te normaliseren, berust mogelijk op een verbeterde bloedvoorziening op capillairbed nivo, waardoor er in het geinfarceerde myocard een tendens tot genezing wordt bewerkstelligd.

Daarnaast zijn de aangetoonde veranderingen en de verschillen in mRNA expressie bij de 2 patientengroepen misschien wel de sleutel tot het bepalen van de functionele status van het falende hart en kunnen deze mogelijk behulpzaam zijn bij het beoordelen van de ernst van de afwijkingen. Daarmee zou het optimale moment bepaald kunnen worden, waarop de LVAD ondersteuning moet beginnen en later, als de mRNA expressie weer is genormaliseerd, weer beëindigd kan worden.

Belangrijker dan de mRNA expressie blijkt echter de posttranscriptionele regulatie door de miR's te zijn. Alleen de invloed van de miR's kan de verschillen verklaren tussen de mRNA expressie en dat wat er in werkelijkheid gebeurt met de eiwitten op de 'werkvloer' van het myocard.

Hoofdstuk 2. Eiwit expressie tijdens LVAD ondersteuning

In deze studie zijn de veranderingen op eiwitnivo in het myocard bij DCM en IHD patiënten gedurende het reverse remodeling proces en de vertaling daarvan naar het morfologisch substraat, onderwerp van onderzoek geweest. Daarbij werd gevonden dat het reverse remodeling proces bij zowel DCM als IHD patiënten geassocieerd is met downregulering van alle 4 de isovormen van de serine protease remmer ACT (anti-chymo-trypsine). Dit acute fase eiwit, dat wordt geproduceerd door macrofagen, cellen die een rol spelen bij de ontstekings- en opruimreactie, is verhoogd in het serum van hartfalen patiënten met een ischaemische hartziekte en verdwijnt gedurende LVAD ondersteuning. Dit kan betekenen dat de ontstekings- en opruimreactie in het interstitium van het hart minder worden gedurende het reverse remodeling proces.

Verder werd er downregulering gevonden van eiwitten, die te maken hebben met de inactiviteit van de cardiomyocyten waarbij er bij de ATPase subunits alfa en beta een verschil in eiwit expressie tussen DCM en IHD patiënten werd gezien. Deze waren bij de DCM groep ge-downreguleerd en bij de IHD patiënten juist andersom. Wat betreft de integriteit van het cytoskelet en de structurele (matrix) eiwitten toonden beide groepen patiënten downregulatie, hetgeen overeenkomt met de substantiële reductie in extracellulaire matrix tijdens LVAD ondersteuning, die ook in de literatuur wordt beschreven. De eiwitten die bij de IHD patiënten waren ge-downreguleerd betroffen vrijwel uitsluitend cytoskelet eiwitten zoals desmine, vinculine en actine.

Interessant genoeg waren er in de groep IHD patiënten ook eiwitten, die een rol spelen bij de glycolyse, het energie metabolisme en de oxidatieve fosforylering ge-upreguleerd, hetgeen suggereert dat door de LVAD ondersteuning de energie productie door de mitochondrien weer op gang komt, dit in tegenstelling tot de DCM patiënten. Deze mitochondrale veranderingen werden ook bij electronen microscopisch onderzoek teruggevonden. Hyperactiviteit van hypertrofische cardiomyocyten gaat gepaard met versterkte activiteit en energieproductie in de mitochondrien, die electronenmicroscopisch een zeer compact crista-patroon blijken te hebben. Inactiviteit van cardiomyocyten bij LVAD ondersteuning resulteeert in minder energie productie door de mitochondrien, electronienmicroscopisch gekenmerkt door zwelling en uiteen zakken van de mitochondriale cristae. Het afbouwen van de LVAD ondersteuning (weaning genoemd) gecombineerd met training van de cardiomyocyten, kan deze mitochondriale respons weer omkeren, reverse remodeling op electronenmicroscopisch nivo. Bij DCM patiënten werden deze morfologische veranderingen in de mitochondrien ook gezien, maar dit ging daar niet gepaard met de opvallende upregulatie van de eiwitten van het mitochondriale energie systeem, zoals bij de IHD patiënten.

Uiteraard behoren de DCM patiënten tot een pathogenetisch buitengewoon heterogene groep, variërend van post-myocarditis tot diverse zeldzame erfelijke en niet-erfelijke enzymdefecten in de mitochondriale stofwisselingsenzymen. Deze onderliggende ziekte kan een belangrijke, vooralsnog onbekende rol spelen bij het reverse remodeling proces en daardoor een heel ander eiwit profiel tot expressie brengen.

Uit deze studie is ook naar voren gekomen dat er tenminste 2 verschillende soorten ziekte-entiteiten zijn, die een verschillend, maar karakteristiek, remodeling-gessocieerd eiwitprofiel tot expressie brengen. De hiërarchische clustering van de betreffende eiwit profielen kan

mogelijk niet alleen een indicatie geven voor de pathogenese van het hartfalen, maar ook iets zeggen over de reactie van de individuele patient op LVAD ondersteuning. Daaruit zouden criteria kunnen voortvloeien met betrekking tot weaning of de noodzaak om op termijn te transplanteren, omdat herstel van het myocard niet waarschijnlijk is.

Hoofdstuk 4. Integrine expressie tijdens het reverse remodeling proces

Integrines zijn eiwitten in de basaalmembraan van cardiomyocyten. Deze structurele eiwitten zorgen voor de in stand houding van de architectuur van het myocard, de mechanotransductie en de verbinding van de cardiomyocyten met de extracellulaire matrix. In deze studie werd met immuunhistochemie (IHC) gekeken naar de veranderingen in de eiwit-expressie van 7 alfa-integrines en 4 beta-integrines tijdens het reverse remodeling proces. Daarnaast werden met behulp van de standaard Q-PCR veranderingen in de mRNA's opgespoord, die coderen voor verschillende integrines. De afname van collageen en het handhaven van laminine in de basaalmembraan van cardiomyocyten was al eerder aangetoond. Wat betreft de immunohistochemische kleuringen werd er geen verschil gezien in de aankleuring van de verschillende integrines voor en na LVAD ondersteuning, ook niet tussen de DCM en de IHD patiënten. Misschien zijn de veranderingen op eiwitnivo te subtiel om te detecteren met behulp van IHC. Wat betreft de integrine mRNA expressie werden er wel veranderingen gezien pre- en post LVAD support. Vooral de mRNA expressie van integrine alfa-6 lijkt hierbij een belangrijke rol te spelen. Dit mRNA toonde in beide groepen patiënten een sterke toename na LVAD ondersteuning. Waarschijnlijk is het reverse remodeling proces zelf hiervoor verantwoordelijk, omdat dit integrine uitsluitend aanwezig is in de wand van de capillairen in het myocard. De inductie van capillair-ingroei in het myocard door de verbeterde bloeddoorstroming, zoals dit ook bij wondgenezing wordt gezien, zou de reverse remodeling dus wel eens direct kunnen aansturen. Het stimuleren van de integrine alfa-6 expressie kan derhalve een aangrijpingspunt zijn om het reverse remodeling proces positief te beïnvloeden, temeer daar dit eiwit in het myocard ook mesenchymale stamcellen aantrekt en bindt. Deze mesenchymale cellen zouden in dit kader dan weer een rol kunnen spelen bij het reparatieproces van het geinfarceerde myocard.

Hoofdstuk 5. Osteopontin als potentiële biomarker bij reverse remodeling

Het was bekend dat de expressie van het extracellulaire matrix eiwit Osteopontin (OPN) in het myocard toeneemt met de ernst van het hartfalen. Daarom werd in deze studie gekeken

of de OPN mRNA expressie in het hart en de OPN eiwit expressie in het plasma iets zeggen over het reverse remodeling proces tijdens LVAD ondersteuning. In de eerste plaats blijkt dat de OPN plasma concentraties bij alle hartfalen patiënten significant hoger zijn dan bij gezonde controles, dat OPN in het plasma niet daalt tijdens LVAD ondersteuning, maar dat het wel normaliseert binnen 3 maanden na harttransplantatie. Deze bevindingen zijn vreemd genoeg in tegenstelling tot wat in het hartweefsel zelf werd aangetoond. De hoeveelheid OPN-mRNA in het natieve (zieke) hart van met name IHD patienten, was significant lager na LVAD ondersteuning en daalde zelfs naar controle nivo. Het zou kunnen betekenen dat er een externe bron (buiten het myocard) is die het OPN in het plasma produceert of dat het remodeling proces en de daarmee gepaard gaande opruimreactie in het myocard het OPN, ondanks de lage expressie van het OPN-mRNA, op de een of andere manier toch nog steeds blijft afgeven. Dit laatste wordt aannemelijk gemaakt doordat binnen 3 maanden na HTX het OPN in het plasma is genormaliseerd. Met in situ hybridisatie op en laser microdissectie van hartweefsel werd gezien dat vooral de cellen in de extracellulaire matrix het OPN-mRNA genereren. Welke cellen dat zijn, alleen mestcellen of ook fibroblasten en macrofagen, kon niet met zekerheid worden vastgesteld. De veranderingen in het fibroseringsproces van de ECM bij LVAD ondersteuning kunnen wijzen op OPN productie door fibroblasten, die bij IHD patiënten veel prominenter aanwezig kunnen zijn in het myocard. Verder waren er opvallende individuele verschillen tussen de hartfalen patiënten. Sommigen hadden een laag OPN nivo en weinig fibrose, bij anderen werd een hoge expressie en veel fibrose gezien Daardoor lijkt het er op dat de OPN plasma concentratie niet alleen een maat is voor de ernst van het hartfalen zelf, maar ook gebruikt kan worden voor de status van het reverse remodeling proces in het myocard tijdens LVAD ondersteuning. Gezien de grote verschillen tussen de individuele patiënten is het zeer waarschijnlijk dat de onderliggende oorzaak van het hartfalen een rol speelt bij het type en de hoeveelheid interstitiele fibrose in het myocard en dus ook bij het reverse remodeling proces, waarvan de OPN-mRNA expressie het resultaat is.

Hoofdstuk 6. Regulatoire microRNA expressie tijdens LVAD ondersteuning

Er is steeds meer bekend over de microRNA's, die een rol spelen bij de pathogenese van allerlei ziekten in het hart. Zo zijn miR-1, miR-208 en miR-133 betrokken bij de regeneratie en hypertrofie van cardiomyocyten bij hartfalen patiënten en miR-1 bij het ontstaan van ventriculaire ritmestoornissen.

In dezelfde patientengroepen met een IHD en een DCM werd in deze studie met behulp van Q-PCR gekeken naar de expressie-profielen van 4 miR's (waarvan al bekend was dat zij in het hart aanwezig zijn) voor en na LVAD ondersteuning. Bij beide groepen patiënten bleek dat alle 4 de miR's een veel lager expressie profiel hadden in vergelijking met de controle harten, maar dat er ook een verschil was tussen de IHD en de DCM groep. De expressie profielen van miR-1, miR-133a en miR-133b waren relatief hoger in de IHD groep dan in de DCM patiënten, die post LVAD op een lagere expressie uitkwamen. Daardoor lijkt het alsof het myocard van de de IHD patiënten het vermogen heeft om het miR expressie nivo gedurende LVAD ondersteuning, althans wat betreft deze 3 miR's, te normaliseren. Voor het 4e miR (miR-208) waren de waarden in beide groepen te laag om te interpreteren.

Dit zou er inderdaad op kunnen wijzen dat deze miR's het reparatie-proces aansturen om het door ischaemie verloren gegane hartweefsel te herstellen. Onafhankelijk van de duur van de mechanische ondersteuning werd het miR expressie nivo in de IHD patiënten nooit meer even hoog als in de controle groep. In de DCM groep werd er geen verbetering van de miR expressie profielen gevonden. Een mogelijke verklaring hiervoor is dat er in deze patiëntengroep geen herstel en reparatie van het myocard kunnen plaatsvinden, omdat er een heel andere pathogenese aan het hartfalen ten grondslag ligt.

De onderzochte regulatoire miR's spelen een fundamentele rol bij de ontwikkeling van het hart tijdens de zwangerschap en het is bekend dat hun expressie profielen afnemen bij patiënten met hartfalen. Nu duidelijk is uit deze studie dat deze miR's zich, vooralsnog gedeeltelijk, kunnen herstellen gedurende LVAD ondersteuning bij hartfalen patiënten met IHD, wijst dit er op dat zij inderdaad een rol spelen bij het reverse remodeling proces in het hart. Dit laatste biedt wellicht in de toekomst mogelijkheden om het herstel van het myocard bij deze specifieke patientengroep medicamenteus te beïnvloeden.

Hoofdstuk 7. Conclusies

Bij patienten met progressief hartfalen treden er grote structurele veranderingen op in de hartspiercellen en in het steunweefsel van het hart daaromheen. Maar het is lastig om er achter te komen wat er zich precies in het myocard op weefselnivo afspeelt en ook hoe dit remodeling proces beinvloed kan worden. Ditzelfde geldt voor het omgekeerde proces (reverse remodeling), dat in gang wordt gezet als de ventrikels 'rust' krijgen doordat er een ondersteunende pomp (LVAD) wordt geimplanteerd. Van tevoren kan niet gezegd worden bij welke patient de hartspier zal herstellen of bij wie uiteindelijk de pomp vervangen zal

moeten worden door een nieuw hart. Het doel van dit onderzoek was om parameters over het (reverse) remodeling proces in handen te krijgen, die gebruikt kunnen worden om patienten met hartfalen van tevoren beter te selecteren, zodat bekend is wie in aanmerking komt voor weaning (het verwijderen van de pomp), voor LVAD destination therapie of voor harttransplantatie. De ontdekking van mogelijkheden om het reverse remodeling proces positief te beinvloeden, was een bijkomend doel.

De interpretatie van de resultaten bij deze toch relatief kleine en heterogene groep patienten, waarvan hartweefsel voor basale research ter beschikking was, heeft aanzienlijke beperkingen. Mogelijk bruikbare biomarkers die aangeven in welke fase het reverse remodeling proces verkeert (Osteopontin, Alfa-1-antichymotrypsine) zijn veelbelovend, maar nog niet klinisch toepasbaar. Uit deze studie is voor het eerst wel het belang van de regulatoire functies van verschillende microRNA's in het reverse remodeling proces naar voren gekomen. De ontwikkeling en de toepassing van anti-miR medicatie kan daardoor een nieuw doel voor research en therapie worden.

Verder wijzen de resultaten van deze studie op de verschillen tussen de patienten met een ischaemische hartziekte (IHD) en een dilaterende cardiomyopathie (DCM). De heterogene groep van DCM patienten reageert met andere verbindweefselingspatronen en herstelmechanismen tijdens LVAD ondersteuning, dan de IHD patienten. Het beinvloeden van het fibrose-proces door in een vroege fase van de ziekte over te gaan tot LVAD ondersteuning zou overwogen kunnen worden, naar analogie van de behandeling van vroege leverfibrose bij hepatitis patienten. Daaruit blijkt dat de fibrose na adequate behandeling in regressie kan gaan. Selectie van DCM patienten die daarvoor in aanmerking zouden kunnen komen, kan alleen als er meer bekend is over de achterliggende oorzaak van hun ziekte.

Toekomst

De essentieel andere pathogenese en etiologie die aan het hartfalen bij DCM patienten in vergelijking met IHD patienten ten grondslag ligt, zal hoogstwaarschijnlijk verantwoordelijk zijn voor de verschillen in het reverse remodeling proces. Bij veel DCM patienten is de oorzaak van de ziekte nog onbekend en ondanks de snelle ontwikkelingen in de moleculaire biologie en uitgebreider genetisch testen, kan maar bij een beperkt aantal DCM patienten een genetisch defect worden aangetoond. Het systematisch bijeenbrengen in een data base van alle relevante informatie over de pathogenese en etiologie van hartfalen, de klinische symptomatologie, diagnostiek en behandeling, het morfologisch substraat in het

hartweefsel en de factoren die het (reverse) remodeling proces beinvloeden, zou de basis kunnen worden voor het ontwikkelen van innovatieve vormen van 'personalized' therapie. De rol van de patholoog daarin is bescheiden, maar essentieel. Het ontrafelen van de pathogenetische mechanismen van hartfalen, het documenteren van de histopathologische afwijkingen, de interpretatie daarvan in het kader van uit de data base naar voren komende etiologische factoren en de verdere bestudering van het (reverse) remodeling proces, zullen voor de cardiovasculaire patholoog in de nabije toekomst een belangrijke uitdaging zijn.

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Epiloog

Het is ondoenlijk om aan het eind van dit traject iedereen persoonlijk te bedanken die hieraan een bijdrage heeft geleverd. Vooral de hele HTx groep, alle mensen die daar de afgelopen jaren deel van hebben uitgemaakt, ben ik buitengewoon erkentelijk voor de onvoorwaardelijke hulp en morele steun, die ik altijd heb gekregen. Anders was dit werk nooit volbracht.

Mijn opleiders, mijn directe collega's, de stafleden in het ziekenhuis, de AIOS die ik op mocht leiden, het personeel van de laboratoria, de secretariaten en de sectie-afdeling, als jullie er niet waren geweest, had ik hier nu niet gestaan.

Met veel plezier denk ik terug aan de afgelopen jaren, het was soms heftig, maar het was de moeite waard en het is nu klaar.

Nooit zal ik de huwelijksaanzoeken op de uitsnijkamer vergeten, het raam waarlangs je naar binnen moest klimmen, als de hoofdingang van de Pathologie op slot zat, de autoritjes naar Tiel en op de terugweg eten bij McDonalds, het altijd maar moeten uitleggen wat het verschil is tussen 'buiten daar' en het resectievlak hier, de AIOS die als pink panters over de afdeling slopen, de collega die de watermeloenkauwgom uit zijn tas toverde, vers uit de VS aangevoerd, met een auto vol mensen racend naar de coupeavond en dan op 1 avond 2 bonnen krijgen voor te hard rijden, de AIOS die de lekkere warme opossumhandschoentjes voor mij meenam uit Nieuw Zeeland, het verschil tussen blader-pathologen en conclusie-jumpers, de MDL AIOS die een revolutionaire anti-obesitas therapie op een congres moest presenteren, waar wij samen de slappe lach van kregen, de colleges waarvan je 15 minuten vantevoren hoorde dat je ze moest geven, het staflid dat met helm en wielrenschoenen op een zonnige zondagochtend koffie kwam drinken en zijn schoenen niet uit wilde doen, het dansje van de degranulerende mestcel met het haloframe voor het afscheidsfeest van de opleider, de telefoontjes met die geheimzinnige 'badmuts' waar nooit iemand iets van begreep, de dwarse patholoog die ook zijn proefschrift dwars liet drukken, de collega die mij een illegale copie van de allermooiste uitvoering van de Matthaeus Passion schonk, de diepgravende gesprekken over 'het leven en de dingen' tijdens het coupes kijken aan de microscoop, de pannetjes met warm eten die thuis werden gebracht toen ik ziek was, alle mensen die altijd voor mij klaar stonden in moeilijke tijden, iedere keer opnieuw die kisten met coupes die gereviseerd moesten worden en tenslotte de bloemen met het overdonderende applaus van de studenten na mijn laatste college. Ik zal dat allemaal vreselijk gaan missen, maar de tijd is aangebroken om stenen te gaan hakken.

Het verleden en de toekomst overdenkend, ergens in de regen op een bankje in het donker bij een station, realiseerde ik mij dat herinneringen niet verloren gaan en dat de afstand tussen mensen zo groot is als de afstand in hun hart.

de afstand tussen mensen is zo groot als de afstand in hun hart

oud Perzisch spreekwoord