Chapter 4.

Left ventricular assist device in end-stage heart failure: persistence of structural myocyte damage after unloading.
An immunohistochemical analysis of the contractile myofilaments

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Abstract

OBJECTIVES – We sought to evaluate the contractile proteins in cardiomyocytes of patients with end-stage heart failure (HF) before and after mechanical support with a left ventricular assist device (LVAD).

BACKGROUND - Improvement of myocyte dysfunction has been suggested after LVAD support.

METHODS - Fourteen patients’ myocardial biopsies taken at the time of LVAD implantation and after explantation, at the time of heart transplantation, were processed for routine hematoxylin-eosin staining and immunohistochemistry using monoclonal antibodies against actin, myosin, tropomyosin, troponin C and T and titin. A grading scale from 1 (abnormal staining of all myocytes, no cross-striation) to 5 (normal fiber anatomy and striation) was used. The cross-sectional area of cardiomyocytes was also measured.

RESULTS - The cardiomyocytes’ cross-sectional area decreased after support, from $519 \pm 94 \mu m^2$ to $319 \pm 53 \mu m^2$ ($p<0.001$). Actin, tropomyosin, troponin C, troponin T and titin at the time of LVAD implantation showed widespread distortion of architecture; their grades were: $1.4 \pm 0.6$, $2.3 \pm 1.0$, $2.1 \pm 0.9$, $2.1 \pm 1.2$, $2.0 \pm 0.6$, respectively. In contrast, myosin morphology was preserved ($4.6 \pm 0.7$). After LVAD support, actin, tropomyosin, troponin C, troponin T and titin showed improvement (grades $2.7 \pm 1.3$ [$p=0.004$], $3.2 \pm 1.2$ [$p=0.021$], $3.3 \pm 0.9$ [$p=0.004$], $3.0 \pm 1.1$ [$p=0.048$] and $3.1 \pm 0.9$ [$p=0.001$], respectively), but no normalization. The myosin pattern deteriorated slightly ($3.6 \pm 1.6$ [$p=0.058$]).

CONCLUSIONS – After LVAD support, during a period of $213 \pm 135$ days in patients with end-stage HF, despite a decrease in the size of the cardiomyocytes, severe structural myocyte damage persisted. This does not support complete recovery of myocyte histologic features.
Introduction

Heart failure (HF) is a growing problem in cardiovascular medicine. Aging of the population, prevention of premature death by improved medical and surgical management as well as better therapy available for the treatment of chronic HF, results in an increasing number of patients with this syndrome. Heart failure may be considered a progressive disorder, initiated after an index event that results in a decline in pumping capacity of the heart. It is accompanied by activation of neurohormonal and cytokine systems, as well as adaptive changes in the myocardium called "remodeling" (1). This process of remodeling includes alterations in left ventricular (LV) geometry, alterations in extracellular matrix and alterations in the cardiomyocytes’ size, function and number. Some of the changes reported in failing human cardiac myocytes are loss of myofilaments and alterations in cytoskeletal proteins (2). One canine study suggests reversibility of myocyte dysfunction, associated with an increase in the number of contractile elements, after treatment with beta-blockade (3). Also, in humans, reversibility of myocyte dysfunction has been suggested after mechanical support with a left ventricular assist device (LVAD) (4-8). This raises the option to use LVAD support as a bridge to recovery of cardiac function in patients with end-stage HF (9-11). The number of patients who have been weaned from the LVAD, however, is limited, and long-term results are not available. Unloading the heart with an LVAD will lead to a decrease in cardiac dimensions and neurohormonal activation (5,12,13). Whether this results in a significant and sustained reversal of myocyte dysfunction, however, is questionable (14,15). Few histologic studies on this subject are available (4-6,16,17). No microscopic studies of the contractile proteins in myocytes have been performed. The aim of this study was to evaluate the contractile proteins in cardiomyocytes by means of routine staining and immunohistochemical analysis of myocardial biopsies taken at the time of LVAD implantation and to compare these with biopsies taken at the time of heart transplantation (HTx). Furthermore the cardiomyocytes’ cross-sectional area before and after LVAD support was compared.

Methods

Patient group
Fourteen consecutive patients (2 women and 12 men) with refractory end-stage HF treated with a pneumatic LVAD (Heartmate, Thoratec, Pleasanton,
California) as a bridge to transplantation were included in this study (Table 1). Eight patients had dilated cardiomyopathy (DCM) and six had ischemic heart disease (IHD). Thirteen patients were successfully transplanted. One patient died of a recurrent cerebral embolism. The mean duration of LV unloading was $213 \pm 135$ days (range 71-455 days). Before HTx, all patients were in New York Heart Association functional class I and demonstrated a very good exercise performance, as reported previously (18). All cardiac medication had been stopped. Written, informed consent was obtained from all patients.

Table 1. Characteristics of patients with an implanted LVAD

| Male (n) | 12 |
| Female (n) | 2 |
| Age (years) | $32 \pm 12$ |
| Duration of HF* (months) | $5.6 \pm 6.1$ |
| DCM / IHD (n) | 8 / 6 |
| LVEF (%) | $13 \pm 5$ |
| CO (l/min) | $3.4 \pm 1.2$ |
| MAP (mmHg) | $64 \pm 13$ |
| IABP/other support (n) | 7 |
| Duration of LVAD support (days) | $213 \pm 135$ |

* Time between onset of heart failure symptoms and implantation of LVAD (months).

**Immunohistochemical analysis**

Myocardial biopsy at LVAD implantation consisted of the LV apical core removed during Heartmate implantation. These biopsies were compared with LV tissue specimens of the explanted heart after HTx, from the apical half of the LV, outside the suture area of the inflow canula. In the one patient who died before transplantation, tissue specimen of the heart at autopsy were used. All biopsies were directly fixed in buffered formalin, embedded in paraffin and routinely processed for hematoxylin-eosin staining on 5-µm sections. Standard immuno-histochemical analysis was performed using a three-step avidin-biotin peroxidase reaction. In brief, the sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked using a solution of methanol and hydrogen peroxide. Primary antibodies were applied to the sections for 1 hr and subsequently demonstrated using a biotinylated horse immunoglobulin G (IgG) anti-mouse IgG and horseradish peroxidase-labeled streptavidin. Immunovisualization was performed using 3,3 diamino benzidine tetra hydrochloride reagents and counterstaining with Mayer’s hematoxylin. The sections were dehydrated, cleared and embedded in Pertex mounting medium.
Contractile proteins

(Histolab, Göteborg, Sweden). Primary monoclonal antibodies used in this study were anti-sarcomere actin (Sigma, Zwijndrecht, the Netherlands), myosin slow (Sigma), tropomyosin (Sigma), troponin T (Sigma), troponin C (Novocastra) and titin (Novocastra, Newcastle upon Tyne, United Kingdom). Antigen retrieval, by boiling sections in a citrate-buffered solution for 15 min, was performed on the sections for all antibodies except myosin. Sections for myosin incubation were proteolytic-digested with pepsin for 15 min.

All slides contained large transmural biopsies and were examined completely by two to four investigators who aimed for consensus. In these biopsies, all longitudinal arranged cardiomyocytes were analyzed. In the case of IHD, only surviving myocytes were studied; the necrotic area was not taken into consideration. Besides providing a description of the histological findings, a grading system was also used for the immunohistochemical data, according to the following scale:

1. Almost all myocytes show a distorted architecture with absence of cross-striation
2. Some normal myocytes with cross-striation are present; most of the myocytes are abnormal, however
3. There are normal myocytes with cross-striation, along with myocytes without cross-striation, or myocytes without antigen staining over the full length of the contractile fiber
4. There are some myocytes with a distorted architecture; most of the myocytes are normal, however
5. Almost all myocytes show a normal architecture and cross-striation

To test for inter- and intra-observer variabilities, grading was repeated in half of the biopsies in a blinded manner, and the kappa value of reliability was determined. The cardiomyocytes’ cross-sectional area was measured on 3 µm slides stained with modified azan, making use of a Videoplan morphometric program (Zeiss Kontron, Eching, Germany). Thirty cells were measured perpendicular to the long axis; oblique sections were excluded.

Control tissue

Before starting this study in patients with a LVAD, we optimized our technique of tissue handling and immunohistochemistry on all kinds of human myocardial biopsies. This was mostly done to rule out the effects of globally warm ischemia on the biopsies. As normal human LV myocardial tissue is not
available, atrial auricles obtained at open heart surgery from patients without
HF were used for comparison.

**Statistical analysis**
Grading of the immunohistochemical data was compared by using the paired
Student-t test. Inter- and intra-observer variabilities were tested using kappa
analysis. A comparison between DCM and IHD was made by using the
unpaired Student-t test. All data were calculated with SPSS version 8.0 for
windows. A p value< 0.05 was considered significant.

**Results**

**Hematoxylin-eosin staining.**
At the time of LVAD implantation, all myocardial biopsies showed widespread
hypertrophy of the myocytes, striking vacuolization and absence of normal
cross-striation. Increased amounts of lipofuscin and interstitial fibrosis were
seen (Fig 1a). After LVAD support, at the time of HTx, the biopsies showed less
hypertrophy and vacuolization and some improvement in cross-striation (Fig
1b). None of the patients, however, showed complete normalization. Interstitial
fibrosis appeared to increase in most patients.

![Figure 1](image.png)

**Figure 1:** Hematoxylin-eosin staining in left ventricular biopsies (original magnification
x50) before and after LVAD support. (A) Before LVAD support, there were
hypertrophic myocytes with varying nuclear sizes, with vacuolization and interstitial
fibrosis. (B) After LVAD support, the myocytes showed less hypertrophy and
vacuolization, together with a decrease in myocyte size and an increase in interstitial
fibrosis.

To rule out the effect of the implantation of the inflow cannula on this fibrosis,
we also examined longitudinal sections of the complete LV. These biopsies showed that the interstitial fibrosis was diffusely present throughout the whole LV. The inflow cannula only caused a small local ring of dense fibrous tissue directly around it. The widespread variation in myocyte histologic features in both series of biopsies was remarkable, both at LVAD implantation and HTx. Areas with almost normal structure were neighbored by areas showing severe morphological disorganization.

**Actin**
The myocardial biopsies at the time of LVAD implantation showed severe distortion of actin-staining pattern in nearly all myocytes, with barely any cross-striation visible (Fig 2a). Within some vacuoles, granular material staining for actin was discerned. At the time of HTx, some improvement in actin pattern was observed, with partly restoration of cross-striation (Fig 2b). Large areas, however, still demonstrated an abnormal architecture.

**Myosin**
Remarkably, biopsies taken at the time of LVAD implantation showed an almost normal myosin architecture (Fig 2c). In the vacuoles, no myosin-positive material was discerned. In contrast to the partial improvement in actin staining, myosin staining demonstrated a slight deterioration at the time of HTx (Fig 2d).

**Tropomyosin, roponin C and T and Titin**
The staining pattern for these antibodies was comparable to that of actin staining, but less pronounced. At the time of LVAD implantation, some cross-striation was seen, but there were also large areas with disorganization and a lack of contractile material. Within individual cardiomyocytes, a wide variability of the staining pattern was occasionally observed. At the time of HTx, biopsies showed some improvement of cross-striation, but areas with disorganization persisted. Within each cardiomyocyte, the staining pattern appeared to be more homogeneous after LVAD support than before it. Between cardiomyocytes, however, the variability in the staining pattern increased. This frequently resulted in alternation of structurally normal myocytes with apparently abnormal myocytes in the same contractile fiber - a peculiar phenomenon not mentioned before in the published data (Fig 3). The titin-staining pattern was comparable to that of the other thin contractile filaments.
Figure 2: A: Actin staining before LVAD support shows a severe distortion of the architecture and absence of cross-striation in nearly all myocytes (grade 1). B: Actin after LVAD support demonstrates some improvement in the staining pattern, with partial restoration of cross-striation (grade 3). C: Myosin before LAVD support displays a normal architecture (grade 5). D: After LVAD support, some deterioration is observed (grade 3). (original magnification x250).

Grading of the contractile proteins
A clear pattern can be delineated (Table 2, Figure 4); the thin, contractile filaments and titin demonstrated widespread disorganization at the time of LVAD implantation and showed improvement after support (p = 0.004 for actin pre-vs. post-support). In contrast, myosin, the main constituent of the thick contractile filament, displayed an almost normal morphology, demonstrating a slight deterioration after LVAD support, although not significant (p = 0.058).

The inter- and intra-observer kappa values were 0.55 and 0.63, respectively, indicating fair agreement. Ischemic heart disease showed a worse staining pattern of the thin contractile filaments at the time of LVAD implantation than DCM (p < 0.05 for actin, troponin C and titin). After LVAD support, this
difference disappeared.

**Figure 3:** Tropomyosin staining after LVAD support show the alternation of histochemical normal myocytes with abnormal ones in the same contractile fiber (original magnification x150).

There was no correlation between the grading of the contractile elements in individual patients and the duration of HF symptoms, the duration of LVAD support, the dose and duration of catecholamine support before LVAD implantation or the use of an intra-aortic balloon pump before support.

**Cross-sectional area of the cardiomyocytes**

The cardiomyocytes’ cross-sectional area decreased significantly, from $519 \pm 94 \mu m^2$ before LVAD support to $319 \pm 53 \mu m^2$ after support ($p < 0.001$), a reduction of $36 \pm 19\%$. Normal values in five control-patients showed a cross-sectional area of $226 \pm 67 \mu m^2$.

**Normal atrial auricle tissue**

The atrial cardiomyocytes of patients undergoing coronary artery bypass graft surgery showed a normal staining pattern of the contractile proteins (Fig. 5).
Table 2. Grading scale of the contractile proteins

<table>
<thead>
<tr>
<th></th>
<th>LVAD implantation</th>
<th>Heart transplantation</th>
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<tbody>
<tr>
<td><strong>Actin</strong></td>
<td></td>
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<tr>
<td>DCM</td>
<td>1.6±0.7</td>
<td>2.6±1.4</td>
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<td>Ischemic</td>
<td>1.0±0.0*</td>
<td>2.8±1.3</td>
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<tr>
<td>All</td>
<td>1.4±0.6</td>
<td>2.7±1.3 (p=0.004)</td>
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<td><strong>Myosin</strong></td>
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<td></td>
</tr>
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<td>DCM</td>
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<td>3.4±1.6</td>
</tr>
<tr>
<td>Ischemic</td>
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<td>4.6±0.7</td>
<td>3.6±1.6 (p=0.058)</td>
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<td><strong>Tropomyosin</strong></td>
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<tr>
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<tr>
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<td><strong>Troponin T</strong></td>
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<td>3.0±1.1 (p=0.048)</td>
</tr>
<tr>
<td><strong>Titin</strong></td>
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</tr>
<tr>
<td>DCM</td>
<td>2.3±0.5</td>
<td>3.3±1.0</td>
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<tr>
<td>Ischemic</td>
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<td>3.0±0.6</td>
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<tr>
<td>All</td>
<td>2.0±0.6</td>
<td>3.1±0.9 (p=0.001)</td>
</tr>
</tbody>
</table>

Data are presented as the mean value ± SD. * p< 0.05 for IHD versus DCM, at the time of LVAD implantation.

**Discussion**

In the present study, for the first time, to the best of our knowledge, immunohistochemical data on contractile proteins in cardiomyocytes are reported in patients with end-stage HF treated with an LVAD. This study showed that the myosin structure was preserved in patients with end-stage HF before LVAD implantation. In contrast, the thin, contractile filaments and titin
demonstrated widespread distortion of the normal staining pattern, with actin displaying the worst degradation. After LVAD support, a partial improvement in morphology of the thin contractile filaments and titin had occurred. In contrast, myosin demonstrated a slight deterioration in histologic features. Furthermore, after LVAD support, the staining pattern within each cardiomyocyte appeared to be more homogeneous. Between different cardiomyocytes, however, the variability in the staining pattern persisted, leading sometimes to the alternation of apparently abnormal myocytes with normal ones in the same contractile fiber. This may affect cardiac contractility in a negative way and may also affect the spread of the electrical impulse, creating a condition for re-entry. Finally, a significant decrease in the cardiomyocytes’ cross-sectional area after LVAD support was demonstrated, although normalization was not complete.

Figure 4: Grading of the contractile proteins in myocytes before and after LVAD support. Myosin staining showed some deterioration (grade 4.6 before LVAD implantation vs. 3.6 after LVAD support; p = 0.058). In contrast, the contractile proteins of the thin filaments and titin showed improvement (grade 1.4 for actin before LVAD implantation vs. 2.7 after LVAD support; p = 0.004). Severe structural myocyte damage persisted, despite a period of LVAD support of $213 \pm 135$ days.
The role of immunohistochemistry in HF

Immunohistocheamic studies on myocardial tissue in chronic HF are scarce and comprise mainly hearts with DCM (2,19,20). With respect to the thin contractile filaments and titin, our results correspond to these studies. In contrast to the study reported by Hein et al. (19) the myosin pattern in our patients with end-stage HF was remarkably preserved. This relative sparing of myosin, however, has been demonstrated before in a study of dogs in whom regional ischemia was induced by ligation of the left anterior descending coronary artery (21).

Figure 5: Human atrial cardiomyocytes show normal cross-striation (actin staining, grade 5; original magnification x250).

Recovery of myocyte function

Despite persistence of widespread areas with a distorted myocyte architecture, as shown in this study, LVAD implantation leads to an impressive improvement in the hemodynamic condition in our patients (18), as well as in those reported in other studies. The decrease in cardiomyocyte size is in accordance with other studies (17,22, 23). Furthermore, LVAD support results in a decrease in cardiac dimensions (4-6,17,24), an improvement of myocyte-(7,25,26) and mitochondrial function (27, 28), an improvement of neurohormonal activation (12,13,17), an increase in beta- receptor density (29) and disappearance of antibodies against beta- receptors (9) as well as a decrease in serum interleukin
(IL)-6 and IL-8 (30). Recently, a decreased susceptibility to apoptosis (31) and a reduction in tumor necrosis factor-alpha activity in nonischemic cardiomyocytes after support were shown (32). Histologic studies after LVAD support showed a reduction of wavy fibers, contraction band necrosis and myocytolysis, accompanied by an increase in myocardial fibrosis (4-6,8,9,16,17), although recently, a reduction in the collagen content was also reported after LVAD support (23).

These alterations, however, probably resulted from unloading of the heart with consequent circulatory recovery and may not be specific for LVAD support at all. Medical therapy (e.g. angiotensin-converting enzyme inhibitors and beta-adrenergic blockers) also improves the circulatory status and slows deterioration of the failing heart, a benefit that is partly due to inhibition of remodeling (14, 33). Furthermore, spontaneous improvement in LV function is well known in myocarditis and in peripartum cardiomyopathy and was also reported in 27% of patients with recent-onset DCM (34). This may partly explain the number of patients being weaned from LVAD support in some studies. It is evident that in these situations, an LVAD can play an important role in keeping a patient alive and gaining time for cardiac improvement. At present, however, there is no evidence available that LVAD support can be a permanent cure for patients with HF (15). The extent of myocardial recovery after LVAD support is highly variable (11,35,36), and there are conflicting data on the amount of fibrosis after support. The increased fibrosis demonstrated in our study and by others (4,6,37), will have a negative impact on systolic and diastolic cardiac function, limiting the chance for complete recovery. Furthermore, the inadequate presence of titin after LVAD support, as seen in our study, can hamper the formation of new sarcomeres, because titin is necessary as a template for the organization of newly synthesized myosin and actin filaments (19).

A new and intriguing strategy that combines mechanical and intensive pharmacological therapy to promote reverse remodeling and uses clenbuterol to induce physiologic hypertrophy has been suggested - the Harefield approach (38, 39). This requires further studies to determine the long-term efficacy and to elucidate the concepts of reverse remodeling (39).

**Study limitations**

In principle, abnormalities observed in cardiac biopsies could partly be caused by global warm ischemia of cardiac tissue after obtaining the biopsies. Diseased human hearts are extremely susceptible to the effects of ischemia,
particularly the contractile proteins (20). Therefore, we optimized our tissue handling so that 90 % of the biopsies were fixed within 10 minutes. The normal architecture of the myosin pattern in the patients with HF argues against global ischemia as a potential mechanism for our findings, because in the study of Hein et al.(20), it was shown that all contractile proteins are equally sensitive to the effect of global ischemia. Furthermore, our control biopsies were also exposed to the potential effect of global ischemia, but showed a normal staining pattern of the contractile proteins. Most importantly, in this study, every patient is its own control. The improvement in the staining pattern of all thin contractile proteins and titin in the biopsies after LVAD support, which are handled in the same way as before LVAD placement, is a strong argument against the effects of global warm ischemia.

Another problem is that biopsies may not be representative of the whole heart. This problem is overcome, in the greater part in this study, by the quantity of available tissue (1-3 cm. biopsies).

All our patients had end-stage HF. It cannot be ruled out that patients with less severe forms of HF may actually show more recovery of myocyte histologic features after LVAD support.

The rather long duration of LVAD support in this study may mean that the optimal period of recovery has been exceeded, because some clinical and animal studies suggest that recovery is complete within a couple of weeks (11, 26, 40).

In our patients with LVAD support, all cardiac medications were stopped. It is not known to what extent this could have influenced recovery; this will be an area for future research.

**Conclusion**

Despite impressive hemodynamic recovery, a decrease in the cardiomyocytes’ cross-sectional area and an improvement in staining pattern of the thin contractile elements and titin, our findings endorse the persistence of severe structural myocyte damage after a long duration of LVAD support (213 ± 135 days). Our findings do not support complete recovery of myocyte histologic features after a period of unloading of the heart by LVAD support.
References

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