ALLOIMMUNOSENSITIZATION IN LEFT VENTRICULAR ASSIST DEVICE RECIPIENTS AND IMPACT ON POST-TRANSPLANTATION OUTCOME

BY

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LIST OF ABBREVIATIONS

ISHLT – International Society for Heart and Lung Transplantation

INTERMACS – International Registry for Mechanically Assisted Circulatory Support

HF – heart failure

MCS – mechanical circulatory support

LVAD – left ventricular assist device

RVAD – right ventricular assist support

TAH – total artificial heart

ECMO – extracorporeal membrane oxygenation

CF – continuous flow

PF – pulsatile flow

MHC – major histocompatibility complex

HLA – human leukocyte antigen

MICA – major histocompatibility class I – related chain

ETAR – endothelin – 1 type-A receptor

AT1R – anti-angiotensin type-1 receptor

GPCR – G protein-coupled receptor

LDH – lactate dehydrogenase

ICAM-1 – intracellular adhesion molecule 1

IL – interleukin

CD – cluster of differentiation

IgG – immunoglobulin G

AP-1 – activator protein 1

Erk ½ - extracellular signal-regulated kinase ½

NF – nuclear factor

CDC – complement dependent cytotoxicity

FCXM – flow cytometry cross-match

ELISA – enzyme-linked immunosorbent assay

IA – immuno-adsorption

PRA – panel reactive antibody

IVIG – intra-venous immunoglobulin

BMI – body mass index

BSA – body surface area

COPD – chronic obstructive pulmonary disease

PRBC – pure red blood cells

FFP – fresh frozen plasma

ACEI – angiotensin-converting enzyme inhibitor

ARB – angiotensin receptor blocker

BP – blood pressure

PGD – primary graft dysfunction

MOF – multi-organ failure

ACR – acute cellular rejection

pAMR – pathology antibody-mediated rejection

CMV – cytomegalovirus

PTLD – post-transplant lymphoproliferative disorder

Chapter 1

INTRODUCTION

1. INTRODUCTION

The main goal of this study is to assess the role of antibodies on the outcome of surgical treatment of patients with end-stage heart failure. Heart failure is a complex clinical syndrome whose management requires an input from various medical specialities. After exhausting all conservative treatment options, heart transplantation remains the ultimate therapy for selected group of patients. In recent years mechanical circulatory assist devices became an established option in bridging patients to heart transplantation and in patients ineligible for transplantation these devices serve as a destination therapy. One of the alleged limitations of mechanical devices is a high degree of antibody production with possible deleterious effect on subsequent heart transplantation outcome. We first did a literature review on the current state of knowledge of possible immunologic mechanisms involved in alloimmunization of left ventricular assist device (LVAD) recipients. We also included new methods of antibody detection, compared various desensitization strategies, and presented an overview of published evidence assessing the impact of sensitization on post-transplantation outcome. In the experimental part of our study we investigated this phenomenon in our clinical practice of a very busy transplant centre with a wide experience in left ventricular assist devices.

Chapter 2

BACKGROUND AND LITERATURE REVIEW

2. BACKGROUND AND LITERATURE REVIEW

2.1 Heart failure and mechanical assist devices

Heart failure (HF) is a major public health problem with a prevalence of over 23 million worldwide, and rising [1]. The growing prevalence of HF might reflect increasing incidence, an aging population, and improvements in the treatment of acute cardiovascular disease, or a combination of these factors. From the 1970s a dramatic increase in the prevalence of HF and the number of HF hospitalizations was observed, and an epidemic was declared. The lifetime risk of developing HF is now one in fife. Despite advances in therapy and management, HF remains a deadly clinical syndrome. Although mortality from HF has improved over the past few decades, it still results in a high 5 – year mortality that rivals that of many cancers.

Traditionally, heart transplantation is considered a gold standard treatment for patients with end stage heart failure. Since the first heart transplant was performed in 1967, the procedure has grown worldwide and up to June 2014 data from approximately 121,000 heart transplant recipients of all ages have been reported to the International Society for Heart and Lung Transplantation (ISHLT) database [2]. After initial rapid increase in the number of procedures in late eighties and early nineties there was a gradual decline between 1993 and 2004 as a consequence of a decreasing number of available donors especially in European countries. The total number of heart transplantations has plateaued in recent years and there were 4477 procedures performed in 2013 (Figure 1). The discrepancy between the limited availability of donor organs and the increasing number of patients with heart failure has led to the development of left ventricular assist devices (LVADs). LVAD technology has revolutionized the management of refractory heart failure and become an established surgical therapy as a bridge-to-transplantation and for selected group of patients also as a destination therapy. The purpose of long-term mechanical circulatory support is to maximize functional capacity and quality of life while decreasing mortality and adverse events associated with advanced heart failure. There have been significant technical improvements in the device design over the recent years leading to longer durability and reliability. The first generation of pulsatile fill-to-empty pumps has now

been replaced by a second generation of either centrifugal or axial continuous-flow pumps (Figure 2).

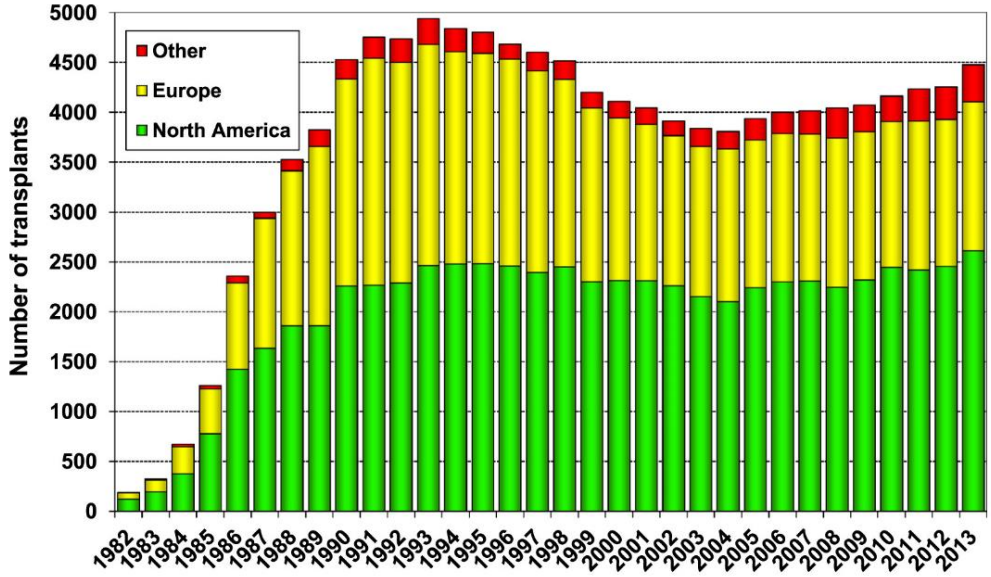

Figure 1. Number of heart transplants (all recipient ages) by year (transplants: 1982 to 2013) and geographic region (The Registry of the International Society for Heart and Lung Transplantation: Thirty-second Official Adult Heart Transplantation Report—2015)

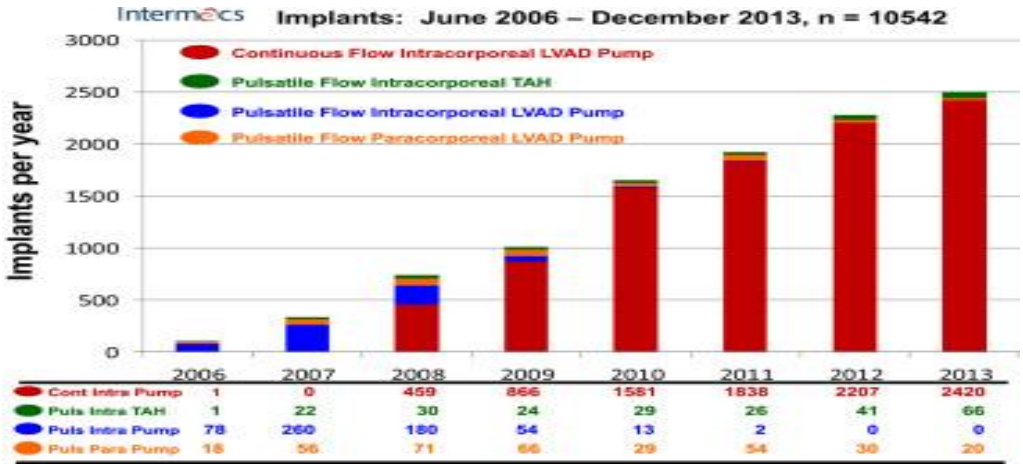

Figure 2. Primary adult implants in the INTERMACS registry by year of implant. LVAD, left ventricular assist device; TAH, total artificial heart (Sixth INTERMACS annual report: A 10,000-patient database)

HeartMate II LVAD (Thoratec Corp., Pleasanton, CA, USA) (Figure 3) and HVAD LVAD (HeartWare Inter., Framingham, MA, USA) (Figure 4) are currently the two most commonly implanted devices worldwide. With the advancements in patient selection, improvements in surgical technique and post-operative management contemporary devices have been proven to provide safe and effective circulatory support with an 80% one year survival (Figure 5). LVADs have also been shown to reduce heart transplantation waiting list mortality and improve the quality of life and survival of heart transplant candidates [3, 4]. In Europe, more MCS systems are implanted than hearts transplanted, and in the near future, this will be the case also in North America [5].

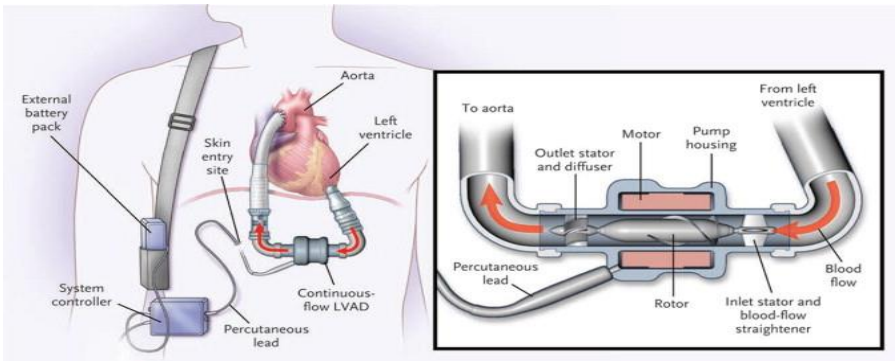

Figure 3. HeartMate II LVAD

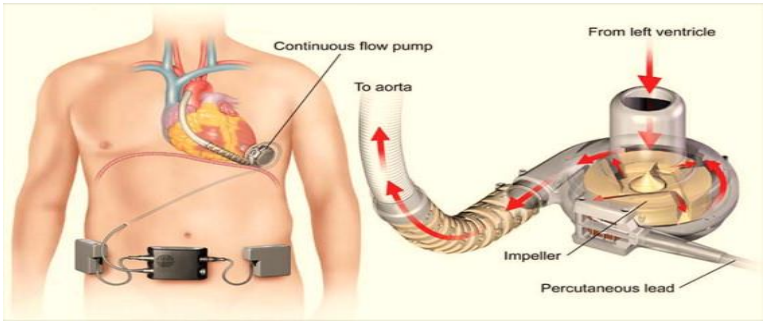

Figure 4. HeartWare HVAD

The number of patients bridged to transplant with MCS has increased from 19% before 2009 up to 35% in 2013 [6]. The widespread use of mechanical devices has led to an increase in the percentage of transplantations of patients from the durable LVADs, reaching 42% in 2013 (Figure 6).

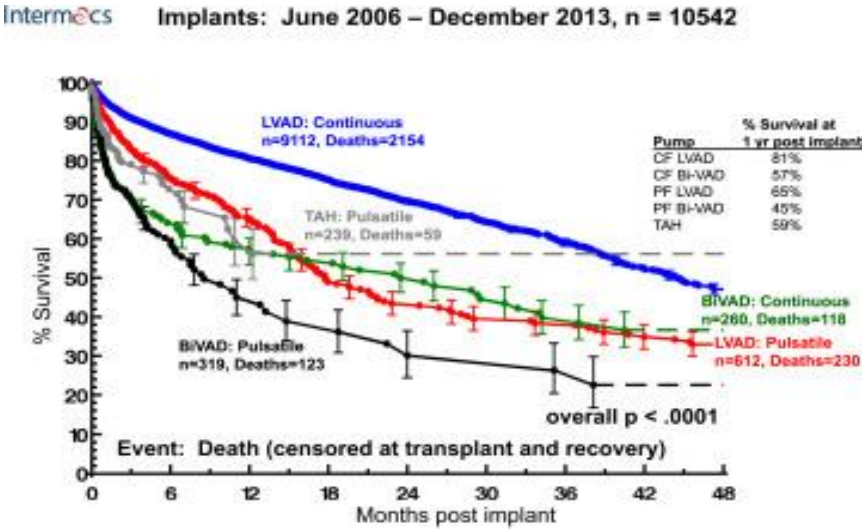

Figure 5. Actuarial survival for primary device implant, stratified by device type. Error bars indicate ± 1 SE. Patients are censored at transplant and recovery. CF, continuous flow; LVAD, left ventricular assist device; PF, pulsatile flow; TAH, total artificial heart (Sixth INTERMACS annual report: A 10,000-patient database)

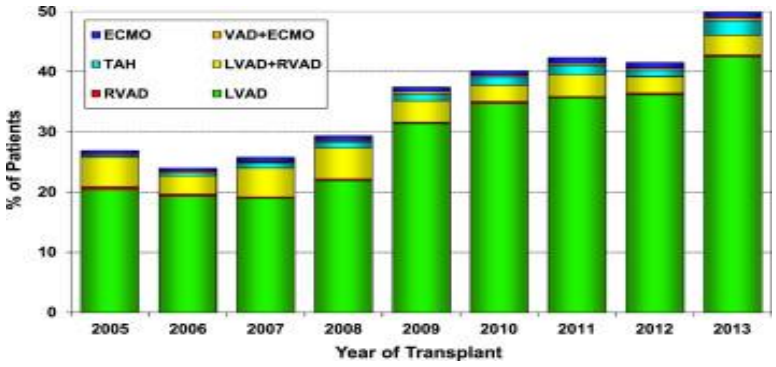

Figure 6. Use of mechanical circulatory support at time of heart transplant by year of transplant. ECMO, extracorporeal membrane oxygenation; LVAD, left ventricular assist device; RVAD, right ventricular assist device; TAH, total artificial heart (The Registry of the International Society for Heart and Lung Transplantation: Thirty-second Official Adult Heart Transplantation Report - 2015)

LVAD use pre-transplant has historically conferred worse post-transplant prognosis, but, in the modern era of continuous-flow devices, this is no longer the case [2]. According to the latest ISHLT Registry Report, pre-transplant bridging with a durable LVAD is not a risk factor for diminished post-transplant survival (Figure 7).

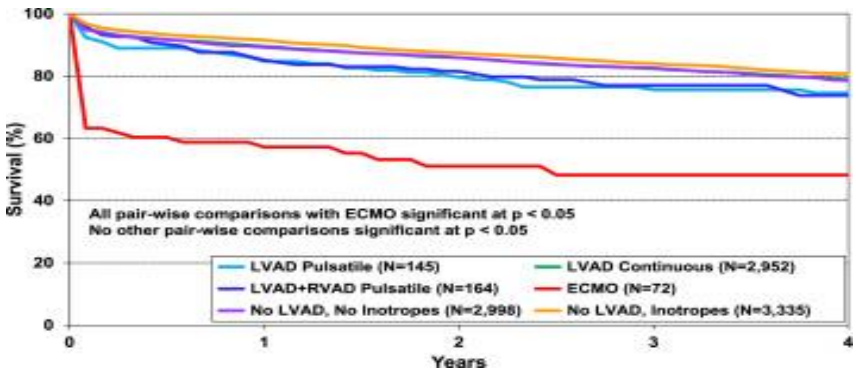

Figure 7. Kaplan-Meier intermediate-term survival by pre-transplant mechanical circulatory support use (The Registry of the International Society for Heart and Lung Transplantation: Thirty-second Official Adult Heart Transplantation Report—2015)

Despite the clinical success of these devices, the anatomic and physiologic consequences of long-term LVAD support have yet to be fully clarified. It has been reported that many patients bridged to transplantation with mechanical support develop circulating antibodies both against human leukocyte antigen (HLA) and various non-HLA antigens. Post-transplantation, these newly developed recipient antibodies interact with donor antigens, potentially compromising the outcome. Transplanting against existing or historic donor-specific antibodies is associated with increased risk of antibody-mediated rejection, graft dysfunction, and decreased survival. The challenge of the sensitized patient awaiting transplantation is that in order to avoid risk of rejection, the donor pool is limited to only compatible donors. Safe transplantation of allosensitized patients is dependent on using prospective crossmatching and antibody titer reduction techniques (desensitization). Strict protocols requiring a negative prospective crossmatch before transplantation result in a decreased donor pool and a longer duration of support in sensitized LVAD recipients with increased inherent morbidity such as infections and thromboembolic complications.

2.2 Description of anti-HLA antibodies

The HLA system is composed of genes (chromosome 6) that encode for proteins located on the surface of cells that are responsible for regulation of the immune system. The HLA complex is vital in distinguishing self from non-self-proteins (antigens). The HLA genes are the human version of the major histocompatibility complex (MHC) genes that are found in most vertebrates. HLAs corresponding to MHC class I present peptides from inside the cell. Foreign antigens presented by MHC class I attract killer T-cells (CD8 positive or cytotoxic T-cells) that destroy cells. MHC class I proteins form a functional receptor on most nucleated cells of the body (Figure 8). There are three major (A, B, and C) and three minor (E, F, and G) MHC class I genes in HLA.

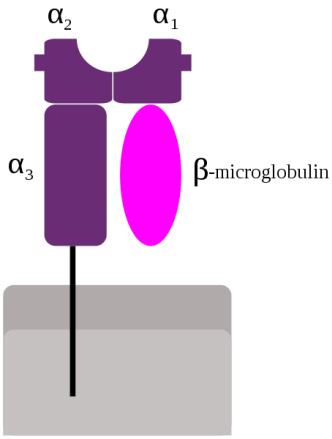

Figure 8. Schematic representation of MHC class I

HLAs corresponding to MHC class II present antigens from outside of the cell to T-lymphocytes. These antigens stimulate the multiplication of T-helper cells, which in turn stimulate antibody-producing B-cells to produce antibodies. Self-antigens are suppressed by regulatory T cells. There are three major (DP, DQ, and DR) and two minor (DM, DO) MHC class II proteins encoded by the HLA. The genes of the class II combine to form heterodimeric (αß) protein receptors that are typically expressed on the surface of antigen-presenting cells (Figure 9).

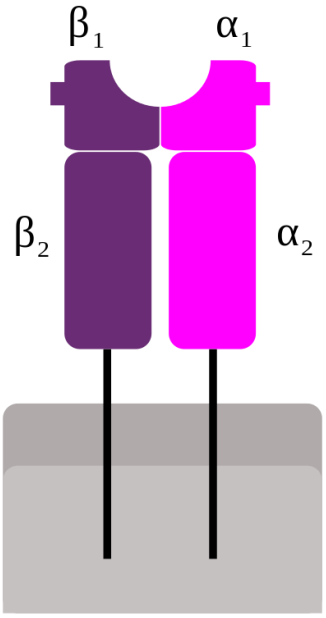

Figure 9. Schematic representation of MHC class II

2.3 Description of antibodies against non-HLA antigens

Apart from antibodies directed against human leukocyte (HLA), several non-HLA antibodies such as major histocompatibility class I-related chain (MICA), autoantibodies against angiotensin II type 1 receptor (AT1R) and endothelin receptor A (ETAR) as well as antibodies to cardiac self-antigens (Myosin and Vimentin) have been associated with an LVAD use [7-10].

AT1R belongs to type A family of G-protein-coupled receptors (GPCRs) with similar structures to rhodopsin and exhibits an extracellular, glycosylated region connected to the seven trans-membrane α – helices linked by three intracellular and three extracellular loops (Figure 10). The human gene for AT1R is located on chromosome 3 and contains four exons. Agonistic antibodies against AT1R were originally found in women with preeclampsia [11]. Anti-AT1R antibodies have also been associated with systemic sclerosis and malignant hypertension [12, 13]. These antibodies have been shown to be the IgG1 and IgG3 subclasses and have the ability to fix complement.

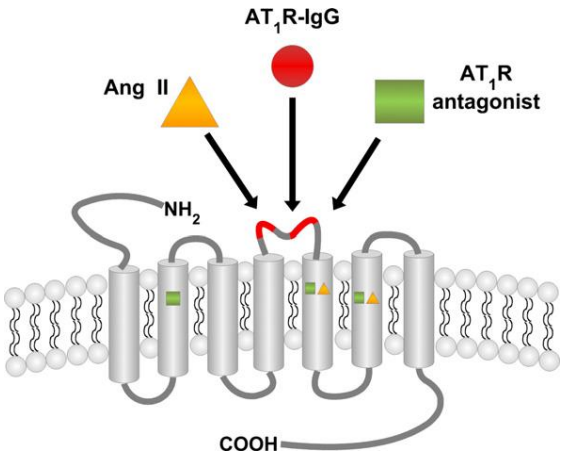

Figure 10. Schematic representation of AT1R

2.4 Antibodies in Transplantation

Antibodies specific for a graft provide an index for immunity and a potential trigger for injury and rejection. Binding of antibodies of the recipient to foreign blood vessels in a transplant activates complement and recruits phagocytic cells leading to vascular injury and various types

of rejection. To a large extent, the injury caused by antibodies, complement, and phagocytic cells on blood vessels in a foreign organ depends on how quickly complement is activated and phagocytes are recruited. Within minutes of binding of antibodies to endothelium of a graft, the process leads to the loss of heparin sulphate proteoglycan, expression of P-selectin, and retraction of endothelial cells, allowing interaction of platelets with underlying matrix. These early events probably cause the condition referred to as hyperacute rejection. During the next period of hours, bound antibodies, activated complement, and activated phagocytic cells change the physiology of blood vessels in ways that promote coagulation, thrombosis, inflammation, and immunity. These changes, which lead to endothelial cell activation, cause a type of rejection variously called ″antibody-mediated rejection″ , ″acute humoral rejection,″ or ″acute vascular rejection.″ Over period of weeks to months, bound antibodies, activated complement, leukocytes, and perhaps other factors induce chronic changes in blood vessels which lead to manifestations of ″chronic rejection.″

There is growing evidence to suggest that antibodies against non-HLA antigens may also contribute to AMR in solid organ transplantation. While anti – HLA antibodies exert their negative effect via complement activation and antibody – mediated cytotoxicity, antibodies against AT1R, act as a natural allosteric receptor agonist. Angiotensin type 1 receptor is a G protein-coupled receptor (GPCR) that mediates physiologic actions of Angiotensin II. Binding of agonistic antibodies to AT1R causes activation of the phosphatidylinositol-calcium second messenger system, phosphorylation of extracellular signal-regulated kinase 1/2 (Erk 1/2), activator protein 1 (AP–1) activation, and increase DNA-binding activity of nuclear factor-κB (NF-κB) pro-inflammatory target genes [14]. Anti-AT1R antibodies also trigger tissue factor induction, as evidenced by intense diffuse tissue staining of epithelial, endothelial and mesangial cells in the renal transplant biopsy specimens obtained at the time of AT1R antibody mediated rejection in the absence of complement activation [15]. Anti-AT1R antibodies derived from preeclamptic patients enhanced promoter activity of tissue factor, an initiator of extrinsic coagulation pathway and a target gene for AP–1 and NF-κB in vitro [16]. Anti-AT1R antibodies developed during pregnancy cause both maternal and fetal pathology via pro-inflammatory,

vasoconstrictive, pro-coagulatory and pro-apoptotic actions on the placenta [17]. There is also evidence that anti-AT1R antibodies promote endothelial micro particles formation through activating p38 mitogen-activated protein kinase pathway. The ´injured´ endothelial micro particles trigger reactive oxygen species production and reduce nitric oxide synthesis in vitro experiments [18]. Zhang et al. [19] investigated in an animal model the association between autoantibodies against AT1 receptor and endothelial dysfunction in vivo. The investigators demonstrated an increased activity of lactate dehydrogenase (LDH) in anti-AT1R positive rats which was regarded as an indicator of cell necrotic death. Functional assessment revealed a decline in the endothelium – dependent relaxation and up – regulation of endothelial intracellular adhesion molecule – 1 (ICAM-1) suggesting that endothelial cells may have inflammatory lesions in anti-AT1R positive rats.

2.5 Pathogenesis of sensitization in LVAD recipients

Antibodies to HLA do not occur naturally; their development requires exposure to foreign (non-self) antigens. Commonly recognized risk factors for allosensitization in all transplant candidates include previous allografts, blood product transfusions, and history of pregnancy [20]. Patients who require mechanical support often receive multiple transfusions because of coagulopathy from hepatic congestion and poor hepatic function, bleeding caused by adhesions from previous surgery, or preoperative anticoagulation therapy. Leukocytes contained in the cellular blood product transfusions have long been implicated as a source of sensitization. Methods of leukofiltration have been more recently adopted to decrease the alloimmunizing effect of transfusions. Sensitization in LVAD recipients may occur as a result of passenger leukocytes that escape filtration. Clinical studies analysing the effect of cellular blood products on sensitization in LVAD recipients have produced conflicting results. Stringham et al. [21] studied the effect of cellular blood products on human leukocyte antigen (HLA) sensitization in seven LVAD recipients. They found that 50% of LVAD recipients who survived the perioperative period and received no cellular blood product developed panel reactive antibody (PRA) levels in excess of 90%. They concluded that avoiding transfusions of cellular blood products in LVAD recipients is safe and well tolerated, but does not universally protect from HLA allosensitization. Similar

results were achieved by Drakos et al. [22] who observed that in a group of 54 patients supported with HeartMate I, who received cellular blood, 35.2% *vs.* 58.8% of non-transfused patients became sensitized (p = 0.15). They concluded that strategies of withholding perioperative transfusions in LVAD recipients have no clear advantage in reducing sensitization as long as leukofiltered blood products are used. McKenna et al. [23] observed that 28% of patients bridged to transplantation with HeartMate I developed HLA antibodies. Patients who developed antibodies received significantly more total peri - and postoperative transfusions than did those who remained negative. In their study, only 10 patients received leukofiltered cellular blood products. Of these 10 patients, 3 (30%) patients subsequently developed HLA antibodies. Interestingly, 3 patients forming antibodies received a mean of 20 units of plasma, whereas the remaining 7 patients received a mean of 10 units. This suggests that plasma may contain a sufficient amount of leukocytes to cause HLA alloimmunization. Platelet transfusions during LVAD implantation have also been shown to be a risk factor associated with the development of HLA class I immunoglobulin G (IgG) antibodies [24, 25]. Itescu et al. [26] sought to determine whether production of anti-HLA antibodies in LVAD recipients was influenced by perioperative transfusion of blood products. Sixty-three percent of patients who received more than six platelet units were found to develop IgG antibodies against HLA class I antigens by 4 months of LVAD implantation compared with 8% of those receiving less than six units ($p < 0.01$). Perioperative red blood cell transfusion did not influence the production of these antibodies, presumably because donor red blood cells contain less contaminating HLA class I-expressing T cells than donor platelets. Development of IgG antibodies against HLA class II antigens was not influenced by either the number of perioperative platelet or red blood cell transfusions. Massad et al. [25] evaluated factors influencing HLA sensitization in 53 patients bridged to transplant with HeartMate I device. From the group of patients who become sensitized during LVAD support, 49.2% received more than 28 units of blood products, whereas 28.4% of sensitized patients received less than 28 units. When examined by the type of blood product, only the number of platelet transfusions significantly increased the peak PRA.

Another mechanism implicated in sensitization of LVAD recipients is the interaction of human body with device biomaterials. Specifically, the textured chamber surface, polyurethane

diaphragm, and polytetrafluoroethylene components of the device have been shown to cause the up-regulation of the immune system and an increased antibody production. Pioneering work and basic research have been done by Itescu et al. [26] who have identified functionally activated monocyte/macrophage lineage type cells within the pseudointima of the HeartMate I textured pumping chamber surface [27]. Another important observation is the aberrant T-cell activation on the LVAD surface *via* interleukin-2 (IL-2) receptor-dependent pathways [28]. Circulating T cells from LVAD recipients showed a heightened state of *in vivo* activation, as defined by surface expression of the activation markers CD95. Pre-activated T cells expressing CD95 are susceptible to activation-induced cell death after triggering via the T-cell receptor complex. Because T cells producing Th1-type cytokines (*i.e.*, IL-2 and interferon-γ) are selectively susceptible to CD95-mediated apoptosis, this process leads to unopposed production of Th2-type cytokines (*i.e.*, IL-10 and transforming growth factor-ß). Predominance of circulating Th2-type cytokines and excessive circulating apoptotic waste is associated with polyclonal B-cell activation *via* CD40-CD40 ligand interactions [29]. Although the proposed mechanism of antibody production has been extensively studied and subsequently validated in clinical studies in patients supported with first-generation pulsatile devices, the same mechanism of antibody production applies in newer-generation axial flow devices that lack biologic chamber valves and possess a substantially smaller inner surface is a matter of some controversy. George et al. [30] hypothesized that axial flow devices would cause less alloimmunosensitization. From the group of 24 patients supported with continuous flow devices, 8% became sensitized *vs.* 28% of 36 patients bridged with pulsatile flow devices (p = 0.02). The authors also noted fewer episodes of acute rejection per patient in the first 9 post-transplant months in the continuous flow group. Garatti et al.[31] came to the conclusion that first year post-transplant incidence of treated rejections was similar in patients supported with pulsatile and continuous flow devices. The same conclusion was reached by Healy et al. [32] who analysed 77 patients supported with pulsatile flow devices and 34 patients supported with continuous flow devices. Although there was no difference in the rejection, patients with pulsatile flow LVADs had more clinically relevant (grades 2–3R) rejection than did patients with continuous flow LVADs. Klotz et al. [33] on the other hand reported that risk of a severe rejection was increased threefold after continuous devices compared with pulsatile-type LVADs.

There are multiple pathways by which anti-AT1R antibodies may appear before transplantation in mechanically supported patients. Protein antigenic determinants may become accessible after injury or surgical stress associated with an LVAD implantation. Inflammatory events might lead to de novo expression of these auto antigens [34, 35]. Anti-AT1R antibodies may also develop through similar pathways as those observed for HLA specific antibodies: transfusions, pregnancies and previous solid organ transplantations.

2.6 Antibody Detection and Monitoring

The presence of antibodies against a given donor can be detected by a cross-match test or against a set of potential donors by one of several ″panel reactive antibody″ assays. The aim is to determine whether a potential transplant recipient has antibodies specific for a given transplant donor, and, if those antibodies are detected, organs from that donor could not be transplanted into the recipient but would be directed to other potential recipients. Present approaches to determining panel reactivity include testing of recipient serum against a panel of cells obtained from individuals of known histocompatibility types. In complement dependent cytotoxicity assay (CDC) the target donor lymphocytes are killed by antibody-activated complement when the recipient has anti-donor lymphocyte antibodies. CDC detects not only donor HLA class I antibodies (by T-cell crossmatch) but also class II antibodies (by B-cell crossmatch). However, the isolation of T or B lymphocyte subpopulations from donor peripheral blood is relatively time-consuming. Apart from HLA, CDC also detects non-HLA antibodies specific for some unknown polymorphic antigens, mismatched between the donor-recipient pair. The disadvantage of CDC assay is that detected antibodies must be cytotoxic and must activate complement. Furthermore, lymphocytes, the target cells used in the CDC assay, may not fully represent the true primary target *in vivo* endothelial cells. In flow cytometry cross-match (FCXM), donor blood cells are incubated with recipient serum followed by an additional incubation with the secondary fluorescence-labelled antihuman immunoglobulin. If the patient has anti-donor antibodies, the second fluorescent antibodies will react to anti-donor lymphocyte antibodies already bound to target cells. Flow cytometry crossmatch assay detects both HLA class I and class II and non-HLA

antigens. As opposed to traditional CDC test, FCXM is not a functional test, and it measures the binding of donor-specific antibody to its potential donor target. Binding is not killing, and positive FCXM does not necessarily mean that detected antibodies have a pathologic effect on target cells.

In HLA antigen-based ELISA, a panel of HLA antigen mixtures (recently replaced with purified recombinant single HLA class I or class II antigens) is coated on the ELISA plates. After the incubation of the coated plates with test serum, bound antibodies are detected using a peroxidase-conjugated antihuman immunoglobulin antibody. Purified HLA antigen-based ELISA is a sensitive method, and antibodies specificities are easily defined. Human leukocyte antigen bead flow cytometry is performed by incubating HLA antibodies present in the test serum bound to the beads, which are then detected by a coloured secondary antihuman immunoglobulin. Theoretically, all the isotypes (IgG, IgM, IgA, etc.) of HLA antibodies may be detected, depending on the type of secondary antibody used. Most recently, Luminex (One Lambda Inc., Canoga Park, CA), a new flow cytometry technology, was introduced for detection of HLA antibody. It is a multiplexed data acquisition and analysis platform of microscope-based assay that performs simultaneous measurements of up to 100 different analytes. Studies comparing flow cytometry with conventional panel reactive antibody (PRA) testing methods have shown that Luminex provides quantitative antibody measurement as well as detailed specificity assessment exceeding that of CDC and ELISA. In a study by Yang et al. [36] 42% of patients who were PRA negative by CDC assay were subsequently reclassified as sensitized in Luminex assay testing. Increased sensitivity of newer solid-based assays raises a question about the clinical relevance of detected antibodies, and further stratification of these antibodies may be necessary to avoid depriving patients of transplants because of antibodies that may not be important. Ho et al. [37] compared the survival of heart transplant recipients who never developed anti-HLA antibodies (n = 390) with that of patients who showed alloantibodies only before but not after transplantation (n = 25), only after but not before transplantation (n = 250), or both before and after and after transplantation (n = 109). The highest 10 year survival (80%) was that of patients with no anti-HLA antibodies either before or after transplantation, and the lowest (61%) was that of patients who developed antibodies only after transplantation. In fact, the survival rate of these patients

was lower than that of patients with antibodies both before and after transplantation (69%), probably because the pre-transplantation antibodies were not donor-specific.

2.7 Desensitization Strategies

The incidence of AMR in un-sensitized patients is less than 5% [38], but it can reach 40 to 90% in sensitized patients [39]. Once a cardiac transplant candidate has become sensitized, traditionally indicated by a PRA of 10% or higher, the time required to wait for a donor who is crossmatch-negative may be prohibitive. Treatment to reduce circulating antibodies before transplantation is called desensitization. The decision to proceed with desensitization therapy should be dependent on the percentage chance that any donor will be available for the sensitized patient. Desensitization therapy is based on several basic principles: 1) removal of circulating antibodies (plasmapheresis or immunoadsorption [IA]); 2) inhibition of residual antibodies (intravenous immunoglobulins [IVIg]); and 3) prevention of formation of new antibodies by suppressing B lymphocytes (rituximab) and plasma cells (bortezomib). Available protocols include different permutations of these principles and have variable success rates. Plasmapheresis means non-selective mechanical removal of proteins. Side effects of plasma-pheresis are necessity of substitution of fresh frozen plasma or albumin, volume contraction, bleeding diathesis, allergic reactions, and pathogen transmission. Plasmapheresis is recently replaced by selective IA because of its more specificity and efficacy as well as superior safety profile. Limitation for wide clinic use of IA is high cost. To reach sufficient decrease of PRA, the patient needs to undergo several cycles of plasmapheresis or IA. Plasma exchange modalities alone are not able to completely eliminate antibodies. Intravenous immunoglobulins are commercially prepared preparations from pooled plasma. Recent studies have suggested that IVIg is an effective modality to reduce allosensitization [40]. Postulated mechanisms include the presence of anti-idiotypic antibodies [41], antibodies against membrane-associated immunologic molecules such as CD4 or CD5, [42] or soluble forms of HLA molecules [43] that bind circulating anti-HLA antibodies. The optimal dose of IVIg for desensitization is debatable. The most commonly used dose of IVIg reported in literature ranges from 100 mg/kg to 20 g/ kg. Rituximab is a chimeric monoclonal antibody to CD20 that depletes B lymphocytes through CDC, antibody-

dependent cytotoxicity, and induction of apoptosis. These effects are associated with higher rate of infectious complications in some patients. Rituximab depletes only the naive B-cell pool but has no effect on antibody-producing plasma cells. It is believed that rituximab prevents *de novo* antibody production by inhibiting antigen presentation. Bortezomib is a proteasome inhibitor. *In vitro* bortezomib leads to apoptosis of alloantibody-producing plasma cells and may lead to modest reduction in anti-HLA antibodies in sensitized patients, but may be not sufficiently efficacious as single agent for desensitization. In conclusion, although several strategies for tackling allosensitization have been advocated, no clear consensus exists on the best modality, and only limited success has been achieved with various desensitization protocols. These techniques are neither universally successful nor standardized and expose the patient to invasive procedures and costly medications with significant risks and potential serious side effects. Given the inherent shortcomings of desensitization strategies, some centers have adopted an alternative approach of transplanting sensitized heart transplant candidates. Lick et al. [44] reported on a cohort of 8 patients with PRA > 70% who received non-cross-matched, ABO-compatible hearts using intraoperative, on-bypass, high-volume plasmapheresis and alemtuzumab induction. Alemtuzumab is a cytolitic antibody against CD52, a stable surface glycoprotein expressed on T and B lymphocytes, macrophages, and monocytes. The difference in survival between sensitized and non-sensitized groups at 1 year or at a mean follow-up of 2.3 and 2.4 years was not significant. There was a trend toward increased risk of cellular rejection per 100 patient-years beyond 1 year in the sensitized group. Risk of antibody-mediated rejection was significantly increased in the sensitized patients. They concluded that transplantation with plasmapheresis and alemtuzumab in sensitized heart transplant recipients does not compromise midterm survival. The expected higher rates of rejection, especially beyond the first postoperative year, demand adjustments in surveillance strategies and immunosuppressive management.

2.8 Virtual Crossmatch: A New Screening Tool

The common practice at many transplant centers is to require prospective (direct) crossmatch for all recipients with a PRA > 10%. Direct crossmatch necessitates transfer of donor

cells to the recipient institution, and the assay itself takes 4 hours to perform. These time constraints may severely limit the donor pool for sensitized patients (essentially limiting to "local" donors only) and thus increase waiting times and hence mortality. Implementation of sensitive and specific solid-phase antibody detection methods improved the ability to detect preformed antibodies and introduced the virtual crossmatch as a screening tool for sensitized patients. Positive virtual crossmatch is defined by the presence of recipient's preformed anti-HLA antibodies to the prospective donor HLA type. Donor HLA typing is routinely performed early in the procurement process because HLA matching is a part of the allocation criteria for some types of organs, namely kidneys. With this in mind, virtual crossmatch could allow a rational screening approach for sensitized patients without further delay in the allocation process. Zangwill et al. [45] have shown that the virtual crossmatch was 100% sensitive in detecting positive flow cytometric crossmatch results for T and B cells. Yanagida et al. [46] assessed the impact of virtual crossmatch on waiting times for heart transplantation. They concluded that in sensitized heart transplant candidates, virtual crossmatch shortens waiting time for heart transplantation without increasing subsequent occurrence of cellular rejection, antibody-mediated rejection, and mortality after heart transplantation.

2.9 Impact of Allosensitization on Survival

The true impact of LVAD sensitization on outcome after heart transplantation is controversial. Although the Registry of the International Society for Heart and Lung Transplantation (ISHLT) continues to identify mechanical support as a risk factor for decreased survival after transplantation, experienced centers report survival outcomes of patients with LVAD similar to those of non-bridged patients, despite the significantly higher immunologic risk caused by sensitization.. Regardless of the cause of allosensitization in LVAD-bridged patients, the clinically relevant question is whether VAD-related immune activation is associated with increased rejection rates and mortality after cardiac transplantation. Hong et al. [47] recently published a direct comparison of post-transplant survival of patients supported with continuous and pulsatile flow devices with patients bridged to transplantation with intravenous inotropes. Unadjusted post-transplant graft survival was similar in all groups. The authors also noted that outcomes among the continuous group improved significantly from the first to the second half of

the study period. John et al. [48] report the overall 30 day and 1 year post-transplant survivals in patients bridged with continuous flow devices of 97% and 87%, respectively. These survival rates are equivalent to that with conventional transplantation. They conclude that improved durability and reduced short- and long-term morbidity associated with axial flow LVADs have reduced the need for urgent cardiac transplantation, which may have adversely influenced survival in the pulsatile LVAD era. These findings are in keeping with the latest ISHLT transplant report. An analysis that focused on the most recent cohort of patients – those who received their allografts between July 2004 and June 2009 – showed that there was no longer a statistically significant difference in survival of patients bridged with pulsatile flow or continuous flow VADs compared with patients not requiring LVAD bridging [6]. A single center report and another report using the ISHLT registry have suggested that although VAD use does increase sensitization, these patients have comparable outcomes with non-bridged patients [49, 50]. A potential explanation for the lack of influence of sensitization on outcome in patients with LVAD is a temporal pattern of HLA sensitization during LVAD support [51]. This temporal pattern consists of a rapid PRA increase followed by rapid progressive decrease. This is also supported by findings of Nwakanma et al. [52] that poorer survival among patients with PRA greater than 25% is no longer significant when the "most recent PRA at transplant" is replaced with "peak PRA at transplant." This would suggest that the most recent PRA at transplant is a more important predictor of outcome, because some patients with high peak PRA at transplant may have their PRA level normalized or reduced at the time of heart transplantation. Massad et al. [53] found in a study group of 53 patients with LVAD that the overall mean PRA level before LVAD increased significantly from 2.1% to 33.5% during LVAD support ($p < 0.0001$) and decreased to 10.2% at transplantation ($p = 0.04$). Despite the higher rate of sensitization, patients bridged to transplantation with LVAD had similar post-transplantation hospital stay, operative mortality, and survival to those patients not requiring support. Pagani et al. [54] compared 32 patients with LVAD with 68 patients without mechanical support. The rejection rates were not significantly different between the LVAD and control groups. Cumulative post-transplantation survival at 1 and 2 years was also not significantly different for the two groups. Interestingly, the group of patients with a high rate of allograft rejection did not have a higher incidence of elevated PRA. In

2003, John et al. [50] retrospectively analysed 521 heart transplant recipients, of whom 105 were supported with LVAD before transplantation. Among LVAD recipients, 66% were sensitized before transplantation, in contrast, only 6% of non-bridged recipients were sensitized ($p < 0.001$). Despite higher rates of sensitization in the LVAD recipients compared with the non-LVAD patients, 5 year survival and development of allograft vasculopathy after transplantation were similar in the two groups. Pamboukian et al. [55] found that patients bridged with an LVAD did not have increased rejection episodes, allograft vasculopathy, or decreased survival after transplantation compared with non-bridged patients, despite higher rates of sensitization. Using the registry of the ISHLT, Joyce et al. [49] compared PRA levels in 7,686 heart transplant recipients to determine the impact of LVAD therapy on humoral sensitization, acute rejection, and mortality. Elevated PRA levels were found in 16.6% of LVAD recipients compared with 7.6% of non-LVAD control subjects ($p < 0.0001$). Despite these findings, LVAD use had no impact on rejection rates. Gonzalez-Stawinski et al. [56] studied the impact of preoperative LVAD support on the development of cardiac allograft vasculopathy. They found that patients with LVAD had a six fold greater chance of having PRA > 10% at the time of transplant ($p < 0.05$) compared with non-VAD patients. Normal coronary anatomy at 3 year follow-up was 80% in VAD group and 62% in non-VAD group. Modified immunosuppression, close surveillance, and a prospective crossmatch for all patients with a PRA ≥ 10% may contribute to better overall post-LVAD and post-transplant care. VAD-supported heart transplant candidates are more likely to receive desensitization therapies before transplantation, and allograft rejection episodes may be treated more aggressively. Post transplantation survival rate in patients supported with VAD is similar to that of non-supported patients, yet the causes of death are different. Up to 75% of post-transplantation mortality in VAD-supported heart transplant recipients is related to infectious complications, whereas rejection may account for 20%. Non-supported transplant recipients commonly die of rejection (38%), ischemic complications (31%), and respiratory failure (23%) [57].

Chapter 3

AIMS AND OBJECTIVES

3. AIMS AND OBJECTIVES

The aims of our project were threefold. First, we assessed the impact of antibodies on outcome of patients implanted with a durable long-term left ventricular assist device HeartMate II. Apart from longer waiting times and associated increased morbidity and mortality, there have been no reports linking anti-HLA antibodies in mechanically bridged recipients to post-LVAD adverse outcomes. While anti-HLA antibodies exert their negative effect via complement activation and antibody – mediated cytotoxicity, antibodies against AT1R, act as a natural allosteric receptor agonist. Given the known potential of these antibodies to activate inflammatory and coagulation cascade we hypothesized that mechanically bridged patients with raised levels of anti-AT1R antibodies may experience increased rate of thromboembolic and infectious complications while on support.

There is sufficient amount of evidence for association of pre-formed anti-HLA antibodies and post-transplant hyper-acute rejection, acute cellular and antibody mediated rejection as well as chronic allograft vasculopathy and organ loss in heart transplant recipients. Little is known about the impact of non-HLA antibodies on post-heart transplantation outcome. Antibodies targeting AT1R have been associated with malignant hypertension, autoimmune diseases and acute rejection and graft loss in kidney transplantation. The objective of the second part of our study was to compare the survival and freedom from acute cellular and antibody mediated rejection in heart transplant recipients bridged with HeartMate II assist device stratified according the pre-transplant presence of anti-AT1R antibodies.

In the third and final part of our analysis, our goal was to evaluate the relationship between pre-transplant alloimmunosensitization against both HLA antigens and AT1R and post-transplantation outcomes in recipients who were either bridged with the durable LVADs or transplanted without prior use of mechanical assist device.

Chapter 4

METHODS

4. METHODS

4.1 Patients

First, we prospectively evaluated the presence of anti-AT1R antibodies in 96 consecutive Heart Mate II recipients at our institution between 2008 and 2012. After excluding 13 patients who died within 60 days of implantation, 83 patients with a mean duration of 375 ± 34 days of support were left for the analysis. On-device survival and various adverse clinical events (device malfunction, major infection, major bleeding and neurologic dysfunction) during the support were compared between antibody positive and antibody negative recipients. Standard INTERMACS definitions were used to classify individual adverse events [58].

Out of a total of 83 patients, 69 eventually underwent heart transplantation, 9 died on support, three were explanted for recovery and two were still alive on support at the last day of follow-up. Sera of all 69 consecutive heart transplant recipients transplanted between October 2008 and August 2014 were tested for the presence of anti HLA Class 1 and Class 2 antibodies and Angiotensin II type 1 Receptor antibodies before Heart Mate II device implantation and at the time of transplantation. Overall survival and post-transplant rejection free survival were compared between antibody negative and antibody positive recipients.

For the third part of our study we compared the survival and rejection in all first-time heart transplant recipients transplanted at our institution between 2009 and 2010. Seventeen patients who were bridged with Heart Mate II device and survived the first year were compared to 60 non-bridged first-year survivors. The impact of the presence of anti HLA and anti-AT1R antibodies on the post-transplantation survival, rejection and immunosuppression related adverse events was compared between antibody negative and antibody positive recipients.

Hospital database and medical records were searched for clinical data on survival and incidence of acute cellular and antibody mediated rejection. Identification and classification of rejection episodes was based on histopathology and immunohistochemistry evaluation of endomyocardial biopsy specimens and followed the International Society for Heart and Lung Transplantation guidelines [59, 60]. Patients with ACR ≥ 2R and pAMR of any grade were

included in the time to event analyses. As per our institutional protocol all heart-transplant recipients received induction therapy with antithymocyte globulin (1.5 mg/kg body weight). Maintenance immunosuppression comprised a combination of calcineurin inhibitor with either cyclosporine (trough level 200 mg/dL) or tacrolimus (trough level 3 – 8 ng/d), antiproliferative agent (mycophenolate mofetil) and steroids (tapering regimen). Follow-up of all transplanted patients ended on 5 April 2015, was 100% complete.

4.2 Antibody Analysis

The presence of HLA-specific antibodies was assessed with CDC assay and a solid phase assay (SPA). Panel reactive antibodies (PRA) were expressed as a percentage of positive tests within a panel of lymphocytes from 30 healthy donors. The maximum PRA (peak PRA) and the last pre-transplant PRA were recorded. The specificity of HLA and MICA antibodies was defined by LABS screen Mixed and Single Antigen class I and class II beads (One Lambda Inc., Canoga Park, CA, USA). Mean fluorescence intensity of 1000 and 2000 units was adopted as a cut-off point for positivity. Sera from patients on LVAD were treated with AdsourbOut (OneLambda Inc.) before analysis due to non-specific binding on polystyrene beads.

The concentration of anti-AT1R IgG antibody in serum was measured by ELISA according to the manufacturer's instructions. The samples were assayed on Angiotensin II type 1-receptor-precoated microtiter plates. Standards and diluted 1:100 samples were added into the wells and incubated for two hours at 2 – 8 °C. After washing steps, anti-AT1R antibody was detected with POD-labelled anti-human IgG antibody (1:100) followed by colour development with TMB substrate solution and, measured at 450 nm, with correction wavelength set at 630 nm. Optical densities were then converted into concentration by standard curve. The detection range of the test was > 2, 5 U/ml with positive value set at 17 U/ml and negative ≤ 17 U/ml.

4.3 Statistical Analysis

Continuous variables are presented as median with 25th and 75th percentile interval. Categorical variables are shown as the percentage of the sample. The $\chi 2$-test and Fisher's exact test were used to evaluate the categorical characteristics. Continuous variables comparisons were performed using Mann-Whitney U test for two study groups and Kruskal Wallis one – way analysis of variance test for multiple group analysis. Survival and time-to-event analyses were assessed by Kaplan-Meier method and the log-rank test was used for comparisons. Heart Mate II recipients were censored for transplantation and LVAD explantation after recovery to calculate estimated on-device survival. For overall survival analysis, all patients were censored on the date of death or at conclusion of the study. Only patients surviving the first 60 days post Heart Mate II implantation were included in the on-device survival analysis. Date of Heart Mate II implantation was set as the time origin for survival and freedom from LVAD associated adverse event analyses and the date of transplantation as the time origin for freedom from rejection analysis. The linearized rate for each adverse event was calculated as total number of observed events divided by total patient-years of follow-up and expressed as episodes per one patient – year (eppy). A p value < 0.05 was considered significant. The statistical analyses were performed with IBM SPSS 18 (SPSS Inc., Chicago, Il, USA).

Chapter 5

RESULTS

5. RESULTS

5.1 The impact of Angiotensin II Type 1 Receptor antibodies on morbidity and mortality in Heart Mate II supported recipients

Anti-AT1R antibodies were observed in 13/83 (16%) of the recipients before Heart Mate II implantation (Table 1). Four of these patients (6%) were also sensitized against HLA antigens. During the support, 50 patients (71%) who were initially anti-AT1R negative became positive (AT1R-/+) and 20 (29%) remained negative (AT1R-). Total amount of Heart Mate II support for all 83 patients was 86.7 patient-years. There were no differences in the duration of support or the amount of the blood products used between LVAD recipients who remained negative and those who became positive. Basic demographic and clinical characteristics of both patients groups are summarized in Table 2. Out of 20 patients who remained negative on the mechanical device, 8 became sensitized to HLA antigens. In a cohort of 50 LVAD recipients who developed anti-AT1R antibodies during the support, 15 recipients also developed concurrent anti-HLA antibodies.

	AT1R positive (N = 13)	AT1R negative (N = 70)	*P* - value
Age, years	50 (40, 59)	45 (33, 58)	0.607
BMI	25.4 (22.9, 27.8)	22.6 (20.3, 25.9)	0.021
Male gender, %	11 (85)	60 (86)	0.918
Ischemic etiology of heart failure, %	3 (23)	24 (34)	0.766
HLA sensitized, %	0	4 (6)	0.477
Previous mechanical support, %	2 (14)	8 (11)	0.822
Previous sternotomy, %	2 (14)	15 (21)	0.660

Table 1. Basic characteristics of AT1R antibody negative versus positive HeartMate II recipients before implantation

	AT1R negative (N = 20)	AT1R positive (N = 50)	*P* - value
Age, years	47 (41, 57)	51 (36, 59)	0.969
BMI	26.5 (23.3, 28.8)	25.0 (22.0, 27.0)	0.326
HMII duration of support, days	324 (137, 470)	246 (129,416)	0.907
PRBC during implantation, units	9 (6, 18)	10 (8, 14)	0.608
FFP, units	26 (15, 32)	26 (22, 34)	0.856
Platelets, units	3 (2, 4)	4 (3, 6)	0.277
Ischemic etiology of heart failure, %	6 (30)	18 (36)	0.696
Previous mechanical support, %	1 (5)	6 (12)	0.730
Previous sternotomy, %	5 (25)	10 (20)	0.800
HLA sensitized, %	8 (40)	15 (30)	0.545
Male gender, %	18 (90)	42 (84)	0.713
Driveline infection, %	4 (20)	13 (26)	0.761

Table 2. Comparison of AT1R negative patients versus those who became AT1R positive during HeartMate II support

5.1.1 Survival

Out of 83 LVAD recipients who survived 60 days post–implantation, 9 additional patients died after a mean duration of support for 462 (minimum 82, maximum 1123) days. Two year estimated on – device survival was 78 ± 12% in AT1R-, 60 ± 23% in AT1R+ and 92 ± 6% in AT1R-/+ group (p = 0.409) (Figure11).

5.1.1 Major adverse events

Freedom from device malfunction, major infection, major bleeding and neurologic dysfunction at two years for AT1R-, AT1R+ and AT1R-/+ was 49 ± 14%, 53 ± 16% and 41 ± 11% (p = 0.875) (Figure 12).

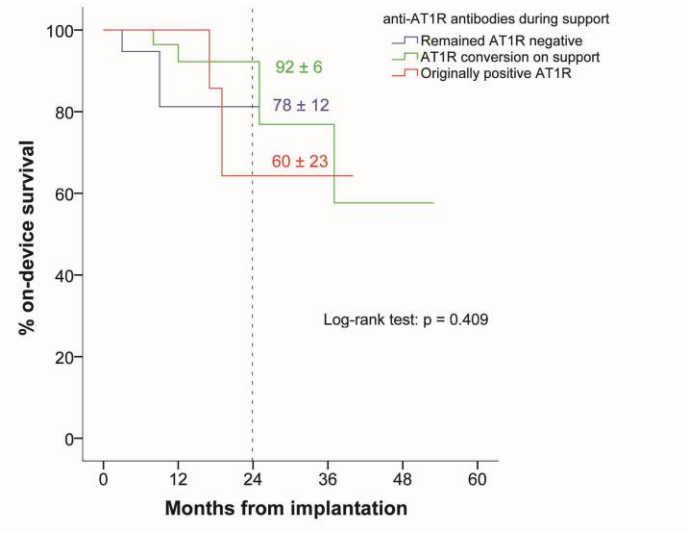

Figure 11. On-device survival of HeartMate II recipients stratified according the presence of anti-AT1R antibodies

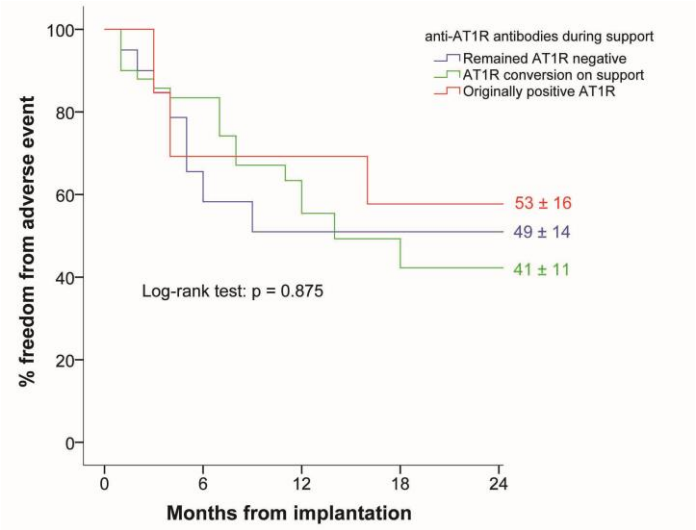

Figure 12. Freedom from HeartMate II related post-implantation adverse events (device malfunction, major bleeding, major infection and neurologic dysfunction)

5.1.2 Device malfunction

Altogether 5 patients (6%) experienced device malfunction in our cohort (0.06 eppy). All episodes were related to pump failure (pump thrombosis in four patients and kinked outflow graft in one patient) and resulted in pump exchange in two patients and death in two patients. One patient with pump thrombosis was successfully treated conservatively and subsequently transplanted. Freedom from device malfunction at 2 years in AT1R+, AT1R- and AT1R-/+ was 100%, 95 ± 5% and 86 ± 8% (p = 0.487).

5.1.3 Major bleeding

Our institutional protocol for patients supported with HeartMate II device is anticoagulation with Warfarin (target INR of 1.8 – 2.2) without antiplatelet therapy. Out of 83, three patients (4%) experienced major bleeding episode after 7 days post implantation (0.03 eppy). The reasons for readmissions for bleeding were epistaxis, retroperitoneal bleeding and GI bleeding. All patients were discharged home following their bleeding episode and all three eventually underwent heart transplantation. Freedom from major bleeding at 2 years in AT1R+, AT1R- and AT1R-/+ was 100%, 100% and 90 ± 5% (p = 0.232).

5.1.4 Major infection

More than one third (27 patients, 33%) of our patients were readmitted due to infection during the course of their mechanical support (0.3). These patients fell into two major categories: infection of a drive - line site (21 patients) and deep sternal wound infection (6 patients). Two patients experienced both drive – line and deep sternal wound infections. One patient with deep sternal wound infection developed sepsis, multi – organ failure and subsequently died as a direct consequence of LVAD infection. Freedom from major infection at 2 years in AT1R+, AT1R- and AT1R-/+ was 54 ± 16%, 62 ± 13% and 51 ± 11% (p = 0.594).

5.1.5 Neurological dysfunction

Altogether six (7%) patients experienced neurological dysfunction. Four patients suffered from haemorrhagic CVA (0.05 eppy) and two from ischemic CVA (0.02 eppy). Two of the patients recovered and were subsequently transplanted, four died as a result of CVA. Freedom from neurologic dysfunction at 2 years in AT1R+, AT1R- and AT1R-/+ was 87 ± 12%, 93 ± 7% and 92 ± 6% (p = 0.997).

5.2 The Impact of Angiotensin II Type 1 Receptor Antibodies on Post – Heart Transplantation Outcome in Heart Mate II Bridged Recipients

Altogether 69 patients were transplanted from the Heart Mate II device at our institution during the study period. The mean time of mechanical support before heart transplantation was 11 months (range 1-53). Anti-AT1R antibodies were present in 8 (11.6%) and anti-HLA antibodies in three (4.3%) patients before Heart Mate II implantation. During the support 44 patients (63.8%) who were initially anti – AT1R negative became positive and 17 (24.6%) remained anti-AT1R antibody negative until transplantation. Out of 67 patients who were not sensitized against HLA antigens before HM II implantation, 6 (9%) developed anti-HLA antibodies during the support. At the time of transplantation there were 13 patients who were antibody negative for both HLA and AT1R antigens (AT1R-HLA-), three patients who were anti-AT1R antibody negative and anti-HLA antibody positive (AT1R-HLA+), 47 patients who were anti-AT1R antibody positive and anti-HLA antibody negative (AT1R+HLA-) and four patients who were sensitized against both AT1R and HLA antigens (AT1R+HLA+). Basic demographic and clinical characteristics of patients stratified according to presence of anti-AT1R antibodies are presented in Table 3.

	AT1R positive before HMII implantation (N = 8)	AT1R positive during HMII support (N = 44)	AT1R negative (N = 17)	*P* - value
Age, years	49 (41,58)	51 (39, 59)	48 (41, 58)	0.875

BSA, m^2	1.81 (1.66, 2.03)	1.97 (1.81, 2.10)	2.01 (1.91, 2.13)	0.118
BMI	21 (20, 25)	25 (23, 27)	27 (24, 29)	0.054
Female gender, %	1 (12.5)	7 (15.9)	1 (5.9)	0.580
Diabetes, %	1 (12.5)	8 (18.2)	3 (17.6)	0.926
COPD, %	1 (12.5)	7 (15.9)	2 (11.8)	0.905
Previous stroke, %	2 (25)	10 (22.7)	2 (11.8)	0.596
INTERMACS I,II, %	2 (25)	27 (61.4)	10 (58.9)	0.158
Ischemic etiology of HF, %	2 (25)	16 (36.4)	5 (29.4)	0.902
HLA sensitized, %	0	3 (8.6)	0	0.354
Previous sternotomy, %	1 (12.5)	8 (18.2)	4 (23.5)	0.792
Previous VA ECMO	0	4 (9.1)	0	0.299
After HMII implantation				
Concomitant procedure, %		14 (31.8)	2 (11.8)	0.110
PRBC, units		10 (7, 14)	9 (7, 17)	0.863
Platelets, units		4 (3, 6)	3 (2, 5)	0.159
FFP, units		24 (18, 35)	26 (16, 31)	0.700
Major bleeding, %		6 (13.6)	0	0.173
Major infection, %		11 (25)	5 (29.4)	0.725
Neurological dysfunction, %		1 (2.3)	0	0.531
Device malfunction, %		1 (2.3)	1 (5.9)	0.478
ARBs during support, %		8 (18.2)	1 (5.9)	0.206
HLA sensitized during support, %		13 (30.2)	7 (41.2)	0.418
Mean BP on support, mmHg		85 (80, 90)	90 (81, 99)	0.048
Duration of support, months		9 (5, 16)	11 (5, 16)	0.705

Table 3. Basic demographic and clinical characteristics of patients stratified according to the presence of anti-AT1R antibodies before Heart Mate II implantation and throughout the support

5.2.1 Survival

Out of 69 transplanted patients 8 did not survive until discharge. Primary graft dysfunction was the leading cause of death, followed by sepsis and neurological complications (Table 4). Four additional patients died after being discharged from the hospital during the follow-up period.

Patient	Survival in days	Anti-AT1R antibody at transplantation	Cause of death
Patient 1	1	negative	PGD
Patient 2	58	positive	Sepsis
Patient 3	19	positive	Ischemic stroke, Sepsis, MOF
Patient 4	26	positive	PGD, ACR
Patient 5	6	positive	PGD, Small bowel ischemia
Patient 6	1	positive	PGD
Patient 7	67	positive	Sepsis
Patient 8	16	negative	PGD
Patient 9	672	negative	CAV
Patient 10	830	negative	Unknown
Patient 11	1417	positive	Ischemic stroke
Patient 12	176	positive	Unknown

Table 4. Survival in days and causes of death of individual patients

Survival analysis of recipients stratified according to the presence of anti-AT1R antibodies before transplantation revealed one and five year survival of 88 ± 8% and 76 ± 10% for anti-AT1R antibody negative and 87 ± 5% and 81 ± 7% for anti-AT1R antibody positive patients ($p = 0.582$) (Figure 13).

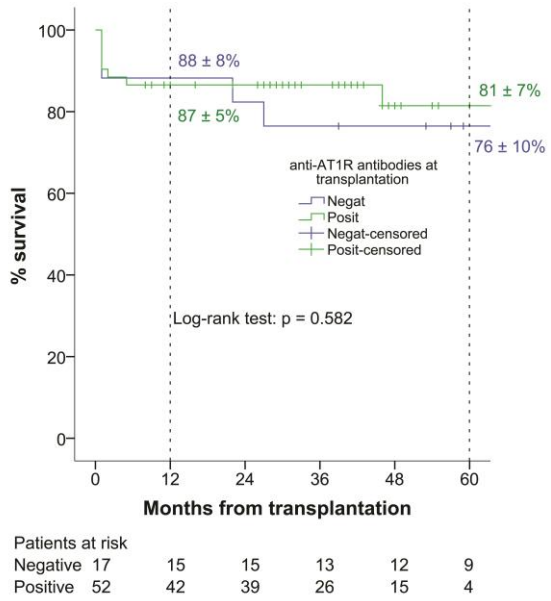

Figure 12. Overall post-heart transplantation survival stratified according the presence of anti-AT1R antibodies before transplantation

5.2.2 Acute Cellular Rejection

Out of 67 heart-transplant recipients who had biopsy results available, 14 (20.9%) were diagnosed with acute cellular rejection with ISHLT Grade ≥ 2R (12 patients 2R and two patients 3R). Patient stratification according to the pre-transplant presence of antibodies against AT1R and HLA antigens with respect to subsequent post-transplant ACR is depicted in Table 4.

ACR ISHLT Grade	AT1R-HLA- (N = 13)	AT1R-HLA+ (N = 3)	AT1R+HLA- (N = 47)	AT1R+HLA+ (N = 4)
0 (N = 31)	7 (53.8%)	3 (100%)	19 (40.4%)	2 (50%)
1R (N = 22)	3 (23.1%)	0	18 (38.3%)	1 (25%)
2R (N = 12)	3 (23.1%)	0	9 (19.1%)	0
3R (N = 2)	0	0	1 (2.1%)	1 (25%)

Table 4. Acute cellular rejection

Both recipients with grade 3R rejection presented with an associated graft dysfunction. The first patient was successfully treated with 1g of intravenous solumedrol administered daily for three days. The second patient required veno-arterial extracorporeal membrane oxygenation (VA ECMO) implanted centrally for severe bi-ventricular graft dysfunction on top of pulse steroid therapy. After 12 days of support the graft function recovered and ECMO was successfully explanted. The median time to ACR episode was 147 days (43, 606) in anti-AT1R antibody negative and 46 days (17, 264) in anti-AT1R antibody positive recipients (p = 0.306). Freedom from ACR at one year was 68 ± 12% for anti-AT1R negative and 75 ± 6% for anti-AT1R positive recipients(p=0.218)(Figure14).

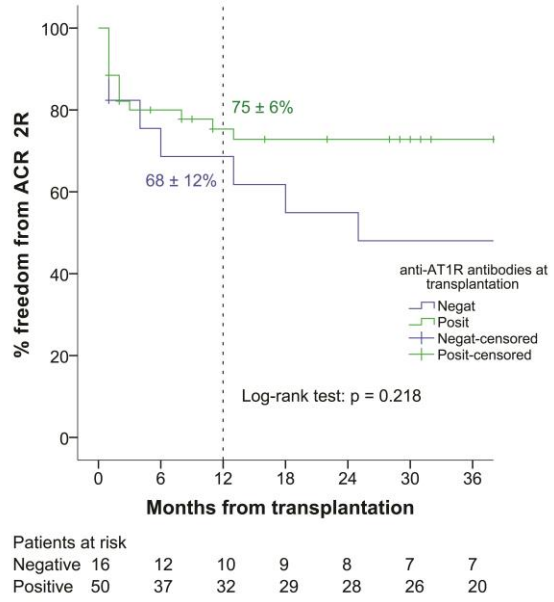

Figure 13. Freedom from acute cellular rejection ISHLT grade ≥ 2R

5.2.3 Antibody Mediated Rejection

Four patients' endomyocardial biopsy specimen yielded histology and/or immunohistochemistry signs of antibody mediated rejection (Table 5).

pAMR ISHLT Grade	AT1R-HLA- (N = 13)	AT1R-HLA+ (N = 3)	AT1R+HLA- (N = 47)	AT1R+HLA+ (N = 4)
0 (n = 63)	13 (100%)	2 (66.7%)	45 (95.7%)	3 (75%)
1i (n = 1)	0	0	1 (2.1%)	0
1h (n = 0)	0	0	0	0
2 (n = 2)	0	0	1 (2.1%)	1 (25%)
3 (n = 1)	0	1 (33.3%)	0	0

Table 5. Pathology antibody mediated rejection

Only patient with Grade 3 pAMR was positive for donor specific antibodies against human leukocyte antigen (HLA) and had concomitant graft dysfunction. Acute rejection was treated with a pulse of steroid that consisted of 1 g of intravenous solumedrol administered for three consecutive days, 10 cycles of therapeutic plasma exchange and intravenous immunoglobulins at 100 mg/kg. After multimodality treatment this patient is now symptom free, showing no signs of rejection in the latest endomyocardial biopsies and the graft function assessed with transthoracic echocardiography is satisfactory. None of the anti-AT1R negative patients presented with pAMR at one year post- transplantation, whereas freedom from pAMR in anti-AT1R positive recipients was 98 ± 2% (p = 0.198) (Figure 15).

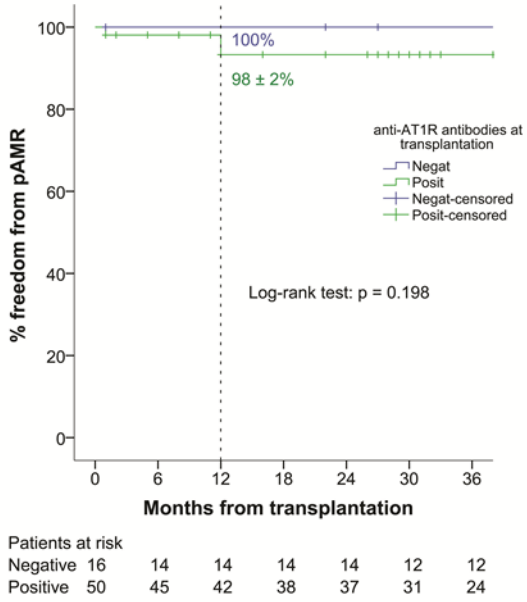

Figure 14. Freedom from pathology antibody mediated rejection of any ISHLT grade

5.3 The impact of anti-HLA and anti-AT1R antibodies on post transplantation outcome in patients stratified by bridging with HeartMate II device

Between 2009 and 2010 altogether 18 patients bridged with HeartMate II device and 68 patients without previous mechanical support underwent first-time orthotopic heart transplantation. One patient from the mechanical support group and 8 patients from the non-supported group died within the first post-transplant year leaving 17 and 60 heart transplant recipients for the final analysis. Median duration of HeartMate II supported patients was 292 days (minimum 59, maximum 736). Apart from the younger age of patients who were transplanted from the HeartMate II device there were no major differences in the baseline demographic and clinical donor and recipient characteristics (Table 6).

Baseline Characteristics	no support (N = 60)	HeartMate II (N = 17)	*P* - value
Recipient			
Age, years	56 (49, 60)	47 (42, 59)	0.042

Female gender	8 (13%)	2 (12%)	0.865
BSA, m^{-2}	1.93 (1.81, 2.09)	1.99 (1.89, 2.11)	0.248
Ischemic Etiology	23 (38%)	8 (47%)	0.304
Previous sternotomy	12 (20%)	17 (100%)	< 0.01
Creatinine, µmol/L	108 (89, 137)	80 (69, 110)	0.05
Bilirubin, mmol/L	15 (11, 27)	13 (10, 26)	0.488
Donor			
Age, years	39 (28, 48)	38 (30, 48)	0.883
Cold ischemia time, min	133 (91, 179)	130 (80, 200)	0.868
Mismatches			
CMV	20 (33%)	5 (29%)	0.761
Gender	14 (23%)	1 (6%)	0.168

Table 6. Basic clinical and demographic donor and recipient characteristics

Although there were no differences in the duration of cardiopulmonary bypass time between the groups (135 minutes for HeartMate II versus 143 minutes for patients without prior support, p = 0.475), the use of blood products (packed red blood cells, fresh frozen plasma and platelets) was significantly higher in patients transplanted from HeartMate II device (Table 7).

	no support (N = 60)	HeartMate II (N = 17)	*P* - value
pRBC, units	4 (2, 9)	8 (6, 12)	0.018
FFP, units	10 (5, 15)	18 (14, 23)	< 0.001
Platelets	2 (1, 3)	3 (2, 5)	< 0.001

Table 7. Use of blood products

Out of 17 patients transplanted from HeartMate II device, 6 (35%) had anti-HLA class I, two (12%) had anti-HLA class II and two (12%) had MICA antibodies before transplantation.

Four out of 6 with anti-HLA class I and all two patients with anti-HLA class II antibodies became sensitized during mechanical support. (Table 8). All but one patient with pre-formed anti-AT1R antibodies from the HeartMate II bridged cohort also became sensitized while on support. When compared to their non-bridged counterparts, recipients transplanted from the device were significantly more sensitized against HLA class I antigens and AT1R (Table 8).

Type of antibody	no HeartMate II (N = 60)	HeartMate II (N = 17)	*P* - value
HLA Class I	3 (5%)	6 (35%)	0.004
HLA Class II	1 (2%)	2 (12%)	0.330
HLA Class I + II	1 (2%)	2 (12%)	0.378
MICA	4 (7%)	2 (12%)	0.619
AT1R	15 (25%)	13 (76%)	0.009

Table 8. Types of antibodies in heart transplant candidates stratified by the pre-transplant presence of mechanical device

5.3.1 Survival

Overall one patient from the HeartMate II bridged and 8 patients from the non-supported group died in the late post-transplant period (median 36 months). The post-transplant survival of patients bridged with HeartMate II device at 1, 3 and 5 years was 100%, 94 ± 6% and 94 ± 6% (Figure 16). This was not significantly different from the survival of non-supported heart transplant recipients with 100%, 95 ± 3% and 81 ± 7% (p = 0.398).

There was no difference in survival of patients with pre-transplant anti-HLA class I and class II antibodies in comparison to non-sensitized recipients at 1, 3 and 5 years post-transplantation (100%, 91 ± 9% and 91 ± 9% for sensitized versus 100%, 95 ± 3% and 83 ± 6% for non-sensitized, p = 0.739) (Figure 17).

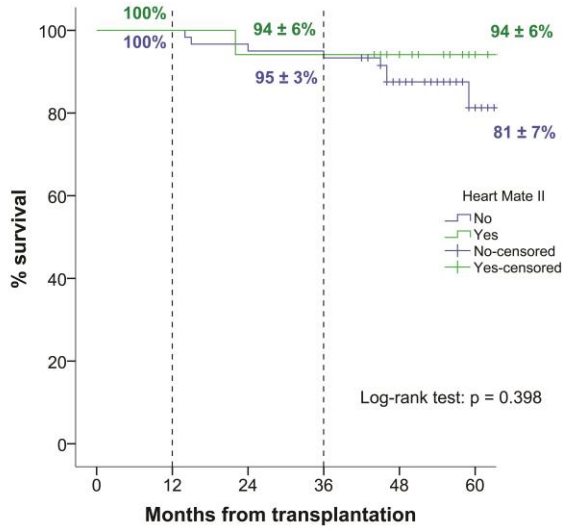

Figure 16. Overall post-transplant survival of bridged versus non-bridged recipients conditional on one year survival

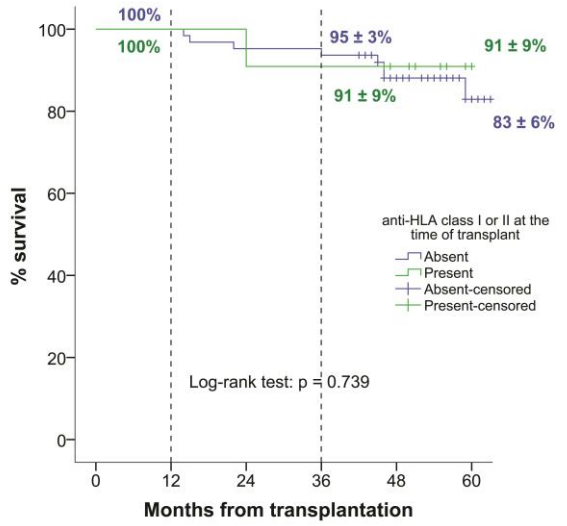

Figure 17. Survival of HLA sensitized versus non-sensitized recipients conditional on one year survival

Patients who had antibodies against AT1R before transplantation had survival of 100%, 96 ± 4% and 92 ± 5% at 1, 3 and 5 years. Anti-AT1R negative recipients' survival was 100%, 97 ± 3% and 78 ± 11% (p = 0.489) (Figure 18).

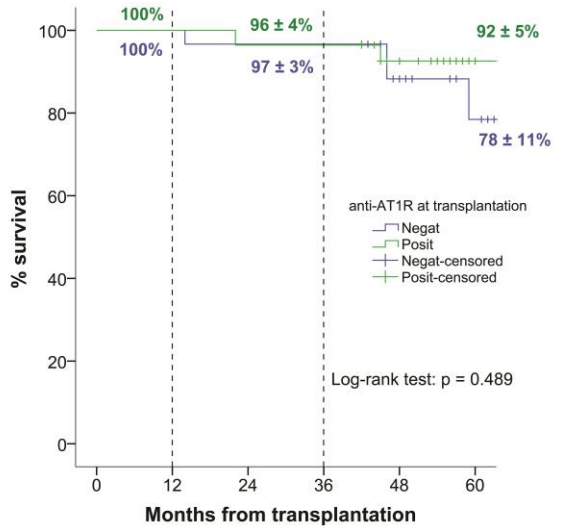

Figure 18. Survival of anti-AT1R antibody positive versus negative heart transplant recipients conditional on one year survival

5.3.2 Immunosuppression related adverse events

Both HeartMate II bridged and non-bridged recipients experienced the same rate of immunosuppression associated adverse events (opportunistic infection, cytomegalovirus disease and post-transplant lymphoproliferative disorder) (Table 9).

Adverse Event	no HeartMate II (N = 60)	HeartMate II (N = 17)	*P* - value
Opportunistic infection	7 (11.7%)	1 (5.9%)	0.676
CMV disease	2 (3.3%)	1 (5.9%)	1
PTLD	1 (1.7%)	0	0.532

Table 8. Types of antibodies in heart transplant candidates stratified by the pre-transplant presence of mechanical device

5.3.3 Acute cellular rejection

Freedom from ACR ISHLT Grade ≥ 2R at one year was 88 ± 8% in HeartMate II and 73 ± 6% in non-bridged recipients (p=0.113). There were no differences in the freedom from ACR between patients with and without pre-transplant non-cytotoxic HLA antibodies at one year (71 ±

17% versus 79 ± 5%, p=0.911) (Figure 19).Freedom from ACR ≥ 2R at one year for anti-AT1R antibody positive patients was 75 ± 8%, whereas for anti-AT1R negative recipients it was 80 ± 7% (p = 0.442) (Figure 20).

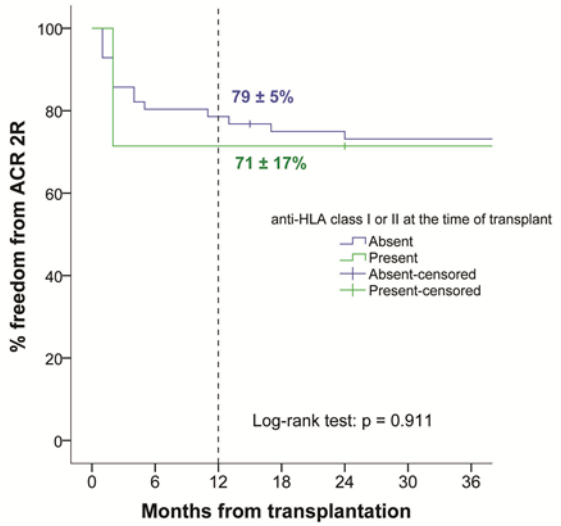

Figure 19. Freedom from ACR ISHLT grade ≥ 2R

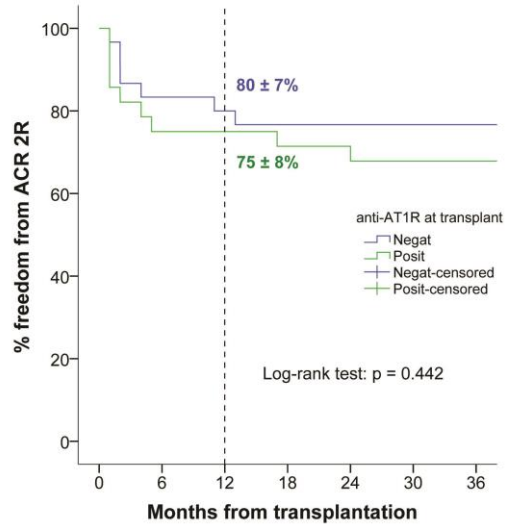

Figure 20. Freedom from ACR ISHLT ≥ 2R in anti-AT1R positive versus negative recipients

5.3.4 Antibody mediated rejection

Freedom from pAMR ISHLT Grade 1 - 3 was 94 ± 6% in HeartMate II and 95 ± 3% in non-bridged patients (p=0.665). Patients with preformed anti-HLA antibodies experienced significantly less freedom from pAMR than non-sensitized recipients (71 ± 17% for antibody

positive versus 96 ± 2% for antibody negative, p = 0.047) (Figure 21). Freedom from pAMR at one year post-transplant was 96 ± 4% in anti-AT1R antibody and 93 ± 5% in anti-AT1R antibody positive patients (p = 0.460) (Figure 22).

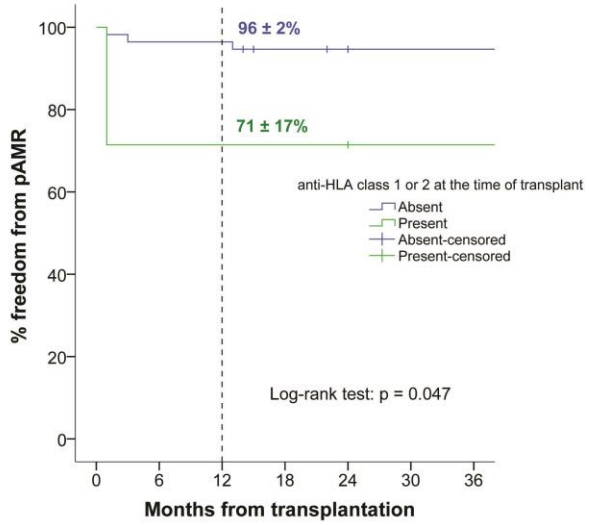

Figure 21. Freedom from pAMR in anti-HLA antibody positive versus negative recipients

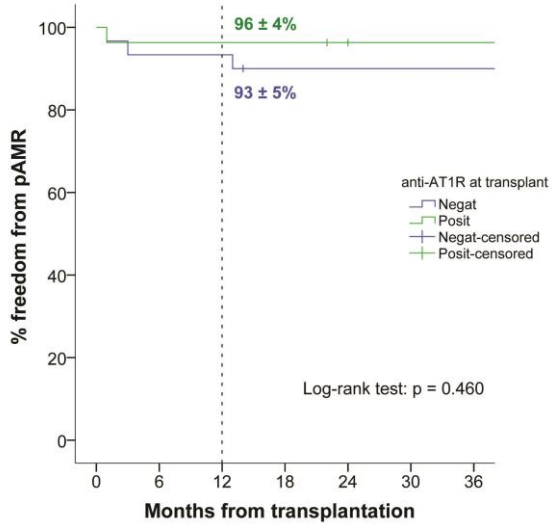

Figure 22. Freedom from pAMR in anti-AT1R antibody positive versus negative recipients

Chapter 6

DISCUSSION

6. DISCUSSION

The question of whether antibodies only mark or also mediate immunity remains a challenging one in medicine today. Antibodies against components of nuclei, insulin, and other components of beta cells and even against the surfaces of extra-vascular cells are commonly observed and taken as evidence of autoimmunity. Yet, many people who have autoantibodies do not manifest autoimmune disease and when disease is present the role of autoantibodies can be difficult to determine.

Whether or not antibodies in the circulation of graft recipients damage transplants, they do predict outcome of transplantation

The proportion of heart transplant candidates who are sensitized to HLA with a PRA > 10% is steadily increasing and has reached a 12% mark in 2011. This trend reflects the increased use of mechanical assist devices in bridging patients to transplantation as left ventricular assist devices are a recognized risk factor for sensitization [22, 23, 25]. LVAD supported patients now constitute a substantial proportion of the heart transplant recipients. Our results showed that approximately 9% of patients were sensitized against HLA antigens and another 16% were sensitized against AT1R even before LVAD implantation. Anti-HLA and anti-AT1R antibodies develop before LVAD implantation through similar pathways: transfusions, pregnancies and prior transplant. Du et al [61] observed in their previous report an increased titer of anti-AT1R antibodies in the sera of congestive heart failure patients with ischemic cardiomyopathy and hypertension. The authors suggested that these antibodies may play an important role in the pathogenesis and myocardial remodelling of heart failure. We did not find any association between basic demographic and clinical characteristics (female gender/ previous pregnancy, history of surgery) and sensitization against AT1R before LVAD implantation.

The exact mechanism of antibody production in mechanically bridged heart transplant candidates is not known. Avoiding leukofiltered red blood cell transfusions in perioperative period does not prevent alloimmunization in LVAD recipients. Plasma may contain sufficient amount of soluble HLA antigens to cause sensitization. There is evidence that platelet

transfusion may be associated with the development of IgG HLA class I antigens but in general there is insufficient evidence to prove causation of blood product use and increased rate of sensitization in LVAD recipients. Studies comparing the rate of sensitization in pulsatile and continuous flow LVADs are of historical value only. By the first half of 2011, more than 99% of LVAD implants were continuous flow devices [58]. In our series we observed that around 24% of previously anti-HLA negative patients became positive during the support.

There is accumulating evidence that LVAD support may be associated not only with an increased anti-HLA but also various anti non-HLA antibodies. Hiemann et al. [62] reported in their pilot study that patients on assist device support before heart transplantation were more likely to develop high anti – AT1R antibody levels (43% of supported versus 18% of non – supported patients, p = 0.021) within 24 hours after heart transplantation, implicating pre – transplant sensitization. Barten el al. [10] found in their study of 29 VAD recipients that 65.5% were positive for anti-AT1R antibodies. Our results confirmed these findings. During the support 71% of the initially negative AT1R patients became positive. There are multiple pathways by which the production of antibodies against AT1R in patients supported with mechanical devices may be initiated. Protein antigenic determinants from targets may become accessible after injury or surgical stress. Inflammatory events might lead to de novo expression of auto-antigens [35]. These autoantibodies are generally of the IgG class requiring T cell help [63]. T cell self-tolerance may be broken by an inflammatory event or hypoxia. We observed no association between pre-operative demographics, blood product peri-operative use or duration of mechanical support and conversion of AT1R negative to AT1R positive status.

6.1 Impact of antibodies on LVAD associated complications

Apart from longer waiting times and associated increased morbidity and mortality, there have been no reports linking anti HLA or anti non-HLA antibodies in mechanically bridged recipients to post-LVAD adverse outcomes. Our theory that anti-AT1R antibodies with their pro-inflammatory and pro-coagulation properties and their ability to cause endothelial dysfunction may lead to an increased rate of thromboembolic and infectious complications in LVAD recipients

was not borne out in our results. There was no difference in the overall survival among patients who were anti-AT1R antibody negative before Heart Mate II implantation and patients who either became positive or remained negative during the support. The incidence of device malfunction, bleeding, infection and neurological dysfunction was not influenced by the presence of anti-AT1R antibodies. There are several possible explanations for the lack of negative impact of AT1R activating antibodies on survival and adverse LVAD related complications in our cohort. Biological impetus regulating At1R antibody injury is fairly complex. Level of AT1R and induction of specific conformations is dependent on individual genetic polymorphisms and the state of local tissue expression influenced by various stressors. AT1R gene has 14 described polymorphisms, and some of them act as gain or loss of function mutations implicated in receptor activation [64]. The most extensively studied A1166C polymorphism is associated with increased responsiveness to Angiotensin II and various cardiovascular and renal pathologies [65]. It is plausible that mechanical circulatory support with the continuous flow creates a unique microenvironment resulting in lower AT1R expression, potentially less susceptible to anti-AT1R antibody mediated actions. There is compelling evidence that the AT1R may also be activated by mechanical stress without the involvement of Angiotensin II [66]. The AT1R is the first recognized mechano-sensitive GPCR [67]. It is plausible that in the situation when the heart is fully unloaded with mechanical assist device AT1R would be down regulated. There may also be other factors that influence the features of anti-AT1R antibodies, changing their agonistic affinity. The tissue damage caused by certain mechanisms prior to anti-AT1R binding may affect the level of AT1R expression, resulting in different degree of anti-AT1R binding efficiency. Several modifiers have been identified thus far: ischemia, inflammatory events, and microbiome. [68, 69].

6.2 Impact of antibodies on post-transplantation outcome

Our data showed no impact of pre-transplant sensitization against HLA antigens on post-transplant survival. These results are in line with previous reports [53-55]. Although several studies evaluated pre-transplant HLA antibodies as detected by SPA in heart transplantation, there is still conflicting evidence regarding their clinical consequences [70-72]. While there was

also no statistically significant difference in the freedom from ACR between anti-HLA positive and negative heart transplant survivors we found that patients with preformed HLA antibodies experienced far less freedom from pAMR than non-sensitized recipients.

Although there is a substantial amount of literature on deleterious effects of anti-AT1R antibodies on post-renal transplantation outcomes, we were only able to find one manuscript in reference to heart-transplantation. Whereas we studied the effect of anti-AT1R antibodies as detected before transplantation, Hiemann et al. [62] evaluated the impact of anti-AT1R antibodies detected immediately post transplantation and during one year of follow-up. The relevant clinical end-points included acute cellular rejection of any grade, antibody mediated rejection and microvasculopathy. Evaluating the results of 30 heart transplant recipients, the authors concluded that elevated post-transplantation levels of anti-AT1R antibodies (cut-off > 16.5 U/ml) are associated with cellular and antibody mediated rejection and early onset of microvasculopathy and should be routinely monitored after heart transplantation. Apart from the difference in the time frame of anti-AT1R antibody evaluation, all our patients were bridged to transplantation with an LVAD and 75% were antibody positive before transplantation. Also, ISHLT standardization of nomenclature of pathologic antibody mediated rejection [59] was only published one year after the study. We believe there are fundamental differences about how the clinical end points were defined and the results of those two studies are therefore difficult to compare. We nevertheless find the concept of increasing titres of anti-AT1R antibodies after transplantation very intriguing and plan to expand on the results of our study by evaluating the post-transplantation sera of all our patients. Another noteworthy aspect of the study by Hiemann et al. [62] is the suggestion of a potential association between anti-AT1R antibodies and post-transplant microvasculopathy. There is also increasing evidence for the active role of angiotensin II type 1 receptor (AT1R) itself in the pathogenesis of chronic allograft rejection explaining the link between acute rejection and subsequent long-term clinical outcome [73]. Yamani et al. [74] observed an increase in mRNA of AT1R in 14 heart transplant recipients who had recurrent acute cellular rejection in comparison to controls. In our study cohort we only had the results of 41 coronary angiograms available and for that reason we did not include cardiac allograft

vasculopathy among the outcome measures in our study. We nevertheless acknowledge the compelling evidence for the immunoregulatory function of the renin-angiotensin system and its role in the pathogenesis of chronic allograft rejection. Comparing the incidence of cardiac allograft vasculopathy between groups of patients stratified by the presence of anti-AT1R antibodies and increased expression of AT1 receptor is a challenge for future studies.

Although anti-AT1R antibodies may belong to complement fixing IgG subclasses (IgG1 and IgG3 isotypes), C4d positive staining was found not to be very frequent in biopsies of renal transplant recipients with anti-AT1R antibody mediated rejections [15, 75] implicating complement independent mechanism of injury. This would explain the lack of association between anti-AT1R antibody status and pAMR in our series. Our results also showed no statistically significant difference in the freedom from acute cellular rejection ≥ 2R between anti-AT1R antibody negative and positive recipients. Given the putative mechanism of action of these antibodies which primarily act on vascular endothelium causing non-specific, non-complement mediated microvascular damage these results are not surprising. When we stratified the patients not only by the presence of anti-AT1R antibodies but also by the anti-ALA antibodies status our results showed that none of the transplant recipients who were both anti-AT1R and anti-HLA antibody negative experienced pAMR or grade 3R ACR. Conversely, 25% of recipients who were sensitized against both AT1R and HLA antigens presented post-transplantation with high grade ACR with associated graft dysfunction and another 25% with pAMR similarly with graft dysfunction. This leads us to believe that knowing the anti-AT1R antibody status on top of standard evaluation of anti-HLA antibodies pre-transplantation adds an incremental value in a risk stratification of post-heart transplantation immunologic related adverse events.

Chapter 7

LIMITATIONS

7. LIMITATIONS

The study has several limitations inherent to the retrospective nature of a single center observational study. Another limitation is a relatively small number of patients with relatively low event rates increasing the probability of Type II error. Another drawback of our study is the fact that all our patients received Heart Mate II device thus limiting the generalization of our results to other types of mechanical devices. Future studies will need to address the question of whether newer generation of devices would show the same high degree of sensitization against HLA and AT1R and asses the role of these antibodies in post-transplantation outcome of mechanically bridged recipients.

Chapter 8

SUMMARY

8. SUMMARY

1. The primary finding of our study is that patients who received a long term LVAD developed a high degree on sensitization against both HLA and AT1R antigens after implantation.

2. Our data showed no impact of anti-HLA and anti-AT1R antibodies in Heart Mate II recipients on the overall survival and incidence of LVAD related complications.

3. We found no association between the presence of preformed anti-HLA and anti-AT1R in the pre-transplant sera and acute cellular rejection in the first post-transplant year.

4. Patients with anti-HLA antibodies experienced less freedom from pAMR than patients without preformed antibodies.

Chapter 9

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9. REFERENCES

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Chapter 10

AUTHOR'S LIST OF PUBLICATIONS

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10.1 Related to the topic with IF

1. Urban M, Pirk J, Dorazilova Z, Netuka I. *How does successful bridging with ventricular assist device affect cardiac transplantation outcome?* Interact Cardiovasc Thorac Surg. 2011;13(4):405-9.
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