

Diploma thesis

**Diagnosis of Cardiac Allograft Rejection by Quantitative
Short Tandem Repeat Analysis of Recipient-derived Cells**

submitted by

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Preface

Heart transplantation is considered to be among the most important breakthroughs in 20th century medicine. After introduction of this therapeutic intervention, patient survival was poor, but the groundwork for further developments and research had been laid. Over the past few decades, technological progress, a better understanding of transplantation immunology and the resulting introduction of new immunosuppressive substances have increased graft survival and patient comfort enormously. Today, heart transplantation is still a valuable treatment option for patients suffering from end-stage heart failure, providing these patients with a literal second chance and the possibility to return to a relatively high quality of life almost equal to that of a healthy person. While the pharmaceutical and device-based therapy of chronic heart failure has made great strides in the past decades, diseases leading to acute heart failure have become the predominating indication for heart transplantation.

However, even though much progress has been made in immunosuppressive therapy, acute allograft rejection still remains a relevant reason for graft failure in the first year after transplantation. The prevention and timely detection of such rejection episodes is one of the cornerstones for the improvement of long-term graft survival, as the summative effect of acute rejection episodes is a major contributing factor for changes associated with chronic rejection, most prominently cardiac allograft vasculopathy.

Unfortunately, the current gold standard for the diagnosis of cardiac allograft rejection has some limitations; several studies suggest only moderate reliability. Incorrect assignment of rejection grades has several negative consequences, such as—most importantly—the generally unfavorable outcome for the affected patient and also the impairment of clinical study comparability.

The establishment of a method providing higher reproducibility and reliability was attempted many times. However, none of these methods are currently used on a regular basis fit to rival histological grading. This study demonstrates the use of a genetic method for the diagnosis of cardiac allograft rejection that leads to objective, observer-independent results and may thus complement the current standard.

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Abstract

Background: The current standard for the diagnosis of cardiac allograft rejection is the histopathological assessment of right-ventricular endomyocardial biopsies (EMB). However, several studies have demonstrated high inter- and intraobserver variability in the histopathological grading, leading to erroneous modifications in immunosuppressive medication and poor comparability of multicenter clinical studies. Quantitative short tandem repeat (STR) analysis has previously been used for the evaluation of donor-cell engraftment after allogeneic hematopoietic stem cell transplantation and for the detection of chimerism after solid organ transplantation. This thesis evaluates the use of the method for diagnosis of cardiac allograft rejection.

Methods: Genomic DNA was extracted from sixty-six right-ventricular endomyocardial biopsies obtained from nine cardiac allograft recipients. Recipient-derived cell content was determined through amplification of nine polymorphic STR loci by multiplex polymerase chain reaction (PCR) and subsequent capillary electrophoresis. Results of quantitative short tandem repeat analyses were set in correlation with the previous histopathological grading according to both the 1990 and 2004 International Society for Heart and Lung Transplantation (ISHLT) grading systems.

Results: The mean percentage of recipient-derived cell content was 15% for grade 0/0R, 28% for grade 1A, 30% for grade 1B, 37% for grade 2, 51% for grade $\geq 3A$ according to the 1990 classification; and 32% for grade 1R, 49% for grade 2R and 54% for grade 3R according to the 2004 classification. Biopsies without signs of rejection (grade 0/0R) displayed significantly lower recipient-derived cell content than biopsies with ongoing rejection of any grade ($P < 0.001$). Time-dependent mean recipient-derived cell percentage was 12% in the first week but remained constant from the first month up to five years after transplantation with a mean share of 22%.

Conclusion: Episodes of allograft rejection are associated with significantly increased recipient-derived cell content. Furthermore, recipient-derived cell content correlates with histopathological grading. Quantitative STR analysis offers a highly reproducible and observer independent method for the assessment of cardiac allograft rejection and may supplement the current histological examination.

Zusammenfassung

Hintergrund: Den derzeitigen Standard in der Diagnostik von Abstoßungsreaktionen allogener Herztransplantate stellt die histopathologische Beurteilung von rechtsventrikulären endomyocardialen Biopsien (EMB) dar. Mehrere Studien haben für diese Methode jedoch eine erhebliche Inter- und Intra-Beobachter-Variabilität nachweisen können. Aufgrund einer fehlerhaften Zuweisung von Abstoßungsgraden kann es in der Folge zu Fehlentscheidungen hinsichtlich der immunsuppressiven Therapie kommen. Darüber hinaus ist die Vergleichbarkeit von Daten aus klinischen Studien eingeschränkt. Quantitative Analyse von short tandem repeats (STR) ist ein bewährtes diagnostisches Verfahren für die Erfolgskontrolle nach Stammzelltransplantationen sowie für Chimärismus-Studien. Diese Arbeit untersucht die Anwendbarkeit von quantitativer STR-Analyse für die Diagnose von Herzabstoßungsreaktionen.

Methoden: Sechsendsechzig endomyocardiale Biopsien von neun herztransplantierten Patienten wurden auf den Empfängerzellanteil untersucht. Nachdem genomische DNA aus den Proben extrahiert wurde, wurden neun polymorphe STR-Loci durch multiplexe Polymerase-Kettenreaktion amplifiziert und anschließend mit Vierkanal-Kapillarelektrophorese quantifiziert. Die Ergebnisse der quantitativen STR-Analyse wurden schließlich mit der histopathologischen Graduierung entsprechend den Klassifikationssystemen der Internationalen Gesellschaft für Herz- und Lungen-Transplantation von 1990 und 2004 verglichen.

Ergebnisse: Die Mittelwerte für den prozentualen Empfängerzellanteil sind 15% für Grad 0/0R, 28% für Grad 1A, 30% für Grad 1B, 37% für Grad 2, 51% für die Grade $\geq 3A$ nach der Klassifikation von 1990; 32% für Grad 1R, 49% für Grad 2R und 54% für Grad 3R nach der Klassifikation von 2004. Biopsien ohne Hinweis auf Abstoßungsreaktion (Grad 0/0R) zeigten einen signifikant geringeren Empfängerzellanteil als Biopsien mit Abstoßungsreaktion aller Grade ($P < 0,001$). Im zeitlichen Verlauf zeigte sich ein Empfängerzellanteil von 12% nach einer Woche, der nach dem ersten Monat bis zum fünften Jahr nach der Transplantation jedoch mit einem Mittelwert von 22% konstant blieb.

Diskussion: Akute Abstoßungsreaktionen sind mit einem signifikant erhöhtem Empfängerzellanteil verbunden. Es wurde außerdem eine Korrelation des Empfängerzellanteils mit der vorhergegangenen histopathologischen Graduierung nachgewiesen. Die quantitative STR-Analyse ergibt hoch reproduzierbare und Beobachter-unabhängige Resultate und könnte die bisherige histologische Beurteilung ergänzen.

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1 Introduction

1.1 *Heart transplantation*

Heart transplantation (HTX) represents an effective therapeutic option for the treatment of selected patients with end-stage irreversible heart failure.² The leading pre-transplant diagnoses of HTX recipients are non-ischemic cardiomyopathy in 53% of cases, followed by ischemic cardiomyopathy in 38% of cases. Congenital heart disease, valvular heart disease and retransplantations each amount to 3%. The worldwide number of HTX peaked in 1995 with almost 4500 transplantations reported to the International Society for Heart and Lung Transplantation (ISHLT). Numbers have decreased over the late 1990s but remained relatively constant during the following decade with over 3000 reported procedures per year.³

For the entire cohort of pediatric and adult transplant recipients from 1982 to 2008, current median survival is 10 years. Mortality risk is highest in the first six months. Notably, patients surviving the first year have a better current median survival of 13 years.³ Exceedingly long single-graft survivals of up to 31 years have also been reported.⁴

The major causes of mortality in the first 30 days post-transplant are primary graft failure (PGF) and infections; these remain the two leading causes of death in the entire post-transplant course. Acute rejection episodes are mainly a problem of the first year after transplantation with almost constant incidence rates after three years and dropping mortality rates. In the later course, consequences of chronic rejection come into effect. These could be either rejection-related, such as antibody-mediated rejection and cardiac allograft vasculopathy, or consequences of permanent immunosuppressive therapy like renal insufficiency and malignancies.³

Donor organ shortage is a major restrictive factor in transplantation medicine. Recent developments in organ procurement include the transplantation of less ideally suited hearts, e.g. from older donors (i.e. > 40 years old) and the toleration of longer cold ischemia times (i.e. > 4 hours). Also because of organ shortage, increasing numbers of transplant recipients on waiting lists receive a ventricular assist device (VAD) as bridge-to-transplant therapy.³ Total artificial heart replacement has proved to be a viable long-term alternative with a current median patient survival of 6 years.⁵

Xenografts, especially of porcine origin, have also been discussed as an alternative for human allografts. Unfortunately, high immunological barriers and concerns about the possible transfection of xenozoonoses have so far barred xenografts from use as a suitable clinical alternative. Recent research into genetically engineered xenografts remains promising as an alternative to the implantation of a VAD, but these methods are not yet ready for routine clinical application, not to mention long-term application.⁶

1.1.1 Historical development of heart transplantation

The road to today's routine HTX was long, stretching back to the experimental heart transplantations performed on dogs by Carrel and Guthrie in 1905 and Carrel's pioneering work in vascular surgery. More transplantation experiments were conducted by many groups over the 1930s to 1950s, furthering the understanding of immunological processes and establishing basic surgical techniques.⁷ Groundbreaking work was done by the Stanford group around Lower and Shumway, who conducted extensive experimental work during the 1960s.⁸⁻¹¹ The results of the Stanford group's research were authoritative for all following efforts.

Another milestone for the technical feasibility of heart transplantation was the introduction of extracorporeal circulation for open human cardiac surgery by Dennis¹² and Gibbon¹³ in 1951 and 1953, respectively.

The first successful human-to-human heart transplantation was performed by Barnard at Groote Schuur Hospital, Cape Town, on December 3, 1967.¹⁴ Over the first postoperative days, the patient recovered well but then his condition deteriorated rapidly and he died after 18 days. However, the next allograft recipient—transplanted only one month later on January 2, 1968—did well and returned home to a relatively normal life, dying 19 months after the operation from complications of chronic rejection. Both transplantations were followed closely by the international press, thus creating an unprecedented level of public awareness of this new era in medicine.

Although historically little-known, the first *de facto* successful heart transplantation into a human was already carried out almost four years earlier by Hardy in 1963.¹⁵ However, the chimpanzee-to-human xenograft failed after just 2 hours due to massive xenograft rejection.

In its first years, HTX numbers peaked at ~100 transplantations in 1968 but until 1971 numbers decreased and then stagnated in the low two-digit number range. Mean survival was poor with 1 week in 1970, 1 year in 1974 and moderate with 5 years in 1978. The introduction of cyclosporine as a new immunosuppressive agent in the early 1980s resulted in a new era of transplantation medicine and resulted in an increase in HTX numbers from < 100 in 1981 to over 3000 in 1989. Mean survival also increased to 8.1 years in 1985 and 9.8 years in 1996.^{16,17}

1.1.2 Operative techniques

The modern operative techniques are very similar to the technique originally established by Lower and Shumway, which was also followed by Barnard in 1967. Since then, only minor changes have been made to the general concept. The procedure can be performed either as an orthotopic or heterotopic cardiac transplantation.¹⁸ Orthotopic transplantation means implantation of the graft in accordance with the normal anatomical situs, including explantation of the recipient heart except for the posterior wall of the left atrium where the pulmonary vein orifices are located and possibly the posterior wall of the right atrium. Heterotopic transplantation means the additional implantation of the donor heart in a manner that both hearts are in some way connected (also known as 'piggy-back' transplantation). Heterotopic transplantation has become rare since the 1980s and will not be elaborated further herein. Thus, orthotopic transplantation became a widely used technique and underwent some further modifications.

Barnard proposed the first modification to the traditional biatrial orthotopic transplantation immediately after his first HTX in 1967. He suggested a slight variation in the incision line of the right atrium of the donor heart, leaving out the area of the sinus node.¹⁹ This modification resulted in sustained function of the sinus node and a decrease of post-transplant arrhythmias.

Blanche et al. introduced another modification for orthotopic transplantation, leaving the right atrium intact and dissection of the superior vena cava (SVC) and inferior vena cava (IVC) at the level of their emergence from the parietal pericardium for later bicaval anastomosis, while the transection of the left atrium remains unchanged.²⁰ This method is now the recommended technique as it yields slightly improved graft survival and lowers the need for implantation of permanent pacemakers.^{21,22}

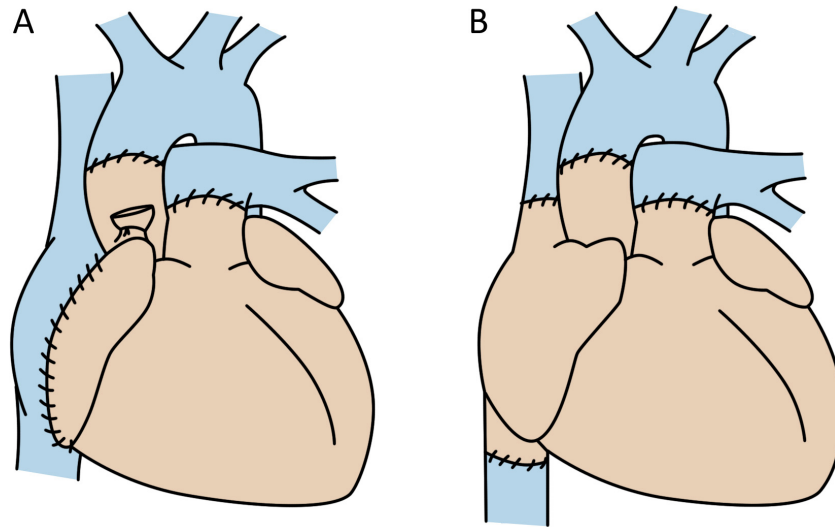


Figure 1-1: Different techniques for orthotopic heart transplantation. **A:** traditional biatrial transplantation. **B:** modern bicaval transplantation.

For organ explantation, the donor heart is exposed through a median sternotomy and pericardiotomy. Before explantation, it is perfused with a cardioplegic solution. The aorta and pulmonary artery are divided above the level of the commissures of the semilunar valves. The left atrium is transected at the level of its atrioventricular groove, posterior of the atrial appendage. The right atrium is left intact, the SVC and IVC are dissected at the level of their emergence from the parietal pericardium. The organ is subsequently cooled down to 2–4 °C.¹⁸

Preparation of the recipient, exposure of the pericardial cavity and institution of extracorporeal circulation is practically identical to that of other open cardiac surgical procedures, although the placement of the SVC and IVC cannulas may be varied slightly. The explantation of the recipient heart is also identical to the procedure described above for donor heart explantation. Implantation of the graft begins with the attachment of recipient and donor left atria, followed by connection of the right atria, the manner of which is depending on the precontemplated technique. For biatrial anastomosis, the cavity of the right atrium of the donor must be opened and trimmed to fit on the posterior wall of the recipient right atrium. Proceedings for bicaval anastomosis may seem much more simply, but necessitate the direct end-to-end anastomosis of the venae cavae. These sutures may be surgically challenging as the relatively thin wall of venous vessels can be damaged easily and strictures must be

avoided. The implantation is completed by reconnection of recipient and donor aorta and pulmonary artery. At that point, rewarming is begun and circulation reestablished. In more than 50% of cases, spontaneous defibrillation of the graft occurs, the remaining cases requiring electrical defibrillation.¹⁸

1.2 Endomyocardial biopsy

In the era before routinely performed HTX, retrieval of myocardial biopsies was a rather rare event, mostly needed when an uncertain infection of the myocardium had to be confirmed histologically. Two methods were commonly used, the first being epimyocardial biopsy, performed during open heart surgery and requiring sternotomy, the second being transthoracic biopsy using a Vim–Silverman needle with considerable procedural risk for transmural perforation and pericardial tamponade.^{23,24}

The first endovascular approach for endomyocardial biopsy (EMB) was introduced in 1962, making use of a special biopsy catheter also known as bioptome.²⁵ This method made harvesting EMBs safer and laid the groundwork for the development of the Caves–Schultz Stanford bioptome by Caves et al. in 1973, which also used the transvascular approach under fluoroscopic control.^{26–28} These developments followed the increasing importance of EMB in the diagnosis of allograft rejection in the emerging field of heart transplantation.

1.2.1 Technique of percutaneous transvenous right-ventricular EMB

While at first the approach was transarterial, sampling the left-ventricular side of the interventricular septum, this soon evolved into a transvenous approach through the neck veins, reaching the right-ventricular side of the septum. The internal jugular or subclavian veins are most commonly used for EMB, although approaches via the femoral or external jugular veins are also possible. Over the last decades, bioptomes were further enhanced; e.g. modern bioptomes have increased flexibility and maneuverability, sheathed catheters allow for procurement of multiple biopsies through the same venous puncture at a single sitting.²⁴

The transvenous technique has proved itself to be a safe method for obtaining EMBs. The rate of serious complications following percutaneous transvenous right-ventricular EMB in patients after HTX is at low 0.3–0.7%.²³ The most commonly found complications are erroneous puncture of the carotid artery, pneumothorax,

right-ventricular perforation, triggering of supraventricular or ventricular arrhythmias and embolization. The emergence of tricuspid valve regurgitation may be caused by a flail tricuspid leaflet when chordae tendineae are ruptured or biopsied during the procedure.^{23,29}

At the local Division of Transplantation Surgery (Department of Surgery, Medical University Hospital Graz, Austria), routine biopsies are obtained through the right internal jugular vein approach. The vein is punctured according to Seldinger's technique under local anesthesia of the puncture site. The bioptome is inserted over a guidewire and dilator and the right ventricle is located under fluoroscopic control. Eight separate tissue pieces are then harvested from the interventricular septum. When acute rejection is suspected, a central venous catheter is placed through the same venous puncture for the subsequent administration of immunosuppressive medication.

1.2.2 Protocol for surveillance biopsies

The protocol for routine post-transplant EMB surveillance varies from center to center. At the transplant center in Graz, the first routine biopsy after HTX is performed on the eighth day postoperatively. The following EMB is performed seven days later. The intervals for all following EMBs after that point are determined by the rule, that the next EMB is performed in as many months as have passed since the transplantation. E.g., when a patient received a HTX eight months ago, the next appointment for an EMB would be in eight weeks.

However, should any of the routine EMBs give a hint of ongoing allograft rejection (i.e. graded > grade 0), the periods between surveillance biopsies will be shorter, depending on rejection severity and response to increased immunosuppressive therapy.

1.3 Immunopathology of cardiac allograft rejection

The recipient's immune reaction toward the allograft tissue involves—to different degrees—both cellular and humoral components and is manifested as hyperacute, acute and chronic rejection.

Acute cellular rejection remains one of the major problems in the first year after heart transplantation.³ Approximately 30% of all patients who underwent heart transplantation between 2003 and 2008 experienced rejection episodes during the

first year. Mortality rates, however, are dropping since the introduction of improved immunosuppressive therapies in the 1990s. As of 2010, acute rejection is responsible for 11% of deaths. Younger recipient age and female donor gender are significant risk factors for early rejection episodes and reduced graft survival.³

Under the modern immunosuppressive regimen, acute rejection episodes are usually clinically silent. Signs of acute rejection may be (sub-)febrile temperature, onset of ventricular arrhythmias or the result of impaired graft function, resembling different grades of congestive heart failure (pulmonary congestion, systemic low-output state).

Vascular changes brought about by chronic rejection are the primary reason for graft failure in the later course. The main manifestation of these changes is known as cardiac allograft vasculopathy (CAV). CAV is closely related to humoral immune processes directed against the graft, and more specifically the endothelium of its blood vessels. It is the primary factor for mortality in the late post-transplant course with an incidence rate of 20% at 3 years, 30% at 5 years and 50% at 10 years.

Clinically, CAV yields the same consequences as the atherosclerotic coronary artery disease (CAD)—with one important limitation: Patients may not experience angina pectoris during coronary ischemia due to unreliable reinnervation of the cardiac allograft.

1.3.1 Mechanisms of cellular rejection

The main cause for allograft rejection is the recognition of alloantigenic molecules on graft cell surfaces by the immune system of the recipient. These molecules were first discovered in experimental skin transplantation in mice, although it is now known that they are physiologically more closely related to the detection and abatement of bacterial and viral infections. These molecules were thus called major histocompatibility (MHC) molecules with regards to their involvement in the mechanism of allograft rejection. The expression of MHC molecules was also detected in man where they are synonymously called human leukocyte antigen (HLA) with the corresponding gene loci on chromosome 6. HLA molecules are heterodimeric glycoproteins anchored in the cell membrane by a transmembrane immunoglobulin-like domain. The N-terminal extracellular domains form a peptide-binding groove for presentation of antigen to T lymphocytes. The exons that encode the peptide-binding groove are

highly polymorphic with hundreds of alleles, accounting for the difficulty of matching donor and recipient for transplantation unless they are closely related. Unfortunately, a good HLA match is a rare event in cardiac transplantation with almost 96% of allograft recipients having more than three HLA mismatches with the donor heart.³ Three classes of HLA molecules have been described:

HLA class I molecules are expressed on all nucleated cells. The class I molecule is composed of a heavy chain (subdivided in three domains $\alpha 1$, $\alpha 2$ and $\alpha 3$) and a $\beta 2$ -microglobulin (non-anchored association with the $\alpha 3$ domain). Together, the heavy chain $\alpha 1$ and $\alpha 2$ domains form a peptide-binding groove. In the absence of cell infection with viral particles, the groove is used for the presentation of endogenous peptides with a length of 8 or 9 amino acids, which fit the groove specifically. HLA class I molecules are genetically sub-classified in three relevant phenotypes by their corresponding gene locus: HLA-A, HLA-B and HLA-C.

HLA class II molecules are constitutively expressed on B-lymphocytes and other antigen presenting cells (APC) such as macrophages and dendritic cells. Their expression may also be induced in endothelial cells, which is important in the pathogenesis of CAV.^{30,31} Class II molecules are of slightly different composition, with two connected transmembrane polypeptide chains α and β (subdivided in $\alpha 1$, $\alpha 2$ and $\beta 1$, $\beta 2$ domains). The $\alpha 1$ and $\beta 1$ domains form the peptide-binding groove, which is comparable to that of HLA class I molecules. Foreign antigens presented by class II HLA molecules have been endocytosed and processed by the APC prior to presentation. They originate from cell membranes and extracellular proteins that are processed in intracellular lysosomes into fragments of 14–18 amino acids length, loaded on the fitting HLA class II molecule and then transported to the cell surface. Phenotypes of HLA class II molecules are also corresponding to certain gene loci and are sub-classified accordingly as HLA-DP, HLA-DQ and HLA-DR.

HLA class III molecules have been described as well. They appear to be connected to the complement system and are believed to be irrelevant to allorecognition.

T-lymphocytes use a specific receptor (T cell receptor, TCR) to interact with HLA molecules, scanning all encountered cell-surfaces for presentation of self/non-self peptides bound to self/non-self HLA molecules. For effectively recognizing these epitopes, T cells require the presence of a co-receptor and cell-adhesion molecules.

The TCR is a membrane-bound antibody-like heterodimer, composed of an α and a β chain, connected by a disulfide bond. Both chains are subdivided in a variable (V) and a constant (C) extracellular domain. However, the TCR does not have two antigen-binding sites like other immunoglobulins, but only one, formed by the $V\alpha$ and $V\beta$ domains. The receptor is directly associated with the CD3 complex, which transduces TCR activation into intracellular signals.

Interaction of the TCR with HLA classes is determined by the co-receptor CD4 or CD8 binding to invariant regions of HLA molecules. CD4⁺ helper T cells recognize only class II HLA molecules, whereas CD8⁺ cytotoxic T cells interact solely with HLA class I. Cytotoxic T cells aim for the direct destruction of cells displaying the target antigen, while helper T cells do not themselves attack target cells but stimulate macrophages and antibody-producing B cells to do so.

Alloantigens are presented either directly or indirectly to alloreactive recipient T-lymphocytes, i.e. to T cells that express a fitting TCR with high affinity for a specific donor antigen. In the direct pathway, recipient T cells recognize donor HLA molecules, which may present donor or recipient peptides. In the indirect pathway, representing the more common way of alloantigen presentation, fragments of donor HLA molecules are presented by recipient APCs (figure 1-3). Although recipient T-lymphocytes have—through the process of thymic education—learned to tolerate the combination self-HLA $\pm$ self-peptide (so-called MHC-restriction), they may still be highly sensitive to self-HLA or donor-HLA loaded with donor peptide.⁴⁸

The graft's endothelial cells play an important role in the initiation and perpetuation of allograft rejection. They present alloantigen to recipient T cells through expression of both HLA class I and II molecules, thus becoming the primary target of rejection as the vascular endothelium is the only barrier between the graft and the recipient's immune system. Activated endothelial cells produce IL-1 α , IL-8, RANTES (regulated upon activation, normal T cell expressed and secreted) and monocyte chemotactic protein 1 (MCP-1), beaconing neutrophils and monocytes to the site of injury.³² They also allow for inflammatory tissue infiltration by enabling adhesion and diapedesis of recipient leukocytes. Endothelial cells constitutively express intercellular adhesion molecule 1 (ICAM-1, CD54) and platelet endothelial cellular adhesion molecule (PECAM, CD31) to enable weak leukocyte adhesion and rolling. The presence of activated T cells—as in the allograft during rejection episodes—and the asso-

ciated secretion of cytokines (IFN γ , TNF α , IL-2), induce the expression of endothelial leukocyte adhesion molecule 1 (ELAM-1 or E-selectin, CD62E) and vascular cell adhesion molecule 1 (VCAM-1, CD106), aiding firm leukocyte adhesion and extravasation.³³ This also accounts for the notable participation of neutrophil and eosinophil³⁴ granulocytes in the more severe inflammatory infiltrates, because these cell types also respond to the chemokine gradient and cytokine secretion emanating from foci of acute allograft rejection.

To become fully activated and differentiate into effector cells, T cells need costimulatory signaling in addition to TCR activation by HLA-peptide-complexes. Such signals may be transmitted by the interaction between B7 family proteins (CD80 and CD86) and CD28 or CTLA-4 (cytotoxic T lymphocyte antigen 4, CD152). Another costimulatory signal is CD40 interaction with CD40 ligand (CD40L or CD154). However, as the process of T cell activation is progressing, necessity of costimulatory signaling is waning, because T lymphocytes—once activated—are able to interact with alloantigens and to perpetuate allograft rejection without these stimuli. E.g., propagation of alloreactive T cells is mediated not by external stimuli but through autocrine/paracrine signaling. An activated T cell secretes interleukin 2 (IL-2), which activates high-affinity IL-2 receptors (IL-2R, CD25) on the same T cell, inducing intracellular signal pathways that procure mitosis, leading to monoclonal expansion of the alloreactive T cell clone.³⁵

When an APC activates a naïve CD4⁺ T helper cell (T_H0), it may differentiate into the T_H1 or T_H2 subtype. The further differentiation of T_H0 cells is depending on the immediate cytokine environment; influence of interferon γ (IFN γ) induces T_H1 differentiation, whereas interleukin 4 (IL-4) induces the T_H2 phenotype.³⁶ T_H1 cells secrete IL-2, IL-12, IFN γ and tumor necrosis factor α (TNF α), activating macrophages and CD8⁺ cytotoxic T cells. T_H2 cells secrete interleukin 3, 4, 5, 6, 10 and 13, activating, in turn, B cells and eosinophil granulocytes. IFN γ was proven to enhance expression of class I and II HLA and CD40 costimulatory molecules in endothelial cells, enabling further alloactivation of resting T cells.³⁷ Also, in connection with secretion of TNF α , it may mediate direct cytotoxic effects on allograft myocytes.⁴⁰

Naïve cytotoxic CD8⁺ T cells are activated by APCs. Target cell destruction can be effectuated by 1) secretion of perforin, a complement factor C9 homologue, polymerizing in the target cell membrane and forming transmembrane channels, 2) serine

proteases (such as granzyme A and B), reaching the intracellular space of target cells through the previously formed perforin pores, indirectly activating the caspase cascade, and 3) direct induction of apoptosis through activation of the fas receptor (fibroblast associated receptor, CD95) by fas ligand (fasL, CD95L), thus initiating the caspase cascade. These functions can—to a lesser extent—also be carried out by CD4⁺ T lymphocytes.^{38,39} Furthermore, CD4⁺ T cells may play a role in the infliction of myocyte damage through the delayed type hypersensitivity reaction (DTH, Coombs & Gell type IV reaction). Primed T_H1 cells respond to a second contact with the alloantigen with the secretion of cytokines and activation of macrophages, which in turn may contribute to cell damage. As mentioned above, neutrophil and eosinophil granulocytes occur during more severe rejection episodes and also may contribute to cell damage by the releasing of proteolytic enzyme- and toxin-filled granules and generating cytotoxic reactive oxygen species (ROS).⁴⁰

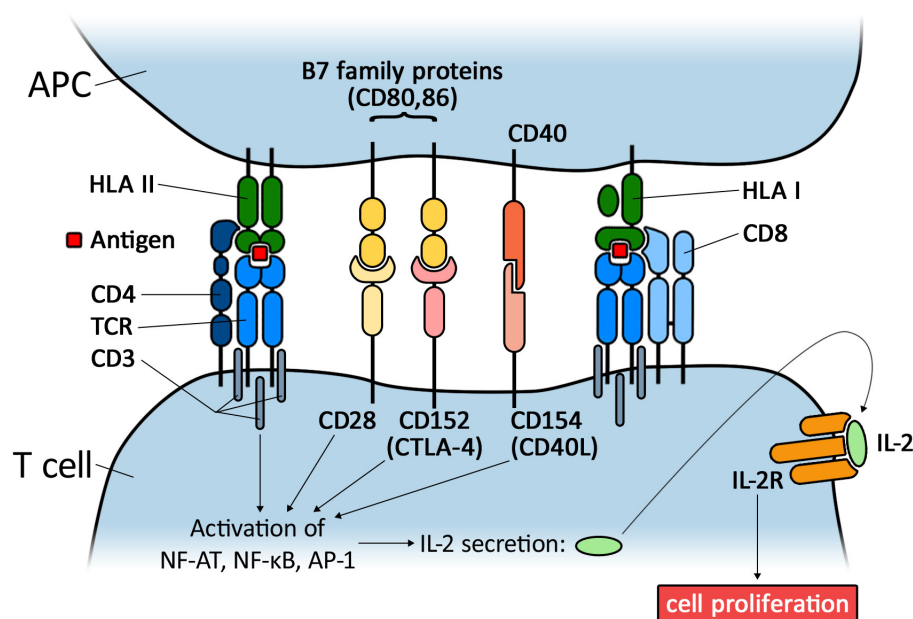


Figure 1-2: Mechanism of T cell activation. TCR-HLA-interaction under participation of the fitting co-receptor (CD4 on T helper cells, CD8 on cytotoxic T cells) constitutes the first step in T cell activation (signal 1). Proper activation of other co-receptors (CD28, CD152, CD154) by their respective ligands is needed for completion of T cell activation (signal 2). Summative effect of activation via the signal 1 and 2 pathways is increased secretion of IL-2, leading to cell proliferation, i.e. cell cycle progression from G₁ to S phase.

Macrophages (M ϕ) are activated either by effector T lymphocytes or by direct contact with alloantigen. Circulating monocytes follow the chemotactic gradient to the inflammatory focus, where they migrate into the tissue and—under the influence of IFN β and IFN γ secreted by T cells—become activated tissue macrophages. They will confront antigen-bearing cells by secretion of pro-inflammatory cytokines such as TNF α , IL-1, IL-12 and IL-18. Target cell detection may be enhanced by opsonizing antibodies and complement factors as macrophages carry opsonin receptors (see ch. 1.3.2). They also carry the fas ligand for inducing target cell apoptosis and the density of macrophage infiltration is correlated with an increased rate of myocyte apoptosis.⁴¹ Activated macrophages show increased iNOS (inducible nitric oxide synthase) activity. NO may play an integral role in myocyte damage and necrosis and is also acting negatively inotropic, possibly compromising graft function.^{42,43}

Natural killer (NK) cells also participate in allograft rejection as a first-responder cell type. Experimental rat HTX without immunosuppressive induction- and maintenance-therapy showed that the inflammatory infiltrate in the first three days post-transplant is composed of macrophages and NK cells.^{44,45} NK cells are a part of the innate immune system. As such, they do not require HLA-TCR-CD8-interaction for allorecognition like cytotoxic T cells, but rather attack all cells that do not sufficiently express ‘self’ HLA class I molecules. Physiologically, this approach is connected to the suppression of tumours and infections as some viral infections or mutation-induced changes force cells to down-regulate HLA expression as a means to escape immune detection. NK cells can also attack target cells that are marked by opsonins (antibodies, complement). The cytotoxic mechanisms are the same as in cytotoxic T lymphocytes (perforin, fas-fasL-interaction). Furthermore, activated NK cells secrete IFN γ and IL-2, promoting T_H1 T cell response.⁴⁰

CD4⁺CD25⁺FOXP3⁺ regulatory T cells (T_{REG}) are a special subtype of T helper cells that do not sustain immune response but rather suppress it. They have become the focus of great attention in approaches to induce tolerance in allograft recipients.⁴⁶

1.3.2 Mechanisms of humoral rejection

The presence of preformed antibodies with high avidity towards allograft epitopes causes a hyperacute form of cardiac allograft rejection. Such antibodies are present in patients who have previously been sensitized for an alloantigen, usually through

blood transfusions, pregnancies or hematopoietic stem cell transplantation (HSCT).⁴⁷ However, the introduction of standard pre-transplant screening assays (blood type crossmatch, panel reactive antibodies) for the detection of anti-donor antibodies has almost eliminated hyperacute rejection.³

The *de novo* production of anti-donor antibodies is always connected to cellular rejection, albeit in different degrees. Antibody-mediated rejection (AMR) may be diagnosed concurrently with episodes of acute cellular rejection or may persist as a form of chronic allograft rejection. Acutely, it leads to endothelial cell activation and perivascular inflammation. If this inflammation should persist, it can lead to fibrous intimal proliferation, known as chronic allograft vasculopathy (CAV). AMR is initiated through the interaction of allosensitized T and B cells. It is primarily targeted against donor HLA molecules expressed on the surface of graft vascular endothelial cells, as these form the first line of contact between circulating antibodies, leukocytes and the allograft.⁴⁸

B lymphocytes recognize their cognate antigen in its native form through membrane-bound clonal IgD and IgM antibodies (mIgD, mIgM). These are also referred to as B cell receptor (BCR). Once a B lymphocyte is confronted with a fitting antigen, this antigen is digested and presented on class II HLA molecules. Efficient production of antibodies requires B cell activation by T_H2 cells through HLA-TCR-CD4-interaction with costimulatory CD40/CD40L signaling, which, in combination, results in secretion of IL-2, IL-4 and IL-5, causing the B lymphocyte to proliferate by clonal expansion and differentiate into alloantigen-specific antibody producing plasma cells (and to a much lesser degree memory B cells). The produced antibody types are mainly IgG and to a lesser extent IgM and IgA antibodies. IgG and IgA are capable of antigen opsonization and complement activation.³⁵

Involvement of the complement system is triggered by the formation of antigen-antibody-complexes (classical pathway of complement activation), resulting in on-site activation of C1, setting off the complement cascade resulting in the formation of further opsonins (C3b, C4b) and the membrane attack complex (MAC).

Phagocytes (i.e. macrophages, neutrophil granulocytes, eosinophil granulocytes and NK cells) carry opsonin receptors that enable them to recognize and bind to cells that have been marked by antibodies and complement factors. The Fc (fragment, crystallizable) region of antigen-bound IgG or IgA antibodies interacts with Fc recep-

tors, while C3b and C4b are recognized by complement receptors (CR). Activated macrophages then produce a plethora of cytokines including NO (through iNOS activation) and several growth factors. Secreted cytokines include IL-1, TNF α , IFN γ , monocyte chemotactic protein 1 (MCP-1) and apoptosis inducing factor 1 and 2 (AIF-1, -2). Some of these substances play a role in inducing typical features of CAV. Macrophages are also capable of inducing target cell apoptosis through fas-fasL-interaction. Granulocytes will respond to opsonin receptor binding with degranulation (antibody-dependent cell-mediated cytotoxicity, ADCC). The endothelial cells—activated by the inflammatory process—participate in perpetuating the cellular component of AMR by increased production of HLA and leukocyte adhesion molecules (as described in ch. 1.3.1).

CAV is the summative result of chronic rejection, with the main etiologic factor being AMR based upon a DTH-like immune response. As elaborated above, AMR also sets in motion some cellular responses that mediate cell damage on graft vascular endothelial cells. The pathogenesis of CAV can be divided into three stages: 1) unspecific endothelial cell injury, 2) reactive alloresponse, and 3) fully developed CAV.⁴⁹ The primary lesion, initiating the immunogenic process leading to CAV, is mostly of immunological (acute cellular and/or humoral rejection) origin, although non-immunological factors may also contribute. These non-immunological factors for the development of CAV are diverse, e.g. donor coronary artery atherosclerosis, hyperlipidemia, hormone status, infection with cytomegalovirus and side-effects of immunosuppressive therapy.²⁹ Macrophages and granulocytes are the first response to the endothelial cell injury. They create a pro-inflammatory cytokine (IL-1, IL-6, IL-12, IL-18, TNF α) and chemokine (RANTES) environment, thereby recruiting other cells to the site of inflammatory response. In consequence, endothelial cells become activated, up-regulating expression of adhesion molecules ICAM-1, VCAM-1, P- and E-selectins.^{48,49}

The stage of alloresponse is marked by macrophages and T lymphocytes (and to a lesser degree granulocytes, plasma cells and mast cells)⁴⁸ that invade the vessel media and perivascular connective tissue. T cells secrete the key cytokine IFN γ , which plays a major role in the CAV pathogenesis as a macrophage-activator and stimulus for T cell activation and proliferation.⁵⁰ As mentioned above, activated tissue macrophages secrete IL-1, IL-6, IL-12, IL-18 and TNF α , perpetuating and intensifying in-

inflammatory reaction. They also produce platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor β (TGF β), basic fibroblast growth factor (bFGF), creating an imperative signal for proliferation of medial vascular smooth muscle cells (VSMC) and production of intercellular matrix.⁴⁸ It has been shown that VSMCs in grafts with developed CAV are different from normal VSMCs in a way that they lost contractile ability and display a more embryonic, synthetic gene expression profile.⁵¹

Fully developed CAV is characterized by concentric VSMC and matrix proliferation, leading to vascular occlusion.⁵²

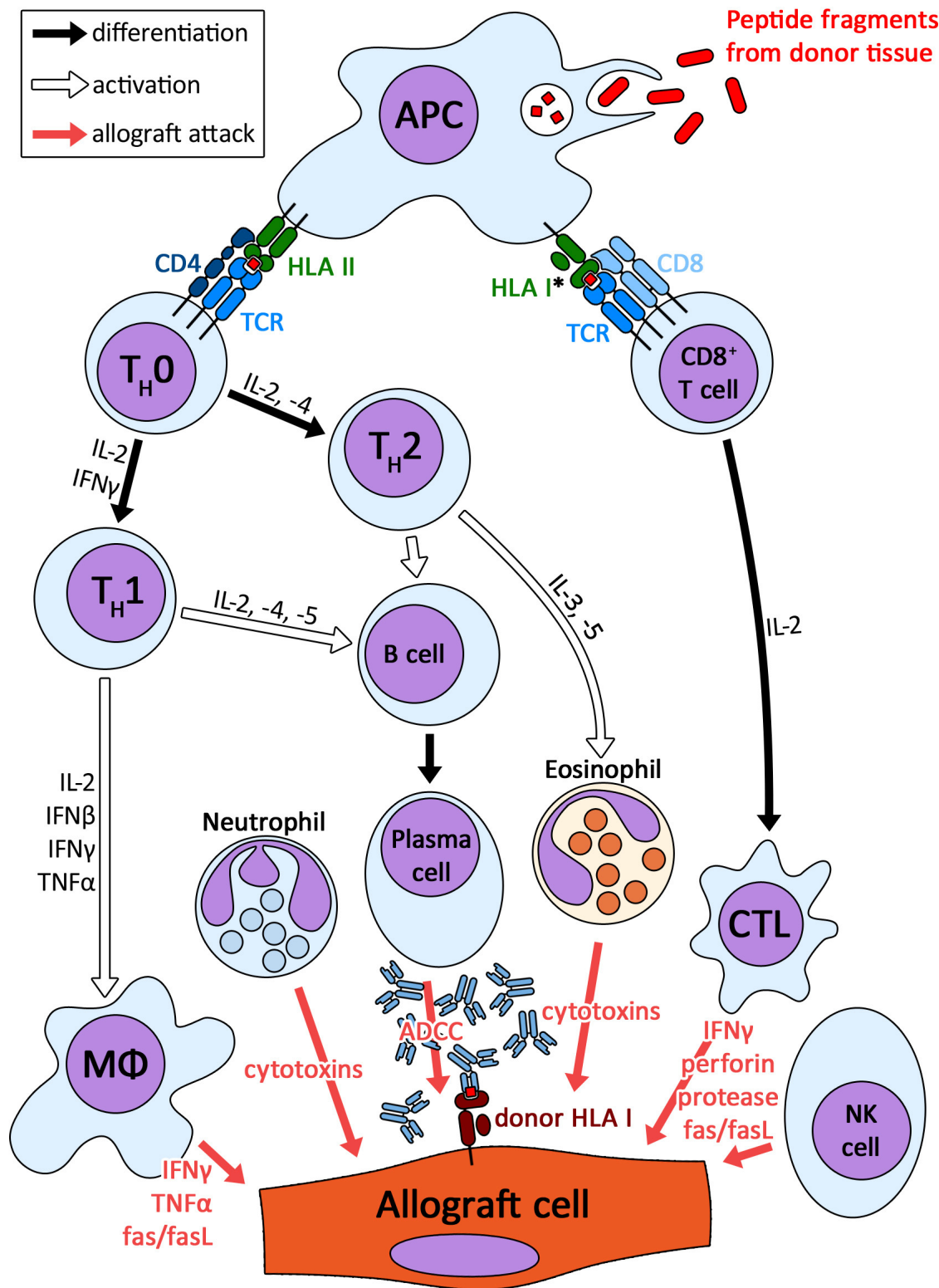


Figure 1-3: Simplified scheme of cellular and humoral mechanisms leading to allograft rejection. CTL: cytotoxic T lymphocyte. *: May also be a donor HLA class I molecule on donor cardiomyocyte or endothelial cell or a class II HLA molecule on a donor APC transplanted along with the graft ("passenger APC").

1.4 Histopathology of cardiac allograft rejection

Cardiac allograft rejection is diagnosed through the histopathological inspection of multiple biopsy slides through light field microscopy. These slides are commonly H&E-stained, although special stains, immunofluorescence and immunohistochemistry may be applied to better differentiate between rejection episodes and degenerative processes of other origin.

According to the current recommendations of the International Society for Heart and Lung Transplantation (ISHLT), at least three biopsy specimens should be harvested from the graft, fixated in formalin and embedded in one paraffin block.⁵³ However, the more tissue pieces are added, the lower the possibility of missing a focus of rejection becomes.^{54,55} Also, at least three serial sections should be prepared to enable better understanding of the infiltrate's three-dimensional expansion.⁵³

According to the current grading classification (see ch. 1.5), rejection is always graded on the worst area of involvement. E.g., six biopsy pieces are examined and graded 0, 1A, 1A, 3A, 0 and 1B, making the overall diagnosis grade 3A rejection, even though the other samples showed no or only mild rejection. The reason for this procedure is that rejection episodes are often distributed heterogeneously in the myocardium. It is, however, good practice to include the individual biopsy grades on the final statement.²⁹

1.4.1 Morphology of acute cellular rejection

Acute cellular rejection is primarily graded through the assessment of pattern and density of the inflammatory infiltrate and the presence of additional effects such as myocyte damage, edema and hemorrhage. Other phenomena and artifacts may influence the accuracy of the assessment.

The **inflammatory cellular infiltrate** is usually comprised of T lymphocytes and macrophages. While T cells constitute the dominating cell type, it has been shown that the number of macrophages increases with rejection severity and can be a large component of the infiltrate.⁵⁶⁻⁵⁹

The ratio of the CD4⁺ and CD8⁺ subsets of T lymphocytes depends on the type of immunosuppressive regimen the patient is receiving and correlates only poorly with histological rejection grades.^{60,61} Under immunosuppression with azathioprine and steroids, the majority of T lymphocytes are of the CD8⁺ phenotype.⁶² Use of cy-

cyclosporine, however, shifts the predominating T lymphocyte phenotype towards CD4⁺, while CD8⁺ T cells and macrophages may still be present in lower numbers.^{63,64}

The occurrence of eosinophil granulocytes in moderate to severe rejection has also increased since the introduction of cyclosporine.^{34,65} Neutrophil granulocytes may be present in severe rejection episodes, although they may also be indicative of infection, healing ischemic injury or humoral rejection.²⁹

As mentioned in ch. 1.3.1, NK cells also take part in the composition of the inflammatory infiltrate,^{44,45,66,67} as well as plasma cells (as indicators of concomitant humoral rejection). It is thought that these cell types do not play an important role during acute cellular rejection⁶⁸ but are important as inductors and promoters.⁵⁶

Myocyte damage occurs in higher-grade rejection (figure 1-7).⁶⁹ It may be inflicted by a multitude of mechanisms (described in ch. 1.3.1). Injured cardiomyocytes may present with a variety of morphologic changes, such as vacuolization, perinuclear halo, nuclear enlargement, ruffling of the sarcoplasmic membrane, irregular myocyte border, increased splitting and branching of myocytes (“Y-sign”) and myocyte encroachment by the inflammatory infiltrate.^{53,70,71} While early myocyte damage is not easily diagnosed by light microscopy on routine H&E slides, Masson’s trichrome stain facilitates its detection (damaged myocytes appear gray-blue instead of the normal red). Necrosis of myocytes is usually indicated by hypereosinophilic sarcoplasm and nuclear pyknosis. Actual myocyte necrosis seems to be a relatively rare event—even during moderate-to-severe rejection episodes—and myocyte damage is mostly reversible.⁷²⁻⁷⁴

Endothelial cells stand on the forefront of rejection, so it is not surprising that these cells suffer injury from acute graft rejection. This injury is primarily inflicted by antibody-mediated rejection (AMR), although a cellular component is also involved, especially in severe cases and during mixed rejection.^{29,48}

The histologically most obvious indicator of AMR is endothelial cell swelling (also called “ballooning”). Increased endothelial cell vacuolization and intravascular aggregates of macrophages and/or neutrophil granulocytes may be observed. Lymphocytic endothelialitis may lead to fibrinoid necrosis of the vascular wall with resulting perivascular hemorrhage and edema.^{75,76}

While routine histology may indicate AMR, immunofluorescence and immunohistochemistry are considered the gold standard for its definitive diagnosis. Evidence of

immunoglobulin (IgG, IgM, IgA) and complement factor cleavage products (C3d, C4d, C1q) deposition in capillaries, as well as positive CD68 staining of intravascular macrophages verify AMR.^{53,77}

1.4.2 Quilty effect and other histological phenomena

The **Quilty effect** is a phenomenon, which was first noticed with the advent of cyclosporine in immunosuppressive regimens. It was named after the first patient in whose biopsies it was described at Stanford University in 1981.^{78,79} No comparable phenomenon has yet been observed in other solid organ transplants, so it seems to be solely affecting cardiac allografts, in which it can be found in 10–50% of cases.^{48,80}

Morphologically, Quilty lesions are subendocardial nodular aggregates of CD20⁺ B lymphocytes with CD21⁺ follicular dendritic cells surrounded by a rim of T lymphocytes, as well as occasional macrophages and plasma cells on a background of dense endocardial collagen and prominent vascularity. Lymphoid-follicle-like structures (germinal centers, mantle zone) are common, suggesting some form of ectopic lymphoid follicle neogenesis.^{81,82} Numerous other hypotheses exist regarding the pathogenesis of this phenomenon, none of which are conclusive, e.g. an idiosyncratic reaction to cyclosporine or a lymphocytic neoplasia induced by the Epstein-Barr virus (EBV).⁸³⁻⁸⁵

Although its biological relevance is still somewhat obscure, and it does not receive therapy itself,^{80,85} Quilty effect seems to be related to both acute and especially chronic allograft rejection.⁸⁶⁻⁸⁹

Quilty lesions can be further divided into two forms. Type A is strictly confined to the endocardium and non-invasive while type B invades into the underlying myocardium and may be accompanied by myocyte damage.^{29,53} Quilty B lesions pose an important pitfall in diagnosis of rejection, as tangential sections of deeper infiltrates do not always depict the infiltrate's connection to the endocardium, making it hard to distinguish from grade 2 focal rejection—especially with concomitant myocyte damage.⁷³ In a study by Fishbein et al. it was in fact shown, that after procuring additional serial sections and immunohistochemically stained slides, 91% of biopsies previously classified as grade 2 rejection were actually Quilty B lesions.⁹⁰ This led to some controversy about the actual existence of grade 2 rejection and the possible consequences the diagnosis of grade 2 had for immunosuppressive therapy.

Separation of Quilty A and B lesions has been abandoned in 2004, as it was proven not to be of any clinical value.⁹¹ The ISHLT Working Formulation (WF) 2004 suggests the general term 'Quilty effect'.⁵³

Ischemic injury is a usual finding in EMBs in the first weeks after transplantation, where it is strongly associated with prolonged total ischemic time⁹² as well as reimplantation damage, air embolism and therapy with highly dosed catecholamines.²⁹

It may mimic myocyte damage, which occurs during rejection, but is distinguishable through some differences. Damaged myocytes and myocyte necrosis (described above) is typically sharply demarked and generally located subendocardially. The inflammatory infiltrate consists of macrophages (pigment-laden in the healing phase) and polymorphonuclear granulocytes. It may be scant or even completely missing, depending on the recipient's immunosuppressive protocol.²⁹

When recent, **previous biopsy sites** can easily be diagnosed by a superficial layer of fresh thrombus and underlying inflammatory granulation tissue. Healing biopsy sites consist of fibrous scar tissue with entrapped myocytes in disarray and possibly a mild inflammatory infiltrate. Old biopsy sites present themselves as thickened endocardial scars.^{29,48}

Opportunistic infections are mostly of bacterial origin (47%), followed by viral (41%) and fungal or protozoal infection (12%).^{93,94} Unusual inflammatory infiltrates composed of granulocytes, plasma cells and macrophages without signs of myocyte necrosis or damage are indicative of processes other than allograft rejection. *Toxoplasma gondii* trophozoites and specific changes induced by cytomegalovirus (CMV) can be identified on heart biopsies.²⁹ Apart from that, analysis of serological markers is now state-of-the-art.

1.4.3 Changes associated with chronic rejection

The most clinically significant entity associated with chronic rejection is caused by repeated inflammatory vascular injury, known as **cardiac allograft vasculopathy** (CAV). It is the leading cause of graft failure after the first year with incidence rates of 8% at 1 year, 20% at 3 years, 30% at 5 years, and more than 50% at 10 years.³ It may be present as early as 3 months after transplantation.⁹⁵

The pathogenesis of CAV encompasses cellular and humoral components as well as non-immunological factors. The graft's endothelial cells express alloantigeneic HLA

class I and class II molecules, and are thus the first target of cellular rejection.⁹⁶⁻⁹⁸ Activated T lymphocytes secrete cytokines, attracting and activating other lymphocytes⁹⁹ and monocytes/macrophages. The humoral component is the production of antibodies against the alloantigenic HLA and endothelial cell antigens.¹⁰⁰ As the endothelial cells are stimulated to express more adhesion molecules,¹⁰¹ macrophages invade the vessel intima and produce cytokines and growth factors that induce concentric thickening of the media through proliferation of VSMC and increased synthesis of extracellular matrix, eventually leading to luminal compromise.⁵² The internal and external elastic membranes tend to remain intact. CAV involves the entire length of all intra-graft vessels. As such, it may be hard to detect in coronary angiography (CAG), making intra-vascular ultrasound (IVUS) or coronary computerized tomography (CT) the preferred diagnostic tools.

1.5 Endomyocardial biopsy grading of allograft rejection

Historically, each transplantation center used its own grading scheme. The most prominent and successful example of the many classification systems is the Stanford-Classification, which was proposed by Billingham and colleagues in 1973.^{26,27} However, many other classification schemes were also used during that time, such as the ten-graded Texas-, Cleveland-, Hannover- and the Brigham-and-Women's-Utah-Classification.^{70,71,79,102} This diversity in rejection grading schemes led to poor statistical accessibility and comparability of multicenter studies.

The era of internationally standardized classification of cardiac allograft rejection began with the adoption of the first official nomenclature by the International Society for Heart and Lung Transplantation (ISHLT) in 1990.¹⁰⁴

In the present diploma thesis the ISHLT-WF1990 is primarily referred to, as it is still a widely applied grading system and all EMBs in this study were originally graded according to it. The revised 2004 nomenclature is provided separately and grade specifications are given as a 1990 grade followed by the corresponding 2004 grade in round brackets, e.g. grade 3A (2R).

1.5.1 1990 ISHLT grading system

The Stanford-Classification evolved into the Billingham Criteria,¹⁰³ which were—with further alterations—adopted by the ISHLT in 1990 as a Working Formulation (WF)

for the Standardization of Nomenclature in the Diagnosis of Heart Rejection.¹⁰⁴ The ISHLT encouraged the use of the new classification and so it was adopted quickly to general acclaim. This classification finally allowed for statistical comparability between centers, studies and the ever more important evaluation of immunosuppressive therapy.

Recommendations were issued for technical aspects, such as the handling and adequacy of biopsy specimens. A minimum of 4 to 6 biopsy pieces was postulated as required for evaluation.

Cellular rejection was categorized into five histologically distinct grades corresponding to three general categories of rejection severity (table 1-1). The main determinant of grade assignment is the density of the inflammatory infiltrate. Grade 1 and 3 were each subdivided with the suffix A or B, corresponding to specific differences in the pattern of inflammatory infiltration.

Grading of rejection was also defined by myocyte damage, which is histologically characterized. Quilty effect is subdivided into the non-invasive type A and the invasive type B. Ischemic damage is classified as either type A for up to three weeks post transplant, or type B for late ischemic damage. Although AMR was mentioned, no clear definition or grading criteria were provided.

Grade	Rejection severity	Criteria
0	No rejection	
1A	Focal mild rejection	Focal perivascular and/or interstitial infiltrate without myocyte damage
1B	Diffuse mild rejection	Diffuse infiltrate without myocyte damage
2	Focal moderate rejection	One focus of infiltrate with associated myocyte damage
3A	Multifocal moderate rejection	Multifocal infiltrate with myocyte damage
3B	Diffuse moderate rejection	Diffuse infiltrate with myocyte damage
4	Severe rejection	Diffuse, polymorphous infiltrate with extensive myocyte damage ± edema, ± hemorrhage ± vasculitis

Table 1-1: 1990 ISHLT standardized cardiac biopsy grading of acute cellular rejection.

1.5.2 2004 revised ISHLT grading system

A revision of the 1990 WF was discussed at the Sixth Banff Conference on Allograft Pathology in 2001.⁶⁹ After almost eleven years since its adoption, the apparent weaknesses of the existing grading system had become sufficiently clear.^{73,74,90,105} A new WF was finalized and approved by the ISHLT in 2004.⁵³ The main goals of the revision were to ameliorate the high interobserver variability in certain grades, a facilitation of clinical decision-making regarding changes in immunosuppressive medication through an integration of the many old grades into fewer new grades, and finally the inclusion of new criteria for the classification of cardiac allograft rejection features that in the meantime had grown in importance, such as AMR.

Minor modifications were made to the technical aspects. A minimum of three biopsy specimens is recommended for microscopic evaluation, each of which must contain over 50% myocardium and exclude previous biopsy sites or scars.

The grading system was simplified (table 1-2). The 1990 grades 1A, 1B and the highly controversial grade 2 were combined into the new common grade 1R for mild rejection. The suffix “R” was added to the new grades to avoid confusion with the 1990 grades. Grade 3A was reclassified as the new grade 2R for moderate rejection. As the histological differentiation between grade 3B and 4 did not have any effect on clinical decision-making, these grades were combined within the revised category for severe rejection, grade 3R.

The controversial characterization of myocyte damage is clarified. Quilty A and B lesions were combined under the new term ‘Quilty effect’, as the differentiation of those two subtypes was of no clinical relevance. Ischemic damage is now labeled as ‘early’, when occurring up to six weeks post-transplant, and ‘late’ with concomitant CAV. New criteria for the diagnosis of AMR were introduced: Presence of histological or immunofluorescent/-histochemical signs of AMR is marked as AMR 1, while their absence may be marked as AMR 0.

Grade	Rejection severity	Criteria
0R	No rejection	
1R	Mild rejection	Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage
2R	Moderate rejection	Two or more foci of infiltrate with associated myocyte damage
3R	Severe rejection	Diffuse infiltrate with multifocal myocyte damage ± edema, ± hemorrhage ± vasculitis

Table 1-2: 2004 ISHLT standardized cardiac biopsy grading of acute cellular rejection.

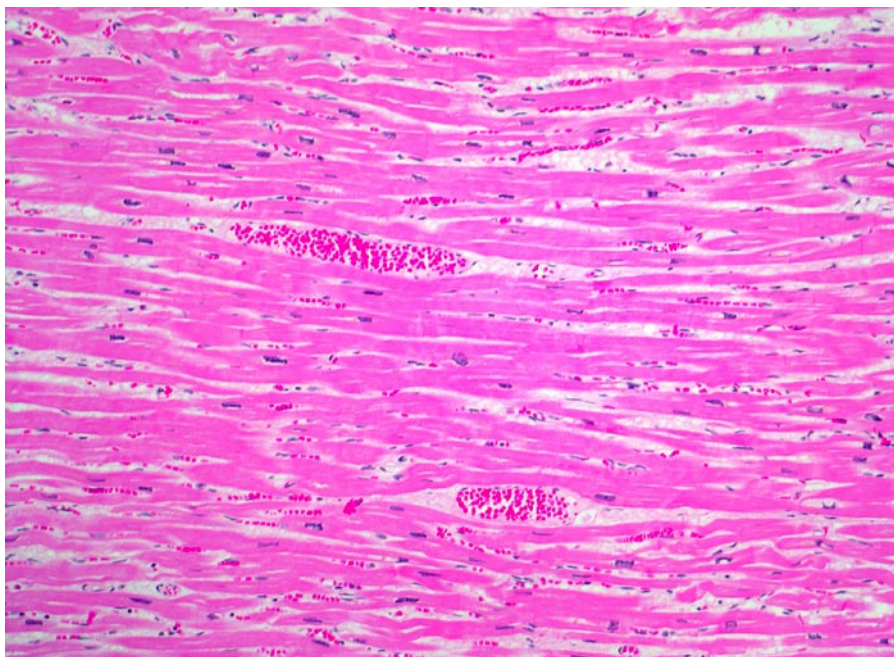


Figure 1-4: Grade 0 (0R). Normal myocardium with sparse interstitial connective tissue. No sign of inflammatory infiltration or myocyte damage (H&E). Used with friendly permission from K. Glatz-Krieger, Basel.¹⁰⁶

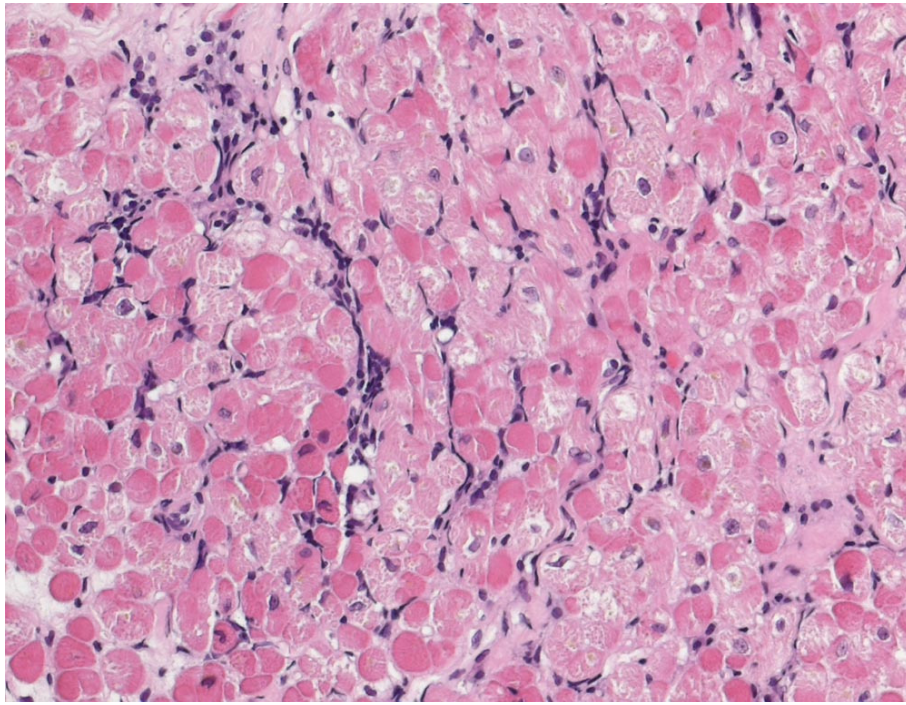


Figure 1-5: Grade 1B (1R) rejection. Intercellular inflammatory infiltrate emanating from perivascular infiltrates without myocyte damage (H&E). Used with friendly permission from K. Glatz-Krieger, Basel.¹⁰⁷

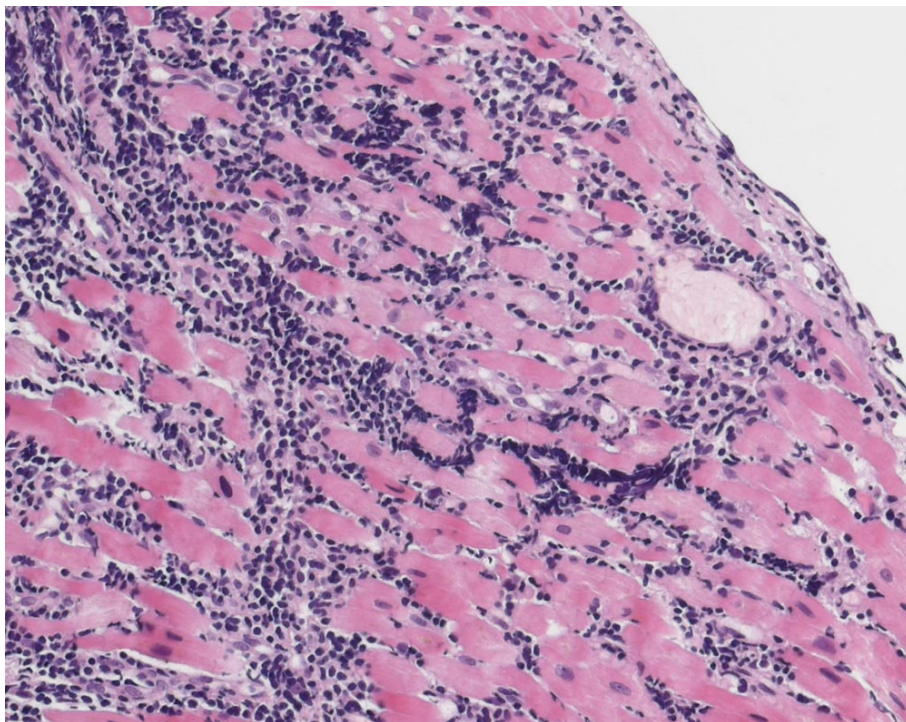


Figure 1-6: Grade 3A (2R) rejection. Diffuse infiltrate with myocyte damage (H&E). Used with friendly permission from K. Glatz-Krieger, Basel.¹⁰⁸

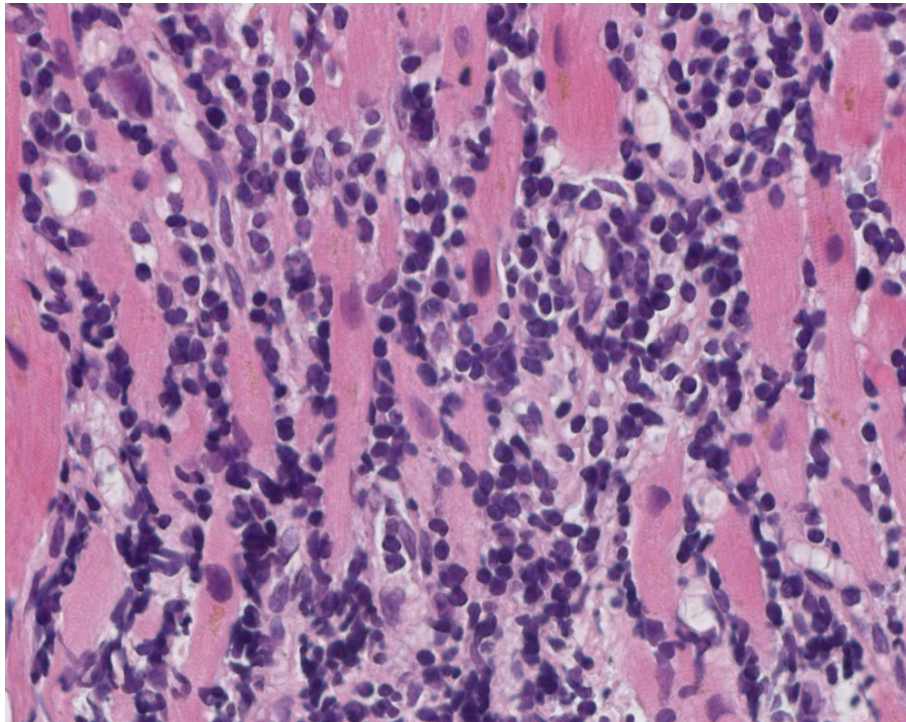


Figure 1-7: Grade 3A (2R) rejection. Same patient as in figure 1-6, further magnified. Note disruption of myocyte architecture and myocyte encroachment by the dense infiltrate (H&E). Used with friendly permission from K. Glatz-Krieger, Basel.¹⁰⁹

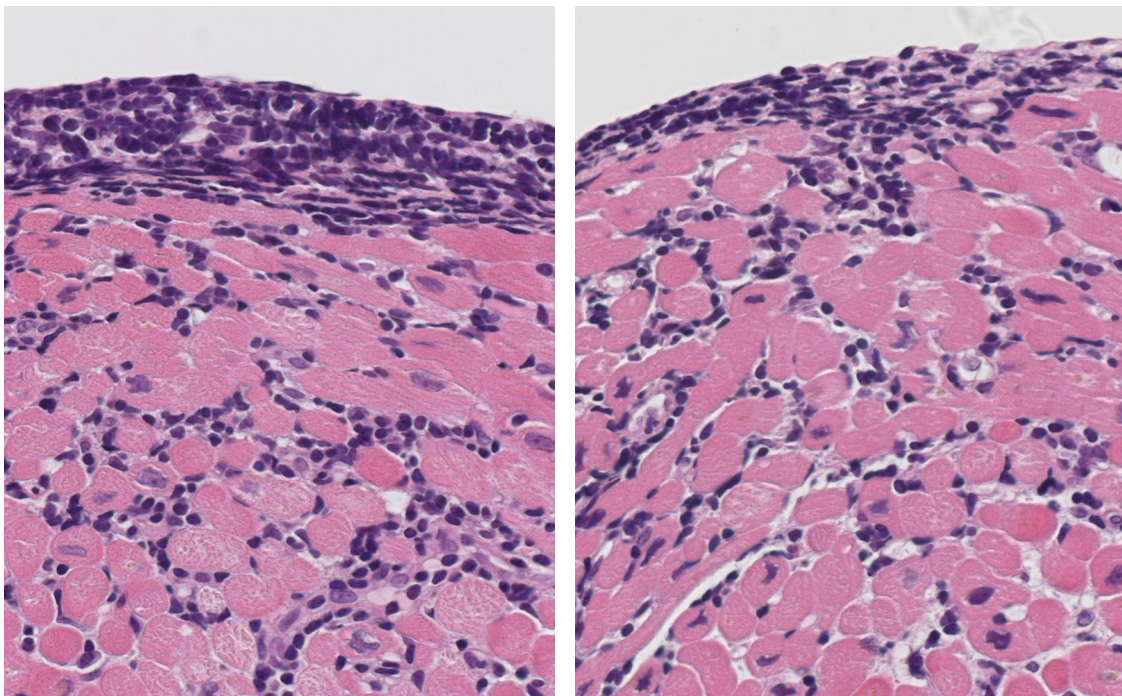


Figure 1-8: Quilty effect, classified as type B according to the IHSIT-WF1990. Dense endocardial infiltrate with invasion into the underlying myocardium that may mimic rejection-related myocyte encroachment. If the tissue section should not show the connection of the infiltrate to the endocardium, it may easily be mistaken for grade 2 (1R) rejection (H&E). Used with friendly permission from K. Glatz-Krieger, Basel.^{110,111}

1.5.3 Inter- and intraobserver variability

As discussed above, grading of acute rejection relies heavily on the estimation of inflammatory infiltrate density. This estimation by the pathologist has turned out to be a major weakness of the grading scheme. For both the 1990 and 2004 nomenclature high interobserver variability was shown for the assignment of some rejection grades.

First results regarding interobserver variability were gathered by Winters and McManus during the Rapamycin Treatment Trial, where 16 experienced trial pathologists were independently presented with 23 selected biopsies of all grades.⁷⁴ While overall interobserver agreement was high ($\kappa = 0.67$), some grading constellations with possible adverse clinical consequences exhibited higher interobserver variability: The main culprits were grade 1A/B vs. 2, grade 2 vs. 3A and Quilty B lesions vs. grade 2/3A. In conclusion, a clarification of the grading criteria for grade 2 and Quilty B lesions was suggested.

During the course of the Cardiac Allograft Rejection Gene Expression Observation (CARGO) study, Marboe et al. have compared the grading of over 800 EMBs between local routine pathologists and three expert study pathologists.⁷³ Concordance rates were poor, especially in the higher grades with a concordance rate for ISHLT grade 2 of only 17% (table 1-3). Intriguingly, even among the three expert pathologists concordance rates were strikingly low for certain grades, with a concordance rate of only 58% for ISHLT grades 3A and 3B.

Grade assigned by local routine pathologist	n	Concordance rate with study pathologists	Concordance rate among study pathologists
0	416	87%	93%
1A, 1B	174	48%	71%
2	159	17%	60%
3A, 3B	78	40%	58%

Table 1-3: Concordance rates for the 1990 ISHLT classification.⁷³

In a more recent study, Yang et al. have taken a close look at improvements of inter- and intraobserver variability between the 1990 and 2004 ISHLT classification.¹¹² 175

biopsies of all grades were reassessed by six independent pathologists and the result of the assessment was analyzed using kappa-statistics.

Calculation of the kappa value is used to quantify observer consensus under mathematical elimination of chance agreement. A kappa value of 1 indicates perfect concordance; a value of 0 indicates purely incidental concordance and negative values mark disagreement among observers.

A slight improvement was found for both inter- and intraobserver concordance rates between the 1990 and 2004 ISHLT classification (table 1-4), yet it was not statistically significant. Lack in interobserver agreement in the 2004 classification was noted to primarily concern the grading of 1R (kappa = 0.397) and 2R (kappa = 0.230) biopsies, for which the high degree of subjectivity in assessment of infiltrate density and myocyte damage were made responsible.

	1990 concordance	2004 concordance	% relative improvement
kappa	0.369 (0.323–0.415)	0.402 (0.349–0.464)	+8.8% (–0.1%–17.8%)

Table 1-4: kappa value improvement between the 1990 and 2004 ISHLT classification for interobserver variability, with bootstrap confidence intervals.¹¹² kappa = 1 indicates perfect agreement, kappa = 0 indicates agreement by chance.

As a consequence of the lingering problem of high interobserver variability, research into new diagnostic methods for detection and grading of cardiac allograft rejection is warranted.

1.6 Immunosuppressive Therapy

When Barnard performed the first successful HTX in 1967, the patient survived for 18 days before succumbing to bilateral pneumonia and severe septicemia. For prevention of allograft rejection, he received local irradiation along with the only immunosuppressive substances available at that time: intravenous and oral corticosteroids, azathioprine and actinomycin C.¹⁴ Although irradiation and actinomycin C no longer play any role, this regimen already implemented the concept of combination maintenance therapy.

ATG/ALG were introduced in the late 1960s and brought significant increase in graft survival. However, long-term survival of cardiac allograft recipients remained

poor over the next decade when maintenance therapy was primarily based on prednisone and azathioprine. The introduction of cyclosporine in 1981 and the advent of the immensely successful triple-drug maintenance therapy (prednisone, azathioprine, cyclosporine), which became the mainstay of immunosuppressive therapy for the next decades, lead to a drastic decline in mortality and allograft rejection.¹¹³⁻¹¹⁵ Another breakthrough was reached in 1986 with the adoption of muromonab-CD3 for induction therapy and treatment of severe rejection in cardiac allografts.¹¹⁶ Muromonab-CD3 was the first monoclonal antibody approved as a drug for use in humans.

The 1990s saw the discovery of a new generation of immunosuppressive substances such as mycophenolate mofetil, tacrolimus, sirolimus and various monoclonal antibodies. These substances further decreased mortality rates, rejection rates and adverse reactions.¹¹⁷

Adverse effects of immunosuppressive therapy are numerous and diverse. The most common problems connected with long-term immunosuppression are opportunistic infections, secondary malignancies and post-transplant lymphoproliferative disorder (PTLD), renal insufficiency due to nephrotoxicity (CyA, tacrolimus), gastrointestinal disorders (CyA, MMF), metabolic dysbalances (CyA, steroids), Cushingoid symptoms (steroids), bone marrow suppression (AZA), cytokine release syndrome and anaphylaxis (poly- and monoclonal antibodies).^{3,23,117,118}

Present developments in immunosuppressive therapy aim at the further reduction of mortality, morbidity from adverse effects and the prevention of CAV. Some steroid-free protocols have been described, but remain controversial.²³ Table 1-5 outlines the current array of substances at disposal for the treatment of allograft rejection.

Furthermore, other substances than immunosuppressive agents contribute to the overall therapy of rejection. Some of these supplementary substances are statins, inotropics, vasopressors, inhalative NO, antibiotics and antiviral substances.¹¹⁸

Class	Substance	Mechanism of action
Steroids	Prednisone, Prednisolone, Methylprednisolone	Inhibition of NF- κ B and AP-1 pathways: M ϕ -function ↓, T cell proliferation ↓, production of cytokines and adhesion molecules ↓, HLA expression ↓.
Calcineurin inhibitors	Cyclosporine (CyA) Tacrolimus (FK-506)	Binds to cyclophilin and blocks dephosphorylation of NF-AT by inhibition of calcineurin in T cells: IL-2 ↓, T cell proliferation ↓. Same as cyclosporine but 10–100 times more potent. Decreased rate of rejection and steroid-sparing effect vs. cyclosporine.
Purine synthesis inhibitors	Azathioprine (AZA) Mycophenolate mofetil (MMF) & mycophenolic acid (MPA)	Acts as purine analogon, inhibiting DNA synthesis: adenine, guanine ↓, T and B cell proliferation ↓. Blocks function of IMPDH, selectively inhibiting T and B cell proliferation. Decreased rate of rejection and mortality vs. azathioprine.
mTOR inhibitors	Sirolimus (Rapamycin) & Everolimus (RAD-001)	Blocks intracellular IL-2R response pathway, inhibiting DNA synthesis and cell cycle progression at G ₁ /S: B and T cells ↓, growth factors ↓, T cell activation ↓.
Monoclonal antibodies	Muromonab-CD3 (OKT3) Basiliximab & daclizumab Alemtuzumab	Specifically binds to TCR-CD3-complex on all T cells, leading to over-activation and subsequent apoptosis: T cell incapacitation and depletion. Acts as an antagonist at CD25 (high affinity IL-2R) on activated T cells: T cell proliferation ↓, B cell activation ↓. Binds to CD52 on circulating mature B and T cells, forming a potent complement activating antigen: B and T cell depletion preserving progenitor cells and bone marrow stem cells.
Polyclonal antibodies	Anti-thymocyte globulin (ATG) Anti-lymphocyte globulin (ALG) Intravenous Immunglobulin (IVIg)	Unspecific antibody mixture directed against circulating human T cells, leading to T cell depletion through induction of cell lysis. Same as ATG but directed against all human lymphocytes. Poorly understood. Pooled human antibodies mediate anti-inflammatory response: anti-donor antibodies ↓, activity of M ϕ , T and B cells ↓.

Class	Substance	Mechanism of action
Others	Plasmapheresis	Used in cases of steroid-resistant humoral rejection. <i>Ex vivo</i> removal of anti-donor antibodies from plasma by centrifugation or filtration and subsequent reperfusion.
	Photopheresis	8-methoxypsoralen inhibits <i>in vivo</i> DNA replication in B and T cells, when activated by UV-A light via selective lymphocytophoresis <i>ex vivo</i> .
	Total lymphoid irradiation (TLI)	Low-dose radiotherapy directed at major lymph node chains, thymus and spleen may reverse cellular rejection in cases of refractory therapy-resistant rejection.

Table 1-5: Overview of immunosuppressive substances used in induction, maintenance and acute rejection therapy.^{23,117,118} NF-κB: nuclear factor kappa B. AP-1: activator protein 1. NF-AT: nuclear factor of activated T cells. IMPDH: inosine monophosphate dehydrogenase. FKBP12: FK-506 binding protein 12. IL-2R: interleukin 2 receptor. UV-A: ultraviolet light type A.

Protocols for induction and maintenance therapy are diverse and vary between centers. Triple-drug immunosuppression is still the most widely used protocol. The aforementioned development of new substances has, however, widened the spectrum of different drug combinations. Tacrolimus, MMF or sirolimus are frequently used as alternatives in contemporary maintenance therapy protocols.¹¹⁸ Induction cytolytic therapy, which is now largely based on basiliximab/daclizumab and ATG/ALG,³ is usually reserved for patients who are expected to be at higher rejection risk. In the first six months of 2009, 54% of patients received induction therapy of any kind. The role of the once widely used muromonab-CD3 has greatly diminished in modern induction therapy, accounting for only 1% of cases.³

The current immunosuppressive treatment scheme at the transplant center in Graz provides a guideline for the perioperative course, combination maintenance therapy and therapy of acute rejection episodes. The recipient is administered a pre-operative loading dose of cyclosporine and azathioprine, followed by a bolus of 500 mg methylprednisolone intraoperatively. During the postoperative course, ATG is administered for 8 d (10 mg/kg/d), cyclosporine, azathioprine and methylprednisolone (4 × 125 mg/d) or prednisolone (15 mg/d).

Maintenance therapy is administered as a standard triple-drug therapy with cyclosporine (5 mg/kg/d), azathioprine (2 mg/kg/d) and prednisolone. The predniso-

lone dose may be reduced by 5 mg after one year, if the first-year surveillance biopsy is free from rejection. Further reductions are possible over the next months, if EMBs remain negative for rejection episodes. Minimum prednisolone dosage is 5 mg/d. Blood substance levels are checked on a regular basis.

Modern alternative substances such as mycophenolate mofetil, tacrolimus, sirolimus and monoclonal antibodies are used to replace classic substances, if intolerance or rejection episodes refractory to therapy should occur.

The following treatment scheme for rejection episodes is divided into stages according to rejection severity. This protocol was followed during the period in which all EMBs used in this study were obtained (1991–2007).

- Grade 1A (1R) is considered inconsequential. No change in immunosuppressive therapy is made.
- Grade 1B (1R): dosage of standard maintenance therapy is increased. Surveillance EMB for evaluation of treatment success after 1 week. If rejection grading is not improved, treatment with methylprednisolone (500–1000 mg/d) is begun for 3–5 d. Switching immunosuppressive maintenance medication may be considered.
- Grades 2 (1R), 3A (2R), 3B (3R) are treated with methylprednisolone (500–1000 mg/d) for 3–5 d.
- Grade 4 (3R) rejection warrants the use of ATG or muromonab-CD3.

1.7 Cardiac allograft chimerism

It has been shown that after allogeneic solid organ transplantation, recipient-derived multipotent mesenchymal stem cells begin to populate the graft.^{119–122} These cells can easily be visualized histologically by chromogenic or fluorescent *in situ* hybridization (CISH/FISH) of the Y chromosome in female-to-male gender-mismatched grafts.¹²³ Special immunostaining techniques or gene expression profiling can be applied for further distinction of cell types.

In the past decade, several studies have examined the immigration of non-inflammatory recipient-derived cells into cardiac allografts. Endothelial cells, VSMCs, cardiomyocytes, fibroblasts or myofibroblasts and glial Schwann cells have so far been made out the as main contributors to cardiac allograft chimerism.

Endothelial cells and VSMCs display by far the highest rates of chimerism. For endothelial cells rates between 14–42% had been reported.^{124–126} All studies concurred that, after the first month, endothelial cell chimerism remains on a constant level. VSMCs were the next largest chimerical cell type with reports ranging from 2.6–3.4%^{125,127} and an extreme finding of up to 60%.¹²⁶ In allograft with developed CAV, 3–15% of VSMCs were found to be of recipient origin.⁴⁹

Cardiomyocyte chimerism was extensively examined, as it was speculated that it might hold perspectives for the treatment of myocardial infarction and heart failure, e.g. through the intramyocardial or intravenous injection of allogeneic mesenchymal stem cells.¹²⁸ Reports for the recipient-derived cardiomyocyte share in allografted hearts vary greatly from none at all¹²⁷ to 30%¹²⁶. However, the majority of studies established cardiomyocyte chimerism at a lower level, ranging from 0.02–2.4%.^{119,125,129–131} Given the high discrepancy of these quantitative reports, concerns towards the methodological validity of some chimerism studies have been formulated, mainly pertaining to shortcomings of classic light/fluorescence microscopy versus the more modern confocal microscopy, favoring reports with higher rates of chimerism.^{129,132}

By using rat models of allogeneic heart transplantation, Wu et al. showed that immigrated mesenchymal stem cells may also differentiate into fibroblasts and myofibroblasts, possibly participating in scar tissue formation and allograft fibrosis.^{133,134}

Sympathetic and parasympathetic reinnervation of the allograft had been observed in transplant recipients and is correlated with improved response of contractile function and heart rate.¹³⁵ Minami et al. evaluated the frequency of chimerical Schwann cells and found 11.6% of these to be of recipient origin.¹²⁵

These above-mentioned observations are largely in concordance with results from CISH analysis of Y chromosome positive cells in gender-mismatched cardiac allografts in our own work, suggesting recipient origin in < 0.05% of cardiomyocytes and 10% of endothelial cells.¹

1.8 Short tandem repeats

Up to 53% of the human genome consist of repetitive DNA sequences. These portions of the genome are sometimes referred to as “junk DNA”, as they apparently do not carry any genetic information for protein-coding genes. They may, however, be in-

volved in the regulation of gene transcription. When further analyzed, these non-coding regions may be characterized as short or long interspersed nuclear elements (SINE/LINE), retroviral-like elements, DNA ‘fossils’, centromere regions, telomere regions, segmental duplications and simple sequence repeats.¹³⁶ The latter can be further distinguished by the length of their core repeat sequences. Minisatellites contain core repeat units of between 5–50 nucleotides in length, while microsatellites—also known as variable number tandem repeats (VNTR) or short tandem repeats (STR)—contain tandem repeats of up to 5 nucleotides in length, e.g. ranging from the shortest dinucleic repeats such as (CA)_n to pentanucleic repeats like (CCAGT)_n. These core repeat units are tandemly repeated from half a dozen to many dozen times. STR display a high rate of polymorphism through variation in the number of tandem repeats (i.e. having a large number of alleles), thus qualifying as excellent genetic markers.¹³⁷

The occurrence of repetitive elements in coding regions of the genome is usually connected to pathological findings in the affected individual. Misplaced trinucleotide repeats are responsible for diseases such as Huntington’s disease, several hereditary ataxias, myodystrophic disorders and the fragile X syndrome.

1.8.1 Nomenclature of STR alleles

Nomenclature of alleles for a specific STR locus is based on the number of n-nucleotide repeats. For each locus a certain set of repeats has been established in population studies. The count of n-nucleotide repeats is equal to the designated allele number. For example, the locus FGA typically has the following count of tetranucleotide repeats: 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30. Each full integer step represents one complete tetranucleotide repeat. Incomplete repeats are designated by the addition of decimal numbers (as in allele 26.2) with the decimal value representing the number of nucleotides added.¹³⁸

1.8.2 Diagnostic applications

With the rise of polymerase chain reaction (PCR) as a standard technique in molecular diagnostics, analysis of short tandem repeat polymorphism (STRP) has become a powerful method for human identity testing, relying on a well-defined set of tetranucleotide STR loci.¹³⁹⁻¹⁴¹ Applications comprise all kinds of genetic fingerprinting, e.g.

parentage testing, identification of disaster victims or missing persons, forensic casework, identification of mixed-up samples in hospitals and so on.¹⁴²

In transplantation medicine, STR analysis offers appealing additional information, as the two genetic fingerprints of donor and recipient can be quantitatively compared to one another. This special situation was first made use of in the evaluation of donor cell engraftment after hematopoietic stem cell transplantation (HSCT)¹⁴³⁻¹⁴⁵ and the diagnosis of graft vs. host disease (GVHD).¹⁴⁶ In solid organ transplantation (quantitative) STR analysis has been profitable in the assessment of graft chimerism.^{120,121}

2 Study objectives

Currently, diagnosis and grading of cardiac allograft rejection relies on the histopathological assessment of right-ventricular EMB. It has been shown, that the evaluation of infiltrate density and myocyte damage by the pathologist is somewhat subjective and prone to low reproducibility. Thus, to improve the reliability of rejection diagnosis and, in consequence, the immunosuppressive treatment of HTX patients, research into new methods is required.

Quantitative STR analysis is a reliable and well-described tool for the quantification of chimerism with frequent use in the clinical setting. This study examines the application of this method to quantify recipient-derived cells in the cardiac allograft and its relation to the current grading practice.

The ultimate goal of this study is the demonstration of quantitative STR analysis as an objective and observer-independent method for the diagnosis of cardiac allograft rejection. Towards this end, we examined the following presumptions:

1. Quantitative STR analysis of formalin-fixated paraffin-embedded (FFPE) EMB material is technically feasible and provides reliable results with sufficiently high sensitivity and specificity.
2. Recipient-derived cell content correlates with histologically diagnosed rejection grades.
3. Quilty effect has no significant influence on recipient-derived cell content.
4. Baseline non-inflammatory recipient-derived cell content remains constant over long periods of time.

Should quantitative STR analysis prove viable for the diagnosis of cardiac allograft rejection, it might represent a promising additional diagnostic tool, complementing the current standard.

3 Methods and materials

3.1 Patients

A total of nine patients were included into the study, of which three were female and six male. Patient median age at the time of HTX was 55 years (range 35 to 69 years); our assessment took place at a median of 9 years (range 7 to 18 years) from transplantation.

Originally, one more patient (UPN 9) was included but had to be removed during the course of analysis due to heavy degradation of the DNA in most samples, which made further analysis impossible (see ch. 3.7).

UPN	Gender (donor)	Age (yrs)	Year of HTX	Primary disease	EMBs	Explant samples
1	f (f)	63	2001	iCMP	7	2*
2	m (m)	76	2002	iCMP	8	1
3	m (m)	44	2000	Myocarditis dCMP	7	1
4	m (m)	60	2001	iCMP	6	1
5	f (f)	66	2002	iCMP	9	1
6	m (m)	65	1999	iCMP	8	1
7	m (m)	78	1991	iCMP	8	1
8	m (m)	77	1992	iCMP	6	3†
9	Excluded from study‡					
10	f (f)	64	1997	iCMP	7	1
subtotal					66	13
total					79	

Table 3-1: Patient data and clinical information. UPN: Unique patient number. m: male. f: female. iCMP: ischaemic cardiomyopathy. dCMP: dilated cardiomyopathy. *: Another explant sample was required for reliable recipient allelotyping. †: Additional rectal and prostate biopsy material had to be obtained from archives for reliable recipient allelotyping. ‡: see ch. 3.7

All patients received their HTX at the local Division of Transplant Surgery (Department of Surgery, Medical University Hospital Graz) and were maintained on a triple-

drug immunosuppressive regimen consisting of life-long intake of cyclosporine, azathioprine and prednisolone. Rejection episodes were treated with a pulse therapy of methylprednisolone (500 mg daily for 3 days) or muromonab-CD3 (formerly OKT3), depending on the duration and severity of rejection and response to treatment. See chapter 1.6 for detailed information regarding the immunosuppressive regimen followed at the local transplantation unit and further background on the pharmaceutical substances.

3.2 Sample management

We retrieved 79 tissue samples from nine patients from the institute's own archives (see table 3-1) for retrospective analysis. All samples were fixated in 10% buffered formalin solution and embedded in paraffin blocks for histological work-up. Sample harvesting took place in the period between 1991 and 2007.

Tissue samples were comprised of:

1. One FFPE sample of recipient origin. Usually, this was a tissue sample taken from the explanted heart. These samples were prepared after a pathological inspection of the heart and originated either from the interventricular septum or the ventricular myocardium.

In one case (UPN 1), samples from both locations had to be analyzed to acquire usable DNA for recipient allelotyping.

In another case (UPN 8) the cardiac tissue sample was analyzed, but no usable recipient DNA could be obtained due to the high grade of DNA-degradation. The recipient DNA was extracted from a rectal and prostate biopsy of the same patient instead.

2. Right-ventricular endomyocardial biopsies ranging from rejection grade 0 (0R) to grade 4 (3R). These were taken either during scheduled routine surveillance examinations following transplantation or whenever an acute event of allograft rejection was suspected.

An experienced pathologist at the Institute of Pathology, MUG, subsequently graded all biopsies through standard light field microscopy of multiple H&E stained slides. Originally, the 1990 ISHLT grading system was applied for all biopsies. A reassessment according to the 2004 revised ISHLT nomenclature was performed before the commencement of our study.

12 of these EMBs were diagnosed positive for Quilty effect (see ch. 1.4). As recommended, Quilty A and B lesions were combined under the new label 'Quilty effect'.⁵³ Its occurrence was registered for later analysis of the effect's impact on the method.

1990 ISHLT nomenclature	Number of EMBs	Quilty effect positives
0	22	none
1A	10	2
1B	9	2
2	8	1
3A	10	5
3B	5	2
4	2	none
total	66	12

Table 3-2: Frequencies of different rejection grades and number of Quilty effect positives according to the 1990 ISHLT nomenclature

2004 ISHLT nomenclature	Number of EMBs	Quilty effect positives
0R	22	none
1R	27	5
2R	10	5
3R	7	2
total	66	12

Table 3-3: Frequencies of different rejection grades and number of Quilty effect positives according to the revised 2004 ISHLT nomenclature

Biopsies for each patient were retrieved according to the following scheme:

- One biopsy taken one week after transplantation. This biopsy was used to determine the donor's genetic fingerprint by subtracting recipient alleles, which were determined previously. All of these EMBs were graded 0 (0R) and were thus expected to display minimal recipient-derived cell share, further increasing the power of recipient/donor-discrimination.

- One biopsy taken one month after transplantation.
- One biopsy taken one year after transplantation.
- One biopsy taken five years after transplantation, if available.
- All other EMBs of each patient for every suspected event of allograft rejection of any grade.

Time point after transplantation	Number of EMBs
1 week	9
1 month	8
1 year	9
5 years	6

Table 3-4: Frequencies of grade 0 (OR) biopsies selected for different time points after heart transplantation

3.3 Sample preparation

For obtaining tissue material from the archival paraffin blocks, we deployed a rotary microtome (Mikrom), which was cleaned meticulously before and after each use for our project. Before commencing work on a new paraffin block, all parts of the microtome that may come into contact with sectioned tissue material (blade, blade fixator, block holder, all immediate surrounding surfaces) were cleaned to avoid contamination with extraneous DNA.

3.3.1 Sample preparation protocol

The following steps for cleaning and sectioning were followed through:

1. The parts of the microtome described above were disassembled and wiped with a fresh absorbent cleaning tissue soaked in *p*-xylene (Sigma-Aldrich) to dissolve adherent paraffin residues.
2. The same parts were wiped freely with the denaturing agent LTK-008 (Biodelta), to assure freedom from DNA, RNA, DNase, RNase, bacteria and phages.
3. The first step was repeated to remove the remaining LTK-008/paraffin emulsion.

4. The blade holder was reassembled and a new microtome blade (Feather) was inserted. It was carefully wiped with LTK-008.
5. For each block 10 sections were made at 7 μm thickness and the material was transferred into a sterile 1.5 ml microcentrifuge tube (Eppendorf), which was previously marked for identification. This step was performed twice for each block, resulting in a sample-pair for each paraffin block. These connected samples are referred to as 'sample pairs' in the following text.
6. The used blade was discarded and after the cleaning procedure described in steps 1–4, a new blade was inserted.
7. Disposable gloves (Sempermed) were changed after each of these work-cycles. Throughout the whole procedure, special care was given not to contaminate the work area. Laboratory windows were closed to minimize chance of aerosol and particle carryover.

An exception was made for the samples used for recipient allelotyping, i.e. the samples taken from the explanted heart. Because of the abundance of tissue material, we made only one section at 7 μm thickness, thus making the tissue volume identical to that of the EMBs. This step also was performed twice per block.

Additional sections were made of a blank paraffin block not containing embedded tissue. These samples were used to check for potential baseline paraffin material contamination and also to obtain a blank value for later spectrophotometry.

The material was stored at room temperature until further processing.

3.4 DNA-extraction and purification

FFPE material is not an ideal source of genomic DNA. During fixation, formalin causes cross-linking, creating DNA-protein-complexes that usually can only be dissolved to a certain degree by heat treatment which, in turn, reduces DNA quality. The age of archival FFPE material also has great influence on extraction efficiency. DNA fragments extracted from FFPE material—even under ideal conditions—are usually not longer than 650 bp.¹⁴⁷

3.4.1 Purification protocol

For purification of the genomic DNA, we employed the QIAamp DNA mini kit (Qiagen). This kit provides an easy method of DNA-extraction and purification through multiple steps of sample washing with different buffers and final elution through the use of elution columns containing silica membranes for specific adsorption of DNA. This system ensures the absence of cell debris, proteins and substances inhibiting PCR or other downstream enzymatic reactions in the final eluate.

For tissue deparaffinization and DNA purification we observed the following protocol:

1. The paraffin sections contained within the microcentrifuge tubes were suspended in 1200 μ l *p*-xylene and vortexed vigorously for 15 sec. The microtubes were then centrifuged at 14,000 rpm for 10 min. The supernatant was pipetted off and discarded. Care was taken not to remove parts of the tissue pellet. This step was repeated twice.
2. 1200 μ l absolute ethanol were applied to each microtube. The pellet was resuspended by pulse-vortexing for 15 sec. The sample tubes were then centrifuged at 14,000 rpm for 10 min. The supernatant was pipetted off and discarded. This step was repeated once.
3. The microtubes were left open and incubated at 37 °C for 30 min until the remaining ethanol had completely evaporated.
4. Each tissue pellet was then resuspended in 180 μ l buffer ATL and 20 μ l proteinase K. The fluid was mixed vigorously by pulse-vortexing for 30 sec. To ensure complete tissue lysis, the microtubes were then placed in a thermomixer (Eppendorf) and incubated over night at 56 °C and 900 rpm mixing speed.
5. The following morning, the microtubes were retrieved and centrifuged at 8000 rpm for 1 min to remove drops from inside the lid.
6. 200 μ l buffer AL were added to each tube and mixed by vortexing for 15 sec. The microtubes were then incubated at 70 °C for 10 min and centrifuged at 8000 rpm for 1 min to remove drops from inside the lid.
7. 200 μ l absolute ethanol were added to each tube. The microtubes were vortexed for 15 sec and centrifuged at 8000 rpm for 1 min to remove drops from inside the lid.

8. **DNA Binding:** QIAamp Mini spin columns were attached to 2 ml collection tubes and the samples from step 7 were transferred into the elution columns. Care was taken not to touch the silica membrane or wetting the rim with the pipette tip. These column-tube assemblies were centrifuged at 8000 rpm for 1 min. Clean 2 ml collection tubes were attached to the elution columns. The used tubes containing the filtrate were discarded.
9. **Washing step 1:** 500 μ l buffer AW1 were applied to each column. Column-tube assemblies were centrifuged at 8000 rpm for 1 min. Clean 2 ml collection tubes were attached to the elution columns. The used tubes containing the filtrate were discarded.
10. **Washing step 2:** 500 μ l buffer AW2 were applied to each column. Column-tube assemblies were centrifuged at 14,000 rpm for 3 min. Clean 1.5 ml collection tubes (Bio Plas) were attached to the elution columns. The used tubes containing the filtrate were discarded.
11. **Elution:** 200 μ l buffer AE were applied to each column for DNA elution and incubated at room temperature for 1 min. The column-tube assemblies were centrifuged at 8000 rpm for 1 min.

After completion of the purification procedure, we measured the DNA concentration for each sample-pair using a NanoDrop 1000 spectrophotometer (Thermo Scientific). For all measurements 2 μ l of sample eluate were placed on the sample pedestal, calibration was checked initially and intermittently through blank measurements with ddH₂O and eluate from blank paraffin samples. DNA purity was determined by measuring the $A_{260/280}$ ratio: dsDNA has a maximum absorbance at 260 nm, while proteins and other contaminants predominately absorb at 280 nm (absorbance maximum of amino acids). For pure dsDNA, the $A_{260/280}$ ratio is ~ 1.8 .

After DNA quantification the samples were stored at -20°C .

3.5 Multiplex PCR and gel electrophoresis

Since its invention by the Cetus Corporation researcher Kary Mullis in 1983, the polymerase chain reaction (PCR) has become one of the most widely used techniques in molecular biology.¹⁴⁸ Today, PCR is an indispensable prerequisite for DNA cloning, sequencing, mutation detection, genome analysis, detection of viral and bacterial infections and countless other applications. As mentioned in ch. 1.8.2, genomic finger-

printing (which includes RFLP- and STR-analysis) is now a standard forensic and clinical method based on PCR and thus also finds use in our study.

3.5.1 Characterization of STR loci

Purified genomic DNA was used as a template for amplification of nine tetrameric STR markers and one gender marker by multiplex STR-PCR using the AmpF ℓ STR Profiler Plus Kit (Applied Biosystems). This kit includes primer-pairs for the amplification of the following polymorphic STR-Loci:

- D3S1358, described by Li et al., 1993 (GeneBank accession number: AC099539)¹⁴⁹
- vWA (von Willebrand Antigen), a polymorphic STR-locus described by Kimpton et al., 1992 (GeneBank accession number: M25858)¹⁵⁰
- FGA (human fibrinogen α), described by Mills et al., 1992 (GeneBank accession number: M64982)¹⁵¹
- D8S1179, described by Oldroyd et al. in 1995 and preliminarily designated D6S502 (GeneBank accession number: G08710)¹⁵²
- D21S11, described by Sharma and Litt, 1992 (GeneBank accession number: M84567)¹⁵³
- D18S51, described by Urquhart et al., 1995 (GeneBank accession number: X91254)¹⁵⁴
- D5S818 and D13S317, both loci were first described by Hudson et al., 1995 (GeneBank accession numbers: G08446, G09017)¹⁵⁵
- D7S820, described by Green et al., 1991 (GeneBank accession number: G08616)¹⁵⁶
- A 6 bp deletion within intron 1 of the Amelogenin (AMG) gene on the X-chromosome homologue, allowing for gender identification¹⁵⁷

The kit manufacturer selected these loci for maximum selectivity, certainty of mendelian inheritance, frequency and polymorphism (based on prior population studies), equal reaction times and temperatures for PCR and relatively same bp-length, frequent heterozygosity as well as the preclusion of preferential amplification.¹³⁸

For general information about the molecular biology of STR, see ch. 1.8.

Locus designation	Chromosome location	Common sequence motif	Size length (bp)*	Dye label
D3S1358	3p	TCTA (TCTG) ₁₋₃ (TCTA) _n	114–142	5-FAM
vWA	12p12-pter	TCTA (TCTG) ₃₋₄ (TCTA) _n	157–197	5-FAM
FGA	4q28	(TTTC) ₃ TTTT TTCT (CTTT) _n CTCC (TTCC) ₂	219–267	5-FAM
Amelogenin	X: p22.1–22.3 Y: p11.2		X: 107 Y: 113	JOE
D8S1179†	8	(TCTR) _n ‡	128–168	JOE
D21S11	21	(TCTA) _n (TCTG) _n [(TCTA) ₃ TA (TCTA) ₃ TCA (TCTA) ₂ TCCA TA] (TCTA) _n	189–243	JOE
D18S51	18q21.3	(AGAA) _n	273–341	JOE
D5S818	5q21–31	(AGAT) _n	135–171	NED
D13S317	13q22–31	(GATA) _n	206–234	NED
D7S820	7q11.21–22	(GATA) _n	258–294	NED

Table 3-5: STR-Loci included in the AmpF ℓ STR Profiler Plus kit.¹³⁸ *: basepair length including 3'-A-addition.

†: Also designated D6S502 in some literature references. ‡: R represents either an A or G nucleotide.

3.5.2 Fluorescent dye labels

Primers were labeled with fluorescent dyes to enable later LIF-detection (Laser-induced fluorescence) of PCR products. For this, the N-Hydroxysuccinimide (NHS) esters 5-FAM, JOE and NED were conjugated to the 5' ends of the primer oligonucleotides.

Fluorescent dye label	Color	Excitation wavelength (nm)	Emission wavelength (nm)
5-FAM	Blue*	494	522
JOE	Green	520	548
NED	Yellow	546	575
ROX†	Red‡	575	602

Table 3-6: Dye colors and wavelengths. *: According to manufacturer specification. Emission wavelength actually lies in the green color spectrum (490–575 nm). †: Used as internal-lane sizing standard in CAE (see ch. 3.6.2). ‡: According to manufacturer specification. Emission wavelength actually lies within the orange color spectrum (585–650 nm).

STR loci with overlapping fragment length ranges were labeled with different spectrum fluorescent dye labels. This allows for same-size locus differentiation in capillary electrophoresis. Excitation and emission wavelengths for the dyes specified above are provided in table 3-6.

Of each primer-pair only one primer is dye-labeled. During capillary array electrophoresis (CAE) the complementary DNA strands are kept separated into ssDNA through the addition of strong denaturants such as formamide and urea, preventing strand reannealing and secondary DNA-conformation phenomena such as intra-strand-looping and intercalation. However, it had been observed that complementary ssDNA strands show differences in electrophoretic mobility during CAE. When evaluating the sensitivity and accuracy of STR genotyping, Mansfield et al. “observed very systematic sequence-dependent migration differences at most STR loci even when CAE separations were run under denaturing conditions.”¹⁵⁸ This effect can produce doublet peaks much like in SSCP studies, hindering reliable allelotype determination. By labeling only one of the complementary ssDNA strands, this problem is avoided.

3.5.3 STR-PCR protocol

DNA amplification was performed with a standard 96-well GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) using a final volume of 26.5 µl/tube consisting of:

- 16.5 µl/tube PCR master mix, which in turn consists of:
 - 10.5 µl/tube AmpF ℓ STR PCR Reaction Mix, containing dNTP (dATP, dCTP, dGTP, dTTP), BSA, 0.05% NaN₃, Tris-HCl (pH 8.3), MgCl₂ and KCl.
 - 5.5 µl/tube AmpF ℓ STR Profiler Plus Primer Set, containing fluorescent dye-labeled primers and non-labeled primers for the STR loci described in Table 3-5.
 - 0.5 µl/tube AmpliTaq Gold DNA polymerase. The manufacturer specifies enzyme activity as 5 U/µl.

The master mix was prepared first and aliquoted to the individual reaction tubes.

- 10 µl/tube template DNA, diluted to four different concentrations per sample (*vide infra*).

- Positive control: 10 μ l AmpF ℓ STR Control DNA 9947A (0.10 ng/ μ l) human cell line DNA in 0.05% NaN₃ and buffer.
- Negative control: 10 μ l TE-buffer. The negative control was prepared as the last step to provide contamination detection for all preceding steps.

The input DNA concentration depended on respective DNA concentration of the purified eluate, which was determined by spectrophotometry. For each sample pair a dilution series of four different input concentrations was arranged around the estimated optimum concentration.

- If initial DNA concentration was high (> 10 ng/ μ l), input range was varied around 5–100 ng. The lower half of the concentration range (5–50 ng) ensured good amplification results of small fragments without the risk of over-amplification, while the higher half (50–100 ng) was useful for amplification of the more heavily degraded longer DNA fragments.

Typical example: 10 ng and 50 ng from the first eluate of the sample pair with slightly higher initial DNA concentration; 25 ng and 75 ng from the second eluate with slightly lower initial DNA concentration.

- Samples with low initial DNA concentration after purification (< 10 ng/ μ l) were varied around 75–300 ng to provide maximum yield in PCR.

For example: 75 ng and 150 ng from the first eluate of the sample pair and 100 ng and 200 ng for the second eluate.

The AmpliTaq Gold DNA polymerase is the recombinant product of the *Thermus aquaticus* DNA polymerase (Taq Pol I) gene produced by and extracted from a hybrid *Escherichia coli* host.¹⁵⁹ It has been chemically altered to remain inactive until being initially incubated at 95 °C for 11 min. This feature prevents the undesirable extension of misprimed primers that can occur at room temperature before the actual PCR is started.¹⁶⁰ Therefore, an initial incubation step was included to activate the AmpliTaq Gold DNA polymerase (hot start PCR) and provide sufficiently high temperatures to suppress primer annealing to non-target sequences.

After 28 cycles of amplification the samples were incubated at 60 °C for 1 hr to ensure complete 3'-adenosine-addition. This addition of a single dATP at the 3' end of the extension product is a characteristic feature of the Taq DNA polymerase enzyme (terminal transferase activity).¹⁶¹

Table 3-7 provides the detailed amplification protocol.

Initial incubation step	28 cycles each			Final extension	Final step
	Melt	Anneal	Extend		
95 °C for 11 min.	94 °C for 1 min.	59 °C for 1 min.	72 °C for 1 min.	60 °C for 60 min.*	25 °C ad inf.

Table 3-7: Thermal cycler amplification protocol. *: Here we deviated from the recommended protocol given in the kit manual and increased the final extension step from 45 min to 1 hr to ensure full 3'-A-addition (see ch. 3.5.3).

3.5.4 Gel electrophoresis

Immediately after amplification, electrophoresis was performed to determine the quantity and quality of the amplified DNA, and especially the efficiency of amplification for the larger fragments (> 200 bp) such as FGA, D18S51, D13S317 and D7S820. Amplification was less effective in these loci, which can be attributed to impaired elongation of degraded template DNA.

As sieving matrix we used 25-slot 12% Poly(NAT) gels (Elchrom Scientific) in 0.75× TAE running-buffer (30 mM).

Electrophoresis was prepared following these consecutive steps:

1. For each gel slot, a 0.2 ml reaction tube was prepared into which 2 µl loading buffer (Elchrom Scientific) were transferred.
2. 1.5 µl 100 bp DNA ladder (GenSura Laboratories) was added and transferred into the first slot of each gel used.
3. 4.5 µl/tube PCR amplificate were added.
4. 4.5 µl positive control was transferred from the corresponding PCR tube. Each gel received one positive control for better comparison of band fluorescence intensity.
5. 4.5 µl negative control was transferred from the corresponding PCR tube.

The remaining PCR product was stored at 6 °C for no more than 24 hrs.

Voltage was applied at 120 V for 1 hr. Gels were detached from the plastic foil backing with a nylon string plate and dyed in 1× SYBR Gold in 1× DMSO (Invitrogen; stock solution stored at –20 °C) and 0.75× TAE buffer solution. For staining, the gel was placed in a container on a platform shaker and agitated gently for 1 hr. SYBR Gold diffuses very well through the thin Poly(NAT) gels and binds to nucleic acids with

high specificity. After staining, the remaining dye was removed by washing the gels in ddH₂O *ad lib.* on a platform shaker for 1 hr.

SYBR Gold has a secondary excitation wavelength maximum in the UV-light spectrum at ~300 nm (the primary maximum being at 495 nm) and emits in the green color spectrum at an emission wavelength of ~537 nm.

The gel was placed on a 312 nm UV transilluminator (Intas, Germany) and fluorescence was photographed using a high-resolution CCD-Camera, applying the appropriate filters as recommended by the dye manufacturer. The image data was stored on a local hard disk and a printout of the image was made.

PCR performance for each sample was evaluated by comparison of band fluorescence intensity for smaller and larger fragments with the positive control serving as standard. The results were used to determine DNA input quantity for capillary electrophoresis (see ch. 3.6.2).

3.6 Capillary array electrophoresis

This method was introduced in 1959 by Hjertén, who laid the groundwork for capillary electrophoresis (CE) of various substances, including nucleic acids.¹⁶² It took until the early 1990s, when advances in capillary design and information technology made it more easily applicable, that this method received a considerable increase of attention. It became a more effective and efficient alternative to slab-gel electrophoresis, yielding much higher resolution and higher throughput capacity than conventional slab gel electrophoresis. Capillary electrophoresis has since become a standard technique for mutation detection (RFLP, SSCP), DNA sequencing, genetic analysis (such as STR analysis) and fragment length analysis.^{162,163}

Whereas CE originally utilized only one capillary, capillary array electrophoresis further increases throughput capacity by running multiple samples simultaneously by using multiple capillaries. These arrays currently can hold up to 96 capillaries.

3.6.1 General principle

For conducting CAE we used the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) with a 50 cm quadruple fused-silica capillary array (Applied Biosystems) and ABI PRISM 3100 POP-6 polymer (Applied Biosystems).

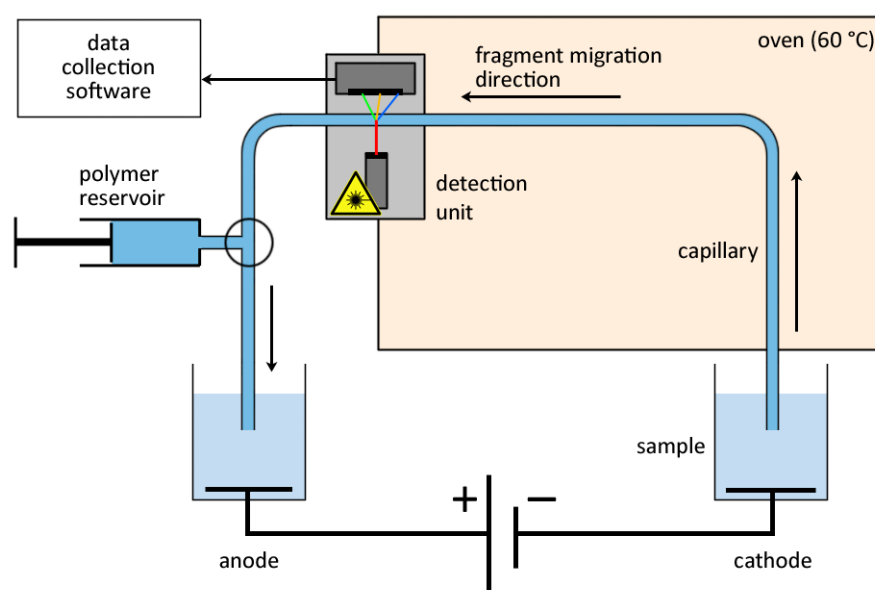


Figure 3-1: General principle of capillary electrophoresis.

Four DNA samples are electrokinetically injected from their corresponding plate wells into four thin fused-silica capillaries (internally uncoated, 50 cm length, 50 μm internal diameter) filled with polymer as sieving matrix. The negatively charged DNA migrates through the polymer following the electric current from cathode to anode and becomes separated according to molecule size. Figure 3-1 shows a simplified scheme of a single-capillary CE apparatus. The POP-6 polymer uses a special silica-adsorbing polymer to suppress two phenomena occurring in capillary electrophoresis: electroosmotic flow (EOF) and the related interaction of analyte with the capillary wall.

EOF occurs in liquids between two electrodes, when an electric current is applied. In CE, it is intensified by buffer-ions reacting with the capillary wall. The internal interface of an uncoated fused silica capillary consists of negatively charged ionized silanol (SiO^-) groups. These attract cationic species from the buffer, depending on buffer pH, forming an electrical double-layer adjacent to the capillary wall. This layer is dividable into a static plane (Stern-Layer or Inner Helmholtz Plane) in direct interaction with the capillary wall, and a mobile plane (Outer Helmholtz Plane). Cations in the mobile plane will move towards the cathode during electrophoresis, effectively moving in the different direction as the negatively charged DNA. However, cations do not move alone; they drag other molecules with them through the formation of hydrogen bonds. This effect can affect the elution time of DNA fragments. Also, the EOF

may not be constant along a capillary, making it inaccessible to prediction. Suppression of EOF is therefore favorable in CE of nucleic acids.^{162,163}

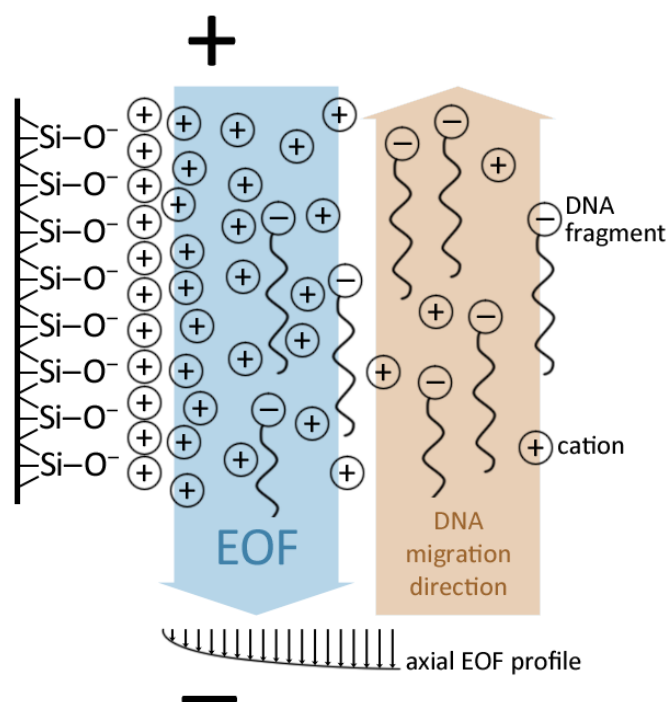


Figure 3-2: Schematic illustration of electrical conditions near the capillary wall, the genesis of electroosmotic flow and its relation to DNA migration.

It has further been demonstrated, that smaller DNA fragments are more likely to directly interact with the capillary wall and the associated electrical double-layer, also negatively affecting elution time of these fragments.¹⁶³

Silica-adsorbing polymers create a coating on the capillary wall that impedes the formation of an electrical double layer or analyte–capillary wall interaction through its high viscosity and binding to ionized SiO^- groups.

The polymer in the capillaries was automatically disposed after every run and the array was filled with fresh polymer for the next run from a reservoir syringe integrated into the system.

At the end of the capillary array, a Laser beam is directed through the capillaries and induces fluorescence of the dye labels attached to the DNA fragments (Laser-induced fluorescence, LIF). This fluorescence is registered separately for each capillary by a photosensor and converted into a digital signal. The signal intensity is plot-

ted against time; the intensity of LIF also allows inference on the quantity of the detected fragment.

The argon-ion Laser used in the detection chamber emits at two primary wavelength maxima, 488 and 514.5 nm, causing the atoms in the dye labels to shift from the ground state with a normal energy level to an excited state with a higher energy level. More specifically, this excitation is caused by the electrons in atomic orbitals that absorb the energy of colliding photons emitted by the Laser and thus jump into higher orbits. After an extremely short period of time (usually nanoseconds) these electrons deexcite, i.e. they fall back to their previously occupied lower orbit and, in turn, emit the excess energy in form of a photon with characteristic wavelength (figure 3-3). Table 3-6 gives an overview of excitation and emission wavelengths of the respective dyes used in our assay. The different emission wavelength maxima allow for a differentiation of the associated dye label, even when different dye labels traverse the excitation beam in the same capillary and at exactly the same point of time.

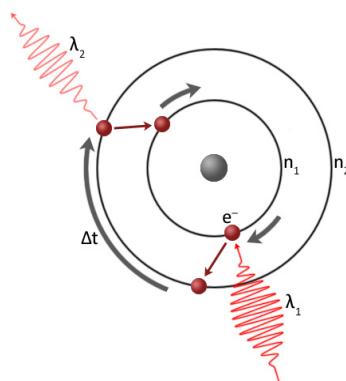


Figure 3-3: Mechanism of excitation and deexcitation. $n_{1,2}$: atomic orbitals. e^- : electron. $\lambda_{1,2}$: excitation and emission wavelengths. Δt : time from excitation to deexcitation.

As figure 3-4 illustrates, the emission spectra for the four dye colors are overlapping, creating an area of uncertainty for specific determination of correct wavelength-to-dye relation. This problem is avoided by application of a predetermined matrix file (Matrix Standard Set DS-32) by the data collection software, which mathematically describes the maximum wavelength tolerance around the emission maximum for each dye label.

The emitted wavelength-conglomerate for each of the four capillaries is filtered in the spectral dispersion device and focused on a CCD photosensor. There it is regis-

tered separately at a sampling rate of 429/min (one measurement every 140 ms) and converted into a digital signal. The raw data is gathered by the data collection software and stored on a hard disk. After run completion, this data can be transferred to a computer workstation for analysis.

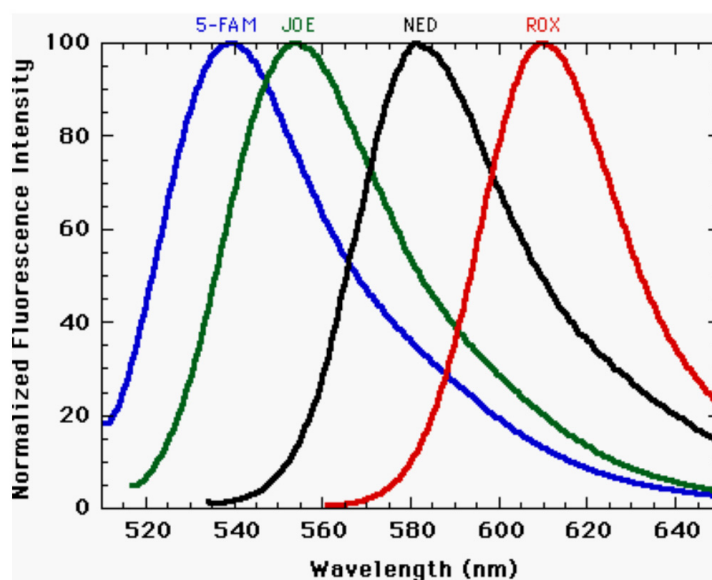


Figure 3-4: Emission wavelength overlap of fluorescent dye labels. Note especially the elongated trailing emission of 5-FAM and its close proximity to the next color label, JOE.¹³⁸

3.6.2 CAE protocol

For capillary gel electrophoresis we prepared a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems) with a previously determined amount of Hi-Di-Formamide (Applied Biosystems) and GeneScan-500 ROX Size Standard (Applied Biosystems).

The amount of PCR product added to each well was determined empirically by the performance of each sample-pair (as described in ch. 3.3.1: two DNA samples were extracted from each paraffin block) in gel electrophoresis. This was evaluated for each sample by band separation, band intensity and comparison with connected dilution series of the same sample. The positive control on each gel provided a standard for orientation.

Input DNA volume was established according to this scheme:

- Strong amplification of short (< 200 bp) and long (> 200 bp) fragments:

- Input volume was varied between 0.5–1.2 μ l.
 - Over-amplificated samples were diluted 1:2 or 1:4 with formamide.
- Strong amplification of short fragments, low amplification of long fragments:
 - Input volume was varied between 1.2–2 μ l.
- Low amplification of short and long fragments:
 - Input volume was varied between 2–3 μ l.
 - The affected sample was either re-run in PCR with a higher template volume, or extracted and purified from scratch.

Highly deionized Hi-Di-Formamide (Applied Biosystems) was used as injection solution, ensuring separation of DNA strands and electrical conduction between cathode, DNA and capillary polymer during sample injection. Formamide was stored at -20°C and thawed at room temperature prior to use.

GeneScan-500 ROX Size Standard (Applied Biosystems) served as internal-lane sizing standard. This dye label is attached to synthetic ssDNA fragments with a defined length of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 bases (figure 3-6). The size standard was run with every sample to normalize lane-to-lane or injection-to-injection fragment migration differences. This allows for accurate size calling of DNA fragments running in the same capillary but labeled with different dyes. ROX 500 was stored at 6°C and vortexed prior to use.

The allelic ladders were mixed together previously. This allelic ladder mix contained AmpF ℓ STR Blue, AmpF ℓ STR Green II, and AmpF ℓ STR Yellow Allelic Ladders containing markers for all alleles in their respective STR loci.

The first 4 wells received only a mix of formamide and ROX 500 with no addition of sample DNA. This provided one blank capillary array run. This run was used to once more rinse the capillaries and check for air bubbles in the polymer column.

Plate preparation was performed as follows:

- 20 μ l/well Hi-Di Formamide
- 0.75 μ l/well ROX 500
- 2 wells allelic ladder mix, receiving 2 μ l and 3 μ l respectively
- 2 wells positive control receiving 1.2 μ l and 1.4 μ l amplified AmpF ℓ STR Control DNA 9947A

- 0.5–3 μl /well sample DNA was transferred according to sample performance in gel electrophoresis as described above. Different dilutions, i.e. input quantities, were applied to a maximum of four wells per sample (figure 3-5).

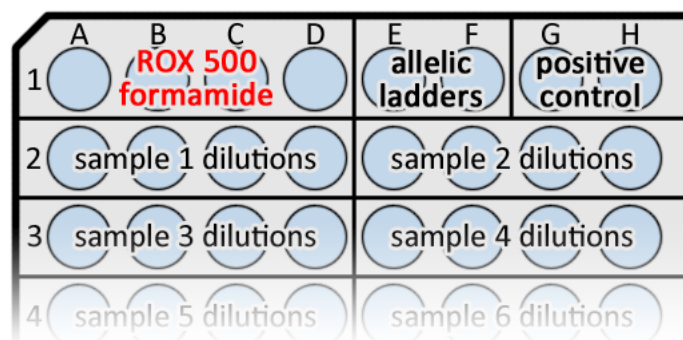


Figure 3-5: CAE plate layout and pipetting scheme.

For DNA denaturizing, the plate was then placed in a Geneamp 2720 thermocycler (Applied Biosystems) at 96 °C for 5 min and subsequently cooled down to 4 °C until retrieval. The added formamide prevented the separated DNA strands from reannealing.

The plate was then placed in a centrifuge and spun down for 10 sec at 600 rpm to remove condensate drops from the plate septa (a fitted PVC covering for prevention of contamination) and placed in the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) for capillary array electrophoresis.

The following parameters were set before initiating CAE:

- A sample sheet was created, containing run data and procedure information, and imported into the data collection software.
- Dye Set: F
- Matrix Standard Set: DS-32 (5-FAM, JOE, NED, ROX)
- Oven temperature: 60 °C. As CE is a temperature-sensitive process, the oven provides the optimal run temperature for fragment length analysis.
- Injection voltage: 15 kV
- Injection time: 10 sec
- Run voltage: 15 kV
- Run time: 28 min

During analyzer runs the ambient temperature was kept at ~21 °C to provide the optimal operating temperature for the analyzer and especially for the incorporated Laser, ensuring steady readings.

3.7 Data processing

Raw data was transferred from the genetic analyzer to a Windows NT (Microsoft) computer workstation (Dell) running the Genescan 3.1 software (Applied Biosystems) for further analysis.

Raw data comprised electropherograms of each capillary run, presenting as x-y-diagrams capturing run sampling instances (equaling run time) on the x-axis and relative fluorescence units (RFU) on the y-axis. For each dye label a separate graph was plotted into the diagram. For analysis, the software automatically separated these different graphs by color, performed fragment size calling and designated alleles accordingly.

Fragment sizing is performed by calculation of sample fragment size between two adjacent sizing standard peaks. The aforementioned internal-lane sizing standard ROX 500 produces peaks for defined fragment sizes (figure 3-6). The size of all other fragments of unknown length can then be accurately calculated by different methods of interpolation, extrapolation, multiple linear regression analysis and fragment migration characteristics (Least square method, cubic spline interpolation, local or global Southern). Local Southern method is recommended by the PCR kit manufacturer and was used in this study. After the application of the size standard, the electropherograms display the accurate base pair size on the x-axis.

The Genescan software then also automatically performed allele designation by mapping the allelic ladder over the now correctly sized sample run. Alleles calls were then designated by fragment size and corresponding allele STR count (see ch. 1.8.1).

DNA concentration has some effects on CAE run performance.¹³⁸ Low DNA input concentration results in low peak readings and high background noise levels. The minimum peak height threshold for quantitative analysis was set at 150 RFU, smaller peaks were considered inaccurate and were thus omitted.

On the other hand, excessive DNA input concentrations produce off-scale data and dye color 'bleed through', a phenomenon that occurs, when off scale data for one dye color produces a 'pull up' of the electropherogram graph in another dye color with an

overlapping emission wavelength spectrum (see ch. 3.5.2 and figure 3-4 for further information of fluorescence wavelength overlap). ‘Bleed through’ peaks may mistakenly be interpreted as additional allele peaks but are usually unmasked by producing unknown allele peaks for a certain STR locus that fit neither recipient nor donor allelotype.

Stutter peaks were frequently detected in capillary runs with high DNA input concentrations. Occurrence of stutter correlates linearly with template DNA concentration in PCR. Research into the genesis of stutter products has yet been somewhat inconclusive. Sequencing of stutter products showed, that they lack one tetranucleic repeat relative to the main product. Slipped strand mispairing has been suggested as a likely mechanism of stutter origin. In this theory, the DNA polymerase falls off the extension strand during PCR, the two DNA strands partially break apart and the extension strand loops out (“slips away”) before reannealing and continued elongation. Stutter products create a characteristic extra peak at $n-4$ bp length (with n being the bp-length of the main peak).¹⁶⁴

The $-A/+A$ phenomenon is another cause of extra peaks.¹³⁸ It can occur, when the extension steps in PCR are too shortly timed so that the Taq DNA polymerase cannot complete 3'-adenosine-addition for all extension products (see ch. 3.5.3). This results in a characteristic extra peak at $n-1$ bp fragment size. The PCR thermal cycling protocol for this study included a final extension step at 60 °C for 1 hr to ensure maximum 3'-adenosine-addition to all elongation products.

Some samples produced no analyzable results in CAE. This was, as discussed before in ch. 3.4, caused by DNA degradation, which accounts for poor PCR output concentrations. These samples were reevaluated at least once:

- If DNA concentration after extraction was sufficient (> 10 ng/ml), PCR was repeated with higher DNA input concentrations and the sample re-run in CAE
- Samples with low initial DNA yield after extraction (< 5 ng/ml) were worked up from scratch, i.e. new tissue sections were made, extracted, purified and amplified in hopes of achieving higher DNA yield in PCR and better results in CAE.

However, in one case (UPN 9) most samples were not analyzable in CAE—even after repeated DNA extraction and several PCR/CAE re-runs. The samples in question were

extracted from 16–21 years old FFPE material, so DNA degradation is likely responsible, as the age of FFPE material has been shown to greatly affect DNA quality.¹⁴⁷ Even though initially one informative STR locus was found, the patient was in consequence excluded from the study *a posteriori*.

3.7.1 Determination of STR profiles

As described in ch. 1.8.1, allele designation and the number of tetranucleotide repeats are interdependent. After correct sizing and allele mapping, donor- and recipient-derived alleles can be differentiated and analyzed.

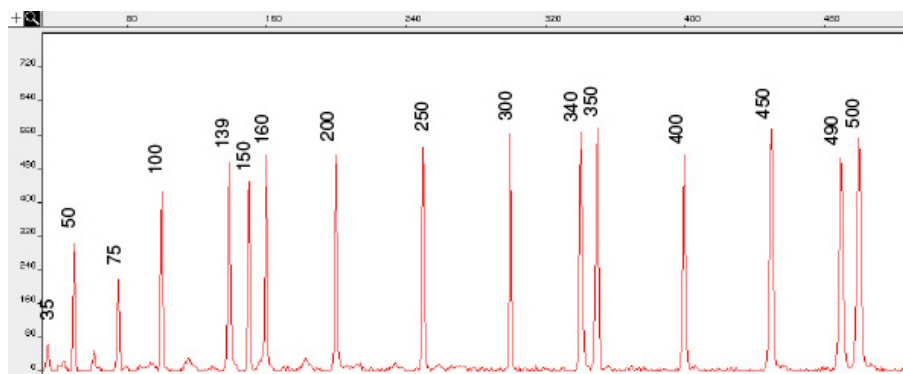


Figure 3-6: Electropherogram for GeneScan ROX-500 internal lane sizing standard.¹⁶⁵

For donor allelotyping we analyzed the EMB harvested in the first week after HTX. None of these biopsies showed high recipient derived cell share (see ch. 4.3.3), all donor alleles were therefore positively identifiable by peak height.

For recipient allelotyping we subtracted the known donor-alleles from the electropherograms. Additional information for allelotyping was obtained from analyzing tissue samples from the explanted recipient heart (figure 3-8). Only loci where donor and recipient shared no alleles were considered informational, as quantitative analysis is not possible for shared alleles.

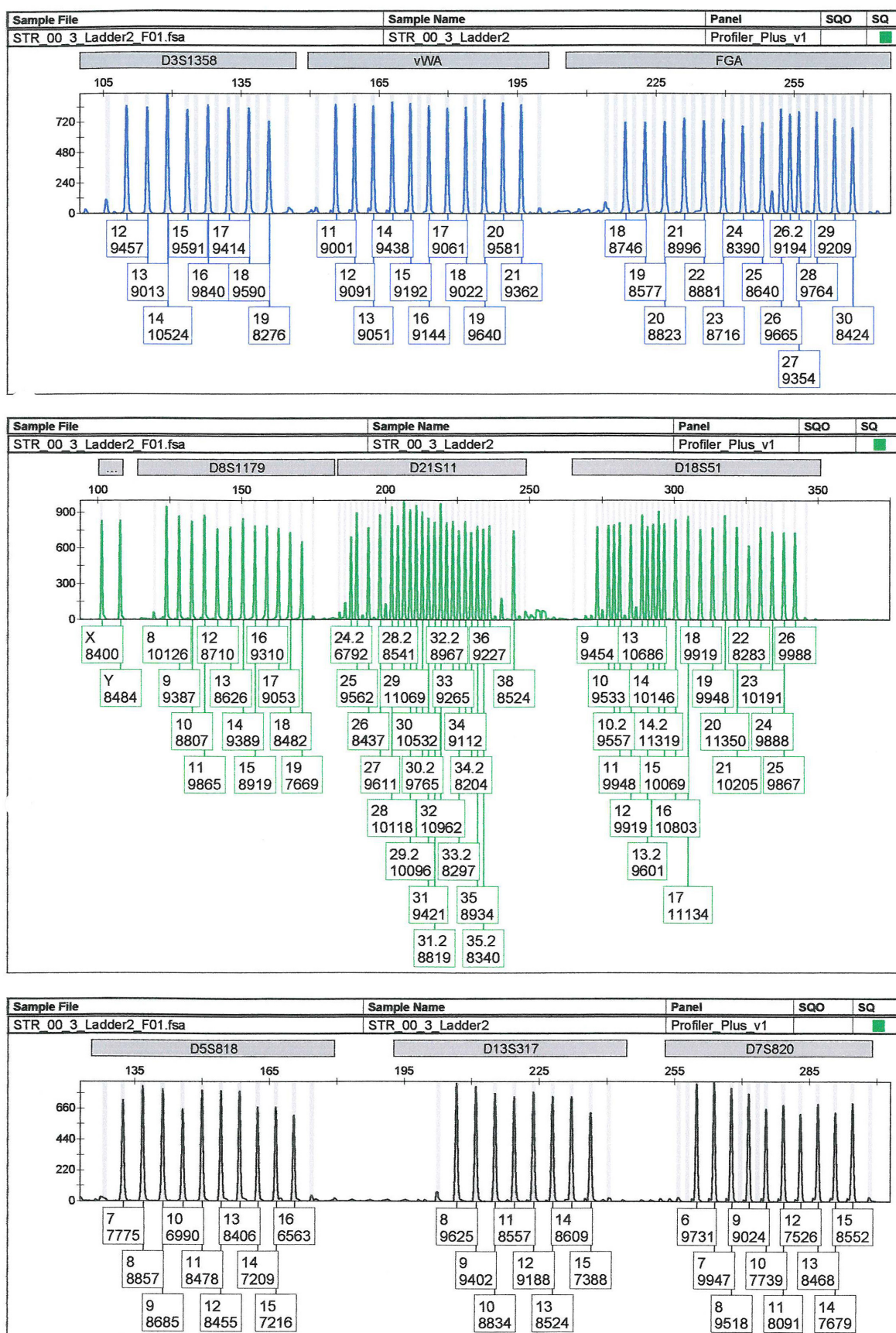


Figure 3-7: Allelic ladders for all loci and dye labels. Alleles are automatically detected and assigned according to bp-length (x-axis) by the GeneScan software. Yellow loci are plotted black for better visibility.

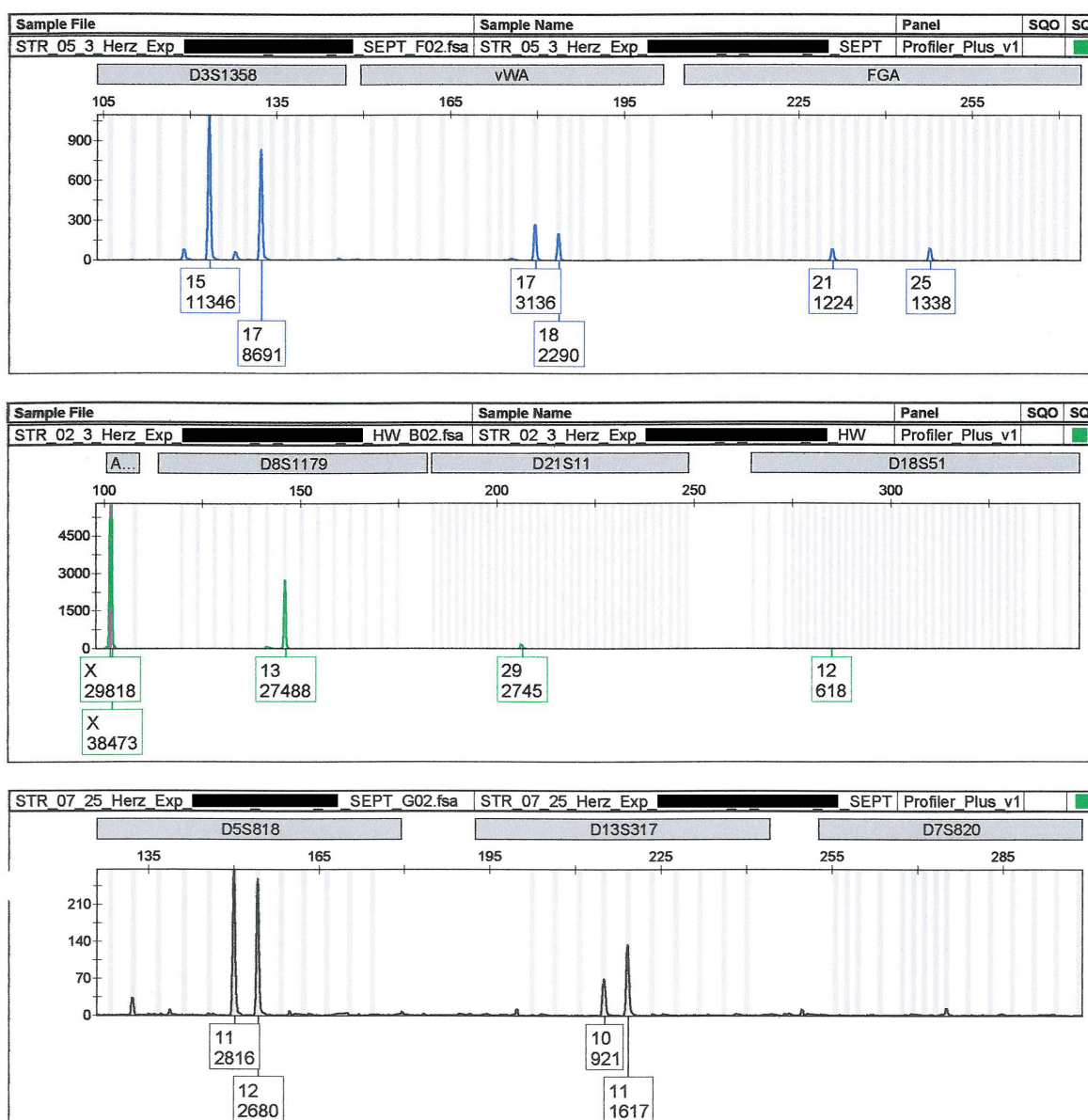


Figure 3-8: Example of recipient allelotype detection for UPN 1 from an interventricular septum specimen of the explanted heart.

3.7.2 Quantitative STR analysis

Loci producing larger fragments (> 200 bp; these include FGA, D18S51, D13S317 and D7S820) were not preferentially used for analysis, even when they were formally considered informational. This decision was based on the observation, that long fragments were often amplified insufficiently by PCR and thus required higher input DNA concentrations for CAE, producing higher background noise in the electropherogram and, as a result, providing less reliable readings. This is caused by DNA degradation and low initial DNA concentration after extraction (see ch. 3.4).

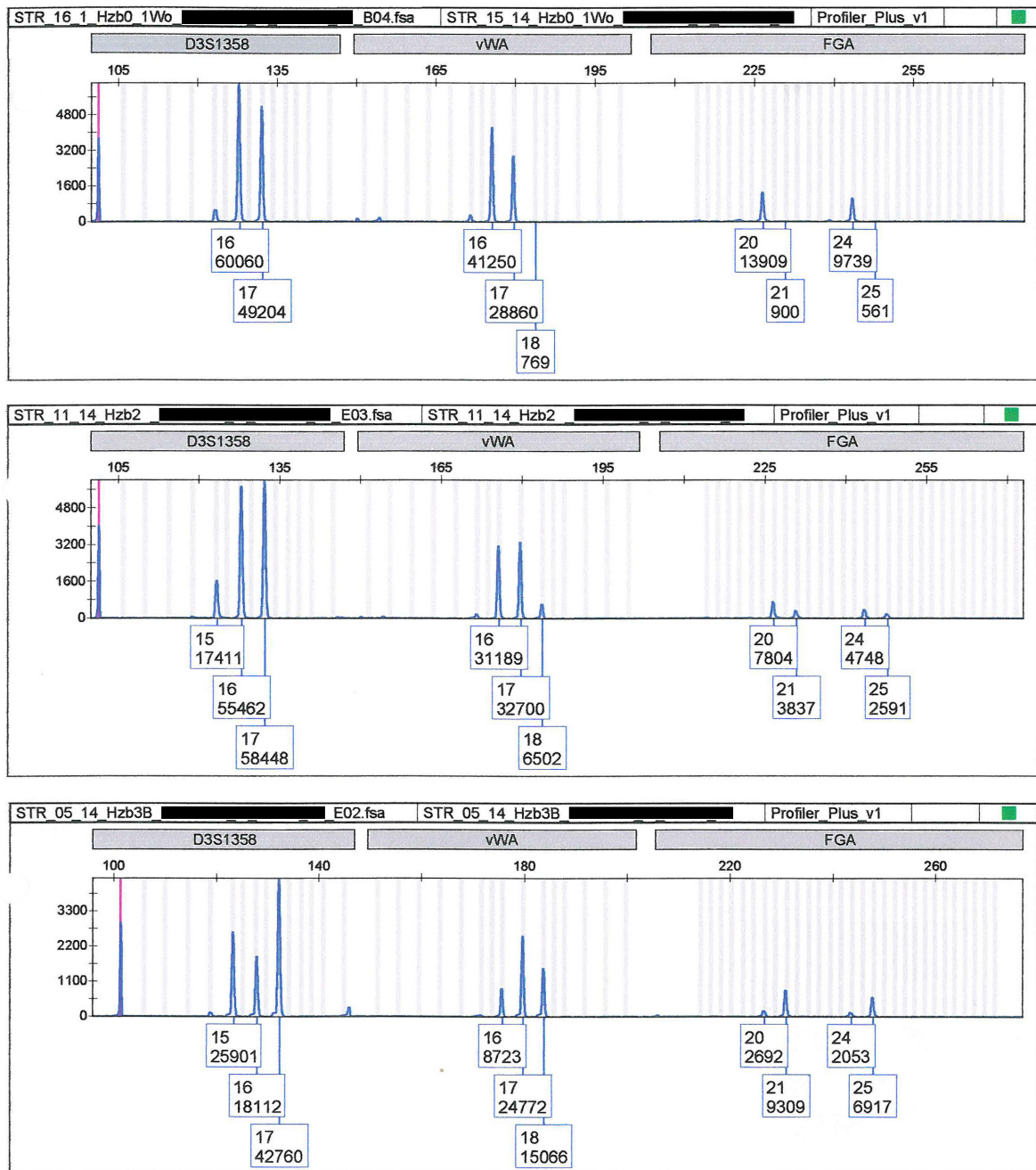


Figure 3-9: Proportion of recipient and donor-derived alleles for three loci labeled with 5-FAM (UPN 1). Locus FGA has no shared alleles and is thus suitable for quantitative analysis. **Top:** Grade 0 (0R) biopsy, first week. Some recipient alleles are barely detectable (informative recipient alleles: 21, 25). The calculated recipient-derived cell content is 8%. **Middle:** Grade 2 (1R) biopsy. Recipient-derived cell content is 30%. **Bottom:** Grade 3B (3R) biopsy. In this biopsy with severe rejection, recipient-derived alleles have come to the fore. Recipient-derived cell content amounts to 77%.

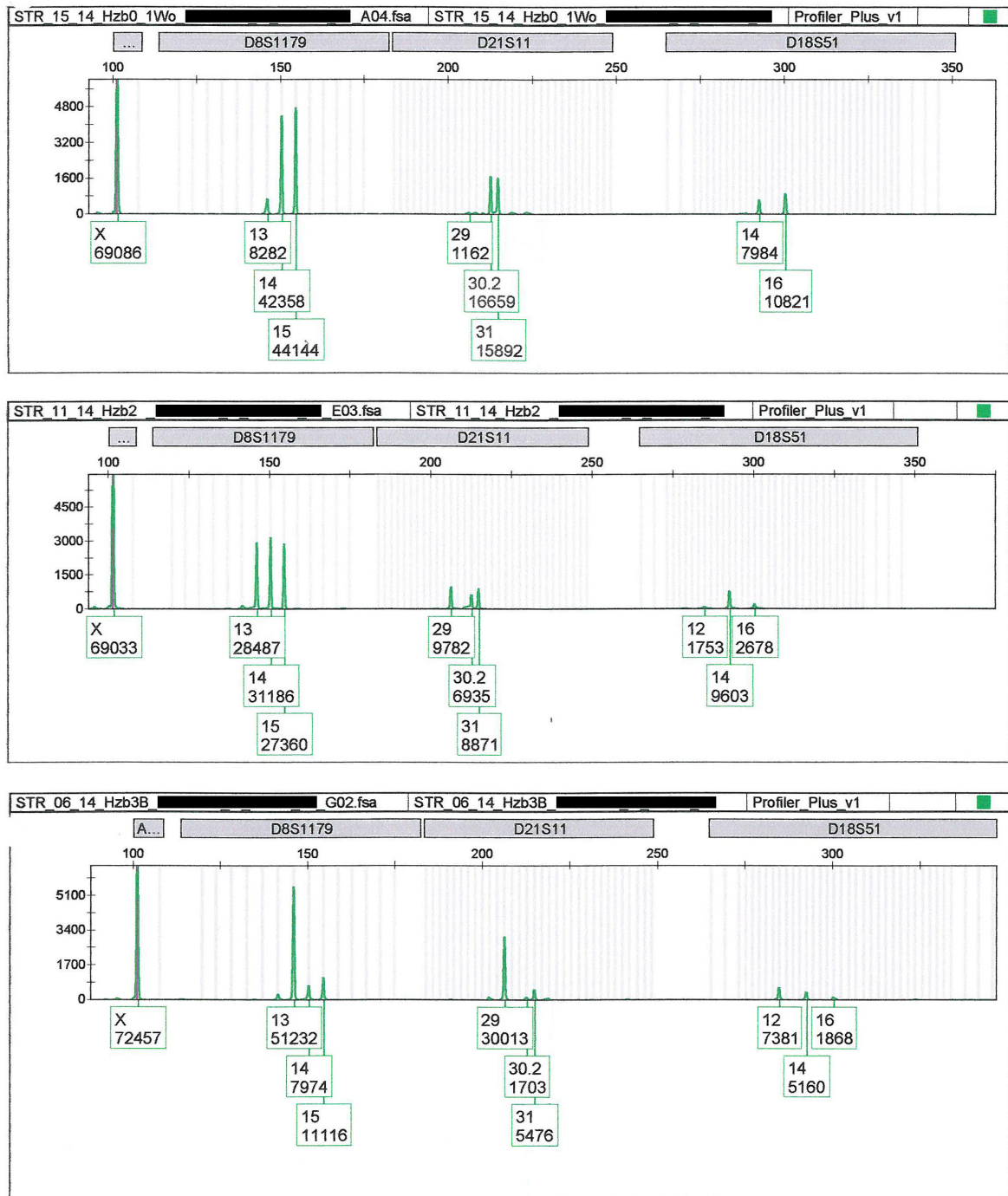


Figure 3-10: Proportion of recipient and donor-derived alleles for three loci labeled with JOE (UPN 1, same biopsies as in figure 3-9). D8S1179 and D21S11 are informative loci for this dye label. **Top:** Grade 0 (OR), first week biopsy. Recipient-derived allele share is low (informative recipient alleles: 13, 29). $q_{\text{rec}} = 8\%$. **Middle:** Grade 2 (1R) biopsy. $q_{\text{rec}} = 30\%$. **Bottom:** Grade 3B (3R) biopsy. $q_{\text{rec}} = 77\%$.

After determination of donor and recipient alleles, the sum of recipient peak areas was divided by the sum of recipient and donor peak areas for each informative STR locus:

$$q_{rec}[\%] = \frac{\sum n_{rec}}{\sum (n_{rec} + n_{don})} \times 100$$

Equation 3-1: Calculation of recipient cell percentage q_{rec} for a given STR locus. n designates allele peak area, the indices rec and don designate recipient and donor origin respectively.

As each sample was run at least four times in different dilutions, four quotients were obtained for each locus and a conclusive mean percentage value was calculated.

The results were gathered and calculated in Excel 2008 (Microsoft) spreadsheets.

3.8 Statistics

For data analysis and conduction of statistical tests, SPSS version 16.0 software was used.

Nonparametric tests (Kruskal-Wallis H , Mann-Whitney U test) were applied where appropriate. Correlation of recipient-derived cell content and rejection grades was examined using Spearman's ρ rank correlation coefficient and linear regression analysis. All P -values are given as two-sided probabilities. The level of significance for all test was predefined at $\alpha = 0.05$.

The threshold values for optimal sensitivity and specificity of recipient-derived cell content for the differential diagnosis of no rejection versus 1990 ISHLT grades $\geq 3A$ and 2004 ISHLT grades $\geq 2R$ were determined by receiver-operating characteristic (ROC) curve analysis.

ROC-curve analysis is a powerful tool for assessment of the accuracy and precision, i.e. sensitivity and specificity, of a diagnostic test by plotting the optimal tradeoff for true-negative ($1 - \text{specificity}$, x-axis) and true-positive (sensitivity, y-axis) rates for every possible cut-off value.¹⁶⁶ Additionally, the area under the ROC-curve (AUC) can be calculated. The AUC can assume values between 0.5 and 1. An AUC-value of 0.5 represents the worst case (no distributional difference between test values in the

positive and negative group; equaling a random guess), while the value 1 is the optimum (100% sensitivity and specificity).

4 Results

4.1 DNA quality and quantity

As discussed previously (ch. 3.4), genomic DNA extracted from FFPE material is subject to degradation and purified fragments are usually well below 650 bp in length. We performed photospectrometric measurements and gel electrophoresis (ch. 3.4.1, 4.1.2) to quantify DNA concentrations and to roughly assess DNA quality, especially in the higher bp-length loci.

4.1.1 Photospectrometry

Photospectrometry showed a mean DNA concentration of 20.16 ng/μl (95% CI, 17.31–23.01 ng/μl) in 185 purified samples with a mean purity ($A_{260/280}$ ratio) of 1.93 (95% CI, 1.88–1.98), indicating a satisfactory level of DNA purity after extraction and purification (solutions containing pure dsDNA produce an $A_{260/280}$ ratio of 1.7–1.9).

UPN	Samples	Mean (ng/μl)	95% CI (ng/μl)	Purity*
1	25	33.18	17.52–48.85	1.90
2	19	15.45	11.53–19.38	1.81
3	19	13.76	6.93–20.59	1.88
4	17	12.20	7.61–16.79	1.79
5	26	15.50	10.71–20.29	1.86
6	22	7.41	5.12–9.71	2.26
7	21	52.17	33.79–70.55	1.80
8	18	15.35	7.65–23.04	1.92
10	18	16.42	3.92–28.93	2.10
total:	185	20.16	17.31–23.01	1.93

Table 4-1: DNA concentration after purification. *: Mean value of $A_{260/280}$ ratios

4.1.2 Gel electrophoresis

Assessment of band fluorescence intensity and migration distance allowed inference on DNA quantity and quality for specific fragment sizes, when compared to a standardized positive control and 100 bp DNA ladder.

The following two associated gel photos (figure 4-1) provide examples for the outcomes of different DNA input concentrations for PCR (a new dilution series of one original sample every 4 lanes, except lanes 5 and 6). They also show the influence of original DNA quality on amplification efficiency.

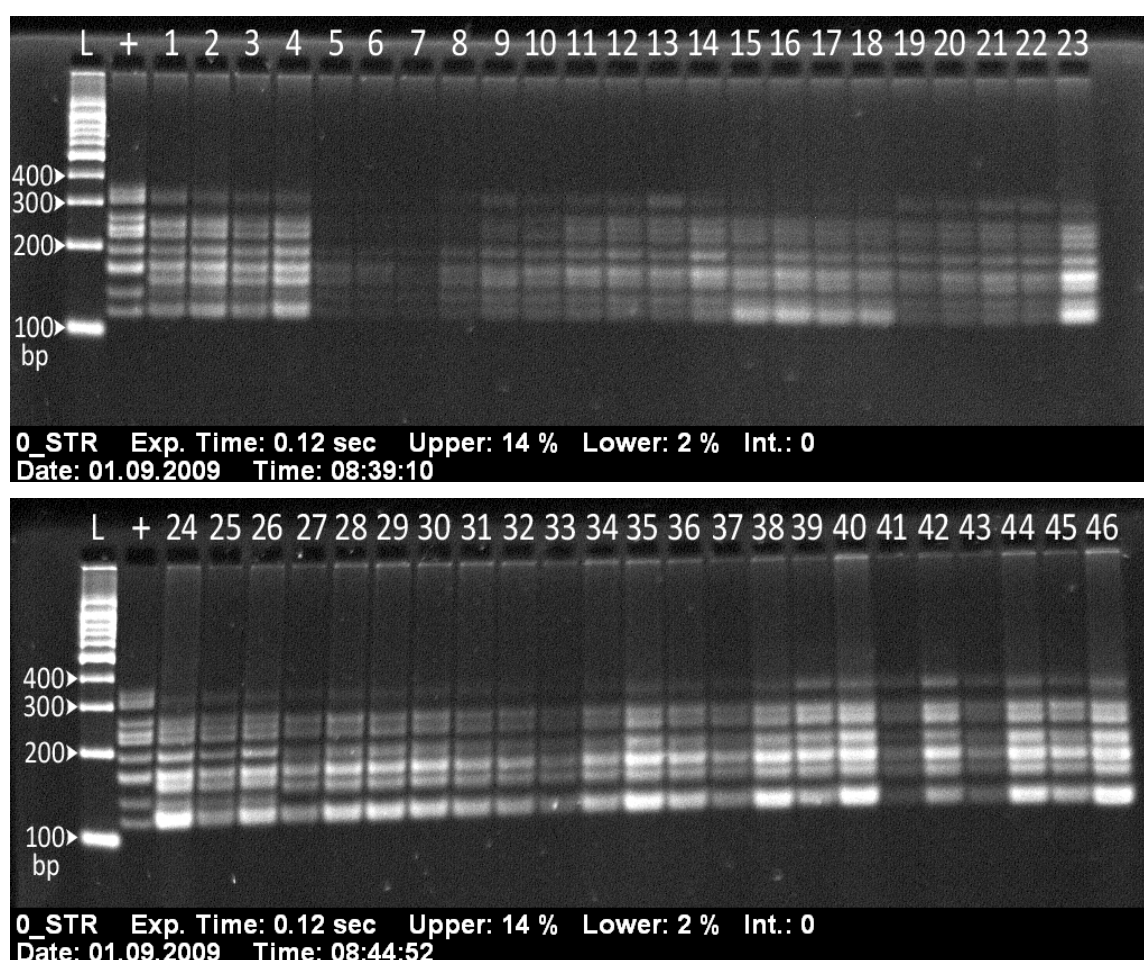


Figure 4-1: Typical result of gel electrophoresis. L: 100 bp DNA ladder; +: Positive control

The intensity of luminescence in lanes 1–4, 25, 31, 32, 39, 42 and 45 is almost matching the positive control (+). These samples are well suited for CAE as they allow analysis of all STR loci. However, larger fragments around 300 bp were not amplified as efficiently as the smaller fragments in most of these samples. This is especially visible in lanes 15–18, where small fragments were amplified well, but the larger

fragments are practically missing. This effect is explained by different degrees of DNA-degradation in the original biopsy material. Lanes 5 and 6 show the result of low amplification due to insufficient DNA input concentration for PCR, while lanes 40 and 46 show the results of over-amplification due to excessive DNA input concentration. These samples were not suitable for CAE, as they would either result in low readings with high background noise or off-chart peak heights and stutter peaks, respectively (see ch. 3.7).

4.2 STR allelotyping

Recipient and donor derived alleles were determined as described in ch. 3.7.1. Table 4-2 and table 4-3 provide an overview of recipient and donor allelotypes for each patient and STR locus. For each patient, at least two informational loci were subjected to quantitative STR analysis.

An exception was made for one patient (UPN 10), for whom only one informative locus was considered reliable. Another locus (FGA) was found to be formally informational, but performed poorly in CAE (peak heights < 150 RFU) and was thus omitted from further analysis.

UPN		D3S1358	vWA	FGA	AMG	D8S1179
1	Recipient	15/17	17/18	21/25	X/X	13
	Donor	16/17	16/17	20/24	X/X	14/15
2	Recipient	15/18	15/18	22	X/Y	11/18
	Donor	17/18	17/20	20/24	X/Y	11/14
3	Recipient	14/15	17	20/25	X/Y	13/15
	Donor	17	18/19	22/23	X/Y	8/14
4	Recipient	17/18	14/18	17/19	X/Y	13/15
	Donor	15/16	16	21/24	X/Y	13/15
5	Recipient	13/17	16	22/23	X/X	13/14
	Donor	14/15	14/16	23	X/X	14
6	Recipient	15/18	16/18	20	X/Y	10/14
	Donor	14/18	17	23/24	X/Y	11/13

7	Recipient	17/18	15/16	21/22	X/Y	13/14
	Donor	16/18	17/18	20/21	X/Y	13/14
8	Recipient	15	17/18	20/24	X/Y	10/14
	Donor	17/18	17/19	22.2/23	X/Y	13
10	Recipient	15	16	20 [?]	X/X	12/13
	Donor	15/17	14/18	21/24	X/X	12/14

Table 4-2: Recipient and donor allelotypes for loci D3S1358, vWA, FGA, AMG, D8S1179. Allele numbers in bold type designate informative allele constellations, single allele numbers signify homozygosity. [?]: Allele numbers were not identified reliably. n.r.: no analyzable result.

UPN		D21S11	D18S51	D5S818	D13S317	D7S820
1	Recipient	29	12/14 [?]	11/12	10/11	10
	Donor	30.2/31	14/16	10/11	11/12	11
2	Recipient	30/31.2	13/18 [?]	11/12	11	9/11
	Donor	30/32.2	14/15	10/11	12/13	9/12
3	Recipient	29	14/18 [?]	11/12	11/12	9
	Donor	28/30	15/16	9/12	8/11	11
4	Recipient	32.2	13/18	12	11/13	10/12 [?]
	Donor	29/32.2	12/17	8/11	12	10
5	Recipient	29/31	12/18	12	11/12	8/12
	Donor	27/30	13/16	12	10/11	10/11
6	Recipient	29/30	14	11/12	8/12	n.r.
	Donor	29/30	12/14	11	11/12	9/12
7	Recipient	29/30	n.r.	10/12	11/13	8/11
	Donor	29/31	12/13	10/12	8/10	10/13
8	Recipient	29/31	n.r.	11/13	11/12	n.r.
	Donor	30	12/16	11/12	12/13	10
10	Recipient	30/31.2	n.r.	10/11	11/12	n.r.
	Donor	30/31	13/14	10/13	8/11	10 [?] /11 [?]

Table 4-3: Continuation of Table 4-2. Recipient and donor allelotypes for loci D21S11, D18S51, D5S818, D13S317, D7S820. See above for legend.

4.3 Quantitative STR analysis

For each EMB, a mean recipient-derived cell percentage was calculated from electropherograms deriving from multiple CAE runs.

Table 4-4 provides a comprehensive compilation of mean recipient-derived cell content (q_{rec}) respective to the number of CAE runs used to obtain the mean percentage value, the 1990 and 2004 ISHLT nomenclature grades, the time point of sample harvesting (if applicable) and the patient number (UPN) for all 66 EMBs.

The highest recipient-derived cell percentage observed was at 76.55% in a grade 3B (3R) biopsy, the lowest at 3.0% in a grade 0 (0R) biopsy.

ID	UPN	1990 ISHLT	2004 ISHLT	Time	CAE runs	Mean q_{rec} (%)
1	8	4	3R		24	55.44
2	3	4	3R		24	44.82
3	1	3B	3R		12	76.55
4	4	3B	3R		12	63.18
5	6	3B	3R		8	62.57
6	5	3B	3R		10	36.36
7	10	3B	3R		4	36.21
8	7	3A	2R		8	65.19
9	2	3A	2R		12	63.87
10	2	3A	2R		12	59.30
11	7	3A	2R		6	58.98
12	5	3A	2R		12	51.22
13	7	3A	2R		8	50.85
14	5	3A	2R		12	43.49
15	5	3A	2R		12	41.41
16	10	3A	2R		4	27.00
17	6	3A	2R		8	24.40
18	7	2	1R		8	56.99
19	2	2	1R		9	52.41
20	4	2	1R		12	50.51

ID	UPN	1990 ISHLT	2004 ISHLT	Time	CAE runs	Mean q_{rec} (%)
21	5	2	1R		12	37.15
22	10	2	1R		4	32.38
23	1	2	1R		12	30.19
24	3	2	1R		12	28.37
25	6	2	1R		10	11.33
26	4	1B	1R		12	40.90
27	7	1B	1R		8	36.62
28	5	1B	1R		8	34.86
29	10	1B	1R		4	34.69
30	2	1B	1R		12	33.65
31	1	1B	1R		12	28.22
32	8	1B	1R		12	24.46
33	3	1B	1R		12	22.88
34	6	1B	1R		8	16.95
35	5	1A	1R	5 years	8	41.83
36	7	1A	1R	1 month	4	38.79
37	8	1A	1R	1 year	8	31.84
38	2	1A	1R	1 month	12	30.71
39	6	1A	1R	1 month	12	28.35
40	4	1A	1R	1 year	12	27.05
41	10	1A	1R	1 month	4	26.04
42	5	1A	1R	1 year	12	25.84
43	10	1A	1R	1 year	4	19.63
44	7	1A	1R	1 year	4	10.77
45	3	0	0R	5 years	2	26.56
46	8	0	0R	5 years	8	18.15
47	6	0	0R	5 years	12	17.96
48	2	0	0R	5 years	12	12.81
49	1	0	0R	5 years	12	9.43

ID	UPN	1990 ISHLT	2004 ISHLT	Time	CAE runs	Mean q_{rec} (%)
50	3	0	OR	1 year	12	29.04
51	2	0	OR	1 year	12	13.14
52	6	0	OR	1 year	12	10.71
53	1	0	OR	1 year	8	8.94
54	4	0	OR	1 month	12	42.73
55	8	0	OR	1 month	8	14.12
56	1	0	OR	1 month	8	13.72
57	3	0	OR	1 month	12	12.61
58	7	0	OR	1 week	8	37.83
59	5	0	OR	1 week	8	28.82
60	1	0	OR	1 week	8	8.13
61	3	0	OR	1 week	12	7.72
62	6	0	OR	1 week	7	7.35
63	2	0	OR	1 week	12	7.32
64	10	0	OR	1 week	4	3.42
65	8	0	OR	1 week	8	3.02
66	4	0	OR	1 week	8	3.00

Table 4-4: Recipient-derived cell content of each sample. Sorted by grades, q_{rec} , and time from HTX. ID: Sample identification digits. UPN: Unique patient number. q_{rec} : Recipient-derived cell content in percent.

4.3.1 Recipient-derived cell content in relation to the 1990 ISHLT grading system

The standardization of nomenclature for heart allograft rejection, introduced in 1990 by the ISHLT Heart Rejection Study Group, describes seven histopathologically distinct grades of rejection respective to five grades of rejection severity. See chapter 1.5.1 for detailed grading criteria. The density of mononuclear infiltration is the key factor in the differentiation of grade assignments, along with the severity of myocyte damage.

Table 4-5 shows the increasing mean percentage of recipient-derived cell content according to rejection grades. Note however, that grade 4 yielded a lower mean value (50.13%) than the next lower grade 3B (54.97%). This may be caused by the very

small number of EMBs in grade 4 and the relative preponderance of myocyte damage, which cannot be assessed through our method (see ch. 1.4.2 and 5.2).

ISHLT 1990	Sample quantity	Mean	95% confidence interval
4	2	50.13%	n.a.
3B	5	54.97%	32.68%–77.26%
3A	10	48.57%	38.25%–58.89%
2	9	37.42%	24.70%–50.13%
1B	8	30.36%	24.43%–36.28%
1A	10	28.09%	21.75%–34.42%
0	22	15.30%	10.39%–20.20%

Table 4-5: Recipient-derived cell content for 1990 ISHLT nomenclature grades. n.a.: not applicable (only two values in grade).

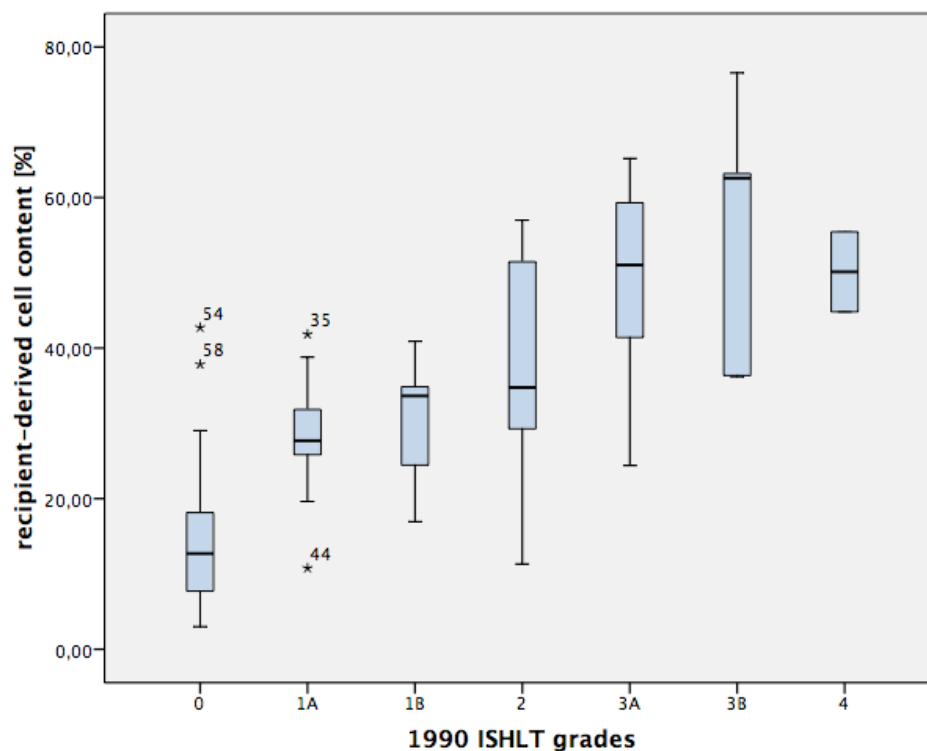


Figure 4-2: Boxplot of recipient-derived cell content for the 1990 ISHLT grading system.

The correlation of mean recipient-derived cell content with 1990 ISHLT grades was found to be highly significant (Correlation coefficient $\rho = 0.747$, $P < 0.001$). The use-

fulness of recipient-derived cell content percentage as predictor of rejection grading was further demonstrated through linear regression analysis (Coefficient of determination $r^2 = 0.568$, $P < 0.001$).

4.3.2 Recipient-derived cell content in relation to the 2004 ISHLT grading system

The revision of the 1990 ISHLT grading system aimed at an increase in histopathological inter-grade differentiability through the regrouping of old grades into fewer new rejection grades, providing greater grading reliability for clinical decision-making. While the old nomenclature based on a separation of rejection reaction into seven grades, the 2004 system relied on four grades identical to rejection severity. See chapter 1.5.2 for detailed information on revised grading criteria and grade regrouping.

As established above for the 1990 grading system, mean recipient-derived cell content is increasing according to rejection grades.

2004 ISHLT	Sample quantity	Mean	95% confidence interval
3R	7	53.59%	39.57%–67.61%
2R	10	48.57%	38.25%–58.89%
1R	27	31.61%	27.21%–36.00%
0R	22	15.30%	10.39%–20.20%

Table 4-6: Recipient-derived cell content for 2004 ISHLT grades.

Similar to the 1990 ISHLT grading system, a significant positive correlation between recipient-derived cell share and 2004 ISHLT grades was established. Correlation showed to be nearly identical to the 1990 system (Correlation coefficient $\rho = 0.737$, $P < 0.001$). Linear regression analysis proved the recipient-derived cell percentage as a usable predictor of rejection grading (Coefficient of determination $r^2 = 0.554$, $P < 0.001$).

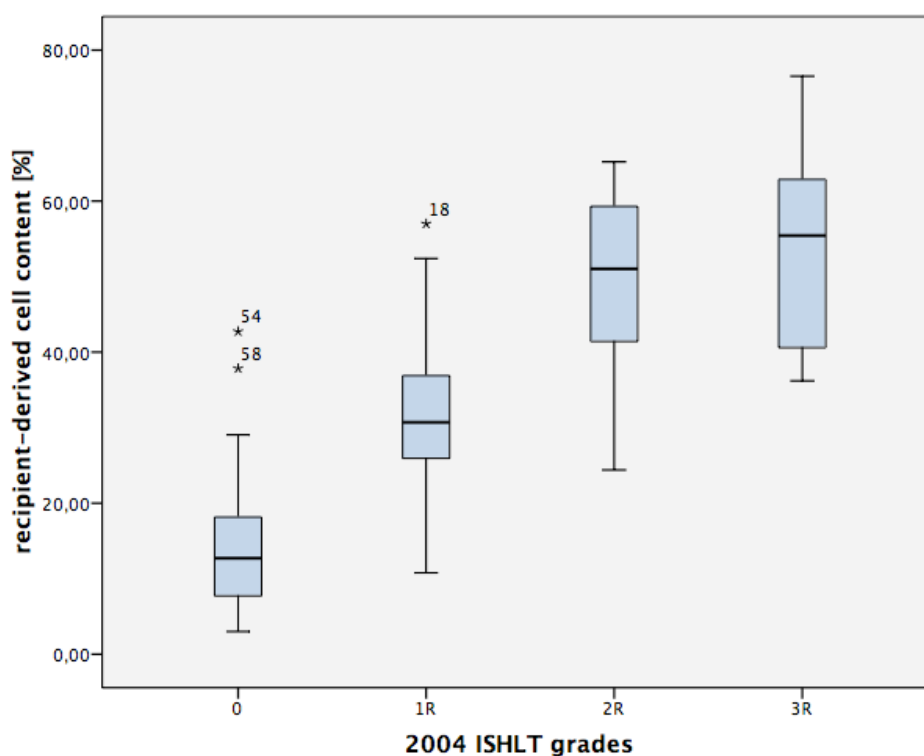


Figure 4-3: Boxplot of recipient-derived cell content for the 2004 ISHLT grading system.

4.3.3 Time-dependent recipient-derived cell content

To establish changes of recipient-derived cell over the course of time, we compared mean percentage values for one week, one month, one year and five years after transplantation (table 4-7, figure 4-4).

Time post HTX	Sample quantity	Mean	95% confidence interval
1 week	9	11.85%	2.19%–21.50%
1 month	8	25.88%	16.18%–35.58%
1 year	9	19.66%	12.76%–26.57%
5 years	6	21.12%	8.86%–33.39%

Table 4-7: Recipient derived cell content at different time points after HTX.

A significant positive correlation of recipient-derived cell content over the course of time was not observed (Correlation coefficient $\rho = 0.315$, $P = 0.079$). Linear regression analysis also did not prove a significant predictive power of recipient-derived

cell percentage for different time points (Coefficient of determination $r^2 = 0.052$, $P = 0.211$).

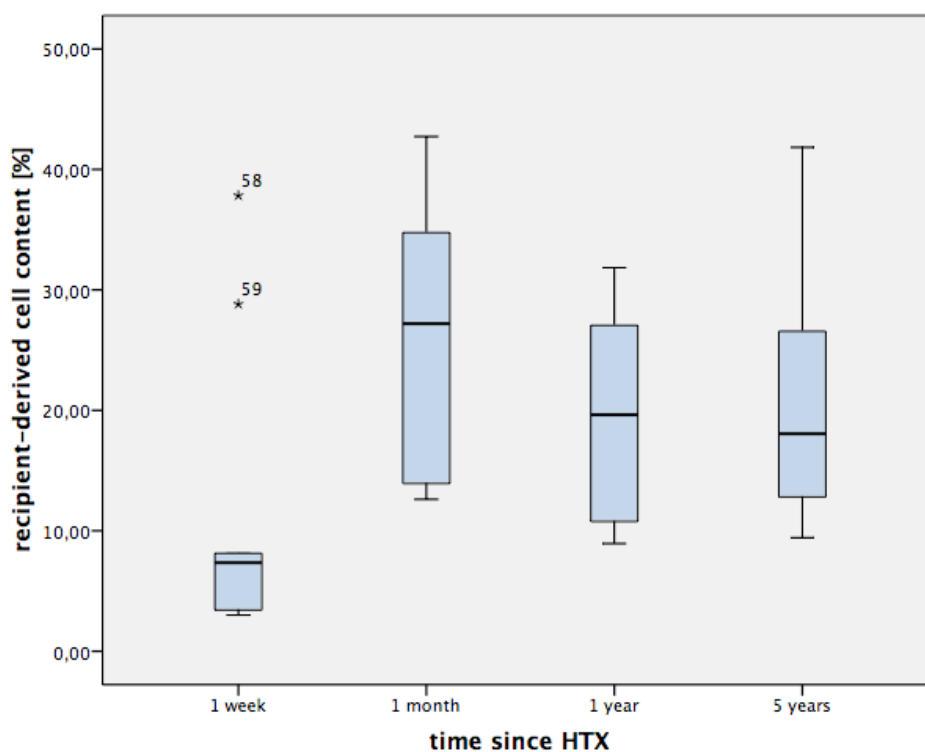


Figure 4-4: Boxplot of recipient-derived cell content at different time points after HTX.

However, a significant increase of recipient-derived cell share was observed between the 1-week biopsy and the following biopsies (table 4-8), although comparison of 1 month, 1 year and 5 years with another showed no significant increase in recipient-derived cell content after 1 month ($P = 0.429$).

1 week		1 month, 1 year, 5 years		P-value
Mean	95% CI	Mean	95% CI	
11.85%	2.19%–21.50%	22.21%	17.64%–26.77%	0.005

Table 4-8: Time-dependent increase in recipient-derived cell content after the first week post-HTX.

4.3.4 Discrimination between rejection and no rejection

To further investigate the relationship of recipient-derived cell percentage and rejection grades, we performed descriptive and inductive statistical methods to quantify this relation and prove statistical significance.

First, we established the power of the method to discriminate between EMBs graded 0 (0R) with no rejection and EMBs graded 1A (1R) and higher. Samples with ongoing allograft rejection showed significantly higher recipient-derived cell content than samples with no rejection. The 95% confidence intervals for both groups showed no overlap (see table 4-9).

No rejection		Rejection		P-value
Mean	95% CI	Mean	95% CI	
15.30%	10.39%–20.20%	38.96%	34.25%–43.68%	< 0.001

Table 4-9: Comparison of recipient-derived cell content in biopsies with no rejection versus ongoing rejection of any grade.

4.3.5 Discrimination between clinically relevant grade groups

For clinical decision-making, separation of certain grade groups is of major importance, as the severity of rejection is the relevant criterion for increasing immunosuppressive medication (see ch. 1.5.1 and 1.5.2).

For the 1990 ISHLT nomenclature a usual treatment threshold was clinically established between grade 2 and 3A corresponding to ‘focal moderate’ and ‘multifocal moderate’ rejection, respectively. Historically, some transplant centers settled upon setting the threshold between grade 1B and grade 2, although treatment of grade 2 rejection was controversially discussed and is now considered unnecessary by general consensus.^{90,105,167-172} Though formally defined as ‘focal moderate’ rejection by the 1990 ISHLT nomenclature, grade 2 has for this reason been incorporated in the ‘mild’ category for the following specific comparison of clinically relevant grade groups.

The threshold severity for intensified treatment has become plainer since the abandonment of sub-classification for moderate rejection through the introduction of the revised nomenclature. The usual treatment threshold for the 2004 grading system

is located between grades 1R and 2R, corresponding to ‘mild’ and ‘moderate’ rejection.

Rejection severity	1990 grades	2004 grades
None	0	0R
Mild	1A 1B 2*	1R
Treatment threshold		
Moderate and Severe	3A 3B 4	2R 3R

Table 4-10: Grouping of grades for recipient-derived cell content comparison under consideration of clinical relevance. *: As grade 2 lesions are not usually receiving treatment, they are placed in the ‘mild’ category for this test.

To assess the power of the method for discrimination between said grades of rejection severity, we compared mean recipient-derived cell content for the appropriate grade groups according to both ISHLT grading systems. First, we examined recipient-derived cell content for ‘no rejection’ (1990 grade 0; 2004 grade 0R) versus ‘moderate’ and ‘severe’ rejection (1990 grades $\geq$ 3A; 2004 grades $\geq$ 2R). As the 1990 and 2004 grade groups for rejection severity converge perfectly (table 4-10), only one mean percentage and *P*-value had to be calculated, which is informative for both grading systems.

Moderate and severe groups showed significantly higher percentage of recipient-derived cells than the no rejection group (table 4-11, figure 4-5).

No rejection		Moderate and severe		<i>P</i> -value
Mean	95% CI	Mean	95% CI	
15.30%	10.39%–20.20%	50.64%	43.19%–58.08%	< 0.001

Table 4-11: Comparison of recipient-derived cell content for no rejection versus moderate and severe rejections, both according to 1990 and 2004 ISHLT grades (0 vs. $\geq$ 3A; 0R vs. $\geq$ 2R).

The next step was analysis of recipient-derived cell content for mild rejection (1990 grades 1A, 1B and 2; 2004 grade 1R) versus moderate and severe rejection (1990

grades $\geq 3A$; 2004 grades $\geq 2R$), with the latter group again reaching significantly higher recipient-derived cell share (table 4-12, figure 4-5).

Mild rejection		Moderate and severe		P-value
Mean	95% CI	Mean	95% CI	
31.61%	27.21%–36.00%	50.64%	43.19%–58.08%	< 0.001

Table 4-12: Comparison of recipient-derived cell content for mild rejection versus moderate and severe rejections, both according to 1990 and 2004 ISHLT grades (1A, 1B and 2 vs. $\geq 3A$; 1R vs. $\geq 2R$).

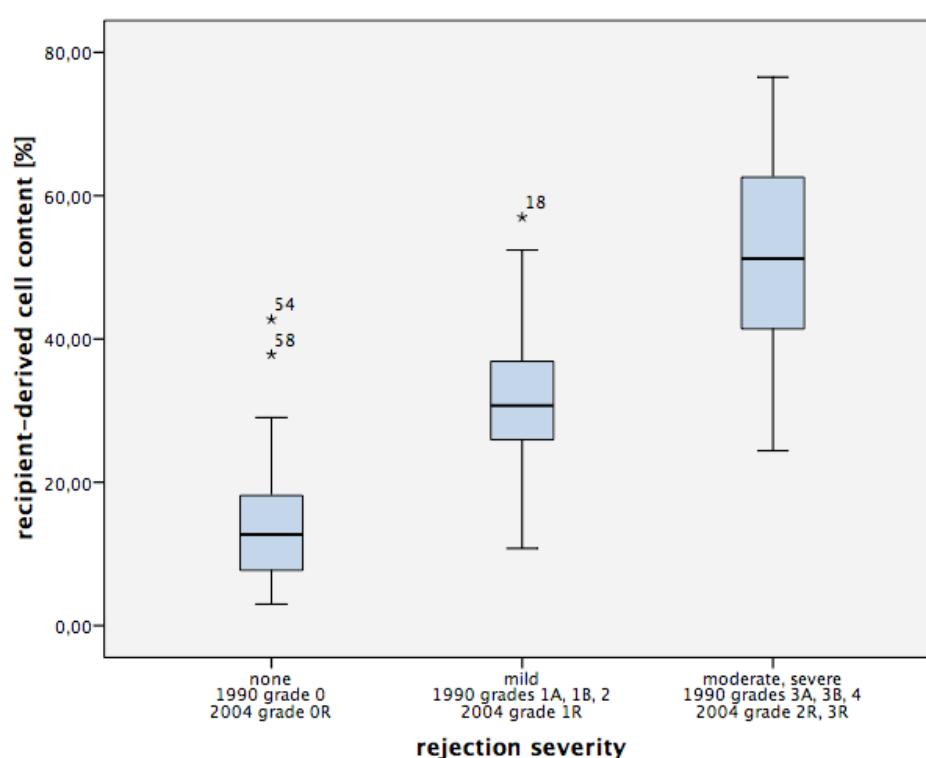


Figure 4-5: Boxplot of recipient-derived cell content for different clinically relevant grade groups according to both the 1990 ISHLT grading system and the revised 2004 ISHLT grading system.

As mentioned above, setting the treatment threshold between grade 1B and 2 was preferred by some transplant centers. For completeness, calculations were repeated with grade two grouped in the ‘moderate and severe’ category.

By comparing ‘no rejection’ (grade 0) versus ‘moderate and severe’ rejection (grades 2, 3A, 3B and 4), a significant increase in recipient-derived cell content was noted in the ‘moderate and severe’ category (Mean values: 15.30% vs. 46.41%, $P < 0.001$).

Comparison of 'mild' (grades 1A and 1B only) versus 'moderate and severe' rejection also resulted in a significant increase of recipient-derived cell share in the latter grade group (Mean values: 29.16% vs. 46.41%, $P < 0.001$).

4.3.6 Influence of Quilty effect on recipient-derived cell content

Quilty effect occurred in 12 EMBs (18%) ranging from grade 1A (1R) to grade 3B (3R). Frequencies of Quilty effect within each grading system are compiled in table 3-2 and table 3-3 for reference.

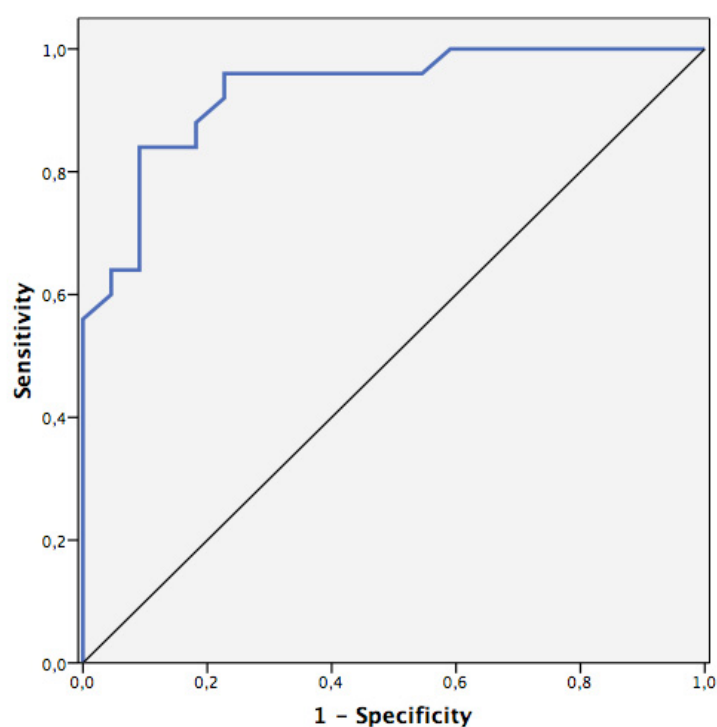
We examined intra-grade differences of recipient-derived cell content between Quilty-negative and Quilty-positive biopsies, both according to the 1990 and 2004 ISHLT grading system. No significant difference was detected, i.e. the P -value for each intra-grade comparison was $P > 0.05$. Influence of Quilty effect on intra-grade mean recipient-derived cell content and the inter-grade discriminatory power of quantitative STR analysis were thus dismissed.

4.4 Sensitivity and specificity of the method

4.4.1 Cut-off values for the 1990 ISHLT grading system

A ROC-curve was plotted (figure 4-6) to determine diagnostic accuracy of the method and establish cut-off values for recipient-derived cell content that enable optimal distinction between 1990 ISHLT grade 0 and grades greater than or equal to grade 2. The area under the ROC-curve (AUC) was 0.932 (95% CI from 0.863 to 1.000, $P < 0.001$). Cut-off values were determined by examination of curve coordinates:

- For screening application, a recipient-derived cell content cut-off value of 21% produces 96% sensitivity and 77% specificity.
- The optimal cut-off value for both maximal sensitivity and specificity was set at 27% and yielded 89% sensitivity and 81% specificity.
- For diagnostic use, a higher specificity is required. The cut-off value may be increased to 29%, producing 84% sensitivity and 91% specificity.



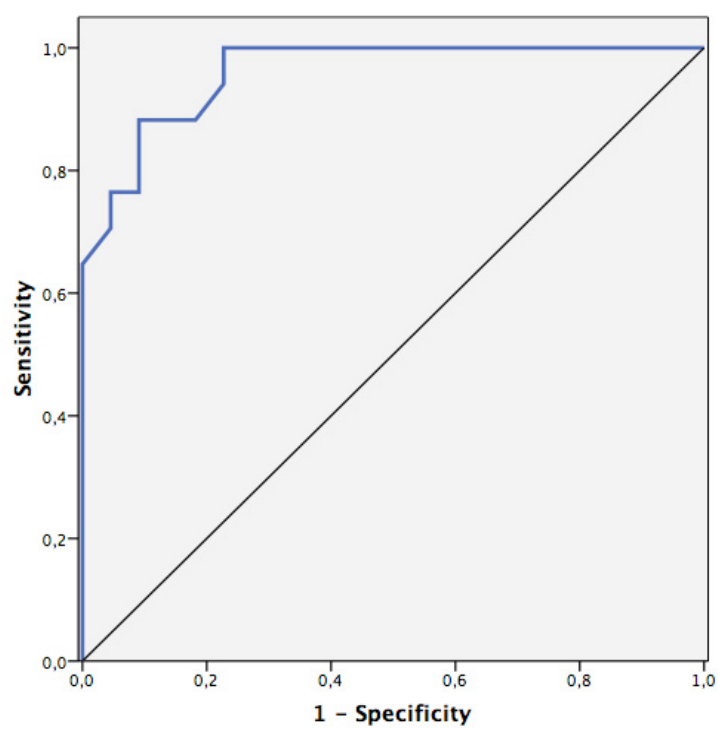
Diagonal segments are produced by ties.

Figure 4-6: ROC-curve for distinction of 1990 ISHLT grade 0 versus grade ≥ 2 .

4.4.2 Cut-off values for the 2004 ISHLT grading system

As described above, another ROC-curve was plotted (figure 4-7) to determine diagnostic accuracy and establish cut-off values for recipient-derived cell content that enable optimal distinction between 2004 ISHLT grade 0R and grades greater than or equal to 2R. The AUC was 0.960 (95% CI from 0.908 to 1.012, $P < 0.001$). Cut-off values were derived from analysis of curve coordinates:

- For screening application, a cut-off value of 21% for the recipient-derived cell content results in 99% sensitivity and 77% specificity.
- The cut-off value for an optimal tradeoff between sensitivity and specificity was determined at 28%, producing 88% sensitivity and 82% specificity.
- For diagnostic use, a cut-off value of 32% may be applied, resulting in 88% sensitivity and 91% specificity. Further increasing the cut-off value to 39% yields an even higher specificity of 96% but at a relatively low sensitivity of 77%.



Diagonal segments are produced by ties.

Figure 4-7: ROC-curve for distinction of 2004 ISHLT grade 0R versus grade $\geq 2R$.

5 Discussion

5.1 *Feasibility and validity of the method*

Quantitative STR analysis is a clinically well-established method after HSCT. Transplant centers are likely to have access to the required laboratory technology if HSCT is practiced locally. Our method shares some workflow aspects with the histopathological examination, such as the reliance on EMB and the tissue sectioning of FFPE material. Thus, it may easily be introduced as a complementing method for histological work-up without the need for additional sample material preparation.

The extraction of genomic DNA from FFPE material was generally successful. After purification, a sufficient mean DNA concentration of 20 ng/ μ l and a mean purity ($A_{260/280}$ ratio) of 1.93 were achieved. As previously described in the literature and as observed in our own study, sample age was correlated with increased DNA degradation. However, other individual factors may also contribute to increased DNA degradation in some biopsies, most importantly shortened tissue fixation times as well as the process of formalin fixation itself, and temperature-induced damage. The consequence of DNA degradation resulted in lower amplification success for longer STR loci, especially for loci with over 200 bp length. Nonetheless, STR allelotyping was successful for all but one patient, who was then excluded from further analysis. For most graft recipients, two to three STR loci were suitable for quantitative STR analysis; one patient yielded one usable STR locus.

Random error was minimized through a number of measures: 1) parallel analysis of at least two separate samples from each unique FFPE specimen, 2) preparation of multiple dilution series from each of these sample pairs, and 3) analysis of multiple CAE runs with different dilutions for obtaining a mean percentage of recipient-derived cell content. Results of STR analysis for each sample proved to be highly consistent and reproducible.

The overall results of this study indicate high sensitivity and specificity of quantitative STR analysis for the detection of cardiac allograft rejection. Also, the proportion of recipient-derived cells correlates well with the histopathological grading of rejection severity (*vide infra*). As a laboratory technique, STR analysis is insusceptible to

observer-related errors, making it an objective tool for the assessment of allograft rejection.

5.2 Limitations of quantitative STR analysis

The application of quantitative STR analysis for the diagnosis of cardiac allograft rejection is limited by a number of factors.

Some processes inherent or related to rejection are not based on cellular mechanisms and therefore are not associated with an increase of recipient-derived DNA concentration in the biopsy. Unfortunately, these processes are “invisible” to quantitative STR analysis, which solely relies on amplification of recipient DNA through PCR. Most importantly, STR analysis is not able to assess and quantify AMR. Unfortunately, this subtype of rejection is an important factor associated with CAV and reduced long-term graft survival. For these changes, immunohistochemistry and radiological imaging techniques constitute the preferred diagnostic method.¹⁸¹ The direct detection of myocyte damage, which is an important indicator of more severe rejection, is also not possible through STR analysis. However, myocardial injury during episodes of acute rejection is primarily inflicted by the aggressive cellular infiltrate, which is accessible to precise quantification by STR analysis.

Another problem is the existence of inflammatory recipient-derived cells of non-rejection causality in the biopsy. These inflammatory cells might infiltrate the myocardium in response to the presence of infective agents—a common side effect of prolonged immunosuppressive therapy. STR analysis cannot distinguish between rejection-related mononuclear infiltrates (lymphocytes, M ϕ) and other infiltrates, such as polymorphonuclear infiltrates (granulocytes) associated with ischemic injury or infections. In this context it should be mentioned that opportunistic infections are rather detected non-invasively through the analysis of serological parameters and antibody-tests and do not necessitate the *a priori* use of EMB.

5.3 Recipient-derived cell content and its relation to histological grading

The main goal of this study was to evaluate the usability of quantitative STR analysis as a tool for the reliable recognition of allograft rejection episodes and to examine the relation of recipient-derived cell content with the histologically assigned rejection grades. The assessment of cardiac allograft rejection by quantitative STR analysis is

based on measuring and comparing DNA amount of different origin. The end result of such analysis is a numerical value, usually expressed as a percentage. This recipient-derived cell percentage correlates well with rejection grades, but is unable to directly replicate histological grades. Because of percentage variability in the single rejection grades, there is always an overlap of 95% confidence intervals, resulting in some fuzziness of grade assignment by quantitative STR analysis alone.

Interestingly, the use of histological grading as an *a priori* standard for our analysis may have a confounding influence on our assessment, because both methods—and even EMB itself—are susceptible to sampling errors. For instance, when a sample was histologically assessed as grade 2 rejection on the basis of several serial sections, the deeper tissue sections obtained for our analysis from the same FFPE material block may contain less or additional rejection foci, rendering the biopsy grade 1A or grade 3A, respectively. As allograft rejection is a patchy process, the analysis of a complete native tissue specimen would increase the reliability of rejection assessment.

Mean recipient-derived cell content for biopsies with no signs of rejection (ISHLT grade 0/0R) was established at 15%, while biopsies with any grade of rejection displayed significantly higher mean recipient-derived cell content of 39%. Thus, quantitative STR analysis is a powerful tool for the diagnosis of allograft rejection. However, current therapeutic protocols require the distinction of nuances in rejection severity as a prerequisite for the adequate adaption of immunosuppressive medication. For that reason, we also evaluated the correlation of recipient-derived cell content with certain categories of rejection severity that are relevant to clinical decision-making. Currently, most transplant centers set the threshold for intensified immunosuppression between grade 2 and 3A, or 2R and 3R respectively.^{29,90,105,167-172} Quantitative STR analysis allowed excellent differentiation of these relevant rejection groups.

5.4 Quantitative STR analysis and non-inflammatory cardiac chimerism

In order for the method to produce valid data regarding the proportion of inflammatory cell content within a given biopsy, it must be precluded that other recipient-derived cells of non-inflammatory origin might affect the mean recipient-derived cell content.

In this study we examined the recipient-derived cell content in biopsies obtained after 1 week, 1 month, 1 year and 5 years after HTX. An analysis of the time-

dependent course of cell proportions showed a significant increase between the first week and the first month after transplantation (12% after the first week), but no significant changes in recipient-derived cell content for the later course from the first month up to 5 years post-transplant (mean value of 22% for that interval). These results suggest that relevant cardiac chimerism (i.e. endothelial cells and VSMCs, see ch. 1.7) occurs rapidly after transplantation but remains constant after the first month. This observation is also in accordance with reports of previous chimerism studies.¹²⁴⁻¹²⁷ Cardiomyocyte chimerism was heavily investigated, but apparently does not have any relevant quantitative impact.^{119,125,127,129-131} The importance of non-inflammatory cardiac chimerism as a potential confounding factor of quantitative STR analysis is greatly diminished by its constant nature.

Occurrence of the Quilty effect was largely made responsible for the high inter-observer variability in the histological assessment of biopsy material. When Quilty-positive and Quilty-negative biopsies were examined for possible differences in recipient-derived cell content in our study, no significant inter- and intra-grade discrepancies were found. In conclusion, these findings suggest that Quilty lesions do not significantly influence the results of quantitative STR analysis.

5.5 Conclusion and future developments

The results of this study demonstrate the use of quantitative STR analysis of recipient-derived cells as a useful tool for the detection of cardiac allograft rejection. The method generates an estimation of rejection severity, which correlates well with the current histological method, but is free from observer-dependent variability. Cellular effects like Quilty lesions (the major cause of high inter-observer variability in histological biopsy assessment) and non-inflammatory cardiac chimerism do not significantly influence the method.

A hypothetical ideal diagnostic test is characterized by achieving optimal accuracy and precision, i.e. 100% sensitivity and specificity or an area under the ROC curve of exactly 1, respectively, while still being cost-efficient and universally practicable. A diagnostic gold standard approximates this optimum as nearly as possible at a reasonable measure of time consumption, technical sophistication, overall work effort and costs. The detection of cardiac allograft rejection by histological examination is a relatively cheap and simple diagnostic tool, but is impaired by the high degree of sub-

jectivity in rejection grade assignment. Quantitative STR analysis is a clinically established method, fitting well into the current workflow for biopsy assessment. It reliably detects rejection episodes that are usually subjected to increased immunosuppressive treatment with high sensitivity and specificity (AUC 0.93 for the 1990 grading scheme, AUC 0.96 for the 2004 grading scheme). Therefore, our work supports the hypothesis that the use of quantitative STR analysis as an additional method for the detection of allograft rejection supplements the current standard in terms of improved objectivity, sensitivity and specificity.

Histopathological examination of cardiac allograft rejection relies on the analysis of tissue samples obtained invasively by endomyocardial biopsy and so does quantitative STR analysis. However, the procedure of EMB harvesting causes patient discomfort and is associated with some rare but severe risks. Novel approaches of diagnosing allograft rejection non-invasively have become an object of extensive research. Among these approaches are computerized imaging techniques,¹⁷³⁻¹⁷⁵ intramyocardial electrograms¹⁷⁶ and echocardiography.¹⁷⁷ Measurement of gene expression of peripheral blood cells is the most promising candidate for the future of diagnosing cardiac allograft rejection.^{178,179} The recent Invasive Monitoring Attenuation through Gene Expression (IMAGE) trial demonstrated non-inferiority of gene-expression profiling for rejection surveillance as opposed to the standard examination of EMBs.¹⁸⁰

Presently, none of these methods has found wide use in clinical routine or was established as an autonomous substitute for EMB. Until such a method is conceived, these alternative methods may, however, decrease the need for regular surveillance EMBs.

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Appendix

Symbols and Abbreviations

Symbols

α	alpha
$\sim$	approximately
β	beta
Δ	delta
=	equals
$\geq$	equal or greater than
$\leq$	equal or lesser than
γ	gamma
>	greater than
<	lesser than
λ	lambda
%	<i>per cent</i>
$\pm$	plus or minus
ρ	roh
$\times$	times
A	
A	adenine, adenosine, absorbance
ADCC	antibody-dependent cell-mediated cytotoxicity
ad lib.	<i>ad libitum</i> , at own discretion
ad inf.	<i>ad infinitum</i> , indefinitely
AIF	apoptosis inducing factor
ALG	anti-lymphocyte globulin
AMG	amelogenin
AMR	antibody-mediated rejection
AP	activating protein

APC	antigen presenting cell
ATG	anti-thymocyte globulin
AUC	area under the curve
AZA	azathioprine
B	
BCR	B cell receptor
bFGF	basic fibroblast growth factor
bp	basepairs
BSA	bovine serum albumin
C	
C	cytosine
CAE	capillary array electrophoresis
CAD	coronary artery disease
CAG	coronary angiography
CAV	cardiac allograft vasculopathy
CCD	circuit controlled device
CD	cluster of differentiation
CE	capillary electrophoresis
$^{\circ}\text{C}$	degrees Celsius
ch.	chapter
CI	confidence interval
CISH	chromogenic <i>in situ</i> hybridization
CMV	cytomegalovirus
CR	complement receptor
CT	computerized tomography
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T lymphocyte antigen
CyA	cyclosporine

D

dCMP	dilated cardiomyopathy
ddH ₂ O	double distilled water
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribo nucleotidephosphate
dsDNA	double strand DNA
DTH	delayed type hypersensitivity

E

e.g.	<i>exempli gratia</i> , for example
EBV	Ebstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELAM	endothelial leukocyte adhesion molecule
EMB	endomyocardial biopsy
EOF	electroosmotic flow

F

f	female
5-FAM	5-carboxyfluorescein
fas	fibroblast associated
Fc	fragment, crystallizable
FFPE	formalin-fixed paraffin-embedded
FGA	human fibrinogen alpha
FISH	fluorescence <i>in situ</i> hybridization
FOXP3	forkhead box P3

G

G	guanine
GVHD	graft versus host disease

H

H&E	hematoxin and eosin
HCl	hydrochloric acid
HLA	human leukocyte antigen
hr, hrs	hour, hours
HSCT	hematopoietic stem cell transplantation
HTX	heart transplantation

I

i.e.	<i>id est</i> , that is
ICAM	intercellular adhesion molecule
iCMP	ischemic cardiomyopathy
Ig	immunoglobulin
IL	interleukin
IMAGE	Invasive Monitoring Attenuation through Gene Expression
iNOS	inducible nitric oxide synthase
INF	interferon
ISHLT	International Society for Heart and Lung Transplantation
IVC	inferior vena cava
IVIG	intravenous immunoglobulin
IVUS	intravascular ultrasound

J

6-JOE	6-Carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein
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K		NAT	n-acryloyl-Tris
KCl	potassium chloride	NF-AT	nuclear factor of activated T cells
kV	kilovolt	NF-κB	nuclear factor kappa-B
L		ng	nanogram
Laser	light amplification by stimulated emission of radiation	NHS	n-hydroxysuccinimide
LIF	laser induced fluorescence	NK	natural killer cell
LINE	long interspersed repetitive element	nm	nanometer
M		NO	nitric oxide
m	male	NYHA	New York Heart Association
MAC	membrane attack complex	O	
max	maximum	OKT3	muromonab-CD3 monoclonal murine anti-CD3 antibody
MCP	monocyte chemotactic protein	P	
MgCl ₂	magnesium-di-chloride	P	statistical P-value
MHC	major histocompatibility complex	p., pp.	page, pages
μl	microliter	PCR	polymerase chain reaction
μm	micrometer	PDGF	platelet-derived growth factor
min	minute, minimum	PECAM	platelet endothelial cellular adhesion molecule
ml	milliliter	PGF	primary graft failure
mM	millimol	POP	performance optimized polymer
MMA	mycophenolic acid	PTLD	post-transplantation lymphoproliferative disorder
MMF	mycophenolate mofetil	PVC	polyvinyl chloride
Mφ	macrophage	Q	
mTOR	mammalian target of rapamycin	q _{rec}	recipient-derived cell content
MUG	Medical University of Graz		
N			
NaN ₃	sodium azide		

R		Taq	<i>Thermus aquaticus</i> DNA polymerase
RANTES	regulated upon activation, normal T cell expressed and secreted	TCR	T cell receptor
		TE	tris-HCl, EDTA
RFLP	restriction fragment length polymorphism	TGF	transforming growth factor
		TNF	tumor necrosis factor
RFU	relative fluorescence units		
RNA	ribonucleic acid	U	
ROC	receiver-operating characteristic	U	units
		UPN	unique patient number
ROS	reactive oxygen species	USA	United States of America
5-ROX	5-Carboxy-X-rhodamine	UV	ultra-violet light ($\lambda=280$ to 400 nm)
rpm	rounds per minute		
S		V	
SD	standard deviation	V	Volt
sec	second	VAD	ventricular assist device
SINE	short interspersed repetitive element	VEGF	vascular endothelial growth factor
SiO ⁻	silanol	VNTR	variable number tandem repeat
SPSS	Statistical Package for the Social Sciences	vs.	versus
ssDNA	single strand DNA	VSMC	vascular smooth muscle cell
SSCP	single strand conformation polymorphism	vWA	von Willebrand antigen
		W	
STR	short tandem repeat	WF	working formulation
STRP	short tandem repeat polymorphism		
SVC	superior vena cava	Y	
		yr, yrs	year, years
T			
T	thymine		
TAE	tris-acetate, EDTA		

Supplementary list of materials

This section provides product additional information and product-numbers for all chemicals and materials used in this study, including minor disposables which were not mentioned explicitly in the text.

Product	Manufacturer	Product number
0.2 ml thin-walled PCR tubes, sterile	Eppendorf, Germany	0030 124.332
1.5 ml microcentrifuge tubes, safe lock, sterile	Eppendorf	0030 120.086
1.5 ml microcentrifuge tubes, screw cap with O-ring, sterile	Bio Plas, USA	4202S
12% Poly(NAT) Wide Mini S-2x25	Elchrom Scientific, Switzerland	3050
50 cm quadruple capillary array	Applied Biosystems, USA	4333466
50 ml conical centrifuge tubes	BD Falcon, USA	352070
96-well Plate Assembly Kit	Applied Biosystems	4316471
100 bp DNA ladder	GenSura Laboratories, USA	
ABI PRISM 3100 Avant Genetic Analyzer with computer workstation	Applied Biosystems	3100-AVANT
ABI PRISM 3100 POP-6 polymer	Applied Biosystems	4316355
Acura manual 2–20 µl adjustable volume pipette	Socorex, Switzerland	825.0020
Acura manual 20–200 µl adjustable volume pipette	Socorex	825.0200
AmpF ℓ STR Profiler Plus kit	Applied Biosystems	4330284
AmpliTaq Gold DNA polymerase	Applied Biosystems	N8080240
Aqua bidestillata “Fresenius”, 100 ml glass bottle	Fresenius Kabi, Austria	
ART 200 µl disposable pipette tips, sterile, hydrophobic filter-plugged	Molecular BioProducts, USA	2069
Biological safety cabinet (for PCR master mix)	Thermo Scientific Heraeus, USA	
Clean bench (for DNA addition)	Thermo Scientific Heraeus	
Disposable gloves	Sempermed, USA	“edition”
Disposable microtome blades	Feather, Japan	R 3 5
DS-32 Matrix Standard Kit, Dye Set F	Applied Biosystems	4345831

Product	Manufacturer	Product number
EDTA-buffer stock solution 10×	Applied Biosystems	402824
Electrophoresis chamber: Sub-Cell Model 96 Cell	Bio-Rad, United Kingdom	170-4503
Electrophoresis loading buffer	Elchrom Scientific	3033
Electrophoresis transformer EV 265	Peqlab, Germany	55-EV265
ep Dualfilter T.I.P.S. 10 µl disposable pipette tips, sterile, hydrophobic filter-plugged	Eppendorf	0030 077.512
ep Dualfilter T.I.P.S. 1000 µl disposable pipette tips, sterile, hydrophobic filter-plugged	Eppendorf	0030 077.571
Ethanol, absolute p.a. ($\geq 99.9\%$)	Merck Chemicals, Germany	1009831011
GeneScan-500 ROX Internal Lane Size Standard Kit	Applied Biosystems	401114
Hi-Di Formamide	Applied Biosystems	4311320
LTK-008	Biodelta, Germany	200-000
MicroAmp Optical 96-well Reaction Plates	Applied Biosystems	N801-0560
Microtube racks with detachable cover	Generic brand	
Mini benchtop centrifuge for spindown	Kisker Biotech, Germany	MZ002-18
Mini Vortexer MV1	VWR, USA	58816-121
Nanodrop 1000	Thermo Scientific, USA	ND-1000
<i>para</i> -xylene, puriss. p.a.	Sigma-Aldrich, USA	95682
Platform shaker	New Brunswick, USA	
QIAmp DNA Mini Kit (50)	Qiagen, Germany	51304
Reference 0.5–10 µl adjustable volume pipette	Eppendorf	4910 000.018
Research plus 20–200 µl adjustable volume pipette	Eppendorf	3111 000.157
Research plus 100–1000 µl adjustable volume pipette	Eppendorf	3111 000.165
Rotary microtome	Microm, Germany	HM 350
Standard freezer –20 °C	Liebherr, Germany	
Standard fridge 6 °C	Bosch, Germany	
SYBR-gold dye	Invitrogen, USA	S11494
TAE-buffer: 40 mM Tris-acetate, 1 mM	Elchrom Scientific	3031

Product	Manufacturer	Product number
EDTA, pH 7.5–8.0		
TE-buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5–8.0	Mixed from stock solution	
Thermocycler (for denaturation): GeneAmp 2720	Applied Biosystems	4359659
Thermocycler (for PCR): GeneAmp 9700	Applied Biosystems	4314879
Thermomixer comfort 24 x 1.5 ml	Eppendorf	5355 000.111 5360 000.111
UV transilluminator and CCD camera	Intas UV-Systems, Germany	“Gel iX Imager”
Z 233 M-2 Microcentrifuge	Hermle, Germany	282.00 V31

Table 6-1: Supplementary list of chemicals and materials.

Curriculum vitae

Jan Beckendorf

Address

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Date of birth

May 28, 1981

Place of birth

Hamburg, Germany

Nationality

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University studies

October 2005 to
July 2011

Medicine at the Medical University of Graz

Vocational training

2004–2006

Biological Laboratory Assistant at the Ludwig-Maximilians-University in Munich, Germany

Compulsory service

2001–2002

Civilian Service at the Paracelsus-Hospital in Kaltenkirchen, Germany

Basic education

June 2001

Abitur (final secondary-school examination)

1993–2001

Gymnasium Kaltenkirchen

1991–1993

Lise-Meitner-Gymnasium Hamburg

1986–1991

Asperloh elementary school in Hamburg

Publications & Conferences

- October 2010 Beckendorf J, Hoefler G, Winter E, Kleinert R, Kniepeiss D, Pichler M. *Quantitative Analyse der Empfängerzell-DNA als Methode zur Beurteilung von akuten Abstoßungsreaktionen in transplantierten Herzen*. Presentation for Young Investigator Award at 2010 Austrotransplant congress. Abstract: Eur Surg. 2010 Aug;42(suppl.237):28-29
- 2010 Pichler M, Beckendorf J, Winter E, Kleinert R, Kniepeiss D, Hoefler G. *Quantitative Short-Tandem Repeat Analysis of Recipient-Derived Cells as an Additional Tool for Diagnosing Cardiac Allograft Rejection*. Transplantation. 2010 Mar 27;89(6):749-55

Memberships

- since 2010 Austrian Society for Transplantation, Transfusion and Genetics

Languages

German as a native-speaker, English fluently (TOEFL-IBT, 2011). Basic knowledge of French, Russian, Latin and Ancient Greek.