Non-invasive Rejection Surveillance after Heart Transplantation

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Abstract

Heart transplantation is a life-saving therapy for patients with end-stage heart failure. Despite excellent outcomes, mainly due to life-long treatment with a cocktail of immunosuppressants, acute cellular rejection (ACR) remains an impending threat in heart transplant patients. Endomyocardial biopsy, a gold standard used in the clinic for ACR diagnosis and surveillance, is associated with risks due to its invasiveness, subjectivity and expense. Moreover, many rejection events on the molecular level could provide early diagnosis, before rejection is seen histologically. The development of molecular biomarkers for the non-invasive diagnosis and surveillance of allograft rejection post-transplantation has been a long-standing challenge in heart transplantation

The profiling of circulating microRNAs in the serum of heart transplant patients provided evidence that changes in these profiles could be used to discriminate between organ rejection and quiescence. MiR-142-3p, which had the best diagnostic ability among the microRNAs tested in the heart transplantation cohort, was enriched in both the serum exosomes and in exosomes shed during *ex vivo* T cell activation. MiR-142-3p takes part in intercellular communication between T cells and endothelial cells by regulating the expression of RAB11FIP2 in endothelial cells, and promoting changes in endothelial physiology by impairing vascular integrity - a molecular hallmark of inflammation, including graft rejection.

By performing proteomic profiling, it was demonstrated that inflammatory fingerprints consisting of T-lymphocyte chemokines and cytokines were increased in the serum of heart transplant patients with ACR, and could provide good diagnostic discrimination of rejection.

In conclusion, this thesis provides evidence of the possibility of using transcriptomic and proteomic strategies for non-invasive diagnosis and surveillance of allograft rejection after heart transplantation. Moreover, the work presented in this thesis has also shed new light on the interaction between the host immune system and the cardiac allograft endothelium during ACR, which could be used as the basis for the development of microRNA-based therapy.

Key words: **Heart transplantation, Acute cellular rejection, Non-invasive monitoring, microRNAs, microRNA biomarker, T cells, Proteomic biomarker.**

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Cover Image: Molecular facet of donor heart. The image was created using Photo Lab Picture Editor FX.

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To Mika
My little heart-warrior

&

Rayan
My day-to-day muse

"Some people never meet their heroes - I gave birth to mine"

“To improve is to change; to be perfect is to change often.”

(Winston Churchill)

Content

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Sukma Dewi I**, Torngren K, Gidlof O, Kornhall B, Ohman J. Altered serum miRNA profiles during acute rejection after heart transplantation: potential for non-invasive allograft surveillance. *The Journal of heart and lung transplantation*. Apr 2013;32(4):463-466.

II. **Sukma Dewi I**, Hollander Z, Lam KK, McManus JW, Tebbutt SJ, Ng RT, Keown PA, McMaster RW, McManus BM, Gidlöf O, Öhman J. Association of Serum MiR-142-3p and MiR-101-3p Levels with Acute Cellular Rejection after Heart Transplantation. *PloS One*. 2017;12(1):e0170842.

III. **Sukma Dewi I**, Celik S, Karlsson A, Hollander Z, Lam K, McManus JW, Tebbutt S, Ng R, Keown P, McMaster R, McManus B, Öhman J, Gidlöf O. Exosomal miR-142-3p is increased during cardiac allograft rejection and augments vascular permeability through down-regulation of endothelial RAB11FIP2 expression. *Cardiovascular research*. Jan 10 2017. pii: cvw244.

IV. **Sukma Dewi I**, Gidlof O, Holander Z, Lam K, McManus J, Tebbutt S, Ng R, Keown P, McMaster R, Ohman J, McManus B, Smith JG. Immunologic serum protein profiles for non–invasive detection of acute cellular rejection in cardiac allograft patients. *Manuscript.*

Abbreviations

ACR	Acute Cellular Rejection
AUC	Area Under the Curve
CARGO	Cardiac Allograft Rejection Gene-expression Observational
CRP	C-reactive protein
EVs	Extracellular Vesicles
FDR	False Discovery Rate
HLA	Human Leukocyte Antigen
HUVECs	Human Umbilical Vein Endothelial Cells
IMAGE	Invasive Monitoring Attenuation through Gene Expression
ISHLT	International Society for Heart and Lung Transplantation
MHC	Major Histocompatibility Complex
miR	microRNA
qRT-PCR	Quantitative Reverse Transcription PCR
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PEA	Proximity Extension Assay
PHA	Phytohemagglutinin
RAB11FIP2	RAB11 Family Interacting Protein 2
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
SAM	Significance Analysis of Microarrays

Abstract

Heart transplantation is a life-saving therapy for patients with end-stage heart failure. Despite excellent outcomes, mainly due to life-long treatment with a cocktail of immunosuppressants, acute cellular rejection (ACR) remains an impending threat in heart transplant patients. Endomyocardial biopsy, a gold standard used in the clinic for ACR diagnosis and surveillance, is associated with risks due to its invasiveness, subjectivity and expense. Moreover, many rejection events on the molecular level could provide early diagnosis, before rejection is seen histologically. The development of molecular biomarkers for the non-invasive diagnosis and surveillance of allograft rejection post-transplantation has been a long-standing challenge in heart transplantation

The profiling of circulating microRNAs in the serum of heart transplant patients provided evidence that changes in these profiles could be used to discriminate between organ rejection and quiescence. MiR-142-3p, which had the best diagnostic ability among the microRNAs tested in the heart transplantation cohort, was enriched in both the serum exosomes and in exosomes shed during *ex vivo* T cell activation. MiR-142-3p takes part in intercellular communication between T cells and endothelial cells by regulating the expression of RAB11FIP2 in endothelial cells, and promoting changes in endothelial physiology by impairing vascular integrity - a molecular hallmark of inflammation, including graft rejection.

By performing proteomic profiling, it was demonstrated that inflammatory fingerprints consisting of T-lymphocyte chemokines and cytokines were increased in the serum of heart transplant patients with ACR, and could provide good diagnostic discrimination of rejection.

In conclusion, this thesis provides evidence of the possibility of using transcriptomic and proteomic strategies for non-invasive diagnosis and surveillance of allograft rejection after heart transplantation. Moreover, the work presented in this thesis has also shed new light on the interaction between the host immune system and the cardiac allograft endothelium during ACR, which could be used as the basis for the development of microRNA-based therapy.

Popularized Summary

Heart transplantation is a life saving treatment for adults and children with end-stage heart failure. Since the first heart transplantation performed in 1967, advances in perioperative care, surgical techniques and immunosuppressive medications have considerably improved post-transplant outcome. Nowadays, adult recipients can expect a median heart transplant survival of more than 10 years and infant recipient can expect a median heart transplant survival that is more than 20 years. However, organ rejection remains a major issue after heart transplantation that lead to the loss of the transplanted organ and morbidity.

Acute cellular rejection (ACR) is the most common form of organ rejection after heart transplantation that is experienced by 20% to 50% of patients at least once during the first year post-transplantation, despite the presence of immunosuppressive medications. The risk of ACR is highest in the first 6 month after heart transplantation. To monitor ACR, an invasive technique called endomyocardial biopsy, where a small sample from heart muscles is taken by using catheter introduced through the vein in the neck or in the thigh, is frequently performed on the patients after heart transplantation. Endomyocardial biopsy is not only invasive, but it is also subjective and expensive. Therefore, developing biomarkers from the peripheral blood that can be used to diagnose ACR from a simple blood test is required in clinical practice.

In Study I, we performed a pilot study, where we performed microRNA profiling in the blood samples of heart transplant recipients with ACR. MicroRNAs are small, single stranded RNA transcripts that are involved in many physiological and disease processes in the human body. Changes in the microRNA profile in tissue or in the peripheral blood have been used earlier as a novel strategy in the biomarker research field. We demonstrated in this study the possibility to use respective strategy to discriminate between heart transplantation patients with ACR and those without.

In Study II, we validated the findings of Study I in a larger group of heart transplant patients. MiR-142-3p and miR-101-3p had the best diagnostic performance among seven microRNAs tested that could discriminate between patients with and without rejection. Moreover, the performance miR-142-3p and miR-101-3p were independent of following important clinical parameters; calcineurin inhibitor levels, kidney function, and general inflammation state.

In Study III, we focused our study in miR-142-3p that was observed in Studies I and II, to be higher in the peripheral blood of ACR patients compared to non-rejection patients. We hypothesized that the presence of miR-142-3p in the peripheral blood during ACR associated with the disease process involving T cells

and endothelial cells, 2 cell populations known for their important roles in orchestrating ACR. We identified in this study the novel mechanism of communication between immune cells and endothelial cells, where miR-142-3p shed by T cells during immune activation or ACR convey a message to endothelial cells resulted in a physiological change in endothelial cells that fit the essential process of ACR.

In Study IV, we demonstrated that proteomic profiling approach employing a cutting-edge technique to find blood-borne protein biomarkers with high sensitivity and high specificity could be used as a new strategy for non-invasive ACR monitoring in heart transplant patients.

In conclusion, this thesis provides evidence of the possibility of using transcriptomic (microRNA) and proteomic (inflammatory proteins) strategies for non-invasive diagnosis and surveillance of allograft rejection after heart transplantation. Moreover, the work presented in this thesis has also shed new light on the interaction between the host immune system and the donor heart endothelium during ACR, which could be used as the basis for the development of microRNA-based therapy.

Populärvetenskaplig Sammanfattning

Hjärttransplantation är i nuläget den enda livräddande behandling som finns för vuxna och barn med grav hjärtsvikt. Sedan den första hjärttransplantationen utfördes 1967 har framsteg inom perioperativ vård, kirurgiska tekniker och immunosuppressiva läkemedel lett till en avsevärd förbättring av mortalitet och livskvalitet hos transplanterade patienter. Förväntad livslängd efter hjärttransplantation är idag ca 10 år för vuxna och ca 20 år för spädbarn. Trots immunosupppressiv behandling är organavstötning, vilket kan leda till förlust av det transplanterade organet, fortfarande ett stort problem efter hjärttransplantation.

Akut cellulär avstötning (ACA) är den vanligaste typen av organavstötning efter hjärttransplantation och drabbar 20% till 50% av patienterna minst en gång under det första året efter transplantation trots behandling med immunosuppressiva läkemedel. Risken för ACA är högst under de första sex månaderna efter hjärttransplantationen. För att övervaka ACA används en invasiv teknik som kallas endomyokardiell biopsi, där ett litet prov från hjärtmuskeln tas med hjälp av en kateter införd genom en ven i halsen eller i låret. Endomyokardbiopsi har, förutom att det är ett invasivt ingrepp, också andra nackdelar. Bedömningen av biopsin är subjektiv, proceduren är kostsam och innebär inte minst ett obehag för patienten. Att hitta andra, icke-invasiva sätt att diagnostisera avstötning med hjälp av ett enkelt blodprov skulle därför vara till stor nytta för patienter efter hjärttransplantation. Syftet med denna avhandling var att hitta s.k. biomarkörer, d.v.s. ämnen i blodet som kan användas för att diagnostisera avstötning, samt att undersöka några av de biologiska mekanismer som initierar avstötningsprocessen.

I det första delarbetet utförde vi en pilotstudie, där vi undersökte mikroRNA-profilen i blodprover från hjärttransplanterade patienter med ACA. MikroRNA är små RNA-transkript som är involverade i många fysiologiska och patologiska processer i människokroppen. Förändringar i mikroRNA-profil i vävnad eller i cirkulationen har nyligen använts som en strategi för att hitta nya biomarkörer för andra sjukdomstillstånd. Vi visade i denna studie möjligheten att använda denna strategi för att kunna identifiera patienter med ACA.

I avhandlingens andra delarbete validerade vi resultaten av Studie I i en större grupp av hjärttransplantationspatienter. miR-142-3p och miR-101-3p uppvisade de bästa diagnostiska egenskaperna bland de sju mikroRNA som testades och kunde användas för att särskilja patienter med och utan avstötning. Nivåerna av miR-142-3p och miR-101-3p var oberoende av flertalet viktiga kliniska parametrar, bland annat njurfunktion, inflammationsnivå och mängden immunsuppressiva läkemedel i blodet.

I det tredje delarbetet fokuserade vi på miR-142-3p, som i studie I och II visade sig ha förhöjda nivåer i blodet hos patienter med ACA. Vår hypotes var att miR-142-3p spelar roll för sjukdomsprocessen genom att fungera som en signalmolekyl mellan kroppens immunförsvar och det transplanterade organet. Vi identifierade i denna studie en ny mekanism där miR-142-3p frisätts från T-celler under immunaktivering och vid ACR, och kan påverka endotelcellerna i kärlväggen hos det transplanterade hjärtat, vilket i sin tur skulle kunna bidra till att påskynda avstötningsprocessen.

I det fjärde delarbetet använde vi avancerad proteomik för att hitta cirkulerande biomarkörer med hög sensitivitet och specificitet för ACA. Resultaten visade att nivåerna av proteinerna SLAMF1 och CXCL10 kunde användas för att identifiera patienter med avstötning.

Sammanfattningsvis är min förhoppning att denna avhandling kan öppna möjligheten att använda mikroRNA och inflammatoriska proteiner för icke-invasiv diagnostik och övervakning av organavstötning efter hjärttransplantation. Dessutom har arbetet med denna avhandling ökat förståelsen för hur kommunikationen mellan patientens immunsystem och det transplanterade hjärtats endotel fungerar under avstötningsprocessen.

Introduction

Heart Transplantation

The world's first human heart transplantation was performed at Groote Schuur Hospital in Cape Town, South Africa, in the early morning of Sunday, December 3rd, 1967, led by a South African surgeon Dr Christian Barnard, who transplanted a donor heart into a 57-year -old patient suffering from ischaemic heart disease[1,2]. Despite initial success, heart transplantation did not gain overall acceptance among healthcare practitioners[3]. The life-saving intervention of heart transplantation in patients with end-stage heart failure was mainly hampered by post-transplantation complications, including allograft rejection, and a high risk of infection resulting from high doses of nonspecific immunosuppressive drugs.

Heart transplantation became the treatment of choice for patients with end-stage heart failure in the early 1980s, when cyclosporine A started to be widely used as an immunosuppressant drug in heart transplant recipients, leading to a substantial improvement in patient survival[4,5]. Since then, over 100,000 heart transplants have been performed worldwide, for the treatment of primary heart diseases including congenital, non-ischaemic, ischaemic and valvular cardiomyopathy, to improve survival and enhance quality of life[6,7]. One-year survival following heart transplantation has improved from 30% in the first era, when heart transplantation was first introduced, to a remarkable number of 90% in the current era[8].

Heart Transplantation in Scandinavia

The first heart transplantation programme in Scandinavia was initiated in Oslo, Norway, in 1983, and was soon followed by programmes in the other Scandinavian countries. The first heart transplantations in Sweden were performed at Sahlgrenska University Hospital in Gothenburg, in 1984, and at Lund University Hospital in 1988. About 50 heart transplantations take place each year in both Lund and Gothenburg, the only two centres in Sweden currently performing heart transplantation.

The Scandinavian countries have 25 million inhabitants, and to improve collaboration among Scandinavian transplant centres, Norway, Sweden, Finland,

Denmark and Iceland share a common database regarding organ allocation within the Nordic organ exchange organization, Scandiatransplant. This means that all Scandinavian patients waiting for a new organ are listed in one common database, which increases accessibility to organ transplantation, as well as ensuring the optimal use of available organs[9,10].

The Nordic Thoracic Transplant Study Group (NTTSG) created a registry within the Scandiatransplant organization, integrating all thoracic transplantations, including heart transplantations, performed in Scandinavia. All transplantation centres participating in the NTTSG, i.e. Aarhus, Copenhagen, Gothenburg, Helsinki, Lund, Oslo and Stockholm, report their data to the NTTSG, and these are subsequently reported to the International Society for Heart and Lung Transplantation (ISHLT) on a yearly basis.

According to NTTSG data for 2015, more than 3000 heart transplantations have been performed in Scandinavia, with relatively good overall survival, in line with the ISHLT registry. The median survival for the whole cohort in the NTTSG 2015 registry was 14.5 years, compared to 10.3 years in the ISHLT registry (Figure 1)[11].

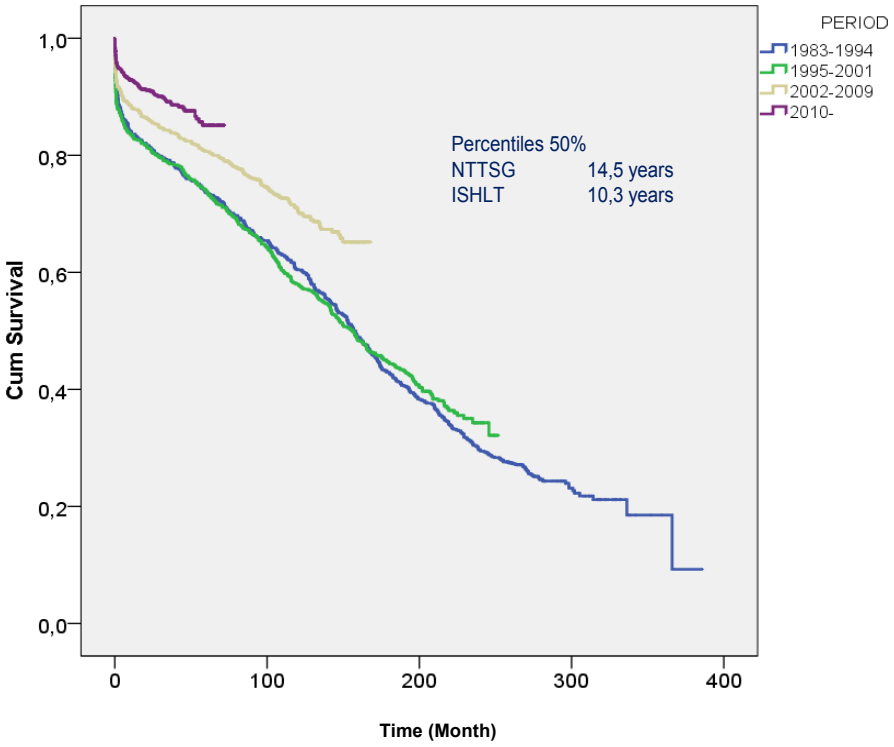

Figure 1. Actual Survival after heart transplantation in Scandinavia 2015. Reprinted with permission from Scandiatransplant.

Acute Cellular Rejection

Acute cellular rejection (ACR), graft failure, cardiac allograft vasculopathy, infection, renal failure and malignancy remain the leading causes of mortality after heart transplantation[8,12]. ACR is experienced by 20% to 50% of patients at least once during the first year post-transplantation, despite of the presence of immunosuppressant[13]. Allograft rejection has been of paramount interest since the first heart transplantation was performed. In a letter to Dr Christian Barnard exhibited in the Heart of Cape Town Museum, praising the world's first human heart transplantation, Dr Norman Shumway, a Stanford cardiac surgeon who performed the first successful human transplantation in the USA, suggested an early biomarker of cardiac allograft rejection. In his letter, dated December 4th 1967, he wrote, "Be certain to watch the R-wave of the ECG during the next several weeks for indices of rejection. It appears to be the earliest herald of important graft invasion." Since 1967, considerable efforts have been made to advance the precision of acute cardiac allograft rejection diagnosis.

Immunology of Transplant Rejection

Rejection is caused by the immune response of the host to donor antigens that are considered to be "non-self"; this recognition of "foreign" antigens is called allorecognition. The most prominent response of alloantigen mediated by host T cells, which recognize the major histocompatibility complex (MHC), an antigen expressed on the surface of donor cells. MHC in graft rejection was first demonstrated in mice and the World Health Organization Nomenclature Committee designated that the leukocyte antigens controlled by the closely linked genes of the human MHC be named human leukocyte antigen (HLA)[14].

Based on the time of onset, rejection can be divided into three general types: hyperacute, acute and chronic rejection. Hyperacute rejection occurs within minutes to hours after transplantation, and is caused by a mismatch of host and donor antigens. However, this type of rejection is very rare, due to advances in knowledge on transplant immunology and pre-transplantation assessment of the immune compatibility of the donor and the recipient, including HLA matching, a crossmatching method that can detect preformed antidonor antibodies, and ABO blood type matching. Acute rejection occurs within days or weeks after transplantation and is mediated by either antibodies (less common) or T cells (most common). In contrast to hyperacute and acute rejection, chronic rejection occurs months to years after transplantation, and is mainly due to the insufficiency of immunosuppression medication.

T cells and endothelial cells play essential roles in the initial events of ACR pathophysiology[15,16], mainly due to their unique locations, which enable direct contact between the endothelial cell lining of the graft vessel and host T cells[17,18]. Endothelial cells initiate rejection by presenting alloantigen to circulating T cells[17]. Allorecognition is then mediated by activated host T cells via two distinct pathways: the direct and the indirect pathways[18]. In the direct allorecognition pathway, T cells recognize intact MHC molecules displayed on the surface of the donor cells. In the indirect allorecognition pathway, T cells recognize the processed donor MHC molecules presented as peptides by self-MHC molecules on the surface of the recipient's antigen presenting cells (APC)[19-22] (Figure 2).

The subsequent events in ACR that lead to the advancement of tissue damage and allograft destruction are also mainly elicited by the interactions between T cells and endothelial cells[23,24]. The recruitment of activated T cells into the graft requires a multi-step process orchestrated by the interplay between endothelial cells and T cells in each of the steps[25]. Moreover, human endothelial cells have also been implicated in modulating T cell subsets, for example, Th17, an Achilles heel in immunosuppression that can mediate proinflammatory immune response, which accelerates allograft rejection, and regulatory T cells, which are important regulators of immune homeostasis in transplantation[26].

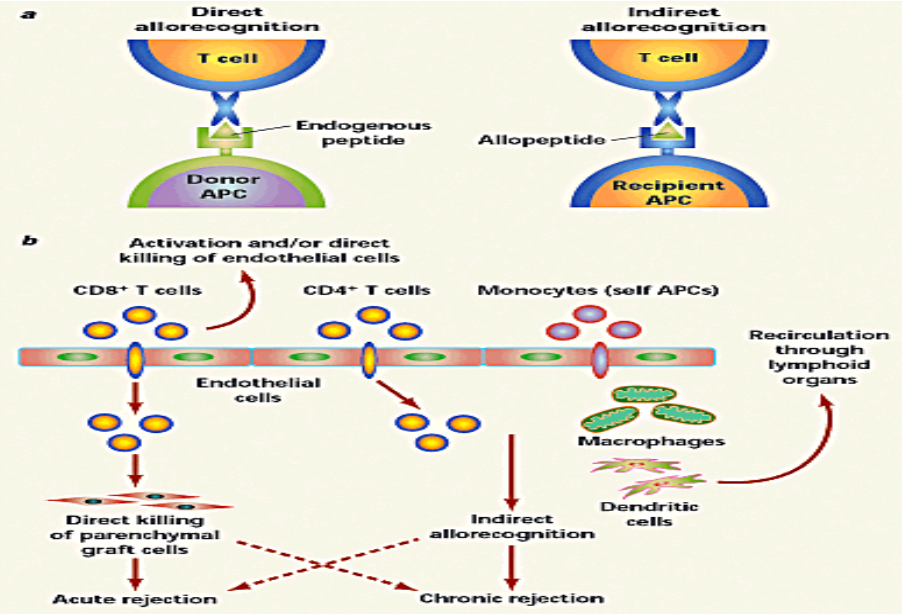

Figure 2. T cell-mediated allograft rejection via the direct and indirect pathways. (Reprinted with permission from Nature Publishing Group. Briscoe DM, et al. A rendezvous before rejection: where do T cells meet transplant antigens? Nature medicine. Mar 2002;8(3):220-222.)

Monitoring of Acute Cellular Rejection

Endomyocardial biopsy or right-heart catheterization remains the gold standard for the diagnosis and rejection surveillance in heart transplant recipient[12,27-29]. Typical surveillance biopsy protocol at heart transplantation centres is once weekly for the first month after transplantation, every 2 weeks in the second month, once monthly between 3 and 6 months and every 2 months between 6 and 12 months. The myocardial tissue is sampled from the right ventricular septal wall using a bioptome introduced through either the jugular or femoral vein using fluoroscopy or 2-dimensional echocardiography to provide information on the course of the bioptome and the site of the biopsy[30-33]. The presence of massive inflammatory infiltration of lymphocytes in the myocardium is the histological hallmark of ACR[34,35].

The ISHLT 2004 guidelines for cardiac allograft biopsy grading are used to diagnose ACR[34](Figure 3). This grading system is a revision of the 1990 ISHLT grading system. The revised (R) categories of cellular rejection are as follows: Grade 0R - no rejection (no change from 1990); Grade 1R - mild rejection (1990 Grades 1A, 1B and 2); Grade 2R - moderate rejection (1990 Grade 3A); and Grade 3R - severe rejection (1990 Grades 3B and 4).

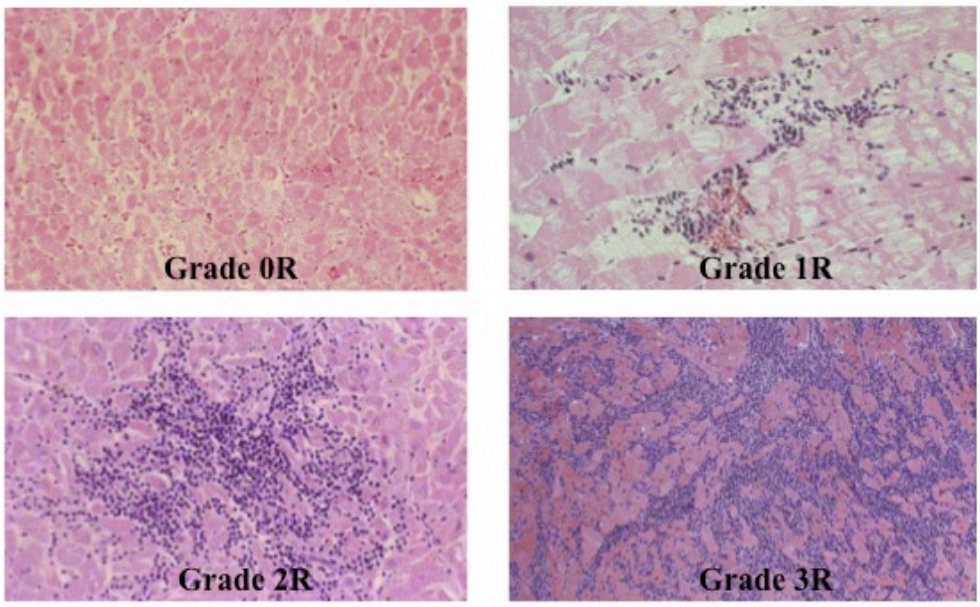

Figure 3. International Society for Heart and Lung Transplantation 2004 grading system for cardiac allograft biopsy. (Reprinted with permission from Elsevier. Stewart S, *et al*. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *The Journal of heart and lung transplantation.* Nov 2005;24(11):1710-1720.)

However, despite its high sensitivity and specificity as the primary monitoring modality for the diagnosis of acute cellular rejection[36], endomyocardial biopsy suffers from several drawbacks. The main problems associated with endomyocardial biopsy lie in its subjectivity, including sampling error, false-negative results and inter-observer variability with the overall, all-grade agreement between pathologists being only 71%[37-40].

Furthermore, endomyocardial biopsy may expose heart transplant patients to discomfort or risks, due to the invasive nature of the technique. There are low but finite risks of morbidity, including arrhythmia and tricuspid regurgitation, as well as myocardial perforation and mortality[27,41-43]. As a result of these risks, the use of endomyocardial biopsy as a cornerstone of the diagnosis and surveillance of acute cellular rejection after heart transplantation has recently come into question. A complementary or replacement surveillance method, preferably a non-invasive one, remains an unmet need in the field of solid organ transplantation.

Non-invasive Rejection Surveillance Strategies

The invasiveness and associated drawbacks of endomyocardial biopsy have led to the search for alternative, non-invasive methods for the diagnosis and surveillance of allograft rejection, for use in routine clinical care. Several non-invasive techniques have been demonstrated, including imaging techniques, such as echocardiography[44-46] and cardiovascular magnetic resonance[47,48], as well as cardiovascular electrophysiological monitoring[49,50].

Research is also being carried out to identify a suitable biomarker in peripheral blood. So far, biomarkers of ACR in peripheral blood have been the most commonly investigated, and there is considerable interest in this field, mainly because peripheral blood is an easily accessible source of patient material, and a wide range of techniques can be used to detect blood-borne biomarkers that can accurately reflect the recipient's immune response to the cardiac allograft (Figure 4). Moreover, a vast clinical diagnostic infrastructure is widely available for the analysis of blood samples, indicating that peripheral blood will remain the preferred diagnostic specimen in the foreseeable future.

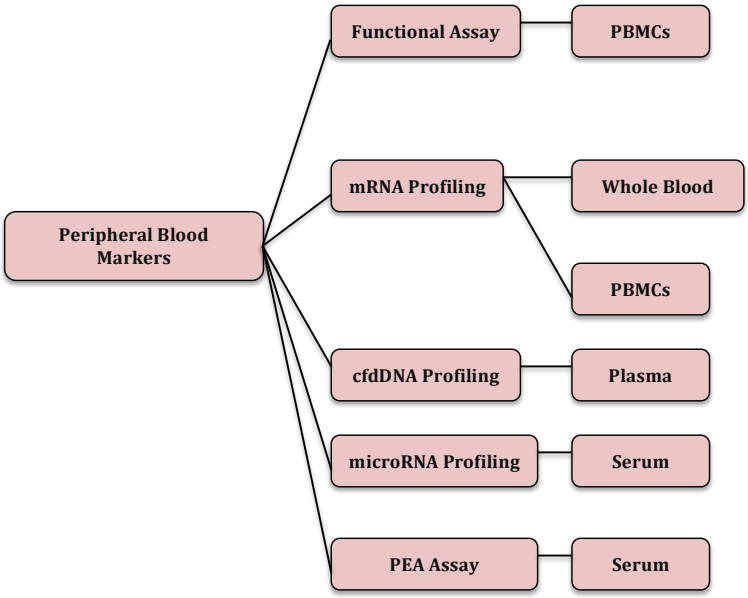

Figure 4. Techniques that have been used to detect biomarkers in the peripheral blood of heart transplant recipients.

Functional Assay

The function of lymphocytes can be determined *ex vivo* by their proliferation ability or by their intracellular adenosine triphosphate (iATP) concentration after phytohaemagglutinin (PHA) stimulation. Both assays can provide information on the status of the cell-mediated immune response in immunosuppressed patients. Studies using the iATP assay in kidney, liver and heart transplantation patients have shown that high CD4+ T cell iATP concentration is associated with a risk of ACR, and that low concentration is associated with the risk of infection[51-55]. The iATP assay has received approval from the US Food and Drug Administration (FDA), and is available commercially as the ImmuKnow assay (Cylex, Columbia, MD, USA).

However, there are some major concerns associated with the implementation of this approach for the diagnosis of ACR, including questions regarding sensitivity and specificity. The ImmuKnow assay mainly gives an indication of the patient's general immune status, not specifically T cell reactivity directed towards the allograft. Furthermore, there is some evidence of inconsistencies in the interpretation of the results when using this technique[56-59].

mRNA Profiling

Gene expression profiling is being performed in the search for peripheral blood biomarkers using a microarray technique that enables broad gene screening and techniques based on the real-time polymerase chain reaction (qRT-PCR) for hypothesis-driven investigations.

Horwitz et al. conducted a case-control study using microarray analysis of the whole blood in a cohort of 189 cardiac transplant patients, and demonstrated that gene transcripts in the expression profile of peripheral blood were correlated with histologically verified ACR[60]. In a multicentre Cardiac Allograft Rejection Gene expression Observational (CARGO) study, a development study designed to test the gene expression profiling hypothesis, Deng et al. developed an algorithm to generate a score ranging from 0 to 40 providing a measure of the expression of 20 genes (11 informative, 9 control and normalization) in peripheral blood mononuclear cells (PBMCs) using qRT-PCR[61]. Lower scores were found to be associated with a very low likelihood of moderate/severe ACR[62]. This gene-expression profiling algorithm has been approved by the FDA, and is commercially available as AlloMap (CareDX, Brisbane, CA, USA).

In the 2010 ISHLT Guidelines for the Care of Heart Transplant Recipients, AlloMap was assigned a Class IIa recommendation Level of evidence B, stating that: "it can be used to rule out the presence of ≥ grade 2R acute cellular rejection in appropriate low-risk patients, between 6 months and 5 years after transplantation"[12]. Although results have been very promising, the study design of the clinical trial of AlloMap, known as the Invasive Monitoring Attenuation through Gene Expression (IMAGE) trial, displayed an essential limitation, as patients who had undergone transplantation less than 6 months previously were not included in the trial, and 85% of the study population was made up of patients who had undergone transplantation between 1 and 5 years previously[63], the period when the overall risk of rejection is low[64].

cfdDNA Profiling

Cell-free DNA (cfDNA) is a short length of double-stranded DNA released from tissues as a result of either normal physiological cell turnover or pathological apoptosis. This cfDNA is found in the circulation at levels typically around 3 ng/mL, and has been used for years for non-invasive prenatal testing of foetal DNA to screen for genetic abnormalities[65,66].

In the field of solid organ transplantation, cell-free donor DNA (cfdDNA) was first detected in recipients of sex-mismatched grafts[67]. Subsequent studies confirmed the hypothesis that cfdDNA is associated with graft rejection[68,69]. A cutting-edge

technique based on next-generation sequencing to detect and measure the amount of cfdDNA in the total cell-free DNA present in the blood of solid organ transplant recipients has been recently introduced under the name AlloSure (CareDX, Brisbane, CA, USA) and is currently being validated for clinical use. In a prospective study on 65 heart transplant patients (565 samples), De Vlaminck et al. demonstrated that measuring cfdDNA enabled the diagnosis of acute cellular rejection/ACR after heart transplantation by discriminating patients suffering rejection from the control group[70].

microRNA Profiling

A novel field in biomarker research aims to detect microRNAs, small non-coding RNA that regulate post-translational gene expression in cells, tissues or body fluids. A change in the microRNA profile during allograft rejection was first observed in 2009 by Anglicheau et al. in a study on renal transplant patients[71]. This study demonstrated changes in microRNA expression in renal allograft biopsies from 33 renal transplant recipients, and it was possible to distinguish rejection from non-rejection. When work on this thesis was initiated, no studies had been performed on the microRNA signature in either intragraft or peripheral blood of heart transplant patients.

Proximity Extension Assay

Emerging proteomic technology using proximity extension assay (PEA) has opened up the possibility of detecting new clinical biomarkers by performing simultaneous analysis of the large sets of circulating proteins in biological specimens with relatively small volumes[72]. Several biomarker studies on different pathological conditions have suggested that PEAs could offer a means of obtaining blood-based molecular fingerprints[73-78]. However, when work on this thesis began, this strategy had not been used in research on solid organ transplantation.

MicroRNA Biomarkers

MicroRNAs are a class of small (19-25 nucleotides in length), non-coding RNAs that bind to the 3′ untranslated region (3′-UTR) of target messenger RNAs (mRNAs). Mature microRNAs can be associated with the RNA-induced silencing complex (RISC), which regulates gene expression by promoting mRNA degradation, or by inhibiting protein translation[79-82]. Alternatively, microRNAs can be released from the cells into the circulation in the non-vesicle fraction by binding to RNA-binding proteins, such as Argonaute-2, or to lipoproteins, and in the vesicle fraction by incorporation into the extracellular vesicles[83-85] (Figure 5).

A single microRNA has the ability to regulate hundreds of mRNAs by recognizing complementary sequences in their target mRNA, and multiple microRNAs can regulate an individual mRNA[86-88]. It has been reported in several studies that microRNAs play an essential role as fine-tuning regulators in the posttranscriptional regulation of gene expression in many pathophysiological processes associated with cardiovascular diseases[89-93] and the modulation of both innate and adaptive immune responses[94-97].

The presence of extracellular RNA in blood was first suggested in 2004[98]. However, the first report of microRNA profiling in human plasma or serum was published in 2008[99,100]. As microRNAs may be expressed in a tissue- or disease-specific manner, and the changes in their expression reflect pathophysiological conditions[101-103], their signatures in the blood can be used as potential non-invasive biomarkers for various diseases.

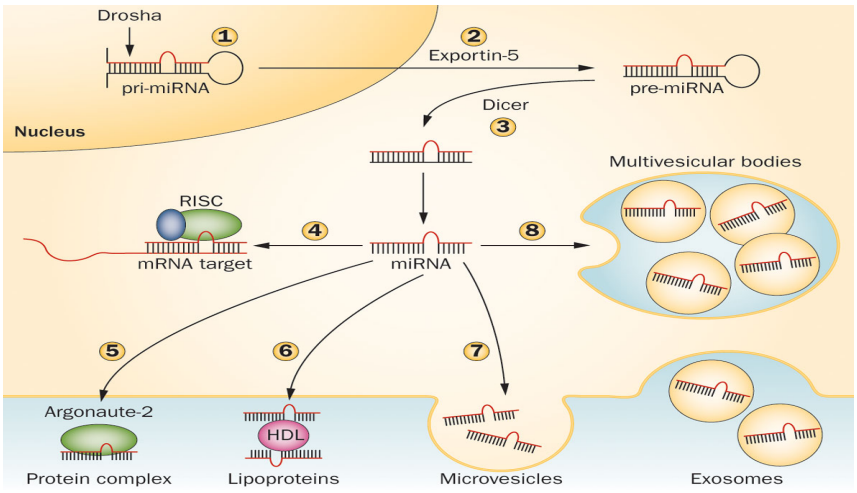

Figure 5. The mechanisms of release of microRNAs to the extracellular compartment. (Reprinted with permission from Nature Publishing Group. Guay C, et al. Circulating microRNAs as novel biomarkers for diabetes mellitus. Nature reviews. Endocrinology. Sep 2013;9(9):513-521.)

MicroRNAs in Liquid Biopsies

The use of circulating microRNAs has emerged as a non-invasive diagnostic biomarker strategy covering a wide range of pathological and biological processes[104,105]. MicroRNA detection in a liquid biopsy, i.e. a liquid biomarker that can be isolated from body fluids such as blood, urine, pleural effusion, amniotic fluid, cerebrospinal fluid and ascites, is a rapidly expanding area of biomarker research due to its capacity to represent the tissue from which it originated[106-113]. The level of extracellular microRNAs detected in liquid biopsy samples may show changes that are correlated to pathophysiological states[114,115], making these molecules excellent candidates for biomarkers of different biological and pathological conditions.

However, the technique is hampered by a number of problems, including low levels of RNA and high levels of RNA inhibitors in the body fluids, as well as the wide range of pre-analytical variables that increase its susceptibility. Despite these drawbacks, microRNAs have some properties that make them suitable for use in routine clinical practice, for example, they are remarkably stable in serum/plasma despite high ribonuclease activity, they are only minimally affected by pH changes and the freezing and thawing cycle, and their stability may be preserved for at least 40 years when frozen at -25°C[100,116-119].

Intercellular Communication via microRNA Transport

A novel mechanism of intercellular communication that involves extracellular vesicles (EVs) has emerged over the past two decades. EVs are a heterogeneous subset of vesicles and, although there is a lack of standard nomenclature (it is still being defined by the EV research community[120,121]), the current nomenclature is generally based on their size and mode of release from the cell of origin[122].

Exosomes, with sizes ranging from 20 to 100 nm, are a subset of EVs with endosomal origin, formed intracellularly within multivesicular bodies, and released into the extracellular space via fusion of these multivesicular bodies with cell surface membrane[123]. Ectosomes (or microvesicles) on the other hand, originate by direct budding from the plasma membrane[124,125]. However, the term “exosomes” is most commonly used in the field of EV research to refer any type of EV[126], as there is no consensus or EV-specific marker that distinguishes the origin of EV subsets once they have been secreted from cells[127,128].

EVs are ubiquitous in body fluids[129-142] and can be recovered from conditioned cell culture medium[143-145]. According to the recommendations of the International Society for Extracellular Vesicles (ISEV), EVs may be isolated by a variety of techniques including ultracentrifugation, filtration, immunoaffinity isolation and microfluidics techniques[146].

The ability of most cell types, including immune cells, neurons, muscle cells and tumour cells, to secrete exosomes or microvesicles has been suggested in several studies[147-153]. Exosomes were first reported to transfer information between cells in a study on immune cells in 1998, and this study provided the basis of the hypothesis that exosomes could take an active part in intercellular communication[154]. Later advances in proteomic analysis of exosome composition allowed researchers to demonstrate the selective intracellular protein content of exosomes, which allowed differentiation between exosomes and membrane vesicles released by apoptotic cells, providing insight into exosomes as a new type of intercellular messenger[155-158].

However, the real breakthrough in the field of EVs came in 2007, when Jan Lötvall's group in Sweden reported the presence of microRNA and mRNA inside these vesicles[159]. Their findings, together with subsequent studies showing that functional nucleic acids (microRNA, mRNA and other RNA species) can be shuttled in exosomes and regulate the recipient cell at a post-transcriptional level[160-162], have fundamentally shifted the paradigm concerning gene regulation.

Intercellular communication via microRNA transfer consists of 3 essential steps: 1) selection of microRNA shuttled in the appropriate EV carrier from the secreting cell; 2) protection of microRNA from circulating RNAses; and 3) the ability of microRNA to repress mRNA target in the recipient cells.

The first evidence of functional microRNA endogenous transfer was reported in 2009, when miR-126 was reported to be shuttled in apoptotic bodies targeting the RGS16 gene in cell-to-cell communication between endothelial cells[163]. However, it was not until a study on functional miR-150 transfer via microvesicles between monocytes and endothelial cells in 2010 that the proposed microRNA-based communication network between immune cells and the vascular endothelium was demonstrated[164]. Subsequent studies have unveiled a variety of "small-talk" involving functional microRNA transfer in both inflammatory and endothelial cells[161].

Role of microRNA in Organ Transplantation

Discovered 24 years ago in *C.elegans*[165], microRNAs have been found to be involved in a wide range of cellular process, as well as playing important roles in regulating diverse functions in normal and diseased states, including transplant immunology. Most research on microRNAs in transplantation so far has concerned renal allografts.

MicroRNAs were first implicated as biomarkers of allograft status in a study on renal transplant patients, where it was found that the intragraft profile of microRNAs was changed in ACR patients compared to non-rejection patients[71]. In that study, miR-155 and miR-142 were among the microRNAs overexpressed in ACR compared to the controls. MiR-155 is one of the key players in the regulation of adaptive immunity and antibody-related T cell response[166]. The expression of miR-142, a haematopoietic lineage-specific microRNA, depends on the activation state of the cells, and can be modulated by the differentiation of Th1 and Th2[167,168], the T-helper cell subsets that mediate graft rejection[169].

MiR-142-3p, a mature microRNA species generated from the 3′ arm of the miR-142 stemloop, is closely associated with allograft rejection. The change in the miR-142-3p expression profile in biopsy samples from patients with ACR has been demonstrated in both humans and a transplantation animal model[170-172]. The fact that miR-142-3p originates from immune cells, not from the graft tissue, raises the possibility of being able to predict rejection prior to organ damage.

Aims and Hypotheses

The overall objective of the work presented in this thesis was to investigate recent advances in genomic and proteomic techniques that could be utilized clinically for the non-invasive diagnosis and surveillance of allograft rejection after heart transplantation, as well as to investigate the biological role of microRNAs in the setting of ACR.

The specific aims and hypotheses of each study are given below.

Study I: The aim of this pilot study was to explore the possibility of using serum microRNAs as biomarkers of ACR after heart transplantation, by comparing serum microRNA profiles in samples collected before, during and after biopsy-proven rejection episodes in heart transplant patients with ACR.

Study II: In this validation study, the aim was to evaluate the levels of seven microRNAs that were found to be increased in serum during ACR in the previous pilot study, in a larger, independent cohort through collaboration with the Prevention of Organ Failure (PROOF) Centre of Excellence (Vancouver, Canada).

Study III: The aim of this study was to investigate the role of intercellular transfer of functional miR-142-3p between T cells and the endothelium in the setting of cardiac allograft rejection.

Study IV: In this study, the aim was to identify inflammatory fingerprints in the serum that could potentially be used as a non-invasive method of rejection surveillance for heart transplant patients with ACR, by profiling inflammation-related protein biomarkers.

Materials and Methods

This chapter provides a brief overview of the materials and methods, some of which are described in detail in the papers included in this thesis.

Heart Transplant Patients (Papers I, II, III and IV)

Subjects

The Lund cohort (Paper I)

All heart transplantations were performed at Lund University Hospital, Sweden. Serum samples from these patients were retrieved from the Microbiology Biobank at the hospital, and were matched in time with endomyocardial biopsy samples from the same patient. Informed consent was given by the patients in accordance with the recommendation and approval by the Regional Ethical Review Board in Lund. The patients included in the study represent different diagnoses (hypertrophic, dilated and ischaemic cardiomyopathy and congenital heart disease) and different age groups (13-69 years at transplantation). The diagnosis of ACR was made according to the ISHLT 1990 grading system in all patients. In 2004, a new ISHLT grading system was introduced to revise ISHLT 1990 grading system and the revised ISHLT 2004 classification has been used in clinical practice since.

The PROOF cohort (Papers II, III and IV)

All heart transplant recipients included in the studies described in Papers II, III and IV were enrolled in the Canadian Biomarkers in Transplantation Trial from 6 Canadian heart transplant centres (QE II Health Sciences Centre, Halifax, NS; Libin Cardiovascular Institute of Alberta, Calgary, AB; St. Boniface General Hospital, Winnipeg, MB; University of Ottawa Heart Institute, Ottawa, ON; Toronto General Hospital, Toronto, ON; St. Paul's Hospital, Vancouver, BC). The patients underwent heart transplantation between February 2009 and September 2013. Each local research ethics committee approved the study. A group of heart transplant patients with endomyocardial biopsy-verified ACR was compared with a control group of heart transplant patients without allograft rejection from the

same centre and within the same time period. All histological samples were blindly reviewed by three expert pathologists. Acute cellular rejection/ACR was defined according to the ISHLT 2004 classification.

Table 1. ISHLT Standardized Cardiac Biopsy Grading for Acute Cellular Rejection.

2004		1990	
Grade 0R	No Rejection	Grade 0	No Rejection
Grade 1R, Mild	Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage	Grade 1, Mild	
		A - Focal	Focal perivascular and/or interstitial infiltrate without myocyte damage
		B - Diffuse	Diffuse infiltrate without myocyte damage
		Grade 2, Moderate (Focal)	One focus of infiltrate with associated myocyte damage
Grade 2 R, Moderate	Two or more foci of infiltrate with associated myocyte damage	Grade 3, Moderate	
		A - Focal	Multifocal infiltrate with myocyte damage
Grade 3 R, Severe	Diffuse infiltrate with multifocal myocyte damage ± edema, ± hemorrhage ± vasculitis	B - Diffuse	Diffuse infiltrate with myocyte damage
		Grade 4, Severe	Diffuse, polymorphous infiltrate with extensive myocyte damage ± edema, ± hemorrhage + vasculitis

Blood Collection (Papers I, II, III and IV)

Blood samples were collected in serum collection tubes and spun down in a refrigerated centrifuge within 2 hours to clot the blood and obtain clot-free serum supernatant. The serum was transferred to a sterile cryogenic vial and then aliquoted into smaller cryogenic vials. The aliquots of serum samples were stored frozen at -80ºC until selected for biomarker analysis.

RNA Purification from Serum (Papers I and II)

Human blood serum is a challenging specimen type for RNA purification due to its high endogenous RNase activity and other pre-analysis variables such as red blood cell haemolysis and centrifugation conditions, which may interfere with the analytical process of microRNA measurement[100,173]. For this reason, each step of the preparation and RNA extraction in serum samples must be specially optimized and delicately handled.

Total RNA, including small RNAs, was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. In addition, MS2 carrier RNA (Roche) was added during the RNA purification steps in order to maximize the RNA yield and minimize the variation in purification variation[174]. As the RNA yield from serum is very low, the RNA input was normalized by using equal volumes of serum, instead of using standard methods for the quantification of RNA concentration by 260 nm spectrophotometry (e.g. Nanodrop)[175].

RNA Purification from Serum Exosomes (Paper III)

The essential criterion to claim the presence of exosomes in the specimens is that they are isolated from extracellular fluids, including body fluids and secreting cells *in vitro*. Several strategies and techniques have been employed to isolate exosomes from extracellular fluids, and no consensus has so far been reached on the gold standard for RNA isolation from exosomes[126].

Most of the studies performed on exosomes from body fluids or culture medium have employed differential centrifugation with or without size filtration to isolate exosomes[143,176-178]. Differential centrifugation was used in the study described in Paper III to pellet the following fractions, consecutively: cell debris/apoptotic bodies, larger EVs (microparticles/microvesicles/ectosomes) and exosomes. Total RNA, including small RNAs, was isolated from the pelleted exosomes obtained in the last centrifugation step using the miRNeasy Mini Kit.

First-strand cDNA Synthesis

First-strand cDNA synthesis was performed using the miRCURY LNA™ universal RT microRNA PCR kit (Exiqon) according to the manufacturer's protocol, where 8 µl of eluted serum RNA was used in 40 µl reverse transcription reactions. A synthetic RNA spike-in, UniSP6 CP, was added prior to cDNA synthesis as a control to monitor the variation in reverse transcription efficiency.

MicroRNA Profiling Assay (Paper I and Paper III)

The profile of microRNAs in serum may reflect blood cell activity[179], but the main challenge of profiling microRNA levels in the circulation is the low abundance of the microRNA fraction, which constitutes only a few percent of the total circulating RNA[100,114]. To address this challenge, a high-sensitivity microRNA quantification platform must be used to quantify the microRNA level in the circulation[173,180]. Previous studies have demonstrated that qRT-PCR-based platforms are the method of choice for microRNA profiling, due to their superior sensitivity and high specificity[181,182]. In fact, qRT-PCR-based platforms have higher sensitivity than microarray-based platforms[180,183].

In the studies presented in Papers I and III, a qRT-PCR-based microRNA-profiling assay for 175 miRNAs, known to be present in human serum, was used. SYBR green-based qRT-PCR for microRNA expression profiling was performed using Serum/Plasma Focus Human microRNA PCR panels (Exiqon), according to the manufacturer's instructions. Seven reference gene candidates (miR-451, miR-16, miR-103, miR-425, miR-423-5p, miR-93 and miR-191) were included in the panel. A global mean expression normalization strategy was used due to the absence of a reference gene for the normalization of serum samples in this patient population, as has been described earlier[184].

Validation of Selected microRNAs (Paper I and II)

Individual qRT-PCR assays (Paper I)

Conventional qRT-PCR using LNA™ microRNA primer sets (Exiqon) was performed to validate the results of the microRNA profiling assay in the serum samples (Paper I). PCR reactions were performed with cDNA synthesized in a different set of reactions from those used for the cDNA in the profiling experiment. Based on data from the profiling assay, miR-451 was selected as a suitable reference gene in the validation experiment, as it showed low inter-sample and inter-group variation. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method[185].

Pick and Mix microRNA qRT-PCR panel (Paper II)

In order to assess the expression of seven microRNAs (miR-326, miR-142-3p, miR-101, miR-144, miR-27a, miR-424 and miR-339-3p), whose profiles were found to be changed in the first study, qRT-PCR reactions were performed using a Pick and Mix microRNA PCR Panel (Exiqon), according to the manufacturer's instructions. Although it is based on the same principle as the individual qRT-PCR validation assay, this panel is more convenient for quality control when handling a

large number of samples. The results obtained from the PCR panel were subsequently analysed using GenEx qPCR Data Analysis Software 2.0 (Exiqon). In brief, the raw data were normalized for run-to-run variations using UniSP3 IPC as an inter-plate calibrator. Delta Ct of (miR-23a-3p - miR-451) was used as a control for cellular contamination and haemolysis. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method and uniSp6 spike-in was used as the reference for normalization.

Proteomic Profiling (Paper IV)

The PEA is a cutting-edge technique in biomarker research for proteomic profiling. This assay allows the analysis of 92 inflammation-related protein biomarkers using 1 µl of serum simultaneously in 96 serum samples, or any other type of biofluid, as has been described previously[72,186].

In the study described in Paper IV, proteomic profiling in serum samples was performed at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University, Sweden, using The Olink Proseek® Inflammation I96×96 multiplex immunoassay. Briefly, for each measured protein, a pair of highly specific antibody probes binds to the targeted protein, and if the two probes are in close proximity, a PCR target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using qPCR (Figure 6).

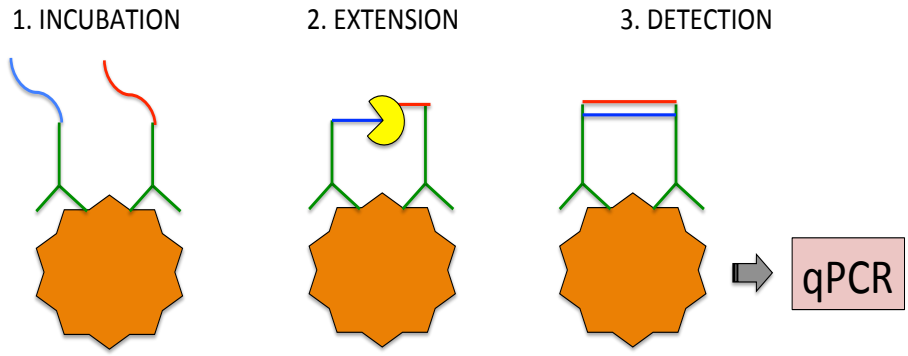

Figure 6. The principle of the proximity extension assay (PEA). Pair-wise binding of probes (DNA oligo-labelled antibodies) that subsequently leads to the formation of a PCR target sequence by DNA polymerase in a proximity-dependent manner, resulting in a sequence that can be detected and quantified using standard real-time PCR.

In Vitro and *Ex Vivo* Cultures (Paper III)

Isolation of Human PBMCs and T cells

PBMCs and T cells were isolated from the blood samples of healthy volunteer donors. Informed consent of the donors included in this study was obtained prior to inclusion, and the study was approved by Lund University Ethics Committee, in accordance with US National Institutes of Health guidelines and the principles outlined in the Declaration of Helsinki.

In brief, the collected blood was centrifuged to obtain the buffy coats. PBMCs were isolated by centrifugation of the buffy coats on the top of Lymphoprep™ (Stemcell Technologies). T cells were isolated from PBMCs by depleting non-T cells to obtain pure and untouched T cells using the Dynabead™ Untouched Human T Cells Kit (Invitrogen) according to the manufacturer's instruction. The T cells obtained were then seeded in cell culture conditioned-medium.

Ex vivo Activation of Human T cells

The function of human T cells can be assessed *ex vivo* by their ability to proliferate during T cell activation. Two different T cell activation methods were used. In the first method, 2% v/v PHA and 10 ng/µl IL-2 were added to the T cell culture medium to activate the T cells[187]. This approach is less physiological than the second method, in which beads coated with anti-CD3 and anti-CD28 were used, in a manner that closely mimics the stimulation of the antigen-presenting cell[188].

Exosome Isolation

The exosome was isolated from the cell culture conditioned-medium using differential centrifugation. Briefly, the cell culture medium was centrifuged at 300 x g for 10 minutes to remove cell debris. The supernatant was then collected and centrifuged at 10,000 x g for 30 minutes to deplete large extracellular vesicles. Finally, the exosomes were pelleted by ultracentrifugation at 200,000 x g for 1 hour. All centrifugation was performed at 4°C. Both the exosome-containing pellets and the cell culture medium without exosomes (supernatant) were collected.

MicroRNA Transfer between T cells and Endothelial Cells

Human umbilical vein endothelial cells (HUVECs, Thermo Fischer Scientific) were used as an in vitro model of endothelial cells. Endothelial cells cultured in conditioned medium were stimulated with exosomes isolated from either serum samples from heart transplant patients with ACR or from activated T cells.

After 24 hours' incubation, stimulated endothelial cells were lysed in Qiazol, and RNA extraction was performed. Subsequently, qRT-PCR was performed for microRNA and mRNA analysis to evaluate the expression of miR-142-3p and its target gene, RAB11FIP1, respectively.

The second line of evidence of microRNA transfer in this study was provided by the blockage of transcription in endothelial cells. Briefly, endothelial cells were treated with 1 μg/ml of actinomycin D (Sigma). The cells were then maintained in culture for 24 hours in the presence or absence of T cell exosomes. MiR-142-3p transfer between T cell exosomes and endothelial cells was analysed by evaluating miR-142-3p expression in the endothelial cells.

Microarray of Endothelial cells

Microarray analysis was performed to predict the potential target of miR-142-3p. The RNA extracted from stimulated endothelial cells underwent quality control using an Agilent Bioanalyzer, and was analysed using the Affymetrix mRNA Gene 2.0 ST Array at the Swegene Center for Integrative Biology at Lund University. A significance analysis of microarray (SAM) *t*-test was performed to determine differentially expressed RNA. A cut-off value of >1.5 fold change and the bioinformatics tools TargetScan, miRanda, miRWalk and miRDB were used to identify potential targets of miR-142-3p

Transfection of Endothelial Cells

Cultured endothelial cells were transfected with 50 nM of either precursor or miR-142 mimic (Life Technologies) for 72 hours, according to the manufacturer's instructions. Scrambled pre-miRNA was used as a negative control. Subsequently, qRT-PCR analysis was performed on transfected cells to evaluate miR-142-3p expression. To confirm RAB11FIP2 as a target for miR-142-3p in endothelial cells on the protein level, the expression of RAB11FIP2 in HUVECs transfected with pre- and anti-miR-142 was evaluated using western blot analysis.

3'UTR Target Plasmid Reporter Assay

The interaction of target mRNA and miR-142-3p was analysed using miTarget miRNA 3′UTR target clones (Genecopoeia). The expression clones were based on the pEZX-MT51 vector containing dual reporter genes, while Gaussia luciferase (GLuc) served as microRNA 3′UTR target reporter, and secreted alkaline phosphatase (SEAP) served as an internal control for normalization of transfection efficiency and cell viability. A miTarget miRNA 3′UTR target clone, containing either of the nucleotides 1-2113 or 2018-4115 of the RAB11FIP2 3′-UTR, was co-transfected with 50 nM of precursor miR-142 (Life Technologies) or scrambled pre-miRNA as a negative control in HUVECs. An empty miTarget vector was transfected as a control. The transfection in HUVECs was performed using a Nucleofector device and its corresponding HUVEC Nucleofector Kit (Lonza). SEAP and GLuc activity were measured 72 hours after transfection using a Secrete-Pair Dual Luminescence Assay Kit (Genecopoeia) on a Glomax 20/20 Luminometer (Promega). Results were expressed as the ratio of GLuc to SEAP (Figure 7).

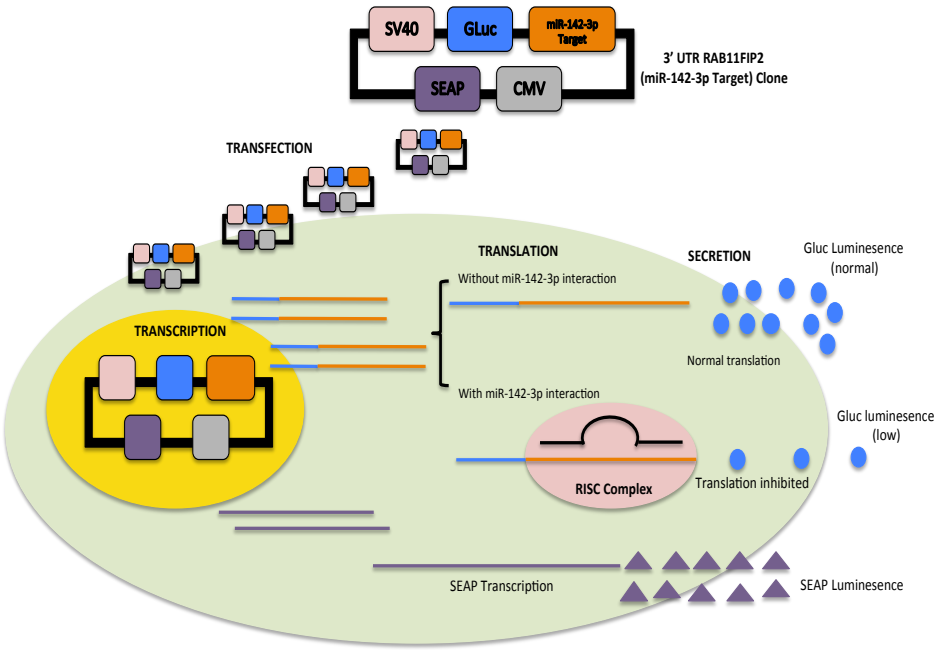

Figure 7 Inhibitory effect of miR-142-3p on the 3′UTR RAB11FIP2 clone expressed as the Gluc/SEAP dual reporter vector system. The 3′UTR RAB11FIP2 sequence is inserted downstream of the Gaussia luciferase (Gluc) reporter gene, which is driven by the SV40 promoter. The secreted alkaline phosphatase (SEAP) reporter, whose expression is controlled by the CMV promoter, is also cloned into the same vector and serves as internal control. The inhibitory effect of the mRNA-microRNA target interaction was analysed by measuring the reporter protein secreted by Gluc and SEAP activity.

Vascular Permeability Assay

The vascular permeability in the endothelial cells was evaluated in connection with knockdown of the RAB11FIP2 expression in the cells, as RAB11FIP2 has previously been shown to take part in the regulation of vascular endothelial barrier integrity and function[189]. Briefly, endothelial cells were seeded on a fibronectin-coated insert of 24-well transwell plate (0.4 μm pore size; Corning). After siRNA-mediated knockdown of RAB11FIP2, 1 mg/ml/L fluorescein isothiocyanate FITC dextran (MW 70 kDa, Sigma) was added to the upper compartment of the transwell plate. The medium from the lower compartment was collected at different times, and the amount of FITC dextran that had passed into the lower compartment was measured with a plate reader (VICTOR3TM, Perkin Elmer).

To provide the second line of evidence that miR-142-3p-containing exosomes may affect the vascular permeability in endothelial cells by targeting RAB11FIP2, exosome transfer in miR-142-3p-inhibited endothelial cells was analysed. Briefly, endothelial cells were cultured on the fibronectin-coated insert of a 24-well transwell plate. Cells were transfected with 50 nM of miR-142 inhibitor (Life Technologies) according to the manufacturer's instructions. After 72 hours' incubation, the cells were treated with activated T-cell exosomes for 24 hours, and subsequently harvested for qRT-PCR or vascular permeability assay analysis.

Statistical Analysis (Paper I - IV)

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA) and SPSS Statistics v.22.0 (IBM Corp., Armonk, N.Y., USA). Visual inspection of the descriptive analysis of histogram and the D'Agostino-Pearson test were applied to test for the normality of the data and to detect a deviation from a Gaussian distribution in the sampled population. Parametric statistical analysis was used for normally distributed data, and the non-parametric approach was used for small sample sizes or non-normally distributed data. A p-value <0.05 was considered to indicate a significant difference.

To establish which diagnostic method gave the best combination of sensitivity and specificity, receiver operating characteristic (ROC) analysis was used to determine the cut-off points and to calculate the area under the curve (AUC).

To account for multiple testing in microarrays (Paper III), microRNA profiling assays (Papers I and III) and proteomic profiling (Paper IV), statistical analysis of microarrays (SAM) as described by Virginia Tusher[190], the Bonferroni correction, and the false discovery rate (FDR) method described by Benjamini and Hochberg[191], were used respectively.

Results

Study I

Altered Serum microRNA Profiles during Acute Rejection after Heart Transplantation: Potential for Non-invasive Allograft Surveillance

A pilot study was conducted to explore the possibility of using serum microRNAs as biomarkers of ACR after heart transplantation, by comparing serum microRNA profiles in samples collected before, during and after histologically verified rejection in patients with cardiac allografts.

Circulating microRNA Profile was Altered during Acute Rejection

A good biomarker of acute cellular rejection should be able to detect small changes in the immune status of the patient before any damage has occurred to the allograft. To investigate whether such changes could be reflected by changes in microRNAs commonly found in human serum, a qPCR-based microRNA profiling assay was applied to 175 microRNAs known to be present in human serum. Ten patients who had experienced histologically verified rejections were selected, and temporally matched serum samples were analysed.

The patients included in this study represent different diagnoses (hypertrophic, dilated and ischaemic cardiomyopathy, and congenital heart disease) and different ages (13-69 years at transplantation). Serum samples were collected from each patient before any rejection had occurred (BR), during rejection (DR), and after rejection (AR) and analysed. The levels of seven microRNAs (miR-326, miR-142-3p, miR-101, miR-144, miR-27a, miR-424 and miR-339-3p) were found to be increased at least 1.5 times during rejection, compared to both before and after rejection.

Six Circulating Serum microRNAs are Significantly Increased During Rejection compared to Before Rejection

Conventional qRT-PCR was used to individually and technically validate the results obtained for the seven selected microRNAs after the initial screening for changes in serum microRNAs. Six microRNAs: miR-326, miR-142-3p, miR-101, miR-144, miR-27a and miR-424, were found to be significantly increased during rejection compared to before rejection (Figure 8).

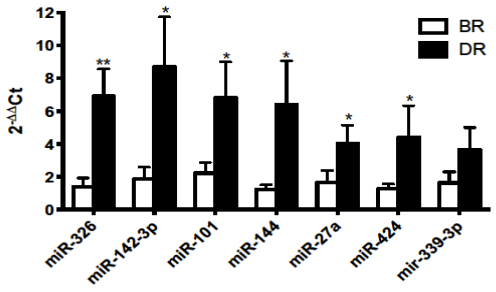

Figure 8. MicroRNA profiling assay in heart transplant patients showing that the level of six microRNAs: miR-326, miR-142-3p, miR-101, miR-144, miR-27a and miR-424, increased significantly during rejection (DR), compared to before rejection (BR).

Evaluation of the Sensitivity and Specificity for miR-326 and miR-142-3p

ROC curves were used to evaluate the relationship between sensitivity and specificity for each microRNA when comparing levels before rejection to levels during rejection. MiR-326 and miR-142-3p showed notable AUC values of 0.86 and 0.80, respectively, which allowed significant discrimination between the normal and diseased state, as determined by histological examination (Figure 9).

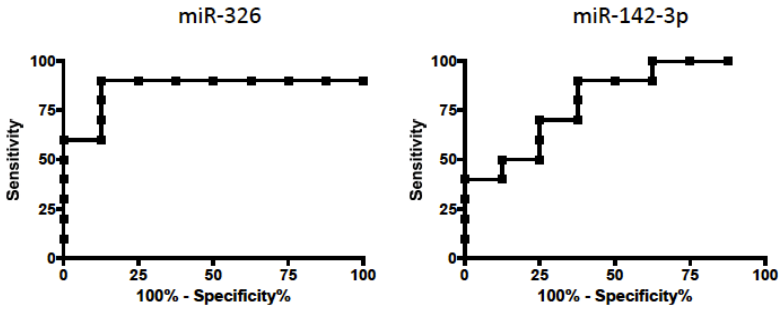

Figure 9. ROC analysis of miR-326 and miR-142-3p showing area under the curve (AUC) values of 0.86 and 0.80, respectively.

Study II

Association of Serum miR-142-3p and miR-101-3p Levels with Acute Cellular Rejection after Heart Transplantation

The results from the pilot study were validated in a larger and independent cohort of heart transplant patients with biopsy-proven acute cellular rejection/ACR. The clinical characteristics of the patients were also evaluated in relation to the changes in level of circulating microRNAs.

Altered Circulating miR-142-3p and miR-101-3p Levels in the Serum of Heart Transplant Patients with ACR

There is consensus in the field of microRNA biomarker research that any clinical association regarding microRNA identified in a small sample of patients must be verified and validated in a larger, independent study.

In this study, seven circulating microRNAs (miR-326, miR-142-3p, miR-101, miR-144, miR-27a, miR-424 and miR-339-3p), whose profiles were changed in serum during ACR in the pilot study, were evaluated in a larger, independent cohort. Heart transplant patients with histologically verified ACR (n=26) were compared to a control group of heart transplant patients without allograft rejection (n=37) from the same centres and within the same time period. Detailed patient characteristics are listed in Table 2.

According to Student's *t*-test using p=0.05 as a cut-off value, the levels of all seven microRNAs were significantly higher in the heart transplant patients with ACR than in the patients with no rejection (NR). MiR-142-3p and miR-101-3p showed the most significant differences, with p-values of 0.0032 and 0.0041, respectively.

Table 2. Clinical characteristics of heart transplant patients included in Study II.

Patient Characteristics	**Acute Cellular Rejection (n = 26)**	**Non Rejection (n = 37)**	***P*-value**
Recipient age (median, $_{IQR}$ <25-75>)	52 (37 – 61)	56 (45 – 61)	0.3
Recipient gender (male, *n* <%>)	17 (65)	29 (76)	0.3
ISHLT Biopsy Grade, n<%>			
- 0R (none)	-	37 (100)	
- 1R (mild)	2 (8)	-	
- 2R (moderate)	24 (92)	-	
Primary heart disease, *n* <%>			
- Ischemic cardiomyopathy	7 (27)	16 (42)	0.2
- Non-ischemic cardiomyopathy	14 (54)	21 (55)	0.9
- Valvular cardiomyopathy	2 (8)	0 (0)	0.08
- Congenital cardiomyopathy	1 (4)	1 (3)	0.8
- Miscellaneous	2 (8)	0 (0)	0.08
Time of Biopsy (Days after Tx) (median, $_{IQR}$ <25-75>)	23 (14 – 105)	37 (20 – 59)	0.09
Creatinine (mean, umol/L)	105.7	133.2	0.055

Circulating microRNA Levels in Serum Discriminate between Patients with ACR and Quiescence

ROC analysis was performed to evaluate the relationship between sensitivity and specificity based on the relative microRNA levels in cases of ACR and controls. All seven microRNA tested could be used to discriminate significantly between ACR and NR (Figure 10). However, according to the ROC analysis, miR-142-3p and miR-101 have the best diagnostic ability among the seven microRNAs tested, with AUC values of 0.78 and 0.75, respectively.

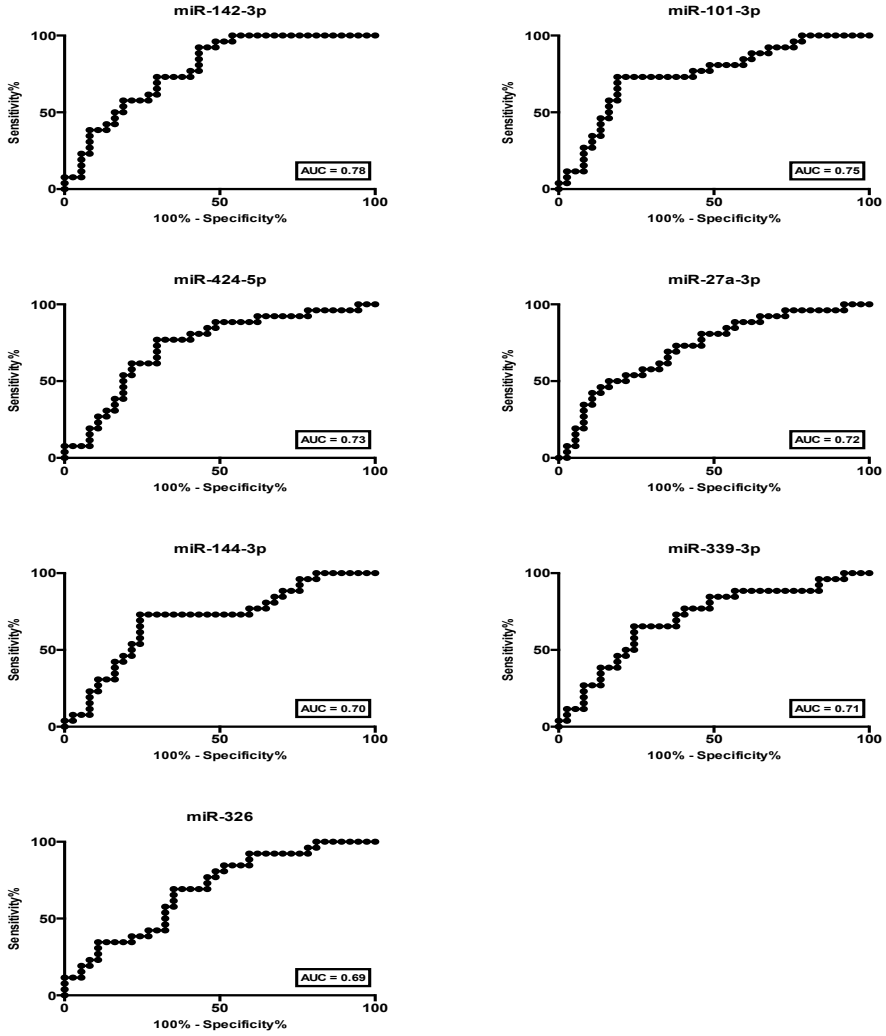

Figure 10. ROC analysis of the seven microRNAs investigated in Study II.

Circulating miR-142-3p and miR-101-3p Levels Reported by Time Post-transplantation

Serum microRNA levels were analysed at the same points in time that typical post-heart transplantation surveillance biopsies are performed, i.e. <1 month, 1-3 months, 3-6 months and 6-12 months after transplantation. The serum level of miR-142-3p was significantly higher in ACR patients than in NR patients in samples collected within 6 months post-transplantation. On the other hand, the serum level of miR-101-3p was significantly higher in the ACR group in samples taken during the first 3 months after transplantation, but not at later times (Figure 11).

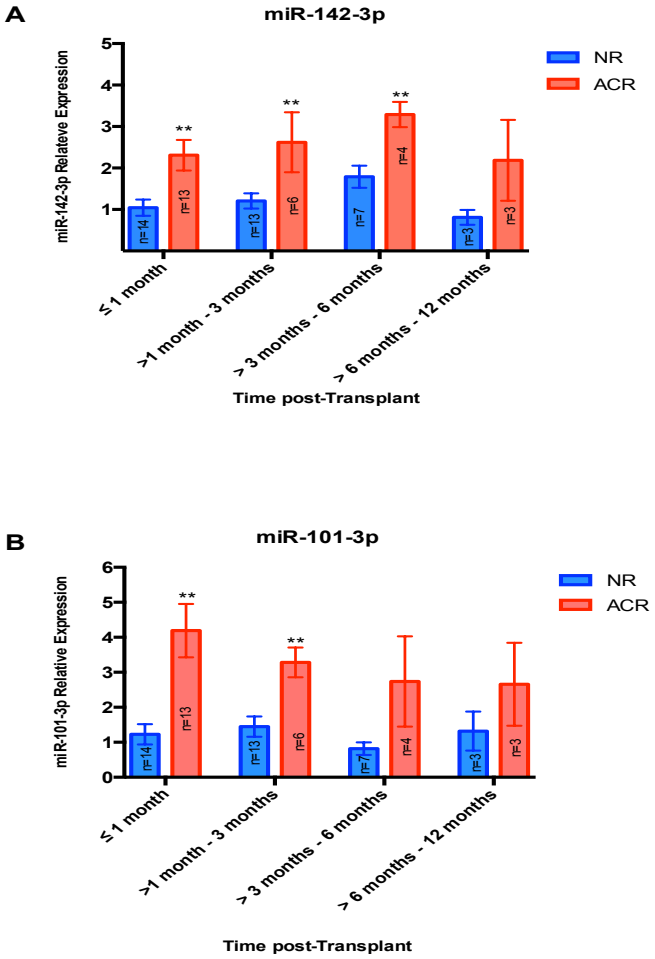

Figure 11. Circulating miR-142-3p and miR-101-3p levels in serum according to time post-transplantation.

Correlation between Circulating miR-142-3p and miR-101-3p and Clinical Parameters

C-Reactive Protein

To investigate whether the changes in the levels of circulating miR-142-3p and miR-101-3p in the serum of heart transplant patients could be indicative of general inflammation, the correlation between these microRNA levels and C-reactive protein (CRP) was investigated. The results showed that there was no correlation between the level of CRP and the levels of miR-142-3p or miR-101-3p. Furthermore, there was no significant difference in CRP levels between the ACR and NR groups.

Immunosuppressive Drugs

Heart transplant recipients are subject to life-long treatment with a cocktail of immunosuppressive drugs to prevent allograft rejection. The aim of this study was to investigate whether the overall intensity of immunosuppression in the heart-transplanted patients could modulate the serum levels of miR-142-3p and miR-101-3p by analysing the correlation between the levels of calcineurin inhibitors, i.e. tacrolimus and cyclosporine, and miR-142-3p or miR-101-3p in the circulation of heart transplant patients. No significant differences were found between the levels of tacrolimus in the blood in ACR group and the NR group, and no correlations were seen between tacrolimus or cyclosporine levels with either miR-142-3p or miR-101-3p levels in serum.

Creatinine

Heart transplant patients that receive the calcineurin inhibitors cyclosporine and tacrolimus in immunosuppressant treatment are at a high risk of developing nephrotoxicity. To assess the relationship between circulating miR-142-3p and miR-101-3p levels and kidney function in heart transplant patients, the correlation between creatinine level and miR-142-3p and miR-101-3p was analysed. The results showed that there was no significant difference in creatinine levels in the ACR group compared to the NR group, and no correlation was found between either miR-142-3p or miR-101-3p and creatinine level.

Study III

Exosomal miR-142-3p is Increased during Cardiac Allograft Rejection and Augments Vascular Permeability through Downregulation of Endothelial RAB11FIP2 Expression

The study was carried out to investigate the role of exosomal transfer of functional miR-142-3p in cell-to-cell communication between T cells and the endothelium in the setting of cardiac allograft rejection.

MicroRNAs are Enriched in the Exosomes of Heart Transplant Patients with ACR

To analyse the exosomal microRNA content in the setting of ACR, qRT-PCR-based microRNA profiling was performed on exosomes isolated from serum of heart transplant patients. The profiling panel consisted of 175 microRNAs with high abundance in the circulation. Differences in microRNA expression were observed between the ACR group and the NR group. Moreover, miR-142-3p, miR-92a-3p miR-339-3p and miR-21-5p were found to be enriched in exosomes from patients in the ACR group, compared to those in the NR group (Figure 12).

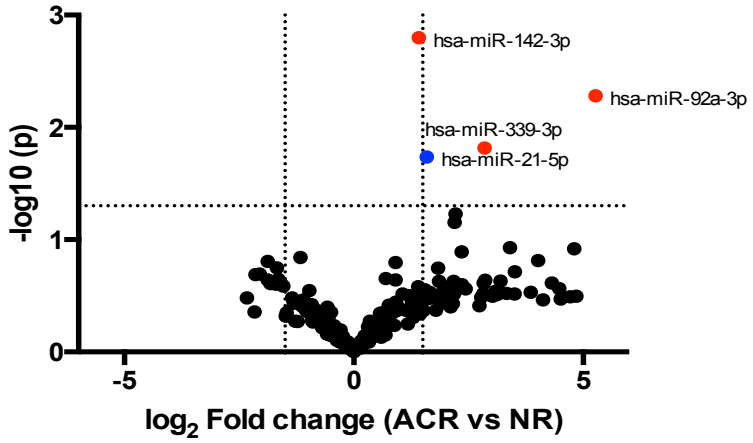

Figure 12. . Volcano plot showing microRNA fold change (ACR versus NR) based on microRNA profiling in exosomes extracted from the serum of heart transplant patients.

MiR-142-3p is Released upon In Vitro Activation of Human T cells

It was demonstrated in this study that miR-142-3p is expressed in human leukocyte subpopulations (monocytes, monocyte-derived macrophages, T cells and B cells); the highest expression being found in monocytes and T cells.

Considering the essential role of T cells in mediating the pathophysiological process of ACR, it was hypothesized that miR-142-3p, which was found to be enriched in the serum of heart transplant patients with ACR, might be shed from activated T cells to the extracellular compartment. Two distinct methods of in vitro activation of human T cells were performed, one using PHA/IL-2 stimulation and the other beads coated with anti-CD3 and anti-CD28 activation. The results showed that both T cell activation methods caused a release of miR-142-3p to the extracellular compartment. Moreover, a decrease was seen in the intracellular levels of miR-142-3p after both treatments (Figure 13).

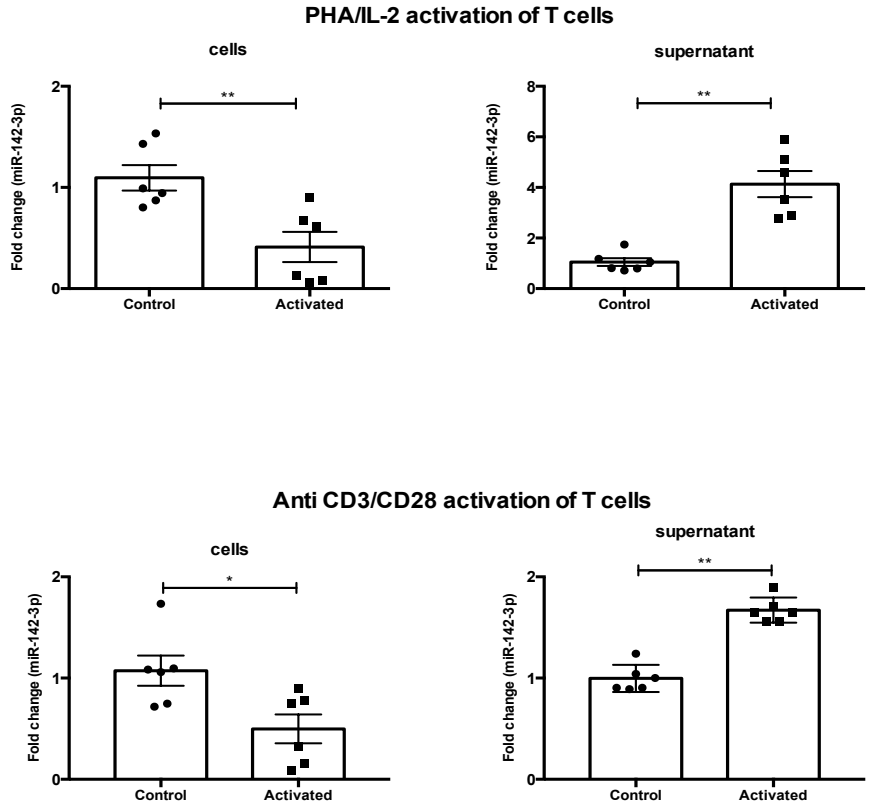

Figure 13. Results of the two human T cell activation methods used in Study III.

Release of miR-142-3p from Activated T cells is Exosome-dependent

Given the novel method of cell-to-cell communication via exosomal transport of functional microRNA, it was hypothesized that the miR-142-3p in the extracellular compartment resulting from T cell activation is shuttled within the exosomes. Ultracentrifugation, the most widely utilized method for exosome isolation, was used to isolate exosomes from the supernatant of activated T cells. The level of miR-142-3p increased significantly in the exosome fraction after T cell activation, but not in the supernatant fraction. Moreover, inhibition of exosome biogenesis by pre-treating T cells with Brefeldin, an inhibitor of exosome formation, before T cell activation, caused complete inhibition of miR-142-3p release.

Endothelial Cells Can Take up miR-142-3p Released from Activated T cells

Given the recent evidence of an intercellular communication network between immune cells and endothelial cells, and that the pathophysiology of ACR is mainly orchestrated by T cells and endothelial cells, it was hypothesized that miR-142-3p shed by activated T cells via exosomes may be taken up by endothelial cells.

Several lines of evidences were provided to demonstrate the uptake of miR-142-3p by endothelial cells. Firstly, the expression of miR-142-3p was increased in endothelial cells treated with supernatant and in exosomes isolated from activated T cells, but not in the exosome-depleted supernatant. Secondly, the primary transcript of miR-142 (pri-miR-142) was also assessed in the stimulated endothelial cells to rule out endogenous expression. Thirdly, miR-142-3p expression was increased in the cells treated with T cell exosomes after the blockage of endothelial transcription by actinomycin D, which inhibits RNA synthesis.

MiR-142-3p Regulates RAB11FIP2 Expression in Endothelial Cells

MicroRNA is a fine-tuning regulator of gene expression that controls many physiological and pathological process. It was hypothesized that the uptake of miR-142-3p by endothelial cells would target a certain gene, leading to biological changes in endothelial cells. Microarray analysis was performed on endothelial cells treated with exosome-containing or exosome-depleted T cell supernatant to gain insight into the effect of the exosome-mediated uptake of miR-142-3p in endothelial cells on a transcriptome-wide level. *In silico* target gene prediction was subsequently employed using five different target prediction tools (miRanda, miRDB, miRWalk, RNA22 and TargetScan) to identify genes with a high probability of being true miR-142-3p targets. Finally, a literature search was conducted on genes predicted by three or more of the prediction tools, which overlapped with the exosome-regulated genes, to find candidate genes associated with vascular biology and thus potentially of importance in inflammation or graft rejection. As disruption of the function of the vascular endothelial barrier is a

molecular hallmark of inflammation as well as graft rejection, the study was focused on RAB11FIP2, which has previously been reported to play an important role in regulation of the endothelial barrier function (Figure 14).

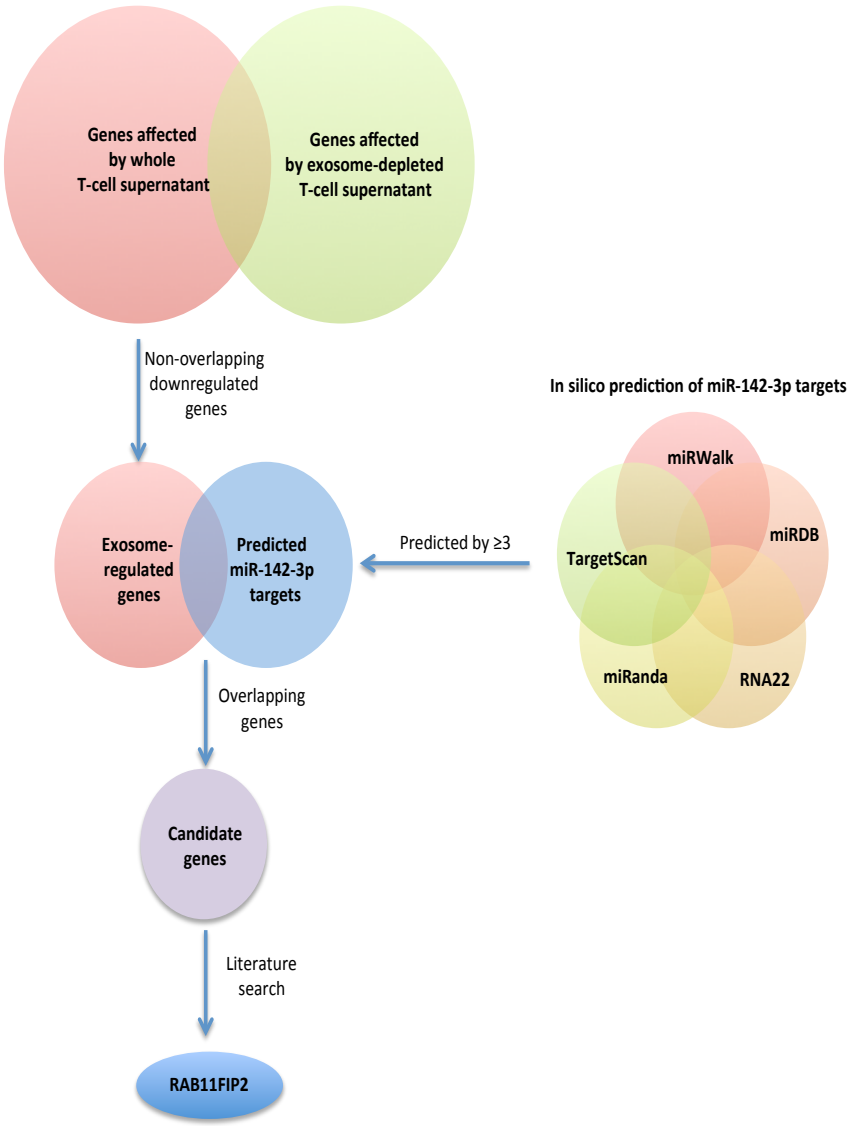

Figure 14. *In vitro, in silico* and literature search for miR-142-3p target genes in Study III.

Interaction of miR-142-3p and RAB11FIP2 in Endothelial Cells Regulates Vascular Permeability

Given the recent evidence concerning the role of RAB11FIP2 in vascular permeability and the cellular mechanism of T cell-mediated cellular rejection being initiated by the increase in vascular permeability, it was hypothesized that the exosomal miR-142-3p shed by T cells upon activation and taken up by endothelial cells would interact with RAB11FIP2 and cause an increase in vascular endothelium permeability. First, siRNA-mediated knockdown of RAB11FIP2 expression in endothelial cells was performed, which resulted in an increase in endothelial permeability, providing evidence of a biological role of RAB11FIP2 in endothelial cells (Figure 15A). It was then demonstrated that inhibiting the transfer of miR-142-3p using the antimiR (antagonist) of miR-142-3p could reverse the effect of exosome treatment on the permeability of endothelial cells (Figure 15B). Finally, a parallel rescue of RAB11FIP2 expression could be observed in endothelial cells pretreated with anti-miR-142 (Figure 15C).

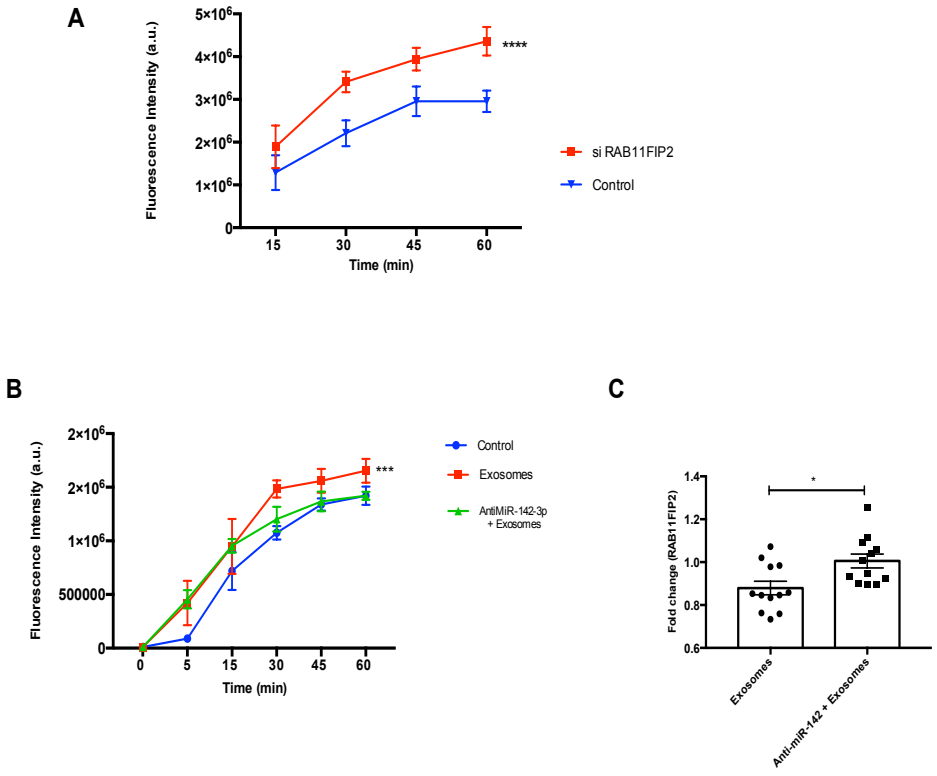

Figure 15. The role of Rab11FIP2 and miR-142-3p in the regulation of endothelial permeability.

Study IV

Immunologic Serum Protein Profiles for Non-invasive Detection of Acute Cellular Rejection in Cardiac Allograft Patients

Recent advances in proteomics and genomics have led to the suggestion of several candidates as non-invasive biomarkers for the early detection of ACR in heart transplant recipients. The aim of this study was to explore the possibility of identifying inflammatory fingerprints in the serum of heart transplant patients with ACR, by profiling inflammation-related protein biomarkers using an emerging technique of proteomic profiling called the proximity extension assay (PEA).

Inflammatory Proteins Can Discriminate between Heart Transplant Patients with ACR and Quiescence

Based on the evidence that ACR of the cardiac allograft is mediated by T cells, it was hypothesized that T cell-associated proteins may be potential protein biomarkers that could be used to diagnostically discriminate between heart transplant patients with patients with ACR and those without. Proteomic profiling assays were performed on serum samples from heart transplant patients, and the results showed that 10 circulating serum proteins were increased in the ACR group, compared to the NR group. At least 7 of these 10 inflammatory biomarkers were T cell chemokines (CXCL9, CXCL10, CXCL11 and CCL19) or cytokines (SLAMF1, LTA and IL12B).

The FDR method was used to account for multiplicity in the protein profiling assay, in which 92 inflammatory proteins were measured simultaneously, and FDR-adjusted *p*-values (referred to as q values) below 0.05 were considered to indicate significant differences. Moreover, ROC analysis of the 10 protein biomarkers suggested that they could diagnostically discriminate between ACR and NR patients (Figure 16).

To determine independently informative predictors of ACR among the protein biomarkers that passed the FDR cut-off values, a stepwise logistic regression analysis with backward elimination was performed. This analysis constructs a final best-fit logistic regression model from protein biomarkers individually associated with rejection. CXCL10 and SLAMF1 were retained in the model with *p*-values of 0.001 and 0.006, respectively.

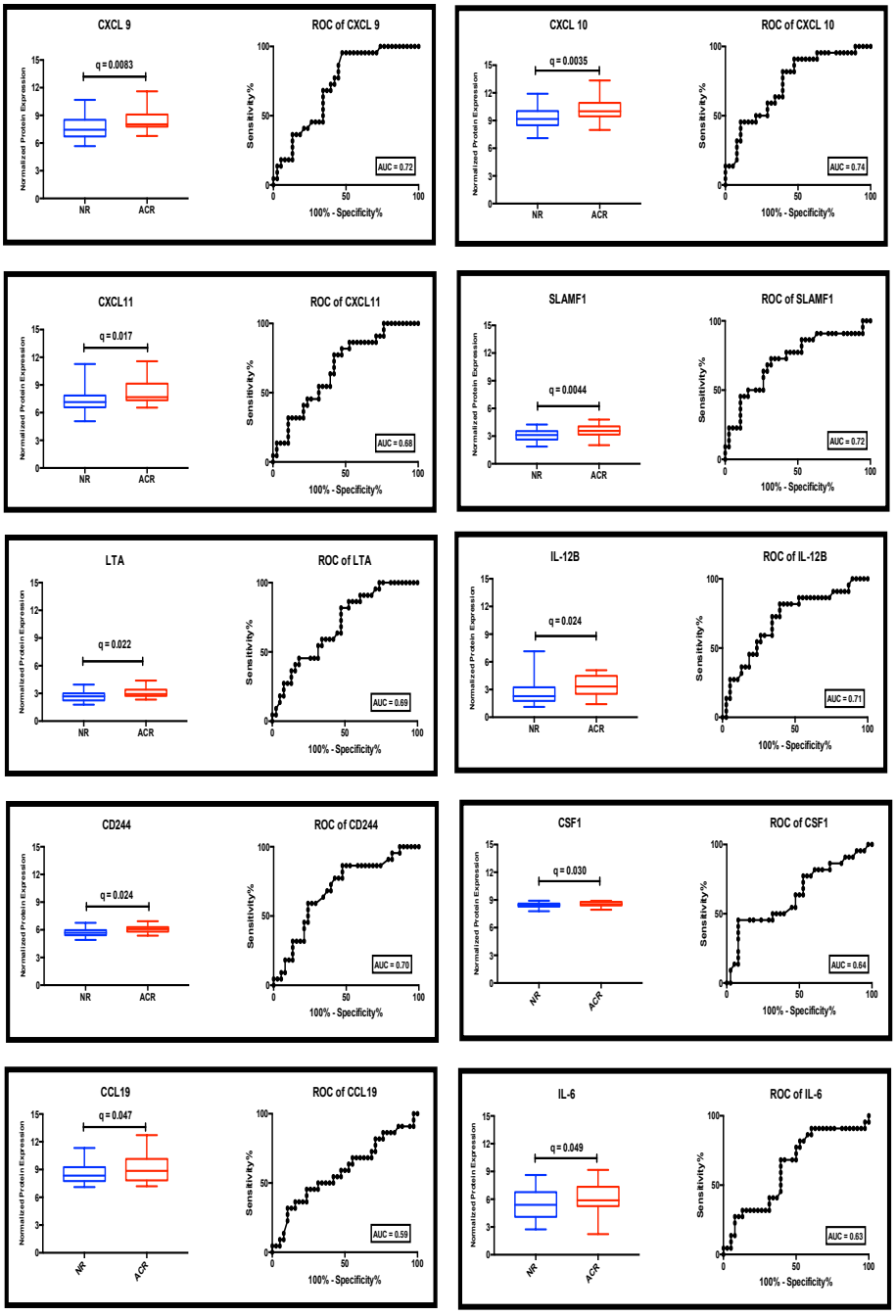

Figure 16. The expression of the 10 most significantly increased proteins in the serum of the ACR group and the NR group and their corresponding ROC curves.

A Multimarker Protein Score can be Constructed from CXCL10 and SLAMF1 Protein Biomarkers

In the context of ACR, it is very unlikely that a single protein biomarker will be able to discriminate between ACR patients and NR patients. Therefore, a multimarker protein score was constructed using CXCL10 and SLAMF1, the two protein biomarkers retained in the final logistic regression model. The multimarker protein score represents the cumulative NPX values of CXCL10 and SLAMF1 obtained from a comparison between the ACR and NR groups (Figure 17A). The performance of this multimarker protein score in identifying allograft rejection was evaluated using ROC analysis, and showed a notable AUC of 0.80 (Figure 17B).

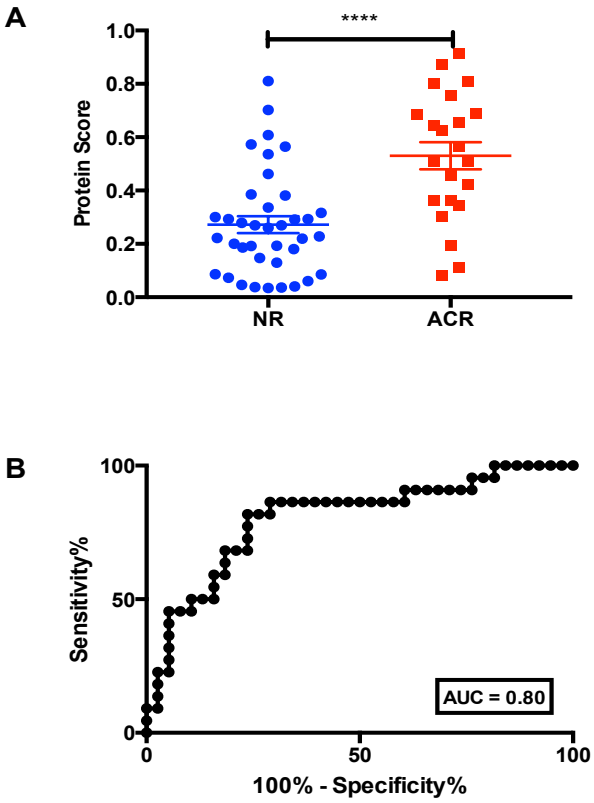

Figure 17. Discrimination between ACR and NR groups using the multimarker protein score.

Discussion

The studies included in this thesis were concerned with two distinct topics of ACR. In Studies I, II and IV, the clinical aspects of molecular biomarkers, i.e. microRNAs and inflammatory proteins, were evaluated to investigate their potential as non-invasive biomarkers after heart transplantation. In Study III, the molecular aspects of acute cellular rejection were assessed by evaluating the functional role of extracellular microRNA and its involvement in vascular permeability, a molecular hallmark of ACR.

Non-invasive Rejection Surveillance – An Unmet Need

Heart transplantation remains a life-saving option for both adults and children with end-stage heart disease in the absence of contraindications [192,193]. Over the past 5 decades, heart transplantation has evolved into a cornerstone therapy for heart failure patients with an excellent outcome, mostly due to advances in surgical techniques, peri-operative care and life-long treatment with immunosuppressant[194]. Unlike other types of solid organ transplantation, such as kidney[195] or liver[196], where a slim possibility of clinical operational tolerance could be expected – defined as life-long stable and acceptable graft function without the need of immunosuppressants – this is not the case in heart transplantation. ACR, which requires immunosuppressants to prevent or treat, would be a lifetime impending threat for heart transplant recipients. Therefore, developing biomarkers matter in heart transplantation and has been a long-standing challenge, not only due to the imprecision of current methods to identify acute cellular rejection, but also for the heart transplant clinician to predict the risk of infection and patient prognosis, as well as to manage the immunosuppressant therapy required for every transplant patient.

Diagnostic advances in the field of heart transplantation have led to the development of endomyocardial biopsy techniques that can be used to diagnose ACR and to obtain endomyocardial biopsy specimens for surveillance. A system for the pathological grading of ACR was proposed by the ISHLT in 1990, and was revised in 2004[34]. However, a number of drawbacks were associated with this

system. The three main issues concerning endomyocardial biopsy as a routine surveillance technique are its invasiveness, subjectivity and expense. These issues have prompted the development of a clinically validated, FDA-approved, gene-expression profiling test, known as AlloMap, to modulate the frequency of biopsy surveillance. However, AlloMap only partially addresses the need for a non-invasive post-heart transplantation method of diagnosis, and does not provide an equivalent alternative to endomyocardial biopsy. In a clinical study in which AlloMap-based surveillance was directly compared with endomyocardial biopsy-based surveillance (the IMAGE trial), the authors acknowledged the limitation of the study, as the patients enrolled had a low risk of rejection[63,197]. Therefore, several efforts to identify new, reliable, non-invasive techniques are still on going in the biomarker research field and waiting to see the light of day.

Early Detection of ACR – Genomic Signatures

The development of a diagnostic assay for the early detection of ACR based on molecular changes in the cardiac allograft is a pressing medical need, as the diagnosis of ACR based on histopathological assessment is not always reliable. The subjectivity involved in evaluating the endomyocardial biopsy is one of the main drawbacks of the histopathological technique. Despite the effort to improve the concordance among the pathologists by revising the ISHLT 1990 classification[198] into a more simplified ISHLT 2004 classification[34], the discrepancies between the results are still common, with the overall agreement among pathologists being 70%[37].

Another significant drawback of histopathological assessment of endomyocardial biopsies in the management of heart transplant patients is that the method is not employed early enough to detect subclinical rejection prior to immune cell infiltration, which leads to graft damage. Being able to identify a rejection signature on the molecular level before histopathological and clinical signs of rejection become evident would be the ideal strategy (Figures 18).

Several methods of finding a molecular signature that can detect ACR earlier than histopathology assessment have already been studied, most of which rely on the microarray technique to characterize the gene expression profile associated with ACR. Horwitz et al. demonstrated that gene expression profiles in peripheral blood could be correlated with histologically verified cardiac allograft rejection[60]. Hollander et al. described a biopsy-targeted blood biomarker discovery approach that could discriminate between patients with and without rejection[199]. Holweg et al. identified both similarities and differences in intragraft gene expression compared to the 1990 ISHLT grading guidelines[200]. Li et al. investigated whether 10 genes, diagnostic of renal allograft rejection in blood, could be used to diagnose

and predict cardiac allograft rejection, and observed a common gene signature in renal and cardiac rejection that could diagnose and predict biopsy-proven rejection[201]. However, it is only the developmental gene expression profiling study by Deng et al.[61] (the CARGO study), followed by the clinical study by Pham et al.[63] (the IMAGE trial) that have made the journey from investigational status to regulatory approval of clinically relevant diagnostic test, called AlloMap.

The slow progress in heart transplantation biomarker research, despite vigorous efforts, reflects the complexity of ACR pathophysiology and the torturous path of discovery and validation of a biomarker, from the laboratory bench to clinical approval.

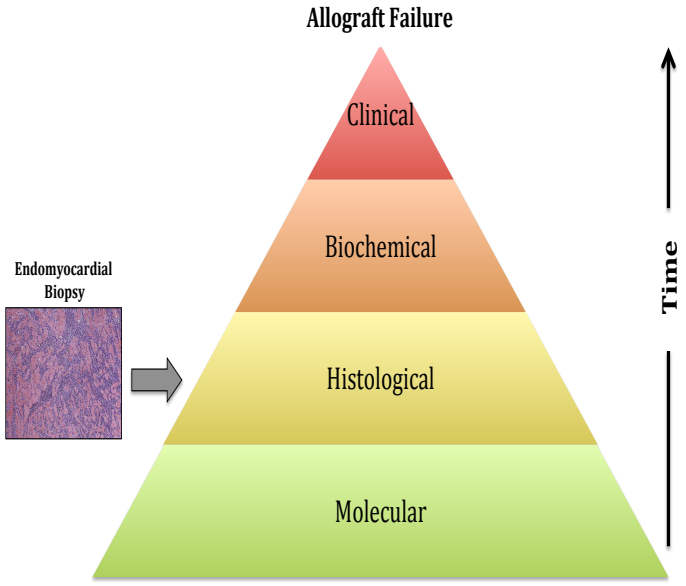

Figure 18. Timeline of allograft failure progression.

Circulating microRNAs as Non-invasive Biomarkers

Although circulating microRNAs are a relatively new platform in biomarker research, they have shown considerable promise[104,115]. The biological information carried by circulating microRNAs may reflect the biological and pathological events in a gene expression network, given that one microRNA can regulate hundreds of mRNAs. However, the main reasons why microRNAs have generated such interest within the field of biomarkers across diverse pathophysiological states are because many pitfalls associated with molecular biomarkers can largely be avoided by using microRNAs, as they are much more stable than mRNA in serum/plasma, and they can be quantified with much greater sensitivity than proteins[100,173].

A good biomarker for allograft rejection will not only reduce healthcare costs and save patients unnecessary discomfort, but should also be able to identify risk patients at the time of transplantation, and thus predict rejection before the graft is damaged. Circulating microRNAs meet these key requirements. The key molecular properties of microRNAs, including their stability at room temperature, their ability to survive unfavourable physiological conditions (such as freeze-thawing cycles and extreme variations in pH), and the fact that they can be frozen for up to 40 years without any significant degradation[100,117,118], make them suitable for use in clinical practice where variations can be expected in the handling of samples. In addition, biomarker analysis should be easy and rapid. MicroRNA levels can be determined with qRT-PCR, a technique available at most clinical diagnostic laboratories. The cost of the analysis can therefore be expected to be reasonable, and the results could be obtained on the same day.

The first evidence of the potential of microRNAs as biomarkers in the field of solid organ transplantation was reported by Anglicheau et al., who demonstrated changes in the microRNA profile in biopsy samples from kidney transplant recipients[71]. However, this does not really satisfy the need for non-invasive rejection surveillance, as the microRNA signature used in their study was on the intragraft level. An attempt was made to address this issue in Study I by performing a pilot study to explore the possibility of detecting changes in the microRNA profile in peripheral blood during ACR. The levels of seven microRNAs in the serum of heart transplant patients were found to be significantly increased during ACR, compared to before and after rejection. Furthermore, two of these microRNAs, miR-142-3p and miR-326, could discriminate between ACR and non-rejection. Despite the small number of heart transplant patients included in Study I, the results indicated the possibility of using microRNA profiles as a new strategy to diagnose ACR after heart transplantation[202]. In a subsequent study of 113 heart transplant patients, Van Huyen et al. showed that changes in the

expression of microRNAs occurring during ACR could be detected in serum, as well as in cardiac allograft tissue[203], suggesting that changes in microRNAs in the peripheral blood reflect intragraft changes.

To identify clinically relevant and optimal biomarkers for ACR, it is essential to integrate information from multiple platforms, such as mRNA, microRNA and protein profiling[204]. Since AlloMap is limited in its ability to distinguish ACR from NR during the first 6 months post-transplantation[63] and is dependent on the time after transplantation[62], it was deemed interesting to determine how microRNA levels react during ACR, regardless of the time after transplantation. The heart transplant patients included in Study II underwent endomyocardial biopsy surveillance according to the following schedule: every week in the first month, every 2 weeks between 1 and 3 months, once a month between 3 and 6 months and every 2 months between 6 and 12 months. The serum level of miR-142-3p was significantly higher in ACR patients than in NR patients during the first 6 months post-transplantation, which is the period during which the overall risk of rejection is highest. This suggests that the serum level of miR-142-3p may be useful in diagnosing ACR during the first 6 months after transplantation. However, the level of miR-101-3p was significantly higher in the ACR group than in the NR group only during the first 3 months post-transplantation.

Technical Considerations

Several issues must be addressed before serum microRNA levels can be used as a clinical diagnostic tool. Firstly, the appropriate microRNA profiling platform must be chosen. Three major microRNA profiling platforms are currently in use: microarray[205-207], qRT-PCR-based method[181,208] and RNA-sequencing[209,210]. Although each platform has its own advantages and disadvantages, the method should allow transplant clinicians to make rapid and appropriate clinical decisions. Therefore, qRT-PCR appears to be a feasible option as it is relatively inexpensive and available in most clinical laboratories.

Secondly, the method of normalization used to compare microRNA levels in different samples using qRT-PCR must be considered. Despite microRNAs being very promising non-invasive candidate biomarkers in diverse molecular diagnostic applications, the normalization of extracellular microRNAs, including serum microRNAs, remains challenging. Considering the low micoRNA yield in the serum (0.1-1 ng/ml)[211], normalization to give the exact quantities of microRNAs is essential. There are several well-established protocols for the normalization of gene expression measurements using housekeeping genes that can be implemented in cellular microRNA. However, there are currently no known or established

housekeeping genes that can be used for the normalization of extracellular microRNA.

Nevertheless, several approaches for the normalization of circulating microRNA levels have been suggested, such as the normalization of serum microRNA expression by input volumes using equal amounts of serum in all samples. This approach has been challenged by the fact that there are considerable variations in RNA quantities extracted from equal volumes of serum from different individuals. Normalization by synthetic RNA Spike-in has also been used to monitor technical variations in RNA purification, cDNA synthesis and qRT-PCR steps. However, RNA spike-in has a limited ability to control the biological variation in the overall serum microRNA expression levels between different individuals. Global mean normalization has also been suggested to be a sensitive and accurate approach for high-throughput microRNA profiling[212]. This method may prove to be the best strategy for clinical samples due to its high appreciation to technical and biological variations of samples.

Intercellular Transport of Functional microRNAs

Extracellular microRNAs are ubiquitous in a diverse range of biological fluids, and are relatively stable, and thus have considerable potential as disease biomarkers and novel therapeutic agents. It has been long believed that intercellular communication takes place through the secretion of soluble molecules, i.e. the paracrine and endocrine mechanisms, or by cell-to-cell contact. The knowledge that functional microRNAs can be selectively transferred using cellular machinery to target gene expression in the recipient cells and alter the cellular phenotype, has led to a new understanding of cell-to-cell communication, and has fundamentally changed the concept of gene regulation.

Immune cells are communication experts that have the ability to convey their messages to each other via genetic information carried by extracellular vesicles during immune response[213]. T cells play an essential role in many facets of immune response. It has been shown that T cell activation causes the secretion of extracellular vesicles[214], and that T cell interactions with B cells[215] or dendritic cells (DCs)[216] result in increased exosome release. Moreover, T cell exosomes undergo preferential sorting of their microRNA content before being transferred to the recipient cells[217], which is in line with the findings in Study III, where enrichment of selected microRNAs was observed in the serum exosome profiles of heart transplant patients with ACR, compared to those without.

Endothelial and T cells ”Small Talk” via Exosomes during ACR

Disruption of the function of the vascular endothelial barrier function is a molecular hallmark of inflammation, including graft rejection. During allograft rejection, endothelial cells advance the recruitment and the activation of alloreactive T cells[23,24]. The accumulation of inflammatory infiltrates, mainly comprised of T cells, in the allograft is initiated by the inflammatory reaction of endothelial cells, which results in an increase in vascular endothelial permeability, leading to endothelial cell death and graft destruction[21].

The role of endothelial cells in the context of organ transplantation has been examined in several studies using HUVECs experimental models[218-220]. A similar experimental model was used in Study III to investigate the intercellular communication between endothelial cells and T cells that involves the transfer of miR-142-3p, a haematopoietic-tissue-specific microRNA that is highly expressed in T cells and has been implicated in several solid organ transplantation studies[170-172,203].

The study revealed that in the setting of acute cardiac allograft rejection, activated T cells communicate with endothelial cells via circulating miR-142-3p carried by T cell exosomes. In the recipient cells, miR-142-3p regulates the expression of RAB11FIP2 and promotes changes in endothelial physiology by impairing vascular integrity.

Although the findings of this study have shed some light on the molecular mechanism behind endothelial cell and immune cell communication during ACR, several matters remain to be further investigated. The selection process of intracellular miR-142-3p shuttled in the activated T cell exosomes remains unknown.

In Study III, several microRNAs were found to be enriched in the serum exosomes during ACR. This could provide a good starting point to examine the involvement of these microRNAs in the pathophysiology of ACR, as well as their respective target genes. Moreover, it would be of great interest to examine the role of miR-142-3p in the progression of ACR and its involvement in graft destruction or tissue damage, as well as which miR-142-3p target genes are involved in the respective processes.

MicroRNAs as Therapeutic Targets

Possible roles of microRNAs in transplant immunology have been suggested in several studies: as therapeutic targets, biomarkers of allograft status, regulators of chronic rejection and organ fibrosis, as well as mediators of organ rejection[221]. Apart from the potential of miR-142-3p as a biomarker for cardiac allograft status, it would also be of great interest to use knowledge concerning the involvement of miR-142-3p in the cellular mechanism of graft rejection as a starting point to develop a microRNA-based therapeutic agent that can modulate the immune response during ACR.

Growing evidence suggests that a new class of RNA therapeutics, i.e. microRNA modulators (such as antagomirs or anti-miRs), could have some advantages in the treatment of human diseases, despite a number of obstacles that must be overcome[222]. In fact, several microRNA therapeutics indicated for a wide range of diseases are currently under preclinical and clinical developmental. The main advantage of microRNA therapeutics is that they enable specific targeting of microRNAs and their respective downstream gene networks *in vivo*, thus influencing the underlying mechanisms of disease and disease progression.

In one of the studies using antimiR, a chemically engineered oligonucleotide to silence endogenous microRNAs, it has been showed that blocking miR-21 expression in a mouse model could inhibit the development of fibrosis in cardiovascular disease. Fibrosis is an endpoint of many pathological conditions, including ACR. The findings of that study, together with the increasing observations of the direct mechanistic involvement of microRNAs in inflammation and transplant immunity, suggest that microRNAs have the potential for clinical use as therapeutic tools, apart from their diagnostic and prognostic features in transplantation medicine.

Serum Protein Biomarkers – Tackling the Challenges

Serum or plasma is the logical choice for the discovery of a new biomarker to reach the ultimate goal of developing a simple blood test. More than 100 assays for different proteins in blood have been developed and are in routine use in clinical laboratories today. Human serum/plasma has been defined as the most comprehensive human proteome, and an appropriate circulating representation of all human tissues in biological or pathological states[223]. However, several challenges remain to be addressed in serum biomarker discovery in order to further development from bench to bed.

Although proteomic biomarker discovery shares many features and similarities with transcriptional profiling, the proteomic-based biomarker discovery in serum/plasma is more complex. Developing quantitative assay for biomarker candidates is the main bottleneck in proteomic biomarker discovery-validation pipeline[224]. It has been suggested that immunoaffinity capture addresses the issue of detecting and quantifying blood-borne protein biomarker candidates. However, there is evidence that this technique is unsuitable for true *de novo* discovery effort[225,226].

Efforts in proteomic-based biomarker discovery are also hampered by the very large range in the concentration of analytes that have to be detected and quantified in serum/plasma, and the absence of techniques to amplify them, as there is no PCR equivalent for protein[227]. In Study IV, an attempt was made to address the detection and amplification issues in proteomic-based biomarker discovery using PEA assay. This is a relatively new platform in proteomic profiling, in which two highly specific antibodies are used for each protein and the pair-wise binding of both antibodies to the target protein allows the formation of a PCR target sequence. This is extended using DNA polymerase and subsequently detected and quantified using qPCR, rendering great sensitivity and specificity to this approach. Given a comprehensive set of highly sensitive and specific protein assays that could be massively parallelized, the process of developing novel protein biomarkers using PEA assay is relatively straightforward.

Conclusions

The major conclusions drawn from the findings of the studies included in this thesis are given below.

- It is feasible to measure circulating microRNA levels in serum samples from heart transplant patients using a qRT-PCR-based microRNA profiling assay (*Study I*).
- The profile of circulating microRNAs is altered in serum samples from heart transplant patients during biopsy-proven ACR, compared to before and after rejection (*Study I*).
- The levels of seven microRNAs (miR-142-3p, miR-101-3p, miR-424-5p, miR-27a-3p, miR-144-3p, miR-339-3p and miR-326) were significantly higher in a cohort of heart transplant patients with ACR, than in a group of patients without ACR (*Study II*).
- The levels of miR-142-3p and miR-101-3p in serum can be used to discriminate reliably between patients with ACR and those without (*Study II*).
- MiR -142-3p is secreted into the extracellular compartment during ACR, as well as during in vitro activation of human T cells (*Study III*).
- MiR-142-3p shed by activated T cells can be taken up by endothelial cells, regulating the expression of RAB11FIP2 and promoting changes in endothelial physiology by impairing vascular integrity (*Study III*).
- Profiling the circulating inflammatory proteins in serum samples from heart transplant patients with ACR can help in the search for novel protein biomarkers that can be used to discriminate rejection from non-rejection (*Study IV*).
- Most of the circulating inflammatory fingerprints that show changes during ACR are associated with T cells (*Study IV*).

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"Coincidence is God's way of remaining anonymous"

(Albert Einstein)

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