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Immunological and non-immunological markers of cardiac allograft vasculopathy amongst heart transplant recipients

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1. ABBREVIATIONS

ACE = angiotensin converting enzyme
ADMA = asymmetric dimethylarginine
AZA = azathioprine
BNP = brain natriuretic peptide
CABG = coronary artery bypass graft
CAV = cardiac allograft vasculopathy
CNI = calcineurin inhibitor
CRP = C-reactive protein
CMV = cytomegalovirus
CSA = cross-sectional area
ELISA = enzyme linked immunosorbent assay
EEM = external elastic membrane
eNOS = endothelial nitric oxide synthase
GFR = glomerular filtration rate
HLA = human leucocyte antigen
HMG-CoA = 3-hydroxy-3methylglutaryl coenzyme A
HTx = heart transplant/heart transplantation
ICAM = intercellular adhesion molecule
IFN-γ = interferon gamma
IHD = ischemic heart disease
IL = interleukin
IVUS = intravascular ultrasound
LC = lumen contour
LDL = low density lipoprotein
MDRD = Modification of Diet in Renal Disease Study
MIT = maximal intimal thickness
MMF = mycophenolate mofetil
NCC = necrotic core component
NO = nitric oxide
NT-proBNP = N-terminal pro-brain natriuretic peptide
OPG = osteoprotegerin
PAV = percent atheroma volume
sTNFR-1 = soluble TNF receptor type 1
T. gondii = *Toxoplasma gondii*
TAV = total atheroma volume
TNF = tumor necrosis factor
VCAM-1 = vascular cell adhesion molecule–1 (VCAM-1)
vWf = von Willebrand factor (vWf)
VH = virtual histology

2. LIST OF PAPERS

Paper I: Arora S, Gullestad L, Wergeland R, Simonsen S, Holm T, Hognestad A, Ueland T, Geiran O, Andreassen A. Probrain natriuretic peptide and C-reactive protein as markers of acute rejection, allograft vasculopathy, and mortality in heart transplantation. *Transplantation.* 2007 May 27;**83**(10):1308-1315.

Paper II: Arora S, Andreassen A, Simonsen S, Gude E, Dahl C, Skaardal R, Hoel I, Geiran O, Gullestad L.Prognostic importance of renal function 1 year after heart transplantation for all-cause and cardiac mortality and development of allograft vasculopathy. *Transplantation.* 2007 Jul 27;**84**(2):149-154.

Paper III: Arora S, Jenum PA, Aukrust P, Rollag H, Andreassen AK, Simonsen S, Gude E, Fiane AE, Geiran O, Gullestad L.Pre-transplant Toxoplasma gondii seropositivity among heart transplant recipients is associated with an increased risk of all-cause and cardiac mortality. *J Am Coll Cardiol.* 2007 Nov 13;**50(**20):1967-1972.

Paper IV: Arora S, Gunther A, Wennerblom B, Ueland T, Andreassen AK, Gude E, Geiran O, Wilhelmsen N, Endresen K, Andersen R, Aukrust P, Gullestad L Systemic markers of inflammation are associated with advanced cardiac allograft vasculopathy and an increased inflammatory component. *Submitted.*

3. INTRODUCTION

3.1 Cardiac allograft vasculopathy – general aspects

Heart transplantation (HTx) is an established and effective therapy for end-stage heart disease. It is estimated that over 5000 HTx procedures are performed annually and the two leading etiological causes are heart failure secondary to ischemic heart disease and non-ischemic cardiomyopathy (41% and 45%, respectively) (1). In contrast to the first HTx performed in 1967 where survival was limited to 18 days, the current median survival is reported to be 10 years (1). Furthermore, the criteria for selection of HTx candidates have evolved with most centers now accepting considerably higher-risk patients with multiple comorbidities (2). Important surgical advances together with optimal medical therapy are responsible for this improvement, but a further reduction in morbidity and mortality remains a significant challenge, particularly, due to the development of cardiac allograft vasculopathy (CAV). This is a unique form of accelerated atherosclerosis occurring in HTx recipients and is characterized by a diffuse, progressive thickening of the arterial intima of both epicardial and intramyocardial arteries of the transplanted graft. According to the Registry of the International Society for Heart and Lung Transplantation (ISHLT), CAV is detectable in 43% of HTx recipients within 8 years after HTx, and accounts for 30% of deaths occurring beyond the first year post-HTx (1).

The pathophysiology of CAV development is not fully clear, but is likely to be mulitfactorial with a range of immunological and non-immunological contributors. Although the optimal therapeutic strategy to prevent or reverse CAV development remains an elusive goal, early and accurate diagnosis of CAV is likely to be critical to further improving outcome post-HTx. Most HTx recipients undergo annual angiographic surveillance to detect the development and progression of CAV. However, it is well established that this technique has insufficient sensitivity to allow accurate CAV detection (3) as the disease is not characterized by discrete lesions easily identified by angiography but by diffuse intimal thickening of both large and small-caliber vessels. Consequently, alternative methods including intravascular ultrasound (IVUS) and measurement of plasma biomarkers are increasingly being considered in routine clinical care and may allow appropriate risk stratification and more individualized management of patients at higher risk of developing CAV. Furthermore, accurate identification of immunological and non-immunological risk factors contributing to CAV development remains an important challenge.

3.2 Pathogenesis of cardiac allograft vasculopathy

3.2.1 Histopathological features

Cardiac allograft vasculopathy is typically characterized by a diffuse concentric intimal thickening of both epicardial and intramural arteries (4). This is in contrast to native atherosclerosis which is typically identified by focal eccentric proliferative lesions of the intima of proximal epicardial coronary arteries (Figure 1). Both CAV and atherosclerosis consist of fibrofatty plaques that are histopathologically indistinguishable as both lesions consist of smooth muscle cell proliferation and accumulation of extracellular lipids (5, 6). However, a key difference is that calcium deposition and disruption of the internal elastic lamina is rare in CAV but occurs frequently in atherosclerosis. Furthermore, fibrous cap thinning together with plaque rupture and formation of complicated plaques (plaques with hemorrhage and thrombotic deposits) rarely occur in CAV but are commonly found in native atherosclerosis (4). Finally, another important difference is that CAV is characterized by an accelerated and rapid progression rate with early intimal changes being evident as early as 1-2 weeks after HTx and development of lipid-filled cells being evident in the following few months after HTx (7).

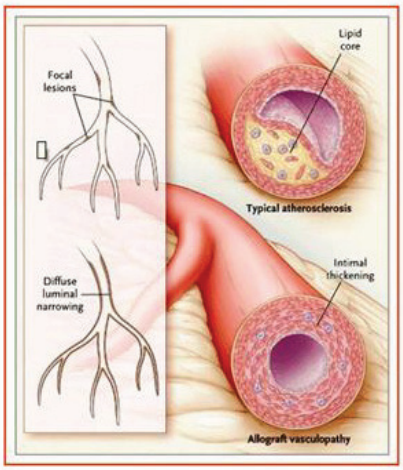

Figure 1. Typical atherosclerosis versus cardiac allograft vasculopathy.
Atherosclerosis is characterized by focal lesions whereas diffuse intimal thickening is the hallmark of allograft vasculopathy.
(Reproduced with permission from NEJM (2003), Massachusetts Medical Society.)

	Cardiac allograft vasculopathy	Native atherosclerosis
Geometry	Diffuse concentric lesions Epicardial and intramural arteries involved Veins may be involved	Focal eccentric lesions Proximal coronary arteries usually involved Veins rarely involved
Plaque composition	Smooth muscle cell proliferation, lipid-filled cells, free lipid deposition Calcium deposition and disruption of internal elastic lamina is rare Fibrous cap thinning, plaque rupture and complicated lesions are rare	Smooth muscle cell proliferation, lipid-filled cells, free lipid deposition Calcium deposition and disruption of internal elastic lamina is common Fibrous cap thinning, plaque rupture and complicated lesions commonly occur
Time progression	Rapid development and accelerated lesion progression (months)	Slow development and lesion progression over years

Table 1. Histopathological features of cardiac allograft vasculopathy as compared to native atherosclerosis.

Diffuse concentric intimal thickening is considered the hallmark of typical CAV development and is often termed negative remodeling as it reduces the lumen diameter. Conversely, positive remodeling (compensatory enlargement of vessel wall) processes may also occur amongst HTx recipients and counteract or delay the development of luminal obstruction. Positive remodeling resulting in an increased vessel caliber can occur by smooth muscle cell turnover and also through extracellular matrix degradation (e.g. via matrix metalloproteinases and other proteolytic enzymes) (8). Both intimal hyperplasia and lack of positive remodeling can reduce lumen diameter but their relative contribution to CAV development remains controversial. Pethig et al. demonstrated that lumen loss is a biphasic process involving early intimal thickening (within the first year) and later constrictive remodeling (9). Another larger IVUS study found that inadequate compensatory enlargement rather than intimal hyperplasia was the major predictor of luminal obstruction (10). Although speculative, it is potentially possible that the predominating remodeling pattern is related to underlying pathophysiological CAV mechanism which may be different in the early versus late stage after HTx.

3.2.2 Immunological factors

The pathogenesis of CAV development is complex and a host of pathophysiological mechanisms are likely to play a contributory role. A range of experimental, animal, clinical and epidemiological studies have established that both immunological and non-immunological factors can be implicated in this process (11-13). A key characteristic of CAV is that intimal hyperplasia and inflammatory cell infiltration are limited to the graft

vasculature with sparing of the host's own arteries and this indicates the presence of a primarily local inflammatory process. Endothelial cells of the allograft are the first donor cells to be recognized as non-self by the recipient's immune system by either the direct or indirect allorecognition pathway. The direct pathway involves identification of donor human leukocyte antigen (HLA) molecules by recipient dendritic cells (13, 14). Indirect recognition occurs when donor antigens are internalized, processed and presented as peptides by host dendritic cells (15) triggering a cellular alloimmune response involving T-lymphocytes and macrophages. There is evidence indicating that whereas direct allorecognition is responsible for acute rejection, the indirect pathway is likely to be critical for development of CAV (13).

Recognition of HLA class II antigens on the surface of donor endothelial cells by circulating CD4 lymphocytes is a key stimulus for initiation of a cellular response against the allograft endothelium (16). This initial event triggers secretion of a range of stimulatory cytokines (interleukin [IL]-2, IL-4, IL-5, IL-6, interferon-gamma [IFN-γ], tumor necrosis factor-α) which in turn induce the expression of endothelial adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1). Adhesion molecules mediate the recruitment and accumulation of macrophages in the intima leading to a sustained inflammatory response where activated cells in the vessel wall produce cytokines and growth factors (platelet derived growth factor, insulin like growth factor-1, fibroblast growth factor, heparin-binding growth factor and transforming growth factor-β) stimulating smooth muscle cell proliferation and extracellular matrix deposition that characterizes CAV (17).

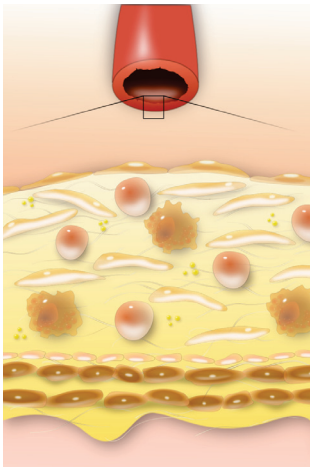

Figure 2. Development of cardiac allograft vasculopathy (CAV). Histological studies reveal that CAV development is characterized by intimal hyperplasia with smooth muscle cell proliferation, accumulation of inflammatory and lipid-filled cells as well as free lipid deposition.

The humoral alloimmune response may also contribute to the development of CAV as it has been shown that circulating anti-HLA antibodies are associated with angiographic CAV (18) and adverse survival (19). Furthermore, it has been reported that patients with rapidly progressive CAV have elevated IgM antibodies against endothelial cell peptides (20). However, the significance of these findings remains unclear as increased antibody formation may be a consequence of B-cell proliferation and stimulation as part of the cellular alloimmune response.

3.2.3 Non-immunological factors

Numerous studies have investigated the role of non-immunological factors for CAV development and it has been reported that older donor age (12), donor gender (12), obesity (21), hyperglycemia (22), hypertension (23), hyperhomocysteinemia (24) and cytomegalovirus (CMV) infection (25) are independent risk factors. However, other studies have reported contradictory results and a further limitation is that most investigators have employed angiography to diagnose CAV despite its lack of sensitivity. According to the ISHLT registry, risk factors for CAV within 5 years following HTx include: era of HTx, donor history of hypertension, donor gender and age and ischemic heart disease as etiology for HTx (1).

Hyperlipidemia and insulin resistance are common metabolic risk factors for traditional atherosclerosis and also occur frequently amongst HTx recipients with a reported prevalence of 50-80% (26). Hyperlipidemia is often related to the use of calcineurin inhibitors whereas hyperglycemia may occur secondary to the use of steroids employed as part of HTx immunosuppressive therapy protocols. It has been demonstrated that total cholesterol, low density lipoprotein (LDL) and triglyceride levels are independent risk factors for increased intimal thickening determined by IVUS (21). Randomized trials have also confirmed that statin therapy early after HTx reduces the incidence and severity of CAV development (27). Similarly, it has also been shown that insulin resistance and glucose intolerance are strong determinants of IVUS determined CAV (22). There is evidence indicating that hyperglycemia and hyperlipidemia contribute to CAV development via upregulated expression of adhesion molecules on endothelial cells (28) triggering a cellular proliferative response associated with CAV.

Studies have also indicated that viral pathogens, particularly CMV infection, may be involved in CAV pathogenesis. For example, it has been shown in observational studies that

CMV infected patients had a significantly higher risk of severe coronary artery obstructive lesions (29) and this is supported by clinical studies demonstrating a beneficial role of CMV therapy on CAV incidence (25). It has been suggested that viral pathogens, such as CMV, can influence CAV incidence and progression via a direct effect on proinflammatory cytokine induction and expression of adhesion molecules (30) or, secondary to impaired vasodilatation of the coronary vasculature due to changes in the endothelial nitric oxide synthase (eNOS) pathway (28).

Endothelial dysfunction refers to abnormal vasoreactivity in response to local acetylcholine administration and there are studies indicating that it can be detected early after HTx and predicts the development of CAV (31, 32). Several proposed mechanisms may mediate endothelial dysfunction, including generation of superoxide anions, increased degradation of nitric oxide (NO) or impaired synthesis of NO secondary to increased levels of asymmetric dimethylarginine (ADMA) (28). Elevated ADMA levels are associated with several traditional risk factors for atherosclerosis, including diabetes, hypertension and renal failure. Consequently, endothelial dysfunction may be a common mediating pathway that links various non-immunological risk factors to the development of CAV.

Overall, it is likely that the development of CAV occurs secondary to a complex interplay of both immunological and non-immunological factors which results in endothelial activation and a chronic inflammatory response causing intimal hyperplasia due to inflammatory cell accumulation, proliferation of vascular smooth muscle cells and deposition of an extracellular tissue matrix (Figure 2).

3.3 Diagnosis of cardiac allograft vasculopathy

Due to cardiac denervation at the time of HTx, development of CAV is often clinically silent and patients present late with silent myocardial infarction, loss of allograft function, arrhythmia or sudden death (33, 34). Identification of CAV is, therefore, an important goal and various invasive and non-invasive methods can be utilized in clinical practice.

3.3.1 Invasive methods

3.3.1.1 Coronary angiography

It has been shown that the diagnosis and progression of CAV determined by coronary angiography is of prognostic significance (35, 36). Although this is the rationale for annual

surveillance angiography of HTx recipients, several studies have demonstrated that this technique has significant limitations and low diagnostic sensitivity (37, 38). This is primarily attributable to the lack of lumen obstruction in early CAV development secondary to compensatory enlargement and vascular remodeling processes (39). Furthermore, the diffuse and longitudinal distribution of CAV may also result in the lack of a normal reference segment resulting in underestimation of disease prevalence. Hence, although a specific diagnostic method, angiography is a relatively insensitive method for CAV diagnosis and surveillance.

I.	**Invasive methods**
•	Coronary angiography
•	Intravascular ultrasound
•	Assessment of coronary vasomotor alteration (e.g. by Doppler flow wire)
•	Myocardial biopsy molecular analysis
-	antithrombin-III
-	HLA-DR
-	intercellular adhesion molecule-1
II.	**Non-invasive methods**
•	Biomarkers
-	Myocyte injury markers (e.g. Troponin)
-	Myocyte stress markers (e.g. BNP or NT-proBNP)
-	Inflammatory markers
i)	*CRP*
ii)	*inflammatory cytokines*
iii)	*anti-inflammatory cytokines*
iv)	*chemokines*
-	Oxidative stress markers (e.g. TBARS, oxidized LDL)
-	Gene expression markers (e.g.AlloMap)
•	Dobutamine stress echocardiography
•	Multidetector CT
•	Single photon emission CT (SPECT)

Table 2. Invasive and non-invasive methods for detection of cardiac allograft vasculopathy.

3.3.1.2 Intravascular ultrasound

Intravascular ultrasound is an advanced imaging modality with an axial resolution of 50-80 μm (39) that generates detailed cross-sectional images of the coronary lumen and entire arterial wall (Figure 3). This allows accurate assessment of intimal wall thickness and vessel and lumen dimensions. According to angiographic evaluation CAV is present in <10% of HTx recipients at one year post-HTx (1) but IVUS studies indicate that the disease can be detected in up to 75% of HTx recipients at the same time point (40).

Normal coronary artery intimal thickness has typically been reported to range between 0.10-0.25 mm (41, 42) and, hence, many investigators consider CAV as intimal thickness >0.3 mm or when the sum of intimal and media thickness exceeds 0.5 mm. Studies have employed IVUS to demonstrate that CAV often progresses most rapidly during the first year after HTx (3). Such rapidly progressive CAV, defined as an increase in maximum intimal thickness (MIT) ≥0.5 mm, is a powerful predictor of all-cause mortality (43) and patients with such intimal thickening are 10 times more likely to experience an adverse cardiac event (44). Although a few studies have shown that intimal thickness demonstrated by IVUS correlates poorly to small vessel disease detected by histological or immunohistochemical analysis (45), this imaging modality remains one of the best available surrogate markers of predicting adverse outcomes related to CAV.

Despite the diagnostic accuracy of IVUS its use has traditionally been limited to research and investigational purposes. This is largely attributable to the financial costs of performing IVUS analysis and technical limitations which include the inability to image vessels with a diameter <1.5 mm (catheter size is ≈1 mm) and selective visualization of the selected coronary artery.

3.3.1.3 Virtual Histology

Virtual histology (VH), developed by Volcano Corporation Inc, Rancho Cordova, California, is a relatively new technique where backscatter radiofrequency data obtained during IVUS pullback is utilised for qualitative plaque assessment. This technique has been shown to have a 94–97% *ex-vivo* and 87–97% *in-vivo* accuracy for characterization of four basic tissue components (fibrous, fibrofatty, calcified and necrotic core components – Figure 3) amongst patients with ischemic heart disease (46, 47). Given our limited understanding of the complex *in-vivo* processes responsible for CAV such an assessment of tissue composition amongst HTx recipients represents a novel and potentially valuable tool that is being increasingly utilised in prospective trials amongst HTx recipients.

3.3.1.4 Other invasive methods

Invasive assessment of epicardial and microvascular endothelial function (e.g. by Doppler flow wire) may also be helpful in detecting significant CAV (48, 49). However, there are conflicting reports (50) regarding the accuracy and utility of this method. Myocardial biopsy molecular analysis is another invasive method that may allow early CAV detection and this

approach includes assessment of various molecules, including, HLA-DR, intercellular adhesion molecule-1 and anti-thrombin III (51-53). However, this technique requires methodological improvement and validation before it can be considered in routine clinical practice.

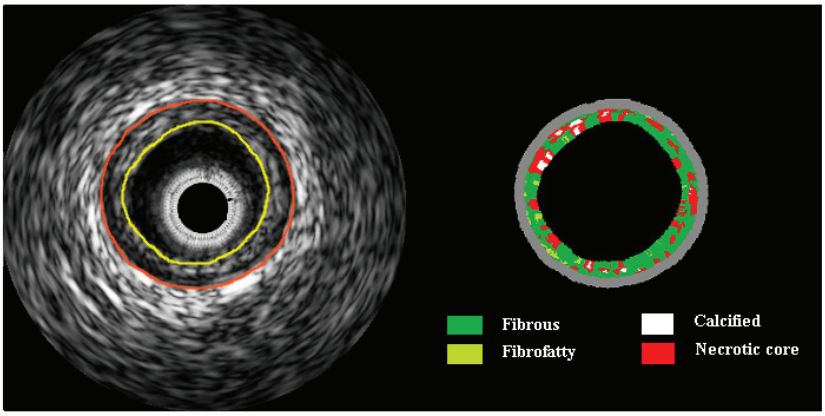

Figure 3. ***Left:*** **Example of an IVUS recording allowing accurate visualization of intimal wall thickening.** Intima and media corresponds to area between the drawn yellow and red contour. ***Right:*** **Example of a Virtual Histology (VH) tissue map generated via analysis of backscatter radiofrequency data obtained during IVUS imaging.**

3.3.2 Non-invasive methods

3.3.2.1 Biochemical markers

Non-invasive detection of CAV by use of simple biomarkers measuring cardiac damage, systemic inflammation or endothelial activation remains a focus of active research. It has been shown that persistent elevation of troponin I levels during the first year post-HTx is associated with an increased risk of CAV progression and graft failure (54). Similarly, elevated C-reactive protein (CRP), a marker of systemic inflammation is also associated with CAV development and graft failure (55, 56). Brain natriuretic peptide (BNP), a neuroendocrinal hormone, is likely to reflect ongoing remodeling of the allograft and elevated levels have been shown to predict the development of CAV (57). Although the positive predictive value of many of these biomarkers is relatively low, they represent relatively cheap and efficient non-invasive parameters that can be measured in routine clinical practice as part of risk-

stratification protocols. Combined measurement of different biomarkers may also allow increased sensitivity and specificity and needs further investigation.

Measurement of inflammatory biomarkers may represent a non-invasive method of CAV detection and chemokines such as, monocyte chemotactic protein-1 (MCP-1), have been shown to be specifically associated with CAV (58). In addition, soluble IL-2 receptor levels have been found to be elevated in CAV diagnosed by angiography early after HTx (59). Another potential biomarker is oxidized LDL which has been shown to be related to angiographic CAV in both retrospective and prospective studies (60, 61). Finally, the AlloMap gene-expression test which has previously been shown to detect acute rejection also appears to be associated with CAV (62). Further studies are now being considered to investigate the value of this genetic biomarker in predicting the development of CAV.

3.3.2.2 Other non-invasive methods

Recent studies have demonstrated that stress echocardiography can be used as a reliable non-invasive method for detecting CAV and myocardial ischemia (63). Although there is good correlation between the presence of CAV and regional myocardial function, it should be noted that the latter is a subjective interpretation resulting in a wide range of reported sensitivities and specificities for this method (63-65). Single-photon emission computed tomography (SPECT) and 64-slice multidetector CT imaging are two promising imaging modalities that are being explored as an alternative to angiography and there is data indicating these methods have moderate to excellent test characteristics for CAV detection (66, 67). However, important limitations are spatial resolution, radiation exposure and the potential difficulty in obtaining good quality images due to the relatively higher heart rate amongst HTx recipients.

3.4 Treatment of cardiac allograft vasculopathy

Given the adverse prognosis associated with CAV, effective treatment is an important goal of post-HTx management. Current strategies largely focus on prevention of CAV by targeting immunological factors and treating or modifying non-immunological risk factors associated with CAV. Advanced CAV may be amenable to interventional treatment but generally has limited prognostic benefits.

3.4.1 Targeting the immune system

Calcineurin inhibitors (CNI), such as cyclosporine (CsA) or tacrolimus, are generally considered essential immunosuppressive medication for HTx recipients as they have been shown to dramatically reduce the frequency of acute rejection episodes and improve survival (68). However, despite the widespread use of these agents, the high prevalence of CAV may suggest that such therapy is not effective in preventing CAV development (13, 69). A potential explanation is that CAV is mediated via pathways not inhibited by CNIs, such as, complement activation (13, 70) or production of antiendothelial antibodies (13, 71). Furthermore, long-term use of CNIs is associated with an increased risk of developing complications, such as renal dysfunction and hypertension, which may accelerate the progression of CAV. Studies have also tried to assess differential effects of CyA versus tacrolimus but results are inconsistent. Klaus et al. (72) found that tacrolimus therapy was associated with increased CAV progression, whereas Meiser et al. (73) reported a non-significant trend towards increased CAV amongst patients receiving CyA therapy.

I.	**Targeting the immune system**
•	Immunosuppression therapy
-	Calcineurin inhibitors
-	Mycophenolate mofetil
-	Proliferation signal inhibitors
•	Immunomodulatory treatment
-	15-deoxyspergualin
-	CTLA-4-Ig
-	interleukin-10 stem cell therapy
II.	**Targeting non-immunological factors**
•	HMG-CoA inhibitors (statins)
•	Vasodilators
-	ACE inhibitor
-	Calcium channel blocker
•	Endothelial protection
-	L-arginine
-	Antioxidants
•	Anti-CMV therapy (ganciclovir)
III.	**Interventional options**
•	Coronary artery bypass grafting (CABG)
•	Percutaneous coronary intervention (PCI)
•	Retransplantation

Table 3. Therapeutic strategies to target cardiac allograft vasculopathy.

Immunosuppressive therapy with CNI is generally complemented with either azathioprine (AZA) or mycophenolate mofetil (MMF). The latter inhibits inosine monophosphate dehydrogenase which is an essential enzyme required for DNA synthesis by lymphocytes. Furthermore, MMF may also exert its effect via inhibition of adhesion molecule glycolysation (74) contributing to reduced inflammatory infiltration of the intima. A randomized controlled trial has demonstrated that patients treated with MMF had a 35% reduction in 3 year mortality or graft loss compared with patients treated with AZA (75). Although no significant difference in angiographic CAV was evident in this trial, reanalysis of the IVUS data (76) revealed that fewer patients receiving MMF developed intimal thickness ≥0.3 mm during the first year post-HTx (43% versus 23%, p=0.005). Consequently, MMF is increasingly replacing AZA in the immunosuppressive protocol employed at many HTx centers.

Sirolimus and everolimus (synthetic derivative of sirolimus) interfere with interleukin-1-mediated signal transduction pathways signals and inhibit proliferation of B and T lymphocytes, fibroblasts and smooth muscle cells (77, 78). These agents may, therefore, inhibit CAV development or progression and represent an attractive replacement for CNI therapy (79). It has been shown that HTx recipients randomized to sirolimus had a lower incidence of a composite endpoint of death, need for angioplasty or bypass surgery, myocardial infarction or a >25% worsening of the catherization score (80). Everolimus has been evaluated in a large trial with over 600 patients that showed reduction of all IVUS endpoints in patients treated with everolimus versus azathioprine (81). However, no improvement in survival was reported and a worse triglycerides and renal function were found in patients receiving everolimus (81). Nevertheless, proliferation signal inhibitors may represent a significant advancement of CAV treatment and a recent multidisciplinary report concluded that the majority of de novo heart transplant recipients could benefit from everolimus therapy (82).

Various novel immunomodulatory agents are also being explored in an attempt to prevent the development of CAV. These agents include 15-deoxyspergualin which suppresses macrophage function and has been investigated in animal models and appear to prevent CAV development (83) as well the costimulation signal inhibitor CTLA-4-Ig which has been shown to prevention posttransplant arteriopathy in mouse aortic allografts (84). Stimulation of anti-inflammatory pathways via administration of IL-10–engineered hematopoietic stem cells may also prolong allograft survival (85). Such specific agents require further investigation with particular attention being given to the role of targeted combination therapy which may allow a

coordinated blockage of central immunological mechanisms responsible for CAV development.

3.4.2 Targeting non-immunological factors

Hyperlipidemia occurs frequently amongst HTx recipients and has a reported prevalence of 93% in the first 10 years after HTx (1). This is likely to be attributable to a range of factors, including cyclosporine therapy and insulin resistance secondary to steroid treatment. Trials have demonstrated that routine therapy with 3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA) inhibitors, or statins, is associated with a beneficial effect on cardiac allograft rejection and survival (27, 86). It has also been shown that this protective effect is evident independent of changes in lipid levels and may, therefore, reflect immunomodulatory effects of statin therapy such as decreased cytokine activity [interleukin-6 and tumor necrosis factor (TNF)-α] or improved coronary endothelial function (87). Statins also reduce levels of CRP amongst HTx recipients (55) and may inhibit T-lymphocyte activation by interfering with IFN-γ mediated expression of HLA class-II molecules on coronary endothelial cells (88).

According to the ISHLT registry, 98% of HTx recipients have hypertension 10 years after HTx (1). Nephrotoxicity secondary to CNI therapy is an important contributing factor (89) and it has been demonstrated that treatment of hypertension early after HTx with calcium antagonists reduces CAV development and improves overall survival at 1 year post-HTx (90). Similarly, treatment with angiotensin converting enzyme (ACE) inhibitors can delay development of CAV (23) via mechanisms that may include improved endothelial dysfunction and oxidative stress (13). Furthermore, it has been shown that the combined use of an ACE inhibitor and a calcium antagonist is more effective than the individual use of either drug alone on CAV development (91). However, further studies are required to validate these results.

Insulin resistance and diabetes are also common comorbidities amongst HTx recipients. Although, a strong risk factor for traditional atherosclerosis, their role in CAV development is less clear. In one study no increased risk of CAV was evident amongst patients with diabetes (92), whereas in another study, hyperglycemia was associated with greater intimal thickness during a follow-up period of 8 years (22). Larger prospective studies are, therefore, required to evaluate the potential benefit of intensive glycemic control for development of CAV amongst HTx recipients.

Given the association between endothelial dysfunction and development of CAV, studies have focused on investigating whether oral L-arginine (a precursor of NO synthase) therapy can reverse endothelial dysfunction (93), buffer increased vascular oxidant stress (94) and, hence, influence CAV development. Although the results are promising, the effect of L-arginine needs to be investigated further in larger randomized, prospective trials. Similarly, there is data indicating that supplementation with antioxidants, such as, vitamins C and E (95) or riboflavin (96) can limit intimal smooth muscle proliferation and retard the early progression of CAV.

Infection with CMV is an important complication post-HTx (97) and studies indicate that this viral pathogen may contribute to CAV development via dysregulation of the NO pathway (98) or cytokine activation resulting in inflammatory cell recruitment associated with intimal thickening (99). There is observational data supporting this hypothesis as well as data from clinical trials demonstrating that patients randomized to receive prophylactic anti-viral therapy with ganciclovir have a lower risk of developing CAV (25). However, it must also be noted that there are other reports concluding that CMV viremia or infection does not affect CAV development (100). Traditionally, HTx recipients with the CMV seromatch status D+/R- have been considered at highest risk of CMV viremia and an increased risk of CAV amongst this group of patients has been shown (101) . However, in a recent study by Hussain et al. (102) it was demonstrated that pretransplant recipient CMV seronegativity is not a risk factor for CAV development. Hence, although CMV may represent a culprit that can be effectively treated, further trials are required to evaluate whether implementing prophylactic CMV therapy as part of post-HTx management protocols can reduce the long-term risk of CAV and associated mortality.

3.4.3 Interventional options

There are limited treatment options for advanced CAV. Pharmaceutical therapy has limited effect on established CAV and interventional options include retransplantation, coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI). However, retransplantation is generally very difficult due to multiple comorbidities and lack of donor organs, while CABG has a high perioperative mortality rate (103). Although PCI is a common therapeutic approach for traditional atherosclerosis, the restenosis rate in the setting of CAV is particularly high (104, 105). Furthermore, no survival benefit has been demonstrated after PCI of HTx recipients (13, 106) and in one study freedom from death or retransplantation

following PCI was only 34% at 5 years (104). Consequently, the indication for such revascularization interventions should be critically evaluated in all patients.

4. AIMS OF THE THESIS

The aims of this thesis were to investigate immunological and non-immunological markers of CAV, particularly focusing on:

i) The role of the neurohormonal biomarker, NT-proBNP, and the inflammatory biomarker, CRP, in identification of HTx recipients at higher risk of CAV and cardiac mortality
ii) The prognostic significance of impaired renal function post-HTx and whether any particular level of decline during the first year post-HTx is associated with an increased risk of CAV and mortality
iii) The potential adverse impact of chronic infection with the parasite *T.gondii* amongst HTx recipients in relation to CAV and cardiac mortality
iv) Utilization of IVUS and VH to allow an accurate evaluation of CAV and measurement of a broad inflammatory biomarker panel to allow identification of novel disease markers.

5. METHODOLOGICAL CONSIDERATIONS

5.1 Patient population

Our institution is the only HTx centre in Norway and all activity regarding preoperative HTx evaluation, surgery and postoperative management is undertaken here. All patients included in this thesis gave informed consent and are part of cohort of 585 patients that have undergone single organ HTx during the period 1983-2007. All studies complied with the Declaration of Helsinki and the locally appointed ethics committee approved the research protocol. The annual number of single organ HTx procedures performed at our centre generally ranges between 25 and 40 and the indication for HTx is in accordance with international guidelines.

All HTx recipients were evaluated for acute rejection via endomyocardial biopsies performed upon clinical suspicion and at the following intervals: weekly the first 2 months after HTx, fortnightly in the third month and then after 6, 12, 24 and 36 months. A standard immunosuppressive protocol is applied consisting of maintenance therapy with CsA or tacrolimus, prednisolone and AZA or MMF (the latter replaced AZA from 2002 onwards). No cytotoxic induction therapy is used and statin therapy was implemented as protocol from 1997 onwards.

All patients attended annual follow-up visits which includes a clinical assessment, biochemical profiling and coronary angiography unless contraindicated. A standardized regime of immunosuppressive medication, rejection surveillance and annual angiography was employed and allowed accurate comparison of patient outcome. Patients included in this thesis were included at the time of HTx (Papers I and III) or at an annual follow-up visit (papers II and IV).

5.2 Measurement of biomarkers

All biomarkers evaluated in thesis were measured in plasma samples obtained by standard venepuncture. Samples were stored on ice in tubes containing EDTA which is the preferred anticoagulant for immunoassays as it appears to inhibit *ex-vivo* production of pro-inflammatory substances (107, 108). Samples were centrifuged within 30 minutes of collection and stored at -80°C as such a standardized processing and storage procedure limits the effect on assay measurements (109, 110). Samples were subsequently thawed at room temperature but repeated freeze-thaw cycles were avoided as this can also influence biomarker measurements (110).

5.2.1 C-reactive protein

CRP is an inflammatory marker primarily synthesized by hepatocytes in response to IL-6 with synergistic enhancement of IL-1 or tumor necrosis factor (TNF) (111). There appears to be a strong relationship between traditional atherosclerosis and high sensitivity (hs) CRP and it is gaining acceptance as a tool for cardiovascular disease risk assessment (112). In this thesis, CRP was measured on a Roche Modular analyzer using the Tina-quant hs-CRP (Latex) HS assay (Roche Diagnostics, Indianapolis, Ind.). Given that CRP is an acute phase reactant we ensured that all patients were free from any acute clinical infection at the time of sampling to avoid erroneous results. This high-sensitivity assay is based on anti-CRP antibodies coupled to latex microparticles that react with sample antigen to form an antigen/antibody complex which is measured turbidmetrically (particle enhanced immunoturbidmetric assay) (113). In our laboratory the interassay variance was <10% in the range 0.1-300 mg/L.

5.2.2 N-terminal probrain natriuretic peptide

Brain natriuretic peptide (BNP) is a 32 amino acid cardiac natriuretic peptide hormone originally isolated from porcine brain tissue (114). It is released into the circulation in response to volume overload, ventricular stretching and hypertrophy and regulates natriuresis, vasodilatation, inhibition of renin and aldosterone production and of cardiac and vascular myocyte growth. This neurohormone is synthesized in ventricular cardiomyocytes as larger molecules (proBNP) that are subsequently cleaved prior to secretion to yield the active peptide hormone (BNP) and the biologically inactive N-terminal peptide fragment (NT-proBNP) (114). In comparison to BNP, NT-proBNP degrades more slowly both *in-vivo* and *ex-vivo*, has a higher circulating concentration, and is more stable, with less biological variability (115).

We measured NT-proBNP using an electrochemiluminescence immunoassay which uses the sandwich principle (Roche proBNP, Roche Diagnostics, Basel, Switzerland). In the first incubation, sample antibody, biotinylated polyclonal NT-proBNP-specific (amino acids 1–21) antibody and polyclonal NT-proBNP-specific (amino acids 39–50) antibody labeled with a ruthenium complex form a sandwich complex (116). In the second incubation, after addition of streptavidin labeled microparticles, the complex produced is bound to the solid phase via biotin-streptavidin interaction. The reaction mixture is aspirated into the measuring cell where microparticles are magnetically captured on an electrode and subsequent

application of a voltage induces a chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve. In our laboratory the lower detection limit was 0.6 pmol/L and the coefficients of variation were 4.5% and 2.5% in low and high ranges of NT-proBNP.

5.2.3 Renal function

Renal impairment is associated with an increased risk of mortality in chronic heart failure patients (117-119) and although there is data suggesting that this also applies to HTx recipients (120, 121), the relationship to development of CAV is not clear. In this thesis renal function was assessed by calculating estimated GFR according to the established Modification of Diet in Renal Disease Study (MDRD) formula that has been used in several large clinical trials and has also been shown to be accurate in HTx recipients (122-124). According to this formula, estimated GFR=186 x serum creatinine$^{-1.154}$ x Age$^{-0.203}$ (x 0.742 if female and x 0.210 if African-American). Estimated GFR was calculated and categorized according to National Kidney Foundation Disease Outcomes Quality Initiative (NKF-K/DOQI) Guidelines which classify GFR <30 ml/min/1.73 m^2 as severe GFR reduction and GFR between 30 and 59 ml/min/1.73 m^2 as moderate reduction (125).

5.2.4 Enzyme Linked Immunosorbent Assays (ELISAs)

The Enzyme Linked Immunosorbent Assay (ELISA) is a highly versatile and sensitive technique that can be used for qualitative or quantitative determination of antibodies or antigen (126). Cytokines are generally measured by sandwich ELISAs which makes use of highly purified anti-cytokine antibodies (capture antibodies) which are non-covalently absorbed ("coated" – primarily as a result of hydrophobic interaction) onto plastic microwell plates. The immobilized antibodies serve to specifically capture cytokine proteins present in plasma samples applied to the plate. After washing away unbound material, a biotin-conjugated detection antibody is added which binds to the captured cytokine antigen. Subsequently, an enzyme conjugated to avidin/streptavidin is added and this binds to the antigen-antibody sandwich (by exploiting the natural high affinity between avidin/streptavidin and biotin). Finally, a substrate solution (e.g. tatramethlybenzidine/hydrogen peroxide) is added and leads to development of a colored product that is proportional to the cytokine assessed and can be measured spectrophotometrically.

We measured plasma levels of soluble tumor necrosis factor receptor-1 (sTNFR-1), IL-6, osteoprotegerin (OPG), soluble gp130 and vascular cell adhesion molecule 1 (VCAM-1) by sandwich ELISAs obtained from R&D Systems (Minneapolis, MN). Plasma levels of neopterin and von Willebrand factor (vWf) were measured by ELISAs provided by Brahms (Henninsdorf, Germany) and DakoCytomation (Oslo, Norway), respectively. Analysis was performed according to the manufacturers' specifications and all intra-assay and inter-assay coefficients of variance were <10%.

Sandwich ELISAs are very useful for cytokine detection and measurement but several limitations regarding data interpretation must be mentioned. As in the case of this thesis, cytokine concentrations are often measured at a single time point and these results do not reflect the concurrent processes of cytokine secretion, uptake by cells and cytokine protein degradation. Also, the presence of soluble cytokine receptors, cytokine antibodies and binding protein may affect the measured cytokine concentrations (127). Hence, ELISAs do not provide any information regarding the biological *in-vivo* potency of the measured proteins. Another important limitation is the unique recognition profile of the antibodies between kits from different manufacturers and this makes absolute comparison of cytokine concentrations unreliable. There is also a considerable potential for run to run variability, although this was minimized in this thesis by performing each cytokine analysis for all included subjects on the same day.

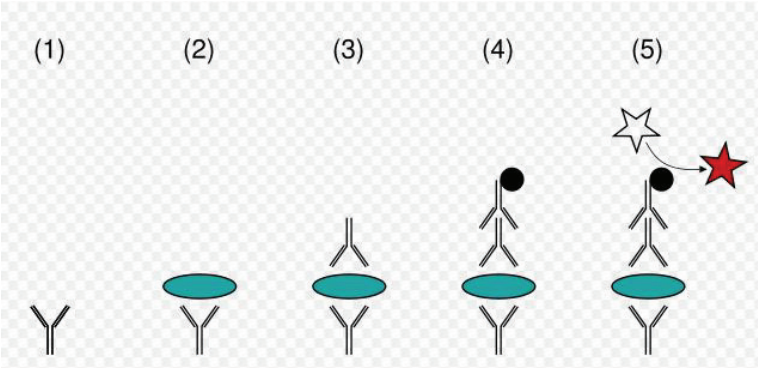

Figure 4. Principles of sandwich ELISA. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by the enzyme to a detectable form.

5.2.5 *Toxoplasma gondii* immunoglobulin analysis

The diagnosis of toxoplasmosis, a zoonosis caused by the protozoan *Toxoplasma gondii,* is based on serological tests which detect immunoglobulin M (IgM) and IgG antibodies. In recent years, several commercial diagnostic kits have been developed allowing automated detection of antibodies to *T gondii* based on ELISA) technology (128). In this thesis, recipient and donor IgG *T.gondii* serostatus were determined by an established IgG (Platelia Toxo IgG TMB, Bio-Rad, Marnes-la-Coquette, France) with the same lot number to ensure diagnostic accuracy.

The Platelia IgG assay is an indirect ELISA where diluted test samples are placed in *T.gondii* antigen coated microplate wells forming anigen-antibody complexes. Unbound antibodies and other serum proteins are removed by washing. Subsequently, peroxidase-labeled monoclonal antibody specific for human IgG is added and this binds to antigen-antibody complexes attached to the microplate wells. Following a second washing cycle, a solution of peroxidase substrate and chromogen tetramethylbenzidine (TMB) is added initiating a color reaction. The enzymatic reaction, read as optical density on a spectrophotometer, is proportional to the quantity of *T.gondii* IgG antibody present in the test sample. The qualitative results are calculated using a standard curve and expressed in international units/ml (IU/ml). We classified an optical density reading > 6.0 IU/ml as *T.gondii* seropositive, according to the manufacturer's instructions and previously published data (129).

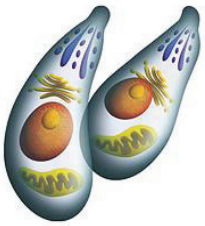

Figure 5. Schematic representation of *Toxoplasma gondii* parasite. Prior infection by T.gondii can be determined using indirect enzyme immunoassays such as the Platelia Toxo IgG assay.

5.3 Definition of endpoints

5.3.1 Mortality

Mortality data for all patients included in the thesis was available from the Norwegian Population Register. This national registry also includes causes of death allowing accurate data analysis and complete follow-up of included patients.

5.3.2 Diagnosis of cardiac allograft vasculopathy

5.3.2.1 *Angiography*

All patients included in the thesis underwent annual angiographic surveillance unless clinically contraindicated. The classification system applied by Costanzo et al. (130) was used to grade CAV as mild, moderate, or severe on the basis of left main involvement, primary vessel stenoses, and branch stenoses. Disease was considered as severe if left main stenosis was > 70% or 2 or more primary vessels stenoses were > 70% or branch stenoses were > 70% in all 3 systems. Angiographic films for all included patients were available for reanalysis if required. Despite the technical limitations of CAV detection by angiography, utilization of such a standardized grading system allowed a more accurate comparison of disease severity amongst the patients included in the thesis.

5.3.2.2 *Intravascular ultrasound and Virtual Histology*

The sensitivity of IVUS analysis in comparison to angiography for CAV detection is well-established. All patients included in Paper IV were evaluated with IVUS to allow such an accurate quantative assessment of CAV. In addition, Virtual Histology was utilized to allow qualitative assessment of these lesions. Formal training in IVUS measurements was provided by an external internationally renowned laboratory (See Appendix 1: Certificate of IVUS training).

IVUS acquisition. IVUS imaging utilizes a high frequency (30-40 MHz) ultrasound transducer on a catheter tip that is placed within the coronary arteries using standard angiographic techniques. The transducer generates high-resolution cross-sectional images of the coronary lumen and entire arterial wall that permit accurate measurement of dimensions of the lumen and vessel wall.

In our study, a major coronary epicardial artery (preferentially the left-anterior descending coronary artery) was imaged using a 20 MHz, 2.9F, monorail electronic Eagle Eye Gold IVUS imaging catheter (Volcano Therapeutics Inc, Rancho Cordova, California) and a dedicated IVUS scanner (Volcano Therapeutics). IVUS examination was performed after routine angiography following intracoronary administration of 200 µg nitroglycerin. The catheter was placed as distal as possible and automated mechanical pullback was performed from this start point to the ostium. Images were acquired at a rate of 30 frames/second and a pullback speed of 0.5 mm/second resulting in 1 mm intervals between every 60 frames. IVUS images were stored on a CD-ROM for later offline 3D volumetric analysis.

IVUS measurements. Semi-automated detection of both the lumen contour (LC) and external elastic membrane (EEM) was performed at intervals of 60 frames using dedicated software (QIVUS Clinical Edition, Medis Medical Imaging, Leiden, Netherlands). The longest possible segment between the most distal and proximal side branch visualized in the IVUS pullback was analyzed for each patient. Following automatic contour detection, borders were edited manually by two independent operators according to the guidelines for acquisition and analysis of IVUS images by the American College of Cardiology and European Society of Cardiology (131) – Figure 6.

The following parameters were recorded for all patients using the mean result of all frames analyzed: (1) lumen cross-sectional area (CSA), (2) vessel CSA, (3) intimal CSA and (4) maximal intimal thickness (MIT). In accordance to established guidelines, the largest distance from the intimal leading edge to the EEM was defined as MIT (131) and advanced CAV was defined as MIT >0.5 mm as this has been shown to accurately predict subsequent mortality and non-fatal major adverse cardiac events related to CAV (132). The CSA measurements were utilized to calculate Total Atheroma Volume (TAV) using Simpson's method as well as Percent Atheroma Volume (PAV) which expresses the summation of atheroma areas in proportion to the EEM area using the equation: $PAV = \sum (EEM_{area} - Lumen_{area})/\sum EEM_{area}) \times 100$. Recent studies suggest that PAV has the smallest coefficient of variability (133, 134) and we, therefore, considered this as an additional endpoint for CAV. Since there is no established PAV value defining advanced CAV, we pre-specified PAV above the mean value as indicative of advanced CAV. In our IVUS laboratory the intra-observer variability for both CAV endpoints (MIT and PAV) was <3%, whereas the inter-observer variability was <5% and this is comparable to other centres (133).

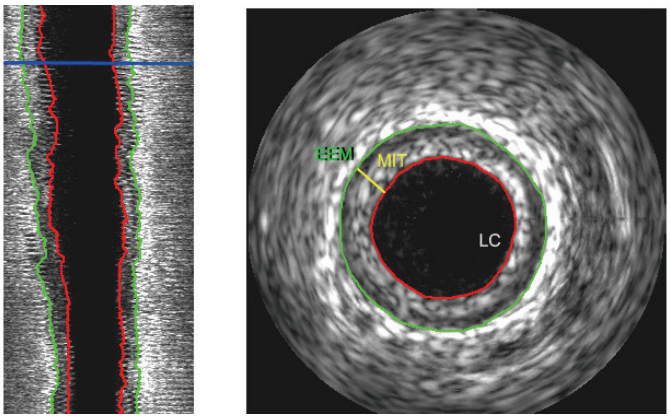

Figure 6. Example of tracing of contours following intravascular ultrasound (IVUS) acquisition. A longitudinal segment of an IVUS recording obtained by the Volcano scanner (left) and an example of a transverse IVUS frame (right) manually edited after semi-automated contour detection. Red marking = lumen contour (LC), green marking = external elastic membrane (EEM), blue line = level for the transverse image, MIT = maximal intimal thickness.

Virtual Histology. IVUS measurements were performed prior to VH analysis and the same segment length and portion that was utilized for CAV quantification (QIVUS software) was utilized for qualitative assessment by dedicated VH software (pcVH, v.2.2, Volcano Corporation). This required initial semi-automated contour detection of all acquired frames captured at the top of the R-wave of the heart cycle. Followed manual editing of contours, stored radiofrequency data was utilized by the software to reconstruct tissue maps with four identifiable major components – fibrous, fibrofatty, calcified and necrotic core components – which were allocated a separate colour coding in digitized images.

Necrotic core component is composed of lipid cells, necrotic and lymphocyte remnants together with tissue microcalcification. Consequently, several previous studies have considered necrotic and dense calcified tissue as one group that is likely to constitute the inflammatory tissue component of CAV (135, 136). In our study we defined an increased inflammatory tissue component as necrotic and dense calcified tissue >30%. This arbitrary cut-off has been utilized in another recent HTx study (136) and is supported by the results of other *in-vivo* and *ex-vivo* VH-IVUS studies performed amongst patients with IHD (137-140).

6. SUMMARY OF RESULTS

Paper I: Probrain natriuretic peptide and C-reactive protein as markers of acute rejection, allograft vasculopathy, and mortality in heart transplantation

In this paper we investigated the individual and combined value of NT-proBNP and CRP as markers of acute rejection, CAV and all-cause mortality in HTx recipients

- The biomarkers NT-proBNP and CRP are not useful as markers of acute rejection after HTx
- Both biomarkers measured at 12 months or more after HTx are independent predictors of all-cause mortality
- Combined measurement of both biomarkers significantly increases their predictive value for all-cause mortality
- The increased risk of mortality amongst patients with both elevated biomarkers is predominantly attributable to a higher risk of cardiovascular death
- Individual measurement of NT-proBNP and CRP does not predict the development of angiographic CAV but patients with both elevated biomarkers have a twofold increased risk of developing CAV

Paper II: Prognostic importance of renal function 1 year after heart transplantation for all-cause and cardiac mortality and development of allograft vasculopathy

In this study we assessed the relationship between renal function at 1 year post-HTx and all-cause and cardiac mortality and development of CAV

- A significant majority of patients have impaired renal function at one year post-HTx and this is an independent determinant of all-cause and cardiac mortality
- Impaired renal function at one year post-HTx is not associated with an increased risk of development of angiographic CAV
- Impaired renal function prior to HTx is not a risk factor for mortality or CAV but a significant proportion of patients experience a steep GFR decline (>25 ml/min/m^2) during the first year post-HTx and this is associated with a higher risk of all-cause and cardiac mortality.

Paper III: Pre-transplant *Toxoplasma gondii* seropositivity among heart transplant recipients is associated with an increased risk of all-cause and cardiac mortality

In this paper we investigated the risk of mortality, CAV and acute cellular rejection among T.gondii seropositive HTx recipients and the 4 donor/recipient seropairing groups

- Recipient *T.gondii* seropositivity is associated with a significantly higher risk of all-cause and CAV mortality
- The increased risk of mortality is primarily evident in the period beyond 4 years after HTx
- An increased risk of developing advanced angiographic CAV is demonstrable amongst *T.gondii* seropositive recipients
- *T.gondii* seropositivity does not influence the number of acute cellular rejection episodes and donor/recipient *T.gondii* seropairing status is not associated with an adverse outcome.

Paper IV: General and specific markers of inflammation are associated with advanced cardiac allograft vasculopathy and an increased inflammatory tissue component determined by virtual histology analysis

In this study we evaluated an extensive profile of clinical variables and immune markers to assess the chronic inflammatory milieu associated with advanced CAV assessed by IVUS and intimal inflammatory tissue determined by VH.

- Elevated levels of CRP, VCAM-1 and neopterin are associated with advanced CAV as defined by the two separate IVUS endpoints, MIT and PAV
- Elevated levels of CRP and VCAM-1 are also associated with an increased inflammatory component determined by VH analysis
- The above biomarkers indicate a pathophysiological role of inflammation, particularly characterized by endothelial cell and macrophage activation, in the development of advanced CAV and potentially vulnerable lesions.

7. DISCUSSION

7.1 C-reactive protein and N-terminal probrain natriuretic peptide

C-reactive protein, a pentameric protein produced by hepatocytes under the influence of inflammatory cytokines, is an established marker of systemic inflammation. Numerous studies have established that CRP provides prognostic information in patients with native atherosclerosis (141-144). Given the central role of immune mediated pathways in CAV development, CRP may allow accurate measurement of chronic inflammation in HTx recipients and, hence, may represent a non-invasive marker of CAV.

It has previously been demonstrated in a cross-sectional study that increased CRP levels are associated with CAV (55) and may also be related to disease progression (145). Eisenberg et al. (56) demonstrated that elevated CRP levels were associated with subsequent allograft failure in 99 HTx recipients that were included at varying times after HTx. In Paper I we evaluated the prognostic value of measurement of this biomarker amongst 210 HTx recipients at one year after HTx to predict the development of both advanced CAV and mortality. Our results indicate that elevated levels at one year post-HTx are not independent predictors of CAV but are associated with an increased risk of mortality that is predominantly attributable to CAV. The significant diagnostic limitation of angiography and the associated possibility of a type II error is likely to explain the negative finding regarding development of CAV. Nevertheless, our positive finding regarding the robust endpoint of mortality supports the hypothesis that chronic inflammation plays an important role in development of CAV and influences long-term survival. Furthermore, as discussed below, we proceeded to demonstrate for the first time that the prognostic information offered by this biomarker can be increased significantly when applied in combination with alternative biomarkers.

The neurohormone, NT-proBNP, is cleavage product of BNP that is secreted by ventricular cardiomyocytes and prior investigations have identified this biomarker as an independent predictor of mortality or cardiovascular events in patients with native atherosclerosis. Mehra et al. (57)have previously shown that NT-proBNP is also a useful marker of CAV. There are several potential explanations for this association including: (i) CAV can result in myocardial ischemia and increased ventricular wall stress which may in turn be a stimulus for release of NT-proBNP (146) or (ii) BNP release is directly influenced by cardiac ischemia (147). Furthermore, along with myocardial stretch and ischemia a range of other factors can stimulate BNP secretion, including, endothelin A (148), angiotensin II

(148) and TNF-α (149) which may also play a pathogenic role in CAV development. Consequently, the interdependence of NT-proBNP with local and circulating inflammatory factors may allow it to serve as a biomarker for identification of patients at higher risk of developing CAV.

We evaluated the predictive value of NT-proBNP for CAV and mortality amongst 210 HTx recipients. We chose to measure this biomarker at one year after HTx as it has been shown that levels of this neurohormone fall rapidly during the first few months after HTx but tend to stabilize by this point in time (150, 151). However, we did not find that NT-proBNP at one year after HTx was an independent predictor of CAV and this may be related to the dynamic and variable timescale associated with CAV development and progression. Hence, measurement of NT-proBNP at a single time point may not be sufficiently reliable to detect the current and impending pathophysiological processes driving CAV. An alternative explanation is the possibility of a type II error related to the limitations of angiography. Similar to our CRP findings, NT-proBNP was found to be an independent predictor of all-cause mortality with a negative predictive value of 90% but a positive predictive value limited to 28%.

Our study found that the combined measurement of CRP and NT-proBNP was an independent predictor of both CAV and all-cause mortality. Patients with both elevated biomarkers had a two-fold risk of CAV and three-fold risk of mortality. Furthermore, amongst patients with elevated CRP, the presence of elevated NT-proBNP clearly identified a group of patients at higher risk of adverse outcomes. Our results indicate that CRP and NT-proBNP are indicators of two distinct, but potentially overlapping, altered immunological and hormonal milieus associated with CAV and increased mortality. Hence, the inherent properties of these two biomarkers allow a more accurate reflection of the complex and multi-faceted pathological processes responsible for CAV. Although the positive predictive value of combined biomarker analysis was limited, the negative predictive value of 70% and 89% for CAV and all-cause mortality, respectively, indicates that these biomarkers can be useful "rule-out" tests and can assist risk stratification of HTx recipients. Although speculative, serial measurement of these biomarkers may yield further prognostic information and herald more individualized patient management with more aggressive surveillance and treatment of patients at highest risk of developing CAV.

7.2 Renal function

It has been established that impaired renal function is a risk factor for cardiovascular mortality in the general population (152-154). For example, the mortality due to cardiovascular causes is 500-fold greater in patients with end-stage renal failure (155) and this elevated risk is also evident in patients with mild renal impairment (153). Although renal impairment is a common complication after HTx, its contribution to CAV is not clear. Hence, in Paper II we evaluated the prognostic importance of renal function at 1 year after HTx for all-cause and cardiac mortality and development of CAV.

Our study revealed that impaired renal function, as assessed by glomerular filtration rate (GFR), at one year post-HTx is a strong predictor of both all-cause mortality and cardiac mortality defined as death due to CAV or sudden cardiac death. This risk increased progressively with increasing severity of renal impairment with a nearly two-fold increased risk of all-cause mortality for patients with moderate renal impairment (GFR 30-60 ml/min/1.73 m2) and three-fold increased risk in patients with severe renal impairment (GFR <30 ml/min/1.73 m2). Furthermore, a steep decline in renal function during the first year post-HTx is associated with an adverse prognosis. We were unable to demonstrate that impaired renal function at one year post-HTx predicted a higher risk of developing CAV and this is likely to be related to the technical limitations of angiography. In addition, our study did not assess the angiographic progression of CAV. A more rapid progression of CAV may occur amongst patients with impaired renal function at one year post-HTx, potentially explaining the increased risk of CAV mortality observed amongst this group of patients.

Multiple lines of evidence have identified exposure to CNIs as a major contributing cause to renal dysfunction amongst HTx recipients (156, 157). The renal histopathological changes causes by cyclosporine consist of arteriolopathy, glomerulosclerosis, interstitial fibrosis and tubular atrophy (158). These changes also contribute to the development of hypertension which is a common comorbidity amongst patients with impaired renal function. However, although hypertension is a known risk factor for atherosclerosis and may also contribute to CAV development (159), there are several other potential pathways that may explain the observed relationship between impaired renal function and cardiac mortality amongst HTx recipients and are discussed below.

It has been demonstrated that patients with impaired renal function have elevated serum levels of inflammatory markers such as C-reactive protein and interleukin-6 and tumor necrosis factor-α (160, 161), indicating potential mechanisms by which renal impairment may

accelerate the chronic inflammatory process associated with CAV development. Furthermore, the adhesion molecules E-selectin, VCAM-1 and intercellular adhesion molecule (ICAM)-1 are elevated in renal impairment (162) and are also believed to mitigate cellular infiltration and intimal thickening characteristic of CAV. Another mechanism may involve the renin-angiotensin system which is known to be activated by multiple pathways in renal disease. It has been shown that angiotensin II stimulates NAD(P)H oxidase and leads to generation of superoxide anions contributing to endothelial dysfunction (163) and upregulatation of a host of inflammatory mediators including cytokines, chemokines, adhesion molecules, and plasminogen activator inhibitor-1 (164-166). These events may promote proliferation and migration of smooth muscle cells and macrophages causing intimal thickening and progression of CAV in HTx recipients with renal impairment. Finally, plasma concentrations of ADMA are often elevated in renal impairment secondary to both increased activity of protein arginine methyltransferase and decreased metabolism by dimethylarginine dimethylaminohydrolase (167) and infusion with ADMA has been shown to directly induce intimal hyperplasia (162). Hence, renal impairment and elevated ADMA levels in HTx recipients may directly, or indirectly via their role in triggering endothelial dysfunction, contribute to development of CAV.

The observed relationship between impaired renal function at one year post-HTx and all-cause and cardiac mortality strongly indicates the importance of implementing strategies to preserve and improve renal function post-HTx. Aggressive monitoring and effective treatment of comorbidities that may worsen renal function, such as hypertension and diabetes, is essential. Equal attention also needs to be given to renal-sparing immunosuppressive regimes, and novel therapies, such as everolimus, that have been shown to improve renal function by more than 50% (168) may contribute to a reduction in morbidity and mortality attributable to CAV.

7.3 *Toxoplasma gondii* seropositivity

Toxoplasma gondii is an obligate intracellular parasite and primary infection is largely asymptomatic in healthy individuals (169). However, following primary infection, parasites are sequestered in various organs throughout the body and latency is maintained by an essential adaptive immune response. In Paper III we hypothesized that the chronic inflammatory response required to maintain *T.gondii* latency amongst HTx recipients may be

associated with an adverse prognosis and evaluated the relationship between pre-transplant *T.gondii* seropositivity and CAV and cardiac mortality.

Our study found the pre-transplant *T.gondii* seropositivity is a risk factor for all-cause and CAV mortality and development of advanced CAV. There was a three-fold risk of developing advanced CAV and a four-fold risk of mortality attributable to CAV amongst seropositive HTx recipients. Our data did not indicate that donor/recipient *T.gondii* seromismatch was a risk factor for any endpoint. Although our study was observational in design and did not measure specific markers of immune activity, data was collected on large number of covariates and appropriate adjustment was performed in the statistical analysis reducing the possibility of spurious results secondary to confounding. Furthermore, the immunological response associated with chronic *T.gondii* infection has been studied in detail previously and there are several plausible biological mechanisms (discussed below) that may explain our observation of an increased risk of CAV amongst seropositive HTx recipients.

It has been established that generation of IFN-γ by adaptive CD4 and CD8 lymphocytes is central to maintaining T.gondii latency (170, 171). The cytokine IL-12 has been identified as critical for driving prompt INF-γ production and proper differentiation of T helper (Th) 1 lymphocytes during the immune response to *T.gondii* (172, 173). With relevance to these findings, it is noteworthy that separate studies have also demonstrated IFN-γ as a key mediator of CAV development (174, 175). In addition, there are investigations demonstrating a correlation between γ-IFN suppression and prevention of CAV (176). Hence, we suggest that in the HTx recipient, production of IFN-γ in response to chronic *T.gondii* infection may play an overall detrimental role by promoting an extensive inflammatory response involving the intima of coronary arteries and development of CAV. An alternative explanation may involve a subset of T-lymphocytes known as T_H17 which may be induced by *T.gondii* parasites via mechanisms involving transforming growth factor-β and IL-6 (177) . T_H17 cells produce a range of pro-inflammatory cytokines, including IL-6, TNF, IL-17 (178), and this cell lineage is now known to be a major pathogenic mediator of organ-specific autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease (179) and may also be associated with reduced graft survival (180). Hence, although speculative, the innate response to *T.gondii* may induce differentiation of T_H17 cells and secretion of a range of inflammatory cytokines which promote development of CAV in seropositive HTx recipients.

Our report of *T.gondii* seropositivity and CAV is a novel finding but the role of other microbial pathogens such as, Hepatitis, CMV and EBV, has been studied by several other

researchers. CMV has probably received the greatest attention and both observational and interventional studies have implicated this viral pathogen in CAV development (25, 181, 182). For example, it has been shown that CMV-negative recipients from CMV-positive donors (D+/R-) are at a high risk of developing CAV (182, 183). We did not find an increased risk of CAV amongst T.gondii D+/R- patients and this may be indirectly attributable to the relative low risk of *T.gondii* transmission via the allograft in the era of universal trimethoprim sulfamethoxazole prophylaxis and this is supported by our finding of only 1 case of *T.gondii* seroconversion after HTx. Hence, although studies have shown that *acute* CMV infection and the subsequent inflammatory response involving endothelial injury are likely to contribute to CAV development (181, 184, 185), our results indicate that acute *T.gondii* infection is rarely encountered in HTx recipients and would not be a significant contributing factor. Another evident difference between the two pathogens is that endothelial cells are an important site of CMV infection and CMV has been shown to contribute directly to CAV via a direct pro-proliferative effect on intimal smooth muscle cells (181), but there is no known data supporting or indicating such a direct role of *T.gondii* parasites on the allograft vasculature. However, despite these differences, both pathogens may also share several pathological mechanisms as it has been demonstrated that CMV can remain latent and cause chronic inflammation in HTx recipients. For example, it has been suggested that allograft endothelial cells harbor low-levels of CMV and are capable of directly eliciting host CD4+ T cell activation and subsequent release of IFN-γ (186) . Furthermore, upregulation of the cytokines TNF-α, IL-6, TGF-β and platelet derived growth factor (PDGF) may also occur in conjunction with low-grade CMV infection (181). Hence, although CMV and *T.gondii* are inherently different pathogens, both microbes can contribute to a pro-atherogenic inflammatory environment in HTx recipients which favors the development of CAV.

7.4. Inflammatory markers

There is increasing evidence indicating that multiple inflammatory pathways contribute to the development of CAV (4, 28, 187). Histopathological studies have demonstrated focal inflammation of the coronary vasculature of HTx recipients (188) and elevation of systemic inflammatory markers is associated with an increased risk for CAV and an adverse outcome (55, 145). However, no studies have evaluated a broad spectrum of general and specific markers of inflammation and their correlation to quantitative and qualitative CAV assessment. Such an approach may allow identification of non-invasive markers of CAV and also increase

our understanding of the inflammatory mediators and pathways contributing to CAV development. Hence, in paper IV we evaluated an extensive panel of immune markers in an attempt to assess the inflammatory milieu associated with CAV assessed by IVUS and VH intimal tissue analysis.

Our study concluded that advanced CAV (MIT >0.5 mm) is associated with increased levels of CRP, VCAM-1 and neopterin. The two former markers were also found to be associated with an increased proportion of inflammatory tissue in the thickened intimal area evaluated by VH analysis. Hence, our data indicates a pathophysiological role of inflammation that is particularly characterized by endothelial cell and monocyte/macrophage activation. We did not find an association between advanced CAV (determined by IVUS or VH) and the other assessed markers of inflammation that included sTNFR-1, IL-6, OPG, soluble gp130 and vWf.

CRP is a systemic inflammatory marker that accurately reflects overall cytokine activity (189) and its prognostic value amongst HTx recipients has been demonstrated in several studies (55, 145). Although CRP may have a direct pathophysiological role in CAV (mediated via its prothrombotic effect) it also reflects activation of several upstream inflammatory pathways. The present study has confirmed that elevated CRP is strongly associated with advanced CAV as measured by two different IVUS endpoints (MIT and PAV), and in addition we show a clear relationship between CRP and inflammatory tissue content. This intimal characteristic is regarded as an inherently vulnerable tissue type due to increased inflammatory cells, higher lipid content, many necrotic cells and foam cells and a poor cellular matrix with a higher risk of thrombosis or rupture (190). Consequently, our results suggest that the systemic marker CRP can be utilized amongst HTx recipients to provide both quantitative and qualitatively information regarding CAV development and lesion susceptibility. Precise identification of intimal composition in HTx recipients in this manner is vital to allow implementation of a more appropriate risk stratification and individualized management protocol that can reduce the current morbidity and mortality attributable to CAV.

A key histological characteristic of CAV development and progression is neointimal proliferation and infiltration by macrophages. Initiation of inflammatory pathways resulting on activation of T-cells and release of IFN-γ causing macrophage activation and production of growth factors, such as, platelet-derived growth factor (191) and insulin-like growth factor-1 (192), can contribute to the proliferation of neointimal cells. Neopterin is a reliable marker of monocyte/macrophage activation and also reflects the IFN-γ mediated activation of these cells

(193). Our cross-sectional study has demonstrated that elevated plasma levels of this inflammatory biomarker are associated with quantitatively advanced CAV but its utility as a marker of disease severity and progression requires further investigation in longitudinal studies. Similarly, the specific triggers for macrophage activation in HTx recipients and development of advanced CAV needs to be explored as it may, ultimately, yield novel therapeutic targets.

The intimal thickening of CAV is characterized by a cellular infiltrate consisting of modified smooth muscle cells, macrophages-monocytes and T lymphocytes. Several studies amongst patients with native IHD have established that adherence of leukocytes to vascular endothelium is a prerequisite for transmigration of these cells and development of atherosclerotic plaques (194, 195). Various adhesion molecules, including the selection family (including P- and E-selectin) and the immunoglobulin family of molecules (including ICAM-1 and VCAM-1), have been studied and shown to play an important role in development of atherosclerosis (196-198). Based on these findings, it is plausible that these molecules may also contribute in a similar manner to CAV development. Indeed, our results have confirmed that elevated levels of VCAM-1, an adhesion molecule most closely related to T-cell recruitment at sites of local inflammation, is associated with advanced CAV verified by IVUS as well as an increased inflammatory component determined by VH analysis. Hence, VCAM-1 appears to be an important mediator of mononuclear cell recruitment and monitoring the expression of VCAM-1 may be a useful surrogate marker for detecting the presence of activated T cells and the associated risk of development of CAV and susceptible inflammatory lesions.

8. CONCLUSIONS

This thesis has investigated the role of immunological and non-immunological markers of CAV amongst HTx recipients and the following conclusions can be drawn:

1. NT-proBNP and CRP are not useful as markers of acute cellular rejection but both biomarkers, individually and in combination, predict long-term mortality amongst HTx recipients. Combined analysis also identifies patients at higher risk of developing angiographic CAV
2. Impaired renal function is apparent in a majority of HTx recipients already at one year post-HTx and is associated with an increased risk of all-cause mortality. Although renal impairment post-HTx does not appear to increase the risk of development of angiographic CAV it is as associated with an increased risk of mortality attributable to CAV
3. HTx recipients seropositive for *T. gondii* are at an increased risk of developing angiographically advanced CAV. Several immunoregulatory changes occur following *T.gondii* infection and these events may potentially contribute to the development of CAV and the increased risk of mortality
4. Advanced CAV, determined by IVUS, is associated with an inflammatory signature comprising of elevated CRP, VCAM-1 and neopterin. Elevated levels of CRP and VCAM-1 are also associated with an increased proportion of inflammatory tissue determined by VH analysis. Routine measurement and monitoring of these inflammatory markers may represent a novel and non-invasive method of post-HTx risk stratification and CAV surveillance.

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July 14, 2008

Thesis Examination Committee

Dear Colleagues

Re: Dr Satish Arora

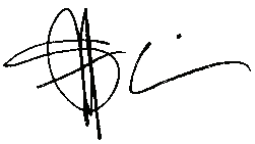
This is to confirm that Dr Satish Arora spent time in my ultrasound laboratory at the Cleveland Clinic during 2007 as part of his doctoral studies. Given that assessment of transplant vasculopathy by use of intravascular ultrasound is a component of his studies, Dr Arora requested to come to the Cleveland Clinic to learn how we perform our ultrasound analysis.

In the time that Dr Arora was in Cleveland he undertook the standard program of analysis training that each of the research technologists in our laboratory are required to complete. Dr Arora quickly learned the procedures and his measurements met with a high rate of accuracy, as assessed by our laboratory supervisor.

In a short period of time it became apparent that he has a very good working knowledge of the area, develops skills quickly and is able to critically think about results. He is a very amicable fellow and was a pleasure to have him as a guest, albeit for a short time, in our laboratory.

We wish him all the best in his future academic endeavours.

Best wishes



Stephen Nicholls

Article 1, 2 and 3 are removed.

Article 4:

Arora S, Gunther A, Wennerblom B, Ueland T, Andreassen AK, Gude E, Geiran O, Wilhelmsen N, Endresen K, Andersen R, Aukrust P, Gullestad L. Systemic markers of inflammation are associated with advanced cardiac allograft vasculopathy and an increased inflammatory component. Submitted.

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Systemic markers of inflammation are associated with advanced cardiac allograft vasculopathy and an increased inflammatory component

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ABSTRACT

We evaluated an extensive profile of clinical variables and immune markers to assess the inflammatory milieu associated with cardiac allograft vasculopathy (CAV) assessed by intravascular ultrasound (IVUS) and virtual histology (VH).

In total, 101 heart transplant (HTx) recipients were included and underwent IVUS/VH examination and measurement of plasma C-reactive protein (CRP), soluble tumor necrosis factor receptor-1, interleukin-6, osteoprotegerin, soluble gp130, von Willebrand factor, vascular cell adhesion molecule-1 (VCAM-1) and neopterin.

Mean Maximal Intimal Thickness (MIT) was 0.61±0.19 mm and mean fibrotic, fibrofatty, dense calcified and necrotic core components were 55±15, 14±10, 15±13 and 17±9%, respectively. In multivariate analysis, CRP >1.5 mg/L (OR 4.6, p<0.01), VCAM-1 >391 ng/mL (adjusted OR 3.2, p=0.04) and neopterin >7.7 nmol/L (OR 3.8, p=0.02) were independently associated with MIT >0.5 mm. In a separate analysis, CRP >1.5 mg/L (OR 3.7, p<0.01) and VCAM-1 >391 (OR 2.7, p=0.04) were independently associated with an increased inflammatory component (dense calcified/necrotic core component>30%).

Advanced CAV is associated with elevated CRP, VCAM-1 and neopterin and the two former biomarkers are also associated with an increased inflammatory component. Forthcoming studies should clarify if routine measurements of these markers can accurately identify HTx recipients at risk of developing advanced CAV and vulnerable lesions.

INTRODUCTION

Cardiac allograft vasculopathy (CAV) is an important complication limiting survival after heart transplantation (HTx). According to international registry data 43% of HTx recipients have developed CAV within the first 8 years after transplantation, and it is the second leading cause of mortality in patients who survive the first year after HTx (1). CAV is characterized by diffuse intimal thickening and although the pathogenesis is not fully understood, there is a consensus that the process is multifactoral and likely to be mediated by a final common immunological and inflammatory pathway (2).

Various studies have demonstrated that both immunological and non-immunological factors, such as impaired renal function (3), hyperlipidemia (4), and cytomegalovirus (CMV) infection (5) are associated with an increased risk of CAV development. It is believed that these various factors are able to trigger inflammatory responses that include cytokine induction and T-cell activation resulting in cell infiltration and intimal thickening. For example, we and others have previously demonstrated that elevated C-reactive protein (CRP) is associated with an increased risk of CAV development diagnosed by angiography (6, 7). Similarly, it has also been shown that antibodies to endothelial cells are associated with a higher risk of CAV (8). Such studies are important, but a single-marker approach gives a limited view of a complex process and does not recognize the interactions of various inflammatory mediators that are likely to occur *in vivo*. A further major limitation of these studies is the use of angiography to determine CAV considering it is well-established that this method grossly underestimates CAV diagnosis (9).

In the present study we aimed to evaluate the relationship between a broad spectrum of immune markers and quantitative and qualitative CAV assessment by intravascular ultrasound (IVUS) and virtual histology (VH) analysis, respectively. Virtual histology is a relatively new technique that utilizes backscatter radiofrequency data obtained during IVUS analysis and has

a 94–97% *ex vivo* and 87–97% *in vivo* accuracy for characterization of four basic tissue components amongst patients with ischemic heart disease (10, 11). Given our limited understanding of the complex *in vivo* processes responsible for CAV, such an assessment of CAV tissue composition amongst HTx recipients represents a novel and potentially valuable tool that merits further exploration.

The soluble inflammatory markers evaluated in this study included more general downstream markers of immune activation such as CRP and neopterin, being the prototypical acute-phase protein and marker of macrophage activation, respectively, markers of endothelial cell activation such as vascular cell adhesion molecule-1 (VCAM-1) and von Willebrand factor (vWf), more specific markers of activity in the upstream inflammatory pathways such as the interleukin-6 (IL-6) (i.e. IL-6 and soluble gp130) and tumor necrosis factor-α (TNF-α) [i.e. soluble TNF receptor type 1 (sTNFR-1)] systems, as well as osteoprotegerin (OPG), a molecule involved in bone remodeling, inflammation and coronary artery calcification. These various inflammatory markers reflect distinct, but potentially equally important, pathophysiological pathways and we sought to evaluate their relationship to CAV as assessed by IVUS and VH analysis.

MATERIAL AND METHODS

Patient population. In total, 101 HTx recipients attending their routine annual follow-up visit between November 2005 and February 2008 were prospectively included. The inclusion criteria were: (1) age >18 years at time of follow-up visit; (2) consent to IVUS and VH examination; (3) estimated glomerular filtration rate >30 ml/minute at the time of follow-up visit (HTx recipients with severe renal impairment do not undergo annual angiography at our centre unless clinically indicated). The study complies with the Declaration of Helsinki, the

locally appointed ethics committee approved the research protocol and informed consent was obtained from all patients.

The immunosuppressive regime at our centre consists of maintenance therapy with cyclosporine, prednisolone and azathioprine or mycophenolate mofetil (the latter was implemented as protocol for patients undergoing HTx after 2001). No induction therapy is used at our centre and all patients receive statin therapy unless clinically contraindicated. Data regarding patient demographics, acute rejection episodes, medication, echocardiography measurements, biochemical profile [including N-terminal probrain natriuretic peptide (NT-proBNP (12)) and uric acid], serological analysis [CMV and *Toxoplasma gondii* (*T. gondii*) seropositivity (13)] was available for all patients (Table 1).

IVUS and VH acquisition. IVUS examination was performed after routine angiography following intracoronary administration of 200 μg nitroglycerin. A major coronary epicardial artery (preferentially the left-anterior descending coronary artery) was imaged using a 20 MHz, 2.9F, monorail electronic Eagle Eye Gold IVUS imaging catheter (Volcano Corporation Inc, Rancho Cordova, CA) and a dedicated IVUS scanner (Volcano Corporation) which also allows simultaneous acquisition of VH (backscatter radiofrequency) data.

The IVUS catheter was placed as distal as possible and automated mechanical pullback was performed from this start point to the ostium. IVUS images were acquired at a rate of 30 frames/second and a pullback speed of 0.5 mm/second resulting in 1 mm intervals between every 60 frames. IVUS images were stored on a CD-ROM for later offline 3D volumetric analysis. VH images were acquired with each heart beat which functions as a trigger for image acquisition and these were stored on DVD-ROM for later offline analysis.

IVUS measurements. Semi-automated contour detection of both the lumen and external elastic membrane (EEM) was performed at intervals of 60 frames using dedicated software (QIVUS Clinical Edition, Medis Medical Imaging, Leiden, Netherlands). The longest possible

segment between the most distal and proximal side branch visualized in the IVUS pullback was analyzed for each patient. Following automatic contour detection, borders were edited manually by two independent operators according to the guidelines for acquisition and analysis of IVUS images by the American College of Cardiology and European Society of Cardiology (14). The IVUS analysis was performed prior to measurement of inflammatory markers to prevent any potential observer bias. Satisfactory IVUS recordings were available for all patients and a total of 4258 IVUS frames were analyzed with a median number of 42 frames per patient.

The following parameters were recorded for all patients using the mean result of all frames analyzed: (1) lumen cross-sectional area (CSA), (2) vessel CSA, (3) intimal CSA and (4) maximal intimal thickness (MIT). In accordance to established guidelines, the largest distance from the intimal leading edge to the EEM was defined as MIT (14) and advanced CAV was defined as MIT >0.5 mm as this has been shown to accurately predict subsequent mortality and non-fatal major adverse cardiac events related to CAV (15). The CSA measurements were utilized to calculate Total Atheroma Volume (TAV) using Simpson's method as well as Percent Atheroma Volume (PAV) which expresses the summation of atheroma areas in proportion to the EEM area using the equation: PAV = ($\sum$EEM $_{area}$ − Lumen$_{area}$)/$\sum$EEM$_{area}$) x 100. Recent studies suggest that PAV has the smallest coefficient of variability (16, 17) and we, therefore, considered this as an additional endpoint for CAV. Since there is no established PAV value defining advanced CAV, we pre-specified PAV above the mean value as indicative of advanced CAV. In our IVUS laboratory the intra-observer variability for both CAV endpoints (MIT and PAV) was <3%, whereas the inter-observer variability was <5% and this is comparable to other centers (16).

VH measurements. IVUS measurements were performed prior to VH analysis and the same segment length and portion that was utilized for CAV quantification (QIVUS software) was

utilized for qualitative assessment by dedicated VH software (pcVH, v.2.2, Volcano Corporation). This required initial semi-automated contour detection of all acquired frames captured at the top of the R-wave of the heart cycle. Satisfactory VH recordings were available for all patients and a median number of 176 VH frames were analyzed per patient. Followed manual editing of contours, stored radiofrequency data was utilized by the software to reconstruct tissue maps with four identifiable major components – fibrous, fibrofatty, dense calcified and necrotic core components – which were allocated a separate color coding in digitized images and expressed as a percentage of total intima area. Necrotic core component is composed of lipid cells, necrotic and lymphocyte remnants together with tissue microcalcification. Consequently, several previous studies have considered necrotic and dense calcified tissue to, at least partly, reflect inflammatory tissue components of the vessel wall being associated with a higher subsequent progression of CAV (18, 19). We defined increased inflammatory tissue component as necrotic and dense calcified tissue >30%. This arbitrary cut-off has been utilized in another other recent HTx study (19) and is supported by the results of other *ex-vivo* and *in-vivo* VH-IVUS studies performed amongst patients with IHD (20-23).

Inflammatory marker analysis. Plasma samples were collected by standard venepuncture prior to angiography and IVUS examination. Peripheral venous blood were drawn into sterile blood collection tubes with EDTA as anticoagulant, immediately immersed in melting ice, and centrifuged within 30 minutes at 2000*g* for 20 minutes to obtain platelet-poor plasma. Plasma specimens were stored at -80°C and were thawed less than three times.

Plasma levels of sTNFR-1, IL-6, OPG, gp130 and VCAM-1 were measured by enzyme immunoassays (EIA) obtained from R&D Systems (Minneapolis, MN). Plasma levels of neopterin were measured by EIA provided by Brahms (Henninsdorf, Germany). CRP and vWf were measured by EIAs as previously described (24, 25). All intra-assay and inter-assay coefficients of variance were <10%.

Statistical analysis. Analyses were performed with the SPSS statistical software (SPSS Inc. Chicago, IL, v.13.0) and a p-value <0.05 was considered statistically significant. Student's t test was used for normally distributed continuous variables and Mann-Whitney test for other continuous variables. One way analysis of variance was used to compare means amongst two or more groups. Categorical variables were compared using the chi-square test and multivariate regression analysis was performed to estimate odds ratios using forward stepwise conditional method. Data is expressed as mean ±SD or as median (interquartile range) as appropriate.

RESULTS

The baseline characteristics of the study population are shown in Table 1.

IVUS measurements and the occurrence of advanced CAV. The mean vessel length analyzed by IVUS examination was 42.0±9.8 mm and an example of an IVUS recording is shown in Figure 1. Mean MIT was 0.61±0.19 mm, and 47 (47%) of patients were found to have advanced CAV defined as MIT >0.5 mm (15). Mean PAV was 32.3±9.5% and normalized TAV was 4.9±2.0 mm^3/mm. A relatively large HTx population was included and time since HTx varied considerably [median 5.0 (IQ range 3.0-9.0) years], but no significant difference in time since HTx was found amongst patients with MIT below and above 0.5 mm (p=0.65). A similar pattern was seen for the other measured IVUS parameters (vessel, lumen and intimal CSA, vessel and lumen volume, normalized TAV and PAV) with no relation to time since HTx (Table 2).

When comparing demographic and clinical characteristics, patients with MIT >0.5 mm (n=47) were older, had a higher serum creatinine, displayed higher NT-proBNP levels and were more likely to be *T. gondii* seropositive (Table 1). In contrast, a past history of significant cellular rejection episodes (≥grade 2R), impaired ventricular function as measured

by echocardiography and CMV seropositivity/mismatch were not associated with this marker of advanced CAV (Table 1).

VH measurements. An example of an analyzed VH frame is shown in Figure 1. Fibrous tissue was the predominant identifiable component of the intimal area (55±15%) followed by very similar proportions of fibrofatty (14±10%), dense calcified (15±13%) and necrotic core (17±9%) tissue. (Table 3). Our data indicated increased fibrotic and less inflammatory tissue (dense calcified and necrotic core) with increasing time since HTx, but this association did not reach statistical significance ($p=0.07$). When evaluating demographic and clinical parameters we noted that increased inflammatory component was also associated with increased serum creatinine ($p<0.01$) and lower HDL levels ($p<0.01$), but not with any of the other parameters outlined in Table 1.

Inflammatory markers. As shown in Figure 2, patients with MIT > 0.5 mm had significantly higher levels of CRP, sTNFR-1, VCAM-1 and neopterin as compared with those with MIT ≤0.5 mm. Similar significant results were found for the above inflammatory markers, in addition to vWf, when using PAV >32% as an alternative CAV endpoint (Figure 3). As for IL-6, OPG and gp130, no significant relationship was found to either MIT or PAV (Figures 2 and 3). Patients with increased intimal inflammatory component (i.e. dense calcified/necrotic core >30%) also displayed significantly higher levels of CRP, sTNFR-1, VCAM-1, vWf and neopterin, but there was no significant relation to the other markers of inflammation (Figure 4).

Multivariate analysis. The variables in Table 1 found to be associated with advanced CAV upon univariate analysis ($p<0.05$) were all included in a multivariate regression analysis to identify independent determinants of advanced CAV (i.e. MIT >0.5 mm). CRP, sTNFR-1, VCAM-1 and neopterin (all significant upon univariate analysis) were included in this analysis and categorized according to median values. This final regression model revealed

that CRP >1.5 mg/L [adjusted OR 4.6 (95% CI 1.7-12.2), p<0.01], VCAM-1 >391 ng/mL [adjusted OR 3.2 (95% CI 1.1-9.7), p=0.04] and neopterin >7.7 nmol/L [adjusted OR 3.8 (95% CI (1.2-11.6), p=0.02], but not sTNFR-1 [adjusted OR 1.3 (95% CI 0.2-4.8), p=0.81], were independently associated with MIT >0.5 mm (Table 4). Similar significant results were found when utilizing PAV >32% as an alternative CAV endpoint (data not shown). Notably, of all the included parameters, CRP was the strongest predictor of advanced CAV, and all the inflammatory markers were stronger predictors of CAV than all the included clinical parameters, except for *T. gondii* seropositivity (Table 4). This latter finding confirms our previously reported association between *T. gondii* seropositivity and angiographically diagnosed CAV (26), further underscoring that this serological risk factor may have been overlooked in previous studies.

A separate multivariate analysis was performed to assess independent determinants of an increased inflammatory component and CRP and VCAM-1 were found to be significant variables with an adjusted OR of 3.7 (95% CI 1.4-9.5, p<0.01) and 2.7 (95% CI 1.1-6.9, p=0.04), respectively (Table 5). Elevated serum creatinine [adjusted OR 4.7 (95% CI 1.6-14.1), p<0.01] and lower HDL levels [adjusted OR 3.7 (95% CI 1.3-10.6), p=0.01] were also independent predictors of an increased inflammatory component. Although univariate analysis revealed significantly elevated levels of sTNF-R1, vWf and neopterin amongst patients with an increased inflammatory component these markers did not retain statistical significance in this multivariate regression model (Table 5).

DISCUSSION

The current study has evaluated the association between a broad profile of clinical and inflammatory variables and both quantitative and qualitative CAV assessment by IVUS and VH analysis, respectively. This is, to the best of our knowledge, the first report that shows that advanced CAV, as determined by IVUS, is associated with increased plasma levels of

CRP as well as VCAM-1 and neopterin, suggesting a pathogenic role of inflammation, including endothelial cell and monocyte/macrophage activation, in the development of this complication post-HTx. Furthermore, we found that elevated levels of CRP and VCAM-1 were associated with an increased proportion of inflammatory tissue in the thickened intimal area evaluated by VH analysis, suggesting a link between systemic and local inflammation within coronary artery in the transplanted heart.

CRP is considered as a stable and sensitive marker of upstream systemic inflammation (27). We and others have previously demonstrated in prospective studies that elevated levels predict development of angiographically evident CAV amongst HTx recipients (28, 29). Hence, it has been suggested that CRP reflects a general inflammatory state associated with CAV development and adverse outcome (30). Our current study extends previous studies by showing that elevated CRP is strongly associated with advanced CAV as measured by two different IVUS endpoints (MIT and PAV). Also, we show a clear relationship between CRP and inflammatory tissue content in the affected intima. This intimal characteristic is inherently regarded as a vulnerable tissue type due to increased inflammatory cells, higher lipid content, many necrotic cells and foam cells and a poor cellular matrix with a higher risk of thrombosis or rupture (31), and our finding in the present study further suggest a link between inflammation and this vascular phenotype. Previously, increased CRP levels have been linked to an inflammatory and higher risk plaque phenotype in atherosclerotic disorders (32, 33), and our finding suggest that a similar association may be present in CAV.

Neointimal proliferation and infiltration by macrophages is a key feature of CAV development and is likely to be driven by several processes including upstream cytokine activation (34). For example, activated T-cells release interferon-γ (IFN-γ) causing macrophage activation and production of growth factors, such as, platelet-derived growth factor (35) and insulin-like growth factor-1 (36), all of which may contribute to the

proliferation of neointimal cells. In the present study we have shown that plasma levels of neopterin, a reliable marker of monocyte/macrophage activation, also reflecting the IFN-γ mediated activation of these cells (37), is associated with quantitatively advanced CAV as well as an increased inflammatory component of the affected intima. Although our findings further support a role for monocyte/macrophage activation in the development of CAV, the triggers for this activation in HTx recipients requires investigation in further studies and may, ultimately, yield novel therapeutic targets.

Endothelial adhesion molecules, including VCAM-1, are known to play an important role in the recruitment of inflammatory cells into vessel wall. Various immunological (e.g. CMV infection) and non-immunological risk factors (e.g. hyperlipidemia and hyperglycemia) are believed to contribute to CAV development via upregulation of adhesion molecule expression (30). Although experimental studies have demonstrated that VCAM-1 is induced on vascular endothelial cells in animal models of CAV (38), this has not been investigated in clinical studies. The current study shows that elevated plasma levels of VCAM-1 are independently associated with advanced CAV determined by IVUS. Furthermore, we noted that VCAM-1 is also associated with an increased inflammatory component determined by VH analysis further suggesting that recruitment of inflammatory cells via this adhesion molecule may play a role in the development of vulnerable and high-risk lesions.

Our study included a heterogeneous group of HTx recipients and collected a wide array of recipient and donor data including acute rejection history, echocardiography data and a range of biochemical measurements. Time since HTx was not found to be a determinant of advanced CAV (defined by MIT or PAV). Although this negative association may seem surprising, it should be emphasized that previous reports of CAV detected in 8%, 32% and 48% of HTx recipients at 1, 5 and 8 years post- HTx (1) are based on angiographic examination. It has been established that these figures are likely to be highly inaccurate as

CAV is reported to be present in up to 75% HTx recipients already at one year post-HTx based on IVUS examinations (39). Tsutsui et al. (40) conducted a 5-year serial IVUS study and found that most intimal thickening occurred during the first two years post-HTx, with no significant increase beyond this period, indicating that CAV development is not a time-dependent linear process. Li et al. (41) performed a similar study concluding that variable vascular remodeling patterns exist post-HTx and can influence and determine the rate of CAV progression. Hence, we believe our IVUS results demonstrate that the incidence and progression rate and composition of CAV is highly variable and individual.The cross-sectional design is a limitation of our study and our results warrant further investigation in prospective follow-up studies. Characterization of tissue composition by VH analysis is a relatively new technological advancement and although it has been validated in several large studies it has important technical limitations. For example, the pathological definition of vulnerable plaque is a necrotic core with an overlying fibrous cap of <65 μm whereas the spatial resolution of VH is approximately 100–200 μm. Nevertheless, we believe our results are robust due to a relatively large patient population, assessment of a broad range of clinical and inflammatory markers and the use of both quantitative and qualitative analysis of CAV severity. However, based on the suggestion that most intimal thickening occurs during the first two years after HTx (42) future studies should also include longitudinal measurements of inflammatory markers to elucidate if an early inflammatory response (i.e. within the first year) is of more importance than a more late inflammatory response for the development of CAV.

In contrast to the lack of association with most of the traditional risk factors for cardiovascular disease, we found that quantitatively advanced CAV, as assessed by IVUS, is associated with an inflammatory signature comprising of elevated CRP, VCAM-1 and neopterin. Although these markers are robust markers of inflammation, reflecting upstream inflammatory activity, they are also, at least partly, complementary and reflect different arms

of the immune system. Furthermore, elevated levels of CRP of VCAM-1 are associated with an increased proportion of necrotic core tissue determined by VH analysis. Forthcoming studies should clarify if routine measurements of these markers can allow accurate identification of HTx recipients at risk of developing quantitatively advanced CAV and vulnerable lesions with an increased necrotic component.

Table 1. Demographic and Clinical Characteristics of the Study Population (n=101).

Characteristic	Overall	MIT ≤0.5mm (n=54)	MIT >0.5 mm (n=47)	p-value
Demographics				
Recipient age (yrs)	60 (52-65)	57 (51-63)	63 (53-67)	**0.05**
Recipient male gender	87 (86%)	45 (83%)	42 (89%)	0.38
Medical history				
Time since HTx (yrs)	5.0 (3.0-9.0)	6.0 (2.8-8.0)	6.0 (3.0-10.0)	0.65
IHD as etiology for HTx	43 (43%)	23 (43%)	20 (43%)	0.99
Hypertension	79 (78%)	42 (78%)	37 (79%)	0.91
Diabetes mellitus	10 (10%)	3 (6%)	7 (15%)	0.12
Current smoker	29 (29%)	16 (30%)	13 (28%)	0.83
Cellular rejection ≥grade 2R	31 (31%)	17 (31%)	14 (30%)	0.85
Medication				
Cyclosporine	99 (98%)	54 (100%)	45 (96%)	0.13
Tacrolimus	2 (2%)	0 (0%)	2 (4%)	0.13
Prednisolone	99 (98%)	52 (96%)	47 (100%)	0.18
Azathioprine	49 (49%)	28 (52%)	21 (45%)	0.47
Mycophenolate mofetil*	42(42%)	23 (43%)	19 (40%)	0.83
Beta-blocker	21 (21%)	9 (17%)	12 (26%)	0.27
Calcium channel blocker	32 (32%)	21 (39%)	11 (23%)	0.10
Angiotensin converting enzyme inhibitor/receptor antagonist	41 (41%)	20 (37%)	21 (45%)	0.44
Diuretic	20 (20%)	7 (13%)	13 (28%)	0.07
Statin	89 (88%)	46 (85%)	43 (92%)	0.33
Echocardiography data				
Left ventricular diameter systole (cm)	3.2±0.6	3.2±0.5	3.1±0.6	0.76
Left ventricular diameter diastole (cm)	4.9±0.6	4.8±0.5	4.9±0.6	0.68
Interventricular septal thickness diastole (cm)	1.1±0.2	1.0±0.1	1.1±0.2	0.23
Left ventricular posterior wall thickness diastole (cm)	1.0±0.2	0.9±0.2	1.0±0.3	0.09
Fractional shortening (%)	35.6±8.1	34.6±7.6	36.8±8.5	0.18
Biochemical data				
Total cholesterol (mmol/L)	5.2±1.0	5.2±1.1	5.2±1.0	0.98
HDL (mmol/L)	1.8± 0.5	1.9±0.6	1.7±0.4	0.06
LDL (mmol/L)	3.1±0.8	3.1±0.9	3.2±0.8	0.74
Triglycerides (mmol/L)	1.4±0.9	1.3±0.7	1.6±1.0	0.07
Serum creatinine (µmol/L)	112±30	106±25	119±33	**0.02**
NT-proBNP (pmol/L)	38 (24-81)	33 (16-61)	41 (29-95)	**<0.01**
Serum uric acid (µmol/L)	425±99	398±99	457±88	0.11
Serological data				
CMV IgG seropositivity	69 (68%)	41 (76%)	28 (60%)	0.08
Recipient/donor CMV mismatch (R-/D+)	18 (18%)	7 (13%)	11 (23%)	0.17
T.gondii IgG seropositivity	27 (27%)	9 (17%)	18 (38%)	**0.02**
Donor characteristics				
Donor age (yrs)	35 (21-43)	28 (20-41)	38 (23-48)	0.08
Donor male gender	35 (35%)	19 (35%)	16 (34%)	0.90
Ischemic time (min)	174 (65-206)	179 (74-206)	169 (59-206)	0.36

Data are reported as mean±SD, median and interquartile range or as percent frequency, as appropriate. IHD=ischemic heart disease; NT-proBNP=N-terminal probrain natriuretic peptide; CRP=C-reactive protein; CMV=cytomegalovirus*; T. gondii=Toxoplasma gondii*.

*Mycophenolate mofetil was first introduced at our centre (as an alternative to azathioprine) amongst patients undergoing HTx from 2002 onwards.

Table 2. Results of IVUS Analysis for the Entire Study Population According to Time after Heart Transplantation (HTx).

		Time since HTx (years)			
IVUS parameter	**Overall**	**≤3 (n=29)**	**4-6 (n=28)**	**≥7 (n=44)**	**p-value**
Segment length (mm)*	42.0±9.8	40.2±8.6	42.2±8.7	43.4±11.1	0.41
Vessel CSA (mm^2)	14.9±5.0	14.2±4.0	14.0±3.2	16.1±6.3	0.13
Lumen CSA (mm^2)	10.2±4.0	9.4±2.9	9.8±3.1	10.9±5.0	0.29
Intimal CSA (mm^2)	4.7±1.9	4.7±1.9	4.2±1.5	5.3±2.2	0.09
MIT (mm)	0.61±0.19	0.61±0.19	0.58±0.18	0.63±0.19	0.45
Total vessel volume (mm^3)	641.8±269.1	590.9±217.5	595.0±182.3	694.9±327.8	0.12
Total lumen volume (mm^3)	436.5±202.7	390.7±150.7	422.4±154.3	466.1±245.0	0.20
Normalized TAV (mm^3/mm)	4.9±2.0	5.0±2.0	4.2±1.5	5.2±2.0	0.09
PAV (%)	32.3±9.5	33.2±9.0	30.5±10.1	32.9±9.4	0.49

*Absolute segment length is equal to number of frames analyzed (slice thickness=1 mm).
CSA = cross-sectional area, PAV = percent atheroma volume, TAV = total atheroma volume, MIT = maximal intimal thickness

Table 3. Results of Virtual Histology Analysis for the Entire Study Population According to Time after Heart Transplantation (HTx).

		Time since HTx (years)			
Tissue type (%)	**Overall**	**≤3 (n=29)**	**4-6 (n=28)**	**≥7 (n=44)**	**p-value**
Fibrous	55±15	54±17	50±16	58±11	0.15
Fibrofatty	14±10	14±9	10±9	16±11	0.12
Dense calcified	15±13	14±13	19±16	12±10	0.13
Necrotic core	17±9	17±10	20±10	14±8	0.07

Table 4. Multivariate Logistical Regression Analysis of Determinants of Advanced Allograft Vasculopathy as defined by Maximal Intimal Thickness > 0.5 mm.

Risk factor	Odds ratio	95 % CI	p-value
Age >60 years	1.4	0.6-3.7	0.43
NT-proBNP >39 pmol/L	2.7	0.9-8.1	0.07
Serum creatinine>112 μmol/L	0.7	0.2-2.7	0.63
Recipient *T. gondii* seropositive status	5.0	1.5-17.3	0.01*
CRP >1.5 mg/L	4.6	1.7-12.2	<0.01*
sTNFR-1 >1996 pg/mL	1.3	0.2-4.8	0.81
VCAM-1 >391 mg/mL	3.2	1.1-9.7	0.04*
Neopterin >7.7 nmol/L	3.8	1.2-11.6	0.02*

CRP=C-reactive protein, sTNFR-1=soluble tumor necrosis factor receptor-1, VCAM=Vascular cell adhesion molecule-1, NT-proBNP=N-terminal probrain natriuretic peptide, *T. gondii= Toxoplasmosis gondii.*
* denotes statistically significant values (<0.05)

Table 5. Multivariate Logistical Regression Analysis of Determinants of Increased Intimal Inflammatory Component (>30% dense calcified/necrotic core component).

Risk factor	Odds ratio	95 % CI	p-value
Serum creatinine >112 µmol/L	4.7	1.6-14.1	<0.01*
HDL <1.8 mmol/L	3.7	1.3-10.6	0.01*
CRP >1.5 mg/L	3.7	1.4-9.5	<0.01*
sTNFR-1 >1996 pg/mL	1.5	0.9-2.0	0.30
VCAM-1 >391 mg/mL	2.7	1.1-6.9	0.04*
vWf > 121 AU	1.2	0.5-2.6	0.58
Neopterin >7.7 nmol/L	1.8	0.8-8.2	0.42

CRP=C-reactive protein, sTNFR-1=soluble tumor necrosis factor receptor-1, VCAM=Vascular cell adhesion molecule-1 vWf= von Willebrand factor
* denotes statistically significant values (<0.05)

FIGURE LEGENDS

Figure 1. Example of an intravascular ultrasound and virtual histology recording.

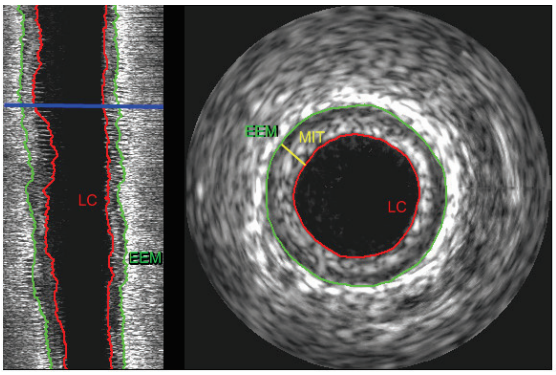
Upper panel: A longitudinal segment of an intravascular (IVUS) recording obtained by the Volcano scanner and an example of a transverse IVUS frame manually edited after semi-automated contour detection. Red marking = lumen contour (LC), green marking = external elastic membrane (EEM), blue line = level for the transverse image.

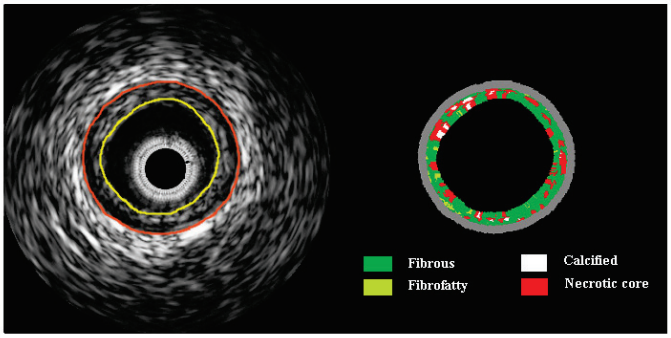
Lower panel: Analysis of a virtual histology frame obtained at the time of IVUS acquisition. Analysis of radiofrequency data following contour detection provides color coded tissue component characterization where green is fibrous, light green is fibrofatty, white is dense calcified and red is necrotic core component.

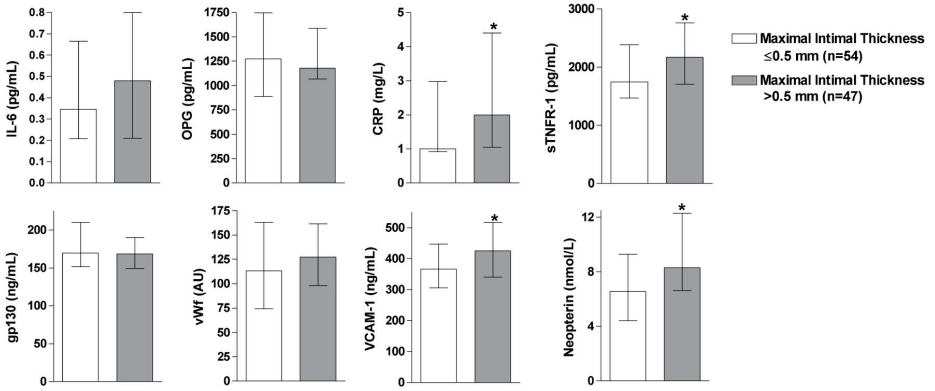
Figure 2. Immune marker profile according to Maximal Intimal Thickness measured by intravascular ultrasound analysis. Advanced cardiac allograft vasculopathy as defined by Maximal Intimal Thickness (MIT) >0.5 mm was associated with elevated levels of C-reactive protein (CRP), soluble tumor necrosis factor receptor-1 (sTNFR-1), vascular cell adhesion molecule–1 (VCAM-1) and neopterin. There was no significant difference in levels of interleukin-6 (IL-6), osteoprotegerin (OPG), soluble gp130 and von Willebrand factor (vWf) between the two groups.

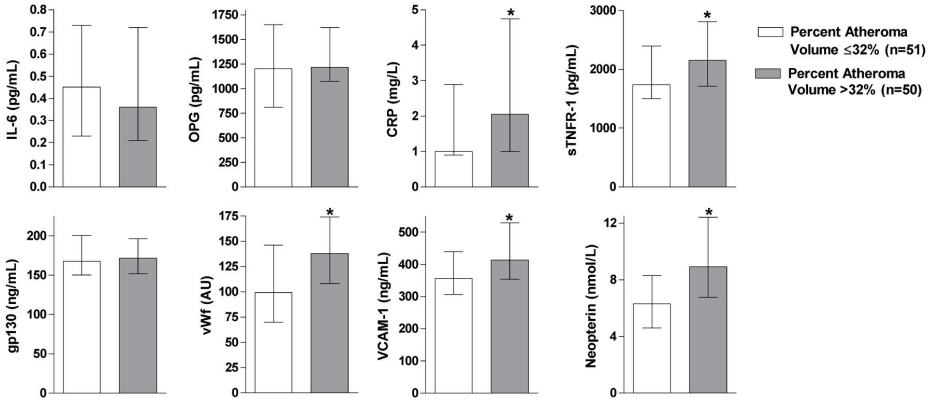
Figure 3. Immune marker profile according to Percent Atheroma Volume measured by intravascular ultrasound analysis. Advanced cardiac allograft vasculopathy as defined by Percent Atheroma Volume (PAV) >32% was associated with elevated levels of C-reactive protein (CRP), soluble tumor necrosis factor receptor-1 (sTNFR-1), von Willebrand factor (vWf), vascular cell adhesion molecule–1 (VCAM-1) and neopterin. There was no significant difference in levels of interleukin-6 (IL-6), osteoprotegerin (OPG) and soluble gp130 between the two groups.

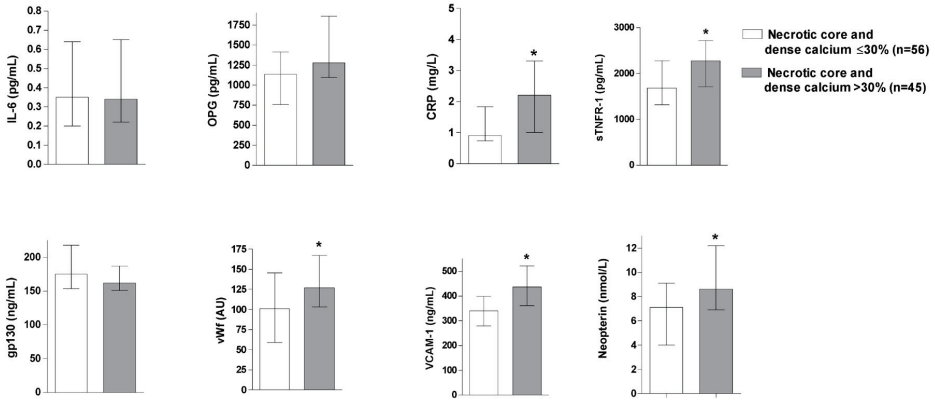
Figure 4. Immune marker profile according to intima inflammatory component determined by VH analysis. An increased intima inflammatory component (necrotic core and dense calcium >30%) was associated with elevated levels of C-reactive protein (CRP), soluble tumor necrosis factor receptor-1 (sTNFR-1), von Willebrand factor (vWf), vascular cell adhesion molecule–1 (VCAM-1) and neopterin. There was no significant difference in levels of interleukin-6 (IL-6), osteoprotegerin (OPG) and soluble gp130 between the two groups.


LC
EEM
EEM
MIT
LC


Fibrous
Fibrofatty
Calcified
Necrotic core


IL-6 (pg/mL)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OPG (pg/mL)
1750
1500
1250
1000
750
500
250
0
CRP (mg/L)
5
4
3
2
1
0
*
sTNFR-1 (pg/mL)
3000
2000
1000
0
*
Maximal Intimal Thickness ≤0.5 mm (n=54)
Maximal Intimal Thickness >0.5 mm (n=47)
gp130 (ng/mL)
200
150
100
50
0
vWf (AU)
175
150
125
100
75
50
25
0
VCAM-1 (ng/mL)
500
400
300
200
100
0
*
Neopterin (nmol/L)
12
8
4
0
*


IL-6 (pg/mL)
OPG (pg/mL)
CRP (mg/L)
sTNFR-1 (pg/mL)
gp130 (ng/mL)
vWf (AU)
VCAM-1 (ng/mL)
Neopterin (nmol/L)
*
Percent Atheroma Volume ≤32% (n=51)
Percent Atheroma Volume >32% (n=50)


IL-6 (pg/mL)
OPG (pg/mL)
CRP (mg/L)
sTNFR-1 (pg/mL)
gp130 (ng/mL)
vWf (AU)
VCAM-1 (ng/mL)
Neopterin (nmol/L)
*
Necrotic core and dense calcium ≤30% (n=56)
Necrotic core and dense calcium >30% (n=45)

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